Handbook of

Analysis of Active Compounds in Functional Foods

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Analysis of Active Compounds in Functional Foods

Edited by
Leo M.L. Nollet
Fidel Toldrá

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This book is dedicated to my fifth grandchild and second grandson, Roel.

I hope he will become a respected and beloved man.

Leo M. L. Nollet

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Preface

There are no such things as applied sciences, only applications of science.

Louis Pasteur

Handbook of Analysis of Active Compounds in Functional Foods is the sixth volume of a series of books on the analysis of foods. The previous five volumes are

Handbook of Muscle Foods Analysis
Handbook of Processed Meats and Poultry Analysis
Handbook of Seafood and Seafood Products Analysis
Handbook of Dairy Foods Analysis
Handbook of Analysis of Edible Animal By-Products

This unique handbook provides readers with a full overview of the analytical tools available for the analysis of active ingredients in functional foods. Readers will find the main types of analytical techniques and methodologies available worldwide.

The book contains 42 chapters arranged in 12 sections. Most chapters describe the substance and its role in food, with detailed information and a critical review of the available methodologies of analysis, their applications, and references. The chapters discuss the analysis techniques of active ingredients in functional foods such as certain dairy products. The discussion on analysis methods for active ingredients is extended to a wide variety of foods.

Section I: *Amino Acids, Peptides, and Proteins*, organized into seven chapters, deals with essential amino acids, peptides, and proteins. Additional chapters on, glutamine, taurine, glutathione, carnitine, and creatine are discussed in detail.

Section II: *Vitamins*, consists of five chapters dealing with different water- and fat-soluble vitamins.

Section III: *Terpenes*, contains two chapters—one on hydrocarbon carotenoids and the other on oxycarotenoids or xanthophylls.

Section IV: *Phenolic Compounds*, organized into seven chapters, discusses flavonoids, flavan-3-ols, proanthocyanidins, stilbenes, resveratrol, anthocyanins, isoflavones, tannins, ellagic acid, and chlorogenic acids.

Section V: *Fibers and Polysaccharides* (five chapters). Here the reader finds details about chitosan, insoluble dietary fiber, fructans, inulin, pectin, and cyclodextrins.

Section VI: *Probiotics*, in one chapter the authors discuss the selection and evaluation of probiotics.

Section VII: *Phytoestrogens and Hormones*, contains two chapters: Anise Oil and Occurrence and Analysis of Melatonin in Food Plants.

Section VIII: *Tetrapyrroles* and Section IX: *Minerals and Trace Elements*, comprise one chapter each on chlorophylls and on minerals, respectively.

Section X: *Lipid Compounds*, there are seven chapters on omega 3 and 6 fatty acids, conjugated linoleic acids, lecithin, sterols, stanols, lipoic acid, and alliin.

There are two chapters in Section XI: *Sweeteners*. They present detailed information on acesulfame-K, aspartame, and saccharin.

Finally, Section XII: *Salt Replacers and Taste-Modifying Compounds*, contains two chapters: one on sodium replacers and the other on triterpene glycosides.

Editing this book was a real challenge. For all chapters, we found skilled and enthusiastic colleagues interested in seizing the opportunity for such a job. For all their hard labor and very interesting end-products, chapters of this handbook, we cordially thank all contributors.

Leo M. L. Nollet
Fidel Toldrá

Editors

Leo M. L. Nollet, PhD, received MS (1973) and PhD (1978) degrees in biology from the Katholieke Universiteit Leuven, Belgium. Dr. Nollet is the editor and associate editor of numerous books. He edited for Marcel Dekker, New York—now CRC Press of Taylor & Francis Group—the first and second editions of *Food Analysis by HPLC* and *Handbook of Food Analysis*. The last edition is a three-volume book. He also edited *Handbook of Water Analysis* (first and second editions) and *Chromatographic Analysis of the Environment,* Third Edition (CRC Press).

Working jointly with F. Toldrá, he coedited two books published in 2006 and 2007: *Advanced Technologies for Meat Processing* (CRC Press) and *Advances in Food Diagnostics* (Blackwell Publishing—now Wiley). In collaboration with M. Poschl, he coedited *Radionuclide Concentrations in Foods and the Environment*, published in 2006 (CRC Press). Along with Y. H. Hui and other colleagues, Dr. Nollet coedited several books: *Handbook of Food Product Manufacturing* (Wiley, 2007), *Handbook of Food Science, Technology and Engineering* (CRC Press, 2005), *Food Biochemistry and Food Processing* (Blackwell Publishing—now Wiley, 2006), and *Handbook of Fruits and Vegetable Flavors* (Wiley, 2010). He edited *Handbook of Meat, Poultry and Seafood Quality* (Blackwell Publishing—now Wiley, 2007).

From 2008 to 2011, in collaboration with F. Toldrá, he edited five volumes in animal products-related books: *Handbook of Muscle Foods Analysis*, *Handbook of Processed Meats and Poultry Analysis*, *Handbook of Seafood and Seafood Products Analysis, Handbook of Dairy Foods Analysis*, and *Handbook of Analysis of Edible Animal By-Products*. Also, in 2011, in collaboration with F. Toldrá, he coedited two volumes: *Safety Analysis of Foods of Animal Origin* and *Sensory Analysis of Foods of Animal Origin* for CRC Press. Dr. Nollet and Hamir Rathore coedited *Handbook of Pesticides: Methods of Pesticides Residues Analysis*, published in 2009. Among other completed book projects are *Food Allergens: Analysis, Instrumentation, and Methods* (in collaboration with A. van Hengel) (CRC Press, 2011) and *Analysis of Endocrine Compounds in Food* (Wiley-Blackwell, 2011).

Fidel Toldrá, PhD, is a research professor in the Department of Food Science, Instituto de Agroquímica y Tecnología de Alimentos (CSIC) and serves as the European editor of *Trends in Food Science & Technology*, and editor-in-chief of *Current Nutrition & Food Science*. He is also a member of the CEF panel at the European Food Safety Authority. He is a member of the editorial board of nine journals including *Food Chemistry*, *Meat Science*, and *Food Analytical Methods*. He has acted as the editor or associate editor of several books in recent years. He was the editor of *Research Advances in the Quality of Meat and Meat Products* (Research Signpost, 2002) and the associate editor of the *Handbook of Food and Beverage Fermentation Technology* and the *Handbook of Food Science, Technology and Engineering* both published by CRC Press in 2004 and 2006, respectively. In collaboration with Leo Nollet, he coedited two books published in 2006: *Advanced Technologies for Meat Processing* (CRC Press) and *Advances in Food Diagnostics* (Blackwell Publishing). Both are associate editors of the *Handbook of Food Product Manufacturing* published by John Wiley & Sons in 2007 and the third edition of *Food Biochemistry and Food Processing* (Wiley-Blackwell, in preparation). Professor Toldrá has edited *Meat Biotechnology* (Springer, 2008) and *Safety of Meat and Processed Meat* (Springer, 2009) and also authored the book *Dry-Cured Meat Products* published by Food & Nutrition Press (now Wiley-Blackwell) in 2002.

In collaboration with L. Nollet, he published five books on analytical methodologies for foods of animal origin from 2009 to 2011: *Handbook of Muscle Foods Analysis*, *Handbook of Processed Meats and Poultry Analysis*, *Handbook of Seafood and Seafood Products Analysis, Handbook of Dairy Foods Analysis*, and *Handbook of Analysis of Edible Animal By-Products*. Also in 2011, in collaboration with

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Fidel Toldrá was awarded the 2002 International Prize for Meat Science and Technology by the International Meat Secretariat (IMS) and the Distinguished Research Award in 2010 by the American Meat Science Association (AMSA). In 2008 he was elected as a Fellow of the International Academy of Food Science & Technology (IAFOST) and in 2009 as a Fellow of the Institute of Food Technologists (IFT).

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Section I

Amino Acids, Peptides, and Proteins

1

Essential Amino Acids

M-Concepción Aristoy and Fidel Toldrá

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1.1 Introduction

Amino acids play a double role on living cells as building blocks of proteins and as intermediates in metabolism. Amino acids are organic compounds which contain both an amino group (p*K* by 9) and a carboxyl group (p*K* by 2) bound to a specific side chain which confers characteristic properties to each amino acid. The precise amino acid content, and the sequence of amino acids, of a specific protein, determines the biological activity of that protein. In addition, proteins contain within their amino acid sequences the necessary information to determine how that protein will fold into a three-dimensional structure, and the stability of the resulting structure. As a result, the characteristics of even a small, relatively simple, protein are a composite of the properties of the amino acids which comprise the protein. That is why each amino acid within a protein is unique and irreplaceable.

In all, 20 amino acids form part of proteins, and the human body can synthesize all of the amino acids necessary to build proteins except for nine called the "essential amino acids." An adequate diet must contain all these essential amino acids. Typically, they are present in meat and dairy products, but if these are not consumed, care must be taken to ensure an adequate supply of an alternative diet. Several studies have reported that most of the weaning foods consumed by children in many parts of developing nations are deficient in essential macronutrients and micronutrients. In view of this nutritional problem, several strategies have been used to formulate weaning food through a combination of locally available foods that complement each other in such a way as to create a new pattern of amino acids that provide the recommended daily allowance for infants (Ijarotimi and Olopade, 2009). A complete diet from the point of view of essential amino acids can be supplied in a natural way by a combined diet containing cereal grains (wheat, corn, rice, etc.) with legumes (beans, peanuts, etc.). Cereals are deficient in lysine but have sufficient sulfur-containing amino acids which are limited in legumes. The alternative is to provide essential amino acid supplements.

The amino acids that we can produce are alanine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, glycine, proline serine, and tyrosine. The distinction between essential and nonessential amino acids is somewhat unclear, as some amino acids, such as tyrosine and cysteine, may be produced from other essential amino acids. Tyrosine is produced from phenylalanine, so if the diet is deficient in phenylalanine as in the case of patients suffering from phenylketonuria (PKU), tyrosine will be required as well. Also cysteine is not classified as an essential amino acid because it usually can be synthesized by the human body under normal physiological conditions if sufficient amounts of other sulfur-amino acids (i.e., methionine and homocysteine) are available. So, for convenience, sulfur-containing amino acids are sometimes considered a single pool of nutritionally equivalent amino acids. Other amino acids are considered "semiessential" or "conditionally essential." Arginine is essential for infants who cannot synthesize it, but not for adults who are able to synthesize arginine from ornithine and citrulline through the urea cycle. Glutamine has also been proposed as a "conditionally essential amino acid" due to its importance under exceptionally severe stress conditions, such as very intense exercise, infectious disease, surgery, burn injury, or any other acute traumas. In all these cases, supplementation with glutamine can be a matter of life or death.

There are nine essential amino acids for which humans do not have the required enzymes for their biosynthesis. They are histidine, lysine, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, and valine. The failure to obtain enough of even one of the nine essential amino acids has serious health implications and can result in degradation of the body's proteins to obtain the one amino acid that is needed. Unlike fat and starch, the human body does not store excess amino acids for later use and thus amino acids must be present in food everyday.

1.1.1 Chemical Properties and Physiological Importance of Essential and Semiessential Amino Acids

Table 1.1 shows the nomenclature and structure of the essential and semiessential amino acids. Some chemical properties are described below and the relationship between the respective amino acid structure and biological role is pointed out. Essential amino acids can be classified by their chemical nature.

Basic amino acids: Are positively charged amino acids at neutral pH: lysine, histidine, and arginine.

Lysine has a positively charged ε-amino group (a primary amine). The ε-amino group has a significantly higher pK_a (10.5) than does the α-amino group (9.0). The ε-amino group is highly reactive and often participates in reactions at the active centers of enzymes. Proteins only have one α-amino group, but numerous ε-amino groups from lysines. Specific environmental effects in enzyme active centers can lower the pK_a of the lysyl side chain such that it becomes reactive. The side chain has four methylene groups, so that even though the terminal amino group will be charged under physiological conditions, the side chain has significant hydrophobic character and that is why lysines are often buried with only the ε-amino group exposed to the solvent.

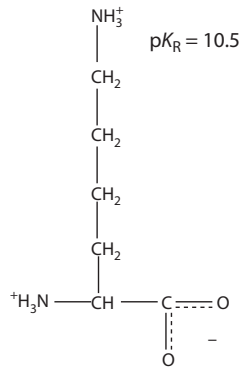
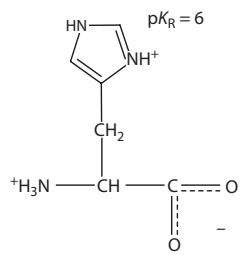
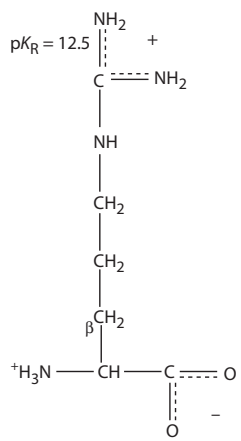
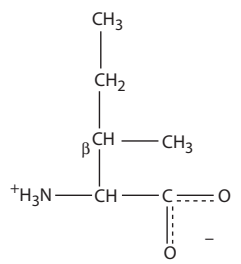
L-Lysine is a necessary building block for all proteins in the body. L-Lysine plays a major role in calcium absorption; building muscle protein; recovering from surgery or sports injuries; and in the production of hormones, enzymes, and antibodies. It is found to be limited in all cereal grains, but is abundantly present in all legumes, potatoes, dairy, meat, and brewer's yeast.

Histidine has a positively charged imidazole functional group. The imidazole side chain of histidine is a common coordinating ligand in metalloproteins and is a part of the catalytic sites in certain enzymes. The residue can also serve in stabilizing the folded structures of proteins. Histidine is a precursor to histamine, a compound released by the immune system cells during an allergic reaction. Also it is a precursor for carnosine biosynthesis. Histidine is considered an essential amino acid in human infants. After several years of age, humans begin to synthesize it, at which point it becomes a nonessential amino acid.

Arginine has a positively charged guanidino group. As a cation, arginine, as well as lysine, plays a role in maintaining the overall charge balance of a protein. Arginine is well designed to bind the phosphate anion, and is often found in the active centers of proteins that bind phosphorylated substrates. Arginine is extremely useful in enhancing the immune system and plays an important role in nitrogen metabolism.

TABLE 1.1

Structure, Chemical, and Physical Properties of Essential and Semiessential Amino Acids

	Abbreviation	N[a]	Chemical Properties	Physical Properties	Structure
Lysine	Lys	K	Basic	Polar Positively charged	
Histidine	His	H	Basic	Polar Positively charged	
Arginine	Arg	R	Basic	Polar Positively charged	
Isoleucine	Ile	I	Aliphatic Branched	Nonpolar	

continued

TABLE 1.1 (continued)

Structure, Chemical, and Physical Properties of Essential and Semiessential Amino Acids

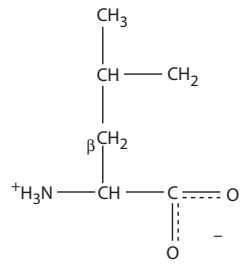
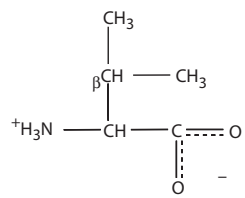
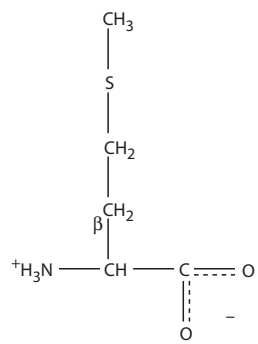
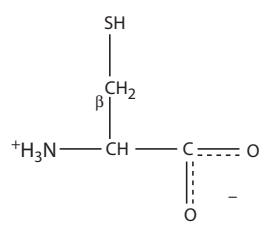
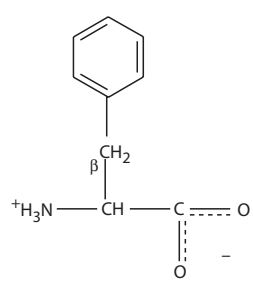
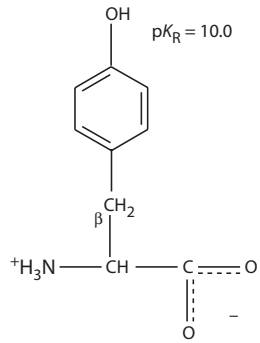
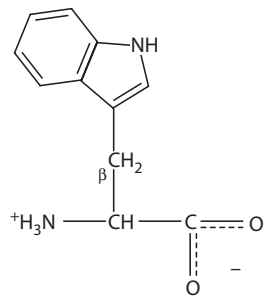
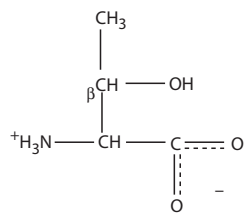
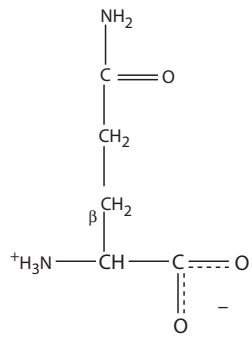
	Abbreviation	N[a]	Chemical Properties	Physical Properties	Structure
Leucine	Leu	L	Aliphatic Branched	Nonpolar	
Valine	Val	V	Aliphatic Branched	Nonpolar	
Methionine	Met	M	Sulfur-containing	Nonpolar	
Cysteine	Cys	C	Sulfur-containing	Polar (uncharged)	
Phenylalanine	Phe	F	Aromatic	Nonpolar	

TABLE 1.1 (continued)

Structure, Chemical, and Physical Properties of Essential and Semiessential Amino Acids

	Abbreviation	N[a]	Chemical Properties	Physical Properties	Structure
Tyrosine	Tyr	Y	Aromatic	Nonpolar	
Tryptophan	Trp	W	Aromatic	Nonpolar	
Threonine	Thr	T	Nonaromatic hydroxyl	Polar (uncharged)	
Glutamine	Gln	Q	Neutral Amide of acidic amino acids R-group	Polar (uncharged)	

[a] One-letter amino acid nomenclature given by Dr. Margaret Oakley Dayhoff (1925–1983).

In the urea cycle, the enzyme arginase cleaves the guanidinium group to yield urea and the l-amino acid ornithine. l-Ornithine is not normally found in proteins and arginine is one of the least frequent amino acids in proteins, but its deficiency is rare.

Branched-chain amino acids are building blocks of all proteins, and, for it, they are essentials for normal growth and tissue repair. They are isoleucine, leucine, and valine. The side chains of these amino acids are

not reactive and therefore not involved in any covalent chemistry in enzyme active centers. However, these residues are critically important for ligand binding to proteins, and play central roles in protein stability. They also are needed during times of physical stress and intense exercise and they should always be taken in balance. Also, it has been reported that supplementation of soy protein with branched-chain amino acids alters protein metabolism in healthy elderly and even more in patients with chronic obstructive pulmonary disease (Engelen et al., 2007). They are hydrophobic and generally buried in folded proteins.

Isoleucine is essential in the formation of hemoglobin. Note also that the β-carbon of isoleucine is optically active, just as the β-carbon of threonine. Ile is usually interchangeable with leucine and occasionally with valine in proteins.

Leucine promotes wound healing of skin and bones, increases insulin secretion, lowers blood sugar level, and is a precursor to cholesterol.

Valine is the third branched amino acid.

Maple syrup urine disease is caused by the inability to metabolize the leucine, isoleucine, and valine. The disease is so named because urine from affected people smells like maple syrup. A deficiency may affect the myelin covering of the nerves.

The *sulfur-containing amino acids*, methionine and homocysteine, can be converted into each other but neither can be synthesized *de novo* in humans. Likewise, cysteine can be made from homocysteine but cannot be synthesized on its own. So, for convenience, sulfur-containing amino acids are sometimes considered a single pool of nutritionally equivalent amino acids. Sulfur belongs to the same group in the periodic table as oxygen but is much less electronegative. This difference accounts for some of the distinctive properties of the sulfur-containing amino acids (Brosnan and Brosnan, 2006).

Methionine is the principal source of sulfur to the body. Unlike cysteine, the sulfur of methionine is not highly nucleophilic, and it is generally not a participant in the binding chemistry that occurs in the active centers of enzymes.

The sulfur of methionine, as with that of cysteine, is prone to oxidation. The first step, yielding methionine sulfoxide, can be reversed by standard thiol-containing reducing agents. The second step yields methionine sulfone, and is effectively irreversible. It is thought that oxidation of the sulfur in a specific methionine of the elastase inhibitor in human lung tissue by agents in cigarette smoke is one of the causes of smoking-induced emphysema.

Methionine is characterized by the presence of a methyl group attached to a sulfur atom located in its side chain. In addition to its role in protein synthesis, large amounts of this amino acid are used for the synthesis of *S*-adenosylmethionine (AdoMet), the main biological methyl donor. AdoMet participates in a large number of reactions, due to its ability to donate all the groups surrounding the sulfur atom. Thus, it is the precursor of biotine, glutathion, cysteine, phospholipids, polyamines, and neurotransmitters through the methionine cycle which also drives methionine regeneration (Markham and Pajares, 2009).

Racemic methionine is sometimes added as an ingredient to pet foods. High levels of methionine can be found in sesame seeds, Brazil nuts, fish, meats, yogurt, and some other plant seeds; methionine is also found in cereal grains. Most fruits, vegetables, and legumes are low in methionine. The complement of cereal (rich in methionine) and legumes (rich in lysine), providing a complete protein, is a classic combination, found throughout the world (rice and beans or rice and lentils), also the combination of sesamo and chickpeas is a famous dish in the Arabian cuisine which is called hummus.

Cysteine is the other sulfur-containing amino acid. It takes part in the active centers of certain enzymes (cystein-proteases). Also the thiol in the molecule is susceptible to oxidation to give the disulfide derivative cystine, which serves an important structural role in many proteins. Insulin is an example of a protein with cystine crosslinking, wherein two separate peptide chains are connected by a pair of disulfide bonds. Due to the ability of thiols to undergo redox reactions, cysteine has antioxidant properties. Cysteine's antioxidant properties are typically expressed in the tripeptide glutathione (GSH). The availability of oral GSH is negligible; so it must be biosynthesized from its constituent amino acids, cysteine, glycine, and glutamic acid.

Cysteine is used in the production of flavors and in cosmetics. For example, the reaction of cysteine with sugars in a Maillard reaction yields meat flavors. L-Cysteine is also used as a processing aid in baking.

Aromatic amino acids include the phenylalanine, tryptophan, and tyrosine which are neurotransmitter precursors.

Phenylalanine is a derivative of alanine with a phenyl substituent on the β-carbon. Phenylalanine is hydrophobic and not very soluble in water. Due to its hydrophobicity, phenylalanine is nearly always found buried within a protein. The π electrons of the phenyl ring can stack with other aromatic systems and often do within folded proteins, adding to the stability of the structure.

It is a direct precursor to the neuromodulator phenylethylamine, a commonly used dietary supplement for its analgesic and antidepressant effects. The genetic disorder PKU is the inability to metabolize the phenylalanine. Individuals with this disorder are known as "phenylketonurics" and must regulate their intake of phenylalanine.

It is contained in most protein-rich foods but good sources are found in dairy products, almonds, avocados, lima beans, peanuts, and seeds.

Tyrosine is also an aromatic amino acid and is derived from phenylalanine by hydroxylation in the *para* position. It is not an essential amino acid, properly, but in absence of phenylalanine, like in phenylketonuric patients, it turns into an essential amino acid.

Tyrosine absorbs ultraviolet radiation and contributes to the absorbance spectra of proteins. The absorbance spectrum of tyrosine will be shown later; the extinction of tyrosine is only about one-fifth that of tryptophan at 280 nm. Tyrosine is a precursor to neurotransmitters (dopamine and norepinephrine) and thyroid hormones, and also to the pigment melanin. Meat, dairy, eggs as well as almonds, avocados, and bananas are good sources of this nutrient.

Tryptophan is the largest of the amino acids. It is also a derivative of alanine, having an indole substituent on the β-carbon. The indole functional group absorbs strongly in the near ultraviolet part of the spectrum and thus tryptophan is responsible for most of the absorbance of ultraviolet light (ca. 280 nm) by proteins.

Tryptophan has been considered a reasonably effective sleep aid, and acts against premenstrual dysphoric disorder (Steinberg et al., 1999) and seasonal affective disorder (Jepson et al., 1999), probably due to its ability to increase the brain levels of serotonin (a calming neurotransmitter when present in moderate levels) and/or melatonin (a sleep-inducing hormone secreted by the pineal gland in response to darkness or low-light levels) (Wurtman et al., 1968; Ruddick et al., 2006). It is also a precursor to niacine (vitamin B_3) and auxin (phytohormone).

Food sources of tryptophan are dairy products, meat, soy, and peanuts. Tryptophan is often supplemented in poultry diets which are based on cereal grains and thus contain low levels of this amino acid (Ravindran et al., 2006).

Threonine is a hydrophilic hydroxyl-containing amino acid similar to serine. It differs from serine by having a methyl substituent in the place of one of the hydrogens on the β-carbon and it differs from valine by replacement of a methyl substituent with a hydroxyl group. Note that both the α- and β-carbons of threonine are optically active. Threonine is involved in the production of antibodies and immunoglobulins. It is an important constituent of connective tissue in tendons and skin, it is glycogenic and participates in lipid metabolism by preventing fatty build up in the liver. It is generally low in vegetarian diets and used to enrich cereal proteins.

Glutamine is considered to be a conditionally essential amino acid during episodes of catabolic stress and malnutrition (Ziegler et al., 1993). Glutamine is the amide of glutamic acid, and is uncharged under all biological conditions. The additional single methylene group in the side chain relative to asparagine allows glutamine in the free form or as the *N*-terminus of proteins to spontaneously cyclize and deamidate yielding the six-member ring structure pyrrolidone carboxylic acid, which is found at the *N*-terminus of many immunoglobulin polypeptides. It causes obvious difficulties with amino acid sequence determination.

In human blood, glutamine is the most abundant free amino acid, with a concentration of about 500–900 μmol/L (Brosnan, 2003).

1.1.2 Recommended Daily Allowance for Essential Amino Acids

The Recommended Dietary Allowance (RDA), nowadays known as Dietary Reference Intake, was developed by the Food and Nutrition Board of the National Academy of Sciences/National Research Council (Washington, DC, USA) and is a collaborative effort between the United States and Canada. The

TABLE 1.2

Recommended Daily Allowance for Essential Amino Acids (mg/kg weight)

	Infant 3–6 Months	Child 10–12 Years	Adults
Histidine	33	—	—
Isoleucine	80	28	12
Leucine	128	42	16
Lysine	97	44	12
S-containing amino acids	45	22	10
Aromatic amino acids	132	22	16
Threonine	63	28	8
Tryptophan	19	4	3
Valine	89	25	14

RDA states the amount of a nutrient that is needed for most people to stay healthy. They are different for children and adults both males and females. The RDA of essential amino acids is shown in Table 1.2, and indicates the required mg/kg weight of each essential amino acid per day.

The nutritional value or quality of structurally different proteins varies and is governed by amino acid composition, ratios of essential amino acids, susceptibility to hydrolysis during digestion, and source and the effects of processing (Friedman, 1996). From this state, the analysis of essential amino acids is a very common goal in many laboratories on food, pharmaceutical, or clinical subjects. Many methods are available; however, the essential ones are described below.

1.1.3 Other Uses for Essential Amino Acids

N-Acetyl-L-methionine and *N*-acetyl-L-cysteine can be used as a source of L-methionine and L-cysteine, respectively, for use in foods for special medical purposes in children over 1 year and adults (EFSA, 2003a,b). These substances improve the palatability of the product and enhance patient compliance with the diet, better than when the L-amino acid is included as such. It does not compromise the biological value of the formulation. *N*-Acetyl-L-methionine and *N*-acetyl-L-cysteine are deacetylated in animals and humans to the respective L-amino acid. The bioavailability of methionine from *N*-acetyl-L-methionine is comparable to that from L-methionine in other sources (EFSA, 2003a).

The use of L-cysteine has been proposed in processed cereal-based foods and foods (specifically baby biscuits) for infants and young children for technological purposes (EFSA, 2006). The function of cysteine as a flour treatment agent is the reduction of disulfide bonds in gluten, thus acting as a dough improver, controlling the rheological properties of the dough and the texture of the final product. So, it acts as a dough improver in biscuit recipes, especially those with a low-fat content because they have increased brittleness, with an associated risk of choking and/or suffocation when the biscuit is broken down into small pieces in the child's mouth.

There are certain salts based on specific amino acids that are used to provide a source of minerals as nutrients in food supplements. The Scientific Committee on Food considered that the use of L-amino acids may be generally acceptable provided the addition to food does not give rise to a nutritional imbalance of the amino acids (SCF, 1990). Magnesium L-lysinate, calcium L-lysinate, zinc L-lysinate as sources for magnesium, calcium, and zinc added for nutritional purposes in food supplements being salts of the amino acid lysine (EFSA, 2008a). Like other salts of organic acids, they are soluble in water and are expected to dissociate. The bioavailability of lysine is expected to be similar to that of other soluble sources. So, in those cases, certain exposure to lysine may be expected from these sources when used. Certain bisglycinates have also been proposed as sources of copper, zinc, calcium, magnesium, and glycinate nicotinate as source of chromium in foods intended for the general population (including food supplements) and foods for particular nutritional uses (EFSA, 2008b).

1.2 Essential Amino Acids Analysis

Essential amino acids are analyzed from a food or a supplement preparation. Methods for this analysis have been widely described in the literature (Aristoy and Toldrá, 2004, 2008, 2009, 2010) and are summarized in this chapter with special attention to some amino acids which present difficulties to their analysis due to their reactiveness or unstability under hydrolysis conditions.

Free soluble amino acids are analyzed with previous sample extraction and deproteinization or by simple sample dissolution, while amino acids forming proteins require the previous hydrolysis of these proteins. The amino acid lysine may be found unavailable in food (mainly in processed foods) for having blocked the imidazole group in the side chain. The hydrolysis of the sample to analyze this amino acid will lead to release of lysine and overestimate the available lysine in the food. Some strategies will be described in Section 1.2.2.1 to overcome this problem.

1.2.1 Sample Preparation

1.2.1.1 Free Essential Amino Acids

The analysis of free amino acids in a food goes through three stages: extraction, clean-up or deproteinization, and proper analysis. The preparation for essential amino acid supplements will require only dissolution. The analysis procedures will be described together with that of the hydrolyzed amino acids.

1.2.1.1.1 Extraction

The extraction consists in the separation of the free amino acids fraction from the insoluble portion of the food matrix. It is usually achieved by homogenization of the ground sample in an appropriate solvent. The homogenization is usually achieved by using a Polytron™, Ultra Turrax™, or Stomacher™ or by means of a simple stirring in warm solvent. The extraction solvent can be hot water, 0.01–0.1 N hydrochloric acid solution, or diluted phosphate buffers. In some cases, concentrated strong acid solutions such as 0.6 N perchloric acid (PCA) (Alonso et al., 1994), 4–5% of 5-sulfosalicylic acid (SSA) (Godel et al., 1984; Arnold et al., 1994; Paleari et al., 2003), 2–5% of trichloroacetic acid (TCA) (Shibata et al., 1991; Periago et al., 1996; Hughes et al., 2002), or rich alcohol-containing solution (>75%) such as ethanol (Qureschi et al., 1984; Nguyen and Zarkadas, 1989; Hagen et al., 1993; Stancher et al., 1995) or methanol (Antoine et al., 1999) have been successfully used as extraction solvents with the additional advantage that proteins are not extracted and, then, there is no need for further cleaning up of the sample. Nevertheless, Periago et al. (1996) found differences in some amino acid extraction according to the extraction method used, thus low recoveries of basic amino acids, when using ethanol 70%, were observed.

Once homogenized, the solution is centrifuged at 10,000*g* under refrigeration to separate the supernatant from the nonextracted materials (pellet) and filtered through glass wool to retain any fat material remaining on the surface of the supernatant.

1.2.1.1.2 Deproteinization

The deproteinization process can be achieved through different chemical or physical procedures. Chemical methods include the use of concentrated strong acids like SSA (Hagen et al., 1993; Arnold et al., 1994; Izco et al., 2000; Yongjin et al., 2007), PCA (Qureschi et al., 1984), TCA (Büetikofer and Ardö, 1999; Scanell et al., 2004), picric (Sugawara et al., 1984; Brüechner and Hausch, 1990; Oh et al., 1995), or phosphotungstic (PTA) (Aristoy and Toldrá, 1991) acids or organic solvents such as methanol, ethanol, or acetonitrile (Sarwar and Botting, 1990; Aristoy and Toldrá, 1991; Antoine et al., 1999; Durá et al., 2004). Under these conditions, proteins precipitate by denaturation while free amino acids remain in solution. Physical methods consist in the forced filtration (mainly by centrifugation) through cut-off membrane filters (1000, 5000, 10,000, and 30,000 Da) that allow free amino acids through while retaining the large compounds (Cohen and Strydom, 1988; Nicolas, 1990; Aristoy and Toldrá, 1991; Hagen et al., 1993; Krause et al., 1995). All these methods give a sample solution rich in free amino acids and free of proteins.

Differences among all these chemical and physical methods are referred to several aspects like the differences in the recovery of amino acids, compatibility with derivatization (pH, presence of salts, . . .)

or separation method (interferences in the chromatogram ...), and so on. Some of these methods, even though promising, give low recoveries of some amino acids as is the case of the PTA which is the most efficient (cut-off is around 700 Da) but causes losses of acidic and basic amino acids, especially lysine. The membrane used can also affect amino acid recoveries (Sarwar and Botting, 1990) and thus prewashing of filters is recommended to improve those recoveries (Krause et al., 1995). It is important to consider that strong acids exert a very low pH in the medium, that can interfere with the precolumn derivatization where high pH is necessary to accomplish the majority of the derivatization reactions. Thus, it is essential either, to completely eliminate this acid by evaporation or extraction or adjust the pH of the sample solution. This is not a problem when the amino acids have to be analyzed by ion-exchange chromatography and postcolumn derivatization; indeed, SSA or TCA have been commonly used prior to ion exchange amino acid analysis because they give an appropriate pH for the chromatographic separation (Godel et al., 1984; Scanell et al., 2004). The interference of SSA in the chromatographic separation is doubtful (Deyl et al., 1986; Schuster, 1988) and low recoveries of some amino acids have been reported (Aristoy and Toldrá, 1991). As 10–12% TCA was used to fractionate cheese (Virgili et al., 1999) or ham (Ordoñez et al., 1998) extracts to study the proteolysis course during ripening, 15% and 12% TCA were used to analyze free amino acids in legumes (Periago et al., 1996) and cured loins, respectively (Scanell et al., 2004). By using 0.6 N PCA, which is easily neutralized by the addition of potassium hydroxide (KOH) or potassium bicarbonate, the deproteinization procedure can be very simple and no-interferences have been described.

The use of organic solvents, by mixing 2 or 3 volumes of organic solvent with 1 volume of extract, has yielded very good results (Schuster, 1988; Sarwar and Botting, 1990; Jansen et al., 1991) with amino acid recoveries around 100% for all of them (Aristoy and Toldrá, 1991, Jansen et al., 1991) with the additional advantage of easy evaporation to concentrate the sample. Some comparative studies on these deproteinization techniques have been published (Blanchard, 1981; Davey and Ersser, 1990; Aristoy and Toldrá, 1991).

1.2.1.2 Total Essential Amino Acids

Sample preparation for the analysis of total amino acids includes the hydrolysis of proteins and peptides as a first step. A quantitative hydrolysis may be difficult to achieve for some essential amino acids. Main hydrolysis methods are described below and some cautions for some specially labile amino acids are pointed out.

1.2.1.2.1 Acid Hydrolysis

Acid hydrolysis is the most common method for hydrolyzing proteins. It consists of an acid digestion with constant boiling of 6 N hydrochloric acid in an oven at around 110°C for 20–96 h or by using a microwave oven (Kuhn et al., 1996). The hydrolysis must be carried out in sealed vials under nitrogen atmosphere and in the presence of antioxidants/scavengers in order to minimize the degradation suffered by some especially labile amino acids (tyrosine, threonine, serine, methionine, cysteine, and tryptophan) in such acidic and oxidative medium. Phenol (up to 1%) or sodium sulfite (0.1%) are typical protective agents and are effective for nearly all amino acids except for tryptophan and cysteine. Even though, considerable tryptophan recoveries have been reported in the presence of phenol when using liquid-phase hydrolysis (Muramoto and Kamiya, 1990) or in the presence of tryptamine when using gas-phase hydrolysis (Molnár-Perl et al., 1993), and absence of oxygen. Hydrolysis with hydrochloric acid may also be improved by optimizing the temperature and time of incubation (Molnár-Perl et al., 1993).

Alternative reagents for acid hydrolysis are 4 M methanesulfonic acid (115°C for 22–72 h or 160°C for 45 min, under vacuum) and 3 M mercaptoethanesulfonic acid (160–170°C for 15–30 min) which have been described to improve tryptophan and methionine recoveries (Simpson et al., 1976; Chiou and Wang, 1989; Stocchi et al., 1989; Malmer and Schroeder, 1990; Molnár-Perl et al., 1993; Csapo, 1994). These acids possess a high-boiling point and thus, only the liquid-phase hydrolysis is possible and after hydrolysis, the hydrolyzed sample will have to be subjected to pH adjustment before analysis. The use of protective reagents like tryptamine (Umagat et al., 1982; Stocchi et al., 1989; Malmer and Schroeder, 1990) or thioglycolic acid (Ashworth, 1987a,b) is also advisable to prevent oxidation.

Cyst(e)ine is partially oxidized during acid hydrolysis yielding several adducts such as cystine, cysteine, cystein sulfinic acid, and cysteic acid making its analysis rather difficult. Several procedures have been proposed to analyze cyst(e)ine after acid hydrolysis. The simplest method consists in subjecting the sample to a performic acid oxidation, prior to the acid hydrolysis (Spindler et al., 1984; Gehrke et al., 1987; Alegría et al., 1996; Akinyele et al., 1999). This process transforms the cyst(e)ine to cysteic acid which is acid resistant and will be analyzed together with at least nine amino acids plus methionine (transformed into methionine sulfone) and lysine with optimized recoveries, as proposed by Gehrke et al. (1987). Nevertheless, tyrosine and histidine and phenylalanine may be more or less destroyed by this oxidation procedure (Spindler et al., 1984; Gehrke et al., 1987; Aristoy and Toldrá, 2009, 2010). Figure 1.1 shows an amino acid chromatogram of hydrolyzed pork meat without and after performic acid oxidation.

The use of alkylating agents to stabilize cysteine before hydrolysis has been used as a valid alternative. Good recoveries have been reported by using 3-bromopropionic acid (Bradbury and Smith, 1973), 3-bromopropylamine (Hale et al., 1994), iodoacetic acid (Pripis-Nicolau et al., 2001), and 3,3′-dithiodipropionic acid (Strydom and Cohen, 1994; Krause et al., 1995; Tuan and Phillips, 1997; Ballin, 2006).

The analysis of bound glutamine after acid hydrolysis is impossible because glutamine turns into glutamic acid. Kuhn et al. (1996) proposed the conversion of bound glutamine into L-2,α-diaminobutyric acid which is acid/heat stable. The protein acid hydrolysis is then achieved using a microwave technique and afterwards the glutamine is dansyl derivatized (see Section 1.2.2.2.1.1) previous high performance liquid chromatography (HPLC) analysis.

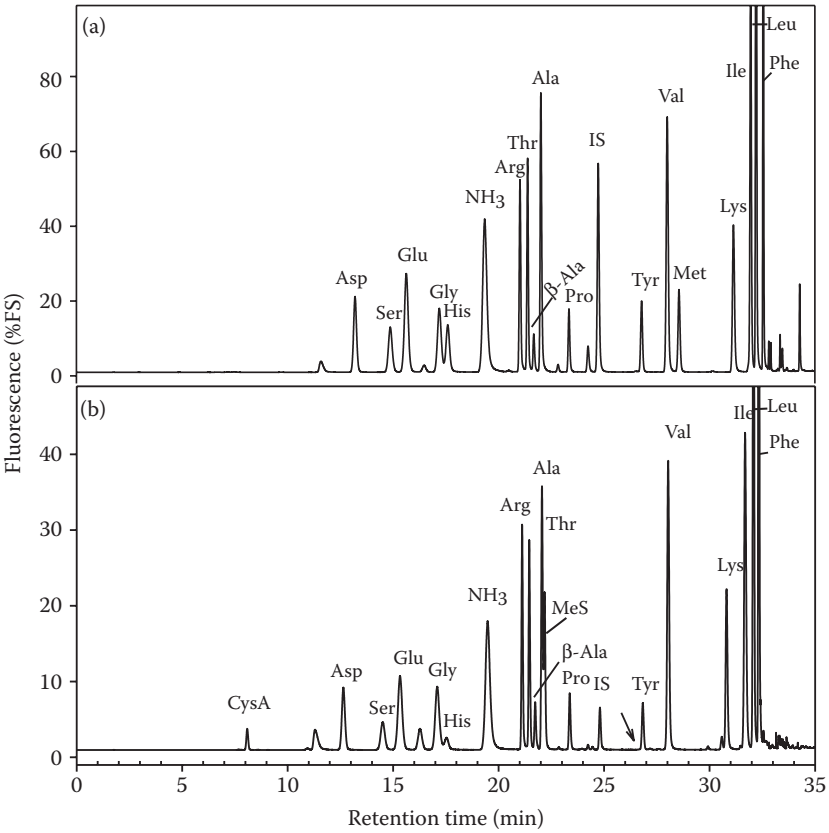


FIGURE 1.1 AQC-amino acids from hydrolyzed pork meat without (a) and after performic acid oxidation (b). Arrow points at cysteine elution position. CysA, cysteic acid; MeS, methionine sulfone; IS, internal standard nor-valine. (Adapted from Aristoy, M.C. and Toldrá, F. 2008. In: *Handbook of Muscle Food Analysis*, pp. 385–398. Boca Raton, FL: CRC Press.)

As can be observed in this section, no single set of conditions will yield the accurate determination of all essential amino acids. In fact, it is a compromise of conditions that offer the best overall estimation for the largest number of amino acids. In general, the 22–24 h acid hydrolysis at 110°C (vapor-phase hydrolysis, preferably), or 2 h at 145°C (Alegría et al., 1996) with the addition of a protective agent like phenol, yields acceptable results for the majority of essential amino acids, being enough for the requirements of any food control laboratory. However, when the analysis of tryptophan, cyst(e)ine, and/or glutamine is necessary, adequate especially hydrolysis procedures as those described above should be performed. When high sensitivity is required, the pyrolysis at 500°C for 3 h (Knetch and Chang, 1986) to 600°C overnight (Stocchi et al., 1989) of all glass material in contact with the sample is advisable as well as the analysis of some blank samples to control the level of background present. The optimization of conditions based on the study of hydrolysis time and temperature, acid-to-protein ratio, presence and concentration of oxidation protective agents, importance of a correct deaeration, and so on has been extensively reported in papers (Lucas and Sotelo, 1982; Gehrke et al., 1985; Zumwalt et al., 1987; Molnár-Perl and Khalifa, 1993; Weiss et al., 1998; Albin et al., 2000).

1.2.1.2.2 Alkaline Hydrolysis

The alkaline hydrolysis with 4.2 M of either NaOH, KOH, LiOH, or BaOH, with or without the addition of 1% (w/v) thiodiglycol for 18 h at 110°C is recommended by some authors (Hugli and Moore, 1972; Zarkadas et al., 1986; Meredith et al., 1988; Stocchi et al., 1989; Slump et al., 1991; Viadel et al., 2000), for a better tryptophan determination. This would be the method of choice to analyze the tryptophan in food samples containing high-sugar concentration like cereals.

1.2.1.2.3 Enzymatic Hydrolysis

Enzymatic hydrolysis with proteolytic enzymes such as trypsin, chymotrypsin, carboxypeptidase, papain, thermolysin, and pronase has been used to analyze specific amino acid sequences or single amino acids because of their specific and well-defined activity. By using this methodology, tryptophan content was analyzed in soy- and milk-based nutritional products by enzymatic (pronase) digestion of the protein to release the tryptophan, which was further analyzed by isocratic reversed-phase liquid chromatography with fluorescence detection. Enzymatic digestion was completed in <6 h and was accomplished under chemically mild conditions (pH 8.5, 50°C), which did not significantly degrade tryptophan (García and Baxter, 1992).

1.2.2 Analysis

After sample preparation, target essential amino acids may be analyzed either by direct spectrophotometric or by chromatographic [HPLC or gas liquid chromatographic (GLC)] methods. The choice mainly depends on the available equipment or personal preferences, because each methodology has its advantages and drawbacks.

1.2.2.1 Direct Spectrophotometric Methods

The direct determinations of tryptophan without separation or even without hydrolysis of the sample are based on the acid ninhydrin method (Pinter-Szakacs and Molnar-Perl, 1990) or on the direct measurement of the tryptophan derivative spectrophotometry (Fletouris et al., 1993).

Another example is the analysis of lysine. During the acid hydrolysis used in amino acid analysis, some lysine molecules, which are found blocked in their native proteins, revert back to the parent amino acid, leading to errors in estimates during the amino acid digestibility. This is a particular concern in damaged food proteins (Moughan, 2005), but also in processed foods. To overcome this fact, methods analyzing free NH_2-lys residues have been developed. In one of these methods, free (reactive) lysine is coupled to fluorodinitrobenzene previous acid hydrolysis permitting the determination of lysine available by direct spectrophotometric measurement at 435 nm (Anderson et al., 1984; Carpenter et al., 1989; Moughan, 2005; Torbatinejad et al., 2005) of the hydrolyzed sample. These analyses are very often used in cereals in which lysine is a limiting amino acid.

Foods submitted to intense heat treatment as drum-drying suffer protein denaturation with the formation of disulfide (S–S) bonds from sulfydryl (–SH) groups and the reduction of the protein and amino acid digestibility. Some special derivatives are proposed to determine cystine and cysteine as an index of this damage. 7-Chloro-4-nitrobenzo-2-oxa-1,3-diazole (Akinyele et al., 1999) and 5,5′-dithio-bis-nitrobenzoic acid (Hofmann, 1977; Opstvedt et al., 1984) will specifically analyze –SH groups (cysteine), and S–S bonds reduced with sodium borohydride ($NaBH_4$) may be determined as –SH groups along with the native –SH groups. Formed derivatives are measured at 410 and 415 nm, respectively. These methods are highly specific for cysteine and do not need a posterior chromatographic separation.

1.2.2.2 Chromatographic Methods

The separation of the individual essential amino acids in a mixture requires very efficient separation techniques like chromatography (liquid chromatography [LC] or gas chromatography [GC]) or capillary electrophoresis. The choice mainly depends on the available equipment or personal preferences, because each methodology has its advantages and drawbacks.

1.2.2.2.1 High-Performance Liquid Chromatography

HPLC is the most versatile and widespreading technique to separate amino acids. Before or after this separation, amino acids are derivatized to allow their separation and/or to enhance their detection.

1.2.2.2.1.1 Derivatization Derivatization is an usual practice in the amino acid analysis. The goodness of a derivatizing agent is evaluated based on the following aspects: It must be able to react with both primary and secondary amino acids, give a quantitative and reproducible reaction, yield a single derivative of each amino acid, have mild and simple reaction conditions, possibility of automation, good stability of the derivatization products and no interferences due to by-products or excess of reagent. It is worthwhile to remark that the use of enough amount of derivatization reagent is of special importance when dealing with biological samples since reagent-consuming amines, although unidentified, are always present (Godel et al., 1984).

Some reports comparing amino acid derivatization methods for the HPLC analysis of biological samples have been published (Fürst et al., 1989; Sarwar and Botting, 1990; Fekkes, 1996; Ordóñez et al., 1998; Aristoy and Toldrá, 2004). Pre- or postcolumn derivatization reagents used in the analysis of free amino acids are also useful for essential amino acids, with some exceptions. Essential amino acids such as histidine, lysine, tryptophan, and cysteine present some difficulties. The most used derivatization methods are described below.

Ninhydrin: It is the most used postcolumn derivatization reagent after amino acid cation exchange chromatographic analysis. The reaction takes place in hot (at pH 6) and renders colored derivatives detectable at 570 (primary amino acids) and 440 nm (secondary amino acids).

4-Dimethyl-aminoazobenzene-4′-sulfonyl chloride (DBS- or Dabsyl-Cl): This reagent forms stable (weak) derivatives with primary and secondary amino acids which are detectable in the visible range of 448–468 nm. The high wavelength of absorption makes the baseline chromatogram very stable with a large variety of solvents and gradient systems. Detection limits are in the low picomole range (Stocchi et al., 1989). The reaction time is around 15 min at 70°C and takes place in a basic medium with an excess of the reagent. The major disadvantage is that the reaction efficiency is highly matrix dependent and variable for different amino acids, being especially affected by the presence of high levels of some chloride salts (Jansen et al., 1991). To overcome this problem and obtain an accurate calibration, standard amino acids solution should be derivatized under similar conditions. By-products originating from an excess of reagent absorb at the same wavelength and appear in the chromatogram. Nevertheless, Stocchi et al. (1989) obtained a good separation of 35 DBS-amino acids and by-products in a 15 cm C18 column packed with 3 μm particle size.

Phenylisothiocyanate (PITC): The methodology involves the conversion of primary and secondary amino acids into their phenylthiocarbamyl (PTC-) derivatives which are detectable at UV (254 nm). The PTC-amino acids are moderately stable at room temperature for 1 day and much longer in the freezer especially under dry conditions. The methodology is well described in the literature (Cohen and Strydom,

1988, Heinrikson and Meredith, 1984, Bidlingmeyer et al., 1984). Sample preparation is quite laborious; and requires a basic medium (pH = 10.5) with triethylamine and includes several drying steps, being the last one necessary to eliminate the excess of reagent which may cause some damage to the chromatographic column. Twenty minutes of reaction time at room temperature is recommended for a complete reaction. The chromatographic separation takes around 20 min for hydrolyzed amino acids and 50 min for physiological amino acids (free). Figure 1.2 shows a reversed-phase high-performance liquid chromatogram (RP-HPLC) of PITC-free amino acids from salmon muscle extract.

The reproducibility of the method is very good, ranging from 2.6% to 5.5% for all amino acids except for histidine (6.3%) and cystine (10%). PTC-cystine shows a poor linearity that makes the quantitation of free cystine nonfeasible with this method (Fürst et al., 1989). Detection limits are in the high picomole range. The selection of the column is critical to get a good resolved separation especially when the analysis of physiological amino acids is involved.

The reliability of the method has been tested on food samples (Bidlingmeyer et al., 1987) and compared with the traditional ion-exchange chromatography and postcolumn derivatization (Bidlingmeyer et al., 1984; White et al., 1986; Davey and Ersser, 1990).

1-Dimethylamino-naphthalene-5-sulfonyl chloride (Dansyl-Cl): Dansyl-Cl reacts with both primary and secondary amines to give a highly fluorescent derivative (λ_{ex} 350 nm; λ_{em} 510 nm). The dansylated amino acids are stable until 7 days at –4°C (Martín et al., 1980), if protected from light. The sample derivatization appears as simple, only needs a basic pH, around 9.5, and a reaction time of 1 h at room temperature (in the dark), or 15 min at 60°C or even 2 min at 100°C. However, the reaction conditions (pH, temperature, and excess of reagent) must be carefully fixed to optimize the product yield and to minimize the secondary reactions (Martín et al., 1980; Prieto et al., 1990). Even so, this will commonly form multiple derivatives with histidine, lysine, and tyrosine. Histidine gives a very poor fluorescence response (10% of the other amino acids), reinforcing the poor reproducibility of its results (Fürst et al., 1989). Another problem is that the excess of reagent (needed to assure a quantitative reaction) is hydrolyzed to dansyl sulfonic acid, which is highly fluorescent and probably interferes into the chromatogram as a huge peak. On the contrary, this methodology reveals excellent linearity for cystine and also cystine-containing short-chain peptides (Mackey and Beck, 1982; Stehle et al., 1988; Fürst et al.,

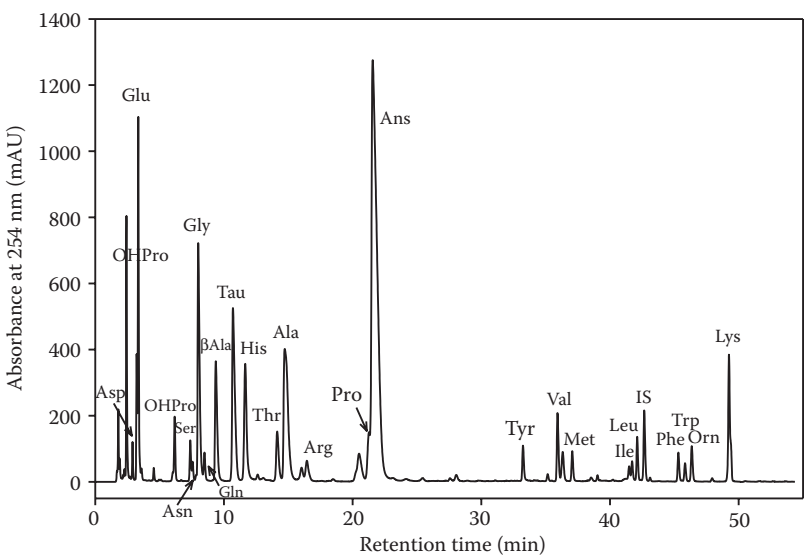


FIGURE 1.2 RP-HPLC chromatogram of PITC-free amino acids from salmon muscle extract. Tau, taurine; Ans, anserine; IS, internal standard nor-Leucine. (Adapted from Aristoy, M.C. and Toldrá, F. 2010. In: *Handbook of Dairy Foods Analysis,* pp 9–32. Boca Raton, FL: CRC Press.)

1989) like the case of GSH. This derivatization reagent has also been used to analyze the taurine (Hischenhuber, 1988).

o-Phthaldialdehyde (OPA): This reagent reacts with primary amino acids in the presence of a mercaptan cofactor to give a highly fluorescent adduct. The fluorescence is recorded at 455 or 470 nm after excitation at 230 or 330 nm, respectively and the reagent itself is not fluorescent. OPA derivatives can be detected by UV absorption (338 nm) as well. The choice of mercaptan (2-mercaptoethanol, ethanethiol or 3-mercaptopropionic acid) can affect derivative stability, chromatographic selectivity, and fluorescent intensity (Bradbury and Smith, 1973; Lookhart and Jones, 1985; Euerby, 1988; Godel et al., 1992; Rutherfurd et al., 2007). The derivatization is fast (1–3 min) and is performed at room temperature in alkaline buffer (pH 9.5). These reaction conditions permit that OPA could be used either for precolumn derivatization, or postcolumn derivatization coupled with cation exchange HPLC (Ashworth, 1987a,b). OPA amino acids are not stable; this problem is overcome by standardizing the time between sample derivatization and column injection by automation. The major disadvantage when applying to essential amino acids is that the yield with lysine and cysteine is low and variable. The addition of detergents like Brij 35 to the derivatization reagent seems to increase the fluorescence response of lysine (Gardner and Miller, 1980; Jones and Gilligan, 1983; Jarrett et al., 1986). Routine quantification of cystine is impossible with OPA due to the formation of a derivative with minimal fluorescence and several methods have been proposed before derivatization. These methods include the conversion of cysteine and cystine into cysteic acid by oxidation with performic acid (see Section 2.1.2.1), or dimethyl sulfoxide (Me_2SO) (Spencer and Wold, 1969; Williams et al., 1979), the carboxymethylation (Gurd, 1972) of the sulfhydryl residues with iodoacetic acid (Pripis-Nicolau et al., 2001) or the formation of the mixed disulfide *S*-2-carboxyethylthiocysteine from cysteine and cystine, using 3,3′-dithiodipropionic acid (Barkholt and Jensen, 1989) and incorporated by Godel et al. (1992) into the automatic sample preparation protocol described by Schuster (1988). In these methods, cysteine and cystine are quantified together. Other proposals (Park et al., 2000) consist of a slight modification in the OPA derivatization method by using 2-aminoethanol as a nucleophilic agent and altering the order in the addition of reagents in the automated derivatization procedure (Schuster, 1988).

6-Aminoquinolyl-N-hydroxysuccinimidyl carbamate (AQC): It reacts with primary and secondary amines from amino acids yielding very stable derivatives (1 week at room temperature) with fluorescent properties (λ_{ex} 250 nm; λ_{em} 395 nm). Ultraviolet detection (254 nm) may also be used. Sensitivity is in the femto mole range. The main advantage of this reagent is that the yield and reproducibility of the derivatization reaction is scarcely interfered by the presence of salts, detergents, lipids, and other compounds naturally occurring in many foods. Furthermore, the optimum pH for the reaction is in a broad range, from 8.2 to 10.0, which facilitates sample preparation. The excess of reagent is consumed during the reaction to form aminoquinoline (AMQ), which is only weakly fluorescent at the amino acid derivatives detection conditions and does not interfere in the chromatogram. Reaction time is short, 1 min, but 10 min at 55°C would be necessary if tyrosine mono-derivative is required, because both mono- and di-derivatives are the initial adducts from tyrosine. Fluorescence of tryptophan derivative is poor and UV detection at 254 nm may be used to analyze it. In this case, the AMQ peak appears very big at the beginning of the chromatogram, and may interfere with the first eluting peak (see Bosch et al., 2006). The chromatographic separation of these derivatives has been optimized for the amino acids from hydrolyzed proteins, but the resolution of physiological amino acids is still incomplete, and needs to be improved (Park et al., 2000) which is the main drawback of this method.

Cysteic acid and methionine sulfone which are the adducts after performic acid oxidation of cystine/cysteine and methionine, respectively, are well separated inside the chromatogram as shown in Figure 1.1.

Fluorescamine, which rends fluorescent derivatives with primary amino acids, has been used in precolumn derivatization of taurine. The column (RP-column) eluent was monitored at 480 nm (emission) after excitation at 400 nm (Sakai and Nagasawa, 1992).

1.2.2.2.1.2 Separation and Detection The HPLC separation techniques more used for the analysis of essential amino acids are cation exchange (CE-HPLC) and RP-HPLC. CE-HPLC is used for the separation of nonderivatized amino acids which are then derivatized postcolumn (ninhydrin or OPA), while RP-HPLC is mainly used to separate precolumn-derivatized amino acids (see the above-described

reagents). The choice of the reversed-phase column is essential to get a good separation because many peaks appear in the chromatogram, especially in the analysis of physiologic amino acids. In the case of hydrolyzates, the sample is simpler and the use of shorter columns is advisable to reduce the time of analysis. Reversed phase has also been used to separate some underivatized amino acids like methionine, which is further detected at 214 nm (Johns et al., 2004) or the aromatic amino acids tyrosine, phenylalanine, and tryptophan that can be detected at 214 nm but also at 260 or 280 nm. Indeed, Phe presents a maximum of absorption at 260 nm; Tyr at 274.6 and Trp at 280 nm. A chromatogram of Tyr, Phe, and Trp from a Manchego cheese extract is shown in Figure 1.3. The separation was achieved by using a gradient between 0.1% trifluoroacetic acid (TFA) in water and 0.08% of TFA in acetonitrile: water (60:40). Absorption spectra from these amino acids are also shown in the same figure.

For the rest of the amino acids, the detector used depends on the chosen derivative, but it is worthwhile to take into account the previous section about derivatization (Section 1.2.2.2.1.1), because certain derivatives from some specific amino acids have a poor response.

There are several different techniques for the analysis of any amino acid. An example of that is tryptophan which was analyzed by cation-exchange chromatography with postcolumn derivatization with OPA and fluorescence detection (Ravindran and Bryden, 2005) or by RP-HPLC without derivatization and UV or fluorescence detection (Delgado-Andrade et al., 2006) or even by RP-HPLC previous derivatization.

1.2.2.2.2 Gas–Liquid Chromatography

GLC technique is, in general, not recommended for some of the essential amino acids such as cysteine, tryptophan, or methionine. A special method of analysis for tryptophan in proteins based upon the GLC separation of skatole produced by pyrolysis of tryptophan at 850°C was developed by Danielson and Rogers (1978). Sample pretreatment for this method is limited to only sample lyophilization to form a dry solid, and hydrolysis is not required.

General GLC methods to analyze amino acids include their previous derivatization to enhance the volatility and thermal stability and thus improve their chromatographic behavior. The main drawback

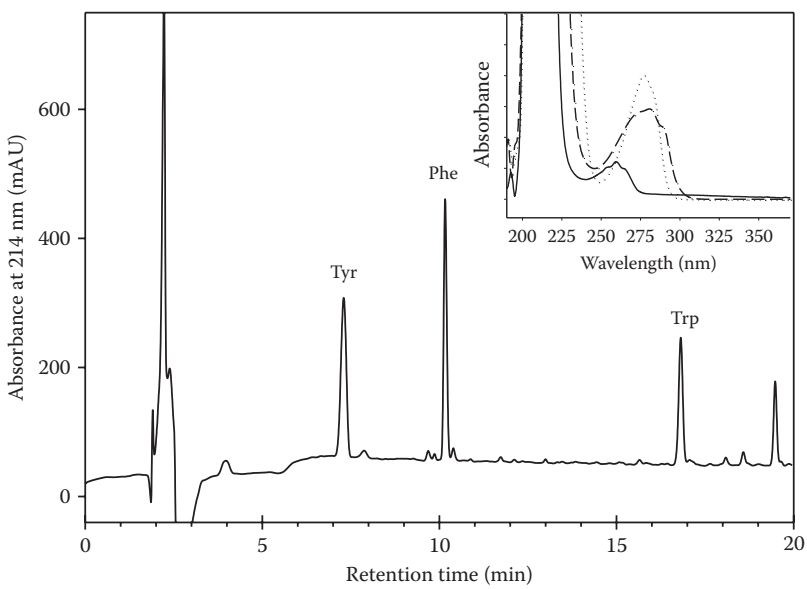


FIGURE 1.3 RP-HPLC chromatogram of underivatized Manchego-type cheese extract showing the aromatic amino acids and their corresponding UV spectra. Chromatographic conditions are described in the text (Section 1.2.2.2.1.2). Tyrosine (····), phenylalanine (——), and tryptophan (- - - -) espectra. (Adapted from Aristoy, M.C. and Toldrá, F. 2010. In: *Handbook of Dairy Foods Analysis*, pp. 9–32. Boca Raton, FL: CRC Press.)

is the different derivatives and derivatization conditions need to accomplish an only derivative for each essential amino acid (Molnar-Perl and Katona, 2000). Nevertheless, Oh et al. (1995) analyzed the protein and nonprotein amino acids from edible seeds, nuts and beans, previous carbonylation (with isobutyl chloroformate), solid phase extraction, and *t*-butyldimethylsilylation. The aforementioned amino acids, except cysteine, were included in this analysis. In this report, nonprotein amino acids were identified by mass spectrometry (MS) coupled to GLC. Some commercial proposals are the kits offered by Supelco (Sigma-Aldrich, Bellefonte, PA, USA), which uses *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide as derivatizing reagent and a short (20 m) capillary column (they give the conditions to separate 24 amino acids in 8 min) or the method EZ:faast which is a patent-pending method (Phenomenex, Torrance, CA, USA) to analyze the protein hydrolyzates and physiological amino acids from serum, urine, beer, wine, feeds, fermentation broths, and foodstuffs. This method includes a derivatization reaction (proprietary) in which both the amine and carboxyl groups of amino acids are derivatized. Derivatives are stable for up to 1 day at room temperature and for several days if refrigerated and are further analyzed by GC/flame ionization detector, GC/nitrogen phosphorous detector, GC/MS, and LC/MS. Results (50 amino acids and related compounds) are obtained in about 15 min (sample preparation included) when using the GC method or 24 min by using the LC method. An example of this application is that of Koutsidis et al. (2008) who analyzed free amino acids from beef. Only arginine was not included in this run and had to be determined by the other method.

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2

Glutamine and Taurine

Colin G. Prosser and Shane M. Rutherfurd

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2.1 Introduction

There is growing recognition that, apart from their role in synthesis of proteins and polypeptides, some amino acids regulate key metabolic pathways necessary for maintenance, growth, reproduction, and immunity. These include arginine, cysteine, glutamine, leucine, proline, taurine, and tryptophan, and are frequently termed functional amino acids. Animal and human clinical research suggests that oral supplementation of these functional amino acids may provide additional health and/or performance benefits under certain conditions or disease states. The following chapter describes some of the food sources and functions of dietary glutamine and taurine, two of the amino acids that may have significant biological function beyond protein synthesis. An overview of the analysis of these amino acids in foodstuffs is also provided.

2.2 Glutamine

Glutamine is considered to be a nonessential amino acid because it is made by the body. As a result, a dietary source is not absolutely required and there is no recommended daily intake of glutamine. Glutamine has multiple functions in the body: (1) acting as a carrier of excess nitrogen produced during

metabolism of amino acids; (2) as a respiratory substrate for energy production in rapidly dividing cells; (3) as a precursor for nucleotides; and (4) for protein synthesis (Young and Ajami, 2001; Duggan et al., 2002; Newsholme et al., 2003). In certain situations, such as stress, illness, or after intense exercise, there may be insufficient glutamine and having a higher dietary intake of glutamine can be beneficial (Lacey and Wilmore, 1990). There are no adverse effects for intakes of glutamine up to 14 g/day (Shao and Hathcock, 2008).

Glutamine in foods is predominantly bound within protein. As will be discussed later in this chapter, this protein-bound glutamine is difficult to quantitate and is generally determined along with its acid derivative (glutamic acid) after acid hydrolysis. As a result values for glutamine in foodstuffs are usually reported as the sum of glutamate and glutamine and there are relatively few data on the concentration of glutamine in foods. For instance, glutamine makes up 45% of the total glutamate and glutamine content of proteins in whole milk (Tsao and Otter, 1999; Baxter et al., 2004), but there are no similar data from other foods. Glutamine also exists in the free form in milk (Agostoni et al., 2000a). While it has been suggested that free glutamine might be more important than protein-bound glutamine, the efficiency of extraction of free or protein-bound glutamine appears to be similar, at least in adults (Boza et al., 2001).

There is some evidence that processing can affect glutamine. Airaudo et al. (1987) reported that as much as two-third of a pure glutamine solution was converted to pyroglutamic acid after boiling in water for 60 min. At extreme pH (<4 or >11), this conversion was even greater. This finding may have implications for processed foods or for foods stored for extended periods and more work on the effects of processing glutamine availability in foods is warranted.

The potential functions of dietary glutamine are discussed below.

2.2.1 Glutamine and Gut

Glutamine is the preferred form of energy for the rapidly dividing cells of the gut (Reeds et al., 2000; Duggan et al., 2002). More than half of the glutamine delivered orally is removed by the splanchnic bed and oxidized as a respiratory substrate for energy production (Haisch et al., 2000). Not surprisingly, glutamine delivered orally has little impact on plasma concentrations of glutamine (Wernerman, 2008), suggesting dietary glutamine may only have a limited role in other organs. The main functional benefit of glutamine in the gut appears to be maintaining gut mucosal integrity, preventing bacterial translocation and infections (Ziegler et al., 2003; De-Souza and Greene, 2005; Tian et al., 2009).

Glutamine might have a therapeutic effect in inflammatory bowel disease. Inflammatory bowel disease is associated with overproduction of inflammatory cytokines and impaired gut barrier function. Results of animal models are consistent with a role of glutamine as a candidate therapy for preventing inflammatory cytokine production and barrier dysfunction (Ziegler et al., 2003; Vicario et al., 2007). In addition, glutamine and arginine can decrease proinflammatory cytokines production by colonic biopsies of patients with Crohn's disease (Lecleire et al., 2008). Glutamine therapy has yet to be tested in patients with inflammatory bowel diseases.

2.2.2 Glutamine and Muscle

Skeletal muscle is the major site of synthesis of glutamine in the human body and glutamine is the most abundant free amino acid in human muscle (Newsholme et al., 2003). Prolonged exercise and fatigue are associated with the depletion of plasma concentrations of glutamine (Parry-Billings et al., 1992; Kingsbury et al., 1998). For this reason, glutamine has been used as a dietary supplement to assist with recovery from sports and exercise. However, the beneficial effect of oral glutamine supplementation on muscle repair after exercise-induced damage has yet to be unequivocally demonstrated (Gleeson et al., 1998; Gleeson, 2008).

2.2.3 Glutamine and Immune Function

Perhaps the most promising role for dietary glutamine lies with prevention of infections, particularly in seriously ill or cancer patients. Parental administration of glutamine reduces rates of mortality and

morbidity in critically ill adult patients and with fewer episodes of sepsis and no toxic effects (Wernerman, 2008; Kuhn et al., 2010). Glutamine supplementation also reduces mortality in preterm infants, although there was no effect on secondary infection (Tubman et al., 2008).

The benefit of glutamine in these situations has been attributed to its role in repair and growth of rapidly dividing enterocytes and the enhancement of the intestinal barrier function (De-Souza and Greene, 2005). The latter helps prevent bacterial translocation and subsequent sepsis. Glutamine may also be used as a source of respiratory fuel, precursors for synthesis of nucleotides, and for regulation of cytokine production and antioxidant activity by lymphocytes and macrophages (Newsholme et al., 2003), but further research on the role of dietary-derived glutamine on immune cell activity is needed to establish whether this forms a part of the protective role of glutamine.

2.3 Taurine

Taurine is a sulfur-containing amino acid and is the most abundant free nitrogenous compound in all cells. Most mammals are able to synthesize taurine endogenously, but some species such as humans are more dependent on dietary sources of taurine. Milk, meat, and shellfish are the main sources of taurine in food (Table 2.1). Because plants are generally very low in taurine, vegetarian diets may be deficient in taurine (Laidlaw et al., 1990). There are no recommended intake levels for taurine. Human clinical trial data have revealed no adverse effects for taurine at supplemental intakes up to 3 g/d for normal healthy adults (Shao and Hathcock, 2008).

Some functions of dietary taurine are discussed below.

2.3.1 Taurine and Infants

Many studies on effects of dietary taurine have been conducted in neonates, partly in recognition of the very high levels of taurine in breast milk (Agostoni et al., 2000b). As taurine is low in cow milk, infants fed unsupplemented cow formula have low plasma taurine (Gaull et al., 1977). In contrast to cow

TABLE 2.1

Taurine Concentration in Some Foods

	Taurine Concentrations	Reference
Milk		
Human	335 μmol/L	Mehaia and Al-Kanhal (1992); Sarwar et al. (1998); Agostoni et al. (2000b)
	301 μmol/L	
	331 μmol/L	
Cow	19 μmol/L	Mehaia and Al-Kanhal (1992); Prosser et al. (2008)
	40 μmol/L	
Goat	1150 μmol/L	Mehaia and Al-Kanhal (1992); Sarwar et al. (1998); Prosser et al. (2008)
	362 μmol/L	
	784 μmol/L	
Camel	13 μmol/L	Mehaia and Al-Kanhal (1992)
Sheep	341 μmol/L	Sarwar et al. (1998)
Egg	Not detected	Zhao et al. (1998)
Shellfish	300–800 mg/100 g edible portion	Zhao et al. (1998)
Meat	30–160 mg/100 g	Zhao et al. (1998)
Plants	Not detected	Zhao et al. (1998)

milk-based formulas, formulas made from goat milk naturally contain high concentrations of taurine and do not require a lot of supplementation (Prosser et al., 2008).

Despite the numerous studies, the function of milk (or formula) derived taurine in infants is still not fully understood. Taurine insufficiency in preterm infants is associated with impaired bile acid secretion and absorption of fat (Galeano et al., 1987), but not in term infants (Wasserhess et al., 1993). One study found a marginal increase in fat absorption in infants given a taurine-supplemented formula, although there was no effect on growth or energy balance (Galeano et al., 1987).

Taurine has been suggested to be involved in the neuronal development of neonates (Sturman and Chesney, 1995). Electroretinographic abnormalities have been shown to develop in children receiving long-term parental nutrition without taurine and these were resolved when taurine was subsequently added to infant diets (Geggel et al., 1985; Ament et al., 1986). Low plasma taurine in infants was also associated with lower Bayleys Mental Development index at 18 months (Wharton et al., 2004). However, a trial with preterm infants did not find any evidence of an effect of taurine supplementation on electroretinographic responses (Tyson et al., 1989) and in term infants, taurine has been associated with delayed auditory maturation (Dhillon et al., 1998).

A review of the clinical studies of taurine supplementation in preterm infants concluded that there is a lack of data of a benefit of taurine supplementation (Verner et al., 2007). Nevertheless, supplementation of infant formula with taurine to achieve plasma levels similar to infants fed human milk is seen as a prudent measure.

2.3.2 Taurine and Fat Absorption

Taurine conjugates of bile acids and duodenal bile acid concentrations are important for bile flow, fatty acid absorption, and protection against hepatotoxicity (Loruenco and Camilo, 2002). In adults, there are threefold more conjugates of taurine than glycine, whereas bile salts in newborn infants are exclusively conjugates of taurine. In humans, the proportion of bile acids conjugated to taurine is directly related to the concentration of taurine in the liver, which, in turn, is a function of its dietary intake (Hardison, 1978). Patients with short bowel syndrome have reduced capacity to absorb taurine-conjugated bile salts and may have increased need for dietary sources of taurine (Schneider et al., 2006). Taurine has been tried as a dietary therapy for patients with cystic fibrosis, who have reduced capacity to absorb fat, but results are mixed (Belli et al., 1987; Thompson, 1988; Smith et al., 1991; De Curtis et al., 1992; Merli et al., 1994).

2.3.3 Taurine and Diabetes

Nutritional interventions are a major part of diabetes management. Taurine supplementation reduces lipid peroxidation, plasma lipids, and retinal lens deterioration in animal models of diabetes (Franconi et al., 2006; Kim et al., 2007) suggesting it may be useful for management of diabetes. One human study showed that taurine supplementation produces a beneficial effect on lipid metabolism in overweight or obese subjects (Zhang et al., 2004a), whereas another did not (Brøns et al., 2004). Thus, further studies will be required to fully determine taurine's possible therapeutic potential for delaying onset or management of diabetes.

2.3.4 Taurine and Muscle

Taurine has been included in energy drinks in combination with performance-enhancing substances, such as creatine and other amino acids, in an attempt to improve exercise performance and recovery (Alford et al., 2001; Little et al., 2008; Ivy et al., 2009). Animal trials show that taurine supplementation raises muscle taurine concentrations, enhances exercise capacity, and helps alleviate muscle fatigue in strenuous workouts (Warskulat et al., 2004; Goodman et al., 2009). Similarly, taurine supplementation attenuated exercise-induced muscle injury in athletes (Dawson et al., 2002; Zhang et al., 2004b). In contrast, taurine supplementation in men for 7 days failed to alter muscle levels of taurine, despite raising plasma taurine, and had little impact on muscle metabolites after exercise (Galloway et al., 2008). The

levels of taurine in energy drinks have been suggested to be below the amounts expected to deliver either therapeutic benefits or adverse events (Clauson et al., 2008).

2.3.5 Taurine and Immune Function

The effects of taurine on the various functions of immune cells have been reviewed extensively (Redmond et al., 1998; Lourenco and Camilo, 2002; Grimble, 2006). In brief, taurine influences the viability, phagocytotic, and antimicrobial activity of immune cells as well as release of proinflammatory cytokines. Some animal studies have implicated a benefit of dietary taurine on immune status. For instance, supplementation of mice with taurine prevented the age-related decline in T-cell number and enhances the proliferative responses of T cells (Grimble, 2006). Unfortunately, as with many of the functions of taurine, there are no clinical data on the effect of dietary intervention with taurine on the prevention of infections in humans.

2.4 Analysis of Taurine and Free Glutamine

While glutamine is an abundant free amino acid in many foods and biological samples, taurine is often present in low concentrations, making interference from other compounds a problem for accurate analysis. The first step to overcome this is to remove as much contaminating material as possible. Usually this consists of a robust extraction and deproteinization. The deproteinated samples are then analyzed, often initially by separating the amino acids using some form of chromatography and then detecting and quantifying either the derivatized or underivatized amino acids.

2.5 Sample Preparation

Numerous methods of sample preparation have been published for the analysis of free amino acids, including taurine and glutamine. An excellent discussion of some of these methods as applied to taurine has been published (Mou et al., 2002). The choice of sample preparation method is often dictated by sample matrix and a summary of these is presented in Table 2.2.

Aristoy and Toldrá (1991) compared acid precipitation using PCA, TCA, phosphotungstic acid, sulfosalicylic acid, picric acid, solvent precipitation with acetonitrile, and ultrafiltration through either a 1 kDa or 10 kDa membrane to extract amino acids from fresh pork muscle and dry-cured ham. Excellent recoveries (99–102%) of glutamine were achieved using PCA, TCA, sulfosalicylic acid, picric acid, acetonitrile, and ultrafiltration. Acetonitrile and ultrafiltration gave the best recoveries of taurine, whereas acid extraction resulted in the presence of interfering compounds. Trichloroacetic acid and PCA were unreliable when applied to ileal digesta, which have high glycoprotein levels (Moughan et al., 1990). Higher taurine yields were obtained from physiological samples when sulfosalicylic acid was used in comparison to PCA and TCA (Hirschberger et al., 1985). Jiang et al. (2004) reported that TCA and acetone precipitation as well as ultrafiltration were the most effective means of separating protein from low-molecular weight compounds in human plasma. Irving and Klein (1980) found that simply boiling the sample for 3 min followed by ion-exchange cleanup was the optimum preparation method for GCMS analysis of taurine isotopes, whereas TCA, acetone, and PCA followed by ion-exchange chromatography all led to compounds that interfered with taurine analysis. Mou et al. (2002) concluded that ion-exchange chromatography using either cation exchange resins or a combination of cation and anion exchange resins would be useful for samples containing large amounts of amino acids other than taurine. For milk samples, defatting may also be necessary (Elmastas et al., 2008).

Acid hydrolysis has also been used to hydrolyze protein to amino acids as a means of removing protein (Fay et al., 1998). Since taurine is acid stable, acid hydrolysis is an effective means of removing protein. However, acid hydrolysis is a tedious process and since taurine may be present in low levels compared to the protein-derived amino acids present, this method may have limited application.

Nuclear magnetic resonance (NMR) has recently been applied to the quantitative determination of taurine in saliva (Takeda et al., 2008) and serum (Wang et al., 2009). The advantage of this technique

TABLE 2.2

Sample Preparation Regimes for Analyzing Taurine in Foodstuffs

Sample	Analysis Method[a]	Deproteinization Method	Reference
Energy drinks	FTIR	None	Triebel et al. (2007)
	Planar chromatography-MS	Dilution	Aranda and Morlock (2006)
	CE	Dilution	Zinellu et al. (2009)
Infant formula	RP-HPLC	Ethanol/centrifugation	Zunin and Evangelisti (1999)
	RP-HPLC	Sulfosalicylic acid	Ferreira (2003); Ferreira et al. (1997)
	Ion-exchange	Ion-exchange clean up	Prusisz and Pohl (2008)
Raw milk	RP-HPLC	Sulfosalicylic acid	Ferreira (2003); Ferreira et al. (1997)
	Ion-exchange	None	Elmastas et al. (2008)
	Ion-exchange	Ion-exchange clean up	Prusisz and Pohl (2008)
	RP-HPLC	Ultrafiltration	Tripaldi et al. (1998)
	Ion-exchange	$K_4Fe(CN)_6$, $ZnSO_4$, $MgSO_4$ then centrifugation	Cataldi et al. (2004)
	RP-HPLC	TCA	Saidi and Warthesen (1990)
	CE	TCA	Zinellu et al. (2009)
Shellfish	RP-HPLC	PCA	Barbarro and Reiriz (2006)
	RP-HPLC	TCA	Lee et al. (2004)
	LCMS	70% ethanol	Chaimbault et al. (2004)
Pork/ham	RP-HPLC	Acid precipitation	Aristoy and Toldrá (1991)

[a] CE, capillary electrophoresis; FTIR, Fourier transform infrared spectroscopy; GCMS, gas chromatography-mass spectrometry; Ion-exchange, ion-exchange-HPLC; LCMS, liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance; RP-HPLC, reverse-phase HPLC.

is that minimal sample preparation is required. NMR has yet to be applied to analysis of amino acids in food.

The above discussion highlights the impact of sample extraction on the subsequent analysis and reporting of levels of taurine in foodstuffs and biological samples. With exception of those workers using NMR or those analyzing energy-promoting drinks, most workers make some attempt to remove protein from the sample matrix. Unfortunately, there is no standard approach for protein removal of all sample matrices and details of the extraction method are not always stated (Altamura et al., 1995) making interpretation of the data more difficult. As only a few studies have made any comparison of protein removal methods for foods, a study comparing an extensive range of methods applied to a variety of food types may be warranted.

2.6 Quantification of Taurine and Glutamine

Amino acids have been analyzed using a range of instrumentation. The most common quantitation method is chromatography, either ion-exchange chromatography or reverse-phase HPLC. These methods require derivatization of the amino acids with a chromophore since, with the exception of histidine and the aromatic amino acids, underivatized amino acids cannot be detected using absorbance. Recently, however, other methods such as capillary electrophoresis (CE), mass spectrometry (MS), and NMR have been attempted (Table 2.3). These methods are discussed below.

TABLE 2.3

Summary of the Methods of Analysis of Taurine and Free Glutamine

	Speed	Sample Preparation	Derivatization
Ion-exchange	>30 min	Extraction/deproteinization	OPA/ninhydrin
RP-HPLC	<20 min	Extraction/deproteinization	OPA, PITC, Dabsyl-Cl, fluorescamine
LCMS	<10 min	Extraction	None required
GCMS	<20 min	Extraction/deproteinization	Dimethylformamide dimethylacetal
CE	<20 min	Extraction/deproteinization	OPA, FITC, 3-(4-bromobenzoyl)-2-quinolinecarboxaldehyde
NMR	<15 min	None	None required

2.6.1 Chromatography

Anion-exchange chromatography was the original method used for separating and quantifying amino acids (Moore and Stein, 1951). This method takes advantage of the amino acids zwitter ion properties (its ability to be either positively or negatively charged depending on the pH). It is particularly suited to analysis of taurine as it elutes early in the run and is well separated from other amino acids. This is important since taurine is often present in levels well below that of other amino acids and can be difficult to quantify if baseline resolution is not achieved. For anion-exchange chromatography, samples are loaded at low pH and amino acids are selectively eluted using a pH gradient. The eluted amino acids are then derivatized with a chromophore or fluorophore in-line. Ninhydrin (Monyano et al., 1998; Elmastas et al., 2008; Bragg and Freeman, 2009) and *o*-phthaldialdehyde (OPA) (Waterfield, 1994) are most commonly used. While run times for the ion-exchange chromatography are generally longer than for other methods, it is the most robust and arguably the most common method in use.

Reverse-phase HPLC is now a popular method for quantifying amino acids due to more rapid run times. This method separates molecules based on their hydrophobicity. As most amino acids, including taurine and glutamine, are hydrophilic, they are derivatized with a hydrophobic chromophore or fluorophore prior to being loaded onto the column. There are a range of reagents available for precolumn derivatization, including OPA (Waterfield 1994; Ferreira et al., 1997; Mühling et al., 1999; Zunin and Evangelisti, 1999; Piepponen and Skujins, 2001; Tcherkas et al., 2001; Ferreira, 2003; Hillenkamp et al., 2004; Lee et al., 2004; Ghandforoush-Sattari et al., 2009), phenylisothiocyanate (PITC) (Tripaldi et al., 1998; D'Eufemia et al., 2007), dabsyl chloride (Babarro and Reiriz, 2006), and fluorescamine (Saidi and Warthesen, 1990). A thorough discussion of derivatizing agents for amino acid analysis has been presented elsewhere (Rutherfurd and Sarwar-Gilani, 2009). Since OPA is by far the most commonly used derivatizing agent for taurine analysis, a brief overview for OPA will be presented here.

OPA was first used by Roth and Hampai (1973) as a postcolumn derivatizing reagent for ion-exchange chromatography. OPA reacts specifically with primary amino acids in the presence of thiol to produce a substituted isoindole ring. OPA fluoresces (λ excitation 350 nm, λ emission 450 nm) when bound to amino acids but not in its free form. The reaction of OPA with amino acids takes only a few minutes, even at room temperature, and can be automated using most modern autosamplers. Recently, and with the advent of ultra-high pressure HPLC's and 1.8 μm pore size columns, ultra-fast runtimes, as low as several minutes for an amino acid analysis profile, have been reported. If the reliability of such rapid methods can be assured, then ultra-fast HPLC using automated precolumn derivatization is likely to render ion-exchange chromatography methods for amino acid analysis obsolete.

2.6.2 Capillary Electrophoresis

Very recently, CE has been applied to the determination of taurine in plasma (Zhang et al., 2009; Zinellu et al., 2009) and beverages (Zinellu et al., 2009). During CE, the sample is loaded into one end of a capillary column, a voltage is then applied across the capillary and the charged amino acids migrate through the capillary. Since taurine is a sulfonic rather than a carboxylic amino acid, it elutes much earlier and is

well separated from the carboxyl amino acids. The detection method of choice for taurine appears to be laser-induced fluorescence and several derivatizing reagent have been used, including OPA (Cellar et al., 2005), 3-(4-bromobenzoyl)-2-quinolinecarboxaldehyde (Zhang et al., 2009), and fluorescein isothiocyanate (FITC) (Zinellu et al., 2009). The main advantage of CE compared to HPLC is that the resolving power of CE is several orders of magnitude greater. Unfortunately, few studies have used CE for analysis of taurine in foods.

2.6.3 Mass Spectrometry

MS is arguably the most commonly used analytical tool for the quantitative analysis of small compounds in the metabolomics field. MS separates compounds based on their mass to charge ratio and has been used to examine the metabolite profile of complex mixtures of foods and drinks (de Person et al., 2005; Marchei et al., 2005; Aranda and Morlock, 2006; Chen et al., 2007). In addition, modern instruments are able to use tandem MS, where the first MS process selects compounds with the mass to charge ratio of the compound of interest, thereby effectively filtering out interfering compounds. While samples can be introduced into the mass spectrometer directly using electrospray ionization, HPLC's and GC's can also make up the front end of the mass spectrometer (LCMS of GCMS) to provide an additional degree of separation prior to sample introduction into the mass spectrometer. This two-dimensional separation of samples makes LCMS or GCMS an extremely powerful analytical tool for complex mixtures.

GCMS requires volatilization of the test compound for detection. Derivatization with dimethylformamide dimethylacetal to produce the volatile *N*,*N*-dimethylaminomethylene methyl esters adduct has been employed for taurine analysis (Irving and Klein, 1980). In contrast, LCMS does not require derivatization of taurine or glutamine. Hydrophilic interaction-LCMS has been used to determine taurine in carbohydrate-rich matrices such as energy-promoting drinks (de Person et al., 2005). The method uses a rapid (15 min) isocratic run to separate underivatized taurine from other interfering compounds before being introduced into the mass spectrometer. These workers reported excellent accuracy (98.3–100.8% recovery of taurine) and precision (1.9–3.3% CV) for this method. Chaimbault et al. (2004) used a rapid (10 min) LC-MS-MS analysis to determine taurine and taurine analogs in marine invertebrates, also with no derivatization. Negative ion mode gave superior results to positive ion mode, which may necessitate the development of alkaline reverse-phase HPLC buffers in order to maintain taurine as a negative ion. Marchei et al. (2005) also used reverse-phase LCMS to determine taurine in dietary supplements and found taurine recovery to be around 80%.

Overall, MS techniques offer considerable promise as a simple and rapid means to determine taurine in complex mixtures such as foods. However, the cost of equipment and specialized training required to run these instruments have limited their application to date.

2.6.4 Nuclear Magnetic Resonance

NMR-based technologies have recently been employed to determine taurine and glutamine in a range of biological samples including urine (Ekman et al., 2007), serum (Wang et al., 2009), saliva (Takeda et al., 2008), and tissue culture cell lines (Lee et al., 2008). As yet, foods have not been analyzed using this method. As previously mentioned, a key advantage of NMR is the minimal sample preparation required. However, NMR instruments are still expensive and require highly trained staff making them outside the reach of many laboratories. Given the ease of sample preparation and short run times, NMR as a routine analytical tool for taurine and glutamine may increase in popularity in the future.

2.7 Analysis of Protein-Bound Glutamine

Unlike taurine, glutamine is found as a free amino acid and also bound within protein. While analysis of free glutamine is relatively straightforward, protein-bound glutamine is considerably more difficult. Samples normally undergo acid hydrolysis to break the peptide bonds that hold the protein together to release the amino acids. During this step, the amide side chain of glutamine is converted into a

carboxylic acid producing glutamic acid. In contrast, if base hydrolysis of the protein is carried out, glutamic acid is converted into glutamine. Several strategies have been put forward to circumvent this issue.

Soby and Johnson (1981) used the reaction with bis(1,1-trifluoroacetoxy)iodobenzene (BTI) to convert protein-bound glutamine into the acid stable 2-4-diaminobutyric acid (DABA) prior to acid hydrolysis. However, due to poor chromatographic separation, these workers were forced to quantify glutamine as the difference in the corresponding acid derivative present in acid hydrolysates without and then with prior BTI treatment. Kuhn et al. (1996) also used BTI derivatization to determine DABA (and therefore glutamine) directly. Recoveries of DABA from a range of BTI-derivatized proteins based on glutamine content determined by sequence analysis were reported to be between 87% and 96%. As BTI reacts specifically with protein-bound glutamine and does not react with free glutamine or when glutamine is present in proteins or peptides in the N-terminal position, it is unlikely to accurately quantify total glutamine levels in foods. Another method for glutamine analysis is to convert the carboxyl groups of glutamic acid residues to methyl esters, then reduce these to stable alcohols prior to acid hydrolysis (Wilcox, 1967). During acid hydrolysis, the glutamine residues are converted into glutamic acid residues which are then determined.

Enzymatic methods have also been used for analysis of glutamine. Tower (1967) used L-glutaminase to quantitatively deaminate glutamine to glutamic acid and then determined the amount of released ammonia. Hediger et al. (1973) used digestion with pronase (1% w/w, 37°C, 24 h) followed by aminopeptidase M (4% w/w, 37°C, 17 h) to enzymatically release the glutamine from protein. Tsao and Otter (1999) used a mixture of pronase (3.5% w/w), aminopeptidase M (1.4% w/w), and prolidase (0.7% w/w) at 37°C for 20 h. The latter workers reported 87–96% recovery of glutamine from milk proteins, but <50% recovery of proline or acidic amino acids residues. This method may warrant further investigation since the range of commercially available enzymes is likely to have increased from those that were available when this study was conducted over a decade ago.

2.8 Conclusion

Although there is much knowledge of the biological functions of glutamine and taurine within the body, there is much less concerning the functions of dietary sources of glutamine and taurine. Many of the benefits described in the literature have been extrapolated from results from *in vitro* or animal studies. Dietary or sports supplements are currently being marketed with added glutamine and taurine, but much work on their role in modulating muscle function during and after exercise are needed to confirm the claims. Happily there are few reports of adverse effects of these two amino acids, even at relatively high intakes, suggesting these drinks are doing no harm at least.

In the future, the most exciting applications for dietary supplements of taurine and glutamine will be nutritional therapies for prevention or recovery from illness. Animal studies of specific disease functions are showing promising results for both glutamine and taurine supplementation as nutritional therapies for diabetes and inflammatory bowel diseases and there is some clinical evidence of a role of glutamine in preventing infection in critically ill or cancer patients.

One issue to be addressed in this research is the determination of the total dietary intake of glutamine. The analysis of glutamine in foods is complicated by the association of the glutamine in proteins, requiring techniques to release the protein-bound amino acids. As the most common processes for these results in conversion of glutamine into glutamate, most reports simply list glutamine and glutamate concentrations combined. Other methods that have been attempted to address this issue are very cumbersome and subject to variable or low recoveries and are seldom used.

Taurine analysis is simpler as it is not associated with proteins and analysis usually consists of an extraction or clean-up step, often involving protein removal, followed by analysis. Different protein removal methods have been used and although sample type has a significant effect on the need for protein removal and the efficacy of the protein removal method, an extrapolation across the studies suggests that sulfosalicylic acid precipitation or possible ultrafiltration is the most effective protein removal method.

HPLC-based methods are still the most common techniques for analysis of amino acids, probably due to the relative cost of the equipment and their ease of use. Other methods, such as LCMS and NMR, are becoming increasingly common and offer considerable promise due to the ease of sample preparation and the fact that a large number of different compounds can be analyzed quickly in a single run.

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3

Bioactive Peptides

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3.1 Introduction

Proteins and peptides from food are essential components of the diet as sources of amino acids. It has been also recognized that dietary proteins and peptides exert many other functionalities *in vivo*, directly or after hydrolysis. Several biologically active peptides have been identified after hydrolysis of food proteins by digestive enzymes during gastrointestinal transit or by fermentation or ripening during food processing. These bioactive peptides have been defined as specific amino acid sequences that may exhibit regulatory functions in the organism beyond normal and adequate nutrition. Once ingested, they may exert different activities in the major body systems: cardiovascular, digestive, endocrine, immune, and nervous system. Although any food protein from plant or animal origin may act as a precursor of these bioactive peptides, to date, most of the bioactive peptides described and commercially used derive from milk proteins. Research in the field of bioactive peptides has intensified in the past two decades (for recent reviews, see for instance [1–3]).

The potential of bioactive peptides to improve consumer's health is a subject of great interest to the scientific community and the food industry. Different chemical and biological methods have been developed and applied to screen their bioactivity, and in some cases, the postulated effects have also been proved in animal and human studies. In addition, the identification and characterization of these peptides is performed using different analytical techniques namely, electrophoresis, chromatography, immunochemical methods, and mass spectrometry in its different modes. A detailed description of these analytical techniques and their application to dairy peptides has been recently reviewed [4]. Research performed to date has found certain structure–activity relationships for bioactive peptides, that is, peptides with a given activity share specific structure cluster or have sequence requirements.

This chapter deals with the methods applied to test the bioactivity of food-derived peptides. The common structural features of peptides exhibiting a particular activity will also be summarized. Special

attention will be focused on the *in vitro* methods used to evaluate bioactivity and the *in vivo* animal models employed.

3.2 Evaluation of Peptide Bioactivity

Evaluation of peptide bioactivity is one of the most important challenges in the search for novel bioactive sequences and usually it cannot be accomplished by the use of a single method. To perform an initial screening of numerous sequences or fractions, rapid and simple *in vitro* methods are required. Once they are liberated, they can act locally, at any point of the gastrointestinal tract, or they have to be absorbed to reach the target organ. In this latter case, bioavailability of the peptides and changes that can occur during digestion, absorption, or distribution have to be evaluated using cell cultures, *ex vivo* organ preparations, or *in vivo* animal models. Finally, there is consent over the fact that health claims must be proved in human studies.

3.2.1 Antihypertensive Peptides

Antihypertensive peptides are probably the most extensively studied bioactive peptides from foods and, in fact, an important progress has been made in the area of blood pressure reduction by the use of food peptides. This can be, at least partially, explained because hypertension is a significant problem worldwide and one of the major controllable risk factors associated with cardiovascular diseases [5]. Inhibition of angiotensin-converting enzyme (ACE) is regarded as the main mechanism of action of the antihypertensive activity of food peptides. ACE plays an important role in the renin–angiotensin system, regulating arterial blood pressure, as well as the salt and water balance. ACE hydrolyzes angiotensin I into angiotensin II, a potent vasoconstrictor, whereas it degrades bradykinin, a potent vasodilator [6]. The measurement of the reduction in the formation of angiotensin II *in vitro* is a common test for evaluating ACE inhibition exerted by different drugs and food peptides. The IC_{50} value (the inhibitor concentration that leads to 50% inhibition) is used to compare the effectiveness of different ACE inhibitory peptides. Once the IC_{50} value has been established, it represents an approximation to the possible antihypertensive effect of these compounds [7].

Several methods can measure the ability of food peptides to inhibit ACE *in vitro.* One of the most commonly used ones was developed by Cushman and Cheung (1971) [8], and later modified by Nakamura et al. [9]. This method employs the compound hyppuryl–histidil–leucine (HHL) as the substrate of ACE, which releases the dipeptide His–Leu and hyppuric acid. The amount of hyppuric acid is quantified spectrophotometrically at 228 nm once it has been extracted with ethyl acetate. However, it is a tedious process that can overestimate ACE activity if nonhydrolyzed HHL is also extracted, although this can be avoided by HHL separation and quantitative determination by high-performance liquid chromatography (HPLC) that also has the problem to be a time-consuming method to evaluate ACE-inhibitory activity [10].

Other spectrophotometric methods have been optimized and validated, such as that based on the release of the dipeptide Gly-Gly (GG) when the enzyme reacts with the substrate furanacryloyl-Phe-Gly-Gly (FAPGG) [11]. This method was later evaluated and it was demonstrated that the level of ACE activity in the assay determines apparent IC_{50} value obtained. Therefore, for comparative purposes, it is necessary to detail the number of enzyme units used and include in the study a standard ACE inhibitor, such as Captopril [12]. Later, a fluorescence assay which employs *o*-aminobenzoylglycyl-*p*-nitrophenylalanylproline as the substrate of ACE was developed [13]. The fluorescence generated by the release of the *o*-aminobenzoylglycyl group is read on a microplate fluorimeter, resulting in a simple, rapid, and sensitive method that allows a continuous monitoring of ACE activity in just one step.

A biochemical assay based on a continuous-flow enzyme–substrate reaction and the subsequent detection of the reaction products by mass spectrometry has been developed and applied to the screening of complex extracts of peptides with ACE-inhibitory activity. The inherent advantage of this method is that the fractionation of peptides for further activity testing is avoided, although an online HPLC-mass spectrometer is needed [14]. Recently, a new turbulent flow cytometry (TFC)/electrospray ionization mass spectrometry (ESI-MS)-based screening method has been developed. In contrast to the methods

previously described, this approach uses the natural ACE substrate angiotensin I, instead of artificial substrates. The ACE assay is carried out in an atypical offline setup by incubation of the samples with ACE and angiotensin I followed by stopping the reaction with acetonitrile containing val5-angiotensin I, which serves as an internal standard. Angiotensin I and the product angiotensin II are extracted from the incubation mixture by TFC applied in backflush mode as online solid-phase extraction and are directly quantified by ESI-MS. The presence of inhibitors is detected by an increase in the angiotensin I signal intensity and a corresponding decrease of angiotensin II signal, as compared to the blank assay. This parallel (two-way) measurement of the inhibitory activity allows the exclusion of false-positive results caused by interfering matrix compounds. No laborious cleanup is required and the incubation mixture can be analyzed directly after stopping the ACE conversion. The whole TFC/ESI-MS analysis takes <5 min making the method also suitable for a rapid screening of a large set of samples [15].

The structure–activity relationship of ACE-inhibitory peptides has been extensively studied and it is accepted that binding to ACE is strongly influenced by the C-terminal sequence of the peptide. ACE-inhibitory peptides seem to share, as a common structural property, the presence of hydrophobic amino acid residues (with aromatic or branched side chains) at each of the three C-terminal positions. Some of the most potent ACE-inhibitory peptides reported have a proline residue at the end of their sequence. In addition, the presence of a residue of proline at the C-terminal and antepenultimate position appears to enhance binding while ACE only binds weakly to peptides that have penultimate proline residues [16,17]. Later on, it has also been confirmed that the presence of another hydrophobic amino acid, such as leucine as C-terminal end helps inhibit ACE [18]. C-terminal lysine or arginine also seems to contribute to the inhibitory activity of the peptides, which suggests a possible interaction between a positive charge of the inhibitor and an anionic-binding site of ACE [19]. For instance, when the C-terminal proline residue of peptide LHLPLP was substituted by arginine (LHLPLR), the resultant peptide was twice more potent than the parent peptide. However, substitution of the C-terminal proline by leucine yielded a similar activity [20]. It is important to note that ACE-inhibitory activity also depends on the conformation of the peptides. It has been shown that the peptide DKIHP containing *trans*-proline exerts a more potent ACE-inhibitory activity than the conformer with *cis*-proline. It was suggested that the change of a *trans*-form into a *cis*-form of proline could cause significant changes in the peptide structure and in its interaction with the active site of the enzyme [21].

Very often, the attempts to corroborate the antihypertensive effect of peptides with ACE-inhibitory properties reveal a lack of correlation between the *in vitro* ACE-inhibitory activity and the *in vivo* action [22]. This is probably because the selection of potentially antihypertensive food peptides is solely based on the *in vitro* determination of the ACE-inhibitory activity, while important aspects such as the physiological transformations that determine the bioavailability of the peptides or the existence of other mechanisms of action are overlooked [23]. In fact, antihypertensive food peptides with mechanisms of action other than ACE inhibition, such as those possessing antioxidant, vasodilator, and opioid activities, have been reported [24–28].

Some peptides derived from food proteins exert direct effects on vascular smooth muscles [25] or modulate the release of endothelium factors that relax or contract vascular smooth muscles [26,28–32]. To study the vasodilator activity produced by different peptide sequences, organ baths are used. Isolated rings from conduit arteries or resistance vessels from normotensive or hypertensive rats are mounted in isolated tissue chambers containing a physiologic solution at 37°C and continuously bubbled with a 95% O_2–5% CO_2 mixture, which gives a pH of 7.4. After an equilibration period, the functionality and the maximum contractility of the rings are tested by adding KCl. The functional integrity of the endothelium can be confirmed by the relaxation response to acetylcholine of segments previously contracted with a vasoconstrictor agent. The absence of relaxation induced by acetylcholine confirms the absence of endothelium. Afterward, the relaxation produced by a single (not >0.1 mM) or several concentrations (0.1 μM–0.1 mM) of the peptide can be evaluated in precontracted segments. The effects of different inhibitors such as a nitric oxide (NO) synthase inhibitor (L-NAME), or a cyclooxygenase inhibitor (indomethacin), on the relaxation induced by the peptides can be studied by their addition 30 min before precontraction of the vessels.

There are very few studies dealing with the effects of food peptides on the vascular function, so that the structure–activity relationship of vasodilator peptides is not as yet well established. In contrast

to the ACE-inhibitory activity, it seems that the N-terminal residue is more important than the C-terminal residue to the vascular-relaxing activity [33]. It has been suggested that amino acids, such as arginine or tyrosine, are important at the N-terminal position [28,34]. In addition, small variations in the amino acids at certain positions could lead to different vasorelaxing mechanisms. This is the case of the hypotensive peptides ovokinin (2–7) (RADHPF) and novokinin (RPLKPW). NO and B2 bradykinin receptors are implicated in the mechanism of action of ovokinin [29,31], while the vasorelaxing activity of novokinin, a potent hypotensive peptide acting through the AT_2 receptor, is mediated by prostacyclin [35].

In any case, to evaluate the antihypertensive activity of these compounds, experiments on animals and humans are necessary and of particular importance. Several studies have been performed using animal models to determine the antihypertensive effects of food peptides after short- and long-term treatments [22,36–42]. Spontaneously hypertensive rats (SHRs), developed from Wistar rats bred at Kyoto University [43], constitute the most widely used experimental model for hypertension, because the basic principles associated with the onset of hypertension in these animals are surprisingly similar to those in humans [44,45]. Wistar–Kyoto rats are the normotensive control of SHRs. The rats' arterial blood pressure is usually measured by a modification of the tail cuff method, a noninvasive method originally described by Buñag [46]. In most studies, the acute effect caused by the oral administration by gavage of a single dose of the peptide is evaluated and the rats' arterial blood pressure is measured before this administration, and then at 2 h intervals for 8 h. Arterial blood pressure is again measured 24 h after administration. For these acute administration experiments, rats with stable arterial blood pressure values (and thus older than 20 weeks) should be used. As an example, Figure 3.1 shows the antihypertensive effect of egg-white-derived peptides after a single oral administration to SHRs (Figure 3.1) [39]. The antihypertensive activity can also be evaluated using radiotelemetric monitoring in conscious rats [47,48]. The telemetric transmitters are implanted into the abdominal aorta by a feasible surgical procedure. This method has the advantage that it avoids the animal stress produced when several measurements are performed in a small period of time.

Hypertension is a chronic pathology which requires chronic treatments; therefore, it is advisable to follow the changes in the arterial blood pressure of SHRs caused by a long-term oral intake of food peptides. In these experiments, the development of hypertension in the rats and the role played by the peptides administered is assessed. In experimental studies of chronic administration to SHRs, arterial

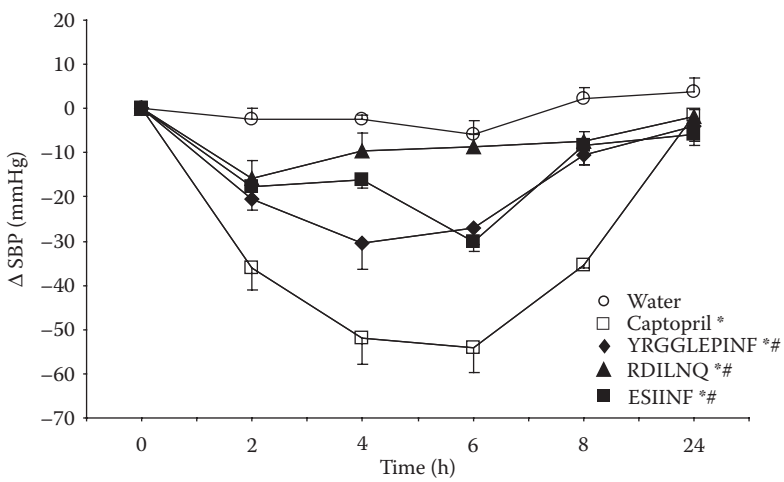

FIGURE 3.1 Decrease in systolic blood pressure (SBP) caused in SHRs by the administration of water (○), captopril (50 mg/kg) (□), and the peptides (10 mg/kg): YRGGLEPINF (♦), RDILNQ (▲), and ESIINF (■). The data represent the mean values ± SEM for a minimum of five animals. *$P < 0.05$ versus water; #$P < 0.05$ versuss captopril; P was estimated by a two-way analysis of variance. (From M. Miguel et al. *J Agric Food Chem.* 55: 10615–10621, 2007. With permission.)

blood pressure is measured at the same time of day (usually in the morning), in order to avoid the influence of the circadian cycle. In these studies, the treatment begins at weaning and the products are usually administered in the animals' drinking water. Continuation of the measurements after withdrawal of the treatment allows the evaluation of the possible reversal of the effects. Food intake, water intake, and body weight gain in the animals can be recorded weekly during all experimental period. At the end of the study, blood and tissue samples of the rats can be obtained after an overnight fasting period, in order to perform biochemical studies to explore the mechanisms of action implicated in the antihypertensive effect of the product.

There are several differences in the bowel structure, function, and microflora between rodents and humans. It is therefore evident that it is necessary to carry out clinical studies in humans to demonstrate the efficiency of the antihypertensive peptides and to guarantee their security. However, only a few human clinical studies have been carried out [49–59]. Recent data do not seem to support a positive role of bioactive peptides in blood pressure regulation. However, beneficial effects in hypertensive subjects from specific populations cannot be discarded, and more research is needed to elucidate mechanisms other than the intervention in the renin–angiotensin system that could be involved [60].

The results of the above-mentioned studies suggest the possibility of using hydrolyzates and peptides from food proteins as health-enhancing ingredients in functional foods, nutraceuticals, and pharmaceutical preparations to reduce the risk of hypertension. Their usefulness may be particularly clear in prehypertensive subjects who do not need antihypertensive medication yet, and who control their blood pressure by dietary means. It is also likely that these products can be of use in hypertensive patients who do not respond well to pharmacological treatments. It should be noted that several of these peptides, which have already proved their safety and effectiveness in hypertensive patients, are currently marketed in functional foods [61].

3.2.2 Antioxidant Peptides

Oxidative stress caused by an abnormal production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in cells has been implicated in the etiology of age-associated chronic diseases, such as cardiovascular diseases, cancer, diabetes, neurodegenerative disorders, and aging [62]. Moreover, it is well known that lipid peroxidation occurring in food products causes deteriorations of food quality and shortening of shelf life. In the past years, the importance of antioxidants in the protection of organisms, tissues, and nonliving systems against oxidative stress has become evident. Artificial antioxidants (butylated hydroxyanisole, butylated hydroxytoluene, and *n*-propyl gallate) exhibit a strong antioxidant activity against several oxidation systems. However, there is growing concern about their safety, particularly their potential carcinogenicity and genotoxicity [63,64]. This, along with consumer preference for natural occurring bioactive components, has prompted researchers to identify and develop novel natural and cost-effective antioxidants. Recent studies have suggested that food proteins hold promise as a potential dietary source of natural antioxidants. Hydrolyzed proteins from many animal and plant sources, such as milk [65,66], soy [67,68], zein [69], rice bran [70], canola [71], egg-white and yolk [24,72], porcine myofibrillar [73], and fish proteins [74–79] have been found to possess antioxidant activity. This activity is believed to be attributed to peptide sequences contained in these proteins and released after enzymatic proteolysis.

The ability of peptide fractions to inhibit deleterious changes induced by lipid oxidation appears to be related to their nature and composition. This seems to be dependent on the specificity of the proteinase used to generate the hydrolyzate [80]. There is evidence that the antioxidant effect of individual amino acids increases when they are incorporated in a peptide, indicating that the peptide bond or the structural conformation have an influence of this activity [81]. Thus, the peptide composition can lead to both synergistic and antagonistic effects with regard to the antioxidant activity of free amino acids. Chen et al. (1996) found that peptides containing a Pro-His-His sequence exhibited the greatest antioxidant activity among a group of tested peptides [67]. In addition, high levels of histidine and of some hydrophobic amino acids were related to peptide antioxidant potency [82]. The activity of histidine-containing peptides is thought to be connected to their hydrogen-donating ability, lipid peroxy-radical trapping, and/or the metal-ion chelating ability of the imidazole group [83]. Other amino acids such as tryptophane and

tyrosine have also been reported to contribute to the antioxidant activity, because of the capacity of the indolic and phenolic groups, respectively, to serve as hydrogen donors [24,66]. The hydrophobicity of the peptide also appears to be an important factor for its antioxidant activity due to increased accessibility to hydrophobic targets (e.g., lipophilic fatty acids) [84].

Generally, the ideal method for determination of antioxidant properties of peptides should assess their effect under reaction conditions that mimic those occurring when oxidative stress is induced *in vivo* by RNS and ROS [85]. As it has been observed for other antioxidants, the activity of peptides depends on the assay considered. The antioxidant properties of peptides have been attributed to the cooperative effect of a number of properties, including their ability to scavenge free radicals, to act as metal-ion chelators, oxygen quenchers, or hydrogen donors, and to the possibility of preventing the penetration of lipid oxidation initiators by forming a membrane around oil droplets. According to these properties, diverse methods have been proposed to measure the antioxidant activity of peptides derived from food proteins.

In the last few years, the Oxygen Radical Absorbance Capacity assay has been widely used to determine the antioxidant capacity of peptides derived from milk-whey proteins [66,81,86], egg [24], soy [87], seafood muscle [88], and fish proteins [89]. In the basic assay, the peroxyl radicals generated react with a fluorescent oxidizable protein substrate to form a nonfluorescent product. Probe reaction with peroxyl radicals is followed by a loss of the intensity of fluorescence with time. This assay measures the antioxidant inhibition of peroxyl-radical-induced oxidations and reflects a classical radical chain breaking antioxidant activity by H-atom transfer [90]. The low cost of the material and the possibility of using an automated fluorescence reader are the two main advantages of this assay. Moreover, it is particularly useful for samples which contain multiple ingredients with antioxidant activity.

The Trolox Equivalent Antioxidant Capacity (TEAC) assay has become one of the most widely used methods in testing the antioxidant capacity of food peptides from different sources. It has been applied to vegetal proteins, such as soy [91,92], zein [93], and rice bran proteins [94], among others. Hydrolyzates from animal proteins, such as milk and fish proteins, have also been analyzed for antioxidant activity with this method [66,95–97]. This assay uses 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), a peroxidase substrate, which when oxidized, generates a metastable radical cation ($ABTS^{\bullet+}$). This radical is intensely colored and can be monitored spectrophotometrically in the range of 600–750 nm. The antioxidant capacity is measured as the capacity of test compounds to decrease the color by reacting directly with $ABTS^{\bullet+}$ radical, and expressed relative to that of 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) [98]. This assay is often used to rank antioxidants to study structure–activity relationships [99]. Because the $ABTS^{\bullet+}$ radical can be solubilized in both aqueous and organic media and it is not affected by the ionic strength, the antioxidant capacity of hydrophilic and lipophilic compounds can be measured [100]. However, this method has two important limitations. On the one hand, the TEAC value actually characterizes the capability of the tested sample to react with $ABTS^{\bullet+}$ rather than to inhibit the oxidative process. On the other, the ABTS radical used in TEAC assays is not similar to the radicals found in biological systems [101].

In the 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Capacity Assay, the purple chromogen radical DPPH is reduced by antioxidant/reducing compounds to the corresponding pale yellow hydrazine. The reducing ability of the antioxidants can be evaluated by monitoring the absorbance decrease at 515–528 nm, until the absorbance remains stable or by electron spin resonance. This assay is technically simple and rapid and it only requires a UV–vis spectrophotometer that might explain its widespread use in antioxidant screening. An important limitation of this method is the interpretation of the role of hydrophilic antioxidants, because DPPH can only be dissolved in organic media [100]. However, many authors have used this assay for measuring the antioxidant activity of peptides from food proteins [87,93,102–108].

The ferric reducing antioxidant power assay is based on the reduction of yellow ferric tripyridyltriazine complex (Fe(III)-TPTZ) to blue ferrous complex (Fe(II)-TPTZ) by the action of electron-donating antioxidants [109]. The resulting blue color, measured spectrophotometrically at 593 nm, is taken as linearly related to the total reducing capacity of electron-donating antioxidants. This assay is rapid, simple, robust, inexpensive, and it does not require specialized equipment. Therefore, it has become one of the preferred methods to determine the antioxidant activity of peptides from several food protein sources [88,96,110].

In general, *in vitro* low-density lipoproteins (LDL) oxidation has been effectively used in several studies to characterize the antioxidant capacity of a number of different phytochemicals [111–114] and food peptides [115–117]. This method artificially induces the autoxidation of linoleic acid or of LDL particles, and the peroxidation of the lipid components is determined through the formation of conjugated dienes, followed by a spectrophotometrical analysis at 234 nm [118]. Basically, there are three mechanisms involved: free radical scavenging activity, binding to critical sites on LDL, and metal chelation [112].

Currently, different methods are being used to analyze the inhibitory properties of food peptides on ROS generation and on lipid peroxidation in different cell lines as the basis of their ability to reduce oxidative stress. For instance, estrogen-independent breast cancer cells were employed by Hernández-Ledesma et al. [92] to evaluate the antioxidative effect of the peptide lunasin in macrophages RAW 264.7.

Many food-derived peptides are effective as *in vitro* antioxidants but, due to the variable reports on their *in vivo* efficacy and the pro-oxidant nature of some antioxidants, it is necessary to perform studies in experimental animal models fed with these components to demonstrate the reduction of *in vivo* oxidative stress by the use of different biomarkers. It is known that compounds that upregulate the production of endogenous antioxidants, such as glutathione and antioxidant enzymes, provide novel approaches for the restoration of redox homeostasis. The values and enzymatic activities of these compounds, as well as the total antioxidant capacity, can be evaluated in the plasma and tissues of animals by enzymatic–colorimetric methods [119–121].

3.2.3 Antimicrobial Peptides

In the past 20 years, there has been an increasing interest in antimicrobial peptides present in men and animals, because they are considered to possess the functions in host defense, inflammation, and tissue generation [122,123]. In addition to these, it has also been demonstrated that several food proteins and peptides exert antimicrobial activities that can also provide protection against infection [124,125].

Numerous studies have been performed to understand the relationship between the structure and the activity of antibacterial peptides, especially of antibiotics peptides that act as components of the host defenses of animals or plants. An amphiphilic, mostly α-helical conformation, and an excess of positive charges are recognized as major structural motifs determining the membrane-disturbing activity of these peptides. These cationic and hydrophobic peptides are demonstrated to act by direct interaction with specific anionic membrane components, orientated toward the exterior of the cell, promoting pore formation and bacterial membrane disruption. Other peptides have membrane-permeabilizing properties and cell-penetrating activity. Some of these structural characteristics are also found in food-derived peptides.

The antibacterial activity of some dietary peptides can be tested by an agar diffusion assay where the peptide diffuses from a paper disc or small cylinder into an agar medium that contains test organisms. Inhibition is observed as the failure of the organism to grow in the region of the peptide. This method has been used, for instance, as a preliminary test to characterize the antibacterial activity of peptides obtained after the fermentation of casein with *Lactobacillus acidophilus* [126] and of peptides from human milk [127]. More frequently, the antimicrobial activity is determined in a liquid medium, commonly by using a microtiter plate assay, and measuring bacterial growth by optical density or bacterial count. Results are compared by establishing the minimum inhibitory concentration of a peptide/hydrolyzate for a given microorganism, that is, the lowest peptide/protein concentration that shows no growth at the end of the experiment. To compare the activity of different peptides or hydrolyzates, the IC_{50} value, defined as the concentration required to obtain 50% inhibition of growth at the exponential phase of growth, is also employed [128]. This assay has been widely used and recent applications include the assay of the antimicrobial activity of human milk against *Escherichia coli* and *Listeria monocytogenes* [129], synthetic porcine lactoferricin against *Staphylococcus aureus* and *Candida albicans* [130], α_{s2}-casein-derived peptides [131], and lactoferrampin [132].

The use of a bioluminescence assay to follow bacterial growth and metabolism has been also applied to investigate the effect of hydrolyzates of α-lactalbumin and β-lactoglobulin against *E. coli* JM103 [133]. Bacterial enumeration through fluorescent *in situ* hybridization has also been used to study the effect of α-lactalbumin and glycomacropeptide on mixed populations of human gut bacteria [134]. In the

latter example, in addition to a batch culture, a two-stage continuous culture model was used, with the advantage of allowing spatial and temporal heterogeneity and the assay of more complex environmental conditions.

Electron microscopic examination of bacterial preparations has also been used for lactoferricin, other lactoferrin-derived peptides [135], and α_{s2}-casein-derived peptides [129]. As an example, Figure 3.2 shows the transmission electron microphotographs corresponding to intact cells of *E. coli* ATCC 25922 cells and cells incubated with bovine α_{s2}-casein f(183–207) or lactoferricin. This technique allows the detection of damage in bacterial membranes and changes in the cytoplasm. With the aim of studying the mechanism of action of these antibacterial peptides, other techniques, such as circular dichroism analysis with micelles or liposomes, peptide binding to microbial cell components, and nuclear magnetic resonance spectroscopy, among others, have been successfully employed, but they fall out of the scope of this chapter.

There exist only a few studies on the *in vivo* activity of antimicrobial dietary peptides performed using animal models or in clinical trials in humans. Studies using animal models have reported a protective effect of orally administered lactoferricin against infections by methicillin-resistant *S. aureus* [136] or by the parasite *Toxoplasma gondii* [137]. Most of these studies were performed to demonstrate the antibacterial effects of the entire protein lactoferrin after oral administration. However, as orally administered lactoferrin is partially degraded to fragments that contain the lactoferricin sequence [138,139], some of these effects can probably be attributed either to lactoferrin fragments or to the combined action of lactoferrin and its derived peptides.

In humans, a study with low birth-weight infants fed with a lactoferrin-enriched infant formula concluded that lactoferrin contributes to the formation of a bifidobacteria-rich flora [140]. Lactoferrin has also demonstrated to be effective in the eradication of *Helicobacter pylori* when used as a supplement

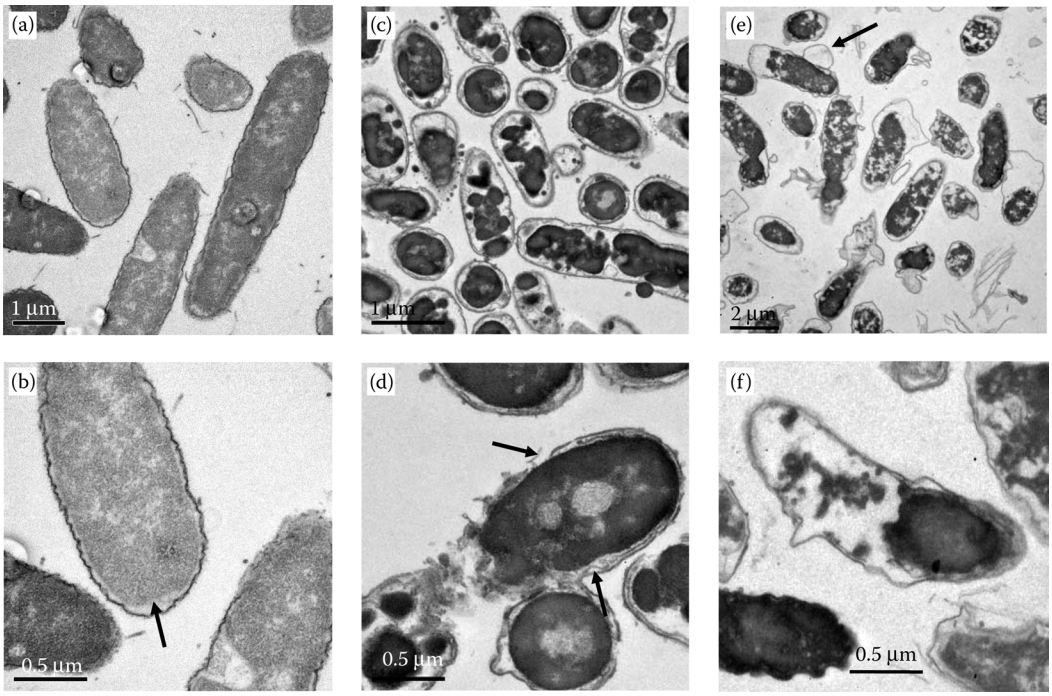

FIGURE 3.2 TEM micrographs of *E. coli* ATCC 25922. (a, b) Untreated cells. The arrow in (b) indicates the periplasmic space. (c, d) Cells treated with bovine α_{s2}-casein f(183–207) at a concentration of 31 µM during 2 h at 37°C. Arrows in (d) indicate the loss of continuity of the membrane and the swollen area between inner and outer membranes. (e, f) Cells treated with lactoferricin-B at a concentration of 31 µM during 2 h at 37°C. The arrow in (e) indicates a blister in the outer membrane. (From I. López-Expósito, L. Amigo, and I. Recio. *BBA Biomembranes* 1778: 2444, 2008. With permission.)

to an antibiotic treatment [141]. Other *in vivo* studies on lactoferrin to investigate its antiviral and immunomodulatory effects, as well as other host-protective activities, such as cancer prevention, and clinical applications have been reviewed [142,143].

There exist some studies on the *in vivo* activity of isracidin, corresponding to α_{s1}-casein f(1–23). In mice, it exerts a protective effect against *L. monocytogenes*, *Streptococcus pyogenes*, and *S. aureus*. Protection of rabbits, guinea pigs, and sheep against *S. aureus* has also been achieved. In cows with mastitis, isracidin obtained a success rate of over 80% in the treatment of chronic streptococcal infection. Furthermore, it has been demonstrated that isracidin possesses immunomodulant properties, with a significant effect in the production of IgG, IgM, and antibody-forming cells and an enhancing effect on cell-mediated immunity when injected to mice [144]. Another interesting finding was made with a tryptic casein hydrolyzate for the treatment and prophylaxis of newborn calf colibacillosis [145]. Recently, a product obtained from bovine colostrum rich in immunoglobulins, growth factors, antibacterial peptides, and nutrients was demonstrated to reduce the number of evacuations of stools per day in patients with HIV-associated diarrhea [146].

3.2.4 Opioid Peptides

Accumulated evidence shows that peptides from food proteins may act as opioid receptor ligands, and thus, they may be regarded as exogenous supplements to the endogenous opioidergic systems of the human organism. They have been referred to as exorphins, to distinguish them from opioid peptides of endogenous origin, called endorphins. Opioid receptors (μ-, δ-, and κ-type) are located in the nervous, endocrine, and immune systems, as well as in the intestinal tract of the mammalian organism and they can interact with their endogenous ligands, as well as with the dietary opioid and opioid antagonists [147]. Orally administered food-protein-derived peptides have demonstrated to influence postprandial metabolism by stimulating the secretion of insulin and somatostatin [148,149], to modulate intestinal transport of amino acids [150], to prolong gastrointestinal transit time, and to exert antidiarrheal action [151]. Recently, it has been proposed that β-casomorphin-7 may contribute to mucin production via a direct effect on the intestinal goblet cells and an activation of μ-opioid receptors [152]. They may also play a role in the reproductive function of the female organism, supporting processes of pregnancy, delivery, and lactation period, while they may exert a cardioprotective function in pregnant or lactating mammals [153].

The common structural characteristic of opioid peptides is the presence of a tyrosine residue at the amino terminal end (except milk-derived α-casomorphins that have an additional arginine residue) and the presence of another aromatic residue in the third or fourth position. The negative potential, localized in the vicinity of the phenolic hydroxyl group of tyrosine, is essential for opioid activity and the presence of a proline residue helps maintain the proper orientation of the tyrosine residue and the other aromatic amino acids [154,155]. The occurrence of aliphatic amino acids in the third position also contributes to the opioid activity in the case of gluten exorphins and neocasomorphin (YPVEPF).

Food-derived peptides can be screened for opioid activity by measuring the adenylate cyclase activity in homogenates of neuroblastoma X-glioma cells and using naloxone as opioid antagonist to reverse the effect [156]. The two bioassays employed more often to test materials with opioid activity are the guinea-pig ileum longitudinal muscle myenteric plexus preparation and the mouse vas deferens preparation. Opioid receptors located on intestinal smooth muscle cells are the targets for exogenous μ- and κ-opioid receptors agonists to inhibit the propulsive peristaltic activity and to increase the smooth muscle tone [157]. The mouse vas deferens are classical preparations for studying the pharmacology of δ-opioid receptors [158]. The activity in these two organ bath preparations can be antagonized with opioid antagonists, such as naloxone. Further, specific radioligand competition assays for opioid μ-, δ-, and κ-sites have also been used for assessment of opioid peptides [159].

Several animal models have been used after oral or intragastrointestinal administration of food-protein-derived opioid peptides, which allowed the demonstration of a number of activities for β-casomorphins, milk casein preparations, gluten exorphins, and gluten or gluten hydrolyzates. In dogs, stimulation of insulin release, reduced gastrointestinal motility, and release of somatostatin are observed after intragastric administration of casein [148,160,161]. In rats, satiety was demonstrated to be influenced by casein or casein hydrolyzate [162]. Prolongation of gastric emptying rate and gastrointestinal transit time are

also observed after intragastric administration of casein [151]. Several studies have also been performed by using calves and cows. For instance, an antidiarrheic effect is found in calves upon oral administration of β-casomorphin(1–4) amide [163]. The behavioral and antinociceptive effects of these peptides are usually tested in mice. The tail pinch method has been employed in the case of a peptide derived from Rubisco [164]. Effects on nociception, locomotor activity, motor coordination, rectal temperature, and duration of pentobarbital anesthesia have been evaluated in mice after intraperitoneal administration of α-lactorphin (YGLF, an opioid tetrapeptide derived from α-lactalbumin). This peptide, in contrast to morphine, elicits no behavioral effect in mice [165]. Interestingly, this α-lactalbumin-derived peptide has the ability to lower blood pressure in hypertensive and normotensive rats after subcutaneous administration [47]. Another α_{s1}-casein-derived opioid peptide, RYLGY, has recently shown antihypertensive activity in SHRs after acute and chronic administration [27,166].

In adult humans, oral administration of β-casomorphin(1–4) amide delays the gastrointestinal transit time [167]. A gluten hydrolyzate also causes a prolongation of gastrointestinal transit time in healthy volunteers, an effect that is blocked by naloxone [168].

3.2.5 Mineral-Binding Peptides

The first reference to bioactive peptides in the scientific literature was made by Mellander in 1950, who suggested that casein-derived phosphorylated peptides enhanced vitamin D-independent bone calcification in rachitic infants [169]. Later, it was demonstrated that these peptides, also named phosphopeptides, by virtue of their high content of negative charges, efficiently bound divalent cations, specially calcium, but also Fe, Mg, Mn, Cu, and Se, forming soluble complexes. It has also been suggested that the mineral-binding activity could be dependent on the histidine content and peptide size [170,171]. These peptides with mineral-binding activity could then function as carriers of various minerals and thus, either enhance or inhibit their bioavailability.

Mineral-binding peptides are considered physiologically beneficial in the prevention of dental caries, mainly because of their role in recalcification of the dental enamel. The use of these peptides in preventing osteoporosis, hypertension, and anemia has also been proposed [172]. Another interesting property associated with phosphopeptides is their potential to enhance mucosal immunity [173]. It is important to note that the high content of negative charges makes these peptides resistant to further hydrolysis [174], and they have been detected in the gastrointestinal tract of animals and humans after oral ingestion [175–177].

Isolation and characterization of peptides with mineral-binding activity requires selective solubilization and precipitation methods [178–184]. Differences in mineral affinity, competition for binding sites, and changes in the pH and ionic strength of the solvent are used to influence mineral binding and release. Mineral-binding peptides can be also isolated using ultrafiltration and hydroxyapatite affinity (HA) chromatography [185–187], or measuring the calcium content of the supernatant after the formation of a Ca–peptide complex using $CaCl_2$ and sodium phosphate buffer [188].

The beneficial properties of phosphopeptides can be estimated *in vitro* by selective precipitation, using a model system based on HA. In this method, the peptides are incubated with a suspension of HA in Tris buffer at pH 7.0, as a replacement for dental enamel and sodium acetate buffer at pH 4.2, is added to simulate the organic acids found in the mouth. After centrifugation, the supernatant is used to measure the levels of calcium and phosphorus dissolved from the HA by the action of the sodium acetate buffer, which represents 100% demineralization. The effectiveness of peptides samples to reduce the demineralization of HA is deduced by comparing calcium and phosphorous concentrations in the protein/peptide supernatant with the values of the controls [189–191].

The measurement of the mineral content is complex and depends on several factors. Among these, the equilibrium status, redox form, solubility, and binding affinity are regarded as some of the most important ones. Sensitivity of the method is also important [174]. For instance, analysis of total calcium, iron, zinc, magnesium, and some trace elements can be performed by atomic absorption spectroscopy [192], colorimetric methods measure the ferrous form of iron [193,194], and chromatography in the presence of chelating agents can be applied as a selective technique [193,195,196]. It is also possible to use radioactive isotopes to follow the binding mechanism [197–199].

Improved *in vitro* methods utilize human colon carcinoma cell lines (Caco-2) [200] to mimic and estimate the uptake and/or transport of mineral elements [201]. Other cell lines can also be employed to assess the potential of peptides to enhance the dietary mineral bioavailability and to modulate the bone formation [202], such as bone-marrow-derived osteoblast precursor cells, to measure the ability of food-derived peptides to stimulate the proliferation of osteoblasts to form osteoclasts [203]. However, these *in vitro* methods only estimate the fraction of elements available for absorption [204], and thus, mineral bioavailability should be evaluated *in vivo* [205], assessing intestinal calcium absorption in rats [206], or enhanced bone calcification [207,208].

3.2.6 Antithrombotic Peptides

It is generally accepted that platelet adhesion and aggregation play an important role in the pathogenesis of thrombosis, particularly arteriothrombosis [209]. Antithrombotic agents are widely used in medicine for the treatment of several cardiovascular events and related diseases, such as coronary angioplasts, coronary thromboembolisms, myocardiac heart attack, pulmonary embolism, and so on [210]. The formation of thrombin consists of the binding of fibrinogen to its platelet receptors after activation by physiological agonists such as adenosine diphosphate (ADP), thrombin, and collagen [211]. One of the sites at which fibrinogen binds to platelet receptors corresponds to the C-terminal sequence of the γ-chain, the 400–411 fragment (HHLGGAKQAGDV) [212]. *In vitro* and *in vivo* experiments have demonstrated that food peptides analog to this sequence, such as casein macropeptide that derives from κ-casein, are capable of inhibiting the binding of the human fibrinogen γ-chain to fibrinogen receptors on the platelets surface, what would otherwise lead to platelet aggregation [213–218]. These peptides have been detected in plasma of new born children after ingestion of breast milk or infant formula, although their *in vivo* significance remains unclear [219].

The antithrombotic activity can be evaluated *in vitro*, by assessing the inhibition of fibrinogen binding to ADP-activated platelets. For this purpose, platelet-rich plasma is incubated with the peptides and, after addition of ADP, the increase in light transmission is monitored using an aggregometer [175,213,220]. The antithrombotic activity can be also studied *in vivo* using a guinea-pig model of arteriolar thrombosis, which is triggered by a laser-induced minimal injury [216,221–223].

3.2.7 Hypocholesterolemic Peptides

Hyperlipidemia, especially hypercholesterolemia, is one of the most important risk factors contributing to the development of cardiovascular diseases [224–227]. Plasma cholesterol levels are influenced by the diet and by cholesterol biosynthesis, uptake, and secretion [228–231]. Considerable evidence indicates that cholesterol lowering stabilizes atheroma plaques reducing cardiovascular events [232–235]. In the last few years, numerous drugs and natural extracts have been explored for their potential in the prevention and treatment of hypercholesterolemia. In this context, food-derived peptides might also lower serum cholesterol levels [229,230,236–239], by increasing cholesterol catabolism, reducing intestinal absorption of dietary cholesterol or inhibiting cholesterol uptake from the plasma into the liver, preventing the deposition of cholesterol into plaques on the arterial wall [240].

Hydrophobicity seems to be responsible for the hypocholesterolemic potential of peptides, especially for their bile acid-binding ability and it has also been suggested that the hypocholesterolemic activity is strongly influenced by the presence of a leucine residue at the N-terminal side [241]. Further work is required to identify hypocholesterolemic peptides, and to clarify the influence of peptide structure on the hypocholesterolemic activity.

Cholesterol is water insoluble and requires a micellar solubilization step prior to intestinal absorption [242]. *In vitro* assays relate the hypocholesterolemic action of proteins and peptides with their ability to decrease the micellar solubility of cholesterol and with their taurocholate-binding capacity. To study micellar solubility *in vitro*, the sample is incubated with either artificially prepared or natural micellar solutions containing sodium taurocholate, cholesterol, oleic acid, and sodium phosphate at pH 7.4 and, after ultracentrifugation, the cholesterol content is measured [236,239]. On the other hand, the taurocholate-binding capacity determines the ability of the peptide to bind bile acids *in vitro* [243,244].

Peptides are incubated with a Tris–HCl buffer containing taurocholate, at 37°C for 30 min, and the content of taurocholate is measured enzymatically after dialysis of the mixture [245,246]. Other *in vitro* methods apply monolayers of Caco-2 cells as a model system to examine *in vitro* cholesterol absorption [231,236,247].

In vivo hypocholesterolemic activity can be studied using different animal models, such as rabbits [248], normal rats [249], diabetic rats [250], diabetic and nondiabetic hamsters, [251], guinea pigs [252], and monkeys [253]. For the induction of hypercholesterolemia, they are fed with a cholesterol-enriched diet, prepared by adding cholesterol and cholic acid to the standard diet, during several weeks [254]. Cholic acid is included because it increases micelle formation and facilitates intestinal absorption of cholesterol. At the end of the experimental study, the serum lipid profile (cholesterol and triglyceride levels) can be determined using commercial kits. It is also useful to measure liver function enzymes, such as the rate-limiting enzyme responsible for bile acid biosynthesis, cholesterol 7α-hydroxylase, and the rate-limiting enzyme responsible for the cholesterol synthesis system, 3-hydroxy-3-methylglutaryl coenzyme A reductase [255,256], or the incorporation of [3H]-cholesterol into the serum, liver, and intestine of the animals [257].

3.2.8 Antiproliferative and Cytotoxic Peptides

Cancer has become one of the most common causes of death in industrialized countries and it has been defined as the medical challenge of our times. An impressive body of evidence supports the notion that prevention can be a major component of cancer control. Epidemiological cell culture and animal studies have demonstrated that a large number of natural compounds present in the diet could lower cancer risk and even sensitizes tumor cells in anticancer therapies [258].

Carcinogenesis is a multistage process with an accumulation of genetic alterations, so that the sequence of events has many phases for intervention with the aim of preventing, slowing down, or reversing the process. Based on various cytochemical studies, there is an increasing evidence for the possible involvement of food-protein-derived peptides as specific signals that can inhibit the viability of cancer cells [259]. These studies are based on the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay as described by Holst and Oredsson [260]. In this assay, MTT is added to the growth medium of the cells, taken up by living cells via endocytosis and reduced to blue formazan crystals inside the cell via complex I in the mitochondrial respiratory chain. The amount of formazan generated is assumed to be directly proportional to the cell number when using homogeneous cell populations [260]. As an example, this method has been used to evaluate the cell proliferation inhibitory properties of peptides from different food protein sources, such as the lactoferricin [261], lunasin from soy protein [262], and lunatusin from lima beans protein [263].

Induction of apoptosis is considered to be one of the important targets in a cancer preventive approach. A regulated form of cell death is a complex process that involves the active participation of the affected cells in a self-destruction cascade and it is defined by a set of characteristic morphological hallmarks, including membrane blebbing, shrinkage of cell and nuclear volume, chromatin condensation, and nuclear DNA fragmentation due to endonuclease activation. Several studies have demonstrated the role of food peptides as apoptosis-inducing agents in different human cancer cell lines [264,265]. As an example, Hsieh et al. [262] have studied recently the effect of the peptide lunasin, alone or in combination with aspirin on the programmed death of estrogen-independent MDA-MB-231 breast cancer cells (Figure 3.3).

New technologies, such as DNA microarrays and serial analyses of gene expression, have created an exciting new frontier for the nutrition and healthcare community. These technologies allow the simultaneous analysis of the expression of thousands of genes and the comparison between normal and abnormal tissues. Therefore, they are being widely used to identify and characterize the response to preventive and therapeutic interventions. Moreover, these techniques are useful for a better understanding of the mechanisms involved in the cancer-preventive actions of food compounds [262,266]. Other techniques are being developed and refined to assist in the identification of the proteome, defined as all proteins present in the cell in a particular cellular time [267]. This type of investigation is very important because of the fact that gene expression does not always correlate with protein expression and the influence of food components may be either translational or posttranslational, rather than at the transcriptional level [268]. Thus, proteomic-based studies, although technically challenging, complement genomic studies,

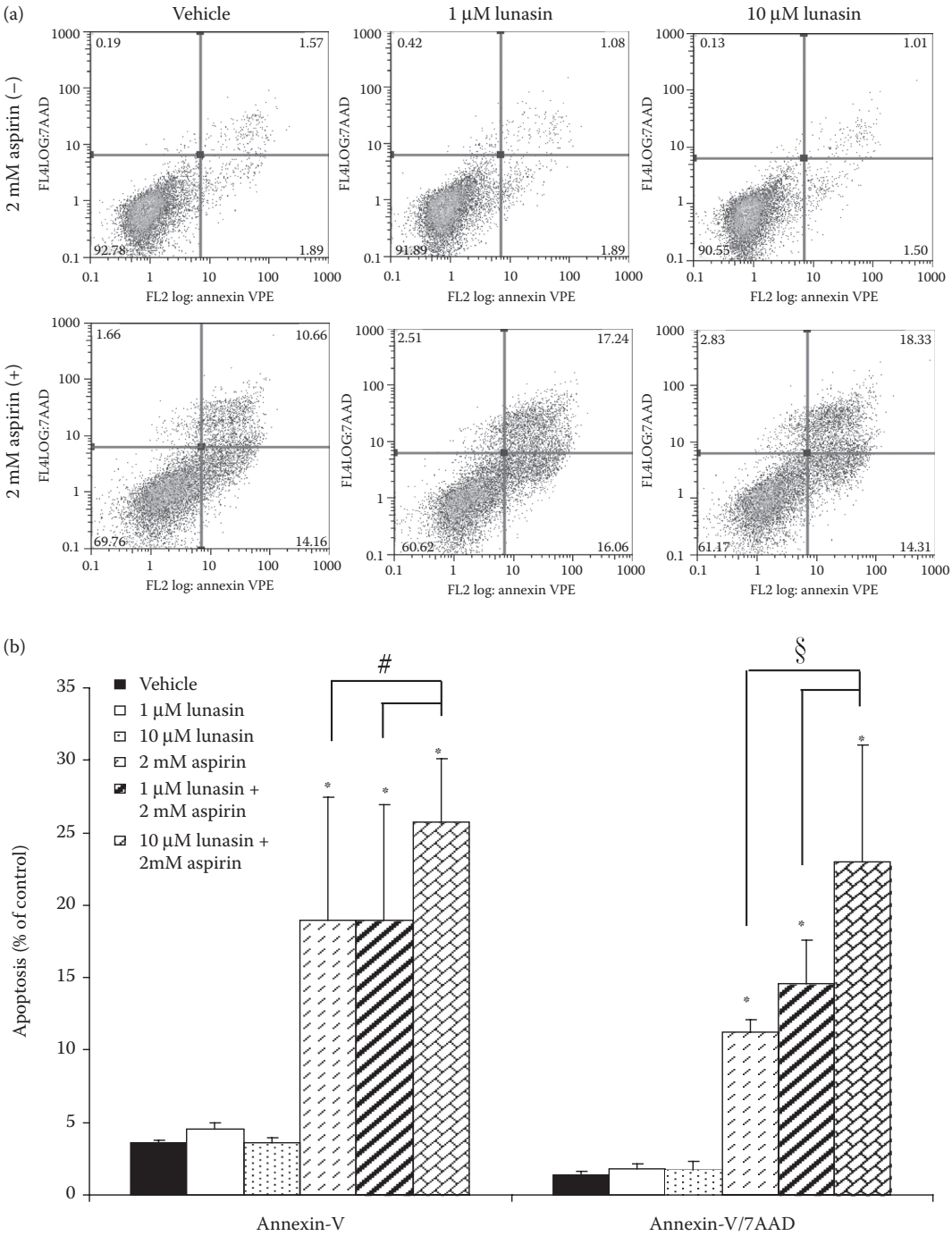

FIGURE 3.3 Lunasin promotes aspirin-induced apoptosis. (a) Flow cytometry-based Annexin V labeling of apoptotic cells. Cells were treated for 48 h with vehicle, 1 μM lunasin, 10 μM lunasin, 2 mM aspirin, 1 μM lunasin + 2 mM aspirin, and 10 μM lunasin + 2 mM aspirin. (b) Early and late stages of apoptosis were identified by Annexin V + /7-AAD− and Annexin V + /7-AAD+, respectively in breast cancer cells treated with aspirin, lunasin, and their combination at different concentrations. Three independent experiments were performed (*$P < 0.0001$ versus vehicle-treated cells; # $P < 0.05$, § $P < 0.0001$ versus aspirin-treated cells). (From C.-C. Hsieh, B. Hernández-Ledesma, and B.O. de Lumen. *Food Chem* 125: 630, 2011. With permission.)

being essential in any comprehensive research strategy aimed at examining the molecular processes and factors involved in modulating cancer risk.

The animal experiments represent the next step to demonstrate the cancer-preventive activity of food peptides. Transgenic and knockout models present evidence that genes markedly influence the response to dietary components, also providing valuable clues about their site(s) of action [269–271]. Other animal models commonly used to evaluate the preventive role of food peptides are those based on chemical and radiation-induced carcinogenesis. As an example, the soybean Bowman–Birk protease inhibitor (BBI) and the BBI concentrate have shown to exert a suppressive effect on 7,12-dimethylbenza(a)anthracene-induced oral cancer [272], 3-methylcholanthrene-induced lung cancer [273], methylbenzylnitrosamine-induced esophageal cancer [274], radiation-induced lymphosarcomas [275], and ovarian sarcomas [276]. BBI and other peptides, such as lunasin, have also demonstrated anticarcinogenic properties against different types of cancer using xenograft mouse models [277–279]. These models are based on the injection of human tumor cells to evaluate the progress of these cells under the effects of chemopreventive peptides. Finally, some peptides, such as BBI, are currently being used in large-scale human trials, which represent the last step in demonstrating their cancer preventive role [280].

3.3 Future Prospects

The known bioactive peptides present in food, or produced upon food processing or ingestion, can improve the health status through reductions in blood pressure, enhancement of human defenses (antimicrobial, antioxidant, and cytomodulatory peptides), or modulation of the absorption of nutrients (mineral and cholesterol absorption). Future research in the field of bioactive peptides will focus on the identification of novel bioactive sequences, on the possible mechanisms of action and on new health benefits. In addition to the search for novel peptide sequences with traditional functionalities, other bioactivities will be explored. Of special interest are bioactivities related with the gastrointestinal tract. For instance, it has been found that peptides can affect gastric hormones that control gastric and intestinal motility and secretions or stimulate the release of satiety hormones, such as cholecystokinin [281,282]. Some of these activities are exerted by modulating and influencing gene expression, being the role of dietary peptides in nutrigenomics an undoubtedly growing field of research [283].

The techniques used to study such benefits have improved over the recent years and unique tools have now become available that facilitate the undertaking of challenges that were impossible only one decade ago. The use of advanced peptidomic analytical tools, mainly tandem mass spectrometry analysis, has led to the identification of minor peptides in complex mixtures and biological fluids. Theoretical predictions and simulations are also considered emerging tools in peptide science. These include structure–activity studies based on *in silico* analysis using chemometric methods and the use of computational chemistry for the creation of sequence databases of bioactive peptides.

New genomic, proteomic, and metabolomic techniques are now enabling the finding of novel associations between diet and chronic diseases through examination of the functional interactions of food components with the genome at molecular, cellular, and systemic levels. Advances in this area will allow the identification of new biomarkers of the physiological effects of peptides that can be employed to evaluate the peptide bioactivity. The mechanisms underlying many of the biological properties of food-derived peptides are, and will be, the subject of increasing interest. Evidence for the beneficial effects of these peptides and demonstration of their mechanisms of action should be conclusive for the approval of a health claim by the authorities. Although some bioactive sequences are already in the market, it is expected that these peptides will be optimally exploited for human nutrition and health when knowledge in these different areas increases.

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4

Glutathione

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4.1 Introduction

An increasingly significant number of studies show a relationship between numerous disorders, including cancer and micronutrients in food (Dragsted et al. 2006, Hushmendy et al. 2009, Manach et al. 2009, Talalay and Fahey 2001). Antioxidants, in particular, stand out among these micronutrients with the number of studies examining the protective role of some foods against oxidative damage steadily increasing (Aruoma 2003, Di Mascio et al. 1991, Farombi et al. 2004, Flagg et al. 1994a, Gale et al. 2001, Herrera et al. 2009, Jiao and Wang 2000, Mayne 2003, Talalay and Fahey 2001, Wang et al. 2005).

Thiols, having an important position among antioxidants, are characterized as being mercaptan and having a sulfhydryl functional group. The most active and reduced form of biological sulfur is thiol (–SH). Biothiols (or biologically derived thiols) protect cells against many kinds of oxidative damage (Dalle-Donne and Rossi 2009, Moran et al. 2001, Moriarty-Craige and Jones 2004, Sahu 2002). Glutathione (GSH, γ-glutamylcysteinylglycine), which was discovered and characterized by Sir Frederick Gowland Hopkins (1861–1947) in 1922, is the most prominent thiol containing the low-molecular weight intracellular antioxidant that is found in millimolar concentrations in tissues of animals, plants, and microorganisms (Brosnan and Brosnan 2009, Demirkol et al. 2004, Jones 1995, Malmezat et al. 2000, Simoni et al. 2002, Townsend et al. 2003, Winters et al. 1995). Although there have been numerous studies on the synthesis and functions of GSH, studies measuring the content of GSH in foods are limited. The GSH levels in foods are affected by many factors, including growing conditions, seasonal and geographical differences, species, and food processing, storage, and preparation (Jones 1995, Kocsy et al. 2002). For example, GSH can be oxidized easily during food storage, processing, and preparation. Besides, because many foods include reactive electrophile components, even if the food is digested, the concentration can be reduced, since the GSH levels in frozen foods are very comparable to those found

in fresh ones (Demirkol et al. 2004, Jones 1995, Jones et al. 1992, Manda et al. 2010). The concentration of GSH in samples of hyperium species has been found to decrease from 0.96 to 0.12 μmol g^{-1} DW when dried with hot air, while no changes were determined in the GSH content of freeze-dried samples (Chen et al. 2009). Another study found that pasteurization removed all of the GSH from Sicilian cactus pear fruit juice (Tesoriere et al. 2005). Similarly, carrot and apple juices stored in cans and bottles lost their GSH (Jones 1995). A study, conducted to determine whether disinfectants affect GSH levels in foods, showed that hydrogen peroxide (H_2O_2) treatment, in particular, eliminated GSH in vegetables (Qiang et al. 2005). Demirkol and Mehmetoglu (2008) showed that asparagus, spinach, green beans, and red pepper grown organically have lower GSH levels as compared to the ones grown traditionally.

Food products contain both GSH and GSSG (oxidized glutathione), and measuring the levels of each one is important because the GSH:GSSG ratio is a well-known oxidative stress parameter. A decrease in GSH levels and an increase in GSSG is an excellent indicator of oxidative stress (Demirkol 2009, Qiang et al. 2005). A change in the intracellular GSH:GSSG ratio could indicate that oxidative stress is responsible for cell proliferation, gene expression, and apoptosis (Rahman et al. 2005).

Additionally, GSSG can form two GSH molecules by the action of glutathione reductase (GR) in the intestine; therefore, the levels of GSSG in food should be factored in when studying the bio-beneficial effects of GSH in food (Ercal et al. 1996, Jones 1995). Qiang et al. (2005) determined the GSH:GSSG ratio in spinach, green beans, red pepper, cucumber, and asparagus. According to their study, spinach had the highest GSH:GSSG ratio.

Ryan et al. (2007) showed that GSH levels decreased significantly (98% of the control level) due to its oxidation to GSSG after 2 h aeration using the methyl iodide fumigation process. However, GSH levels returned to the control level 24 h after the termination of the aeration process.

Recent studies have examined GSH for use in the food industry. Oms-Oliu et al. (2006) showed that GSH prevents enzymatic browning in fresh-cut pears at 4°C for 21 days. Alandes et al. (2009) monitored the changes in structural texture, microstructure, acidity, soluble solids, color, pectin methylesterase activity, and specificity of microflora in fresh-cut fruits after applying a solution containing *N*-acetyl-L-cysteine (NAC), GSH, and malic acid. This study indicated that the GSH precursor, NAC, could be used to increase the shelf-life of food. Molnar-Perl and Friedman (1990) showed that GSH is as effective as sodium sulfite in preventing both enzymatic and nonenzymatic browning in apples and potatoes.

There are other factors to consider when determining the practicality and advantages of GSH usage in the food industry. For example, nicin, the only food additive/bacteriocin deemed acceptable by the United States Food and Drug Administration (USFDA) and generally recognized as safe (GRAS) by the World Health Organization (WHO), can bind with GSH and form conjugates, thereby decreasing GSH concentrations in raw meat and meat products. This decrease was not observed in cooked meat and meat products (Bouttefroy and Milliere 2000, Ganzle et al. 1999, Nel et al. 2004, Rose et al. 1999, Stergiou et al. 2006).

This chapter reviews the techniques used to determine the amounts of GSH in food samples.

4.2 Oxidative Stress

The number of studies on the relationship between reactive oxygen species (ROS) and various disorders has rapidly increased over the years. ROS, strictly defined, indicates free radicals that contain oxygen (O_2), and a free radical is an atom or molecule that has an unpaired electron. The simplest free radical is a hydrogen-centered atom, that is, a proton with an unpaired electron (Aruoma et al. 1991, Fang et al. 2002). In living organisms, free radicals include hydroxyl (•OH), superoxide ($O_2\bullet^-$), nitric oxide (NO), and peroxyl ($RO_2\bullet$) radicals. Hypochlorous acid (HOCl), (H_2O_2), O_2, and ozone (O_3) are not free radicals; however, they can easily be converted into free radicals. Therefore, scientists use ROS not only for •OH and $O_2\bullet^-$ but also for HOCl, O_2, O_3, and H_2O_2 (Aruoma 1991).

ROS are naturally formed during aerobic metabolic processes, while chemicals, drugs, and pollutants are external sources. ROS can induce oxidative damage to lipids, proteins, and DNA that can lead to biochemical and physiological lesions, resulting in cell death. Under normal physiological conditions, endogenous ROS levels are controlled by antioxidant mechanisms (Sahu 2002, Wang and Jiao 2000).

Oxidative stress is a term used to define the imbalance between oxidants and antioxidants. Oxidative stress occurs when there are increased levels of ROS, decreased levels of antioxidants, or both, and can result in DNA oxidation, protein oxidation, and lipid peroxidation. Cellular proteins, especially thiol-containing proteins, are easily damaged by oxidative stress and enzyme activity can, as a result of the damage, be reduced (Aruoma et al. 1991, Moriarty-Craige and Jones 2004, Sahu 2002).

Lipid peroxidation due to oxidative damage may inactivate cellular macromolecules, causing cellular damage similar to the damage attributed to aging. Protein oxidation specified by formation of carbonyl groups is a very serious event and it may occur when there is no lipid peroxidation. Modification of proteins by ROS has been identified as an etiological factor in several physiological and pathological conditions (Kamat et al. 2000).

Oxidation of a macromolecule (DNA, lipids, and proteins), if the oxidative damage is not controlled, may be the cause of many disorders. For example, it is well accepted that oxidized LDL is a factor in the development of cardiovascular disorders, oxidation of DNA may lead to cancer, and cataracts may be due to the photo-oxidation of lens proteins (Mayne 2003).

Oxidative stress may be a factor in the development of age-induced atherosclerosis, chronic lung disorders, paralysis, rheumatoid arthritis, age-induced molecular degeneration, and Alzheimer's disease (De Flora et al. 1991, Moriarty-Craige and Jones 2004).

Diets rich in vegetables and fruits, according to recent studies, may decrease the risk of the aforementioned disorders. The protective role of vegetables and fruits may be linked to the Vitamin C, Vitamin E, beta carotene, flavonoid, and thiol which they contain (Dragsted et al. 2006, Fang et al. 2002, Herrera et al. 2009, Prior 2003, Zhang et al. 2008).

4.3 Biological Functions of Glutathione

GSH is synthesized in cytosol by a two-step process. In the first step, L-glutamate and L-cysteine ligation by γ-glutamylcysteine synthetase makes γ-L-glutamyl-L-cysteine. In the second step, GSH synthetase catalyzes addition of glycine to γ-L-glutamyl-L-cysteine to form γ-L-glutamyl-L-cysteinyl-L-glycine (GSH) (Basu et al. 2004, Camera and Picardo 2002, Pasternak et al. 2008). This tripeptide is found in both GSH and GSSG forms in cells. GSH is an important intracellular reductant, serving as the first line of defense with its functional group, sulfhydryl (-SH), functioning as an innate antioxidant. The protective role of GSH against ROS is carried out by the interaction of glutathione peroxidase (GPx) and GR (Luberda 2005). An optimal ratio of GSH:GSSG is very critical to maintaining cellular viability and preventing homeostatic imbalance. Intracellular fluctuations of GSH:GSSG could affect gene expression and apoptosis, and depletion of GSH could place cells at risk of oxidative damage (Rahman et al. 2005, Schafer and Buettner 2001). Therefore, it is not surprising to see abnormal fluctuations in the levels of GSH in disorders, such as cancer, neurodegenerative disorders, cystic fibrosis, HIV, and aging. An increase in ROS, including H_2O_2 and superoxide $O_2^{\bullet -}$, is toxic for the cells. Therefore, the system that metabolizes and eliminates these toxic compounds is important and carefully controlled within the cells. GPx, working with catalase (CAT) and superoxide dismutase (SOD), protects cells against ROS-induced damage. GPx and GSH (the latter an important electron donor in reductive reactions) are very effective in eliminating (both directly and indirectly) ROS, including hydroxyl, peroxynitrite, lipid peroxyl, and H_2O_2. GSSG, formed as a result of this GPx reaction, is reduced to two molecules of GSH by nicotinamide adenine dinucleotide phosphate (NADPH)-dependent GR (Townsend et al. 2003, Wu et al. 2004) (see Figure 4.1).

Oxidative stress affects cells differently, depending on the type of cell. The stability and proliferation of cells are under control as long as the level of oxidative stress is normal and balanced. Programmed cell death (apoptosis) can be triggered by increased ROS and results in the engulfment of dead cells by apoptotic microphages. A large body of evidence indicates that thiols protect cells against ROS- and oxidative stress-induced apoptosis, while a deficiency of GSH results in necrosis (Higuchi 2004, Moran et al. 2001).

The glutathione-*S*-transferase (GST) enzyme uses GSH in its detoxification process, with GSH catalyzing the conjugation of various toxins, metals, herbicides, xenobiotics, and so on (see Figure 4.1). For example, studies show that GSH protects plants, animals, and humans against heavy metal toxicity (Ercal et al. 2001). GSH also plays a role in other cellular reactions, such as thiol:disulfide exchange by

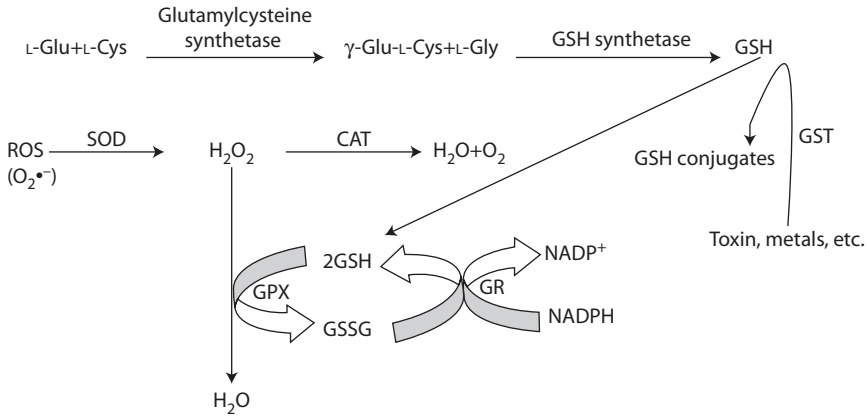

FIGURE 4.1 Glutathione synthesis and utilization.

gene expression, protein regulation, synthesis of RNA, and the glyoxalase system (Lu 2009, Townsend et al. 2003, Xiang et al. 2001, Zechmann et al. 2008). GSH is a substrate for the formaldehyde dehydrogenase enzyme and this enzyme forms *S*-formyl-glutathione with formaldehyde (Wu et al. 2004).

GSH plays a crucial role in protecting intestinal cells against oxidative damage caused by toxic substances in food. Further, GSH is a necessary thiol compound for the normal development and function of the intestinal cells. A positive correlation has been found between the depletion of GSH in human plasma and HIV infection, premature birth, protein energy malnutrition, and alcoholic cirrhosis (Moriarty-Craige and Jones 2004).

GSH also plays an important role in synthesizing protein, DNA, and leukotrienes (inflammation markers), and in maintaining various proteins in their reduced states (Malmezat et al. 2000, Townsend et al. 2003). GSH is a substrate for GSH peroxidase which reduces organic peroxides and protects DNA against oxidative damage (Wierzbicka et al. 1989). GSH is also involved in the reductive process in protein and DNA synthesis. It is required for the transfer and storage of cysteine (CYS), working as a cofactor in this process (Meister 1974, Meister and Anderson 1983).

GSH is released from the cells in viral infections. This is especially true for cancer, AIDS, and hepatitis B. There is a positive correlation between GSH release and hepatocyte viral infection. It has been observed that adding GSH early in the process inhibits the mutagenic effect of nitrosylation as well as, or even better than, either NAC or vitamin C. In addition, only GSH and NAC were found to be effective after the completion of a nitrosylation reaction, by reacting with the nitrate and preventing the formation of mutations (De Flora et al. 1991).

4.4 Glutathione Content in Food

Several studies have found a correlation between diet and the risk of cancer development. These studies indicate that the consumption of fruits and vegetables protect humans from cancer and cardiovascular and cerebrovascular disorders (Cao et al. 1998). In one study, Flagg et al. (1994a) showed that the GSH obtained from a diet containing fruits and vegetables decreased the risk of getting mouth cancer and, in another study, that more than 50% of dietary GSH came from fruits and vegetables and <25% from the meat in a diet (Flagg et al. 1994b). Ueno et al. (2002) showed that GSH in a diet prevented renal and neuronal dysfunctions induced by oxidative damage in diabetic rats.

Aw et al. (1991) found that oral GSH administration significantly increased the levels of GSH in kidneys, heart, lungs, small intestine, and skin after the administration of a well-known GSH synthatase inhibitor (L-buthionine-*S*,*R*-sulfoximine) (BSO, 80 μmol/day) to mice for 5 days.

Balanced amino acid intake, such as from protein, had an important effect on GSH balance. Glutamate, glycine, and thiol-containing amino acids also had to be available in sufficient amounts as they played a

critical role in the efficient synthesis of GSH (Wu et al. 2004). Some reactive electrophilic chemicals (i.e., acrylamide and hydrazine) in food may react with GSH and decrease the biobeneficial effects of dietary GSH (He et al. 2004). However, because GSH may detoxify the chemicals, it can be used as a protectant. For example, it protects organisms against a potential mycotoxin patulin by conjugating with patulin (Mahfoud et al. 2002). Dietary GSH may decrease lipid peroxidation and absorption, according to some studies (Jones 1995).

Dairy products, eggs, fats, and most drinks and grains are poor sources of GSH, while fresh vegetables, fruits, freshly cooked meat, and some spices have very high concentrations of GSH (Demirkol et al. 2004, Jones 1995, Manda et al. 2010) (see Table 4.1).

TABLE 4.1

GSH and GSSG Concentrations of Some Food Reported in the Literature

Food	GSH	GSSG	Reference
Asparagus	21.8 100 g/mg	[a]6.5 100 g/mg	Jones et al. (1992)
Asparagus	349 nmol/g	—	Demirkol et al. (2004)
Asparagus	627 nmol/g	1.0 nmol/g	Qiang et al. (2005)
Spinach	11.4 mg/100 g	[a]0.8	Jones et al. (1992)
Spinach	313 nmol/g	—	Demirkol et al. (2004)
Spinach	377 nmol/g	91.8 nmol/g	Qiang et al. (2005)
Cucumber	3.5 100 g/mg	[a]0.8	Jones et al. (1992)
Cucumber	123 nmol/g	—	Demirkol et al. (2004)
Cucumber	68.8 nmol/g	0.3 nmol/g	Qiang et al. (2005)
Parsley	17 nmol/g	—	Demirkol et al. (2004)
Tomato	7.5 mg/100 g	[a]1.5 mg/100 g	Jones et al. (1992)
Tomato	64 nmol/g	—	Demirkol et al. (2004)
Red sweet pepper	5.5 mg/100 g	[a]0.4 mg/100 g	Jones et al. (1992)
Red pepper	42 nmol/g	—	Demirkol et al. (2004)
Red pepper	72.8 nmol/g	1.33 nmol/g	Qiang et al. (2005)
Green bell pepper	3.4 mg/100 g	[a]2.1 mg/100 g	Jones et al. (1992)
Green pepper	8 nmol/g	—	Demirkol et al. (2004)
Okra	11.3 mg/100 g	[a]0.7 mg/100 g	Jones et al. (1992)
Cauliflower	4.0 mg/100 g	[a]5.1 mg/100 g	Jones et al. (1992)
Potato	5 nmol/g	—	Demirkol et al. (2004)
Avocado	20.6 mg/100 g	[a]7.1 mg/100 g	Jones et al. (1992)
Avocado	339 nmol/g	—	Demirkol et al. (2004)
Green bean	230 nmol/g	—	Demirkol et al. (2004)
Green bean	309 nmol/g	9.7 nmol/g	Qiang et al. (2005)
Orange	4.8 mg/100 g	[a]2.5 mg/100 g	Jones et al. (1992)
Orange	5 nmol/g	—	Demirkol et al. (2004)
Orange Juices	11.6 μmol/L	—	Kubalczyk and Bald (2009)
Lemon	4.8 mg/100 g	[a]3.9 mg/100 g	Jones et al. (1992)
Lemon	5 nmol/g	—	Demirkol et al. (2004)
Grapefruit	6.5 mg/100 g	[a]1.4 mg/100 g	Jones et al. (1992)
Grapefruit	13 nmol/g	—	Demirkol et al. (2004)
Mango	4.3 mg/100 g	[a]0.5 mg/100 g	Jones et al. (1992)
Mango	59 nmol/g	—	Demirkol et al. (2004)
Papaya	5.8 mg/100 g	[a]0.6 mg/100 g	Jones et al. (1992)
Papaya	136 nmol/g	—	Demirkol et al. (2004)
Strawberry	39 nmol/g	—	Demirkol et al. (2004)
Strawberry	6.9 mg/100 g	[a]0.2 mg/100 g	Jones et al. (1992)

continued

TABLE 4.1 (continued)

GSH and GSSG Concentrations of Some Food Reported in the Literature

Food	GSH	GSSG	Reference
Strawberry	62.8–81.7 nmol/g	9.4–11.2 nmol/g	Wang and Lin (2003)
Blackberry	78.7 nmol/g	16 nmol/g	Jiao and Wang (2000)
Peach	5.0 mg/100 g	[a]2.4 mg/100 g	Jones et al. (1992)
Banana	3.3 mg/100 g	[a]0.8 mg/100 g	Jones et al. (1992)
Apple	1.5 mg/100 g	[a]1.8 mg/100 g	Jones et al. (1992)
Pear	3.3 mg/100 g	[a]1.7 mg/100 g	Jones et al. (1992)
Cantaloupe	6.1 mg/100 g	[a]0.8 mg/100 g	Jones et al. (1992)
Watermelon	5.0 mg/100 g	[a]1.6 mg/100 g	Jones et al. (1992)
Carrot	5.9 mg/100 g	[a]2.0 mg/100 g	Jones et al. (1992)
Lettuce	1.1 mg/100 g	[a]1.5 mg/100 g	Jones et al. (1992)
Grape	2.7 mg/100 g	[a]0.3 mg/100 g	Jones et al. (1992)
Grape juice	1.28 mg/L	—	Park et al. (2000)
Grape juice	36.5–39.6 mg/L	—	Maggu et al. (2007)
Nectarines	4.9 mg/100 g	[a]2.5 mg/100 g	Jones et al. (1992)
Nuts and seeds-walnuts	3.7 mg/100 g	[a]11.4 mg/100 g	Jones et al. (1992)
Lupine	100 nmol/g fwt	—	Vázquez et al. (2009)
Legume	188 nmol/g	—	Matamoros et al. (2003)
Wheat flour	18–89 nmol/g	12–22 nmol/g	Schofield and Chen (1995)
Wheat flour	32–280 nmol/g	—	Levavasseur et al. (2006)
Fish	1.7 μg/mg protein		Gopal et al. (2009)
Fish	0.58 μmol g/tissue	0.055 μmol g/tissue	Monteiro et al. (2010)
Chicken/Turkey	7.7 mg/100 g	[a]1.0 mg/100 g	Jones et al. (1992)
Beef (fat trimmed off)	12.3 mg/100 g	[a]1.1 mg/100 g	Jones et al. (1992)
Pork (fat trimmed off)	18.9 mg/100 g	[a]4.7 mg/100 g	Jones et al. (1992)
Beer	1.1 mg/100 g	[a]0.1 mg/100 g	Jones et al. (1992)
Table wine, red	0.7 mg/100 g	[a]0.9 mg/100 g	Jones et al. (1992)
Table wine, white	2.3 mg/100 g	[a]0.6 mg/100 g	Jones et al. (1992)
Wine	5.1 mg/L	—	Park et al. (2000)
Mustard	820 nmol/g dry weight	—	Manda et al. (2010)
Fenugreek	519 nmol/g wet weight	—	Manda et al. (2010)
Ginger	1076 nmol/g wet weight	—	Manda et al. (2010)
Turmeric	41 nmol/g wet weight	—	Manda et al. (2010)
Coriander	35 nmol/g wet weight	—	Manda et al. (2010)
Cardamom	112 nmol/g wet weight	—	Manda et al. (2010)

[a] GSSH values are from Jones et al. (1992), recalculated in total GSH-reduced GSH.

4.5 General Preparation Methods for the Determination of Glutathione in Food

4.5.1 Sample Preparation

The weakest point in the GSH analysis is that GSH can be easily autooxidized nonenzymatically in pH > 7. Because of this, it has been strongly recommended that the pH of the medium be kept acidic (Camerea and Picarda 2002, Monostori et al. 2009). Ethylenediamine tetraacetic acid (EDTA), as an anticoagulant and chelator, prevents the formation of some oxidative reactions by binding to many transition metals (including Fe^{2+}). Use of an important Fe^{2+} chelator, diethylenetriaminopentaacetic acid (DETAPAC), is also suggested for the same purpose (Demirkol et al. 2004, Manda et al. 2010, Monostori et al. 2009, Toyo'oka, 2009, Winters et al. 1995).

4.5.2 Reduction of Disulfides

Reduction of disulfide bonds between GSH and GSSG and other thiols and proteins are necessary before the analysis of GSH and GSSG. GSH, formed as a result of a reduction process, could be reoxidized before the derivatization. The unpredictableness of these types of reactions may result in false detection. This phenomenon can be prevented by 2-vinyl pyridine (2-VP) or iodoacetic acid (IAA) thiol masking agents (Camera and Picardo 2002, Monostori et al. 2009, Pastore et al. 2003). This property of 2-VP was used to identify GSSG in foods. The GSSG can be analyzed by blocking the existing GSH by 2-VP and reducing the GSSG by glutathione reductase enzyme (Monostori et al. 2009, Qiang et al. 2005, Winters et al. 1995). Choosing the right reducing agent is of critical importance for performing the experiment. Sulfhydryl-containing reducing agents like dithioerythritol, dithiothreitol (DTT), or 2-mercaptoethanol (2-ME) might negatively affect the determination of free or bound GSH. These aforementioned reagents may react with monobromobimane (MBB) or *o*-phthalaldehyde (OPA). Sodium and potassium borohydride are other kinds of reductants, but they are not soluble in water and they make unstable solutions. At high concentrations, reduction can be completed in a few minutes, whereas this time could be as high as 30 min at lower concentrations (40–100 mM). Adding monophosphoric acid (MPA) or a surface-active agent (e.g., octanol) can prevent formation of gas or foam during reaction (Camera and Picardo 2002, Iwasaki et al. 2009, Monostori et al. 2009).

Dithionite (DT, sodium hydrosulfite) is another reductant used in reduction of disulfide. DT takes one equivalent thiol instead two from the disulfites treated with reducing agents (Baeyens et al. 1988, Camera and Picardo 2002, Davey et al. 2003, Monostori et al. 2009). Among the very strong reductants are trialkylphosphines in which tributylphosphine (TBP) and triphenylphosphine (TPP) are the specific ones. In order to complete the disulfide reduction, a slight molar excess of reagents is sufficient. Most of the trialkylphosphines do not react with thiol-specific reagents. The other reagent in this group is tris (2-carboxyethyl) phosphine (TCEP). TCEP has the highest solubility in water and does not vaporize and it is neither sensitive to moisture nor to air. However, TCEP may prevent the actions of disulfite containing derivatizing agent 5,5-dithio-bis-2-nitrobenzoic acid (DTNB) (Camera and Picardo 2002, Iwasaki et al. 2009, Kubalczyk and Bald 2009, Monostori et al. 2009).

4.5.3 Protein Removal

Proteins should be eliminated from the samples because they decrease the analysis effectiveness and life of GSH and GSSG. Only a few methods, like NMR, do not require the elimination of proteins in the samples. Deproteinization by addition of organic solutions like acetonitrile, acetone, or methanol or ultracentrifugation is sufficient to eliminate the proteins (Monostori et al. 2009). On the other hand, using membrane filters are advantageous over using acid and organic solvents which could affect separation, derivatization, and detection. Separation of GSH and GSSG could be obtained by using membrane filters (Camera and Picardo 2002).

The acidic agents used in GSH analysis include 5-sulfosalicylic acid (5-SSA), trichloroacetic acid (TCA), trifluoroacetic acid (TFA), perchloric acid (PCA), or MPA. If a mass spectrometer is used as a

detector, then organic solvents are preferred since they do not affect autooxidation of GSH in the presence of acid. Moreover, thiol concentrations fall quickly in neutral-alkali pH if thiol-masking agents are not used in acidic samples. For this reason, derivatization of aminothiols requires chromophores and fluorophores treatment. These mixtures contain IAA, OPA, ammonium 7-fluoro-2,1,3-benzoxadiazole-4-sulfonate (SBDF), and 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F) (Camera and Picardo 2002, Iwasaki et al. 2009, Monostori et al. 2009, Pastore et al. 2003).

4.5.4 Derivatization

There are numerous techniques for determining the presence of GSH and other thiols. Almost all methods are based on derivatization, except for *in vivo* analysis, electrochemical and tandem-mass spectrometry detection (Monostori et al. 2009, Pastore et al. 2003). However, Davey et al. (2003) used an improved method to measure GSH and GSSG in food without derivatization by using a short rocket power column with a diode array detector (DAD).

The best derivatization agent should be able to react with thiols to form absorbance and fluorescent yields at very low concentrations. Also, the ideal reagent should not show any absorption or fluorescent characters by itself. They should form stable and fast reactions with thiols (Monostori et al. 2009, Pastore et al. 2003).

Limit of detection (LOD) could be improved with a suitable derivatizing agent for the detection of GSH and GSSG because GSH, GSSG, and their analogs do not have innate chromophores or fluorophores. Besides, their indirect analysis is possible. Experiments using indirect measurements are based on the modification (decrease) in the uniform signal in the background. Extended path-length UV (Ultraviolet) absorbance detectors give satisfactory detection limits for the derivatized GSH and GSSG. GSH analysis with an electrochemical detection (ECD) system does not depend on derivatization (Monostori et al. 2009). Demirkol et al. (2004) modified the technique developed by Winters et al. (1995), in which GSSG was reduced by GR, followed by derivatization of newly formed GSH with the fluorophore reagent *N*-(1-pyrenyl)-maleimide (NPM) while protecting (blocking) the existing GSH 2-VP. 2-VP does not inhibit GR; therefore, it is not necessary to remove it from the sample. In this method, NPM is used to derivatize GSH and this derivatization reaction is complete within a minute (Qiang et al. 2005, Ridnour et al. 1999).

Reaction of thiol compounds with OPA and 2-aminoethanol yields high fluorescent compound isoindole and its derivatives. A thiol coreagent is not necessary when GSH reacts with OPA. The primary amino group in GSSG may react with OPA. The thiol functional group has to be available for derivatization after the reduction of GSSG (Camera and Picardo 2002, Iwasaki et al. 2009, Monostori et al. 2009, Pastore et al. 2003).

The order of the addition of a reagent affects the yield of reaction, as Park et al. (2000) stated. For example, maximum sensitivity is achieved when a thiol sample is added to OPA first, then 2-aminothiol is added to the mixture. The 2-aminothiol addition first resulted in a peak area that was approximately 40% smaller.

1-Fluoro-2,4-dinitrobenzene (FDNB) is the colorimetric reagent that is most commonly used to determine the GSH content in foods. The amino group in FDNB reacts with GSH and forms derivatives of *S*-carboxymethyldinitrophenyl (DNP) (Monostori et al. 2009, Wierzbicka et al. 1989). He et al. (2004) compared the use of dansyl chloride and FDNB in the HPLC-fluorescence detector (FLD) method to analyze and determine the GSH in foods and found that dansyl chloride presented very sensitive results.

Bimanes like MBB easily reacts with various thiols and forms thioethers at pH 8.0 at room temperature. Some of the disadvantageous of this reagent include its ability to be self-fluorescent character and its ability to photodegrade and to form hydrolysis products (Baeyens et al. 1988, Iwasaki et al. 2009).

One other reagent in this group to be used in the analysis of food is DTNB (Elman's reagent). DTNB, a classic spectrophotometric method developed by Tietze (GR-coupled enzymatic recycling or GSH-recycling assay) was used to derivatize GSH and GSSG. DTNB was used as a UV label for thiols (Toyo'oka, 2009).

4.6 Methods

4.6.1 HPLC Methods

Many HPLC methods for determining GSH and related compounds use ultraviolet–visible (UV–Vis), DAD, ECD, mass spectrometry (MS), and FLD systems (Camera and Picardo 2002). A summary of the HPLC methods reviewed in this chapter is given in Table 4.2.

TABLE 4.2

Summary of Recent HPLC Methods for the Determination of GSH in Food

Instrument	Food Samples	Derivatization	Procedure	Column	Reference
UV	Fruit and fruit juices, vegetables, meat, fish, eggs, poultry, breads, cereals, legumes, nuts	IAA and FDNB	DTT reduction	C18 amine column	Jones et al. (1992)
			Reacted in excess sodium bicarbonate at room temperature for 15 min (IAA)		
			Reacted at room temperature for 4 h in the dark (FDNB)		
DAD	Apple	—	Blending half of the fruit slices in 2 volumes (mL/gfw) of chilled	Short Rocket column	Davey et al. (2003)
			Extraction solvent (6% MPA/2 mM EDTA/2% PVPP)		
UV	Wheat flour	IAA and FDNB	Reacted in excess sodium bicarbonate at room temperature for 15 min (IAA)	Amino bonded phase silica column	Schofield and Chen (1995)
			Reacted at room temperature for 4 h in the dark (FDNB)		
UV	Fruit and vegetables, meat, fish, dairy products, cereal grains, and processed and miscellaneous foods	IAA and FDNB	Reacted in excess sodium bicarbonate at room temperature for 15 min (IAA)	Ultrasil-NH_2 column	Wierzbicka et al. (1989)
			Reacted at room temperature for 4 h in the dark (FDNB)		
UV	Wheat flour	FDNB	Extraction with 5% (w/v) PCA, deproteinization with DTT (pH 8) alkylation with IAA	Amino bonded phase silica column	Li et al. (2004)
FLD	Grape juice and wine	OPA and 2-aminoethanol	Sample diluation with sodium acetate buffer (pH 4) containing 0.1 mM EDTA	C18	Park et al. (2000)

continued

TABLE 4.2 (continued)

Summary of Recent HPLC Methods for the Determination of GSH in Food

Instrument	Food Samples	Derivatization	Procedure	Column	Reference
ND	Lupine	DTNB	Grounding sample in 0.1 N HCl (1 ml g/tissue). Incubation on ice for 10 min, mixing with 2 mM NAC	N.D	Vázquez et al. (2009)
LC-MSMS	White grape juices and wines	—	For grapes: crushing under CO_2, adding SO_2 and AA For wine: Evaporating at 40°C under vacuum	C18	du Toit et al. (2007)
LC-MSMS	Wheat flour	Dansyl chloride	Reduction with TCEP, treated with IAA	C18	Reinbold et al. (2008)
DAD	Fish and shellfish	ND	ND	Analytical Supelcosil NH_2 column	Passi et al. (2002)
FLD	Skim milk, 2% milk, biscuit, and pork sausage	Dansyl chloride	Reacted with 1 mmol/L DTT	ND	He et al. (2004)
ECD	Wheat flour	—	Homogenized with *m*-phosphoric acid in an ice, centrifuged at 4°C	ND	Levavasseur et al. (2006)
ECD	Grape juices	—	—	C18	Maggu et al. (2007)
FLD	Legume	MBB	Extracted with MSA containing DETAPAC	ND	Matamoros et al. (2003)
FLD	Fruits and vegetables	NPM	Homogenized in serine borate buffer, pH 5.0, Reacted at room temperature for 5 min	C18	Demirkol et al. (2004)
FLD	Spices	NPM	Homogenized in serine borate buffer, pH 5.0, Reacted at room temperature for 5 min	C18	Manda et al. (2010)

Note: ND—not described.

HPLC techniques are more frequently used to analyze the content of GSH and other thiols in foods than other methods. HPLC methods are fast, have high specificity, and are very reproducible (Pastore et al. 2003). As indicated in the Pastore et al. review article (2003), the DTNB-based HPLC-UV method for GSH analysis was first developed by Reeve and Kuhlenkamp. This was then modified by Katrusiak et al. In addition, Reed et al. (1980) have further modified the method making possible oxidized forms of GSH and related thiols analyses, free thiols blockage by IAA, and derivatization of amino groups with FDNB (Iwasaki et al. 2009, Pastore et al. 2003). Detection with colorimetric reagents and UV absorbance are not sensitive, but it is simpler when compared to FLD or ECD (Monostori et al. 2009). This method is widely used for the determination of GSH and GSSG in foods (Jones et al. 1992, Reed et al. 1980, Schofield and Chen 1995, Wierzbicka et al. 1989).

The method that Davey et al. (2003) developed can detect ascorbic acid (AA), dehydroascorbate, and both reduced and oxidized GSH by using a short Rocet HPLC column and DAD in as short a time as 6 min. Various other studies have used the same method (Davey and Keulemans 2004, Davey et al. 2004).

HPLC-FLD is the most commonly used method for detecting GSH, GSSG, and other thiols because of its high sensitivity and specificity (Iwasaki et al. 2009). The derivatizing agents utilized in this method react preferentially with the amine groups. On the other hand, when NPM is used to measure

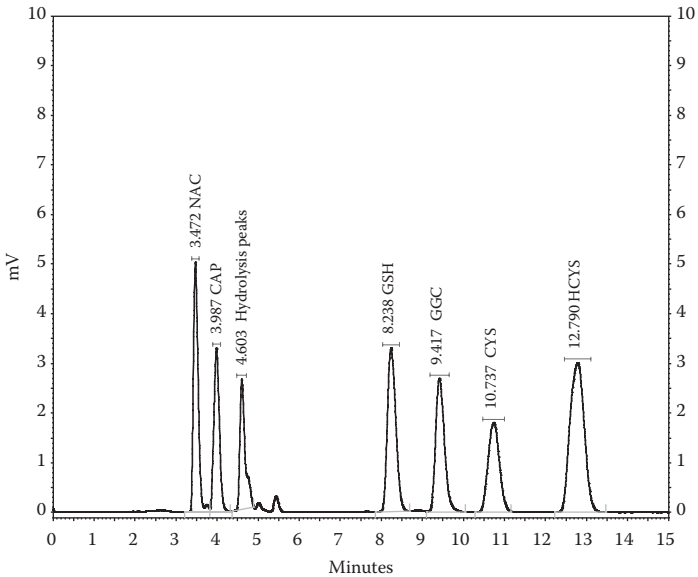

FIGURE 4.2 HPLC chromatogram of NPM derivatives. NAC, *N*-acetyl-L-cysteine derivative; CAP, captopril derivative; GSH, glutathione derivative; GGC, γ-L-glutamyl-L-cysteine derivative; CYS, cysteine derivative; HCYS, homocysteine derivative. (Reproduced from Qiang Z. et al. 2005. *Journal of Agricultural and Food Chemistry*, 53(25), 9830–9840. With permission.)

GSH concentrations in foods, it reacts with the sulfhydryl functional group in GSH during analysis. HPLC-FLD assays for GSH and GSSG have been described on the basis of the reaction between NPM and the sulfhydryl groups of thiols (Demirkol 2009, Demirkol et al. 2004, 2008, Kullman et al. 2000, Manda et al. 2010, Qiang et al. 2005, Winters et al. 1995). This method allowed the determination, not only of GSH and GSSG, but also the other thiols such as captopril (CAP), NAC, CYS, homocysteine (HCYS), and γ-glutamyl cysteine (GGC) (Figures 4.2 and 4.3) (Qiang et al. 2005).

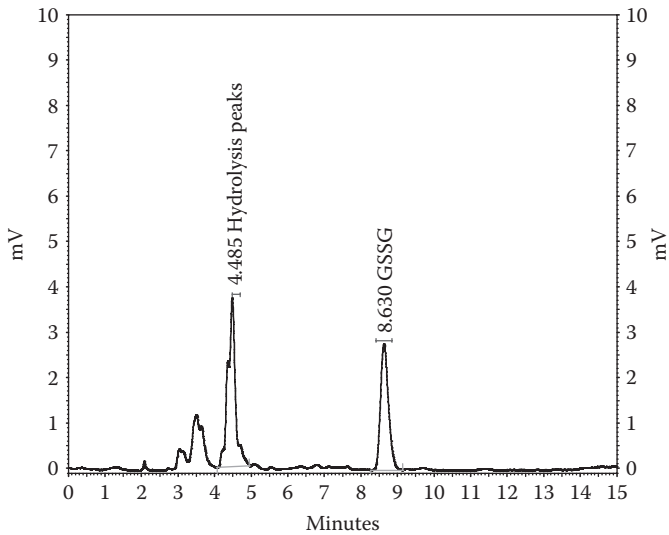

FIGURE 4.3 HPLC chromatogram of NPM derivative. GSSG, oxidized glutathione. (Reproduced from Qiang Z. et al. 2005. *Journal of Agricultural and Food Chemistry*, 53(25), 9830–9840. With permission.)

HPLC with ECD is a very important method for the analysis of redox reactive compounds like thiols and disulfides, including GSH. The very first study performed by Saetre and Rabenstein in 1978 measured the concentration of GSH in the juice of fruits and vegetables. In this study, the total amount of GSH was detected. The separate analyses and detection of GSH and GSSG were done by using ECD. The content of GSH and GSSG in some vegetables was measured by using dual ECD in the aforementioned method (Mills et al. 1997). The first reason why the ECD method provided improved thiol analysis was that thiol oxidation requires slow and generally wide overpotentials (≥+ 1.0 V) with hard electrodes. This problem was solved by using mercury or mercury amalgams with a direct detection method. Thiols interact with mercury-based electrodes and form stable mercury thiolate complexes. Alternatively, recent studies showed that the requirement of mercury-based electrodes is eliminated by using modified electrodes with electrocatalysts such as cobalt phthalocyanine, Prussian blue, ruthenium cyanide, aquocobalamin, copper hexacyanoferrate, and bidoped in PbO_2 (Inoue and Kirchhoff 2000).

The advantages of HPLC-MS detection are that it (1) is automated, (2) requires a small sample size, and (3) is extremely sensitive and specific (Monostori et al. 2009). The content of GSH and GSSG was analyzed in must and wine by the LC-MS/MS method developed by du Toit et al. (2007). It is possible to analyze the samples without a derivatization step by using this method. However, usage of LC-MS/MS is limited because of its expensive instrumentation and complexity of operation (Iwasaki et al. 2009, Pastore et al. 2003).

4.6.2 Spectrophotometric Methods

The most popular spectrophotometric method for determination of GSH was developed by Owens and Belcher, based on an enzymatic recycling reaction identified by Tietze. This method, which determines the total GSH, is more specific than the spectrophotometric method using Ellman's reagent. This method, known as either the "GR-coupled enzymatic recycling assay" or the "GSH-recycling assay," reduces GSSG to GSH in the presence of DTNB, GR, and NADPH. The reaction with DTNB yields chromophore 5-thionitrobenzoate. The change in color at 412 nm is used to analyze GSH. GSSG measurement is possible when GSH is blocked with NEM. This method is still commonly used for the direct determination of GSH and GSSG in foods. Its simplicity, satisfactory sensitivity, and cost are important advantages of this method (Ates et al. 2009, Gopal et al. 2009, Jiao and Wang 2000, Monostori et al. 2009, Monteiro et al. 2010).

4.6.3 Capillary Electrophoresis Methods

Capillary electrophoresis is known as a highly effective and ultra-small-volume analytical method. For example, direct measurement of GSH and GSSG in red blood cells can be completed in <90 s. The lack of automation and inability to measure GSH bound to protein are some of its disadvantages (Pastore et al. 2003). Ercal et al. (1996) directly measured GSH and GSSG in biological samples by using the capillary zone electrophoresis (CZE) method. Total GSH and CYS were derivatized with 2-chloro-1-methylquinolinium (CMQT), followed by CZE-UV, and measured in orange juice samples by Kubalczy and Bald (2009). This method is based on reduction of thiols by TCEP (Toyo'oka, 2009).

4.7 Conclusion

GSH, one of the most prevalent thiol compounds, has an important role as an intracellular antioxidant. It is converted to GSSG by both enzymatic and nonenzymatic processes. At present, the GSH levels in food can be measured by various methods. Among these methods, HPLC-FLD and UV are the most preferred techniques; spectrophotometric and CZE are the other techniques. However, the number and variety of studies that measure GSH levels in food are limited when compared to the ones used to measure GSH in biological samples (cells, blood, and tissue). Given the importance of GSH in biological functions that

are necessary for a healthy life, presently available methods should be refined and new, simpler methods should be developed to measure the concentrations of GSH in foods.

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5

L-Carnitine

Jean Demarquoy, Caroline Rigault, and Françoise Le Borgne

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5.1 Introduction

L-Carnitine (LCAR) is mainly known as a compound essential for the production of energy from fat. LCAR is a cofactor for several enzymes implicated in various metabolic pathways involving fatty acids such as the mitochondrial and the peroxisomal oxidation of fatty acids and the regulation of the pool of

coenzyme A (CoA). LCAR is also involved in the acetylation of proteins and the scavenging of free radicals. Due to this implication in critical pathways, LCAR can be regarded as an important compound.

Extensive research has suggested potential application of LCAR supplementation in various situations such as weight management, infant nutrition, improvement of sport performance, reproductive ability, and wellness. In this chapter, we review the information available on origin of natural LCAR, LCAR physiology and discuss on a potential interest for LCAR supplementation in humans.

5.2 Historical Background

LCAR is a natural compound synthesized in almost all living cells from bacteria to human with the exception of several insects and young mammals. LCAR was first isolated from flesh in 1905 (Gulewitsch and Krimberg, 1905) and its name was derived from Latin *carnis* that can be translated in English by meat. Carnitine is a derivative of lysine and methionine. LCAR composition and chemical structure were determined in the early twentieth century. Its chemical name is β-hydroxy-γ-*N,N,N*, trimethylaminobutyrate or 3-hydroxy-4-trimethyl ammoniobutanoate. Its molecular weight is 161.2 and its structure presented in Figure 5.1.

Even after its isolation, LCAR remained about half a century without known physiological function until the work of Fraenkel in 1957. This author showed that LCAR was implicated in the development of the worm, *tenebrio molitor* (Fraenkel and Friedman, 1957) and subsequently, LCAR was considered as a vitamin and was called vitamin Bt. Several years later, it was described that mammals, including humans, can synthesize LCAR. After this discovery, LCAR was not called vitamin anymore. However, as its biosynthesis is dependent on the food supply of various compounds including ascorbic acid, LCAR is sometimes regarded as a vitamin-like compound as lipoic acid and coenzyme Q10 (CoQ10). In the middle of the twentieth century, biochemical and physiological roles for LCAR were identified.

In the human body as well as in food, LCAR is present either as free LCAR or as acyl-carnitine (acyl esters of LCAR). The length of the acyl chain may vary, from acetyl to very long-chain acyls. Acyl-carnitines are produced by a family of enzymes: the acyl-carnitine transferases. Several acyl-carnitine transferases have been characterized; they exhibit different cellular localizations and acyl chain-length affinities (Ramsay et al., 2001). The ratio between free and esterified carnitine varies in the cell according to its energy metabolism.

5.3 LCAR in Human Body

In the human body, LCAR is found in all organs and tissues but most of the carnitine is concentrated in muscles. L-Carnitine concentration is 50–200 times higher in muscle than in plasma. In the blood, LCAR concentration ranges from 40 μM in females to 50 μM in males, that is, 8.06 mg/L. Sex hormones may influence the regulation of LCAR plasma concentration (Takiyama and Matsumoto, 1998). However, in adults, these values are grossly independent of the nature of the diet, suggesting that a fine balance exists between dietary LCAR and biosynthesis to maintain adequate levels (Rebouche, 2004). In infants, the plasma concentration of LCAR increases during the first years of life reaching the adult concentration at the age of puberty.

LCAR is excreted from the body in the urine and the bile. These losses are low, usually lower than 60 mg/day and can be even lowered in case of low intake of LCAR (Rebouche, 2006).

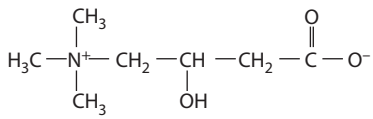

FIGURE 5.1 Chemical structure of carnitine.

LCAR present in humans comes from both exogenous origin and endogenous biosynthesis. Due to this duality of origin, primary deficiencies in LCAR are rare. They may occur when intracellular transport of LCAR is inhibited or inefficient. Primary carnitine deficiency is caused by an alteration in the plasma membrane carnitine uptake causing urinary carnitine wasting and in fine systemic carnitine depletion. Drugs used for AIDS treatment, valproic acid used for the treatment of epilepsy, and kidney dialysis may cause a secondary carnitine deficiency usually by impairing renal tubular reabsorption of carnitine or blocking LCAR uptake in muscle or nerve cells. These deficiencies lead to anomalies in muscle and/or nerve functions (Tanphaichitr and Leelahagul, 1993).

5.4 Dietary Carnitine

LCAR is commonly present in edible products but levels of carnitine are especially high in meat, dairy products, and in some fishes. Fruits and vegetables are usually poor in LCAR.

5.4.1 Methods for Measuring LCAR Levels

Various methods using either radioactive or cold reagents can be used for the determination of LCAR and acyl-carnitine levels in tissues, organs, and food. Total carnitine refers to the sum of free and esterified carnitine.

Methods using classical spectrophotometric measurement of LCAR can be used. These assays are usually based on the reaction, catalyzed by the carnitine acetyl transferase (EC 2.3.1.7), of transesterification of LCAR with acetyl CoA to form acetyl l-carnitine (ALCAR) and free CoASH. To monitor this reaction, the free CoASH formed in the reaction is estimated using thiol-group color reagents such as 5,5′ dithiobis-(2-nitrobenzoic) acid or an enzymatic reaction (De Palo et al., 1987; Deufel, 1990). These techniques can be done either manually or can be automated (Galan et al., 1998).

Radioactive methods include techniques similar to those described above except that the formation of ALCAR is monitored using radioactive compounds (Cederblad et al., 1990; Demarquoy et al., 2004). Kossak described in 1992 (Schmidt-Sommerfeld et al., 1992) a radioisotopic exchange/high-performance liquid chromatography method that identifies LCAR in urine. A few years ago, an assay using (2)H(31)-palmitate and electrospray ionization tandem mass spectrometry (ESI-MS/MS) was developed for the acyl-carnitine profiling in human skin fibroblasts (Law et al., 2007).

ESI-MS/MS is currently a method widely used for the determination of acyl-carnitine in various tissues and samples. In 2002, Okun described a method for quantitative acyl-carnitine profiling in human skin fibroblasts using ESI-MS/MS and unlabeled palmitic acid. The same kind of technique was used to follow urinary acyl-carnitine profiles in several clinical conditions (Kobayashi et al., 2007). More recently, Smith and Matern (2010) described a method that can be used to measure acyl-carnitine species with several carbon chain lengths in various biological samples, including plasma, dried blood, bile, and also urine. This method used derivatization to butylesters and an ESI-MS/MS.

Spectrophotometrical methods are easy to use, costless but are limited in terms of sensitivity. Methods using radiochemical compounds are usually sensitive, not very expensive but need the ability to use radioelements. Methods such as ESI-MS/MS are sensitive, reliable but need a strong financial investment. Using either of these methods, LCAR content was estimated in various foods.

5.4.2 L-Carnitine in Meat

Levels of carnitine in raw meat products are comprised between 6 and 166 mg/100 g. Among regular meat products, veal and beef appeared to be the best sources for LCAR intake. Slight differences are found between the various cuts but the amount of LCAR in these food samples was always higher than 60 mg/100 g of wet weight (Table 5.1). Pork and lamb seem to have lower amounts of LCAR than bovine meat. The level of LCAR in these meat samples is comprised between 20 and 40 mg/100 g. This is less than half of what is found in beef products. Kangaroo appears also as a good (probably the best) source for LCAR. Horse meat is also rich in LCAR (Demarquoy et al., 2004; Seline and Johein, 2007). Broilers

TABLE 5.1

Carnitine Level in Meat Products

	Carnitine (mg/100 g)
Beef	
Steak	65
Tenderloin	79
T-bone	84
Veal	
Shoulder	78
Sirloin	133
Lamb	
Chop	40
Pork	
White ham	33
Shoulder	21
Leg	18
Others	
Chicken muscle	10
Duck muscle	27
Horse (muscle)	117
Kangaroo steak	166
Pigeon (muscle)	16
Quail	17
Turkey	21

Source: Data from Seline, K.-G. and Johein, H. 2007. *Food Chem*, 105 (2): 793–804; Demarquoy, J. et al. 2004. *Food Chem*, 86 (1): 137–142; and unpublished results.

Note: LCAR concentration in different meat products expressed in mg/100 g of food (wet weight).

have relatively low levels of LCAR, mostly around 20 mg/100 g. Most significant data regarding carnitine content in various meat products are presented in Table 5.1.

Meat is usually not eaten crude but previously cooked or frozen and cooked. Cooking is known to induce a loss of some nutrients. This may happen because some compounds such as vitamins are thermosensitive or because they are lost during the cooking process, for example, in the water after boiling, as for instance vitamin C (Nursal and Yucecan, 2000). This is also the case for some minerals (Gokoglu et al., 2004). Boiling, frying, grilling, baking, steaming, or even microwave cooking did not modify LCAR content in meat (Rigault et al., 2008).

5.4.3 L-Carnitine in Fish

Fishes contain about 10 times less carnitine than meat. In fishes, the amount of LCAR was found to be between 1 and 6 mg/100 g of raw or cooked fish (Table 5.2). Among the best sources of LCAR, one can find salmon. However, in salmon, LCAR content is 10–12 times less than in beef cuts. Cooking salmon does not modify LCAR content except the smoking process that seemed to induce a complete loss of LCAR (Rigault et al., 2008).

5.4.4 LCAR in Dairy Products

LCAR is also present in milk and dairy products. In milk, a concentration of 2–3 mg/100 mL was recorded (Demarquoy et al., 2004; Woollard et al., 1999). In cheeses, the concentration of LCAR ranges from 4 to 20 mg/100 g (Table 5.3). Camembert, goat cheese, and Gruyere are good sources for LCAR. Butter, a special dairy product made from the fat portion of milk, is very poor in LCAR.

TABLE 5.2

Carnitine Level in Fishes

	Carnitine (mg/100 g)
Cod (raw)	2
Hake (boiled)	3
Whiting (boiled)	2
Salmon (raw)	6
Tuna (white, boiled)	1

Source: Data from Seline, K.-G. and Johein, H. 2007. *Food Chem*, 105 (2): 793–804; Demarquoy, J. et al. 2004. *Food Chem*, 86 (1): 137–142; and unpublished results.

Note: LCAR concentration in different meat products expressed in mg/100 g of food (wet weight).

5.4.5 LCAR in Fruits and Vegetables

LCAR content is low in vegetables and fruits. Some fruits and vegetables are even free of LCAR, asparagus and tomato belong to this group (Table 5.4). On the other hand, herbs such as sage or chive are usually relatively rich in LCAR with up to 5 mg of LCAR/100 g of product. However, the quantitative importance of herbs in the total food intake is low and herbs should not participate significantly in LCAR supply (Table 5.5).

5.4.6 Bioavailability and General Metabolism of LCAR

The bioavailability of dietary carnitine has been found ranging from 54% to 87% (Evans and Fornasini, 2003; Rebouche and Chenard, 1991) depending on carnitine content and the composition of the food (Rebouche and Chenard, 1991). While bioavailability of LCAR is relatively high when coming from the diet, absorption of LCAR from supplements is much lower and only 15% of LCAR efficiently absorbed (Evans and Fornasini, 2003). LCAR from supplements appeared to be less absorbed, highly cleared by the kidneys, poorly up taken by the tissues (Rebouche and Engel, 1984). Even when high doses are given

TABLE 5.3

Carnitine Level in Dairy Products

	Carnitine (mg/100 g)
Milk dry	10
Milk 2% fat	2.9
Butter	1.3
Brie	
Camembert	14.4
Comté	12.2
Goat cheese	15.3
Gruyère	6.5
Munster	19.8
Reblochon	4.4
Yogurt	12.5
Fromage blanc	1.8

Source: Data from Seline, K.-G. and Johein, H. 2007. *Food Chem*, 105 (2): 793–804; Demarquoy, J. et al. 2004. *Food Chem*, 86 (1): 137–142; Woollard, D.C., Indyk, H.E., and Woollard, G. A. 1999. *Food Chem*, 66 (1): 121–127; and unpublished results.

Note: LCAR concentration in different meat products expressed in mg/100 g of solid food (wet weight) or 100 mL of liquid.

TABLE 5.4

Carnitine Level in Fruits and Vegetables

	Carnitine (mg/100 g)
Fruits	
Apple	0.2
Apricot	0.5
Banana	0.2
Kiwi	0.2
Guava	0.2
Mango	0.8
Pear	0.3
Vegetables	
Asparagus	0
Carrot	0.4
Chanterelle	1.3
Cauliflower	0.4
Corn	0.2
Cucumber	0.1
Lentil	0.2
Noodle	0
Olive	0.5
Onion	0.7
Pea	0.2
Potato (boiled)	0
Rice (boiled, white)	0
Spinach	0
Tomato	0
Zucchini squash	1.1

Source: Data from Seline, K.-G. and Johein, H. 2007. *Food Chem*, 105 (2): 793–804; Demarquoy, J. et al. 2004. *Food Chem*, 86 (1): 137–142; and unpublished results.

Note: LCAR concentration in different meat products expressed in mg/100 g of food (wet weight).

TABLE 5.5

Carnitine Level in Spices, Herbs, and Nuts

	Carnitine (mg/100 g)
Chive	4.7
Garlic	1.3
Ginger root	0.2
Hazel	0.2
Manioc	0
Mint	0.8
Peanut	0.2
Pinion	2.2
Raisin	0.8
Taro	1.8

Source: Data from Demarquoy, J. et al. 2004. *Food Chem*, 86 (1): 137–142; and unpublished results.

Note: LCAR concentration in different meat products expressed in mg/100 g of food (wet weight).

orally, only a very moderate increase in LCAR plasma concentration is observed (Evans and Fornasini, 2003; Rebouche, 2004). For therapeutic application, intravenous administration of LCAR appeared to be much more efficient (Brass et al., 1994; Harper et al., 1988). It is not clear if an increase in plasma LCAR level may induce an increase in intracellular levels of LCAR.

Average intake of free LCAR per day and per capita was estimated (Mitchell, 1978) and recently reevaluated in Demarquoy et al. (2004). As LCAR is mainly found in meat products, the individual intake is dependent on food preferences and varies from 10 to 100 mg/day. A regular diet provides around 80 mg of carnitine/day, 60 out of 80 mg is provided by meat products. About 15% are supplied by dairy products and eggs. Fruits and vegetables provide the remaining (between 5% and 10%).

Healthy humans excrete carnitine at a rate of 5 μmol/kg/day; which for a 70 kg male is 57 mg/day (Sahajwalla et al., 1995), the kidney reabsorbing free carnitine at a rate of 98–99% (Sahajwalla et al., 1995). Thus under omnivorous diet the intake and the losses of LCAR are balanced.

There is no official recommendation for LCAR intake in any countries. However, several reports recommend an intake ranging from 23 to 136 mg/day of LCAR for a 70 kg male. The average or mean recommendation is around 60 mg/day.

A vegetarian diet provides less LCAR than an omnivorous diet. A vegetarian diet supplies from 4 to 20 mg of carnitine/day. Again this value depends on the precise nature of the diet: strict or regular vegetarians. Thus, a vegetarian relies almost entirely on its carnitine biosynthesis to cover its need in terms of carnitine. A vegetarian needs an efficient LCAR biosynthesis and thus adequate supplies in vitamin C and iron, two cofactors essential for carnitine biosynthesis. As a vegetarian diet is usually low in iron (Hua et al., 2001), vegetarians may have increased risk for LCAR deficiency.

5.5 LCAR Biosynthesis

In human, LCAR is synthesized from lysine and methionine by a series of reactions occurring mainly in the kidney, the liver, and the brain (Rigault et al., 2006). Carnitine is synthesized in four reactions from trimethyllysine (TML). TML is, at least in part, generated by the methylation of lysine residues in proteins and a subsequent protein hydrolysis. The methyl donor for this reaction is the *S*-adenosyl-methionine and this reaction is catalyzed by several protein lysyl methyltransferases (EC 2.1.1.n, $n = 43$, 59, or 60). The lysyl backbone of LCAR is provided by several proteins including histones, cytochrome *c*, and calmodulin.

Once released from proteins, TML is hydroxylated in the mitochondria into 3-hydroxy-*N*-trimethyllysine. This reaction is catalyzed by the TML dioxygenase also called epsilon-*N*-trimethyllysine hydroxylase (EC 1.14.11.8), a mitochondrial enzyme (Monfregola et al., 2005). The gene has been extensively studied; it shows a fine regulation process (Monfregola et al., 2007). This enzyme is found in the kidney, the liver, the heart, the muscles, and the brain. Iron is a cofactor of this enzyme and to maintain iron in the ferrous state, vitamin C is required (Figure 5.2).

In the next step, 3-hydroxy-*N*-trimethyllysine is cleaved into gamma-trimethylaminobutyraldehyde. This reaction is a pyridoxal 5′-phosphate-dependent cleavage catalyzed by a cytosolic enzyme called hydroxyl *N*-trimethyllysine aldolase (HTMLA). The precise nature of this enzyme remains to be determined. It has been suggested that this enzyme is the glycine hydroxymethyltransferase (EC 2.1.2.1 also called serine hydroxymethyltransferase) (Chave et al., 1997; Henderson et al., 1982). This enzyme is not involved only in carnitine metabolism, it plays a crucial role in the channeling of various metabolites between amino acid and nucleotide metabolisms (Rao et al., 2000). This enzyme is found in the cytosol and the mitochondria (Rao et al., 2000). However, recent studies carried out on worms suggested that HTMLA could be an enzyme belonging to the threonine aldolase family (Strijbis et al., 2010).

γ-Trimethylaminobutyraldehyde is dehydrogenated to γ-butyrobetaine in the cytosol, a reaction catalyzed by the 4-trimethylammoniobutyraldehyde dehydrogenase (EC 1.2.1.47). In humans, this enzyme is located in the brain and the liver and during this reaction, NAD^+ is converted into $NADH^+$ H^+ (Hulse and Henderson, 1980).

During the following step, γ butyrobetaine is converted into LCAR. This reaction is catalyzed by the γ-butyrobetaine dioxygenase (EC 1.14.11.1), a cytosolic enzyme requiring ascorbate and Fe^{2+} as cofactors (Holme et al., 1982). A possible location of this enzyme in the peroxisome has been suggested several

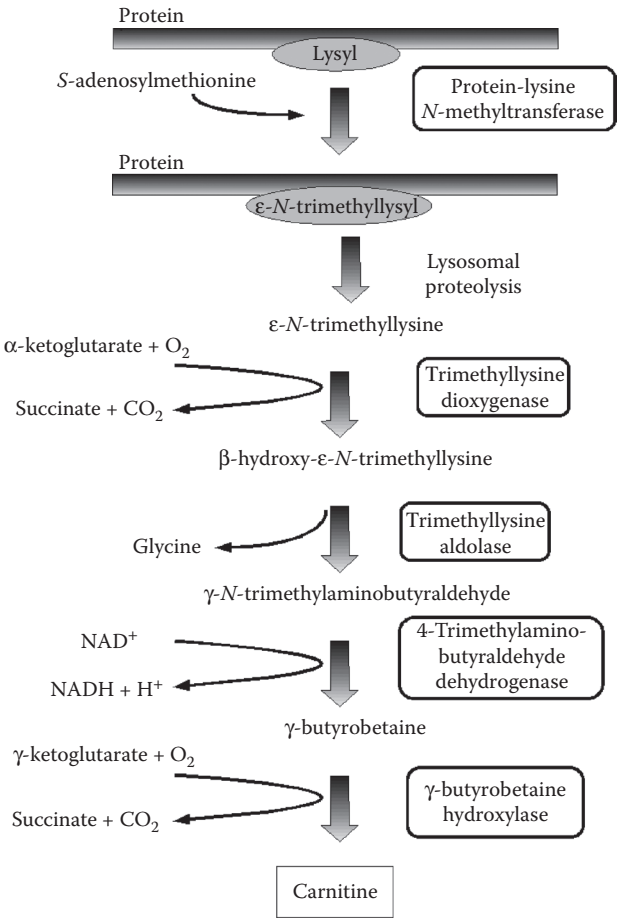

FIGURE 5.2 Representation of LCAR biosynthesis.

years ago (Paul et al., 1992). In human, this enzyme is found only in the liver, the kidney, and the brain (Rigault et al., 2006).

The different steps of this biosynthesis take place in various tissues and organs, inside the cells, the enzymes involved are located in the nucleus, the mitochondria, the peroxisome, and the cytosol and require several cofactors and nutrients such as vitamin C and iron (Galland et al., 2001). The enzymes responsible for γ-butyrobetaine synthesis are ubiquitously distributed in human tissues. On the other hand, the location of the γ-butyrobetaine dioxygenase, the enzyme that catalyzes the last reaction of this pathway is restricted to only three organs (Rigault et al., 2006). Once synthesized, LCAR enters the circulation and is taken up by other tissues via transporters. Pharmacokinetics studies have suggested that humans synthesize between 1 and 2 μmol of carnitine/kg/day (Sahajwalla et al., 1995).

Carnitine is found in various tissues and organs in the human body but most of the LCAR is concentrated in the muscles. In humans, skeletal muscle contains between 3 and 6 μmol of carnitine/g (wet weight), while the liver contains only 0.5–1 μmol of LCAR (per g of tissue w.w.). LCAR present in the muscle does not seem to be exchanged with the plasma; conversely, carnitine contained in the liver may be quickly released in the plasma. In the body, half-life of carnitine was estimated at 66 days (Galland et al., 2001). In mammals, there is no direct catabolism of LCAR. On the other hand, in prokaryotic cells, three possible routes are possible for LCAR catabolism. Some bacteria such as *Pseudomonas* species degrade L-carnitine to provide carbon and nitrogen (Kleber, 1997). In other species, LCAR degradation leads to the formation of trimethylamine (Seim et al., 1982). In *Escherichia coli*, carnitine may be converted into γ-butyrobetaine with the intermediary formation of crotonobetaine (Eichler et al., 1994).

None of these pathways has ever been found in humans. LCAR is eliminated from the body in urine in the free or a conjugated form.

5.6 LCAR Import

As previously mentioned, LCAR is either synthesized in the liver, the kidney, or the brain in humans (Rigault et al., 2006) or absorbed in the digestive tract and is subsequently concentrated in muscles.

LCAR homeostasis is maintained thanks to membrane transporters involved in absorption, distribution, and elimination of LCAR. Different transporters, responsible for LCAR transport, have been characterized. Most of them are involved in the transport of several substrates and belong to the Organic Cation Transporter (OCT) family. These transporters are involved in the transport of cationic and zwitterionic substances and are dependent on ATP. They are primarily involved in the elimination of cationic drugs and xenobiotics. Among the OCT transporters, OCTN1, OCTN2, and OCTN3 are involved in LCAR transport (Lahjouji et al., 2001). Another transporter, $ATB0^+$ is involved in LCAR uptake. All these transporters differ by their tissue specificities, subcellular localizations, affinity for LCAR, and transport capacity (Nakanishi et al., 2001). The specificities of each transporter are detailed below.

5.6.1 OCTN1

OCTN1 (SLC22A4) is a 551-amino-acids protein with 11 transmembrane domains. OCTN1 is involved in the active secretion of cationic compounds across the renal epithelial brush border membrane. This transporter is also found in trachea, bone marrow, sperm cells, in ocular epithelium, and in basolateral membrane of human proximal tubule. The main substrate of this pH-dependent proton antiporter has been discovered only few years ago: OCTN1 is mainly involved in the transport of ergothioneine (Grundemann et al., 2005). Though this group could not detect any LCAR transport activity by OCTN1, another group described OCTN1 as a transporter exhibiting a high affinity, but low transport capacity of LCAR (Lash et al., 2006). OCTN1 has been localized to mitochondria in mammals, suggesting a role for OCTN1 in intracellular carnitine homeostasis (Lamhonwah and Tein, 2006).

5.6.2 OCTN2

OCTN2 (SLC22A5) is the most important LCAR transporter, and mutations in this transporter cause primary systemic carnitine deficiency (Longo et al., 2006). OCTN2 is a protein containing 557 amino acids organized in 12 transmembrane domains. Localized to the plasma membrane, OCTN2 is involved in the transport of LCAR and of several organic cations and cationic drugs. It can transport LCAR and its acyl derivatives (acetyl- and acyl-carnitine), LCAR precursors (e.g., butyrobetaine), but also xenobiotics and cationic drugs such as verapamil, valproate, quinidine, or pyrilamine (Ohnishi et al., 2008). Some of these drugs can have an inhibitory effect on LCAR uptake and can cause secondary carnitine deficiencies. Transport of LCAR by this transporter is dependent on sodium. In contrast, transport of organic cations by OCTN2 is independent on sodium. The transmembrane domains 1–7 are responsible for transport of organic cations and sodium dependence, the transmembrane domain 10–12 are necessary for LCAR uptake (Inano et al., 2004).

OCTN2 is widely expressed. It is expressed in the heart (myocardium, valves, and arterioles), skeletal muscle, kidney (proximal and distal tubules, glomeruli), placenta, small intestine, brain (cortex, hippocampus, and cerebellum), mammary glands, and sperm cells (Lahjouji et al., 2001; Ramsay et al., 2001). In the renal apical membrane, OCTN2 allows the coupling of LCAR reabsorption with the secretion of organic cations. OCTN2 expression in brain capillary endothelial cells allows LCAR and ALCAR to cross the blood–brain barrier (Kido et al., 2001).

Several mutations in OCTN2 have been identified, and most of these mutations are responsible for an autosomal recessive disease: primary systemic LCAR deficiency (MIM603377). Defect in LCAR transport by OCTN2 causes very low concentrations of LCAR in serum and tissues (especially muscle, heart, and liver) and decreased reabsorption of LCAR by the kidney. This causes cardiomyopathy, progressive

skeletal muscle weakness, nonketotic hypoglycemia, hyperammonemia, limited capacity of fatty acid oxidation, particularly during fasting. Mutations in OCTN2 have been analyzed. Some mutants allow a normal maturation and localization of OCTN2 to plasma membrane. Other mutations are responsible for retention of OCTN2 in the cytoplasm (Amat di San Filippo et al., 2006).

5.6.3 OCTN3

OCTN3 has been identified as the peroxisomal carnitine transporter. OCTN3 is localized at the peroxisomal membrane and it has been shown that cellular OCTN3 level is reduced in peroxisomal biogenesis disorders such as Zellweger syndrome (Lamhonwah et al., 2005).

5.6.4 ATB0$^+$

ATB0$^+$ (SLC6A14) has also been characterized as a low-affinity/high-capacity transporter for LCAR in the intestine. Initially, ATB0$^+$ has been identified as an amino-acid transporter, dependent on sodium and chloride. But this transporter is also able to transport LCAR, acetylcarnitine, and propionylcarnitine. LCAR transport by ATB0$^+$ is inhibited by amino acid substrates. It is expressed in the intestinal tract, lung, and mammary gland. In contrast to OCTN2, affinity for LCAR is low, but concentrative capacity is higher than OCTN2 (Nakanishi et al., 2001).

5.7 Biological Activities of LCAR

Once in the cell, LCAR is primarily used as a cofactor for several enzymes.

Today, in humans and other mammals, most if not all characterized functions of carnitine involve energy production, LCAR being a cofactor for a family of enzymes: the acyltransferases (Ramsay and Zammit, 2004). These enzymes catalyze the esterification of the 3-hydroxyl group of carnitine on acyl groups. Numerous acyl-carnitine transferases have been described; they present chain-length specificities, different cellular localizations, and metabolic functions (Ramsay et al., 2001). It is possible to distinguish three major families of carnitine acyltransferases. The first group is represented by the carnitine acetyltransferase (CrAT, EC 2.3.1.7). These proteins are in charge of short-chain acyl and are present in the cytosol, the mitochondrion, the peroxisome, and the endoplasmic reticulum. The human enzyme is located in various organs and tissues including the heart, the brain, the kidney, the sperm cells, and the liver (Bloisi et al., 1990). The carnitine octanoyl transferase (COT, EC 2.3.1.137) is a peroxisomal enzyme that acts on a wide range of acyl-CoAs with an optimal activity for acyls with a length of 6 or 8C. The enzyme is a monomer present in fibroblasts and in hepatic cells (Ferdinandusse et al., 1999). The last group comprises a group of enzymes called carnitine palmitoyltransferases (CPT) (EC 2.3.1.21). These enzymes have a broad specificity for acyl groups whose length ranges from 8 to 18, the optimal activity being found with palmitoyl-CoA. Two subgroups of CPT have been described, the CPT1 enzymes are located in the mitochondrial outer membrane and according to their tissue distribution, three different types have been described, CPT1a initially described in the liver, CP1b in the skeletal muscle, and CPT1c in the brain. CPT2 is located on the mitochondrial inner membrane. Together CPT1, CPT2, and carnitine acyl-carnitine translocase (CACT, SLC25A20) represent the carnitine system involved in the channeling of fatty acids into the mitochondria. More details on these enzymes and their activity are given below.

5.7.1 Mitochondrial Uptake of Long-Chain Fatty Acids

In most tissues, fatty acids represent the major source of energy. Long-chain fatty acids are oxidized in the mitochondrial matrix through the mitochondrial β-oxidation pathway. This leads to the formation of ATP. Fatty acids that enter the cell or are present in the cell are activated by the acyl-CoA synthases to form acyl-CoA. These enzymes are located either in the microsomes or in the outer membrane of the mitochondria. Mitochondrial membranes are impermeable to these acyl-CoAs. To cross the membranes

of this organelle, these acyl-CoA are taken in charge by a multienzymatic system that will allow their transfer. This system named carnitine system involves three enzymes and a cofactor: the LCAR.

This system includes three enzymes that are all dependent on LCAR. It includes the CPT1 (EC 2.3.1.21), located on the external face of the outer membrane of the mitochondria (Murthy and Pande, 1987). This enzyme catalyzes the exchange of the CoA with LCAR. This leads to the formation of acyl-carnitine that is taken in charge by the CACT and crosses the inner mitochondrial membrane. Once in the mitochondrial matrix, acyl-carnitines undergo a reaction that releases acyl-CoA and carnitine. This reaction is catalyzed by the CPT 2 (EC 2.3.1.21), and takes place in the inner membrane of the mitochondria. All together, the three enzymes of the carnitine system take in charge an acyl-CoA from the cytosol to the mitochondrial matrix, with a conversion in LCAR ester and a subsequent restitution of the free LCAR.

Once in the mitochondrial matrix, acyl-CoA enters the β-oxidative pathway that allows the production of acetyl-CoA that can be converted into ketone bodies or into CO_2 allowing the production of energy (Bartlett and Eaton, 2004).

5.7.2 Modulation of the Intramitochondrial ACYL-COA/COA Ratio

In the mitochondrial matrix, various metabolic pathways that use CoA take place. Among these reactions one can list the β-oxidation, the oxidative decarboxylation of pyruvate, the tricarboxylic cycle, and the metabolism of branched amino acids. All these pathways require free CoA (Brass, 1994). Under balanced conditions, the ratio between free and acylated CoA is stable and is independent of the LCAR status (Brass and Hoppel, 1980a). But, in various physiopathological situations, such as diabetes, the ratio between free and acylated CoA becomes unstable and is compensated by the buffering effect of LCAR. In this case, to maintain optimal level of free CoA, the formation of acyl-carnitine is increased (Brass and Hoppel, 1980b). This induces an increased elimination of acyls from the mitochondrial matrix and allows increase in the amount of free CoA.

5.7.3 Peroxisomal Metabolism and Peroxisome

Carnitine is also involved in the peroxisomal oxidation of fatty acids. This pathway involves very long-chain fatty acids (VLCFA) (Schrader and Fahimi, 2008). It requires the entry of the fatty acids in the peroxisomal matrix; this step is not dependent on LCAR contrarily to the mitochondrial β-oxidation; it requires ATP-binding cassette transporters. In the peroxisome, VLCFA are oxidized through a series of reactions that leads to the formation of acetyl-CoA at each cycle of oxidation and of shortened acyls. The peroxisomal β-oxidation of fatty acyl groups is not complete and leads ultimately to chain-shortened fatty acyl CoAs and acetyl CoA. In the peroxisome, two LCAR-dependent enzymes are present: the CrAT (EC 2.3.1.7) and the carnitine octanoyltransferase (CrOT, EC 2.3.1.137). These enzymes transfer the acyl group from the acyl-CoA to carnitine. CrAT uses short-chain acyl groups (C1–C4) as a substrate while medium-chain acyl-CoA ester are transesterified by the CrOT. It is suspected that these enzymes are respectively involved in the exit of the acetyl-CoA and the medium chain acyl-CoAs from the peroxisomal matrix to the cytosol.

5.7.4 Other Metabolic Roles of LCAR

The oxidation of branched-chain amino acids, especially the oxidation of leucine and valine derivatives, requires LCAR (van Hinsbergh et al., 1980). Through its role in the activity of CrAT, LCAR is also implicated in the acetylation of histones (Madiraju et al., 2009). Probably in relation with its role in controlling oxidative metabolism, LCAR also seems to limit the production of reactive oxygen species (ROS) and the oxidative stress (Ye et al., 2010).

5.8 LCAR Supplementation Safety

The safety for LCAR supplementation is difficult precisely since this compound is both synthesized endogenously and comes from food. LCAR belongs to a group of molecules sold as a nutritional

supplement but also as a prescribed drug. In 1983, a review entitled "Health Effects of Dietary Carnitine" was published by a conjoint effort of the food and drug administration and the Federation of American Societies for Experimental Biology in which safety of oral carnitine was reviewed. When 1–15 g of LCAR is given as an oral supplement, the only possible side effect observed was transient diarrhea. The lethal dose 50 for carnitine was also determined, based on experiments carried out on rodents; it was found to be around 1.4 g/kg b.w. for intravenous injection and 8.9 g/kg b.w. when given subcutaneously (this corresponds to 630 g/day in a 70 kg male!). No oral LD50 was reported.

The safety of LCAR supplementation has also been extensively reviewed more recently (Hathcock and Shao, 2006). Looking at dozens of clinical intervention trials in which L-carnitine was used, these authors concluded at the no toxicity of LCAR and its derivatives whatever the amount used in the food, at least up to 6 g/day. Among LCAR supplementations, several forms of LCAR are available: LCAR, ALCAR, and propionyl-L-carnitine (PLCAR). These three forms were used in several clinical intervention trials and no differences in terms of safety were observed. This is likely due to the fact that the human body is able to remove the acetyl or the propionyl groups of ALCAR and PLCAR leading to the free LCAR (Siliprandi et al., 1990). No adverse effects of LCAR, ALCAR, or PLCAR have been established. The only side effect was in some individuals an unpleasant body or urine odor. Thus, a Lowest Observed Adverse Effect Level cannot be defined and no No Observed Adverse Effect Level can be established.

5.9 Is There a Need for LCAR Supplementation?

Partly due to its pleiotropic effects and its safety, LCAR supplementation has been suggested as positive in various physiological and physiopathological statuses. LCAR deficiency is known to induce metabolic and functional alterations in muscle, nerve, and also in the reproductive function.

5.9.1 LCAR Function in Muscle

LCAR supplementation may, under certain circumstances, ameliorate either performance or recovery. Many studies carried out in humans looked for a beneficial role of LCAR in muscle performance. Several papers have reviewed all these studies. Even if there are sometimes criticisms that can be made on the making of these studies, the general conclusion is that LCAR does not allow an increase in performance whatever the sport and the duration of the physical exercise (Brass, 2004; Le Borgne and Demarquoy, 2003).

On the other hand, LCAR appeared to limit the early onset of fatigue during physical exercise. While the precise origin of fatigue is still controversial, many authors suggested that two factors can cause fatigue during physical exercise in muscle: lactic acid accumulation and muscle glycogen depletion. Interestingly, several reports indicate that LCAR supplementation may decrease lactic acid accumulation and may protect glycogen stock. LCAR may therefore play a role in delaying fatigue; this may have indirectly a positive effect on performance as it may allow for longer training period without fatigue (Brass et al., 1993; Dutta et al., 2008). This aspect has to be clearly explained and confirmed.

5.9.2 LCAR and Aging

Aging is marked by a decrease in several physiological functions and also by a reduction in the amount of LCAR in several tissues and especially in the muscles (Costell et al., 1989).

Aging is a complex phenomenon which causes various detrimental changes in the organism, aging is associated with a decrease in physiological functional capacities and increases the probability of suffering degenerative diseases (Trifunovic, 2006). The origins of the negative effects of aging are not well defined and several hypotheses have been enunciated. Most of them linked aging to the mitochondria and a dysregulation of the mitochondrial metabolism (Trifunovic, 2006). Aging is marked by a decrease in muscle mass and muscle oxidative capacities. This leads to an increase in fat gain and a decrease in lean mass. Brain function is also affected by aging. The number of neurons decreases and the blood

supply to the brain decreases. Furthermore, with age, the amount of free radicals increases and the activity of the antioxidant system decreases. This may contribute to the onset of the aging process (Crentsil, 2010).

In several animal models, as well as in a few studies conducted in humans, LCAR supplementation in old animals or individuals has several beneficial effects. LCAR supplementation allows for a restoration in carnitine levels in the plasma and the muscles (Bernard et al., 2008). It also restores several mitochondrial functions in muscle cells and reduced ROS production (Gulcin, 2006).

5.9.3 Sperm and LCAR

Male infertility is a relatively common problem. Men infertility may be associated with a reduction in the number, the motility, or the shape of their spermatozoids. Furthermore, the sperm is very rich in LCAR and this high concentration of LCAR plays a crucial role in energy metabolism and sperm quality and mobility (Bartellini et al., 1987). A reduction in LCAR concentration induces both sperm parameters abnormalities and a subsequent infertility (Matalliotakis et al., 2000). Several studies, recently reviewed by Zhou et al., (2007) led to the conclusion that the administration of LCAR (as free or ALCAR) may be efficient in improving pregnancy rate and sperm features in patients affected by male infertility. However, all these investigations and reviews also concluded that further investigations are needed to explain the underlying mechanisms.

5.9.4 LCAR and Obesity

LCAR is directly involved in fatty acid oxidation and fat metabolism. A decrease in LCAR bioavailability induces lipid deposits and alteration in plasma lipid profile (Almog et al., 1979). LCAR may thus appear as an efficient compound for fighting obesity as one may think that increasing LCAR intake will lead to increased oxidation, increased fat utilization, and a reduction of the obesity.

Today, overweight and obesity are serious health problems in Western countries. It affects more than 60% of the adult population in the United States (Wang and Beydoun, 2007). LCAR may appear as a beneficial agent for obesity.

There is increasing evidence that carnitine supplementation may be beneficial in obesity (Cave et al. 2008). In obese rats, carnitine supplementation improved glucose tolerance and increased total energy expenditure. CPT1 is a key enzyme in fatty acid oxidation pathway and a potential target for the treatment of obesity. Modulation of CPT-1 affects energy metabolism and food intake (Lopaschuk et al., 2010).

Stimulation of brain CPT1c was reported to decrease food intake and body weight (Aja et al., 2008). CPT1c knockout (KO) mice exhibit decreased food intake and a lower body weight than the wild-type control animals. In contrast, when fed a high-fat diet, CPT1c KO mice exhibit increased body weight and body fat while their food intake remained the same than control animals. All these findings suggest that CPT1c and LCAR play a major role in controlling food intake and energy homeostasis, but not through modification in fatty acid oxidation in the brain. The stimulation of the brain-located CPT1c could be a new target for weight management but further clarification of its precise involvement is needed.

5.9.5 Alzheimer's Disease and LCAR

Alzheimer's disease is the most prevalent form of dementia. It represents 50–70% of all cases of dementia. Alzheimer's disease initially alters memory and progressively will destroy the mind. Brain atrophy and amyloid deposits are also found in patients and deterioration of the nerve growth factor (NGF) metabolism (Cuello et al., 2007). Conventional medicine can, with a limited efficiency, slow the progression of the disease, but no fully efficient treatments are available yet (Kidd, 2008). The principal class of drugs used for the treatment of Alzheimer's disease are aimed at increasing acetylcholine levels in the central nervous system. Other pharmacological targets include, reducing calcium entry in cells, reducing chemical changes in tau protein, and regulating secretase activities (Tomita and Iwatsubo, 2006). Besides pharmacological approaches, nutrients may help slow the neurodegenerative process. Among those are found: phosphatidyl serine, omega-3 fatty acids. and also ALCAR. In rat, it has been shown that ALCAR

supplementation was able to maintain NGF receptors in aging brain (Angelucci et al., 1988). The usefulness of ALCAR supplementation in early Alzheimer's disease was investigated with a meta-analysis of double-blind, placebo-controlled prospective studies of at least 3 months. ALCAR supplementation with daily dose varying from 1.5 to 3.0 g/day had a significant positive effect on both clinical scales and psychometric tests (Montgomery et al., 2003).

5.9.6 What About LCAR in Vegetarians

While it is clear that vegetarians have low intake of LCAR, the consequences of this low intake on health have received basically no attention. In vegetarians, LCAR presence relies only on the endogenous synthesis. There are reports suggesting a decrease in LCAR levels in vegetarians and also many reports showing no differences in plasma LCAR levels due to vegetarian habits (Delanghe et al., 1989; Lombard et al., 1989).

5.9.7 LCAR and Dialysis

The kidney is a key organ in LCAR metabolism. Patients with end-stage renal disease have dramatic alteration in LCAR homeostasis. The loss of the renal parenchyma induces an important decrease of carnitine synthesis, and the dialysis procedure leads to an increased nonselective clearance of LCAR and a subsequent carnitine deficiency (Hedayati, 2006). Many studies investigated for a potential effect of LCAR supplementation on several dialysis-related symptoms (hypotension, heart failure, muscle weakness, cramps). Many of these studies are suffering a lack of randomization and inadequate determination of symptom improvement. The main conclusion of the only meta-analysis actually available is that actual studies do not show a beneficial effect of LCAR supplementation on muscle cramps or hypotension. The authors of this meta-analysis, however, concluded that additional rigorous randomized trials are needed (Lynch et al., 2008).

5.10 Conclusion

LCAR is present in many tissues and organs and its role is not restricted to fatty acid transport in mitochondria. LCAR is also present in other cell compartments such as the cytosol, the nuclei, the endoplasmic reticulum, and the peroxisome and LCAR appeared to be involved in various processes that are not only related to energy production. LCAR is involved in the aging process, the fight against free radicals and the acetylation of proteins and many other functions.

Thus, even if LCAR is not an essential element, it is a critical product for cell physiology, its deficiency has many deleterious effects on various cell types, including heart cells, muscles cells, and neurons. In most, if not all cases, restoring LCAR levels allows for a (at least partial) restoration of functions that were altered by the deficiency. It appears that supplementing people that may have a decrease in their levels in LCAR might be useful, this could be the case for aging people, sportsmen (and women), vegetarians, people suffering from kidney or liver diseases. However, there is still a lack of rigorous epidemiological studies to assess the potential beneficial role of LCAR on healthy individuals.

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6

Creatine

Leticia Mora and Fidel Toldrá

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6.1 Introduction

In recent years, much attention has been paid to the search of natural substances in foods that can be beneficial to the human body in preventing or treating one or more diseases or improving its physiological performance. Creatine (*N*-[aminoiminomethyl]-*N*-methyl glycine, Cr) is a nonessential dietary element found in high abundance in meat and fish. Currently, Cr is widely used as an ergogenic nutritional supplement by athletes, and it is well tolerated in this specific sector of consumers. It has been estimated that more than one-third of athletes regularly use Cr to improve muscular performance. On the other hand, recent findings have indicated that Cr supplementation has a therapeutic role in preventing several diseases characterized by atrophic conditions, weakness, and metabolic disturbances. Thus, Cr is an emerging nutritional supplement used not only by athletes, but also by elderly people as well as aged patients presenting different diseases.

6.1.1 Creatine Metabolism

Cr is a nitrogenous organic acid that is naturally synthesized in the liver, kidneys, and pancreas from three amino acids—glycine, arginine, and methionine (see Figure 6.1). Methionine is an essential amino acid that cannot be produced by the body, so Cr formation is limited when methionine is not sufficiently taken in the diet. As can be observed in Figure 6.1, Cr formation begins in the kidney with the transfer of the amidine group from arginine to glycine to form guanidoacetate and ornithine. Finally, guanidoacetate is methylated in the liver with the methyl group of *S*-adenosyl methionine to form Cr. Approximately 95% of the human's body total Cr is located in the skeletal muscle, whereas the remaining 5% of the Cr pool is located in the brain, liver, kidney, and testes (Wyss and Kaddurah-Daouk 2000). Cr can also be obtained from dietary supplements and from animal foods such as meat and fish.

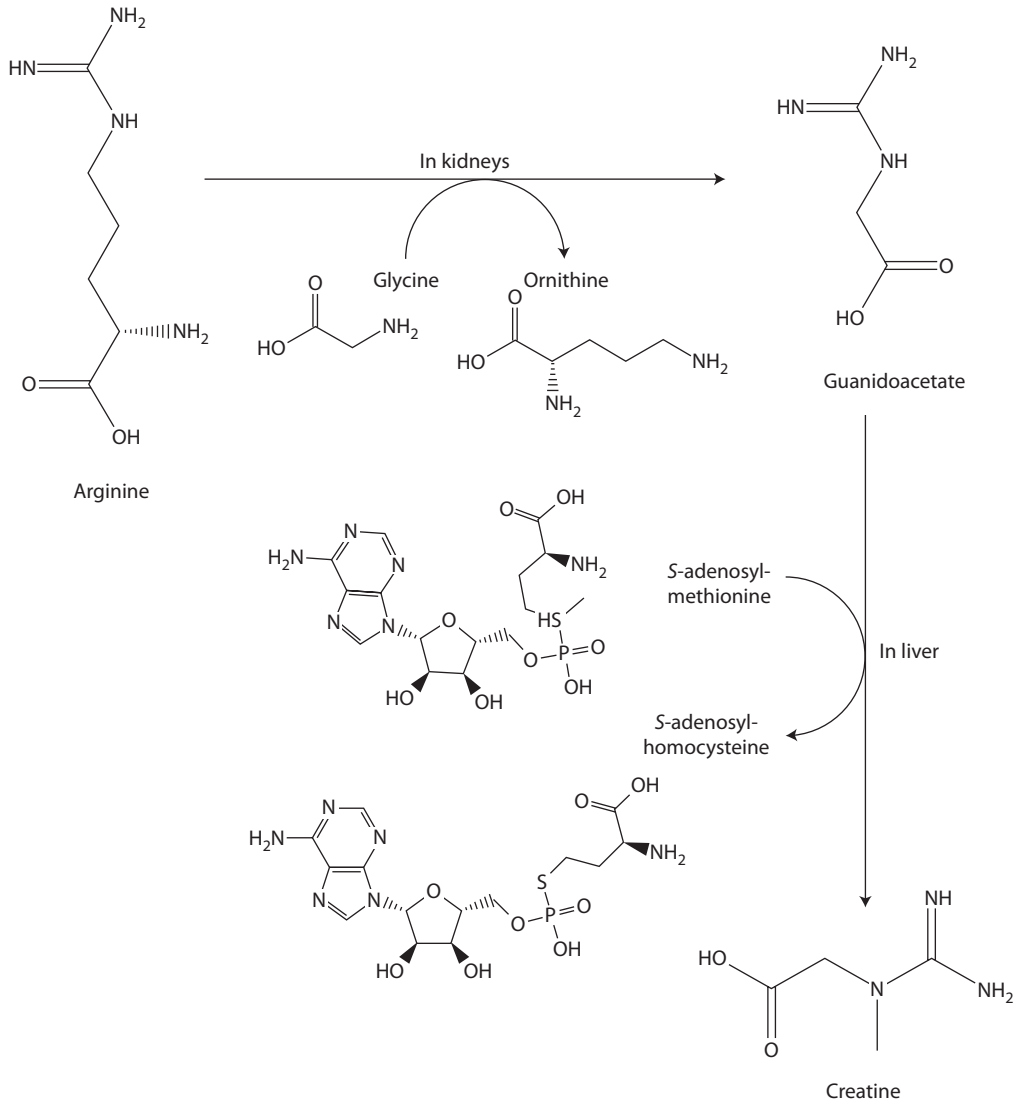

FIGURE 6.1 The pathway for the synthesis of Cr in the mammalian body. The first step of Cr biosynthesis occurs in the kidney, whereas the liver is likely to be the main organ accomplishing Cr synthesis.

Cr plays an important role in rapid energy provision during muscle contraction involving the transfer of *N*-phosphoryl group from phosphocreatine (PCr) to adenosine diphosphate (ADP) to regenerate adenosine triphosphate (ATP) through a reversible reaction catalyzed by creatine kinase (CK) (see Figure 6.2). This reverse reaction provides an alternate pathway—besides glycolysis—for the regeneration of ATP. The CK/Cr/PCr system serves as an energy buffer by connecting the mitochondrial sites of energy production with cytosolic sites of energy consumption in tissues with high-energy demand such as brain and muscle (Wyss and Kaddurah-Daouk 2000).

6.1.2 Creatine Conversion to Creatinine

Cr turns into creatinine (Cn) in muscle due to a nonenzymatic conversion by the removal of water and the formation of a ring structure (Figure 6.3). This conversion is influenced by pH, temperature, and time

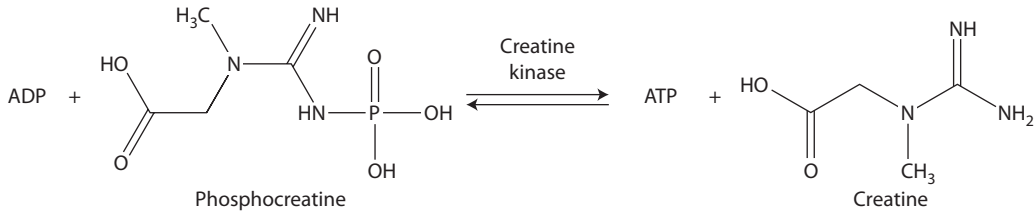

FIGURE 6.2 Schematic representation of the main reaction involved in Cr and Cn metabolism.

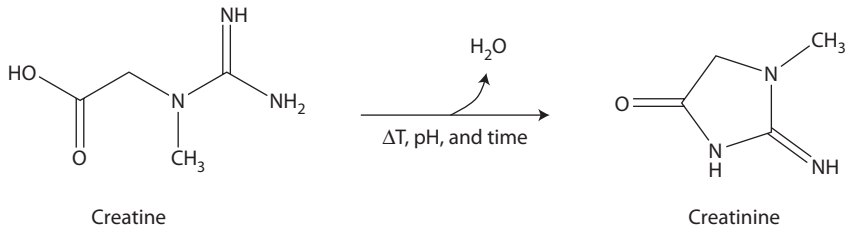

FIGURE 6.3 Schematic representation of Cr conversion into Cn influenced by temperature, pH, and time, as well as initial Cr amounts.

(Edgar and Shiver 1925; Fuller and Elia 1988). This conversion takes place easily under heating conditions such as cooking (Mora et al. 2008b). In fact, there is a general agreement in the literature that Cr contents decrease after cooking, while Cn contents increase, presumably due to the heat-induced conversion of Cr into Cn (Macy et al. 1970; Purchas et al. 2006). It has also been reported that the increase in Cn concentration with cooking is appreciably greater than the decrease in Cr (Macy et al. 1970; Pegg et al. 2006).

The presence of Cr and Cn in meat has also been associated with negative aspects because both can act as important precursors of heterocyclic amines (HAs), which can be formed on the surface of meat when cooked at high temperatures using dry-heating methods such as roasting, frying, and grilling (Knize et al. 1994; Skog et al. 1995; Pais et al. 1999; Sugimura et al. 2004).

Cn is a waste product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body, depending on the muscle mass. The 24 h Cn content of urine remains roughly constant for an individual, and is often used as a normalization factor when measuring other urinary components. Cn is mainly filtered from the blood by the kidneys, although a small amount is actively secreted by the kidneys into the urine. In cases of kidney deficiencies in filtering, Cn blood levels would rise. Thus, Cn levels in blood and urine can be used in the measurement of renal function. On the other hand, urinary Cn levels can also be used to estimate total body mass of striated muscle (Gábor et al. 1984) and to measure the nutritional status of surgical patients (Bistrian et al. 1975).

6.2 Creatine as a Dietary Supplement

Most people consume approximately 1–2 g of Cr in their daily diets (Deldicque and Francaux 2008). In this respect, people who eat large amounts of red meat ingest more Cr, whereas vegetarians consume less. Cr is also synthesized within the body, primarily in the liver, at a rate of about 1 g/day (Wyss and Kaddurah-Daouk 2000). In addition to these sources, Cr may also be consumed as a supplement. Synthetic supplements consist of creatine monohydrate or salts such as creatine citrate or creatine pyruvate (Jager et al. 2008), although Cr is usually supplied as a monohydrate salt due to its ready absorption from the gut (Harris et al. 1992).

Studies examining the Cr plasma concentration obtained over 6 h after the ingestion of 2 g of Cr either in meat or in solid form concluded that Cr is readily absorbed but may result in slightly lower peak concentration than when the same dose is ingested as a solution (Harris et al. 2002).

The effect of Cr supplementation on exercise performance (Harris et al. 1992; Kreider 2003) as well as its potential clinical benefits (Gualano et al. 2010) have been widely studied during the past decades.

6.2.1 Effects of Creatine Supplementation on Exercise Performance

During high-intensity exercise, the energy supplied to rephosphorylate ADP to ATP is determined largely by the amount of PCr stored in the muscle (Hultman et al. 1996). As PCr stores become depleted, performance is likely to rapidly deteriorate because of the inability to resynthesize ATP at the rate required (Harris et al. 1993). Since the availability of PCr stores in the muscle may significantly influence the amount of energy generated during brief periods of time requiring high-intensity exercise, it has been hypothesized that increasing muscle Cr content may increase the availability of PCr and thus may allow for an accelerated rate of resynthesis of ATP during and following high-intensity, short-duration exercises (Harris et al. 1992; Balsom et al. 1994; Hultman et al. 1996).

Cr supplementation in humans has been reported to elevate muscle total Cr and PCr concentration, increasing the total Cr content in skeletal muscle by 10–40% and the intramuscular Cr in the form of PCr by 20–40% when repeated large doses of 20 g/day of creatine monohydrate for 2–7 days were applied (Harris et al. 1992; Balsom et al. 1994; Hultman et al. 1996). On the other hand, the administration of smaller doses of Cr for longer periods such as 30 days also resulted in increases in the total muscle Cr store (Balsom et al. 1994). This availability of Cr and PCr plays a significant role in contributing to energy metabolism particularly during intense exercise. In fact, as has been previously described, increasing the availability of PCr would enhance cellular bioenergetics of the phosphagen system that is involved in high-intensity exercise performance (Williams and Branch 1998; Kraemer and Volek 1999) as well as the shuttling of high-energy phosphates between the mitochondria and cytosol via the creatine phosphate shuttle that may enhance both anaerobic and aerobic capacity.

In support of these hypotheses, studies indicate that Cr loading may improve high-intensity exercise performance in rowing (Nagasawa et al. 2001; Syrotuik et al. 2001; Chwalbinska-Moneta 2003), running (Chilibeck et al. 2007; Esmaeeli et al. 2009; Howatson and Milak 2009; Sweeney et al. 2010), cycling (Birch et al. 1994; Kingsley et al. 2009; Van Thienen et al. 2009), swimming (Hopwood, et al. 2006; Silva et al. 2007; Juhasz et al. 2009; Yildiz et al. 2009), and resistance exercise (Earnest et al. 1995). For this reason, Cr has also been marketed as an ergogenic dietary supplement. An ergogenic supplement serves to increase performance capacity, the efficiency to perform work, the ability to recover from exercise, and/or the quality of training. Ergogenic effects of Cr have been especially described in high-intensity, short-term exercises, in which the PCr–CK system plays an essential energetic role. In fact, athletes commonly take Cr supplements to reduce fatigue and enhance performance of anaerobic exercises. This performance enhancement occurs due to a reduction in the ATP degradation during maximal exercise due to elevated muscle PCr content, particularly in type II muscle fibers.

Some studies relate Cr supplementation with an increase of weight gain and lean body mass without affecting body fat. Some authors suggested that it could be because of stimulating the protein synthesis (Earnest et al. 1995; Kreider et al. 1998) and/or fluid retention. In this respect, it has been described that the increase in body mass was primarily retained water due to the observed decrease in the urinary volume (Balsom et al. 1994; Hultman et al. 1996).

Supplementation of creatine monohydrate in the diet increases Cr and PCr cellular levels in muscle tissue enhancing muscular strength. The most obvious effects of Cr are observed after several weeks of supplementation in combination with a resistance exercise program. In fact, it has been proven that a combination of creatine monohydrate and conjugated linoleic acid together with supervised resistance exercise during 6 months enhances strength gains and improves body composition in older adults (Tarnopolsky et al. 2007). On the other hand, the effect of 2 and 5 days of Cr loading on muscular strength in athletes was also tested, finding that a 5 day Cr-loading regime coupled with resistance training also resulted in significant improvements in average anaerobic power and back squat strength (Law et al. 2009). However, Cr supplementation is not so effective in aerobic exercise performance (Chwalbinska-Moneta 2003).

The ingestion of Cr together with other food supplements can promote and enhance its effect as ergogenic dietary complement. It has been described that the ingestion of carbohydrate with Cr enhances

intramuscular Cr uptake and glycogen deposition (Green et al. 1996a,b), whereas ingestion of glucose and sodium with Cr would theoretically provide additional ergogenic effect.

6.2.2 Creatine Related to the Quality of Meat

Although most studies related to Cr have been carried out in humans, the effect of creatine monohydrate on meat quality has also been studied. In pig muscle, creatine monohydrate provides ATP for muscle contraction and metabolism, increasing the amount of available energy for ATP production. This fact improves anaerobic performance and delays lactate formation in the muscle under the anaerobic post-mortem conditions. A delayed lactate formation will postpone the pH decline, potentially improving the water-holding capacity, and the quality of the meat, reducing the incidence of pale, soft, and exudative (PSE) tissues although this effect was not found on meat from purebred Landrace pigs and Danish cross-bred pigs (Rosenvold et al. 2007). Cr supplementation in pork diets prior to slaughter may affect post-mortem muscle metabolism and improve pork quality. On the other hand, the effect of creatine monohydrate supplementation on pork growth performance and carcass characteristics has also been studied (James et al. 2002).

The determination of Cr and Cn can be used to detect the presence of meat extract in food products. Raw meat has a high Cr content whereas the Cn content is low. The total Cr plus Cn level could be used as an index of the quality of meat products and the Cr/Cn ratio as a quality indicator of processed meat products (Mora et al. 2008b, 2010).

6.2.3 Treatment of Diseases

Recent findings have indicated that Cr supplementation has a therapeutic role in several diseases characterized by atrophic conditions, weakness, and metabolic disturbances. In fact, evidences suggest that Cr supplementation is capable of attenuating the degenerative state in some muscle disorders as inflammatory myopathies or muscular dystrophies (Santos et al. 2004; Gualano et al. 2010), as well as bone and metabolic disturbances such as osteoporosis and type II diabetes (Chilibeck et al. 2005). Cr has also been tested in several animal models and, more recently, in humans as a potential neuroprotective agent in neurodegenerative diseases like Parkinson's (Bender et al. 2008b), Huntington's (Bender et al. 2005; Martin et al. 2008), and Alzheimer's (Gualano et al. 2010), as well as myotrophic lateral sclerosis (Zhang et al. 2003). Cr long-term supplementation in aged mice results in prolonged lifespan, better performance on memory tasks, and a decreased accumulation of the age-related pigment lipofuscin in the hippocampus (Bender et al. 2008a).

Patients with gyrate atrophy present lower levels of PCr in skeletal muscle since ornithine inhibits the rate-limiting step of Cr biosynthesis (Heinanen et al. 1999). Thus, the most used therapy for gyrate atrophy includes diet modification introducing Cr supplementation to reduce the plasma ornithine (Sipila et al. 1981). Finally, Cr has also shown promise in human studies for the treatment of traumatic brain injury, reducing headache, dizziness, and fatigue in patients (Sakellaris et al. 2006, 2008).

6.3 Analysis of Creatine

In recent years, ingestion of Cr has become popular with many athletes who consume it for muscle building and performance enhancement as well as with elderly people for the treatment of different diseases mainly related to the degeneration of muscles. Cn results from the irreversible, nonenzymatic dehydration and loss of phosphate from PCr. The determination of Cn remains one of the most important routinely performed clinical assays since Cn is the most widely used clinical marker to assess renal function.

6.3.1 Extraction of Creatine and Creatinine

In a clinical setting, the extraction of Cr and Cn is usually done from samples of urine, plasma, or serum. In those cases, sample preparation is very easy: urine is diluted using deionized water, whereas plasma

needs a previous centrifugation and a filtration. Finally, the extraction of these compounds from blood sera needs a deproteinization step that is to be carried out with trichloroacetic acid (TCA).

When Cr and Cn compounds are extracted from more complicated matrices such as food, different extraction procedures are used depending on the subsequent analytical techniques. A typical extraction procedure for the analysis of meat and fish samples by reversed-phase chromatography consists of the addition of 3–5 vol. cold 0.6 M perchloric acid to 5 g of tissue sample previously frozen and minced. Once the perchloric acid is added, the tissue is homogenized using a stomacher for a few minutes under cold conditions. After centrifugation (15,000*g* for 20 min), the supernatant is filtered through glass wool and neutralized to pH 6.5–6.8 by adding solid potassium carbonate. This neutralized extract is kept in an ice bath for 15 min and centrifuged again (15,000*g* for 10 min). Supernatant is filtered through a 0.2 μm membrane filter and stored in cold until use. This extraction procedure has also been described in the measurement of these compounds using hydrophilic interaction chromatography (HILIC) (Mora et al. 2010) as well as when subsequent analyses using capillary electrophoresis (CE) are required (Dillon and Sears 1998).

Another extraction method described in the analysis of raw meat and cooked ham samples (Mora 2007, 2008a,b) consists of the homogenization of 5 g of sample tissue with 20 mL of 0.01 N HCl in a stomacher for 15 min and further centrifuged in cold (4°C) at 12,000*g* for 20 min. The supernatant is filtered through glass wool, and 200 μL of this solution is deproteinized by adding 3 volumes of acetonitrile, standing at 4°C for 20 min. Finally, samples are centrifuged at 12,000*g* for 10 min in cold and the supernatant directly analyzed. These extracts are mainly used in HILIC chromatography.

In the development of flow injection methods in cooked meat products (del Campo et al. 1995, 1998) or isotachophoretic determinations in meat and meat products (Kvasnicka and Voldrich 2000), some authors have described extraction methods that consist of the homogenization of the sample (5 g) with 80–100 mL of 5% TCA. Finally, enzymatic determinations allow Cr and Cn extraction using distilled water (typically 2 mL of water per 0.5 g of freeze-dried sample) (Purchas and Busboom 2005).

6.3.2 Creatine and Creatinine Determination

Cr and Cn are commonly determined by the Folin method, based on the reaction that takes place between Cn and alkaline pikrate known as the Jaffé reaction. This method consists of the previous conversion of Cr into Cn by prolonged heating in acid medium. This technique is the basis of some official methods for the spectrophotometric determination of Cr and Cn in meat (Association of Official Analytical Chemists 1984). Another method for Cr analysis is based on the reaction of Cr with 1-naphtol and 2,3-butanedione, with satisfactory results if preliminary ion-exchange chromatography is carried out (Mussini et al. 1984). Although the analysis of Cr and Cn is mainly carried out in urine or plasma matrices for medical uses, these reactions have also been used to develop various flow injection methodologies for the simultaneous determination of Cr and Cn in meat and meat products (del Campo et al. 1995).

As an alternative to the above-mentioned techniques, enzymatic biosensors that have enhanced specificity for Cr have also been developed. However, these methods are not all free of interferences; in fact, carbonyl compounds, dopamine, cephalosporines, and bilirubin interfere with the Jaffé reaction, whereas bilirubin, Cr, dopamine, ascorbic acid, and sarcosine interfere with the enzymatic methods in the determination of Cn. For this reason, other specific methodologies such as high-performance liquid chromatography (HPLC) or CE have been proposed to allow Cr and Cn determination.

6.3.2.1 Enzymatic Assays

Biosensor systems have many advantages over other techniques used to analyze Cr and Cn. Enzymatic assays reduce the time, complexity, and cost of routine analysis in many areas for the analysis of Cr and Cn in blood, urine, or even food matrices. The development of these sensors has followed two paths; systems based on amperometric or potentiometric detection. Potentiometric devices are mainly based on the catalysis of Cn by creatinine iminohydrolase (CIH, creatinine iminohydrolase, EC 3.5.4.21) at the surface of an ammonia-sensing ion-selective electrode (Erlenkötter et al. 2002). These systems have the advantage of requiring only a single enzyme for the detection of Cn as well as avoid Cr interferences when blood or urine is being analyzed. Recently, a new potentiometric biosensor for the determination

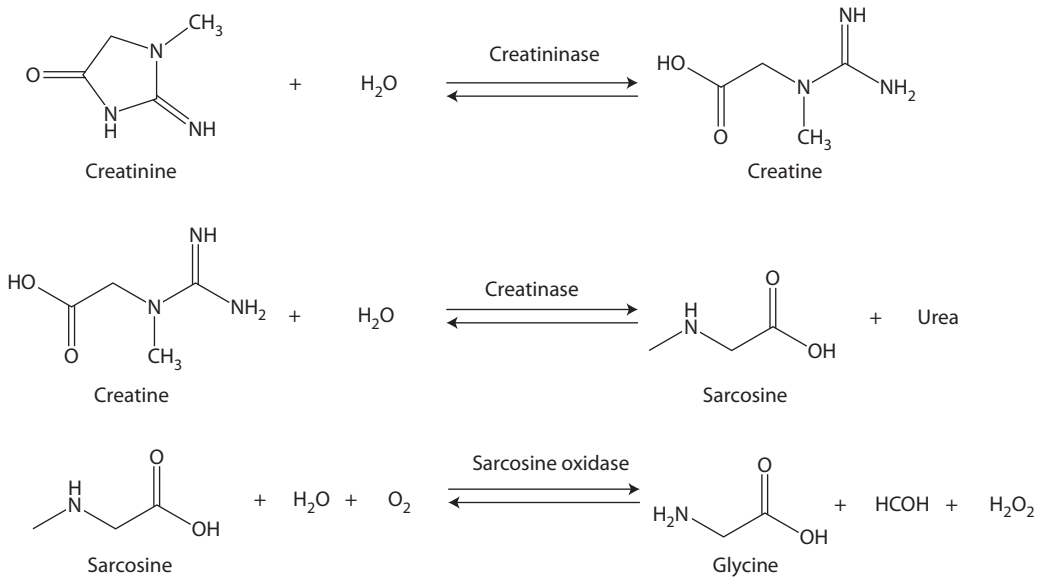

FIGURE 6.4 Three stage enzymatic catalysis of Cn to glycine and the electrochemically detectable hydrogen peroxide (H_2O_2).

of Cr in commercial Cr powder has been tested by immobilizing urease and creatinase. Results were in good agreement with those obtained from Jaffé method (Karakus et al. 2006). Amperometric biosensors for the analysis of Cn are mainly based on the conversion of Cn into Cr using creatininase (creatinine amidohydrolase, EC 3.5.2.10), Cr to sarcosine using creatinase (creatine amidino hydrolase; EC 3.5.3.3), and sarcosine to glycine using sarcosine oxidase (EC 1.5.3.1). In this final stage, consumption of oxygen and liberation of hydrogen peroxide occurs (see Figure 6.4). Two sensors are usually required for the sustractive determination of Cn from a combination of Cr and Cn. The concentrations of both compounds are determined using all three enzymes, whereas a second sensor that omits creatininase, measures Cr. Thus, Cr concentration can be easily determined by subtraction since concentration of either Cr or Cr and Cn is proportional to the amount of H_2O_2 generated. The presence of the three enzymes decreases the system sensitivity whereas the necessity of the two sensors adds complexity, variability, and errors in the system. In spite of this, this methodology has been widely used in the determination of Cr (Erden et al. 2006; Ramanavicius 2007) and Cn in pharmaceutical products (Staden and Bokretsion 2006), commercial Cr powder (Karakus et al. 2006), serum, and urine samples (Sugita et al. 1992; Madaras et al. 1996; Karakus et al. 2006; Staden et al. 2006). Concentrations of Cr in beef and lamb samples have also been determined using an enzymatic assay based on the conversion of urea into ammonia at a rate that is proportional to the initial concentration of creatine (see Figure 6.4). The same authors also determined the Cn amounts using an enzymatic kit based on the Jaffé method (Purchas et al. 2004).

6.3.2.2 Chromatography

Due to the numerous possible interferences detected using enzymatic assays, chromatographic techniques have also been developed for use in clinical and food quality settings. The main chromatographic techniques used for Cr and Cn determination include ion-pairing applied to reversed-phase chromatography (RP-HPLC), ion-exchange chromatography (IE-HPLC), and hydrophilic interaction chromatography (HILIC).

Ion-exchange chromatography has also been a method of choice for the analysis of Cr and Cn in clinical settings although ion-exchange is also very often used as a preparative technique (Yokoyama et al. 1992). This technique has been described for Cn determination in serum by comparing three ion-exchange methods (Kagedal and Olsson 1990), and in serum and urine for the determination of Cn over

a wide linear range from 1 to 2000 μmol/L (Harmoinen et al. 1991). A simple and versatile cation-exchange chromatography technique for the simultaneous determination of urinary Cr, Cn, as well as UV-absorbing amino acids using a binary dual-mode gradient eluting system has been recently developed (Yokoyama et al. 2005).

RP-HPLC has been an indispensable technique for the isolation of these compounds, having as main advantage, apart from its powerful resolving capacity, the use of volatile mobile phases that avoid the need for sample desalting. This technique has been widely used in clinical settings for the determination of Cr and Cn together with other metabolic compounds in urine (Yang 1998), serum (Werner et al. 1990), and plasma, as well as in rat heart biopsies (Ally and Park 1992; Volonte et al. 2004), rat hippocampus (Tranberg et al. 2005), and muscles from different animals (Dunnett et al. 1991; Shirai et al. 1997).

Although RP-HPLC is a powerful separation technique, one major limitation is the low retention of polar molecules. In this case, normal-phase liquid chromatography (NPLC) has been traditionally used to separate polar compounds with nonaqueous mobile phases, but one important limitation is the difficulty to dissolve hydrophilic compounds, in these phases. Moreover, this methodology is not environment-friendly. For these reasons, the application of NPLC in the analysis of Cr and Cn is limited and rarely used for separating these compounds.

HILIC can be an interesting alternative RP-HPLC, IE-HPLC, and NPLC for the determination of Cr compounds for many reasons: it implies the use of volatile buffers avoiding a desalting step, it has clear advantages with regard to the solubility of biological compounds, and there is no need of compound derivatization. This chromatographic mode is similar to NPLC since polar compounds are retained longer than the nonpolar ones and the polar component of the mobile phase (usually water) is the strong solvent used to elute the compounds. However, regarding the solubility of analytes in the mobile phase and matrix compatibility, HILIC is superior to NPLC, as the nature of mobile phases that are used is comparable to reversed-phase separations. For these reasons, HILIC chromatography has been used during the last decades in the determination of Cr, Cn, and other compounds in pork raw muscles of different metabolic type, cooked ham, and dry cured ham samples. Simultaneous determination of carnosine, anserine, balenine, Cr, and Cn in pork loin and chicken breast meat was recently developed using an Atlantis silica column from Waters (Mora et al. 2007). This chromatographic method was subsequently applied to seven pork muscles of different metabolic type (Mora et al. 2008a). On the other hand, HILIC methodology has also been employed to evaluate the effect of different cooking methods on Cr conversion into Cn in cooked ham samples (Mora et al. 2008b). Results allowed the establishment of a correlation between the Cr/Cn ratio and the effectiveness of the heat treatment in cooked ham processes. Cr conversion into Cn is not only influenced by temperature, also time has a very important effect on the conversion. A recent study clearly demonstrates that time of dry-cured processing has a significant effect on the contents of Cr and Cn that reach a plateau at about 7 months of processing. This fact indicates that Cr and Cn contents could be a good estimation of a minimum time of ripening (Mora et al. 2010). The chromatographic separation of Cr and Cn in raw meat and 200 days dry-cured ham is shown in Figure 6.5. In this case, extraction was performed with 0.01 N HCl as previously described.

Some studies using gas chromatography–mass spectrometric assays have also been recently described for the detection of Cr and Cn in several matrices such as human fluids (Kasumov et al. 2009; Prieto et al. 2009) as well as nutritional supplements (Stepan et al. 2008).

6.3.2.3 Capillary Electrophoresis

Despite chromatographic techniques allowing the isolation and identification of Cr and Cn compounds without interferences, in recent years, CE has become a useful and powerful separation technique that often exhibits higher resolutions and shorter analysis times than HPLC (Zinellu et al. 2006) even though not many authors have described the use of this type of electrophoresis for the analysis of Cr and Cn in food, probably due to the complexity of these matrices.

The pK_a values for Cn are 4.8 and 9.2, while those for Cr are 2.6 and 14.3 (Smith-Palmer 2002). Thus, CE can be employed for the analysis of Cr and Cn at pH below 2.6 and 4.8 or above pH 14.3 and 9.2, respectively. However, when using acidic pH, the coelution of many acidic metabolites occurs (Xu et al. 1994); whereas using basic pH, UV-absorbing substances migrate with Cn (Jia et al. 1998). Recently, a

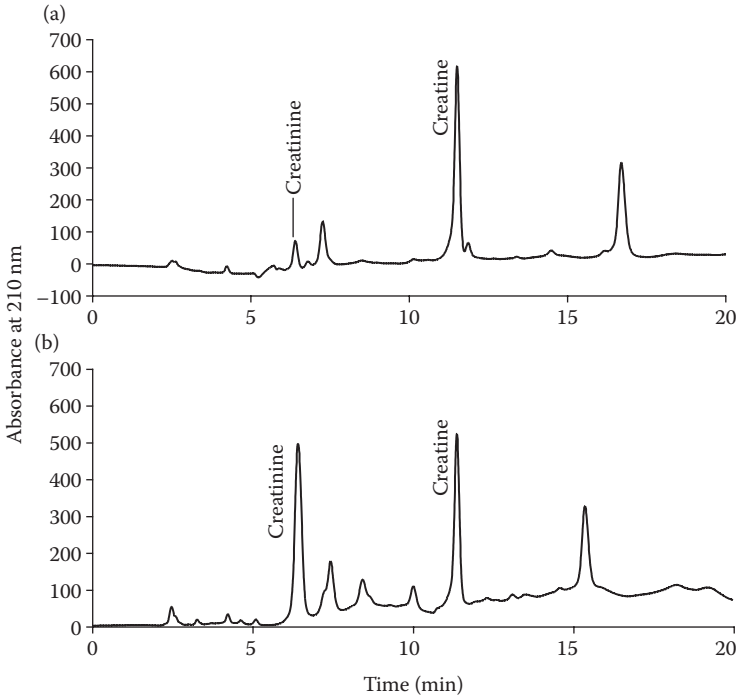

FIGURE 6.5 Chromatograms corresponding to dry-cured ham samples with 0 days (raw meat) (a), and 200 days of ripening (b). The column was a ZIC pHILIC (SeQuant), 150 × 4.6 mm, 5 μm. Chromatographic conditions are detailed in Mora et al. (2010). (From Mora, L., Sentandreu, M.A., and Toldrá, F. 2008a. *Meat Science* 79: 709–15. With permission.)

method for the simultaneous determination of guanidine acetic acid, Cn, and Cr in plasma and urine by CE and UV detection was developed. It consists of a modification of a previous assay of the same authors and results in an improved method able to perform the simultaneous determination (Zinellu et al. 2006). In order to separate Cn from other UV-absorbing neutrals, the use of micellar electrokinetic capillary electrophoresis (MEKC), which consists of the additional micellar phase, has been developed in the clinical analysis of urine and plasma (Miyake et al. 1991; Tran et al. 1997). A capillary isotachophoretic method (CITP) to determine Cn and Cr concentration in meat and meat products under acidic conditions was developed and tested in raw chicken leg, cooked ham, and salami (Kvasnicka et al. 2000).

Other techniques like nuclear magnetic resonance (NMR) have been used for the analysis of Cr and Cn, although not so good results were obtained (Unitt et al. 1992).

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7

Analysis of Bioactive Peptides and Proteins

Prithy Rupa and Yoshinori Mine

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7.1 Introduction

Advances in the research field of bioactive proteins and peptides are driven by a molecular understanding of biological processes and various analytical techniques are a critical component of this understanding. In the past, biochemical functional analysis of peptide and protein activity was focused on the analysis of single molecules. Different up-to-date methods, including peptide synthesis and immunochemistry, have been applied to the characterization of bioactive peptides recently. Although this approach has served well, the rapid pace of discovery of new gene products by large-scale genomic and proteomic initiatives has necessitated the design of alternative strategies for analyzing protein and peptide function as compared to traditional analytical methods. Bioactive peptides have been studied by means of the

following investigation techniques: establishment of an assay system to determine biological activity, hydrolysis of proteins by digestive enzymes, the isolation of peptides and the determination of the structures, and the synthesis of peptides for the verification of activity. Bioactive peptides usually contain 3–20 amino acid residues per molecule.

Molecular genetic screening techniques involving easily manipulated model organisms such as *Escherichia coli*, *Saccharomyces cerevisiae*, and *Drosophila melanogaster* have increased the number of proteins that can be analyzed in a single experiment. These studies tend to yield multiple subjects—genes, proteins, or mutants for additional rounds of experimentation on a molecule-by-molecule basis. Thus, the challenge is to develop high-throughput approaches to systematic and critical global protein analysis that place functionally unclassified proteins into a biological context.

7.2 Quantitation of Peptides and Proteins

Estimation and quantitation of protein concentration are necessary and important in protein purification, electrophoresis, cell biology, molecular biology, and other research applications. Spectroscopic methods are routinely used to determine concentration of protein in a solution and five of them are currently in practice [1]. These include measurement of the protein's intrinsic ultraviolet (UV) absorbance and the other four methods that generate a protein-dependent color change: Lowry [2], bicinchoninic assays (BCA) [3], Bradford dye [4], and Dumas assays. Although a wide variety of protein assays are available, one should consider the relevant technique depending on each application required. Each method has its own advantages and limitations and often it is necessary to obtain more than one type of protein assay for research and practical applications.

7.2.1 UV Light

Proteins absorb UV light at absorbance 280 nm at a maximum in solution. Amino acids with aromatic rings are the primary reason for the absorbance peak at 280 nm. Peptide bonds are primarily responsible for the peak at 200 nm wavelength. Proteins and peptides absorb UV light in proportion to their aromatic amino acid content and total concentration. Secondary, tertiary, and quaternary structures of the protein all affect the absorbance; also, factors such as pH, ionic strength, and so on can alter the absorbance spectrum to a great extent. The UV absorbance also requires that a pure protein with known extinction coefficient be used, in a solution free of interfering substances as a control. Various proteins have different extinction coefficients at both 280 and 205 nm, and concentration estimates obtained this way should take this factor into consideration [5,6]. This assay requires that the protein solution be free of other UV-absorbing particles, and measurements are made using a quartz cuvette.

7.2.2 Lowry Method

The Lowry protein assay method for determination of protein concentration is one of the most widely used protein assays due to its acceptability to estimate the proteins in almost all circumstances in which protein mixtures or crude extracts are involved. The Lowry method was described in 1951 by Lowry et al. [2]. Both Lowry and BCA assays are copper-ion-based protein assays based on reduction of Cu^{2+} to Cu^{+} by amides. Under alkaline conditions, copper complexes with the protein. When folin phenol (phospho-molybdic-phosphotungstic) reagent is added, this reagent binds to the protein. The bound reagent is slowly reduced and the color changes from yellow to blue. The strong blue color depends partly on the tyrosine and tryptophan content. Sensitivity of the method is down to about 0.01 mg of protein/mL, and is best used on solutions with concentrations that range 0.01–1.0 mg/mL of protein. Modification of this method is reported to increase the sensitivity of the assay, such as vortexing between adding Folin's reagent in two portions results in 20% increase in sensitivity [7] and addition of dithiothreitol after addition of Folin's reagent increases the sensitivity to about 50% [8]. These methods are more accurate than the traditional UV absorption method; however, they require preparation of several reagent solutions. Also, the protocol involved is lengthy and incubations are closely controlled with various temperatures

and immediate absorbance measurements of the unstable solutions. The assay may be affected by other substances frequently present in biochemical solutions, including detergents, lipids, buffers, and reducing agents. It also requires that the assay includes a series of standard solutions, each with a different, known concentration of protein having the same composition as the sample solutions.

7.2.3 Bicinchoninic Assay

The BCA assay is a highly sensitive colorimetric assay based on the same princple as the Lowry's method. The BCA protein assay was introduced by Smith et al. in 1985 [3]. One advantage of the BCA method is that unlike other methods available, the BCA protein assay is compatible with samples that contain up to 5% detergents and involves two steps. In the first step, the peptide bonds in the sample reduce Cu^{2+} ions to Cu^{+}. The amount of Cu^{2+} reduced is directly proportional to the amount of protein present in the solution. In the next step, each Cu^{+} ion forms a complex with two BCA molecules, forming purple color that strongly absorbs light at 562 nm. The amount of protein present in the solution can be quantified by measuring the absorption spectra and comparing with protein solutions with known concentrations between 0.02 and 2 mg/mL. In addition, the BCA assay responds more uniformly to different proteins than the Bradford method. Also, a modification of the BCA assay has been described that overcomes lipid interference when measuring the lipoprotein protein content [9].

7.2.4 Bradford Method

The Bradford protein assay method was described first by Bradford [4]. This method is simpler, faster, reliable, and more sensitive than the Lowry's method and subjected to less interference by reagents and nonprotein components of other biological samples. This dye method is based on the equilibrium and binding of Coomassie Blue G-250 dye to the protein. The linear range of the assay is between 5 and 25 μg/mL, the more the protein present, the more the Coomassie binds. Upon binding to protein, however, it is most stable as an unprotonated, blue form. The binding shifts the absorption maximum of the dye from red to blue. Quantity of the protein can be estimated by determining the amount of dye in the blue ionic form. Absorbance of the solution is measured at 595 nm. The Bradford method does not measure the presence of peptide bonds but detects specific amino acids, such as arginine, which is responsible for the binding of the dye to the protein. The Bradford assay is faster, involves fewer mixing steps, does not require heating, and gives a more stable colorimetric response than the other assays described above. Like the other assays, however, the response is prone to influence nonprotein sources, particularly detergents. Modification to the Bradford procedure has been suggested to reduce this variability [10].

7.2.5 Dumas Method

The Dumas method (also known as nitrogen by combustion) serves as a more precise and efficient alternative to Kjeldahl. In the food market, the total Kjeldahl nitrogen methodology is used for protein determination. In the early nineteenth century, Jean-Baptiste Dumas developed the most reliable combustion procedure for estimating the protein content by total nitrogen. This nitrogen combustion method that is used to determine the molecular weight of a substance is more accurate than Kjeldahl, in that the sample is directly related to determinations of a pure amino acid standard, while Kjeldahl requires manual standardization of titration solutions. Also, repeatability and replicate measurements are reliable in this case. However, both the methods lack analytical selectivity for measuring the protein concentration because they measure protein based on the sample nitrogen content.

7.3 Separation of Peptide and Proteins

Separation of proteins and peptides has been a challenge to researchers for many years due to the complexity of biological matrices, such as plasma and tissues, to the level of single-cell analysis. Also, physical instability is a major issue which is affected by temperature, ionic strength, pH, and so on. Identification

and separation of peptides and proteins by various chromatographic and mass spectrometric methods are routinely used in the conformational analysis of peptides and small proteins in solution by circular dichroism (CD), infrared (IR), and so on.

7.3.1 Solid–Liquid Extraction

The term extraction refers to processes involving partitioning of two compounds with two immiscible phases, wherein extraction from a solid raw material is done. Solid–liquid extraction, also known as leaching, is a method which consists in treating a solid substance, from which a few components are to be extracted, with a solvent, or a mixture of two or more solvents, through a number of serially arranged vessels. The vessels are kept during the entire extraction stage under a condition of constant stirring. The desired component solute is washed by the solvent leaving the insoluble solid undissolved. A prominent feature of this system is the supply of a continuous flow of fresh solvent to the solid in the extraction vessel which promotes efficient extraction in the shortest time. Efficient extraction may depend on the mechanical means used to increase the surface area of contact of the solid raw material and the extracting medium. While there are advantages in terms of obtaining the solids, there are several disadvantages, including factors such as temperature, pressure, interfacial or surface area, organic solvents, addition of denaturants and detergents, and so on. These disadvantages can affect the yield.

7.3.2 Precipitation

All precipitation methods for separation of proteins and peptides, except those depending on ionic interaction with oppositely charged material, are most effective at the isoelectric point of the protein. The most common general process of protein precipitation is salting out at high concentration of a salt, usually with ammonium sulfate [$(NH_4)_2SO_4$]. Protein precipitation by removal of the shell of hydrating water, as in ammonium sulfate or polyethylene glycol precipitation, is generally reversible; but lyophilization or even solvent precipitation is irreversible. Low pH can also precipitate a protein isoelectrically, that is, as the net charge goes to zero the protein and others with the same isoelectric point (p*I*), associate and precipitate, without necessarily denaturing. Another method is solvent precipitation. When large amounts of a water-miscible solvent such as ethanol, trichloric acetic acid, or acetone are added to a protein solution, proteins precipitate out. If the protein of interest can be precipitated fairly quantitatively without denaturing, and then can be redissolved at another pH and is active, then one has a reliable method.

7.3.3 Membrane Separation

Membrane-based separation techniques include dialysis and ultrafiltration. Both ultrafiltration and dialysis are conducted to purify peptides and proteins using a membrane. Dialysis is mainly used for separation of large molecules such as plasma proteins; separation is the result of differences in transport rates of analytes or solvent molecules through a membrane forced by a concentration gradient. In case of ultrafiltration, the membrane is a sieving device and not a barrier between two liquid phases with different characteristics. The solvent is driven through the membrane by means of pressure, which can be applied either using a pump or via gravitational force. By this method, proteins can be purified depending on the molecular weight cutoff membrane used for sieving.

7.3.4 Chromatography Techniques

Chromatographic techniques include various methods such as high-performance liquid chromatography (HPLC), reverse-phase LC, ion-exchange, size exclusion, affinity chromatography, and so on. HPLC continues to be the standard method of choice for protein and peptide analysis. Reverse-phase liquid chromatography (RP-HPLC) separates proteins based on their relative hydrophobicities. This technique is highly selective but requires the use of organic solvents. Some proteins are permanently denatured by solvents and will lose functionality; therefore, this procedure is not recommended for all applications.

Ion-exchange chromatography refers to separation of proteins based on relative charge. Columns can either be anion exchange or cation exchange based. Anion-exchange columns contain a stationary phase with a positive charge that attracts negatively charged proteins. Cation-exchange columns have the reverse. Elution of the target protein is derived by pH change, which results in a change or neutralization of the charged functional groups of each protein. Gel filtration or size-exclusion chromatography is a process in which larger proteins are separated from the smaller ones via the crosslinked polymer in the chromatography column. Affinity chromatography is a very useful technique in which beads in the chromatography column are crosslinked to ligands that bind specifically to the target protein. This method generates the purest results and highest specific activity compared to all other techniques.

7.4 Characterization of Peptides and Proteins

Protein characterization is critical to the functional endpoint use of proteins and peptides. Various factors such as aggregation phenomena, self-assembly, stability, and molecular weight influence the functional aspect of the molecule. Protein characterization plays an important role in two key aspects of proteomics. The first is the quality assessment of any produced protein preparation. Obtaining protein of sufficient quality for structural and/or functional studies is one of the major bottlenecks of most projects. The second is the determination of various protein properties such as domains, oligomeric state, post-translational modifications, and protein–protein and protein–ligand interactions. Various techniques that include thermal analysis, CD, chromatography, electrophoresis, and mass spectroscopy are used to characterize the peptides and proteins on a regular basis.

7.4.1 Thermal Analysis and Differential Scanning Calorimetry

Development, production, and storage of stable proteins with full functionality are critical for industries. Thermal analysis is based on enthalpy (detection of changes in the heat content) or the specific heat of a sample with temperature. The specific heat of a material changes slowly with temperature in a particular physical state, but alters discontinuously at a change of state. With increase in the sample temperature, the supply of thermal energy may induce physical or chemical processes in the sample, for example, melting or decomposition, accompanied by a change in enthalpy, the latent heat of fusion, heat of reaction, and so on. Such enthalpy changes may be detected by thermal analysis and related to the processes occurring in the sample. Differential scanning calorimetry (DSC) is an important technique of thermal analysis to characterize and investigate protein stability, and is combined with other biophysical methods to link thermodynamics, structure, and function. The principle of DSC is to compare the rate of heat flow to the sample and to an inert material which are heated or cooled at the same rate. Changes in the sample are associated with absorption or evolution of heat causing a change in the differential heat flow which is then recorded as a peak. The area under the peak is directly proportional to the enthalpic change and its direction indicates whether the thermal event is endothermic or exothermic.

7.4.2 Circular Dichroism

Circular dichroism (CD) is a spectroscopic technique where the CD of molecules is measured over a range of wavelengths and measures the differences in the absorption of left-handed polarized light versus right-handed polarized light which arise due to structural asymmetry. A primary use of CD is in analyzing the secondary structure or conformation of macromolecules, particularly proteins. Secondary structure can be determined by CD spectroscopy in the "far-UV" spectral region (190–250 nm). A representative image of the CD spectrum of an egg-white ovalbumin protein is given in Figure 7.1. The absence of regular structure results in zero intensity, while an ordered structure results in a spectrum that can contain both positive and negative signals. CD is reported in units of absorbance or ellipticity. Each of these can be normalized for molar concentration of the sample. CD measurements have two major advantages: they can be made on small amounts of material in physiological buffers and they provide one of the best methods for monitoring any structural alterations that might result from changes in environmental conditions, such

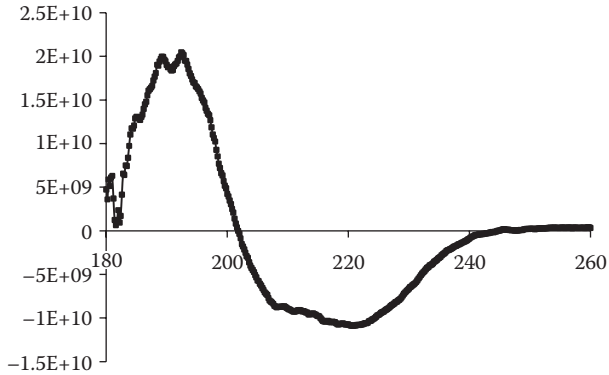

FIGURE 7.1 CD spectrum of egg-white ovalbumin protein.

as pH, temperature, and ionic strength [11]. One of the other most useful applications of CD is monitoring the protein denaturations, which can be initiated either thermally or chemically.

7.4.3 Fourier Transform Infrared and Near-Infrared Spectroscopy

Fourier transform infrared spectroscopy (FT-IR) is a technique that provides information about the chemical bonding or molecular structure of materials, whether organic or inorganic. This technique works on the fact that bonds and groups of bonds vibrate at characteristic frequencies. A molecule that is exposed to IR rays absorbs IR energy at frequencies which are characteristic to that molecule. During FT-IR analysis, a spot on the specimen is subjected to a modulated IR beam. The specimen's transmittance and reflectance of the IR rays at different frequencies is translated into an IR absorption plot consisting of reverse peaks. The resulting FT-IR spectral pattern is then analyzed and matched with known signatures of identified materials in the FT-IR library. This technique can be applied to minute quantity of materials and is used to study protein–ligand interactions [12]. The secondary structure of proteins can also be detected by using near-infrared spectroscopy technique (NIR) [13]. Quantitative methods are possible where changes in the response of the NIR spectrometer are proportional to changes in the concentration of chemical components, or in the physical characteristics (scattering/absorptive properties) of samples undergoing analysis. The use of NIR as a technique mostly for identification, quantitative analysis, and structure-correlation assessment of NIR functional group frequencies is a powerful and valid one. For a wide range of NIR applications, particular attention is given to the appearance of methyl, methylene, methoxy, carbonyl, and aromatic C–H groups; hydroxy O–H; and N–H from amides, amines, and amine salts.

7.4.4 High-Performance Liquid Chromatography

HPLC analysis of peptides and proteins has been a useful tool in the field of biological sciences enabling rapid and sensitive analysis of peptide and protein structure through the exquisite speed, sensitivity, and resolution that can be easily obtained. HPLC with UV detection is basically a highly improved form of column chromatography. It is a pivotal technique used in the characterization of peptides and proteins and currently plays a critical role in both our understanding of biological processes and in the development of peptide- and protein-based pharmaceuticals. In RP-HPLC, compounds are separated based on their hydrophobic character. Compounds stick to RP-HPLC columns in high aqueous mobile phase and are eluted from columns with high organic mobile phase. The separation depends on the hydrophobic binding of the solute molecule from the mobile phase to the immobilized hydrophobic ligands attached to the stationary phase, that is, the sorbent. The extensive use of RP-HPLC for the purification of peptides, small polypeptides with molecular weight up to 10,000 kDa, and related compounds of pharmaceutical interest has not been replicated to the same extent for larger polypeptides (>10 kDa) and globular proteins. RP-HPLC is extremely versatile for the isolation of peptides and proteins from a wide variety

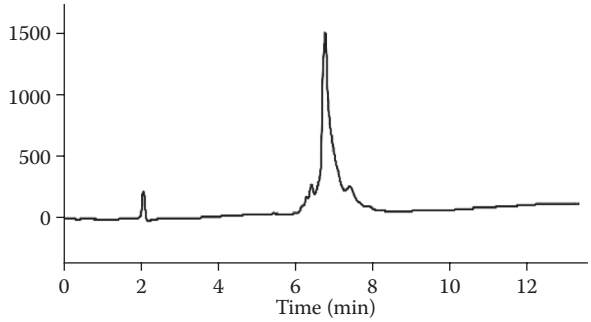

FIGURE 7.2 Representative image of an HPLC chromatogram.

of synthetic or biological sources and is used for both analytical and preparative applications. The representative image of an HPLC chromatogram image is shown in Figure 7.2.

7.4.5 Electrophoresis (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis)

The separation of macromolecules in an electric field is called electrophoresis. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a basic support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The most commonly used system is the Laemmli method after U.K. Laemmli, who was the first to publish a paper employing SDS-PAGE in a scientific study [14]. The purpose of SDS-PAGE is to separate proteins according to their size. Proteins are separated largely on the basis of polypeptide length, and hence the molecular weight can also be estimated. SDS-PAGE can also separate DNA and RNA molecules. SDS denatures proteins, causing multimeric proteins to dissociate into their subunits, and all polypeptide chains are forced into extended conformations with similar charge: mass ratios. Chains that differ in molecular weight by <10% can be separated by this technique. A representative image of immunoglobulin E separated from porcine serum sample on an SDS-PAGE gel is given in Figure 7.3. The molecular weight of a protein can be determined by comparison of a protein ladder or molecular weight ladder which is run on the same gel.

7.4.6 Immunochemical Analysis (Western Blot and ELISA)

Antibodies are useful tools to specifically detect many substances. Methods such as enzyme-linked immunosorbent assays (ELISA), enzyme immunoassays (EIAs), Western blotting, radio immunoassays (RIAs), protein-arrays, immunohistochemistry, or the immuno-polymerase chain reaction (Immuno-PCR) belong to this category of the immuno detection technology. Even if they are highly specific, they all

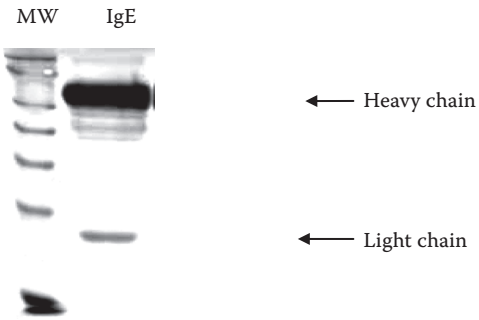

FIGURE 7.3 A representative image of immunoglobulin E separated from porcine serum sample and visualized on an SDS-PAGE. MW represents standard molecular weight markers.

have in common to be disturbed by unwanted cross-reactivities. Immunochemical technical analysis of proteins, for example, blotting, is an analytical method that involves the immobilization of proteins on membranes followed by detection using monoclonal or polyclonal antibodies. There are different blotting protocols (dot blot, 2D blot) and one of the most powerful one is the Western blotting. It is traditionally used to detect low amounts of proteins in complex samples or to monitor protein expression and purification. The use of electrophoresis to transfer proteins from a gel to a membrane was first described by Towbin et al. [15]. Through spatial resolution, this method provides molecular weight information on individual proteins and separates isoforms from processed products. After proteins have been transferred onto a suitable membrane, they can be stained for visualization or directly be identified by N-terminal sequencing, MS, or immunodetection. Western blotting is considered to be a reliable confirmatory diagnostic test and is reported to be the most sensitive, unequivocal, and simple test system available, with the highest complexity of information obtained. ELISAs are plate-based assays designed for detecting and quantifying the substances such as peptides, proteins, antibodies, and hormones. Other names, such as EIAs, are also used to describe the same technology. In an ELISA, an antigen must be immobilized to a solid surface and then complexed with an antibody that is linked to an enzyme. Detection is accomplished by assessing the conjugated enzyme activity via incubation with a substrate to produce a measureable product. ELISAs are used for detecting and quantitating the substances such as peptides, proteins, antibodies, hormones, haptens, and drugs of abuse and their metabolites. ELISAs are typically performed in 96, 384, and even 1536-well polystyrene plates and are easily robotized and adapted to high-throughput screening.

7.4.7 MALDI-TOF-Mass, LC-MS/MS, and ESI-Mass

Mass spectrometry (MS) is a crucial tool in the analysis of peptides and proteins. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are the two dominant ionization methods, and coupling of ESI to separation techniques is now standard. The sensitivity of these two instruments utilizing MALDI and ESI makes them useful tools for monitoring the processes in cell culture. MS is a useful analytical technique and is widely used within structural proteomics consortia. Analysis of both intact proteins and protein digests allows for the verification of construct along with any modifications such as methylation as well as giving information on posttranslational modifications [16]. It is widely used within structural and functional proteomics for a variety of tasks including protein quality assessment, identification, and characterization and routinely used for the determination of the total mass of proteins, including *N*-glycosylated proteins, crystal content verification, and analysis of *N*-glycosylation site occupancy.

7.4.8 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance spectroscopy, commonly referred to as NMR, has become the preeminent technique for determining the structure of proteins. Of all the spectroscopic methods, it is the only one for which a complete analysis and interpretation of the entire spectrum is normally expected. Although larger amounts of sample are needed than for mass spectroscopy, NMR is nondestructive, and with modern instruments data may be obtained from samples weighing less than a milligram. NMR spectroscopy is unique among the other methods available for three-dimensional structure determination of proteins and nucleic acids at atomic resolution, since the NMR data can be recorded in solution. In NMR experiments, solution conditions such as the temperature, pH, and salt concentration can be adjusted so as to closely mimic a given physiological fluid. In addition to protein structure determination, NMR applications include investigations of dynamic features of the molecular structures, as well as studies of structural, thermodynamic, and kinetic aspects of interactions between proteins and other solution components.

7.5 Proteomics and Peptidomics

The role that proteins and peptides play in health and disease is termed as proteomics and peptidomics (also known as comprehensive analytical chemistry) and is a key research area in systems biology. It is

the branch of biology that studies the full set of proteins/peptides derived from a given genome. Identification of proteins and peptides in their multidimensional interactions in complex biological processes can be characterized using proteomics and peptidomics. A restricted window of the proteome that consists of peptides and small proteins not easily manageable by conventional gel electrophoresis prompted the development of separation methods based on liquid chromatography. This new research field termed peptidomics, together with proteomics, is supported by sophisticated bioinformatics tools, to the discovery of new diagnostic and therapeutic targets. The techniques of biophysical separation, MS, and bioinformatics form the basis of mining the proteome even from microgram amounts of protein extracts derived from body fluids and tissues. Proteomics and peptidomics techniques enable us to identify and quantify all proteins and peptides, respectively, present in a biological sample derived from a cell, tissue, body liquid, or whole organism. For proteomics research, the sequencing of amino termini is fundamental to protein or peptide identification. Complementary and often superior to modern MS in terms of defining protein structure and identity, classical Edman degradation chemistry remains the definitive technique for N-terminal amino acid sequencing.

7.6 Biotechnology

7.6.1 Peptide Mapping

Quantitation is generally expressed as an area or height percent of the native peptides. In this way, the peptide map can provide information on the mixture of protein forms in each sample so that safety and efficacy of the preparation may be assured. The method must, therefore, exhibit excellent sensitivity and linearity for quantitative work. The strategy of peptide quantification includes adding a known amount of a specific peptide (peptide standards) to an actual protein digest to test the estimates of quantification. This peptide serves as a surrogate, illustrating the behavior of modified peptides in the digest. Peptide mapping is used to confirm the primary structure of a protein, to identify posttranslational modification, to demonstrate generic stability, and to analyze potential impurities. Critical factors involved in peptide mapping include robustness, the limit of detection, specificity, linearity, range, accuracy, and precision. Any difference in the structure of a protein should be reflected in a change in retention time for the peptide containing the modification. The relative amounts of the peptide with and without a particular modification are used to measure the fraction of the protein in the particular sample that carries that modification.

7.6.2 Complimentary DNA Technology

Development of technologies facilitates the generation of a complete set of full-length human complementary DNAs (cDNA) as well as other mammalian cDNAs. Access to high-quality mammalian cDNA clones and sequences is critical for the rapid progression of biomedical research. cDNA technology can be used to analyze the changes in genome-wide patterns of gene expression [17,18]. Either cDNA microarrays or oligonucleotide-based chips may be used for gene-expression analysis. Oligonucleotide-based DNA chips are also used for analyzing the sequence variations in genomic DNA for screening the individuals for DNA mutations and polymorphism variations. Analysis of the expression of thousands of genes in one experiment allows investigators to address some important biological questions that have not been easily addressed with traditional expression-based technologies, such as Northern blots, *in situ* hybridizations, or RNase protection assays, which examine gene expression changes of only a few genes at a time.

7.6.3 Protein and Peptide Expression

Protein or peptide expression occurs when a related protein or peptide is produced by transforming a host cell with a nucleic acid encoding the related protein to form a transformed cell resulting in a recombinant protein. Molecular methods have been developed to express proteins and peptides in various host cells such as yeast [19], bacteria [20], plants [21], fungi [22], and algae [23]. Bacterial expression systems are

usually the starting point for expression of heterologous proteins. Bacterial fermentations are inexpensive and can reach high cell densities resulting in high volumetric yields of the target protein. The distadvantage is producing a recombinant protein similar to the native form, since posttranslational modifications are not taken into account in this case. Mammalian cell expression has become the system of choice for production of complex, glycosylated biotherapeutic proteins such as antibodies, growth factors, and fertility hormones. Baculovirus/insect cell systems have found wide application for the expression of highly recalcitrant proteins such as protein tyrosine kinases. Yeast offers relatively inexpensive growth media and high-density fermentation and the capability of carrying out limited posttranslational modifications such as disulfide bond formation and glycosylation.

7.6.4 Modification of Proteins

Modification of proteins occurs by crosslinking, fragmenting, denaturing, reducing disulfides, or attaching various prosthetic groups (e.g., PEGylation) to allow manipulation and study of protein function and interactions in any environment. While protein synthesis, or translation, is the beginning of a protein's specific lifecycle, there are numerous other changes that can occur to individual protein molecules as they carry out their specific cellular function. Many modifications add small and large chemical groups to proteins at specific sites in the amino acid chain; there are also chemical modifications that modify the inherent structure of individual amino acids [24]. The major types of protein covalent modifications, such as phosphorylation, acetylation, glycosylation, methylation, and ubiquitylation, can be classified according to the type of amino acid side chain modified, the category of the modifying enzyme, and the extent of reversibility. Chemical events such as protein splicing, green fluorescent protein maturation, and proteasome autoactivations also represent posttranslational modifications [25].

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Section II

Vitamins

8

*Vitamin K**

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8.1 Introduction

Vitamin K, a fat-soluble vitamin, is an enzyme cofactor for posttranslational modification of a select group of proteins. In the presence of vitamin K, specific glutamate residues are converted into γ-carboxyglutamic acid (Gla) residues by a vitamin K-dependent (VKD) carboxylase. The VKD coagulation proteins, factors II, VII, IX, and X, and proteins C, S, and Z, are synthesized in the liver (Berkner, 2005). The extra-hepatic VKD proteins include osteocalcin (Hauschka et al., 1975; Price et al., 1976), Matrix Gla-protein (Price et al., 1983), Gla-rich protein (Viegas et al., 2008), periosin (Coutu et al., 2008), Tgfbi (Ahmed et al., 2007; Zhang et al., 2009), and Gas-6 (Shearer, 2000; Berkner and Runge, 2004).

Vitamin K is historically identified for its role in activation of blood coagulation factors. Variability in vitamin K intake is often cited as a risk factor for instability of oral anticoagulant therapy. There are numerous case reports citing individual food items as potential causes of over- or under-anticoagulation in warfarin patients, and observational data to support an interaction between dietary vitamin K and stability of warfarin therapy (Rohde et al., 2007). Restriction and/or monitoring of vitamin K in the diet are often used as a strategy to minimize the dietary fluctuations that cause instability of oral anticoagulation. Dietary counseling requires accurate food composition data, which has created a need for accurate analytical methods for the measurement of vitamin K in a wide variety of foods.

* Any opinions, findings, conclusions, or recommendations expressed in this publication are those of the authors and do not necessarily reflect the view of the US Department of Agriculture. No authors report a conflict of interest.

Over the past two decades, it has been proposed that vitamin K has multiple roles beyond coagulation (Booth, 2009). Vitamin K deficiency is linked to a variety of age-associated conditions (McCann and Ames, 2009), including loss of bone mineral density or increased fracture risk (Bugel, 2008; Heiss et al., 2008; Shea and Booth, 2008), osteoarthritis (Neogi et al., 2006), arterial calcification or cardiovascular disease (Berkner and Runge, 2004), chronic kidney disease (Holden and Booth, 2007; Pilkey at al., 2007), insulin resistance (Yoshida et al., 2008a,b), and inflammation (Shea and Booth, 2008; Shea et al., 2008). Vitamin K is also involved in inhibition of cancerous cell growth and synthesis of sphingolipids independent of its role as an enzyme cofactor (Lamson and Plaza, 2003; Denisova and Booth, 2005). Moreover, *in vitro* studies indicate that vitamin K can enhance bone mineralization and decrease bone resorption through steroid and xenobiotic receptors (SXRs)/pregnane X receptors (PXRs) that modulate gene transcription (Tabb et al., 2003; Ichikawa et al., 2006). As mechanisms for vitamin K independent of its action as a cofactor for the carboxylase enzyme are elucidated, it is likely that interest in the role of vitamin K as a modifiable factor in health promotion will expand.

Since the high-performance liquid chromatography (HPLC) technique has been applied to analyze vitamin K content in several foods, there is now a significant and growing database for vitamin K contents in vegetables, fruits, prepared foods, and animal products. This chapter describes the physical properties, food sources of vitamin K, and vitamin K dietary recommendations, and reviews the available methodologies for vitamin K analysis in foods.

8.2 Physical Properties of Vitamin K

8.2.1 Chemical Structures

Vitamin K refers to a family of compounds that contains a napthoquinone ring and a side chain. The side chain differs in length and degree of saturation. Phylloquinone (vitamin K_1) is a 2-methyl-1,4-napthoquinone ring with a phytyl group at the 3-position (Figure 8.1). Natural phylloquinone occurs entirely as the *trans*-isomer, but synthetic phylloquinone used for food fortification contains a mixture of *cis*- and *trans*-isomers. The *cis*-form of phylloquinone has little or no biological activity (Matschiner and Bell, 1972; Knauer et al., 1975). The majority of menaquinones (vitamin K_2) are endogenously synthesized and differ in structure from phylloquinone in their 3′-substituted unsaturated multiprenyl group (Figure 8.1). The primary menaquinones, menaquinone-4 (MK-4) through menaquinone-10 (MK-10), contain 4–10 repeating isoprenoid units on their side chain, respectively. MK-4 is formed by tissue-specific conversion from phylloquinone (Davidson et al., 1998) or menadione (vitamin K_3) (Okano et al., 2008), and is not endogenously synthesized by bacteria (Thijssen and Drittij-Reijnders, 1994; Thijssen et al., 2006). Menadione, which is the 2-methyl-1,4-napthoquinone ring common to all forms of vitamin K, can function as an enzyme cofactor in the prevention of subclinical vitamin K deficiency. More recently, menadione has been identified as a metabolite of vitamin K formed during absorption (Thijssen et al., 1996; Okano et al., 2008). Hydrogenation of plant oils, which are rich sources of phylloquinone, results in the conversion of phylloquinone into 2′,3′ dihydrophylloquinone, which differs from its parent form by the

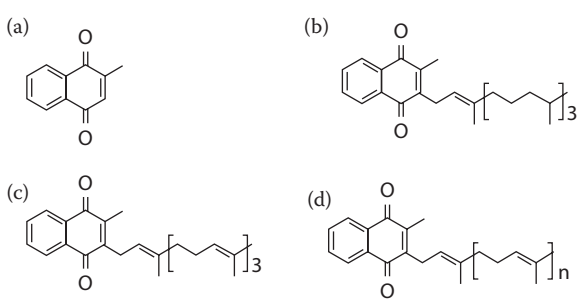

FIGURE 8.1 Vitamin K structures. (a) Menadione; (b) phylloquinone; (c) MK-4; (d) menaquinones (MKn).

saturation of the 2′,3′ double bond in the side chain (Davidson et al., 1996). Dihydrophylloquinone is present in the US food supply (Elder et al., 2006; Ferreira et al., 2006), but has less biological activity than phylloquinone (Booth et al., 2000; Troy et al., 2007).

8.2.2 Stability

Phylloquinone and presumably menaquinones are quite stable to heating, food processing mode, and preparation conditions (Ferland and Sadowski, 1992). However, phylloquinone is unstable in daylight and to alkalinity (Seifert, 1979). Instability to alkalinity precludes the use of saponification for extraction from foods. In contrast, menadione is not as stable as other forms of vitamin K. Menadione sodium bisulfate is the form of vitamin K used in synthetic animal feed. The menadione concentration in animal feed stored for 125–280 days was significantly lower (30–70% lost) compared with that stored for 2–16 days (Billedeau, 1989). Hu et al. (1995) reported that menadione was lost upon drying the extraction solution by a nitrogen steam. Consistent unpublished data from the author's laboratory indicate that more than 90% of menadione is lost under vacuum evaporator during sample processing. One possible reason for this instability is adsorption on the polar surface of the tubes (Hu et al., 1995).

8.3 Food Sources of Vitamin K

Our current understanding of vitamin K suggests that it naturally exists in two dietary forms, phylloquinone and menaquinones. Phylloquinone is found in green leafy vegetables and vegetable oils, which, in the US food supply, provide 40–50% of the total intake (Booth and Suttie, 1998). Milk and dairy products, meats, eggs, cereals, and fruits are low, but consistent sources of vitamin K in the US diet (Booth et al., 1996; Dismore et al., 2003; Elder et al., 2005). An overview of dietary intakes of vitamin K and the vitamin K content of foods is reviewed elsewhere (Suttie, 2009). Table 8.1 also summarizes phylloquinone concentrations of common foods in the US food supply.

MK-4 is generally in lower concentrations in food compared to phylloquinone (Elder et al., 2005). However, it is the primary form of vitamin K in certain tissues, such as glands, brain, and pancreas. MK-4 is unique to the menaquinones because it is an endogenously produced form of vitamin K synthesized from phylloquinone. The bacterial produced long-chain menaquinones (MK-8 and MK-9) are found mainly in cheese, and the amounts reported are quite variable (Schurgers et al., 1999; Dumont et al., 2003). Another menaquinone that has recently received considerable attention is menaquinone-7 (MK-7). A traditional Japanese-fermented food (*natto*) contains significant amounts of MK-7. In certain Asian cultures, *natto* may be an important source of vitamin K (MK-7) for high consumers of this traditional food (Kamao et al., 2007a).

The 2′,3′-dihydrophylloquinone is most prevalent in margarines, infant formulas, and prepared foods, and can represent a substantial portion of the total vitamin K in certain dietary patterns that include high consumption of processed foods (Cook et al., 1999; Peterson et al., 2002; Dumont et al., 2003; Weizmann et al., 2004; Ferreira et al., 2006). With a general trend toward a reduction in hydrogenation of plants oils, it is expected that there will be an overall reduction in dihydrophylloquinone in the US food supply.

8.4 Dietary Recommendations

There is currently no recommended dietary allowance for vitamin K. Dietary reference intake values for vitamin K were generated from the Third National Health and Nutrition Examination Survey (NHANES III, 1988–1994), and the adequate intake (AI) was based on the median intake in the United States for each age and gender category (Institute of Medicine, 2001). The AIs for adult men and women are 120 and 90 μg/day, respectively. The guidelines in the United Kingdom are 1 μg/kg body weight/day (Department of Health, 1991), and the AI in Japan is set at 75 μg for adult men, 60 μg/day for women, aged 18–29, and 65 μg/day for women aged 30 and over (Kamao et al., 2007). The true requirement based on the role of extra-hepatic VKD proteins is still unknown. Estimates of phylloquinone intake in

TABLE 8.1

Phylloquinone Concentration in Common Foods[a]

Food Item	Common Measure	Content (μg) Per Measure	Food Item	Common Measure	Content (μg) Per Measure
Vegetables			Prepared foods		
Collards, frozen, cooked	1 cup	1059.4	Pizza, chesses topping	1 serving	4.2
Kale, cooked, without salt	1 cup	1062.1	Apple pie	1 piece	4.1
Spinach, cooked	1 cup	888.5	Doughnuts, cake-type	1 medium	2.5
Brussels sprouts, cooked	1 cup	299.9	Salad dressings	1 tbsp	2.2
Corn, sweet, cooked	1 ear	97.2	Potato chips	1 oz	2.0
Broccoli, raw	1 cup	89.4	Muffins, corn	1 muffin	1.3
Cucumbers, with peel, raw	1 large	49.4	Cheese, cheddar	1 oz	0.8
Cabbage, raw	1 cup	42.0	Fats and oils		
Celery, raw	1 cup	35.2	Margarine, regular	1 tbsp	13.2
Lettuce, green leaf, raw	1 cup	35	Olive oil, cooking	1 tbsp	8.1
Asparagus, cooked, boiled	4 spears	30.4	Soybean oil, cooking	1 tbsp	3.4
Cauliflower, raw	1 cup	16.0	Butter, salted	1 tbsp	1.0
Carrots, raw	1 cup	14.5	Fruits		
Protein sources			Blackberries, raw	1 cup	28.5
Soybeans, mature, cooked	1 cup	33	Peaches, raw	1 cup	4.4
Lentils, mature seeds, cooked	1 cup	3.4	Apples, raw, with skin	1 apple	3.0
Egg, whole, cooked	1 large	2.6	Pineapple, raw	1 cup	1.1
Beef, cooked	3 oz	1.9	Bananas, raw	1 banana	0.6
Milk, whole, 3.25% fat	1 cup	0.5	Strawberries, raw	1 strawberry	0.3
Fish, salmon, smoked	3 oz	0.1			

[a] US Department of Agriculture's National Nutritional Database for Standard Reference, Release 22 (http://www.nal.usda.gov/fnic/foodcomp).

various populations are probably more accurate than intakes of other forms of vitamin K, and there is a substantial difference in the reported intakes from a number of countries, which seems to be related to food consumption practices in different areas (Schurgers et al., 1999; Thane et al., 2002; Yan et al., 2004; Duggan et al., 2007; Kamao et al., 2007a). For example, the mean total vitamin K intake of young Japanese women (mean 21.2 years, $n = 124$) was about 230 μg/day (Kamao et al., 2007). The estimated phylloquinone intakes in northern China, England, and Scotland were 247, 103, and 70 μg/day, respectively (Bolton-Smith and Shearer, 1997; Yan et al., 2004). Furthermore, tolerable upper intake levels are not established, since no adverse effects have been reported at high intake levels.

8.5 Measurement of Vitamin K in Foods

8.5.1 Overview

Early food composition data for vitamin K were generated using chick bioassays that were more qualitative than quantitative (Suttie, 1995). Subsequent analytical methods used for phylloquinone analysis included thin-layer chromatography and gas chromatography (GC) (Seifert, 1979). In the past three decades, more sensitive and precise methods for vitamin K analysis in foods have been developed using HPLC with various detection techniques, such as ultraviolet (UV), electrochemical (EC), and fluorescence (FL) detection (Booth and Sadowski, 1997). Recently, some new techniques, such as gas chromatography mass spectrometry (GC/MS) (Fauler et al., 1996; Dolnikowski et al., 2002; Erkkila et al., 2004; Jones et al., 2006), liquid chromatography mass spectrometry (LC/MS) (Kurilich et al., 2003), and HPLC-tandem mass spectrometry with atmospheric pressure chemical ionization (LC-APCI MS/MS) have been used (Suhara et al., 2005; Kang et al., 2007). Moreover, the development of reliable chromatographic

procedures has resulted in greater sensitivity and accuracy of menaquinones measurements in foods (Koivu-Tikkanen et al., 2000; Hojo et al., 2007).

8.5.2 Sampling

As is true for analysis of all nutrients, the aim of sampling is to obtain a manageable, yet representative portion of the material that satisfactorily represents the content of the nutrient of interest. In the case of vitamin K, all procedures involving sample processing and preparation should preferably be done under subdued or yellow lighting because vitamin K compounds are sensitive to photooxidation (Booth and Sadowski, 1997). As this is often not feasible, all possible efforts should be made to avoid direct light.

It has been shown that geographical growth location, climate, soil conditions, and stage of maturation can influence the phylloquinone content of green vegetables (Bolton-Smith et al., 2000). Since phylloquinone is primarily found in the photosynthetic tissue of plants, the phylloquinone concentration can also vary within a given vegetable. The outer leaves of cabbage and Brussels sprouts have been shown to contain more phylloquinone than the inner leaves (Ferland and Sadowski, 1992). Cooking by boiling or microwaving may disrupt the plant cell wall, thereby releasing phylloquinone available for detection by HPLC. Potential factors affecting phylloquinone concentrations also include various types of leafy vegetables (Damon et al., 2005). The foods analyzed for vitamin K, as represented in the US Department of Agriculture Food Composition Database (http://www.nal.usda.gov/fnic/foodcomp), were sampled using a sophisticated sampling plan, as described in detail elsewhere (Pehrsson et al., 2000). A similar approach, albeit on a much smaller scale, has been described for analysis of vitamin K in foods sold in the vicinity of Helsinki, Finland (Koivu et al., 1997, 1999; Piironen et al., 1997; Piironen, 2000).

8.5.3 Extraction Techniques

The extraction of vitamin K from foods is usually achieved by first denaturing the vitamin K-lipoprotein complex with a polar solvent (e.g., ethanol, 2-propanol, or methanol), followed by extraction into a nonpolar solvent (e.g., hexane). Solid-phase extraction (SPE) with silica cartridges is the most commonly used technique for further purification of the extract. However, gel permeation chromatography and semipreparative liquid chromatography also provide cleanup necessary for interference-free chromatography.

Human milk and infant formulas contain a large amount of lipids in fat globules that are encased in protein–phospholipid membrane. Consequently, the quantitation of the fat-soluble phylloquinone is an analytical challenge, and the removal of the lipids is a crucial step. The AOAC Official Method 999.15 is an enzyme hydrolysis and LC method for determination of vitamin K in milk and infant formulas (Hwang, 1985). The use of lipase hydrolysis has been proposed as a primary extraction step, with the use of a nonspecific lipase to hydrolyze the lipids in infant formula and dairy products as the initial step in the extraction of all fat-soluble vitamins (Barnett et al., 1980). Lipases usually include Type III from *Candida cylindraces* (Indyk and Woollard, 1997), *Candida rugosa* (Jones et al., 2006), porcine pancreas lipase Type II (Lambert et al., 1992), and cholesterol esterase (from *Pseudomonas*). These treatments reduce chromatographic interferences by removing the lipid components.

Sonication and heating collectively contribute to disperse and extract vitamin K from mixtures (Booth and Sadowski, 1997; Piironen, 2000). SPE is a more contemporary technique for sample preparation and has been widely adopted. The phylloquinone-containing fraction bound to a silica column is subsequently eluted with a wash of 3.5–4% (v/v) diethyl ether in hexane (Booth and Sadowski, 1997; Pérez-Ruiz et al., 2007; Fu et al., 2009). High lipid-containing samples, such as oils, require an additional purification step using a C_{18} SPE column (Booth and Sadowski, 1997). Semipreparative HPLC purification is another popular method for further cleanup; C_{18} μ-bondapak column (Otles and Cagindi, 2007), 5-μm cyanosilane-bonded column (McCarthy et al., 1997), and μ-porasil column (Piironen et al., 2000) have all been reported to successfully separate vitamin K from other lipophilic compounds. Jakob and Elmadfa (1996) also improved the efficacy sample cleanup by liquid–liquid extraction and without SPE or any semipreparative chromatographic step. The summary of extraction and purification methods for different food is shown in Table 8.2.

TABLE 8.2

Summary of Extraction and Purification Methods Tested for Groups Different Foods

Food Groups	Measurements	IS	Extraction	Purification	Reference
Vegetables/Fruits/Cereal Products					
	K_1, MK-4	$K_{1(25)}$	Sonication and isopropanol/hexane (3:2, v/v) extraction	SPE on a silica column. The K_1 is eluted with 3.5% ethyl ether in hexane.	Dismore et al. (2003); Ferreira et al. (2006); Booth and Sadowski (1997)
	K_1	MK-4	Boiling water bath, isopropanol/hexane extraction	Pass through a 0.45 μm filter. Semipreparative HPLC purification	Koivu et al. (1997, 1998)
	K_1	No	Sonication and hexane extraction	Pass through a sintered glass funnel. SPE on silica column. The K_1 is eluted with 4% diethyl ether in hexane	Pérez-Ruiz et al. (2007)
	K_1	dK	Dichloromethane/methanol (2:1, v/v) extraction; hexane/ methanol–water extraction	No	Jakob and Elmadfa (1996)
Milk and Infant Formula					
	K_1	$K_{1(25)}$	Combined sonication and an enzymatic treatment, hexane extraction	SPE on a silica column	Lambert et al. (1992)
	K_1	No	Lipase treatment and ethanol/methanol extraction	SPE on a silica column. The K_1 is eluted with 4% diethyl ether in hexane	Pérez-Ruiz et al. (2007)
	K_1, MK-4	No	Lipase treatment and hexane extraction	Pass through 0.45 μm filter	Indyk and Woollard (1997)
Oil and Margarines					
	K_1, dK	MK-4 or dK	Hexane extraction	Pass through a 0.45 μm filter. Semipreparative HPLC purification	Koivu et al. (1997); Piironen et al. (1997); Otles and Cagindi (2007)
	K_1, dK	$K_{1(25)}$	Sonication and isopropanol/hexane (3:2, v/v) extraction	SPE on a silica column. The K_1 is eluted with 3.5% ethyl ether in hexane. C_{18} SPE is performed to further purify the sample	Peterson et al. (2002)
	K_1	MK-4	Lipase treatment and ethanol/hexane extraction	SPE on a silica column. The K_1 is eluted with 4% diethyl ether in hexane	Gao and Ackman (1995)
	K_1	dK	Hexane extraction, hexane/ methanol–water extraction	No	Jakob and Elmadfa (1996)
Tea and Coffee Brew					
	K_1	$K_{1(25)}$	Sonication and isopropanol/hexane (3:2, v/v) extraction	SPE on a silica column. The phylloquinone is eluted with 3.5% ethyl ether in hexane	Booth et al. (1995)
Dairy and Fast Food/Animal Products/Meat/Cheese					
	K_1, MK-4, dK	$K_{1(25)}$	Sonication and isopropanol/hexane (3:2, v/v) extraction	SPE on a silica column. The K_1 is eluted with 3.5% ethyl ether in hexane. If the residue appeared oily, C_{18} SPE was performed to further purify the sample	Elder et al. (2005)

TABLE 8.2 (continued)

Summary of Extraction and Purification Methods Tested for Groups Different Foods

Food Groups	Measurements	IS	Extraction	Purification	Reference
	K_1, MK4-10	$K_{1(25)}$	Boiling water bath, isopropanol/hexane extraction (fat >10%, lipase hydrolysis)	Pass through a 0.45 μm filter. Semipreparative HPLC purification	Koivu-Tikkanen et al. (2000)
	K_1, MK4-10	$K_{1(25)}$	Acid hydrolysis method, boiling water bath, diethyl ether/petroleum ether extraction (fat >10%, lipase hydrolysis)	Pass through a 0.45 μm filter. Semipreparative HPLC purification	Koivu-Tikkanen et al. (2000)
	K_1	dK	2-Propanol/hexane (3:1, v/v) extraction; hexane/methanol–water extraction	No	Jakob and Elmadfa (1996); Majchrzak and Elmadfa (2001)
Nuts	K_1	$K_{1(25)}$	Sonication and isopropanol/hexane (3:2, v/v) extraction.	SPE on a silica column. The phylloquinone is eluted with 3.5% ethyl ether in hexane. If the residue appeared oily, C_{18} SPE is performed to further purify the sample	Dismore et al. (2003)

Note: IS: internal standard; K_1: phylloquinone; dK: 2′,3′ dihydrophylloquinone; SPE: solid-phase extraction.

8.5.4 Internal Standards

$K_{1(25)}$, a synthetic analog of phylloquinone (produced by the substitution of a 25-carbon side chain to menadione), and MK-4 have both been used as an internal standard to determine vitamin K concentration in vegetables or oils (Gao and Ackman, 1995; Koivu et al., 1997; Piironen, 2000). Since it is not a suitable internal standard for animal products which contain MK-4, $K_{1(25)}$ and dihydrovitamin K have successfully been used to analyze the animal products. There are three reasons that support the use of $K_{1(25)}$ over dihydrovitamin K for food analysis. First, dihydrophylloquinone has the same retention time as other compounds. Second, there is no evidence to support the existence of $K_{1(25)}$ in food, whereas dihydrophylloquinone is abundant in the US food supply. Finally, $K_{1(25)}$ has a recovery similar to that of phylloquinone (Lambert et al., 1986). Synthesized deuterium- or O^{18}-labeled forms of vitamin K are also used as internal standards in vitamin K analysis using MS methods. Fauler et al. have developed a GC/MS method to determine phylloquinone using 2-methyl-d_3-3-eicosa-2-ene-l,4-naphthoquinone (VK-d_3) as an internal standard (Fauler et al., 1996). VK-*d*4 was also synthesized for use as an internal standard in a vitamin K absorption study (van Hasselt et al., 2009). Similarly, VK – ^{18}O, MK-4-^{18}O, and MK-7-^{18}O were used in vitamin K analysis by LC-tandem mass spectrometry (LC/MS/MS) (Suhara et al., 2005).

8.5.5 Standard Reference Materials

Primary stock solutions: Pure vitamins that are commercially available (phylloquinone, menadione, and MK-4) are either an oil or a powder. $K_{1(25)}$ is commercially available as an oil (GL synthesis, Worcester, MA). The other forms of vitamin K are currently not commercially available. To prepare stock solutions, the pure vitamin is weighed out and dissolved in hexane. The hexane solutions seem to be stable indefinitely. For example, concentrations of solutions prepared up to 16 years ago and stored in foil-covered large test tubes at –15 to –20°C are unchanged (unpublished data).

Secondary stock solutions: The primary stock solution is diluted with methanol to a given concentration suitable for preparing working standards. The concentration of vitamin in the secondary standard is determined spectrophotometrically using molar absorption coefficients summarized in Table 8.3. The secondary stock solutions are stored in flasks covered with foil at either –15°C or 5°C.

TABLE 8.3

Vitamin K Molar Absorption Coefficients

Form of Vitamin K	MW	$E_{1cm}^{1\%}$	Maximum Abs (nm)
Phylloquinone	450.7	420	248
Dihydrophylloquinone	452.7	420	270
MK-4	444.7	439	248
$K_{1(25)}$	521.7	420	248

8.5.6 Vitamin K Analysis

8.5.6.1 Gas Chromatography

Gas chromatography was used during 1960 and 1970 for vitamin K analysis (Seifert, 1979). However, the obtained retention times were long, and lower detection limits were too high for routine analysis of foods. With the introduction of new methodologies such as SPE and solid-phase microextraction (SPME), the GC method is of interest again. A new method using SPME-GC with flame ionization detector has been developed for the analysis of vitamin K in leaves, but the lower limit of detection is still high, 0.16 and 0.07 mg/L for vitamin K_1 and K_2, respectively (Reto et al., 2007). Fauler et al. (1996) developed a quick and accurate detection method for measurement of vitamin K using GC/electron impact mass/spectrometry. The detection limit is 1 pg, which allows for measurement of phylloquinone in very low concentrations. However, the derivatization with heptafluorobutyryl ester is required. Dolnikowski et al. (2002) developed a stable isotope-labeled technique using GC/MS and HPLC to measure the deuterium-labeled phylloquinone enrichment in human serum after ingestion of deuterium-labeled broccoli and collard greens, which has utility for human absorption studies (Dolnikowski et al., 2002; Erkkila et al., 2004). Among the methods using GC/MS, technical challenges were reported in plasma samples containing high concentrations of lipophilic compounds (e.g., in nonfasting samples), which precluded the determination of phylloquinone concentrations in these samples (Dolnikowski et al., 2002; Erkkila et al., 2004). Enzyme hydrolysis and subsequent derivatization of phylloquinone before analysis were used to resolve this problem (Jones et al., 2006, 2008). However, the disadvantages of this approach included the complicated and labor-intensive nature of sample preparation, and the contamination of the lipase with phylloquinone (unpublished data).

8.5.6.2 High-Performance Liquid Chromatography

HPLC has become the standard method for the determination of vitamin K concentrations in a wide variety of food samples. Reverse-phase chromatography is the most commonly used technique in that it provides good separation of vitamin K, and various forms of C_{18} is the most common column packing material. Columns packed with C_{30}-coated silica particles are used when it is necessary to separate the *cis*- and *trans*-isomer of vitamin K_1 (Cook et al., 1999; Woollard et al., 2002). The flow rates and composition of the mobile phase are highly variable.

Here, four methods are summarized, which have been used to detect vitamin K using HPLC analysis namely, UV spectrometry, EC, spectrofluorimetry, and MS detection.

UV spectrometry: The earlier LC methods for vitamin K detection were primarily based on UV detection. The K vitamins exhibit maximum UV absorbance at the range of 240–250 and 260–270 nm. A major drawback of the traditional methods using UV detection has often been the inadequacy of the systems employed to detect the low concentrations of K vitamins. These problems have largely been overcome by the introduction of more sensitive detection systems. Recently, Otles and Cagindi (2007) developed a new method to detect vitamin K in olive oil and vegetables using HPLC with UV–vis detector at 248 nm. Similar results were obtained compared with those reported in previous studies using HPLC with fluorescence detection (Jakob and Elmadfa, 2000).

EC detection: In the early 1980s, amperometric EC detection was used in the reduction of phylloquinone to phylloquinone hydroquinone (Shearer et al., 1982; Hart et al., 1984). Reductive EC detection was found to be about threefold more sensitive than UV detection. However, reductive EC detection is problematic because oxygen needs to be completely absent in the mobile phase in order to remove the high background and the chromatographic baseline drift (Hart et al., 1984). In contrast, the use of a dual-electrode detector provided a redox-mode EC detection of phylloquinone, which increased the sensitivity 10-fold compared to the reductive method (McCarthy et al., 1997). Piironen and colleagues determined the vitamin K concentrations in Finnish foods using HPLC-EC detection. In these methods, the analytical Coulochem EC cell is operated in the redox mode with an upstream potential of –1.1 V and a downstream electrode potential of 0 V (Koivu et al., 1997; Piironen et al., 1997).

Fluorescence detection: In the 1980s, analytical methods were published incorporating reductive conversion of the quinones to highly fluorescent hydroquinones (Booth et al., 1994; Booth and Sadowski, 1997; Davidson and Sadowski, 1997). This development provided a significant advance for vitamin K analysis. HPLC with fluorescence detection of the K vitamins exploits the highly fluorescent nature of vitamin K hydroquinone produced either by postcolumn zinc reduction or EC reduction. A number of chemical reduction methods have been employed, including use of sodium borohydride ($NaBH_4$) (Ikenoya et al., 1979), tetramethylammonium octahydriborate [$(CH_3)_4NB_3H_8$] (Lambert et al., 1992; Woollard et al., 2002), zinc metal (Booth and Sadowski, 1997), platinum oxide catalyst (MacCrehan and Schönberger, 1995), or platinum black catalysts (Shino, 1988; Kamao et al., 2005). Recently, online photochemical fluorimetric assays for phylloquinone and menadione have also been used (Pérez-Ruiz et al., 2007, 2006). The measurement of phylloquinone in foods by HPLC with fluorescence detection is summarized in detail elsewhere (Booth and Sadowski, 1997). Fluorescence is monitored using fluorescence detector with excitation wavelengths of 243–325 nm and emission wavelengths of 418–430 nm (Lambert et al., 1992; Jakob and Elmadfa, 1996; Booth and Sadowski, 1997; Indyk and Woollard, 1997; Majchrzak and Elmadfa, 2001; Woollard et al., 2002).

Mass spectrometry: The interfacing of LC and MS produced a powerful analytical tool for quantification and structural confirmation of the vitamin K. APCI, a "soft" and highly efficient method suitable for analysis of polar, ionic, high molecular mass, has greatly increased the popularity of LC/MS. Since most of the ionization resides in a single ion, selected ion monitoring (SIM) of the [MH^+] for each K vitamins affords a more specific method of detecting these compounds. The availability of vitamin K labeled with stable isotopes has greatly expanded our knowledge of the absorption, deposition, and metabolism of vitamin K in humans (Dolnikowski et al., 2002; Kurilich et al., 2003; Fu et al., 2009). A sensitive method has been developed for measurement of isotopes of phylloquinone (produced by growing vegetables with heavy water) using LC-APCI/MS (Fu et al., 2009). To determine the relative bioavailability of phylloquinone from different food sources, phylloquinone obtained from green, leafy vegetables were compared with phylloquinone obtained from *Spirulina* (blue-green algae). Figure 8.2 depicts the enrichment

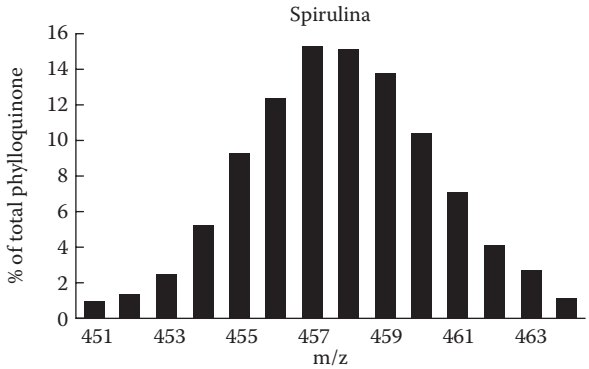

FIGURE 8.2 Enrichment profile of phylloquinone in deuterium-labeled spirulina determined by an LC-APCI/MS (unpublished data).

profile of phylloquinone in deuterium-labeled *Spirulina* determined by LC-APCI/MS (unpublished data). Single-ion monitoring was used to detect the isotopomer of phylloquinone in spirulina, the predominant isotopomers being *m/z* 451 for unlabeled phylloquinone and *m/z* 452–464 for labeled phylloquinone.

More recently, the analysis of vitamin K has been achieved with LC/MS/MS (Suhara et al., 2005; Kamao et al., 2007b; Kang et al., 2007; van Hasselt et al., 2009). The technique uses selective multiple reaction monitoring, which is a tandem mass spectrometric method designed to measure the parent/product ions. The mass transitions are *m/z* 445.9 → 187.1 for MK-4, *m/z* 461.5 → 311.9 for MK-4 epoxide, *m/z* 451.7 → 187.2 for phylloquinone, and *m/z* 649.5 → 187.2 for MK-7 (Suhara et al., 2005; Kamao et al., 2007). The LC/MS/MS methods are more sensitive and faster than GC/MS methods available for phylloquinone (van Hasselt et al., 2009). Derivatization, which is necessary for GC/MS analysis, is not required in LC/MS/MS methods, which confers an advantage in terms of sample preparation and run time.

8.6 Summary

A wide range of analytical techniques are available for the detection, quantitation, and evaluation of vitamin K in foods. The methods vary from simple to complex depending on extraction, separation, identification, and detection of the analyte. Among the extraction methods applied for vitamin K analysis, SPE with silica is a more common technique for sample preparation. Among the methods surveyed, HPLC is used extensively for the analysis of vitamin K in foods as it combines relatively high separation efficiencies with low detection limits. Among the different detection modes utilized for vitamin K analysis, both EC and fluorescence detection provide sensitive and accurate detection techniques. In particular, LC/MS/MS can provide selected ion mode for detecting deuterium- or C^{13}-labeled vitamin K in vegetables. Future investigations are needed to extend these techniques and expand their use into the field of food analysis.

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9

Methods for the Simultaneous Quantitative Analysis of Water-Soluble Vitamins in Food Products

Olivier Heudi

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9.1 Introduction

The water-soluble vitamins (WSVs) group includes diverse compounds (Figure 9.1) with respect to structure, molecular weight, chemical properties, and biological activity: thiamine (vitamin B1), riboflavin (vitamin B2), pantothenic acid (vitamin B5), vitamin B6 vitamers (pyridoxal, pyridoxine, and pyridoxamine), cyanocobalamin (vitamin B12), L-ascorbic acid and L-dehydroascorbic acid (vitamin C), niacin (nicotinic acid) and its amide (nicotinamide, vitamin PP), folic acid (vitamin B9), and biotin. In addition, other biologically active compounds have been recognized as pseudovitamins. The WSVs play an important role in human health and their deficiency in the diet can cause serious health issues. Thus, there is a need to have fast and reliable analytical methods for their quality control in different types of food products. Several methods are used to measure these vitamins in supplements and food products. Blake (2007) and Konings (2005) made excellent reviews on the analysis of WSVs by microbiological assays (MBAs), high-performance liquid chromatography (HPLC), and biosensor/ELISA. As Blake pointed out, MBAs are no longer considered as the gold standard in vitamin analysis. Thus, different HPLC-UV methods with variable wavelength or diode array detection (AlbalaHurtado et al., 1997; Amidzic et al., 2005; Blanco et al., 1994; Chen et al., 2009; Chen and Wolf, 2007; Cheng et al., 2001; Engel et al., 2010; Ivanovic et al., 1999; Klejdus et al., 2004; Kucukbay and Karaca, 2010; Lebiedzinska et al., 2007, Li, 2002; Li and Chen, 2001a,b; Markopoulou et al., 2002; Marszall et al., 2005; Vidovic et al., 2008, Vinas et al., 2003; Woollard and Indyk, 2002; Zafra-Gomez et al., 2006), capillary electrophoresis (CE), and microemulsion electrokinetic chromatography (Delgado-Zamarreno et al., 2002; Fotsing et al., 1997; Okamoto et al., 2002, 2003; Svidritskii et al., 2010) have emerged for the quantitative analysis of WSVs in food products. Also, fluorescence detection (FLD) has been used for the determination of naturally fluorescent vitamins such as B2 (Chen et al., 2009) and B6

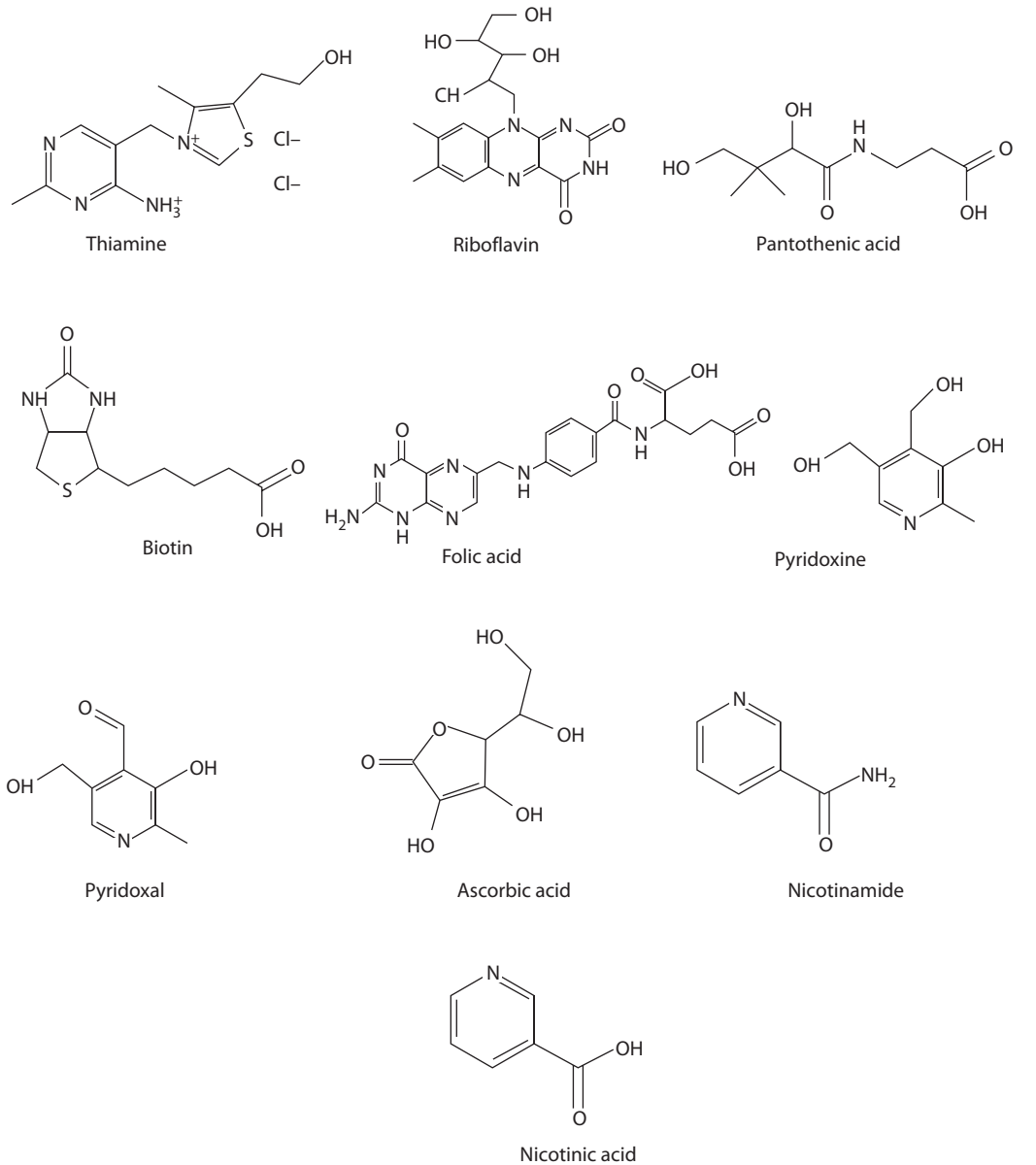

FIGURE 9.1 Structure of the different WSVs discussed in this chapter.

(Gatti and Gioia, 2005) or those that become fluorescent after derivatization, B5 (Pakin et al., 2004) and B12 (Pakin et al., 2005). Only few of these methods dealt with the simultaneous quantitative analysis of more than five WSVs in food products. The reason for this is that the applied methods were not selective and sensitive enough to detect low amounts of WSVs in complex matrices. The introduction of atmospheric pressure ionization techniques greatly increased the number of compounds and matrices that can be analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS). With the development of electrospray ionization (ESI) interface, it is now possible to combine the high selectivity of MS detection with the better sensitivity when operating in multiple reactions monitoring (MRM) mode. LC-MS/MS provides the capability of performing multiple analyte measurements simultaneously. This method has been demonstrated to have widespread applications in many areas, including food and nutrient analysis (Guy and Fenaille, 2006). Some LC/MS works have focused specifically on the measurement of one WSV (Luo et al.,

2006; Mittermayr et al., 2004; Rychlik and Roth-Maier, 2005). During the last 5 years, LC-MS/MS has been expanded to the simultaneous quantitative analysis of WSVs in food products.

The aim of this chapter is to discuss the different methods used for the simultaneous quantitative analysis of WSVs in all types of food products. After reviewing the HPLC method coupled to UV-FLD and CE detections, the interest will be focused on LC-MS/MS methods which have been increasingly used for the simultaneous quantification of WSVs in food products within the past couple of years. Different points such as multivitamins extraction, recovery, and the selectivity and sensitivity related to each analytical method will be discussed in depth. The simultaneous determination of WSVs will be presented in food products such as vitamins supplements, premixes, fortified products (infant formulae (IF), baby food), and in nonfortified products namely honey and pasta.

9.2 HPLC-UV, HPLC-FLD, and Capillary Zone Electrophoresis Methods for the Simultaneous Quantitative Analysis of WSVs in Foodstuffs

9.2.1 Application to the Food Supplements and Premixes

A dietary supplement, also known as food supplement or nutritional supplement, is a preparation intended to supplement the diet and provide nutrients, such as vitamins, minerals, fiber, fatty acids, or amino acids, that may be missing or may not be consumed in sufficient quantities in a person's diet. Some countries define dietary supplements as foods, while in others they are defined as drugs or natural health products. Premixes are raw materials that are used to fortified food products such as IF and baby foods. Except vitamins, these products can also contain minerals. Both supplements and premixes contain high amounts of vitamins and represent less complex matrices as compared to finished products such as milk or chocolate-based products.

A selective HPLC method has been developed for the simultaneous determination of three WSVs (B1, B6, and B12) in multivitamin tablets (Markopoulou et al., 2002). The vitamins were extracted in water as the excipients present in the dosage forms did not interfere with the peaks of interest. The method uses a reversed-phase column and gradient elution over 15 min. The aqueous mobile phase contained 0.015% triethylamine adjusted to pH 2.7 with 1 N sulfuric acid and acetonitrile. Separation and quantitation were achieved by changing the proportion of the system linearly with a time-schedule program. Detection was carried out using a dual-beam UV detector set at 280 and 350 nm. Capillary zone electrophoresis (CZE) in uncoated fused silica capillaries and UV detection has been applied to the quantification of six WSVs (B1, B3, B2, B6, C, and B5) in a pharmaceutical formulation (Fotsing et al., 1997). The influence of different parameters, such as the nature of the buffer anionic component and buffer concentration on the CZE separation of vitamins, was investigated using four vitamins of the B group as model compounds. A good compromise between resolutions, analysis time, and analyte stability was obtained by using a 50 mM borax buffer of pH 8.5. This CZE method was found to be very useful for the separation of more complex samples, a mixture of 10 WSVs being completely resolved in about 10 min. However, Vitamin B12 could not be separated from vitamin B3 in this CZE system, as the two compounds are in uncharged form at the pH used. These two compounds could easily be resolved by micellar electrokinetic chromatography, the anionic surfactant dodecylsulfate being added to the running buffer at 25 mM concentration. In the pharmaceutical formulation, some excipients were found to be adsorbed to the capillary surface, giving rise to a progressive decrease of the electroosmotic flow and consequently to a simultaneous increase of analyte migration times. Thus, good sample preparation and capillary washing were essential to minimize these effects. In two other HPLC-UV methods, Li and Chen (2001a) and Heudi et al. (2005) described a separation of nine WSVs: (B1, C, PP, B6, B5, B9, B12, B2, and B8) in multivitamin tablets and in polyvitaminated premixes. The separation of all nine WSVs was achieved within 17 min (Figures 9.2 and 9.3) and the UV-detection was performed at different wavelengths. The multivitamins were extracted in a phosphate buffer and the mineral content of premixes did not influence the determination of vitamin C. A disadvantage of the methods mentioned above is that sample composition has to be known in advance. According to European legislation, for example, foods might be fortified with riboflavin phosphate or thiamin phosphate, vitamers which were not included in the above-described

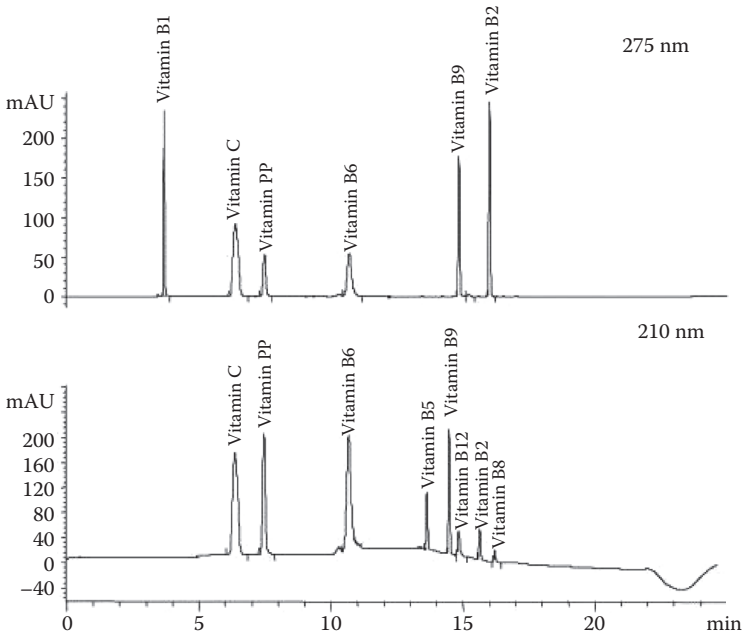

FIGURE 9.2 Baseline separation of WSVs monitored at two different wavelengths. (From Heudi, O., Kilinc, T., and Fontannaz, P. 2005. *J. Chromatogr. A*, 1070, 49–56. With permission.)

methods. A supermodified simplex method has been developed to optimize the mobile phase used for separation of seven WSVs in multivitamin tablets by gradient micellar liquid chromatography with UV detection at 254, 295, and 361 nm (Ghorbani et al., 2004). In this method, vitamin B12 was included. However, the LC-separation of each WSV peak was not optimal.

9.2.2 Application to Fortified Food Products

The simultaneous determination of WSVs in premixes or in multivitamin tablets has been successfully achieved with either HPLC-UV or HPLC-FLD method. However, compared to premixes or multivitamin tablets, fortified food products represent a higher matrix complexity. Thus, the determination of vitamins

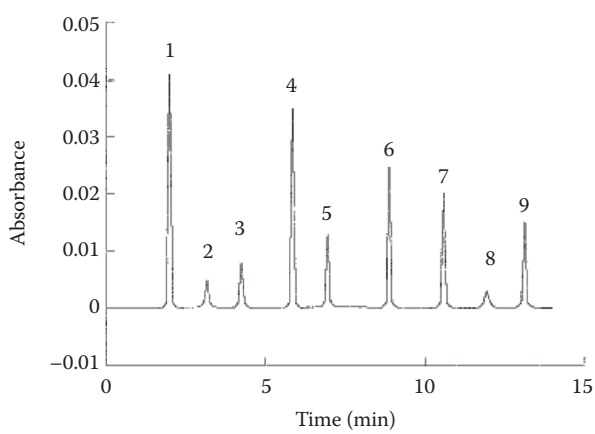

FIGURE 9.3 Chromatogram of vitamins in the multivitamin tablets (2); (1) Vitamin C, (2) biotin, (3) folic acid, (4) nicotinamide, (5) pantothenate, (6) vitamin B1, (7) vitamin B6, (8) vitamin B12, and (9) vitamin B2. (From Li, H. B. and Chen, F. 2001a. *J. Sep. Sci.* 24, 271–274. With permission.)

in this type of food represents a daunting challenge. Several problems are encountered during the development of analytical method for the simultaneous determination of WSVs in fortified food stuffs: (1) different structures and chemical properties of vitamins make the development of a single method for their simultaneous determination difficult; (2) vitamins are often present in food at trace levels; (3) foods are complex matrices; and (4) some vitamins are instable to light, air, and heat. This latter problem can result in underestimation of analytes due to degradation processes occurring during the sample preparation procedure. The extraction and cleanup represent the rate-limiting stage in almost all vitamin analyses. Each WSV requires specific conditions for its extraction from foods. The choice of these conditions depends on the stability of each vitamin (i.e., on its sensitivity to pH, temperature, exposure to light, oxygen partial pressure, ionic strength, and occurrence of heavy metal ions) and on its bond to the food matrix. Also, it is important to know which form of vitamin is determined (free or bound). All the abovementioned points will be addressed for each method developed discussed below.

Multivitamin methods have been recently developed for the quantification of WSVs in fortified food products especially in IF. With this approach, different samples preparation involving protein precipitation with zinc acetate and phosphotungstate (Zafra-Gomez et al., 2006), perchloric acid (Chase et al., 1992), or trichloroacetic acid (AlbalaHurtado et al., 1997; Woollard and Indyk, 2002) followed by centrifugation were tested. In a recent published method for the isolation and simultaneous determination of the vitamins B1, B2, B3, B5, B6, B9, B12, and vitamin C in IF, infant milk, and vitamin-enriched milks, vitamins B1, B3, B5, B9, B12, and C were determined by HPLC-UV detection, whereas vitamins B2 and B6 were determined by HPLC-FLD (Zafra-Gomez et al., 2006). The authors have shown that available fast methods for enriched food products, such as protein precipitation with trichloroacetic acid, lead to poor extraction capacity for some vitamins, such as vitamin B3 or vitamin C. Meanwhile, they have noticed the appearance of impurities that interfere with the chromatographic determination of vitamins B5 and B9, possibly due to the use of strong acids, such as sulfuric acid, hydrochloric acid, or trichloroacetic acid for protein precipitation. The chromatographic separation was carried out on a C18 column with ion-pair reagent and the vitamins were monitored at different wavelengths by either fluorescence or UV–visible detection. Representative chromatograms of a supplemented commercial milk sample for WSVs are given in Figure 9.4. The reliability of the proposed method for the determination of WSVs in milk and IF was confirmed by using a certified reference material milk powder, CRM 421. There were no significant differences between the results obtained with this chromatographic method and the certified values (the test performed at a significance level of $\alpha < 0.05$). These data also confirm the efficiency of the sample treatment procedure for the recovery of all studied vitamins from the samples. Klejdus et al. (2004) described an LC-UV method for the simultaneous determination of 10 WSVs in different types of food products including soya milk, fortified IF, and infant milk food. The sample preparation involved dilution in a mixture of 0.010% TFA:methanol (50:50 v/v) followed by the filtration and LC-analysis on a Polaris C18 column, at the flow rate of 0.7 mL/min. The analysis time was about 10 min and the most two suitable detection wavelength for simultaneous vitamin determination was 280 nm. The combined isocratic and linear gradient profile of the mobile phase consisting of 0.010% TFA of pH 3.9 and methanol allowed determination of the vitamins in three distinct groups—polar (isocratic elution at 95:5 in 6 min), low-polar (positive linear gradient elution between 6th and 14th min), and nonpolar (negative linear gradient elution, longer than 15 min). A baseline separation with good resolution of all vitamins in each group was achieved in a single run for the fortified food products tested. With this method, the data obtained by the authors for selected WSVs on certified materials were in good agreement with the declared values. However, the authors found that vitamin–vitamin, vitamin–mobile phase, and vitamin–column adsorbent interactions could cause unknown and unexpected signals and spurious results for some vitamins, especially the more polar one.

Most of the methods for the simultaneous determination of WSVs have been developed with a reversed-phase column. With such a column, very polar WSVs (vitamins B1 and C) are not well retained and this could lead to problems of low reproducibility in the retention times of these vitamins. Vinas et al. (2003) have developed a liquid chromatographic method for the separation and determination of nine WSVs: B1, B2, PP (niacinamide and nicotinic acid), B6 (pyridoxine and pyridoxal), B9, and B12 in different IFs, cereals, and fruit products. The procedure was based on the use of a new amide-based stationary phase, which avoids the need of using the ion-pair technique, leading to

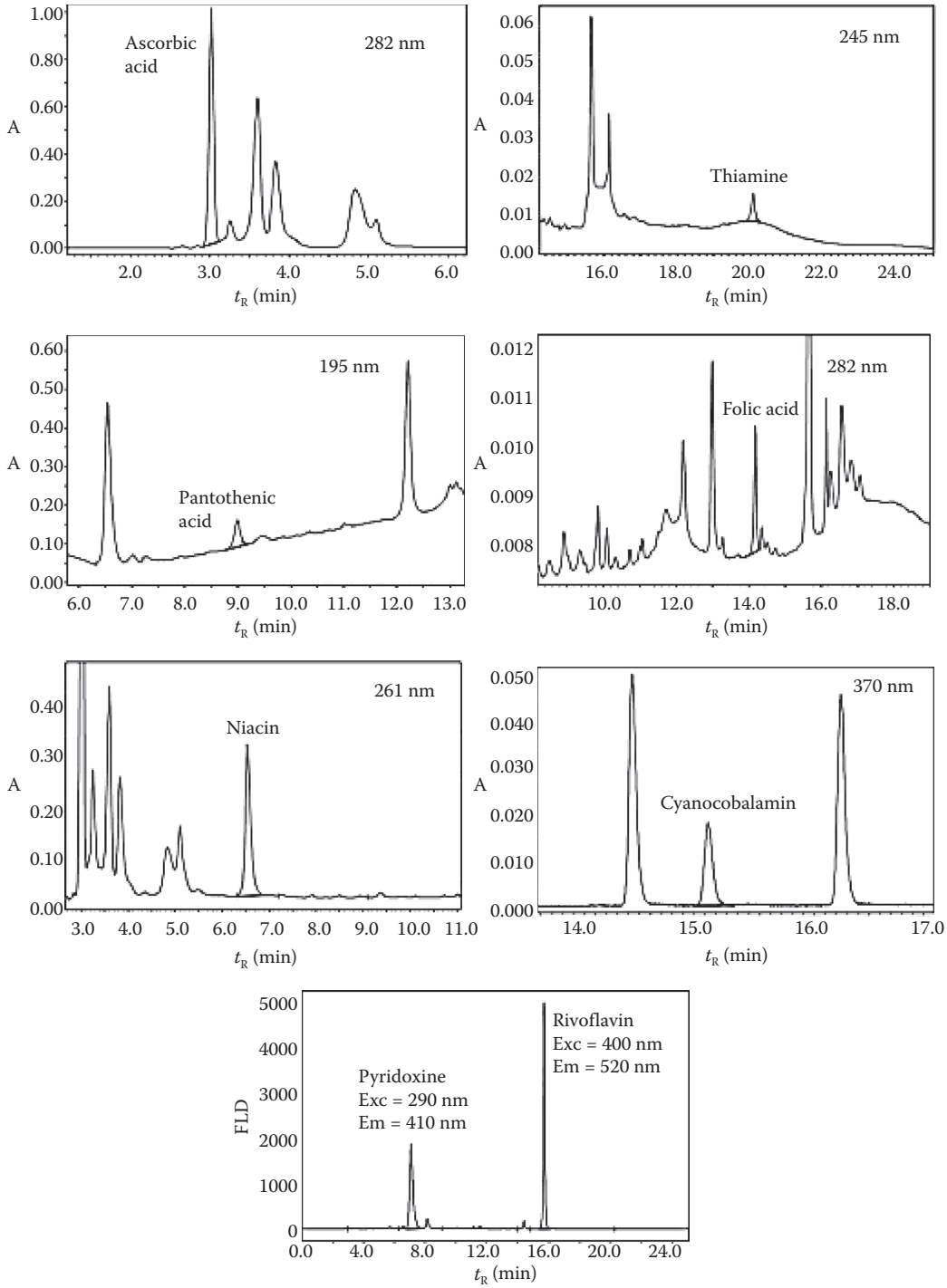

FIGURE 9.4 Representative chromatograms of a supplemented commercial milk sample reference. (From Zafra-Gomez, A. et al. 2006. *J. Agric. Food Chem.* 54, 4531–4536. With permission.)

TABLE 9.1

Determination of WSVs in Baby Food Samples

	Results Found[2] (Labeled) (μg g^{-1})					
Vitamin	**Infant Formula (Starting Milk)**	**Infant Formula**	**Infant Formula**	**Cereals with**	**Multifruits**	**Rice Cream**
NA	ND	ND	ND	ND	ND	ND
PL	2.0 ± 0.12 (9.6)	1.1 ± 0.03 (3.8)	0.75 = 0.03 (3.2)	ND	ND	ND
PN	6.2 ± 0.1	3.2 ± 0.09	2.6 = 0.03	3.5 ± 0.22 (3.6)	1.8 = 0.03 (2.6)	3.3 ± 0.06 (3.6)
T	8.0 ± 0.09 (7.2)	3.6 ± 0.2 (3.6)	3.1 = 0.05 (3.2)	6.8 ± 0.16 (6.0)	4.0 = 0.06 (5.5)	5.5 ± 0.17 (6.0)
N	139 ± 3 (130)	51 ± 2 (51)	73 = 3 (71)	44 ± 2 (50)	69 ± 2 (70)	56 ± 2 (50)
IN	ND	ND	ND	ND	ND	ND
RA	1.1 ± 0.07 (1.4)	0.5 ± 0.01 (0.46)	0.91 = 0.03 (0.8)	0.6 ± 0.01 (0.5)	0.56 = 0.01 (0.4)	0.61 ± 0.02 (0.5)
CC	ND	ND	ND	ND	ND	ND
RF	11.4 ± 0.5 (11.0)	8.5 ± 0.12 (7.6)	8.8 = 0.1 (7.9)	ND	5.7 = 0.1 (4.79)	ND

Source: Adapted from Zafra-Gomez, A. et al. 2006. *J Agric. Food Chem.* 54, 4531–4536.

Note: Thiamine (vitamin B_1, T), riboflavin (vitamin B_2, RF), nicotinamide (vitamin B_3, N), nicotinic acid (NA), pyridoxine (vitamin B_6, PN), pyridoxal (PL), folic acid (FA), and cyanocobalamin (vitamin B_{12}, CC).

narrower peaks and a simpler mobile phase. Analyses were performed by gradient elution with acetonitrile–phosphate buffer as the mobile phase. With this new type of column, the authors demonstrated that the residual silanol groups react more strongly with the vitamins and thus lead to good retention of very polar WSVs. The detection was optimized using a photodiode array detector. In this method, the sample preparation combined multiple extraction steps involving acid protein precipitation, enzymatic digestion to release protein-bound sample, and centrifugation prior the LC analysis. With this method, the authors were able to determine WSVs in several types of foods. The results obtained from the simultaneous determination of WSV in some food products are given in Table 9.1. In addition, the reliability of the method was verified using two certified reference materials as demonstrated by the values summarized in Table 9.2.

9.2.3 Application to Nonfortified Food Products

An RP-HPLC-UV method was developed and validated for the simultaneous determination of five WSVs (B2, B3, B5, B9, and C) in honey (Ciulu et al., 2011). The method provides low detection and quantification

TABLE 9.2

Determination of Selected WSV in Certified Reference Materials (CRM421 and CRM487)

	Milk Powder (CRM 421)		Pig's Liver (CRM 487)	
Vitamin	**Results Found[2] (μg/g)**	**Certified (μg/g)**	**Results Found[2] (μg/g)**	**Certified (μg/g)**
PN total	6.40 ± 0.24	6.66 ± 0.85	17.7 ± 1.1	19.3 ± 2.9
T	6.08 ± 0.09	6.51 ± 0.48	9.1 ± 0.4	8.6 ± 1.1
N	70.3 ± 0.9	68.0 ± 2.0		Not certified
FA	1.24 ± 0.05	1.42 ± 0.14	11.6 ± 0.5	13.3 ± 1.3
CC	Not detected	0.034 ± 0.005	1.17 ± 0.06	1.12 ± 0.09
RF	14.4 ± 0.1	14.5 ± 0.6	105.2 ± 3.3	106.8 ± 5.6

Source: Adapted from Zafra-Gomez, A. et al. 2006. *J Agric. Food Chem.* 54, 4531–4536.

Note: Thiamine (vitamin B1, T), riboflavin (vitamin B2, RF), nicotinamide (vitamin B3, N), folic acid (FA), and cyanocobalamine (vitamin B12, CC).

limits, very good linearity in a large concentration interval, very good precision, and the absence of any bias. The WSVs were extracted in the phosphate buffer prior to their analysis. The chromatographic method was adapted from the one previously developed by Heudi et al. (2005) for the premixes analysis.

9.3 LC-MS/MS Methods for the Simultaneous Quantitative Analysis of WSVs in Foodstuffs

Although LC-MS/MS methods are selective and sensitive, there is still a need, when using this approach, to have an appropriate internal standard (IS). Most of the reported applications of LC-MS or LC-MS/MS for quantification of WSVs have utilized either external standard or IS that is not structurally related to the vitamin being determined. Such methodologies may fail to account for analyte losses during sample preparation, something that can be significant for WSVs such as vitamin B12 that is present at relatively low concentrations and may require multiple steps for isolation prior to their analysis. The use of nonlabeled standards or analogs can provide some improvements but the later cannot always match the analyte's behavior during the sample processing or the LC-MS/MS analysis. Thus, the use of stable isotope labeled (SIL) is anticipated to be superior in accounting fully for sample losses during preparation as well as for any potential matrix effects on ionization when mass spectrometry is used. In this review, both approaches for simultaneous determination of WSVs with nonlabeled and labeled IS are discussed.

9.3.1 Methods with Nonlabeled IS for the Simultaneous Quantitative Analysis of WSVs in Premixes, Multivitamins Supplements, and Fortified Products

An LC-MS/MS method was proposed for the simultaneous determination of WSVs of seven WSVs (B1, B2 B3, B6, B8, (inositol), B5, and B9) in multivitamin/multimineral daily supplements (Chen and Wolf, 2007). This approach utilizes a reversed-phase column with a gradient mobile elution profile, performed at a flow rate of 0.25 mL/min. The seven vitamins were analyzed in 17 min. This method was initially applied to determine WSVs in representative multivitamin/multimineral tablets following the extraction of ground samples with a phosphate buffer (10 mM, pH 2.5). Using the same detection method, with a slight modification of the gradient, the same authors were able to analyze vitamin C together with six other WSVs (Chen et al., 2009). For these two methods, no sample cleanup/preconcentration steps except centrifugation and filtration were required after the extraction, which was performed in phosphate buffer as for the HPLC methods. Although good chromatographic separation of ascorbic acid was obtained in extracts from multivitamin/multimineral supplements, the latter could not be quantified properly due to rapid oxidation catalyzed by the minerals. Quantitation was done by external standard calibration and by standard addition and no SIL vitamin was used. Another HPLC/ESI (electrospray ionization)-MS method for the simultaneous determination of 10 WSVs including vitamins B1 B2, B5, B6, B8, B9, C, and PP (nicotinamide and nicotinic acid) in multivitamin tablets was developed and validated (Chen et al., 2006). The separation was accomplished on a Johnson Spherigel reversed-phase column with methanol in an aqueous solution of heptafluorobutyric acid (5 mM) as the mobile phase under gradient elution mode. The detection of target vitamins was performed by ESI-MS switching continuously from positive-ion (PI) mode to negative-ion (NI) mode. All the vitamins except folic acid and vitamin C were analyzed in the PI mode. In this method, hippuric acid was used as IS. The typical mass spectra of the analyzed vitamins are shown in Figure 9.5. In general, the LC-MS/MS methods that were developed and applied to the simultaneous determination of WSVs in multivitamin tablets showed good accuracy and precision and were able to target more than nine WSVs without applying a complex sample preparation. In addition, most of the vitamins were analyzed in <25 min. Despite the high selectivity and sensitivity of the LC-MS/MS approach, vitamin B12 could not be determined together with the other WSVs in a single run. Thus this vitamin was determined separately (Luo et al., 2006).

Recent years have seen the introduction of a new generation of HPLC instruments that surpass the pressure limitation of conventional equipment (400 bar) and are capable of operating at back pressures up to 1000 bar. These separations have been termed ultra-high-performance liquid chromatography

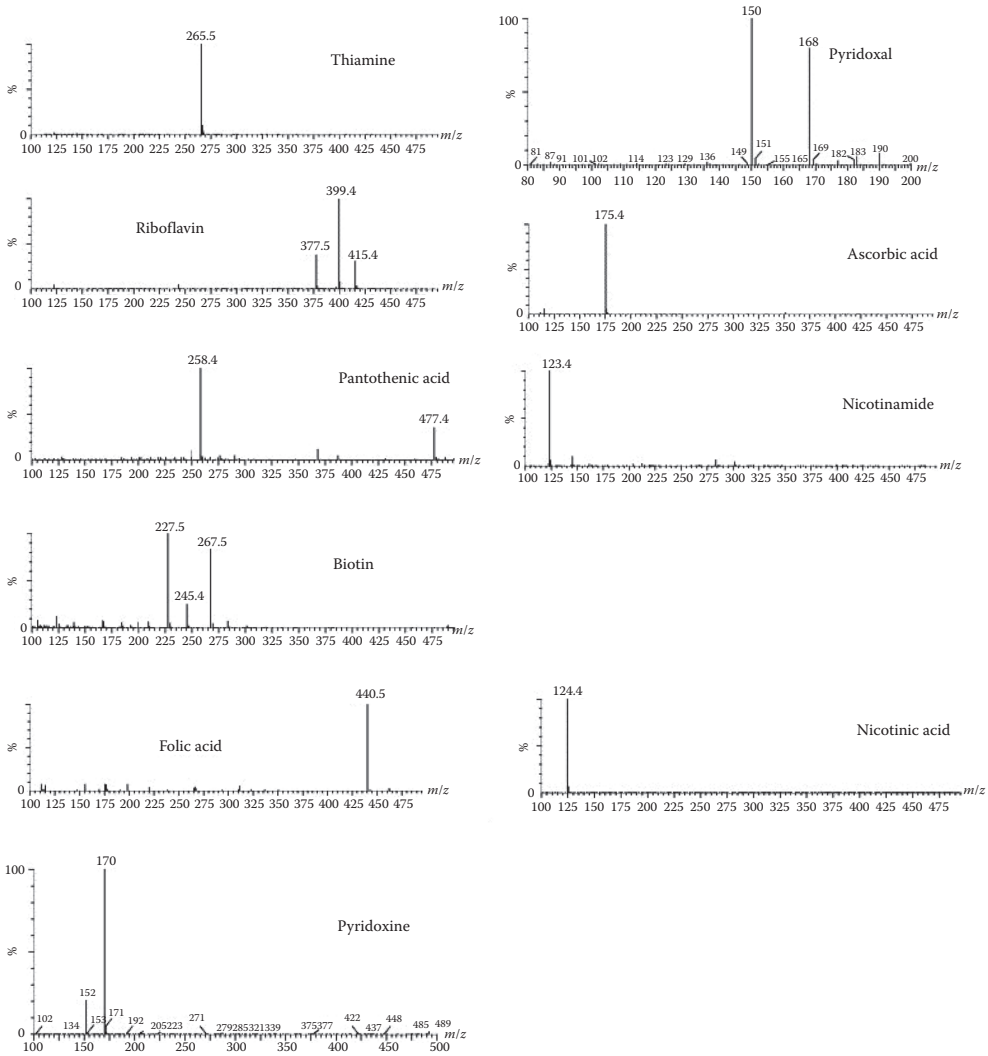

FIGURE 9.5 ESI mass spectra of different WSVs. (From Chen, Z., Chen, B., and Yao, S. Z. 2006. *Anal. Chim. Acta*. 569, 169–175. With permission.)

(UHPLC). This technology has enabled the use of small particle size, ≤2 μm to obtain very high-resolution separations or, in conjunction with high linear mobile phase flow, very fast separations. UHPLC has been used in the food area for the analysis of WSVs (Lu et al., 2008). In this method, four WSVs, vitamin B5, vitamin B8, vitamin B9, and vitamin B12, were simultaneously analyzed in fortified infant foods. Methotrexate was used as IS and the detection was performed by ESI under the MRM mode. All five vitamins were baseline separated and the LC-time was <10 min. Intra- and interday precisions for the determination of the four vitamins are better than 6.84% and 12.26% in relative standard deviations, and recoveries for the four vitamins are in the range of 86.0–101.5%.

9.3.2 Methods with Labeled IS for the Simultaneous Quantitative Analysis of WSVs in Premixes, Multivitamins Supplements, and Fortified Products

Recently, an isotope dilution mass spectrometry (IDMS) method was developed for the simultaneous determination of five WSVs (B1, B2, B3, B5, and B6) in multivitamin/multielement tablets (Phinney

et al., 2010). The compounds used as IS were labeled as follows [$^{2}H_{4}$]B3, [$^{13}C_{3}$]B1, [$^{13}C_{3}$,^{15}N]B5, and [^{13}C4]B6. Vitamin B2 was quantified using the labeled vitamin B6 as the IS. The vitamins were extracted from the tablets in an aqueous solvent containing the IS. Components in the sample matrix may either enhance or suppress ionization of the species of interest. These matrix effects can be minimized through selection of appropriate sample preparation procedures and chromatographic resolution of the analytes from potential interferences. The use of isotopically labeled IS has been suggested as the preferred approach to minimize the impact of matrix effects on quantification by LC-MS/MS. Indeed, these ISs are likely to behave in the same manner as the unlabeled analytes. During sample preparation and analysis, they are anticipated to compensate for any sample losses and/or matrix effects that may occur. The results of this work demonstrate the advantages of the methodology in terms of specificity and sensitivity. Also, the values obtained for multivitamin tablets with this LC-MS/MS method were in good agreement with results from LC-UV. Another method has been developed for the simultaneous measurement of five WSVs (B1, B2, B3, B5, and B6) in IF by LC-MS/MS (Huang et al., 2009). The parameters used for the MS measurements are summarized in Table 9.3. It should be noted that most abundant reactions with product ions were used for quantification. In this method, SIL ISs of vitamins B1, B3, B5, and B6 were used. The vitamins were extracted with acidic solvent, followed by protein precipitation at a pH range of 4.5–5.5, and filtered. This simplified procedure eliminates many of the potential sources of laboratory error and facilitates rapid and efficient analysis. With this method, quantification of highly polar vitamins such as vitamin B1 suffers from a huge signal depression. Additional steps such as SPE could potentially be used to separate the analytes from IF matrices that contain a high amount of potassium, calcium, and other inorganic constituents. Thus, the use of SIL for B1 appears essential to have accurate results for this vitamin. In the later work, a comprehensive evaluation of the analysis of the standard reference material and good spike recovery of the vitamins (100 +/– 6%) demonstrates the accuracy of the method. Finally, the results for commercially available IF samples were compared with those obtained using the current microbiological method. A recent work described the simultaneous analysis of seven WSVs (B1, B3, B6, B5, B9, B2, and B8) in IF (SRM 1849) by IDMS (Goldschmidt and Wolf, 2010). Chromatograms were obtained using the Phenomenex Hydro RP C18 column and RP chromatography. All seven WSVs were reliably determined using this RP approach; nevertheless, alternative approaches are worth exploring and may offer some advantages, especially when using detection methods less selective than MS/MS. With this method, vitamin B1 determination can be problematic, as it is barely retained under typical RP conditions and can exhibit considerable tailing or peak splitting. The isotopically labeled versions of the vitamins used in IDMS were obtained as follows: [$^{13}C_{3}$]B1, [$^{13}C_{4}$]B6, [D_{4}]B3, [$^{13}C_{6}{}^{15}N_{2}$]B5, [$^{2}H_{2}$]B8, [^{13}C4$^{15}N_{2}$]B2, and [$^{13}C_{5}$]B9. The different transitions used for each WSV and SIL compound are summarized in Table 9.4. As it can be seen from this table, most of the WSVs depicted their protonated molecular ions under the MS conditions investigated. The sample extraction was performed in phosphate buffer without any protein precipitation and enzymatic digestion. Due to the high cost of SIL and the complexity of IDMS methods, this approach may generally not be suited for routine analysis. However, the authors have shown that this could be an excellent approach for establishing reference values and can be useful in investigating and validating WSV methods.

9.3.3 Methods for the Quantification of WSVs in Other Types of Food Products

The simultaneous determination of WSVs in nonfortified food products appears more challenging when compared to that of the fortified products. Typically, the determination of WSVs in fortified products is essentially based on the determination of the free form which represents the majority of vitamins. In nonfortified products, WSVs can be in the free or bound form or exits in different forms. As an example, in foodstuffs, the vitamins B1, B2, and B6 may be present in free (thiamine, riboflavin, pyridoxine, pyridoxal, and pyridoxamine) and phosphorylated forms (essentially thiamine pyrophosphate, riboflavin-50-phosphate (FMN), riboflavin-50 adenosyldiphosphate (FAD), pyridoxal-50-phosphate, -phosphate, and pyridoxamine-50-phosphate). Furthermore, they may be bound tightly but noncovalently to proteins and polysaccharides. Vitamin B5 may be found in foodstuffs in free (pantothenic acid) and bound forms (coenzyme A (CoA) and acyl carrier protein). Its release from the bound forms cannot be

TABLE 9.3

Parameters Used for MS Measurement

Analyte	Productions	Q1, amu[a]	Q1, amu[b]	DP[c]	EP[d]	CE[e]	CXP[f]
B_1	122.1[g]	265.20	122.10	35.0	10.0	21.0	22.0
	144.2						
	81.2						
B_2	243.2[g]	377.20	243.20	70.0	10.0	35.0	15.0
	359.2						
	99.1						
B_3 (acid)	80.2[g]	123.10	80.00	60.0	10.0	30.0	14.0
	78.2						
	106.1						
B_3 (mide)	80.1[g]	124.10	80.00	60.0	10.0	30.0	14.0
	78.0						
	96.0						
B_5	90.1[g]	220.20	94.00	40.0	10.0	22.0	16.0
	202.2						
	184.2						
B_6	134.1[g]	170.20	134.10	45.0	9.00	31.0	7.00
	152.2						
	124.2						
Pyridoxamine	134.1[g]	169.10	134.10	38.0	4.00	18.0	9.00
	152.1						
	124.1						
Pyridoxal	150.1[g]	168.10	150.10	35.0	4.00	16.5	8.80
	122.0						
	94.0						
Pyridoxic acid	148.0[g]	184.10	148.00	43.0	4.00	28.0	8.21
	166.1						
	138.0						

Source: From Huang, M. et al. 2009. *J. AOAC Int.* 92, 1728–1738. With permission.

[a] Q1 = Precurs or ion.

[b] Q2 = Product ion.

[c] DP = Declustering potential.

[d] EP = Entrance potential.

[e] CE = Collision energy.

[f] CXP = Collision cell exit potential.

[g] Most abundant reactions with product ions were used for quantification.

performed by chemical hydrolysis, since the amidic bond of pantothenic acid is sensitive to low and high pH. The naturally active form of vitamin B8 is D-(þ)-biotin, which occurs in foodstuffs both as free and covalently bound to proteins. Acid hydrolysis (1–3 M H_2SO_4 by autoclaving at 121°C) is the treatment most used to break these bonds (Kucukbay and Karaca, 2010; Lebiedzinska et al., 2007) while enzymatic hydrolysis with papain is considered less effective (Kucukbay and Karaca, 2010). In food (especially in vegetables), folic acid is mainly bound to oligo-g-L-glutamates made up of 2–6 glutamic acid residues. To determine the total content of vitamin B9, polyglutamates forms have to be cleaved by the use of folic acid conjugase which releases the monoglutamate compound. The major problems during extraction of vitamin B9 are its lability to oxidation, thermal treatment, and acidic media. All these examples show that the sample preparation for the simultaneous determination of nonfortified foodstuffs is essential and will depend on the type of analyzed food.

TABLE 9.4

MRM Transitions Monitored for Natural and Labeled Forms of WSVs

WSV	Transition of Natural Form (*m/z* parent → *m/z* Fragment)	Transition of Labeled Form (*m/z* parent → *m/z* Fragment)	Labeled Compound Structure
Vitamin B1	265 → 122	268 → 122	[$^{13}C_3$] B1
Vitamin B2	377 → 243	383 → 249	[$^{13}C4^{15}N_2$] B2
Vitamin B3	123 → 80	127 → 84	[$^{2}H_4$] B3
Vitamin B5	220 → 90	224 → 94	[$^{13}C_6{}^{15}N_2$] B5
Vitamin B6	170 → 152	174 → 156	[$^{13}C4$] B6
Vitamin B8	245 → 227	247 → 229	[$^{2}H_2$] B8
Vitamin B9	442 → 295	447 → 295	[$^{13}C_5$] B9

Source: From Goldschmidt, R. J. and Wolf, W. R. 2010. *Anal. Bioanal. Chem*. 397, 471–481. With permission.

Recently, a sensitive LC/MS/MS method with an ESI source was developed for the simultaneous analysis of 14 WSVs (B1, B2, two B3 vitamers, B5, five B6 vitamers, B8, B9, B12, and C) in various food matrices, namely: maize flour, green and golden kiwi fruit, and tomato pulp (Gentili et al., 2008). The LC-MS/MS conditions were optimized for all of the WSVs analyzed, and the precursor ion for most of the analyzed vitamins corresponds to the parent ion. The relative abundance of the different fragment ions are summarized in Table 9.5. In order to improve the selectivity of the method, two transitions were used per analyzed vitamins. This allows the baseline separation of all WSVs investigated with an excellent *S/N* ratio in food matrices as can be seen on the LC-MS/MS chromatogram obtained in kiwi fruit.

WSVs were separated by ion-suppression reversed-phase liquid chromatography in <10 min and detected in positive ionization mode. Sensitivity and specificity of this method allowed two important results to be achieved: (1) limits of detection of the analytes at ng/g levels (except for vitamin C); (2) development of a rapid sample treatment that minimizes analyte exposition to light, air, and heat, eliminating any step of extract concentration. The sample preparation was adjusted for each food matrix. In fact, matrix solid-phase dispersion did not work as the whole mixture exhibited uniform color. The samples were extracted in a methanol solution, and the solvent removal was performed in a thermostatic bath heated at 34°C. This procedure allowed the achievement of recoveries exceeding 77% for some vitamins (pyridoxal, vitamins B3, B5, B8, and B12) but it was the cause of severe losses of pyridoxal 5′-phosphate, pyridoxamine 5′-phosphate, vitamin B9, and vitamin C. Difficulties were encountered with the determination of vitamin C which was degraded under some extracting conditions especially in maize flour under these conditions. WSV recovery depends on the type of matrix. In particular, recovery of the analytes in maize flour was ≥70%, with the exception of vitamin C, pyridoxal-5′-phosphate, and vitamin B9 (ca. 40%); with tomato pulp, recovery was ≥64%, except for vitamin C (41%); with kiwi fruit, recovery was ≥73%, except for nicotinamide (ca. 30%). Overall, this simple method enables the simultaneous determination of WSVs in various types of food products with good accuracy and precision data. However, in this work, the authors did not use IS, and the influence of the food matrix on the quantititative determination of WSVs was not discussed.

Most of the published LC-MS/MS methods used for the simultaneous analysis of WSVs utilized the ESI source either in positive or negative ion mode. However, it is well known that ionization in ESI is subjected to matrix effect which can lead to erroneous data. Thus, the use of IS appears essential with this mode of ionization to compensate for any signal variation during the MS analysis. Atmospheric pressure chemical ionization (APCI) is another ionization mode which is less sensitive to matrix effects. This ionization mode was recently applied for the simultaneous quantification of several WSVs B_1, B_2, B_6 (pyridoxine, pyridoxal, and pyridoxamine), B3 (nicotinamide and nicotinic acid), B5, and B9 in Italian pasta (Leporati et al., 2005). In this work, the authors have investigated the WSV-MS responses after ESI and APCI ionizations. They found that all tested WSVs except folic acid could be efficiently ionized by both ESI and APCI, with the production of protonated or deprotonated molecules in positive ion

TABLE 9.5

LC-MS/MS Parameters for the Identification of WSV

Analyte	Retention Time (min)	Qualifier and Quantifier MRM Transitions[a]	Declustering Potential (V)	Collision Potential (V)	Relative Abundance[b] Mean ± SD
Pyridoxamine	2.89	169/134	24	18	0.42 ± 0.02
		169/152		30	
Pyridoxamine 5P	2.94	249/134	24	20	0.86 ± 0.02
		249/232		31	
Vitamin B1	3.39	265/144	16	19	0.151 ± 0.006
		265/122		13	
Vitamin C	5.10	177/95	22	12	0.59 ± 0.05
		177/141		20	
Pyridoxal 5P	5.83	248/122	45	23	0.202 ± 0.005
		248/150		32	
Pyridoxal	5.83	168/122	21	16	0.103 ± 0.008
		168/150		28	
Pyridoxine	5.84	170/134	30	20	0.69 ± 0.02
		170/152		31	
Nicotinamide	5.84	123/78	33	32	0.24 ± 0.05
		123/80		32	
Nicotinic acid	5.84	124/78	35	29	0.90 ± 0.03
		124/80		32	
Pantothenic acid	7.00	220/184	23	17	0.65 ± 0.02
		220/202		19	
Vitamin B9	7.80	442/176	28	20	0.257 ± 0.008
		442/295		52	
Vitamin B12	7.90	678/359	38	62	0.28 ± 0.01
		678/147		36	
Vitamin B2	8.58	377/198	56	34	0.213 ± 0.005
		377/243		52	
D-biotin	9.12	245/123	32	20	0.164 ± 0.008
		245/227		38	

Source: From Gentili, A. et al. 2008. *Rapid Commun. Mass Spectrom.* 22, 2029–2043. With permission.

[a] The first line reports the least intense MRM transition (qualifier) and the second line the most intense one (quantifier).

[b] The relative abundance is calculated as the ratio of qualifier intensity/quantifier intensity; the results are reported as the arithmetic average of six replicates plus the corresponding standard deviation (SD).

ionization (PI) and negative ion (NI), respectively. In general, the PI spectra were 100- to 1000-fold more intense than the corresponding NI ones (except for folic acid). Mass spectra were acquired in both PI and NI mode. In terms of sensitivity, ESI was about two- to five-fold higher for all vitamins except PP vitamers, for which APCI produced a better response.

9.4 Conclusions

Nowadays, the importance of vitamins as essential nutrients, therapeutic aid, and consumer product is unquestionably defined. Furthermore, vitamins show an increased importance also from the legislation point of view. Therefore, there is a growing demand for reproducible analytical methodologies that can test a wider range of foodstuffs. The chemical and structural diversity of WSVs makes it difficult to find

extraction and chromatographic conditions suitable for their simultaneous analysis. Several analytical methods have been proposed for the simultaneous determination of WSVs in food products with satisfactory accuracy and precision data. Also, the sensitivity and the selectivity of the proposed assays were satisfactory for the simultaneous determination of WSVs in a wide range of food products. Since most of the WSVs are low-molecular-weight compounds, their determination by MS is now possible. With such an approach, the sample preparation appears less time consuming, thanks to the great sensitivity and selectivity of MS determination especially in MRM mode. However, the problem of the matrix effect associated with MS detection under the ESI mode can give rise to erroneous data in complex food matrices. The use of IS preferably SIL which is essential for the MS determination is not in common use as their cost is prohibitive. Knowing that matrix effects are less pronounce with APCI or even APPI sources, their use for the MS analysis of WSVs could be of great interest.

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10

Tocopherols, Tocotrienols, and Their Bioactive Analogs

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Vitamin E is a lipid-soluble vitamin naturally synthesized by photosynthetic organisms (Yerin et al., 1984). The term "vitamin E" refers to a group of chemical compounds, tocols, and tocotrienols that act as antioxidant compounds and maintain the stability of cell membranes against oxidative stress. The antioxidant activity of vitamin E seems to be correlated with their capacity to quench free radicals and, in particular, reactive oxygen species produced by the cell metabolism. Vitamin E was discovered by Evans and Bishop (1922) and called "substance X" because it was essential to maintain rat fertility. The most active vitamer of vitamin E is α-tocopherol (from the Greek *tokos* = child, *phero* = to bear, and *-ol* indicating that the substance is an alcohol), isolated for the first time from wheat germ oil (Evans et al., 1936).

10.1 Chemical Structures of Tocopherols and Tocotrienols

Tocochromanols are characterized by a 6-hydroxychroman ring and a terpenoid side chain constituted by four isoprenic units. They can be divided into two main classes, tocopherols and tocotrienols, and each class includes four derivatives (α, β, γ, and δ), differing in the number and position of the methyl groups on the chromane ring.

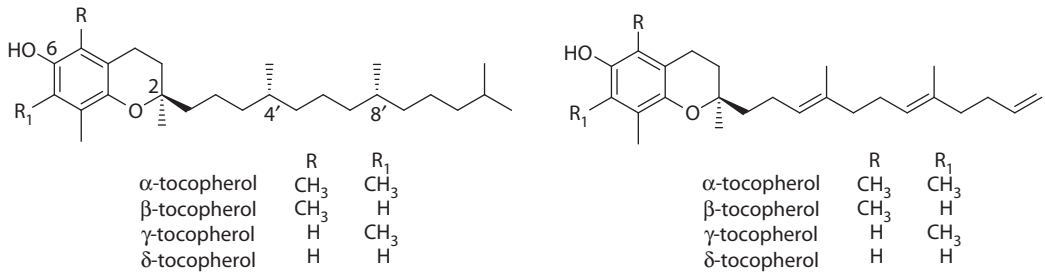


FIGURE 10.1 Tocopherols and tocotrienols.

Tocopherols (Figure 10.1) have three chiral carbons, C-2, C-4′, and C-8′. The naturally occurring stereoisomer of α-tocopherol has configuration 2*R*,4′*R*,8′*R* and its recommended trivial name is *RRR*-α-tocopherol, formerly known as *d*-α-tocopherol. Its epimer at C-2, obtained by synthesis using phytol, is the 2-*epi*-α-tocopherol, formerly known as *l*-α-tocopherol. The term "2-*ambo*-α-tocopherol" is referred to as the mixture of the two epimers (IUPAC, 1982).

Tocotrienols are characterized by the presence of three double bonds in the side chain at the C-3′, C-7′, and C-11′ carbons. The naturally occurring tocotrienols have *R* configuration at the C-2 carbon and *E*,*E* configurations at the C-3′ and C-7′ double bonds. The α-tocotrienol hydrogenation produces four diastereoisomers called 4′-*ambo*-8′-*ambo*-α-tocopherol. Finally, the totally synthetic vitamin E, produced without any stereochemical control, is called *all-rac*-α-tocopherol.

Although tocopherols and tocotrienols are the most common and widespread vitamers of vitamin E, other minor analog compounds have been reported from natural sources.

Tocomonoenols (Figure 10.2) are natural metabolites characterized by the presence of only one double bond in the side chain.

Matsumoto et al. (1995) reported the α-tocomonoenol from palm oil and attributed this metabolite to a biosynthetic intermediate along the reductive pathway from α-tocotrienol to α-tocopherol in higher plants. δ-Tocomonoenol was discovered in *Actinidia chinensis* fruits (Fiorentino et al., 2009). This compound, more abundant in the peels than in the flesh, was able to reduce the DPPH radical (2,2-diphenyl-1-picrylhydrazyl) and the anion superoxide radical, quite similar to δ-tocopherol.

An isomer of α-tocomonoenol, called marine-derived tocopherol, characterized by the presence of an unusual methylene unsaturation at the isoprenoid-chain terminus, was isolated from the lipophilic fraction of salmon eggs (Yamamoto et al., 1999). The antioxidant activity of this compound was found to be identical to that of α-tocopherol.

Qureshi et al. (2000) isolated desmethyl tocotrienol and didesmethyl tocotrienol (Figure 10.3) from stabilized and heated rice bran.

These unusual tocotrienols significantly lowered serum total and LDL cholesterol levels and inhibited HMG-CoA reductase activity in chickens. They had much greater *in vitro* antioxidant activity and greater suppression of B16 melanoma cell proliferation than α-tocopherol and other known tocotrienols.

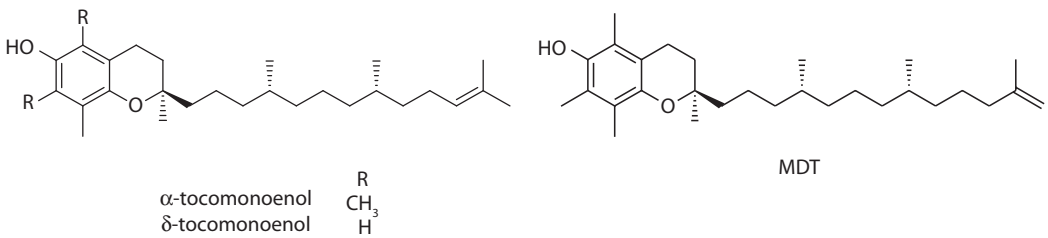


FIGURE 10.2 Tocomonoenols.

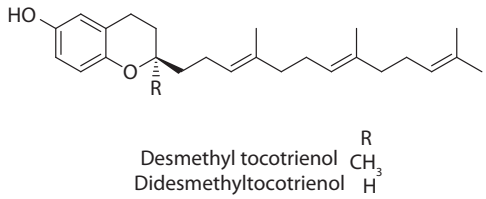


FIGURE 10.3 Desmethyl tocotrienol and didesmethyltocotrienol.

10.2 Biosynthesis of Vitamin E

The biosynthetic pathway of tocopherols was unveiled in higher plants and algae three decades ago from precursor and product studies using radio tracer intermediates. These lipid-soluble antioxidants are only synthesized by photosynthetic eukaryotes and various oxygenic cyanobacteria (Soll et al., 1985). In plants, the site of tocopherol biosynthesis is located in the inner envelope membrane of the plastids along with the site for the synthesis of the multifunctional family of lipid-soluble compounds called prenyl-lipids including phylloquinone and plastoquinone. These prenyllipids share a structural resemblance that contains hydrophobic isoprenoid tails of various lengths attached to aromatic head groups.

The tocopherol synthesis pathway was unraveled by Soll and Schultz (1980) and the corresponding genes from *Arabidopsis thaliana* (DellaPenna, 2005 and references cited therein) and *Brassica napus* (Endrigkeit et al., 2009) have been cloned.

The plant tocopherol biosynthetic pathway uses cytosolic aromatic aminoacid metabolism for synthesis of the tocopherol head group and the plastidial isoprenoid pathway called nonmevalonate or 1-deoxy-D-xylulose-5-phosphate pathway for synthesis of the hydrophobic tail.

The first step in tocopherol synthesis involves the production of the aromatic head group, homogentisic acid (HGA), from *p*-hydroxyphenylpyruvic acid (HPP) and molecular oxygen. HPP can be synthesized either from prephenic acid or from tyrosine by the shikimate pathway (Figure 10.4).

The formation of HGA is catalyzed by the cytosolic enzyme *p*-hydroxyphenylpyruvic acid dioxygenase (HPPD), a member of the large family of nonheme iron α-ketoglutarate-dependent dioxygenase. The enzyme catalyzes a complex reaction consisting of the oxidative decarboxylation of the α-keto acid side chain of *p*-hydroxyphenylpyruvic acid, the hydroxylation of the aromatic ring and a 1,2-shift of a carboxymethyl group. The overall reaction yielding HGA shortens the pyruvate side chain to acetate, moves the acetate side chain into a *meta*-position relative to the original hydroxyl group, and adds the novel hydroxyl group at the *para*-position, the former position of the pyruvate side group (Fritze et al., 2004). The reaction is irreversible (Figure 10.5).

HGA is then subjected to prenylation with saturated phytyl diphosphate, a product of the reduction of unsaturated double bonds of geranyl–geranyl diphospate by geranyl–geranyl diphosphate reductase, to yield 2-methyl-6-phytyl-1,4-benzoquinone (MPBQ) (Figure 10.6). The condensation of HGA and phytyl diphosphate is catalyzed by the homogentisate prenyltransferase (HPT) that fuses the phytyl chain to the

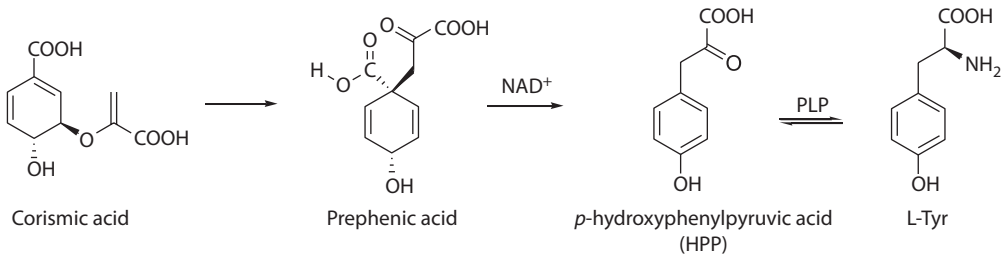


FIGURE 10.4 Shikimate pathway.

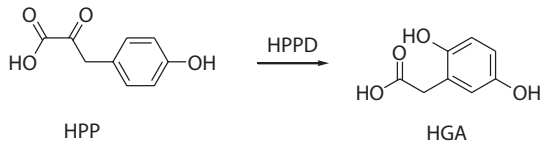


FIGURE 10.5 Formation of homogentisic acid.

aromatic ring in the C-6 position and decarboxylates the acetate group to yield a methyl group in the C-2 position of the aromatic ring (Collakova and DellaPenna, 2001; Savidge et al., 2002).

MPBQ is the substrate for ring cyclization and ring methylation reactions. The first reaction, catalyzed by a tocopherol cyclase (TC), allows to convert MPBQ into δ-tocopherol (Figure 10.7); the second one is catalyzed by 2-methyl-6-phytylplastoquinol methyltraspherase (MPBQ MT) and results in the formation of a 2,3-dimethyl-5-phytyl-1,4-benzoquinone (DMPBQ) derivative (Shintani et al., 2002). DMPBQ is then converted into γ-tocopherol by methylation via SAM (S-Adenosylmethionine) of the substrate (Figure 10.8).

In this case too, the reaction is catalyzed by TC that plays a key role in the formation of the chromanol ring structure of all the tocopherols. The acid promoted cyclization takes place in two steps (Stocker et al., 1994). In order to synthesize the oxygen-containing heterocyclic ring, the ring closure proceeds by the *si* protonation of the double bond of DMPBQ followed by a reattack of the phenolic oxygen atom to trap the intermediate carbocation (Figure 10.9). The enantioselectivity of the enzyme implies that the enzyme induces and immobilizes a single enantiomeric conformation of the substrate during the reaction (Manetsch et al., 2004). Studies on substrate specificity revealed that TC recognizes three main features: the OH group at C-1 of the hydroquinone, the *E*-configuration of the double bond, and the length of the lipophilic side chain.

Finally, γ-tocopherol methyltransferase (γ-TMT) adds a methyl group to the sixth position of the chromanol ring converting δ- and γ-tocopherols to β- and α-tocopherols, respectively (Figure 10.10).

Tocopherols and tocotrienols are made by the same biosynthetic pathway. Homogentisate geranylgeranyltransferases (HGGT) catalyze the transfer of geranylgeranyl diphosphate to HGA.

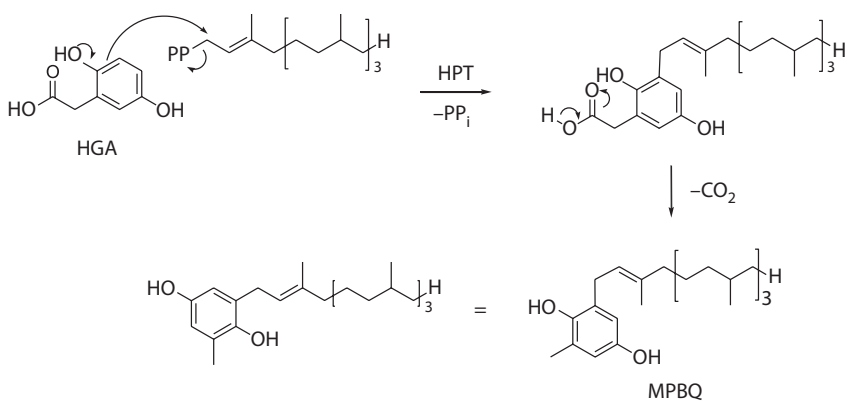


FIGURE 10.6 HGA prenylation.

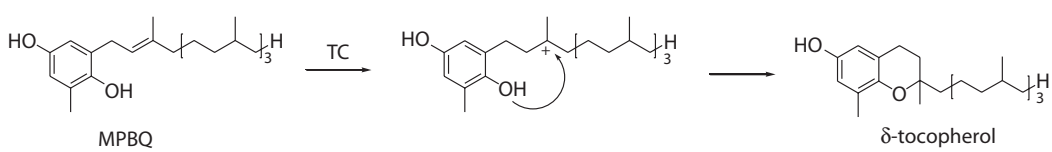


FIGURE 10.7 δ-tocopherol biosynthesis.

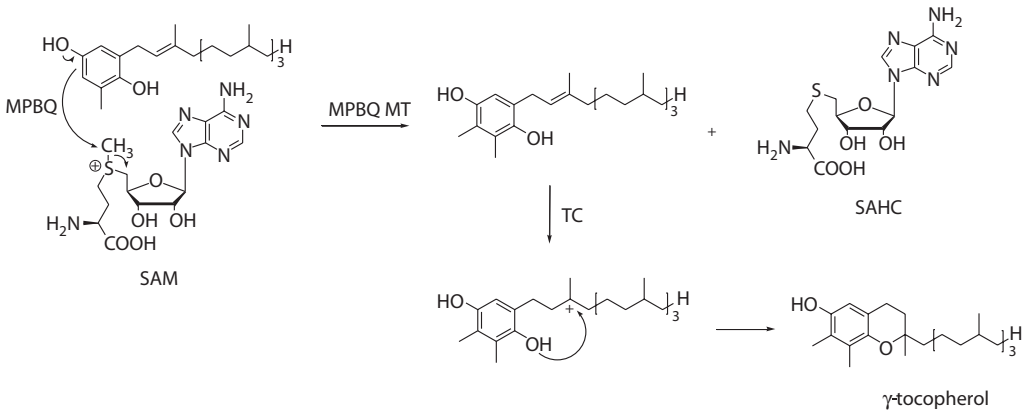


FIGURE 10.8 γ-tocopherol biosynthesis.

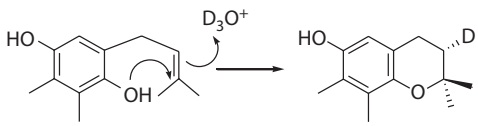


FIGURE 10.9 TC substrate specificity.

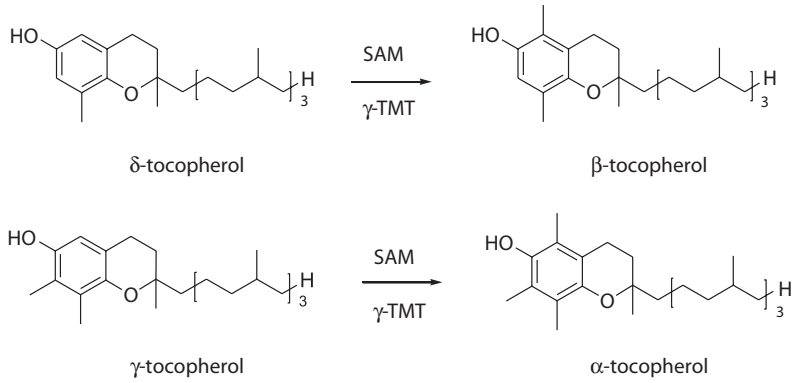


FIGURE 10.10 Conversion of γ- and δ-tocopherols into α- and β-tocopherols by γ-TMT.

10.3 Synthesis of Vitamin E

All naturally occurring tocochromanols are single-isomer products. The determination of the vitamin E activity by the fetal resorption–gestation test in rats shows that *RRR*-α-tocopherol has the highest value of the eight naturally occurring compounds and also of its eight stereoisomers. Due to its prominent biological activity, most efforts have been directed to *RRR*-α-tocopherol. But despite the rapid advances in stereoselective synthesis and the considerable efforts in approaches to this product, no economically commercial total synthesis of naturally identical *RRR*-α-tocopherol has been realized thus far. Although biologically less active than natural *RRR*-α-tocopherol, *all-rac*-α-tocopherol is industrially the most important product, manufactured in about 35,000 tons per year worldwide, mainly applied as its acetate derivative (Netscher, 2007).

The large-scale industrial synthesis of *all-rac*-α-tocopherol consists of three major parts: the preparation of the aromatic building block trymethylhydroquinone, the production of the side chain component *all-rac*-isophytol or a corresponding C_{20} derivative, and the condensation reaction.

The first synthesis of *all-rac*-α-tocopherol by an acid-catalyzed condensation reaction of *all*-racemic isophytol with 2,3,5-trimethylbenzene-1,4-diol was described by Karrer and Isler (1946) which resulted in the first Vitamin E production at F. Hoffmann-La Roche in the early 1950s (Figure 10.11).

Several classical Lewis and Brønsted acids, or combinations thereof, work well in this reaction. Typical examples are $ZnCl_2$/HCl, BF_3, or $AlCl_3$, applied in various organic solvents. Currently, a large number of new catalysts and new reaction conditions are being developed in order to find environment-friendly and more efficient procedures. Examples of novel catalysts are clays, ion-exchange resins, rare earth and indium metal halides and triflates, heteropolytungsten acids, various polyfluorinated compounds (imides, methides), and boron and phosphorous compounds.

The second form of industrially produced "vitamin E," about 10% of the total amount, is isomerically pure *RRR*-α-tocopherol, which is obtained by the enrichment and purification of mixtures of the four tocopherol homologs from soybean deodorizer distillates. To increase the value of the vitamin E concentrate, β-, γ-, and δ-homologs have to be converted to biologically more active α-tocopherol (only ca. 5% in the original mixture) by permethylation with subsequent reduction. As permethylation methods, the halo-, the amino-, or the hydroxymethylation reactions are employed.

The first synthesis of *RRR*-α-tocopherol and *SRR*-α-tocopherol was published by Mayer et al. (1963). All other approaches, which had been described in the literature until then, had given the racemic C-2 product with trimethylhydroquinone and natural phytol as starting materials. The problem of generating the right configuration at C-2 was solved by optical resolution.

In the preceding years, many synthetic schemes had been applied for the stereoselective synthesis of isomers of α-tocopherol, in particular *RRR*-α-tocopherol. Four general strategies had been followed: (1) classical optical resolution, which mainly delivers chroman building blocks; (2) biocatalysis (by microorganisms and isolated enzymes), which gives access to chiral intermediates; (3) asymmetric catalysis, which opens the way to a variety of products; and (4) chiral pool starting materials and chiral auxiliaries in stoichiometric proportions are also applied.

For large-scale applications, many of these methods are characterized by complexity, limited space–time yield, and formation of excessive amounts of waste material, which is the reason why an economic industrial synthesis of *RRR*-α-tocopherol has not yet been found.

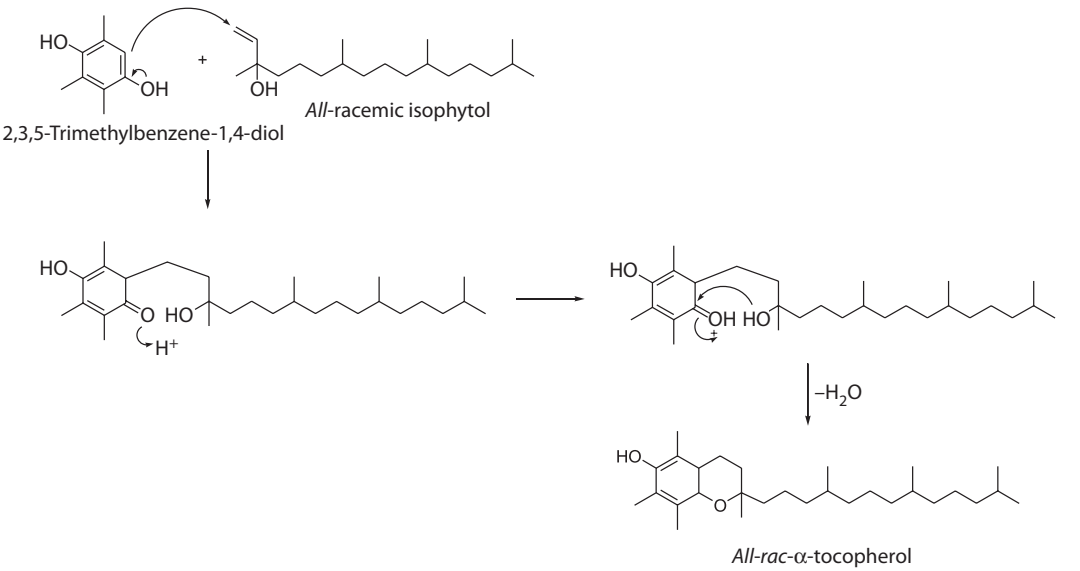


FIGURE 10.11 Synthesis of *all-rac*-α-tocopherol.

10.4 Bioavailability and Metabolism of Vitamin E

Commercially available vitamin E supplements usually contain only α-tocopherol, provided either unesterified or as its acetate, succinate, or nicotinate ester. These vitamin E forms are hydrolyzed by a pancreatic esterase leading to free tocopherols that are absorbed in the intestine, packed into chylomicrons, together with cholesterol and other lipids. Thus, vitamin E enters the circulation via the lymphatic system (Scherf et al., 1996). During chylomicron lipolysis, part of vitamin E is distributed to extrahepatic tissues; the remainder is captured by the liver where α-tocopherol is specifically recognized by the α-tocopherol transfer protein (α-TTP). Thus, in the liver it is incorporated into very low-density lipoproteins, released into human plasma, and consequently delivered to peripheral tissues (Ricciarelli et al., 2001). The high action selectivity of α-TTP, which recognizes only 2*R*-α-stereoisomers as having the phytyl side chain, brings about the excretion of the other forms of vitamin E such as (2*S*)-isomers, γ-tocopherol or tocotrienols as bile, or by the urine (Birringer et al., 2002).

Hepatic oxidation of chroman moiety can occur. The main oxidation product is α-tocopheryl quinone derived from the reaction of the tocopheroxyl radical with a peroxyl radical that can be reduced to α-tocopheryl hydroquinone by NAD(P)H-dependent microsomal and mitochondrial enzymes (Brigelius-Flohé and Traber, 1999). Both quinone and hydroquinone (Figure 10.12) have been found in biological membranes treated with azo-bis(amidinopropane), a peroxyl radical generator.

As regards the urine excretion pathway, although for decades the only known urinary metabolites of α-tocopherol were the so-called Simon metabolites, first described in 1956, it has been established that ω-hydroxylation of α-tocopherol, followed by β-oxidation, catalyzed by cytochrome P450 CYP4F2 produced 2,5,7,8-tetramethyl-2-(29-carboxyethyl)-6-hydroxychroman (α-CEHC; Figure 10.12). The intact chroman structure of this metabolite indicates that α-CEHC is derived from α-tocopherol that has not reacted as an antioxidant. Therefore, it has been concluded that the excretion of α-CEHC could be taken as an indicator of an adequate or excess α-tocopherol supply. A similar mechanism was discovered for tocotrienols degradation, together with a reduction of the unsaturated chain by CoA-reductases.

There is still much controversy concerning the absorption, retention, and metabolism of tocotrienols (Schaffer et al., 2005). Cell culture studies suggest that tocotrienols and tocopherols are similarly metabolized, that is, by ω-oxidation followed by β-oxidation of the side chain, although tocotrienols were found to be degraded to a larger extent than tocopherols. The plasma levels of α-, β-, and γ-tocotrienols increased markedly with food intake. Thereafter, tocotrienol concentrations in the plasma dropped rapidly initially followed by a more gradual decline. Tocotrienols were found in various tissues of rats, especially adipose tissues, skin, and heart, after oral application, which suggests that tocotrienols are absorbed and distributed *in vivo*. Moreover, oral supplementation in female rats produced a moderate

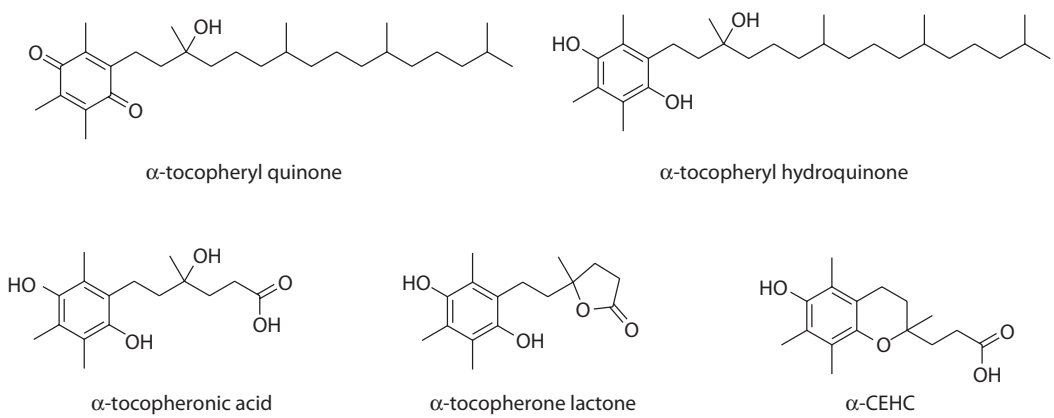


FIGURE 10.12 Oxidation products of α-tocopherol.

accumulation of tocotrienols in the brain and an even significantly higher tocotrienol concentration in the brain of rat pups.

10.5 Antioxidant Activity

Antioxidant compounds are health benefit factors against oxidative degradation of biomolecules and can be divided into two broad classes: *preventive* antioxidants, which reduce the rate of chain initiation, and *chain-breaking* antioxidants.

The greatest interest in the biological activity of vitamin E is its ability to prevent lipid peroxidation, the oxidative process whereby free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. In particular, it is widely reported that vitamin E functions as a chain-breaking antioxidant that prevents the propagation of free radical reactions. The importance of this function is to maintain the integrity of long-chain polyunsaturated fatty acids and thus maintain their bioactivity. These bioactive lipids are important signaling molecules. Changes in their amounts, loss due to oxidation, or their oxidation products are the key cellular events responded to by cells. This hypothesis is supported by studies in plants where the genes for vitamin E synthesis are missing.

To understand the action mechanism of these vitamers, it is essential to recall that lipid peroxidation occurs in biological circles. Lipoperoxidation proceeds by a free radical chain reaction mechanism. The first step for each chain involves the production of a lipid-radical ($R^{\bullet}$) by the reaction of a lipid (RH) with a reactive oxygen species (Figure 10.13).

In the chain oxidative process, the lipid peroxyl radical ($ROO^{\bullet}$) serves as a chain carrier. Tocopherols (TOH) trap peroxyl radicals forming highly resonance-stabilized radical species: tocopheroxyl radicals ($TO^{\bullet}$; Figure 10.14).

Burton et al. (1985) determined the rate constant k_1 for H-atom abstraction by peroxyl radicals from α-tocopherol and structurally related phenols. The relative magnitudes of k_1 values for phenols, which are structurally closely related and have an oxy substituent *para* to the hydroxyl group, can be correlated with the degree of stabilization of the phenoxyl radical. Stabilization depends on two factors: (1) the extent of orbital overlap between the 2p-type lone pair on the *para*-oxygen atom and the aromatic π-electron system and (2) the electron-donating or withdrawing character of the group bonded to the *para*-oxygen atom. 4-Methoxy-2,3,5,6-tetramethylphenol, which was expected to show a high k_1 value, was 10 times lower than that of α-tocopherol (Figure 10.15). This difference was explained by the greater stabilization of the phenoxyl radical formed from α-tocopherol, due to the better overlapping of the lone-pair orbital of the oxygen *para* to the OH, with the semioccupied molecular orbital (SOMO) in the

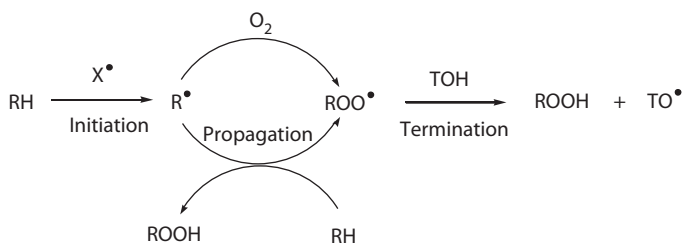


FIGURE 10.13 Lipoperoxidation mechanism.

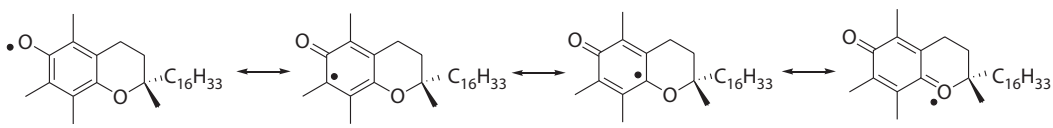
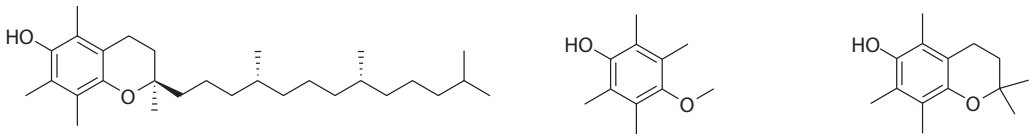


FIGURE 10.14 Resonance stabilization of the tocopheroxyl radical.



$k_2 = 3.2 \times 10^6\ M^{-1}s^{-1}$ $k_2 = 3.9 \times 10^5\ M^{-1}s^{-1}$ $k_2 = 3.8 \times 10^6\ M^{-1}s^{-1}$

FIGURE 10.15 Rate constant k_1 for H-atom abstraction by peroxyl radicals from α-tocopherol, 4-methoxy-2,3,5,6-tetramethyl, and chromanol.

radical. Maximum stabilization was reached when the angle θ between the lone pair and the SOMO approaches 0°, and minimum stabilization corresponded to θ = 90°. X-ray analysis of 4-methoxy-2,3,5,6-tetramethylphenol revealed that θ = 89°, meaning that its radical was not stabilized by the oxygen lone pairs. This would explain the low rate constant k_1 in comparison with α-tocopherol and simple chromanol that showed θ = 17°, which could be extrapolated to α-tocopherol.

Tocopheroxyl stable radicals do not continue the chain; they can be destroyed by reaction with a second peroxyl radical or with two electron oxidants (Figure 10.16). This yields 8α-substituted tocopherones, which are readily hydrolysed to 8α-hydroxy tocopherones which can, in turn, rearrange to form α-tocopheryl quinones. In an alternative pathway, epoxy-α-tocopheryl quinones are also formed.

Furthermore, a redox cycle mediated by a number of antioxidants, including vitamins A and C and coenzyme Q, can regenerate, in plant and animal tissues, tocopherols from the tocopheroxyl radicals, thus extending their biological potency to a great extent.

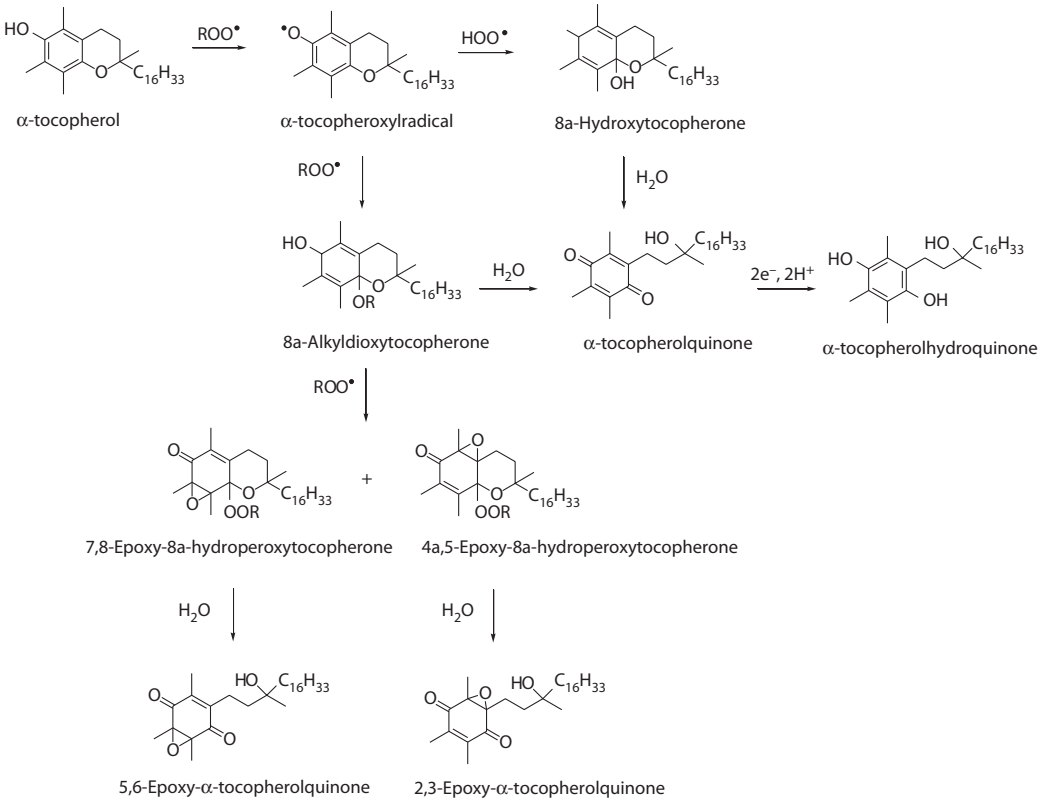


FIGURE 10.16 Reaction of α-tocopherol with peroxyl radicals. Oxidation products of α-tocopherol.

It has been shown that in male Sprague–Dawley rat livers perfused with 2 mM *tert*-butylhydroperoxide, supplementary vitamin E decreases lipid peroxidation and metabolic changes induced by the oxidant species. The antilipoperoxidative effect is accompanied by a similar extent and distribution of α-tocopherol oxidation products in whole liver and isolated mitochondria in the vitamin E-supplemented and vitamin E-unsupplemented animals. These data suggest that the "extra" vitamin E prevents mitochondrial dysfunction in the face of severe oxidative stress (Ham and Liebler, 1997).

The reaction rate of α-tocopherol with peroxyl radicals is 10^7 M^{-1} s^{-1} and 10^5–10^6 times faster than that of unsaturated lipid with peroxyl radicals. Thus, tocopherols take away the radicals from oxidizing fatty acids to prevent further radical chain reaction. One tocopherol molecule can protect about 10^3–10^8 polyunsaturated fatty acid molecules at low peroxide value (Kamal-Eldin and Appelqvist, 1996). The varying ability to donate phenolic hydrogen to lipid free radicals determines the different biological activity of vitamers and their derivatives. Specific structural determinants are responsible for the antioxidant effects: (1) the methylation degree of the chromanol ring ($\alpha > \beta = \gamma > \delta$); (2) the stereochemistry at C-2; and (3) the length of the side chain. The fully methylated chromanol ring of α-tocopherol prevents adduct formation at the positions in the non-α-tocopherols that are not methylated. For example, the formation of 5-nitro-γ-tocopherol has been touted as a marker of the benefit of γ-tocopherol scavenging reactive nitrogen species (Hensley et al., 2004). The degree of unsaturation at the side chain, for instance, in tocotrienols, can also affect the solubility in membranes and ultimately contribute to improving their antioxidant capacity. The α-tocotrienol has been shown to be more effective than α-tocopherol to scavenge free radicals and reduce lipid peroxidation in a model membrane system (Serbinova and Packer, 1994; Packer et al., 2001). The significantly greater antioxidant potency of tocotrienols versus tocopherols results from several factors. First, tocotrienols display a much greater recycling efficiency than tocopherols. Furthermore, tocotrienols display a uniform distribution within the microsomal membrane lipid bilayer and a more efficient interaction with lipid free radicals, as compared to tocopherols.

10.6 Functions of Tocochromonols in Plants

The conservation of vitamin E synthesis during the evolution of oxygenic photosynthetic organisms suggests that this molecule performs one or more critical functions (Sakuragi et al., 2006). α-tocopherol has an obligatory function in plants in protecting photosystem II structure and function and its D1 protein by scavenging the singlet oxygen produced in the PSII reaction center. This obligatory function becomes apparent in high-light conditions, when other singlet oxygen quenching (like carotene bound to the reaction centre) or scavenging (in the degradation mechanism of the D1 protein) cannot cope with the amount of $^1O_2$ produced. It has also been shown that it is not the change of concentration of tocopherol that is indicative of functional significance but rather its turnover rate (Krieger-Liszkay and Trebst, 2006). In the quenching reaction, physical deactivators, such as α-tocopherol, donate an electron to the singlet oxygen to form a charge transfer complex that dissociates into α-tocopherol and molecular oxygen after intersystem crossing (Thomas et al., 1998). The α-tocopherol could deactivate up to 120 $^1O_2$ molecules by resonance energy transfer before being degraded itself. Additionally, tocopherols may, to a limited extent, prevent the generation of $^1O_2$ by deactivation of the triplet excited endogenous membrane photosensitizers *in vivo* in a way similar to β-carotene (Fryer, 1992). Studies on the role of α-tocopherol in photosynthetic electron transport have indicated that cyclic electron transport around photosystem II is inhibited by α-tocopherol and stimulated by α-tocopherol quinone. Kruk and Strzalka (2001) showed that α-tocopherol quinone efficiently oxidizes the reduced cyt b559, and is important in cyclic electron flow around photosystem II, when the photosynthetic electron transport chain is overreduced. Moreover, addition of exogenous α-tocopherol decreases the membrane permeability to the small ions involved in the generation of a transmembrane electrochemical gradient for ATP synthesis in mitochondria and chloroplasts (Krieger-Liszkay and Trebst, 2006). Therefore, α-tocopherol also plays a role in the dissipation of excess energy in thylakoids for the protection of the photosynthetic apparatus.

Tocopherols can affect the plant development and stress responses not only by controlling the redox state of chloroplasts, but also by regulating the amounts of jasmonic acid, known to be involved in

intracellular signaling (Munne-Bosch and Alegre, 2002). Jasmonic acid is synthesized as the secondary oxidation product of lipid hydroperoxides. Thus, tocopherols indirectly regulate the concentration of jasmonic acid in cells by controlling the accumulation of lipid hydroperoxides (Schaller, 2001). The intracellular signaling pathway of jasmonic acid is involved in the regulation of gene expression in the nucleus affecting photosynthesis, and anthocyanin and antioxidant metabolism, in turn (Creelman and Mullet, 1997). Jasmonic acid was shown to regulate some genes of the tocopherol biosynthetic pathway (Falk et al., 2002). Induction of such genes for jasmonic acid synthesis may explain the increased tocopherol content in plants under stress conditions. Therefore, α-tocopherol could control its own synthesis by regulating lipid peroxidation in chloroplasts and jasmonic acid content within the cell.

10.7 Membrane-Stabilizing Actions of Tocochromanols

The absence of tocopherols in cell membranes was found to have an influence on membrane permeability and, in turn, make them susceptible to degradation by endogenous phospholipases *in vivo*. The main experimental approach to explain the functions of vitamin E in membranes has been to study its effects on the structure and stability of model phospholipid membranes (Wang and Quinn, 2000). According to the hypothesis of Diplock and Lucy (1973), tocopherols promote membrane stability by physical interactions between the phytyl side chain of tocopherol and polyunsaturated fatty acyl chains of membrane phospholipids. In contrast to this hypothesis, Urano et al. (1987), measuring ^{13}C Spin-lattice relaxation times (T_1) of ^{13}C-labeled α-tocopherol in three kinds of liposomes, varying in their contents of arachidoyl residues, by ^{13}C-NMR spectroscopy, proved that the segmental motion of isoprenoid side chain of α-tocopherol tends to increase with an increase in the distance from the chromanol moiety, and that three methyl groups attached to the aromatic ring have some affinity to unsaturated fatty acid residues rather than those of the isoprenoid side chain. The same authors investigated the effects of vitamin E (α-tocopherol) and its model compounds on the fluidity of liposomes composed of dipalmitoylphosphatidylcholin and fatty acids by measuring the fluorescent polarization (P) using 1,6-diphenyl-1,3,5-hexatriene as a probe (Urano et al., 1988). Although all tocopherols decreased the fluidity of liposomes, which was perturbed by the inclusion of an unsaturated fatty acid having more than one double bond, α-tocopherol was more effective than the others. The fluidity in arachidonic acid-containing liposomes was highly impaired in the presence of α-tocopherol and was significantly reduced by model compounds having a side chain consisting of at least one of isoprene units or a long straight chain rather than isoprenoid side chain. However, the chromanol with a methyl group instead of the above side chain, and phytol, having no chromanol moiety, had no effect. These results show that a structural requirement for membrane stabilization is to be either the chromanol moiety with methyl groups formed on its aromatic ring or a side chain of appropriate length. It has also been demonstrated that vitamin E forms complexes with the lysophospholipids and free fatty acids liberated by the action of membrane lipid hydrolysis. Both these products form 1:1 stoichiometric complexes with vitamin E and, as a consequence, the overall balance of hydrophobic:hydrophillic affinity within the membrane is restored. In this way, vitamin E is thought to negate the detergent-like properties of the hydrolytic products that would otherwise disrupt membrane stability (Wang and Quinn, 1999). Studies of model membrane systems consisting of phospholipids dispersed in aqueous systems and using a variety of biophysical methods have shown that α-tocopherol intercalates into phospholipid bilayers with the long axis of the molecule oriented parallel to the lipid hydrocarbon chains. The molecule is able to rotate about its long axis and diffuse laterally within fluid lipid bilayers. The vitamin does not distribute randomly throughout phospholipid bilayers but forms complexes of defined stoichiometry, which coexist with bilayers of pure phospholipid. α-tocopherol preferentially forms complexes with phosphatidylethanolamines rather than phosphatidylcholines, and such complexes more readily form nonlamellar structures. Recently, Hincha (2008) reported the use of liposomes resembling plant chloroplast membranes to investigate the effects of α-tocopherol on vesicle stability during freezing and on lipid dynamics. α-tocopherol had a pronounced influence on membrane dynamics and showed strong interactions in its effects on membrane stability during freezing with the cryoprotectant sucrose. α-tocopherol showed maximal effects at low concentrations (around 2 mol%), close to its contents in chloroplast membranes.

10.8 Effects of Tocochromanols at the Cellular Level

Available evidence suggests that tocopherols and tocotrienols inhibit tumor development and growth by modulating a multiple intracellular signaling pathway involved in mitogenesis and apoptosis. Cell proliferation and differentiation, along with apoptosis, are important cellular regulatory mechanisms that must be closely controlled. Protein kinase C (PKC) is a key signaling molecule involved in this regulation of growth and differentiation. Azzi et al. (1998), studying the role of vitamin E in cellular signaling, found that α-tocopherol inhibits PKC perhaps by the activation of protein phosphatase 2A, increasing PKC-α-dephosphorylation. This function, as well as the capability to inhibit smooth muscle cell proliferation and to control expression of the α-tropomyosin gene (Brigelius-Flohe and Traber, 1999), is not related to vitamin E antioxidant action because β-tocopherol, which has a similar antioxidant activity, does not perform any of these. Alternatively, α-tocopherol could also act on the diacylglycerol pathway by activating diacylglycerol kinase and consequently decreasing diacylglycerol and PKC activation (Koya et al., 1997). The change in the physical properties of a membrane due to tocopherols was suggested to have inhibitory effects on phospholipase A2 activity (Grau and Ortiz, 1998). It was shown that β-, γ-, and δ-tocopherols are weaker inhibitors than α-tocopherol because they are located progressively deeper within the membrane.

10.9 Functions of Tocochromanols in Transcriptional Regulation

The function of tocopherol in regulating gene transcription has gained considerable attention. Dietary supplement of α-tocopherol resulted in an inhibition of the liver collagen a1(I) gene expression (Chojkier et al., 1998). Human skin fibroblasts exhibit an age-dependent increase in collagenase expression that can be diminished by α-tocopherol (Ricciarelli et al., 2002). The α-tocopherol triggered downregulation of the transcription of the oxidized low-density lipoprotein (LDL) scavenger receptors SR-A and CD36 in smooth muscle cells, monocytes, and macrophages, while α-tocopherol was ineffective (Ricciarelli et al., 2000). α-tocopherol can also weakly induce the expression of α-Tropomyosin (Villacorta et al., 2003).

α-tocopherol-associated protein was identified as a ligand-dependent transcription factor, which was translocated into the nucleus on binding with α-tocopherol (Yamauchi et al., 2001). Both α- and γ-tocopherols decrease platelet aggregation and delay intra-arterial thrombus formation (Saldeen et al., 1999). That γ-tocopherol is significantly more potent than α-tocopherol suggests that a simple antioxidant mechanism is not applicable to these effects.

Thus, numerous events are related to nonantioxidant properties of tocopherols, both at transcriptional and posttranscriptional levels. However, whether α-tocopherol acts by a pleiotropic mechanism, or binds to a receptor capable of regulating different reactions, still remains unknown.

10.10 Vitamin E and Reproduction

Vitamin E was detected as a micronutrient that was indispensible for proper fetal development in rats. Vitamin E supplementation has become a routine procedure to promote the growth of farm animals. Despite its history, studies in which the underlying mechanism was investigated are surprisingly scarce (Brigelius-Flohé et al., 2002). Conditions associated with impaired vitamin E absorption lead to embryonic lethality in mice. In apo B knockout mice, α-tocopherol was undetectable in embryonic tissue. There was an apparent defect in lipid export from the visceral endodermal cells in the yolk sac, which may have caused vitamin E deficiency in the embryos.

During pregnancy, the blood α-tocopherol concentration increases in association with the increase in blood lipid concentration. In abnormal pregnancies, blood α-tocopherol concentrations are lower than those in normal pregnancies at a corresponding gestational age. It is assumed, though unproven, that vitamin E requirements increase during pregnancy. For this reason, vitamin supplements designed for pregnancy usually contain small doses (50 mg) of vitamin E, but adverse effects have not been observed

with higher levels of supplementation. It is well established that there is no clear relation between maternal and fetal blood concentrations of vitamin E. Newborns have significantly lower plasma vitamin E concentrations than do their mothers. Moreover, when plasma vitamin E concentrations were standardized for phospholipids or total lipids, significant differences were not seen. Short-term supplementation of pregnant women before delivery significantly enhanced the vitamin E status of the mother only, suggesting that vitamin E does not pass efficiently through the placenta to the newborn's circulation. What regulates the placental transfer remains unclear. An α-tocopherol-binding protein was isolated from human placenta, whereas the presence of α-TTP in human placenta has not been firmly established. *RRR*-α-tocopherol, however, is preferentially transferred to the cord blood. This observation points to a role for α-TTP in regulating the transfer of tocopherols through the placental barrier.

10.11 Vitamin E in Neurological Disorders

Vitamin E deficiency in human beings is characterized by very low levels of tocopherol in plasma that cause severe debilitating spinocerebral lesions. An autosomal-recessive neurodegenerative disease called ataxia with isolated vitamin E deficiency (AVED) is a rare form of vitamin E deficiency in which patients have an impaired ability to incorporate α-tocopherol into lipoproteins in liver (Gotoda et al., 1995). Reduced α-TTP gene expression might result in a reduced plasma level of α-tocopherol (Wu et al., 1997). A number of mutations identified in α-TTP gene of AVED patients have been defined as molecular basis of the AVED syndrome that causes reduced α-tocopherol concentrations in plasma and tissue (Traber and Arai, 1999). These low levels of α-tocopherol in plasma are elevated by dietary vitamin E supplementation, which resulted in stabilization or improvement of the neurologic functions (Schuelke et al., 1999). It is not known as to whether the degenerative neurological symptoms in patients with vitamin E deficiency syndrome are the result of an insufficient protection by antioxidant effects or of a lack of specific and nonantioxidant effects mediated by α-tocopherol. Studies in HT4 hippocampal neuronal cells showed that activation of c-Src kinase and phosphorylation of extracellular signal-regulated kinase, both central events in glutamate-induced cell death, are blocked by nanomolar concentrations of α-tocotrienol (Sen et al., 2000). For α-tocopherol, however, no such effects were found. Khanna et al. (2003) further explored the underlying mechanisms of tocotrienol-mediated neuroprotection. Lowering the levels of intracellular GSH triggers the activation of 12-lipoxygenase (12-LOX), which facilitates cell death by the production of peroxides and calcium influx. Primary cortical neurons were resistant to glutamate-induced cell death in the presence of physiologically relevant concentrations of α-tocotrienol. Additional experiments indicated that α-tocotrienol controls 12-LOX activity by hindering the access of arachidonic acid to the catalytic site of 12-LOX. Comparative studies with tocopherols, however, are not found. Both studies suggest that α-tocotrienol prevents glutamate-induced neurotoxicity by modulating the activity of important proteins involved in cell death signal transduction.

10.12 Vitamin E and Cardiovascular Disease

Of the many ideas on the etiology of cardiovascular disease (CVD), two theories related to antioxidant defense have been generated to explain the initiation of the atherogenic process: the oxidation theory and the response-to-injury theory. In both theories, the oxidative modification of LDL is considered to be a key step in the initiation and progression of the disease. The development of atherosclerosis proceeds with the accumulation of oxidized LDL in the arterial wall, which is scavenged by specific scavenger receptors on macrophages. Subsequently, macrophages are converted into lipid-laden foam cells due to the uptake of oxidized LDL and are deposited as fatty streaks on the artery wall (Witztum and Steinberg, 1991). Tocopherol's ability to decrease lipid peroxidation of LDLs has been assumed to be the mechanisms for the reduction of the incidence of cardiovascular death and nonfatal myocardial infarction in patients with angiographically proven coronary atherosclerosis supplemented with vitamin E (400–800 IU/day). To date, the clinical trials undertaken have mostly enrolled patients with established

atherosclerosis or at high risk of developing the disease, and these studies failed to unequivocally show that vitamin E reduces the progression of the disease (Brigelius-Flohé et al., 2002). The outcome of these clinical trials is apparently contradictory to the role of vitamin E. It is evident that the selection of the population in terms of age, sex, smoking habit, and diet may have strongly affected the possible outcome on the efficacy of vitamin E. In studies using experimental animals, F2-isoprostanes, a marker of *in vivo* free radical generation and oxidative lipid damage, increased in plasma and tissues as a result of vitamin E deficiency. Furthermore, in an animal atherosclerosis model (the apoE-deficient mouse), vitamin E supplementation not only suppressed F2-isoprostane production but also decreased atherosclerotic lesion formation. 8-*epi*-Prostaglandin F2a (8-iso-PGF2a) is one of the more abundant F2-isoprostanes produced *in vivo* in humans. 8-iso-PGF2a excretion is depressed in humans by antioxidant vitamins. Although much less is known about γ-tocopherol than about α-tocopherol, much evidence suggests that γ-tocopherol may be important in the defense against CVD (Jiang et al., 2001). Several investigations found that plasma γ-tocopherol concentrations are inversely associated with increased morbidity and mortality due to CVD. In a 7-year follow-up study of 34486 postmenopausal women, Kushi et al. (1996) concluded that the intake of dietary vitamin E (mainly γ-tocopherol), but not of supplemental vitamin E (mainly α-tocopherol), was significantly inversely associated with increased risk of death by CVD. Furthermore, these researchers showed that dietary vitamin E was associated with a reduced incidence of death from stroke in postmenopausal women. Finally, it was reported that the regular consumption of nuts, which are an excellent source of γ-tocopherol, lowers the risk of myocardial infarction and death from ischemic heart disease (Sabate, 1999).

Nonantioxidative functions of α-tocopherol have gained considerable interest and suggested that it may also act in the prevention of atherosclerosis by the following mechanisms: (1) inhibition of monocyte-endothelial cell adhesion; (2) inhibition of platelet adhesion and aggregation; (3) inhibition of cyclooxygenase-2 and 5-lipoxygenase, and (4) inhibition of SR-A and CD36 (Schneider, 2005).

Theriault et al. (2002) reported that α-tocotrienol markedly inhibited not only the surface expression of cell adhesion molecules (vascular cellular adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1, E-selectin) in human umbilical vein endothelial cells (HUVECs) but also monocytic cell adhesion of THP-1 cells. Both effects were more pronounced for α-tocotrienol than for α-tocopherol. Moreover, in a direct comparison of various tocotrienol analogs, α-tocotrienol displayed the highest efficiency toward the prevention of adhesion molecule expression and monocytic cell adhesion. Changes in protein prenylation, via interference with the HMG-CoA reductase-controlled mevalonate pathway, might be responsible for the observed effects of tocotrienols (Chao et al., 2002).

In hypertension, another important risk factor for the development of CD, endothelium-dependent vascular relaxation is impaired. Reduction of eNOS activity and increased removal of NO in blood vessels were postulated to mediate the observed impairment of vascular relaxation. In spontaneously hypertensive rats, Newaz et al. (2003) observed a significant reduction of systolic blood pressure after a 3-month intervention with γ-tocotrienol (15 mg/kg diet). Additionally, γ-tocotrienol treatment improved eNOS activity, leading to a significant negative correlation with systolic blood pressure.

10.13 Vitamin E and Cancer

Vitamin E protects cell membranes against the detrimental effects of free radicals, which may lead to the development of chronic diseases such as cancer. Human observational studies provide further support by showing, on the one hand, that oxidant stress increases with clinical progression of breast cancer and, on the other hand, that a diet rich in antioxidant-containing food reduces the risk of certain cancers. As a cancer-preventive agent, vitamin E acts in synergy with selenium, preventing cell transformation by x-irradiation, suggesting its use in protecting normal cells against the potential late effects of secondary cancers following radiotherapy. Vitamin E has been found to act selectively as an anticancer drug, alone or in combination with chemotherapy and radiation.

An analog of vitamin E, α-tocopherol succinate, has been shown to inhibit growth of a variety of cancer cells (Prasad et al., 2003). The molecular action of α-tocopherol succinate includes the induction of apoptosis by inhibition of PKC via increasing protein phosphatase 2A activity (Neuzil et al., 2001).

The apoptotic effect was more efficient in certain human prostate cancer cell lines. Additional treatment with the selenium agent, methylseleninic acid, resulted in synergistic effects (Zu and Ip, 2003). Zhang et al. (2004) showed that α-tocopherol succinate could inhibit cell invasiveness in three different prostate cancer cell lines possibly due to reduced levels of matrix metalloproteinases involved in the proteolysis of the basement membrane during invasion. Studies on the anticancer roles of α-tocopherols demonstrate that α-tocopherol has the ability to scavenge the mutagenic oxidant peroxynitrite by forming stable carbon-centered adducts and is involved in the downregulation of cyclins D1 and E to inhibit the cell cycle progression of prostate carcinoma cells (Galli et al., 2004).

In the view of the suggested protective effects of vitamin E seen in observational studies, several clinical and intervention trials were carried out to evaluate the anticancer roles of tocopherols. The most persuasive evidence for a protective role of vitamin E is in carcinomas of the prostate and gastrointestinal tract (Hartman et al., 1998). There was less supportive indication for the beneficial role in breast, ovarian, lung, pancreatic, or urinary tract cancers.

The anticancer effects of tocotrienols are well established *in vitro* using the tocotrienol-rich-fraction (TRF) of palm oils (McIntyre et al., 2000). TRF is obtained from the extraction of all forms of vitamin E from palm oils. The composition of TRF is approximately 20.2% α-tocopherol, 16.8% α-tocotrienol, 44.9% γ-tocotrienol, 14.8% δ-tocotrienol, and 3.2% of a nonvitamin E lipid-soluble contaminant. Initial studies showed that supplementation of culture media with 0–120 microM TRF significantly inhibited mammary tumor cell proliferation and induced cell death in a dose-responsive manner. Since TRF contains a mixture of α-tocopherol and α-, γ-, and δ-tocotrienol, it was unclear as to whether the anticancer effects of TRF were mediated by one or all of these vitamin E isoforms.

Direct comparisons between individual tocopherol and tocotrienol isoforms showed that tocotrienols were significantly more potent than tocopherols (McIntyre et al., 2000).

Furthermore, the relative antiproliferative and apoptotic biopotency of specific isoforms displays a consistent relationship corresponding to δ-tocotrienol > γ-tocotrienol > α-tocotrienol > α-tocopherol >> γ- and δ-tocopherol. Moreover, cells with the greatest degree of malignancy also displayed the greatest sensitivity to the antiproliferative and apoptotic actions of tocotrienols, as compared to normal mammary epithelial cells. One reason explaining the greater biopotency of tocotrienols is the finding that tocotrienols are more easily accumulated or taken up into normal and neoplastic mammary epithelial cells than tocopherols. It is believed that the unsaturated phytyl chain produces a less planar molecular conformation that facilitates less restricted transmembrane passage of tocotrienols into the cell (Sylvester and Shah, 2005). Since there is greater uptake, there are higher concentrations of tocotrienols at intracellular sites of action and are thereby able to induce a greater biological response. However, treatments that produce comparable intracellular levels of tocopherols did not elicit the same antiproliferative and apoptotic effects as tocotrienols. These findings demonstrate that tocotrienols are inherently more potent than tocopherols in antiproliferative and apoptotic activity. Tocotrienols display potent anticancer activity at doses that have little or no effect on normal cell growth or function (Sylvester et al., 2002). Tocotrienols' antitumor activity is independent of antioxidant activity. Recent studies have shown that tocotrienols specifically inhibit epidermal growth factor (EGF)-dependent mitogenesis in preneoplastic and neoplastic mammary epithelial cells. The mitogenic actions of EGF are mediated through specific membrane-bound receptors that contain an extracellular ligand-binding domain, a transmembrane hydrophobic segment, and a cytoplasmic catalytic domain that presents intrinsic tyrosine kinase activity. EGF activation of the EGF receptor results in receptor autophosphorylation of C-terminal tyrosine residues that are required for interaction and tyrosine phosphorylation of various intracellular substrates associated with mitogenic signal transduction. The present findings show that the antiproliferative effects of tocotrienols in preneoplastic mammary epithelial cells do not result from a reduction in EGF receptor mitogenic responsiveness, but from the inhibition of early postreceptor downstream events associated with cAMP production and cAMP-dependent kinase signaling. Comitato et al. (2009) proposed, for the first time, a novel mechanism for tocotrienols' activity that involves estrogen receptor (ER) signaling. *In silico* simulations and *in vitro* binding analyses indicate a high affinity of tocotrienols for ERβ but not for ERα. In addition, in ERβ-containing MDA-MB-231 breast cancer cells, they demonstrated that tocotrienols increase the ERβ translocation into the nucleus which, in turn, activates estrogen-responsive genes (MIC-1, EGR-1 and Cathepsin D) as demonstrated by cell preincubation with the ER inhibitor, ICI

182.780. Furthermore, they observed that tocotrienols treatment is associated with alteration of cell morphology, DNA fragmentation, and caspase-3 activation. Hence, although *in vitro* studies gave evidence of the protective role of vitamin E, the efficacy of vitamin E is yet to be determined in clinical trials.

10.14 Tocopherol and Tocotrienol Contents in Foods

Due to their apolar characteristics, tocopherols and tocotrienols are present mainly in oils. The main source of α-tocopherol is wheat germ oil, but high contents of tocopherols and/or tocotrienols have been detected in other vegetal oil and dried fruits. Vitamin E is present in trace amounts in milk, cheese, and other animal-derived products.

The Recommended Daily Amount (RDA) of U.S. Dietary Reference Intake (DRI) for adults is 15 mg α-tocopherol/day. The DRI for vitamin E is based on the α-tocopherol form because it is the most active form as originally tested (Dietary Guideline for Americans, 2005). European legislation for dietary additives accepts four tocopherols: E306, a natural extract enriched in tocopherols, E307 synthetic 2-*ambo*-α-tocopherol; E308 synthetic 2-*ambo*-γ-tocopherol; E309 synthetic 2-*ambo*-δ-tocopherol (EFSA, 2008).

Rupérez et al. (2001) reviewed the tocopherol and tocotrienol content of various edible matrices. The review includes well-referenced tables providing in-depth summaries of methodology for the chromatographic analysis of tocochromanols in foods, pharmaceuticals, plants, animal tissues, and other matrices. The tocopherol and tocotrienol content of 14 vegetable and nine industrial fats and oils has been determined (Schwarts et al., 2008). Wheat germ and sunflower oil showed the highest α-tocopherol content (192 mg and 59 mg/100 g of oil, respectively). The richest γ-tocopherol sources were camelina (72 mg/100 g), linseed (52 mg/100 g), and rapeseed oil (51 mg/100 g). Total tocochromanol content was measured in the range between 4.2 mg/100 g, for coconut oil, to 268 mg/100 g, for wheat germ oil. Ninety selected virgin olive oils from different cultivars and regions all over Greece were analyzed and high concentrations of α-tocopherol were observed in most of the samples, ranging from 98 to 370 mg/kg of oil (Psomiadou et al., 2000). Edible aromatic plants as well as microalgae have been investigated in the α- and γ-tocopherol content, as the potential source of natural antioxidants to utilize in industrial food processes (Gómez-Coronado et al., 2004). The values obtained ranged from 3.42 mg/100 g of dill to 132.2 mg/100 g fresh bay for α-tocopherol, and from 0.14 mg/100 g of spearmint to 3.45 mg/100 g of parsley for γ-tocopherol.

The role of tocochromanols, from various cereal sources and cereal-based products, on human health has been reviewed by Tiwari and Cummins (2009). In fact, food-manufacturing procedures could incorporate by-products of cereals, which are rich in tocochromanols, and hence increase the nutrition and health benefits of the end product (e.g., bread). In cereal grains including barley, oat, wheat, and rye, tocopherols and β-tocotrienol are mainly concentrated in the germ, while tocotrienols are present in the pericarp, aleurone and subaleurone layers, and endosperm fraction (Peterson, 1994, 1995) that generally accumulate during grain development (Falk et al., 2004). The level of tocochromanols in cereals is influenced by various factors including genotypic, environmental, and agronomic factors. These factors along with growing conditions may have a positive or a negative impact on the tocochromanol levels. Furthermore, Andersson et al. (2008) reported that barley grains, one of the richest sources of tocotrienols among cereals, generally contained all eight vitamers of vitamin E, with α-tocopherol being the major one contributing up to 45% of total tocochromanols. Vitamin E composition was studied in 175 genotypes of different wheat types. The average total content of tocopherols and tocotrienols was about 0.5 mg/100 g of dry material, but a large variation in tocol contents was noted among the investigated genotypes (Lampi et al., 2008).

The mean tocopherol contents of commercial peanut cultivars was 10.5 mg/100 g which was 26.7% greater than the imputed value for peanuts provided by USDA National Nutrient Database for Standard Reference (Shin et al., 2009). Reasonable amounts of tocochromanols were detected in wild *Capparis spinosa* plants. α-tocopherol (20.2 mg/100 g fresh weight) was the main tocochromanol present in the leaves, while both α- and γ-tocopherols were found in buds and flowers in 48.1 and 27.8 mg/100 g fresh weight, respectively (Tlili et al., 2009).

Recently, the seeds of 17 cultivars of Saskatoon berries (*Amelanchier alnifolia*) have been evaluated for their tocochromanol contents. These berries, native to the North America, were mostly consumed

fresh, baked in pies, or processed into jams and spreads. The total tocopherol content ranged from 105.3 to 175.4 mg/100 g of seed oil, while α-tocopherol was the major vitamer in all berry seed oils accounting for 87% of total tocols (Bakowska-Barczak et al., 2009).

Furthermore, 62 edible plants were investigated for their tocopherol content. The highest α-tocopherol content was in *Sauropus androgynus* leaves (42.68 mg/100 g edible portion), followed by *Citrus hystrix* leaves (39.83 mg/100 g), *Calamus scipronum* (19.38 mg/100 g), starfruit leaves *Averrhoa belimbi* (16.83 mg/100 g), red pepper *Capsicum annum* (15.54 mg/100 g), local celery *Apium graveolens* (13.64 mg/100 g), sweet potato shoots *Ipomoea batatas* (13.01 mg/100 g), *Pandanus odorus* (13.15 mg/100 g), *Oenanthe javanica* (14.68 mg/100 g), black tea *Camelia chinensis* (18.33 mg/100 g), papaya *Carica papaya* shoots (11.13 mg/100 g), wolfberry leaves *Lycium chinense* (9.44 mg/100 g), bird chili *C. frutescens* leaves (9.54 mg/100 g), drumstick *Moringa oleifera* leaves (9.0 mg/100 g), green chili *C. annum* (8.7 mg/100 g), *Allium fistulosum* leaves (7.46 mg/100 g), and bell pepper *C. annum* (7.10 mg/100 g) (Ching and Mohamed, 2001).

10.15 Tocopherol and Tocotrienol Extraction Methods

Vitamin E extraction methods include liquid–liquid-phase extraction, solid-phase extraction, and supercritical fluid extraction (Hu et al., 1996; Mendes et al., 2002; Chen and Bergman, 2005; Puoci et al., 2007; Alves et al., 2009). Tocopherols and tocotrienols are easily oxidized and oxidation losses can be incremented by heat, light, and by the presence of antioxidants. This is especially critical in samples in which vitamin E can be oxidized during the extraction processes and/or in the extract until its final analysis.

As the main and most active vitamer is α-tocopherol, official methods have been standardized for its analysis as natural α-tocopherol or α-tocopheryl acetate. The AOAC Official methods (AOAC Official method 971.30 and 948.26; 2000) for analysis of α-tocopherol and α-tocopheryl acetate suggest different extractive methods to be used for the determination of vitamin E in foods and feeds in the several forms in which it may occur. In general, α-tocopherol in unsupplemented aliments is extracted and the residue hydrolyzed. α-tocopherol is determined colorimetrically after TLC purification. The foods and feeds supplemented with α-tocopheryl acetate are analyzed in the same way, but to determine tocopheryl acetate, reducing substances are removed by oxidative chromatography and the analytes are saponified before α-tocopherol analysis. The AOAC Official method 975.43 allows the identification of natural *RRR*-form or synthetic *all-rac*-α-tocopheryl acetate in drugs and food or feed supplement polarimetrically. This calculation is based on the fact that optical rotation of oxidation products of α-tocopherol is negligible for *all-rac*-form and positive for *RRR*-form. To analyze the mixed tocopherol vitamers, test portion (AOAC 988.14) is derivatized with propionic anhydride and analyzed by gas chromatography (GC) with flame ionization detector (FID). Finally, α-tocopheryl acetate in supplemental vitamin E concentrates is extracted by Soxhlet apparatus in *n*-hexane and analyzed by GC-FID.

Extraction and pretreatment of tocopherols and tocotrienols present in different matrices have been reviewed by Rupérez et al. (2001). The different ways to perform sample pretreatment and analysis of vitamin E, related to sensitivity and simplicity of matrices, have been critically reviewed by authors.

A small-scale sample preparation method was developed for reliable and economic analysis of tocopherols and tocotrienols in rye and other cereals by Ryynänen et al. (2004). Three critical factors, time, temperature, and amount of potassium hydroxide, were optimized for hot saponification. In order to examine the effect of solvent polarity on the recovery of tocopherols and tocotrienols after saponification, the extracting solvent mixtures were studied. The optimized sample preparation method includes saponification with 0.5 mL KOH at 100°C for 25 min followed by extraction with *n*-hexane-ethyl acetate (4:1). The work is carried out under subdued light, ascorbic acid, and in nitrogen atmosphere to avoid destruction of labile vitamers and the recovery was above 90%.

Sánchez-Pérez et al. (2000) reported a rapid and automated method for α-, γ-, and δ-tocopherols in vegetable oils. The determination of different vitamers of vitamin E is performed after continuous extraction of the analytes from the matrices using a silicone nonporous membrane coupled online to the chromatographic system. Supercritical fluid extraction (Ibanez et al., 2000) was used to recover tocopherols from olive by-products (pomace). Szymańska and Kruk (2008) analyzed leaves of selected edible

and nonedible plant species for their α-tocopherol content and composition of other vitamers. Plant material was ground in a mortar with acetonitrile/methanol/water (72:8:1) and the extract was analyzed by high-performance liquid chromatography (HPLC) after centrifugation.

10.16 Analysis of Tocopherols and Tocotrienols

Analysis of tocopherols and related compounds is most challenging considering their relatively low abundance in most food matrices. Chromatographic techniques have been widely used: the separation of mixtures of tocopherols can be obtained by paper chromatography (Russel and Ward., 1953), thin-layer chromatography (Stowe, 1963; Rybakova et al., 2008), and column chromatography (Dicks-Buthnell, 1967). GC and HPLC are, doubtless the most important and useful tools, allowing quantitative and qualitative analyses with high accuracy and able to separate the different vitamers when the appropriate analysis conditions are used. It is indeed possible to accurately analyze all forms of tocopherols, tocotrienols, and tocomonoenols with modern chromatographic methods (Ryynnanen et al., 2004).

The development of analytical techniques for the determination of vitamin E began in 1938 (Emmerie, 1938). The very first reports concerned colorimetric methods for the quantification of vitamin E activity. These methods were based on the reducing power of α-tocopherol against ferric chloride. The ferrous salt formed in ethanol was determined with α,α´-dipyridyl: the formed complex had a red color and was measured spectrophotometrically.

Due to the discovery of different forms of vitamin E and their different biological activities, methods to separate and analyze qualitatively and quantitatively α-, β-, γ-, and δ- tocopherols, and corresponding tocotrienols and tocomonoenols, were needed. Quantification of only α-tocopherol is not enough to predict the total vitamin E activity and so the determination of each single compound is important, hence the development of different GC and HPLC methods.

The choice of adequate analytical tool would depend on the final goal of the analysis as well as on the kind of matrices to be analyzed.

The method historically used to identify and quantify tocochromanols is GC-FID or, more recently, GC-MS, but it is not really handy because it requires samples pretreatment, which is time consuming and can be a source of errors; so HPLC is preferable for routine analysis. On the other hand, GC is much more sensitive than HPLC. However, in current practice, analysis of vitamins E is usually carried out by HPLC, coupled with fluorescence, ultraviolet (UV), or sometimes electrochemical or light-scattering detection.

HPLC is a faster, more selective, simple, and robust technique (Ruperez et al., 2001), and its advent as an analytical tool has provided a good method with advantages in speed, accuracy, and specificity over the other methods. The separation of β- and γ-tocopherol is the most difficult to achieve because they are only positional isomers featured by very similar polarity. The first report on the separation of α-, β-, γ-, and δ-tocopherol by normal-phase HPLC appeared in 1973 (Van Niekerk, 1973). This system involved direct injection of oils and the separation of tocopherols on silica columns (Corasil II) using a mixture of hexane/diisopropyl ether (19:1) as the mobile phase and fluorescence detection with an excitation wavelength of 295 nm and an emission wavelength of 340 nm. Since then, other HPLC systems have been explored with good results.

A huge number of protocols concerning tocopherols analysis have been published, but fewer data are available for other vitamers, due to the more recent discovery and awareness of their different biological activities.

GC has been used to analyze tocopherols and related compounds since the 1970s, but nowadays it is less used due to the greater advantages of HPLC. GC analysis of chromanols often implies derivatization to volatile trimethylsilyl ethers (Parcerisa et al., 2000), acetates, propionates, or trifluoroacetates (Stoggl et al., 2001) prior to chromatographic injection. These procedures are laborious and can cause errors. Analyses can be carried out isothermally or, usually, with temperature gradient (Riss et al., 1994; Parcerisa et al., 2000). However, with appropriate stationary phases and gradients, it is also possible to analyze tocols together with other classes of compounds (Du and Ahn, 2002).

The stationary phases are usually polymethylsiloxane columns (Liebler et al., 1996). For detection, GC can be coupled not only with FID (Flame ionization detector), but also with mass spectrometer (MS)

(Liebler et al., 1996); and this has the advantage of allowing a better identification of eluted compounds. The carrier gas used depends on the detector: for MS, helium is preferred, whereas nitrogen can be used when the analyses are carried out by FID (Kadioglu et al., 2009).

HPLC analysis of tocopherols and related compounds can be performed in both the normal phase and the reverse phase.

Although reverse-phase columns are known for their better stability and longer durability, normal phase is generally preferred for many reasons. Normal-phase analysis is very easy because it allows the separation of all the vitamers, especially of β- and γ-tocopherols and the use of organic solvents which solubilize vitamin E. One important issue is the possibility of analyzing oils by direct injection after dilution with an organic solvent: silica columns can commonly accommodate up to 2 mg of fat per injection being capable of tolerating high loads of lipids that are easily washed out by nonpolar solvents. For this reason, it is preferred to reverse-phase HPLC in oils analysis, avoiding time-consuming operations. Furthermore, most reverse-phase HPLC procedures for the analysis of vitamin E involve evaporating samples to dryness to dissolve them in a suitable solvent. This operation can induce oxidation and it can avoid working with normal-phase HPLC.

It is also worth noting that new-generation silica columns give better results in terms of reproducibility (Kamal-Eldin et al., 2000).

In some HPLC methods, higher temperatures (Gimeno et al., 2000; Tasioula-Margari and Okogeri, 2001; Korchazkina et al., 2006) can be useful to obtain better separation than the one obtained at room temperature.

In normal-phase HPLC, the vitamers are usually eluted in descending order according to the number of methyl groups on the chroman ring and so of the increasing polarity (This depends on the number of methyl groups: the more they are, the less polar the compound is.) with α- followed by β-, γ-, and δ-tocopherols. Furthermore, the number of double bonds on the side chain also has an influence on the behavior of the vitamers. Tocotrienols are eluted after tocopherol analogs.

Since the normal-phase HPLC technique has been shown to give better separation of all tocopherols and related compounds, many researchers have used it, mostly with silica columns.

Silica columns are used in both isocratic or gradient elution typically with binary mixtures as the mobile phase composed of an alkane (usually hexane) together with a variety of organic modifiers, such as alcohols (the most common one is 2-propanol, but sometimes also methanol (Vatassery et al., 1993) or ethanol can be used), ethers (diisopropyl ether, *tert*-butyl methyl ether, diethyl ether (Thompson and Hatina, 1979), and 1,4-dioxane or tetrahydrofuran (THF). The amount of the second solvent is usually very low (0–1% of the total mobile phase).

Ethers can contain peroxides that may damage tocopherols during the analysis, while alcohols have to be used in very small amount, and hence the difficulty to have accurate mobile-phase proportion, which is of crucial importance for the reproducibility of retention times.

Chase et al. reported the separation of *all-rac*-α-tocopherol acetate, and α-, γ-, and δ-tocopherols from different matrices (Chase et al., 1987; Chase et al., 1998; Chase et al., 1999) using silica columns eluting isocratically with a mixture of hexane and 2-propanol (199:1). This system also allows the separation of α-, β-, γ-, and δ-tocopherols and α-, β-, γ-, and δ-tocotrienols (Hachicha et al., 2009; Hassanein and Abedel-Razek, 2009) and less common vitamin E forms as desmethyl tocotrienol and didesmethyl tocotrienol (Qureshi et al., 1997). The same system, using these solvents in a mixture (99:1) has been reported (Katsanidis and Addis, 1999; Gonzales et al., 2001) and was previously used by Indyk to separate tocopherols and α-tocopherol acetate (Indyk, 1988). The same author achieved the separation of a mixture of cholesterol, phytosterols, and tocopherols on a silica column using a solution of hexane, 2-propanol (999:1) (Indyk, 1990). Several papers have been published reporting the use of this method changing only a few proportions between the two solvents involved (Ye et al., 1998; Qureshi et al., 2000; Seppanen et al., 2003; Mitei et al., 2009).

Actually, one of the problems related to tocopherols analysis is the existence of coanalyzed compounds (such as cholesterol, chlorophylls, carotenoids, and ubiquinons, depending on the matrix) (Podda et al., 2003). To obtain a good separation, gradient elution can be very helpful using the hexane and 2-propanol mixture (Psomiadou and Tsimidou, 1998; Salo-Vaananen et al., 2000; Panfili et al., 2003) or other mixtures (Cayuela et al., 2003) as the mobile phase.

The solvent system preferred for the analysis of tocopherols on silica columns is usually the one that is described above (Chun et al., 2006), but other mixtures are also suitable. Isopropanol can be substituted by 1,4-dioxane (Amaral et al., 2005; Chuna et al., 2006) obtaining good results also in the simultaneous determination of vitamin E and A. Ethyl acetate can also substitute propanol and the substitution of hexane with octane into a mixture with 2-propanol has been reported. Cort et al. (1983) reported an effective method for separation and quantification of eight vitamers (α, β, γ, δ tocopherols and tocotrienols) using a silica column isocratically eluting with a mixture of isooctane and THF (195:5).

Ternary mixtures using silica columns have also been exploited, giving very good separations. In particular, a mixture of hexane, dioxane, and 2-propanol (100:40:5) (Sookwong et al., 2007) has been reported as well as hexane, ethyl acetate, and acetic acid (97.3:1.8:0.9) (Gasior et al., 2009). Alternatively, ternary mixtures composed of hexane, THF, and methanol (97.35:2.5:0.15) or slightly different ones (Gapor et al., 1986) can be used.

Stoggl et al. (2001) analyzed different mobile phases to separate tocopherols and tocotrienols and they found a mixture of isooctane, diisopropyl ether, 1,4-dioxane (95:4:1) to be the best. They also compared normal-phase HPLC with reverse-phase HPLC, finding the normal phase to be more highly sensitive. Amino columns and polymer-bonded columns have also been reported to be useful in separating tocopherols. Some authors suggest that NH_2 columns have more advantages than silica and reverse-phase columns, as they are more stable than the former and giving better resolution than the latter, so somehow giving benefits of both. Nevertheless, there are not so many papers employing these kinds of columns.

Amino and Cyano columns gave good results with the same solvent mixture used on silica columns (Guzman and Murphy 1986; Lammi-Keefe, 1986) or sometimes with different mixtures as reported by Rammel and Hoogenboom (1985) (amino–cyano columns with a mixture of hexane and THF) and by Rushing et al. (1991) who used a cyano column with a mobile phase composed by hexane, isopropanol, and glacial acetic acid obtaining the separation of a mixture of vitamins A and E. Also, diol columns have been used, mostly with binary solvents composed by a hydrocarbon with an alcohol or an ether (more or less the same mixtures described above for the other stationary phases) giving consistent and reproducible separation of all four tocopherols and four tocotrienols (Abidi and Mounts, 1994). The diol column behaves as a normal-phase column, with the advantage of more stability. Indeed, one of the most common problems encountered when working with silica columns is their instability, with occasionally low yield and low reproducibility. The diol column eliminated this problem. Once again we have mixtures of hexane and 2-propanol (Kramer et al., 1997) or of isooctane (or hexane) and *tert*-butyl methyl ether (94:6) (Konings et al., 1996).

The separation of a mixture of standards (tocopherols and methylated tocols) on a cyclodextrin-bonded silica column using hexane or cyclohexane, in combination with alcohols, ethers, or esters in binary and ternary mixtures, as the mobile phase, with excellent results has been reported (Abidi and Mounts, 1994). They studied the chromatographic behavior of tocopherols using γ- and β-CDS phases under various HPLC conditions. This stationary phase behavior was very similar to that of a silica column as regards the elution order of analytes. Once again the best results were obtained in the presence of hexane binary mobile phase especially when used on a β-CDS phase.

Also, polyamide columns have been used (Barros et al., 2008): the separation was carried out at 30°C using a mixture of hexane and ethyl acetate (7:3).

It is worth pointing out that not all the normal-phase columns give the same results. Kamal-Eldin et al. (2000) demonstrated that newer types of silica columns provide a very good separation of the vitamers, eliminating also the problem of reproducibility, which is often found with old-generation silica columns due to inhomogeneous surface. They also compared the use of different mobile phases and found that the best one was a mixture of 1,4-dioxane at 4% or 5% in hexane. They tried a weaker polarity modifier: *tert*-butyl methyl ether, but the separation was not good. Diisopropyl ether or 1% isopropanol in hexane were not able to separate all the compounds either.

In the meantime, they showed that some other silica columns were unable to give such a good result; so the choice of stationary phase is very important. They also utilized diol columns that are considered to have higher reproducibility than silica ones, but they found the results to be less reproducible. With amino columns, there were different results. Some of them, used with 5% dioxane in hexane, gave

good separation with a chromatographic profile comparable to that obtained on a silica column, while others also afforded a good separation of all vitamers, but eluted in a different order. The best mixture in this case was of hexane, *tert*-butyl methyl ether, THF, and methanol (79:20:1:0.1). However, for amino columns also, we have to consider that they are less stable than silica ones due to ionization and wash out of the amino groups, influencing further separation and retention times, and giving peak broadening.

The use of reverse-phase HPLC is less common, especially in food analysis, due to its inability to separate β- and γ-tocopherols. Using this kind of stationary phase, it is usually necessary to dry the sample and dissolve it in an appropriate solvent. On the other hand, reverse-phase HPLC offers faster equilibration time and better reproducibility of retention times than the normal phase. Reverse-phase HPLC is usually suitable when analyzing the animal samples: β-tocopherol is not contained, or is in such small amounts in these tissues that it can be ignored. Furthermore, there have been good improvements in reverse-phase HPLC field, such as the introduction of C30 columns which are able, if coupled with the appropriate solvent system, to separate all tocopherols (Puspitasari-Nienaber et al., 2002).

Reverse-phase systems show separation based on the saturation of the phytyl chain with the less saturated being eluted first. Between α-, β-, γ-, and δ-tocopherols, the order of elution is exactly the opposite of that described for the normal phase. The stationary phase commonly used is C18.

As chromanols are quite apolar, strong solvents are needed because they are well retained from the stationary phase. The mobile phase usually preferred is methanol with small amounts of water (up to 10%) (Barbas et al., 1997; Carlucci et al., 2001) or with other solvents (e.g., acetonitrile), (Li et al., 1996) sometimes performing gradient elution (Schwarts, 2008).

When analyzing only α-tocopherol, pure methanol or ethanol on C18 columns can be used.

When the extract is achieved in other compounds different from tocopherols, more complex mixtures are needed (Karppi et al., 2008; Williamson et al., 2008), and sometimes a gradient elution is more powerful (Silva et al., 2009) in separating all the components.

As mentioned above, reverse-phase HPLC is not able to separate β- and γ-isomers. Several groups have reported the analysis of tocopherols (or tocopherols and related compounds), analyzing both compounds (Hogarty et al., 1989; Gimeno et al., 2000; Gliszczynska-Swiglo and Sikorska, 2004).

However, there are also reports of the separation of these isomers on a pentafluorophenyl-bonded phase using methanol–water mixtures. A good separation has already been obtained with a mixture of methanol and water (98:2), but mixtures with a higher water content might be necessary to improve it (92:8) (Richheimer et al., 1994). On this kind of column, the elution order for γ- and β-tocopherols was in contrast to other reverse-phase methods, whereas α- and δ-tocopherols were eluted in the same order.

C30 columns afford good separation of all vitamers. The enhanced shape recognition of the C30 phase has been attributed to the high order of the alkyl chains in the stationary phase (Strohschein et al., 1998; Strohschein et al., 1999) and has been developed especially to separate carotenoids isomers. The stationary phase has been used, with good separation of all the tocopherols, using 100% methanol: especially advantageous for qualitative analysis. The mobile phase is critical for reproducibility of retention times and as it is difficult to always have the same proportions in mixtures, pure solvent is preferable.

Additionally, a method for the separation of tocopherols stereoisomers using a chiral phase was described by Ueda et al. (1993).

The introduction of monolithic columns is also important. These are made of a single piece of porous silica (in contrast with particulate silica columns), allowing high flow rates with relatively lower backpressure. These kinds of columns appear to be more efficient. They have been used for the separation of retinol, α- and γ-tocopherols in human serum (Miller and Yang, 1985; Krcmova et al., 2010).

In recent years, supercritical fluid chromatoghraphy (SFC) has been developed as a separation technique. (It is a bridge between GC and HPLC.) SFC has been applied to the analysis of tocopherols (Yarita et al., 1994; Ibanez et al., 2000; Xu and Godber, 2000). When only supercritical carbon dioxide is used as the mobile phase, peak shapes are poor. When methanol is added as the modifier, the peak shapes are improved. The column used is C18 and the elution order is the same as that of reverse-phase HPLC, but γ- and β-tocopherols can be separated. The use of methanol, however, makes these two vitamers elute together as a unique peak.

For the detection of tocochromanols, electrochemical (amperometric or coulometric; Sanchez-Perez et al., 2000), fluorescence (Cort et al., 1983), UV (Korchazkina et al., 2006), and light-scattering detection can be employed (Kramer et al., 1997).

Electrochemical detection is the most sensitive and specific detection method, while evaporative light scattering and UV are unselective and less sensitive. Only the reverse phase is compatible with electrochemical detection (Ruperez et al., 2001), due to the requirement of an electrolyte in the eluent solution to support the redox reactions that are the basis for detection, since only reverse-phase eluents are sufficiently polar to carry electrolytes.

UV detectors for tocopherols analysis are usually set at 280 nm, while for fluorescence detection, the excitation wavelength is usually around 290 and the emission is around 350.

Under optimum instrumental conditions, fluorescence detectors are capable of higher sensitivity and greater selectivity than their UV counterparts (Lehmann and Martin, 1982). They are also so selective that interfering peaks occur very rarely. Hence, fluorescence detection is usually preferred to the others. Even though the objective of the analysis is to purify tocols, UV is recommended, as it avoids contaminations. A fluorescence detector is preferred to a UV detector in analyzing these compounds in complex food matrices because of its specificity and sensitivity.

It is necessary to point out that fluorescence detection is enhanced when used together with reverse-phase HPLC analysis: signal intensity is greater with methanol than with a hydrocarbon as the solvent.

It is also possible to associate HPLC to online MS (Rentel et al., 1998; Lauridsen et al., 2001) detection and sometimes to nuclear magnetic resonance (Strohschein et al., 1999).

For quantitative analyses, considering the possibility of loss of sample during pretreatment, when required, it is preferable to use internal standards. α-Tocopherol acetate (Hogarty et al., 1989) and tocols (Lehmann and Martin, 1982) are most frequently referred to as suitable for this purpose. Sometimes, 5,7-dimethyl tocol is also reported (Westerberg et al., 1981).

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11

Vitamin C

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11.1 Introduction

An accurate and specific determination of micronutrient content in food is becoming extremely important to describe the relationship between dietary intake and human health. In addition, it is very important to know the exact concentration of micronutrients in different food substances, so as to consume the right quantity that can be assimilated. Vitamin C is a natural antioxidant in food and biological systems with important nutritional benefits for human health related to the effect against scurvy, cardiovascular disease, and cancer (Versari et al., 2004). Vitamin C is also used as an index of the nutrient quality of fruits and vegetable products. This is because it is much more sensitive to various modes of degradation in food processing and subsequent storage as compared to other nutrients. Beverages and fruit juices are currently fortified with L-ascorbic acid (L-AA) for both nutritional purposes and potential health benefits. In addition, L-AA has been widely used in the pharmaceutical, chemical, and cosmetic industry because of its bioactivity and antioxidant properties.

11.2 Properties

11.2.1 Chemistry

The term "vitamin C" is used as a generic descriptor for any compound exhibiting full or partial biological activity of L-AA. It includes esters of L-AA such as ascorbyl palmitate (100% relative activity), synthetic forms such as 6-deoxy-L-AA (33% relative activity), and the primary oxidized form of L-AA, dehydroascorbic acid (L-DHAA) (80% relative activity) (Eitenmiller and Landen, 1999). L-AA is a lactone with an enediol group on carbons 2 and 3 (cyclic ester of a hydroxyl carboxylic acid). The oxidized product

of L-AA, L-DHAA, is a 2,3-diketal, which exists predominantly as a biological hydrated form in solution. The common substitutes for L-AA in most food uses are the stereoisomer d-arabosacorbic acid (more commonly known as d-isoascorbic acid, D-IAA) and d-erythorbic acid (D-EA), which have little antiscorbutic activity. L-AA and L-DHAA are relatively small molecules with molecular weights of 176.12 and 174.11, respectively. Both are readily soluble in water, but less soluble in organic solvents such as ethanol and acetonitrile, and insoluble in less polar organic solvents (Budavari et al., 1989).

11.2.2 Stability

Crystalline L-AA is highly stable in the presence of oxygen when water activity remains low. In solution, the strong reducing properties of the vitamin can lead to rapid and excessive oxidative changes. The first product of L-AA oxidation is the radical monodehydroascorbate, also known as semihydroascorbate, or ascorbate free radical. If allowed to persist though, two molecules of monodehydroascorbate will also spontaneously break down disproportionately into AA and DHAA. DHAA itself is unstable and undergoes irreversible hydrolytic ring cleavage to 2,3-diketogulonic acid in aqueous solution, which possesses no vitamin C activity (Davey et al., 2000). The mechanism of the anaerobic degradation of L-AA has not been widely studied; however, it seems that 2,3-diketogulonic acid is formed by hydrolysis of the keto tautomeric forms of L-AA. Diketogulonic acid degradation products, xylosone and 4-deoxypentosone, are then converted into ethylglyoxal, various reductones, furfural, and furancarboxylic acid (Belitz et al., 2004). Further reactions in L-AA degradation beyond 2,3-diketogulonic acid are of no nutritional consequence but contribute to the flavor and color changes associated with browning reactions.

The stability properties of vitamin C vary markedly as a function of environmental conditions such as pH, light, and temperature, as well as the concentration of trace metal ions, oxygen, and degradative enzymes (Gregory, 1996). Delocalization of the π-electrons over the C2–C3-conjugated enediol system stabilizes the molecule of L-AA and causes the hydrogen of the C3 hydroxyl to become highly acidic, and to dissociate with a pK_a of 4.13. Therefore, at physiological pH, L-AA exists as a monovalent anion (L-ascorbate). Dissociation of the second hydroxyl takes place at pH 11.6 (Davey et al., 2000). Maximal stability usually occurs between pH 2 and 4. AA and DHAA are known to be susceptible to degradation by light. Iwase (2000a) demonstrated that degradation of L-AA is affected by both natural and UV light (265 nm). After 1 h of the experiment, the initial concentration of L-AA was decreased to 79.7% under the influence of UV, whereas the content was reduced to 84.2% under the influence of natural light. The rate of metaL-catalyzed L-AA degradation reactions is often several orders of magnitude greater than those of uncatalyzed reactions. Among metal ions commonly found in foods, cupric and ferric ions have been found to be the most potent catalysts of the L-AA oxidation. L-AA can be oxidized by metal ions in two-sequential one-electron transfer mechanism. The formation of an ascorbate–M^{n+} complex allows a one-electron transfer to yield the ascorbic radical anion–$M^{(n-1)+}$ complex, which dissociates readily. The resulting radical anion then undergoes a second one-electron transfer by complexing with another metal ion (M^{n+}) to give the final product, L-DHAA (Wong 1989). Metal ions can also catalyze the autoxidation of AA. Although the availability of O_2 is one of the main factors affecting vitamin C degradation in fruits and vegetables, high CO_2 levels appear to have a negative effect on the vitamin C content of fresh-cut pears (Soliva-Fortuny and Martín-Belloso, 2003). Agar et al. (1999) reported that high CO_2 levels stimulated AA oxidation and inhibited the reduction of DHA to AA. According to Tudela et al. (2002), high CO_2 levels in fresh-cut potatoes increased vitamin C loss by accelerating ascorbate peroxidase-catalyzed oxidation processes. The presence of degradative enzymes has been described as one of the key factors that significantly influence the stability of AA and DHAA in solution. The enzymes found in food that oxidize vitamin C are AA oxidase, cytochrome oxidase, and peroxidase. However, in food processing, losses of vitamin C due to enzymatic destruction are minimal. Cooking losses depend upon the degree of heating, leaching into the cooking medium, the surface area exposed to water and oxygen, pH, the presence of transition metals, and any other factor that facilitates the oxidation of L-AA and its conversion into nonbiologically active forms. High temperatures during processing can greatly affect the rates of AA depletion through an aerobic pathway. Vitamin C can be reduced up to 21% in heat-pasteurized juices (90°C, 1 min) compared to fresh juices. Different studies have proved the effectiveness of intense-pulsed electric field (PEF) treatments, as a nonthermal treatment, in achieving juices with higher vitamin C

retention in comparison to heat treatments (Elez-Martínez et al., 2006; Odriozola-Serrano et al., 2008a,b). Lower processing temperatures reached during processing ($T < 40°C$) would explain the higher retention of vitamin C in PEF-treated juices compared to the heat-processed ones. Even mild thermal treatments, such as blanching prior to freezing, have been shown to result in high losses of AA. In addition to the effects of pH, oxygen, and metal ions, the stability of AA has been found to be affected by other food compounds. Various sugars and their corresponding sugar alcohols have been shown to exert equivalent protective effects against the aerobic destruction of AA. This action was attributed to the ability of carbohydrates to bind metal ions. In contrast, sugars have been shown to enhance the rate of anaerobic degradation of AA (Wong, 1989).

11.3 Biological Role

Vitamin C, including L-AA and L-DHAA, has many biological activities in the human body. Vitamin C is widely known for its role in preventing scurvy. In addition, one of the most clear functions of vitamin C in both plant and animal metabolisms is to modulate a number of important enzymatic reactions. Collagen is a protein that contains large amounts of proline and hydroxyproline amino acids. In its formation, vitamin C activates the enzyme prolyl hydroxyproline, which leads to the conversion of proline into hydroxyproline. Likewise, vitamin C effects the conversion of lysine into hydroxylysine, another amino acid that is an essential constituent of collagen. Vitamin C also plays a vital role in the conversion of 3,4-dihydroxyphenylethylamine to noradrenaline. Vitamin C increases the absorption of iron by converting Fe^{3+} into Fe^{2+} that is absorbed more readily and is required in the metabolism of the amino acid tyrosine (Burini, 2007). In addition, vitamin C keeps the membrane-bound antioxidant α-tocopherol in the reduced state, acts as a cofactor when maintaining the activity of a number of enzymes (by keeping metal ions in the reduced state), and has a role in stress resistance in plants (Arrigoni and De Tullio, 2002). Vitamin C is also an important antioxidant that can reduce or eliminate superoxide, hydroxyl radical, hypochlorous acid, and other free radical and oxidants. Free radicals are very unstable molecules, which can damage DNA, proteins, and membrane structures. Therefore, these compounds have been indicated as probable pathogenesis determinants of many degenerative and chronic diseases that develop with age, such as cancer, cardiovascular disease, cataract, and inmunosystem dysfunction (Peng et al., 2008). Vitamin C may prevent free radicaL-induced damage to DNA-quenching oxidants, which overcome cell dysfunction and decrease low-density lipoprotein-induced leukocyte adhesion.

11.4 Dietary Sources

Vitamin C is one of the most important antioxidants provided by fruits and vegetables. Lee and Kader (2000) reported that fruits and vegetables as well as their products provide more than 85% of vitamin C in human diets. Vitamin C is especially abundant in guava (230–300 mg/100 g), blackcurrant fruits (125.2–151.1 mg/100 g), broccoli (113 mg/100 g), and Brussels sprouts (87–109 mg/100 g). Other significant sources include gourds (185 mg/100 g), peppers (120 mg/100 g), kiwis (60 mg/100 g), citrus fruits (30–50 mg/100 mg), spinach (51 mg/100 g), strawberries (29–48 mg/100 g), potatoes (30 mg/100 g), peas (25 mg/100 g), tomatoes (20–25 mg/100 g), pineapples (12–25 mg/100 g), bananas (10–30 mg/100 g), and melons (10–35 mg/100 g). Not all fruits and vegetables contain such amounts; onions, lettuce, carrots, root crops, apples, pears, and plums represent a very modest source of vitamin C (3–5 mg/100 g) (Hägg et al., 1995; Davey et al., 2000). Liver (23 mg/100 g) is a good source of L-AA and fresh milk contains 2 mg/100 g. Fresh meat provides only traces but with offal supplies sufficient to prevent scurvy (Davidson et al., 1986). If vitamin C does not occur in natural products, it can be added to food as an antioxidant. AA is the antioxidant most extensively used to avoid enzymatic browning of fruit and vegetables due to the reduction of the *o*-quinones, generated by the action of the polyphenol oxidase (PPO) enzymes, back to their phenolic substrates (McEvily et al., 1992). AA is considered by the U.S. Food and Drug Administration as a generally recognized as safe antioxidant for being used to prevent browning. However, this treatment may not be completely effective to control enzymatic browning of fresh products,

since once the AA is completely oxidized to L-DHAA, *o*-quinones are no longer reduced and darkening may occur due to the formation of melanins (Nicolas et al., 1994).

11.5 Methods of Analysis

Various methods have been reported for the determination of L-AA and L-DHAA in foods, including spectroscopic, electrochemical, enzymatic, chromatographic, and capillary electrophoresis methods. Unfortunately, in many cases, these methods have been developed for the analysis of specific tissue types, and considerable caution should be exercised in the transfer of these methods to the analysis of different matrices.

11.5.1 Extraction Techniques

Due to the labile nature of vitamin C, preparation procedures are designed to avoid loss of vitamin. The sample is normally homogenized with the extracting solution and then centrifuged and filtered before L-AA determination. Extraction solution should maintain an acidic environment, chelate metals, inactivate AA oxidase, and precipitate starch and proteins. The choice of the extraction solution depends upon the sample matrix and the determination procedure. Diluted solutions of oxalic, trichloroacetic, perchloric, and *m*-phosphoric acid (3–6%) alone or in combination are generally used for the extraction of AA from foods. These acids are chosen because of their ability to prevent catalysis of the oxidation of AA by copper or iron ions. Nevertheless, *m*-phosphoric acid is the most widely applicable reagent because of its ability to precipitate proteins and inhibit L-AA oxidase (Eitenmiller and Landen, 1999). However, L-AA from plant tissues is the most stable in *m*-phosphoric acid (3–5%) containing ethylenediaminetetraacetate (EDTA) (1 mM) (Davey et al., 1996; Liao et al., 2001). Short-chain alcohols, such as methanol and ethanol, added to *m*-phosphoric acid precipitate solubilized starch (Russell, 1986). The metal chelator EDTA has also been used in combination with ethanol or methanol to extract AA from fruits such as orange, kiwi, tomato, strawberry, peach, and apple (Zapata and Dufour, 1992; Pérez et al., 1997). It has also been demonstrated that replacing *m*-phosphoric acid as a dissolving agent by potassium phosphate with EDTA (Iwase and Ono, 1997, 1998), monosodium L-glutamate (Iwase, 2000a), or disodium guanone-5′ monophosphate (Iwase, 2000b), or by using L-methionine (Iwase, 2003) may increase the stability of AA. Inorganic salts such as potassium phosphate are often used in the extract solution for the determination of AA in foods by direct injection with electrochemical or chemiluminescence detection (Dãnet et al., 2000; Florou et al., 2000). However, Ma et al. (2002) used a calcium chloride solution instead of an inorganic buffer to decrease interference of oxalic acid in vegetables. Special techniques of extraction have been reported for the simultaneous analysis of AA and sulfur diode in wines (Cardwell and Christophersen, 2000) or AA and phenolic compounds in tomatoes (Peng et al., 2008). In contrast, several authors have determined AA in juices and beers without previously mixing with a stabilizing extracting solution (Moll and Joly, 1987; Yuan and Chen, 1999; Gökmen et al., 2000; Furusawa, 2001; Rodríguez-Comesaña et al., 2002).

11.5.2 Spectrophotometric Procedures

A number of methods based on spectrophotometrical determinations have been developed for AA analysis. The majority of these methods are based either on the oxidation–reduction properties of AA or on the ability of AA to couple diazotized aniline derivatives. In the standard method for measuring AA in foods, 2,6-dichloroindophenol is reduced by L-AA to a colorless solution from the deep blue color of the oxidized dye (AOAC 967.21). L-AA in food can also be measured by redox reactions in which Fe (III) is reduced to Fe (II) by L-AA, followed by the formation of a chromogenic ferrous iron complex, using a chelating agent such as 2,2-dipyridine, 2,4,6-tripyridyL-5-triazine, and ferrozine (Pachla et al., 1985). Unfortunately, these spectrophotometric methods often lack specificity and are subject to interference from other reducing agents such as ferrous and cuprous anions, sugars, glucuronic acid, sulfite, tannins, betanin, cysteine, glutathione, and reductones generated by nonenzymatic browning. In addition, the

vitamin C content of foods containing appreciable quantities of DHAA can be underestimated because only the AA content is determined. Several modification methods have been introduced to eliminate or minimize the effects of interferences (Verma et al., 1996; Ortega et al., 1998; Paim and Reis, 2000). Recently, two simple and sensitive spectrophotometric methods for the determination of AA in fruit juices and vegetables have been described by Revanasiddappa and Veena (2008). One method is based on the oxidation of AA by a known excess of Se(IV) in hydrochloric acid medium and subsequent determination of unreacted Se(IV) by reacting it with iodide in the same acid medium to liberate iodine. This compound is reacted with starch to form a stable blue-colored iodine–starch complex, which shows maximum absorbance at 590 nm. The second method is based on the oxidation of AA by known excess of Cr(VI) in a sulfuric acid solution and the determination of unreacted Cr(VI) with diphenyl carbazide under the same acidic medium to produce a stable red-violet-colored species, which shows a maximum absorbance at 550 nm. More recent approaches to metal redox applications for L-AA have entailed flow injection techniques (Molina-Díaz et al., 1998; Luque-Pérez et al., 2000). Another widely used procedure is the oxidation of L-AA to DHAA by 2,6-dichloroindophenol, followed by the reaction between ketone groups of DHAA with 2,4-dinitrophenylhydrazine under acidic conditions to form a red osazone derivative. This method is useful for the analysis of vitamin C if appreciable quantities of sugar are not present in the product. Fluorimetric determinations are based on the oxidation of AA to DHAA, condensation of DHAA with *o*-phenylenediamine, and fluorescence detection of a highly fluorescent quinoxaline product (AOAC 927.22). The AA content can be determined by substration after second analysis that omits the oxidation of AA in the sample. This assay is subject to potential interferences, including the presence of α-keto or *cis*-hydroxyl groups, fluorescence properties of the DHAA quinoxaline derivative, and presence of starch in food.

11.5.3 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) methods have some advantages, compared to direct measurement techniques, regarding specificity, sensitivity, or easy operation (Gökmen and Acar, 1996). Because of the ionic character of AA, many different supports have been used. Reversed-phase (Dennison et al., 1981; Speek et al., 1984; Moll and Joy, 1987; Vanderslice and Higgs, 1990; Zapata and Dufour, 1992; Daood et al., 1994; Vinci et al., 1995; Ali and Phillipo, 1996; Arella et al., 1997; Iwase and Ono, 1997, 1998; Cheng and Tsang, 1998; Gökmen et al., 2000; Iwase, 2000a,b, 2003; Sánchez-Mata et al., 2000; Furusawa, 2001; Kafkas et al., 2006; Iglesias et al., 2006; Romeu-Nadal et al., 2006; Burini, 2007; Odriozola-Serrano et al., 2007; Gazdik et al., 2008), amino bonded-phase (AbouL-Enein et al., 1999; Bognár and Daood, 2000; Valls et al., 2002; Odriozola-Serrano et al., 2007), ion-pair reversed columns (Kall and Andersen, 1999), ion-exchange (Kacem et al., 1986; Pérez et al., 1997; Rodríguez-Comesaña et al., 2002), and ion-exclusion (Kim, 1989; Yuan and Chen, 1999) have been the most commonly employed columns for vitamin C analysis (Tables 11.1 through 11.4). Mobile-phase selections suitable for vitamin C analysis are as varied as the support available. A pH equal to or lower than 5 is necessary in the mobile phase to maintain the stability of vitamin C during the analysis. Several authors used isocratic mobile phases consisting of buffer phosphate and/or methanol (Dennison et al., 1981; Vanderslice and Higgs, 1990; Ali and Phillipo, 1996; Gökmen et al., 2000; Burini, 2007), which may include and ion-pair reagent (Zapata and Dufour, 1992; Daood et al., 1994; Arella et al., 1997) or acetonitrile (AbouL-Enein et al., 1999; Valls et al., 2002; Odriozola-Serrano et al., 2007). Acetate (Gazdik et al., 2008) or citrate (Moll and Joly, 1987) buffers have also been used for the determination of AA by HPLC using electrochemical detection. Isocratic phases with sulfuric acid have been effective to resolve AA in juices, fruits, and vegetables (Pérez et al., 1997; Yuan and Chen 1999; Sánchez-Mata et al., 2000; Odriozola-Serrano et al., 2007). Depending on the type of detection technique used, HPLC methods are generally more sensitive. AA can be easily detected by UV at wavelengths between 243 and 265 nm. However, sensitivity is still an important problem for the direct measurement of DHAA by HPLC, using the common detection system UV–Vis detector and no response to electrochemical detection. In order to increase the sensitivity for DHAA, derivatization prior to or after the chromatographic separation is necessary. Prior to HPLC, DHAA should be reduced to AA in samples. Various sulfhydryL-containing compounds such as homocysteine (Dennison et al., 1981), dithiothreitol (Gökmen et al., 2000; Furusawa, 2001;

TABLE 11.1

HPLC Methods with UV-Detector for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Stationary Phase	Mobile Phase	Detection	References
Vitamin C (AA + DHAA)	Beverages	Reduction with homocysteine, filtration	μ-Bondapak-C_{18}	0.25% KH_2PO_4 (pH 3.5):methanol (50:50)	UV 244 nm	Dennison et al. (1981)
AA	Fresh oranges, kiwi fruit and tomato juice, soft drink, beer	Sample dilution with methanol:water (5:95), centrifugation, pH 2.2–2.45 adjustment, cleaning up on C18 cartridge, mixing with *o*-phenylenediamine, filtration	μBondapak C_{18}	Isocratic phase Methanol:water (5:95) with 5 mM hexadecyltrimethylammonium bromide and 0.05 M potassium dihydrogen orthophosphate pH 4.59	UV 261 nm	Zapata and Dufour (1992)
AA	Fresh fruit and vegetables	Extraction with *m*-phosphoric acid (3%), filtration	Sperisorb ODS-2	Isocratic phase 10 mM potassium dihydrogen orthophosphate: tetrabutylammonium hydroxide:methanol (970:1:30) pH 2.75	UV 244 nm	Daood et al. (1994)
AA	Avocado, pear, babaco, feijoa, grapefruit, kiwi, kumquat, litchi, mango, papaya, passion fruit, pineapple	Homogenization, dilution with water, centrifugation and filtration	Supelcosil LC-18	Isocratic phase Binary solvent of tetrabutyl ammonium hydroxide (2×10^{-3} M, pH = 5.0 by 1% formic acid): acetonitrile (75 : 25, v/v)	UV 254 nm	Vinci et al. (1995)
Vitamin C (AA + DHAA)	Orange juice, soup, powdered milk, breakfast cereal, fruit preserves	Extraction with water containing 4% L-cysteine pH 7.0–7., add *m*-phosphoric acid pH 2.5–2.8, filtration	Licrospher 100 RP	Isocratic phase Methanol: 100 mM potassium dihydrogen orthophosphate with 5 mM cetyltrimethylammonium bromide (90:10)	UV 265 nm	Arella et al. (1997)
AA, sugar, and other organic acids, simultaneously	Strawberries, peaches, apples, kiwi fruit, bananas	Homogenization with 95% ethanol, reduction to dryness, residue dilution in 0.2 N sulfuric acid with 0.05% EDTA, cleaning up on C18 SPE cartridge	Ion 300 cation-exchange polymer	Isocratic phase 8.5 mN sulfuric acid	UV 245 nm	Pérez et al. (1997)
AA	Canned vegetables	Centrifugation, supernatant decantation, filtration	Spherisorb ODS-2	Isocratic phase Methanol (20%) with 10 mM octylammonium orthophosphate	UV 254	Cheng and Tsang (1998)

L-AA, D-AA simultaneously	Oranges, grapefruit, strawberries, green peppers, fortified fruit drinks	Extraction with *m*-phosphoric acid (10%)	LC-NH_2	Isocratic phase Acetonitrile: 25 mM potassium dihydrogen orthophosphate:phosphoric acid (800:200:7.5 v/v)	UV 268 nm	AbouL-Enein et al. (1999)
AA	Fruit juice and fruit drinks	Dilution with water, filtration	Aminex HPX-87H hydrogen form	Isocratic phase Acetonitrile:5 mM sulfuric acid (16:84 v/v)	UV 254 nm	Yuan and Chen (1999)
AA Vitamin C (AA + DHAA)	Fruit and vegetables	*AA*: dilution with water, filtration *AA + DHAA*: dilution with water containing dithiothreitol (1%), homogenization and filtration	Hi Chrom C18	Isocratic phase 0.2 M potassium dihydrogen phosphate pH 2.4	UV 254 nm	Gökmen et al. (2000)
Vitamin C (AA + DHAA)	Orange, pineapple and apple juice, soft drink	Reduction with 0.5 mM dithiothreitol, centrifugation, filtration	J'sphere ODS-H80	Isocratic phase Acetic acid (2%)	UV 243 nm	Furusawa (2001)
AA	13 beverages	Homogenization	Ion-exchange Kromasil NH_2	Isocratic phase Acetic acid in water 0.1 M	UV 250 nm	Rodríguez-Comesaña et al. (2002)
AA	Cooked sausages	Extraction with *m*-phosphoric acid (5%)	Spherisorb NH_2	Isocratic phase 0.02 M potassium phosphate buffer solution (pH 3.6) and acetonitrile (40:60l)	UV 248 nm	Valls et al. (2002)
AA, organic acids	Blackberry	Extraction with *m*-phosphoric acid (5%)	Ultrasphere ODS	Isocratic phase *m*-phosphoric acid (0.5%)	DAD 300–600 nm	Kafkas et al. (2006)
AA Vitamin C (AA + DHAA)	Human milk	Extraction with *m*-phosphoric acid (0.56%), centrifugation and *AA*: filtration *AA + DHAA*: reduction with dithiothreitol (100 mM) and filtration	C18 Spherisorb ODS 2	Isocratic phase Acetic acid in water 0.1% and methanol (95:5)	UV 254 nm	Romeu-Nadal et al. (2006)
AA Vitamin C (AA + DHAA)	Strawberries, tomatoes, apples	Extraction with *m*-phosphoric acid (4.5%), centrifugation and *AA*: filtration *AA + DHAA*: reduction with, • Dithiothreitol (0.4%) • Dimercaptopropanol (0.2%) and filtration	1. C18 Spherisorb ODS 2 2. NH_2-Spherisorb S5	Isocratic phase 1. Sulfuric acid (0.01%) pH 2.6 2. 10 mM potassium dihydrogen phosphate buffer adjusted to pH 3.5:acetonitrile (60:40)	UV 245 nm	Odriozola-Serrano et al. (2007)

TABLE 11.2

HPLC Methods with Fluorescence Detection for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Stationary Phase	Mobile Phase	Detection	References
DHAA Vitamin C (AA + DHAA)	Fruits and vegetables	Extraction with 0.3 M trichloroacetic acid	3 μ-ODS Hypersil	80 mM KH_2PO_4 (pH 7.8): methanol (50:50)	Postcolumn reaction: oxidation with ascorbate oxidase and derivatization with *o*-phenylenediamine ex. 355 nm em. 425 nm	Speek et al. (1984)
DHAA Vitamin C (AA + IAA + DHAA + DHIAA)	Meat-based food products	Extraction with *m*-phosphoric acid (3%) containing acetic acid (8%), cleaning up with hexane, filtration	PLRP-S	200 mM phosphate buffer pH 2.14	Postcolumn reaction: oxidation with $CuCl_2$ and derivatization with *o*-phenylenediamine ex. 350 em. 430	Vanderslice and Higgs (1990) Ali and Phillipo (1996)
Vitamin C (AA + IAA + DHAA + DHIAA)	Vegetables, fruit juices, fortified fruit juice, pork liver, sausage, milk	Extraction with *m*-phosphoric acid (3%) containing acetic acid (8%), addition of acetonitrile to obtain acetonitrile: *m*-phosphoric acid-acetic acid (2:1 v/v)	Grom-Sil NH_2	Acetonitrile: 50 mM ammonium dihydrogen orthophosphate pH 5 (7:3, v/v)	Postcolumn reaction: oxidation on in-house slurry-packed activated charcoal column and derivatization with *o*-phenylenediamine (0.4%) in *m*-phosphoric acid (3%) and acetic acid (8%) at pH 5.2 ex. 350 em. 430	Bognár and Daood (2000)
DHAA Vitamin C (AA + DHAA)	Muscle fish	Extraction with *m*-phosphoric acid (4.5%), homogenization, centrifugation	Waters Symmetry C18	Methanol (0.01%): *m*-phosphoric acid (80:20)	Postcolumn reaction: oxidation with oxidation with iodine derivatization with 4,5-dimethyL-*o*-phenilenediamine (0.3%) and a posterior liquid–liquid extraction with isobutanol ex. 350 em. 422	Iglesias et al. (2006)
DHAA Vitamin C (AA + DHAA)	Foods	Extraction with *m*-phosphoric acid (1%), homogenization, centrifugation	NovaPack C18	0.08 M KH_2PO_4 (pH 7.8): methanol (50:50)	Postcolumn reaction: oxidation with 2,2′-azobis(2-amidinopropane)dihydrochloride derivatization with 4,5-dimethyL-*o*-phenylenediamine (0.1%) ex. 355 em. 425	Burini (2007)

TABLE 11.3

HPLC Methods with UV/V Is and Fluorescence Detection for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Stationary Phase	Mobile Phase	Detection	References
AA, DHAA simultaneously	Fruits and vegetables	Extraction with *m*-phosphoric acid (3%), cleaning up on C18 cartridge (Set-Pack)	Alltech NH_2	0.05 M KH_2PO_4 pH 5.9	*AA*: UV 254 nm *DHAA*: Postcolumn derivatization with *o*-phenylenediamine ex. 348 nm, em. –nm	Kacem et al. (1986)
AA + IAA, DHAA + DHIAA simultaneously	Fruits and vegetables	Low starch sample extraction with *m*-phosphoric acid (1%) and oxalic acid (0.5%) pH 2 High starch sample extraction with *m*-phosphoric acid (2%): oxalic acid (1%) pH 2 (50:50 v/v); duplicated dilution with *m*-phosphoric acid (1%)	Ion-pair Jupiter C_{18}	2.3 mM dodecyltrimethyl-ammonium chloride, 2.5 mM Na_2EDTA containing 66 mM phosphate and 20 mM acetate buffer adjusted to pH 4.5	*AA + IAA*: UV 247 nm *DHAA + DHIAA*: Postcolumn derivatization with 12 mM trisodium citrate and 55 mM EDTA buffer pH 3.7 containing 28 mM *o*-phenylenediamine ex. 350 nm, em. 430 nm	Kall and Andersen (1999)
AA, DHAA, Vitamin C (DHAA + AA)	Green beans	*AA* and *AA + DHAA* extraction with *m*-phosphoric acid (4.5%) and *AA*: vacuum filtration *AA + DHAA*: reduction with dithiothreitol (0.4%) and vacuum filtration *DHAA* extraction with *m*-phosphoric acid (3%) with acetic acid (8%), filtration, oxidation with activated charcoal (2 g) and dilution with sodium acetate (50%)	Sphereclone ODS	Sulfuric acid (0.01%) pH 2.6	*AA*: UV 245 nm *AA + DHAA*: UV 245 nm *DHAA*: Postcolumn derivatization with *o*-phenylenediamine ex. 350 nm, em. 430 nm	Sánchez-Mata et al. (2000)

TABLE 11.4

HPLC Methods with Electrochemical Detection for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Stationary Phase	Mobile Phase	Detection	Reference
AA	Beers	Ultrasonic degassing	μBodapak C18	Citrate buffer pH 4.4 with 0.5 mM EDTA and 1 mM *N*-methyldodecylamine	Electrochemical with glassy carbon electrode at 600 mV vs. Ag/AgCl electrode	Moll and Joly (1987)
AA	Fruits, fruit drinks, vegetables	Extraction with 0.02 M sulfuric acid, 0.05 M phosphate buffer (pH 7) and 0.01 M dithiothreitol centrifugation, filtration	SCX sulfonated polystyrene-co-divinylbenzene resin	20 mM sulfuric acid	Electrochemical dual electrode with glassy carbon electrode at 600 mV at 800 mV vs. Ag/AgCl electrode	Kim (1989)
AA	Powdered corn, potage, and vegetable soup	Dilution with 100 mM phosphate buffer pH 3 with 1 mM EDTA	Inertsil ODS-3	100 mM phosphate buffer pH 3 with 1 mM EDTA	Electrochemical electrode at 400 mV vs. Ag/AgCl electrode	Iwase and Ono (1997, 1998)
AA	Athlete food nutritional supplement, infant milk, Japanese radish	Dilution with 20 mM monosodium L-glutamate pH 2.1, filtration	Inertsil ODS-3	20 mM monosodium L-glutamate pH 2.1	Electrochemical with glassy carbon electrode at 400 mV vs. Ag/AgCl electrode	Iwase (2000a)
AA	Orange juice, natsumikan juice, Japanese radish	Dilution with 20 mM guanoside-5′-monophosphate pH 2.1, filtration	Inertsil ODS-3	20 mM guanoside-5′-monophosphate pH 2.1	Electrochemical with glassy carbon electrode at 400 mV vs. Ag/AgCl electrode	Iwase (2000b)
AA	Athlete food nutritional supplement	Extraction with mobile phase containing 20 μM methionine	Inertsil ODS-3	Phosphoric acid (0.2%) pH 2.1	Electrochemical with glassy carbon electrode at 400 mV vs. Ag/AgCl electrode	Iwase (2003)
AA	Oranges, apples, and pharmaceutical preparation tablet	Dilution with water, homogenization, filtration	Metachem polaris C18A RP	Trifluoroacetic acid:acetonitrile (3:97)	Electrochemical with glassy carbon electrode at 100 mV vs. Ag/AgCl electrode	Gazdik et al. (2008)

Romeu-Nadal et al., 2006), mercapto compounds (Odriozola-Serrano et al., 2007), and L-cysteine (Arella et al., 1997) have been studied for the reduction of DHAA to AA. However, Odriozola-Serrano et al. (2007) reported higher recoveries for vitamin C determination when using DL-1,4-dithiotreitol as a reducing agent compared to 2,3-dimercapto-1-propanol. Derivatization of DHAA with *o*-phenylenediamine to form the fluorophore 3(1,2-dihydroxyethyl)furo[3,4-b]quinoxaline-1-one, which shows fluorescence at 348–355 nm (excitation) and 422–430 nm (emission), has also been used (Speek et al., 1984; Kacem et al., 1986; Vanderslice and Higgs, 1990; Ali and Phillipo, 1996; Kall and Andersen, 1999; Bognár and Daood, 2000; Sánchez-Mata et al., 2000; Iglesias et al., 2006; Burini, 2007). However, the depletion of DHAA by reaction with *o*-phenylenediamine induces the oxidation of AA to DHAA in order to preserve the equilibrium between these forms (Sánchez-Mata et al., 2000). Several enzymatic methods using ascorbate oxidase (Speek et al., 1984) and chemical methods based on charcoal (Bognár and Daood, 2000; Sánchez-Mata et al., 2000), iodine (Iglesias et al., 2006), or 2,2′-azobis(2-amidinopropane) dihydrochloride (Burini, 2007) are also employed for the AA oxidation. In recent years, more attention has focused on HPLC methods involving electrochemical detection due to recent advantages in detector technologies. Electrochemical detection is valuable in the analysis for trace amounts of compounds in complex matrices because of its excellent sensitivity and selectivity. The detection limit for AA determination with electrochemical detection procedures can be as low as within the pmol range. Recently, different simple, selective, rapid (≤8 min), and reproducible HPLC methods with electrochemical detection have been proposed by Iwase and Ono (1997, 1998) and Iwase (2000a,b, 2003) to the routine analysis of AA in different food matrices.

IAA is not found in natural products but may be present in vitamin C-enriched food products or in products where IAA is added as an antioxidant. Therefore, some HPLC methods for the accurate assay of AA and IAA and their primary oxidation products (L-DHAA and L-IAA) have been developed (Vanderslice and Higgs, 1990; Ali and Phillipo, 1996; Kall and Andersen, 1999; Bognár and Daood, 2000).

11.5.4 Flow Injection Analysis

Flow injection analysis has been applied to determine AA in a large number of samples, including foods and pharmaceutical and biological samples, by using spectrophotometric (Jain et al., 1995; Molina-Díaz et al., 1998; Fan et al., 1999; Luque-Pérez et al., 2000; Ensafi et al., 2002; Noroozifar and Khorasani-Motlagh 2003), fluorimetric (Pérez-Ruíz et al., 1997; Ensafi and Rezaei, 1998), electrochemical (Cardwell and Christophersen, 2000; Florou et al., 2000), and chemiluminescence (Alwarthan, 1993; Pérez-Ruíz et al., 1995; Zhang and Qin, 1996; Agater and Jewsbury, 1997; Qin et al., 1997; Dãnet et al., 2000; Ma et al., 2002; Kato et al., 2005) detection systems (Tables 11.5 through 11.8). The big advantages of flow injection analyses are short analysis time, instrument simplicity, and reproducibility. The kinetic spectrophotometric method is one of the most attractive approaches for trace determination of some species because of its high sensitivity. Therefore, some rapid, reproducible, and sensitive flow injection spectrophotometric systems have been developed based on reduction of Fe(III) by AA, which can be conventionally measured after its complexation with ferrozine (Molina-Díaz et al., 1998) or 1,2-phenanthroline (Luque-Pérez et al., 2000). Ensafi et al. (2002) proposed a highly selective and sensitive flow injection spectrophotometric method for the determination of AA at 0.5 μM levels based on the inhibitory effect of AA on the reaction of pyrogallol red by potassium iodate in acidic media. Iodine solid-phase reactor has been used extensively for the flow injection determination of AA in oranges, tomatoes (Fan et al., 1999), and fruit juices (Noroozifar and Khorasani-Motlagh, 2003). The amperometric determination of AA is based on its electrochemical oxidation. A chemically modified electrode consisting on glassy carbon modified with a cellulose acetate polymeric film bearing 2,6-dichlorophenolindophenol has been proposed by Florou et al. (2000) to determine AA in fruit juices, sport drinks, and carbonated beverages using flow injection procedures. A flow injection method for the simultaneous determination of AA and sulfur dioxide in wines and fruit juices has been reported by Cardwell and Christophersen (2000). The method, which is based on the oxidation of both analytes in a dual-channel electrochemical detection system, was more rapid and had similar reliability to an ion-exclusion chromatographic method.

Chemiluminescent methods in flow systems have involved the oxidation of AA by Fe(II) (Alwarthan, 1993; Qin et al., 1997) or hexacyanoferrate(III) (Zhang and Qin, 1996; Dãnet et al., 2000), followed by

TABLE 11.5

Flow Injection Analysis Methods with UV-Detector for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Flow Injection Conditions	Detection	References
AA	Soft drinks and fruit juices	Mixing with mercaptoethanol (6 mg/L) and filtration	1. 2-Mercaptoethanol is added to protect AA 2. Sodium hydroxide destroys AA Sample throughout = 30/h	UV 245 nm	Jain et al. (1995)
AA	Orange and lemon juice	Centrifugation and dilution	1. Fe(III) is reduced to Fe(II) by AA 2. Fe(II) is complexed by ferrozine at pH 5.5 to colored complex Sample throughout = 90/h	UV 562 nm	Molina-Díaz et al. (1998)
AA	Oranges and tomatoes	Squeezing and dilution	1. Potassium dichromate reacted with potassium iodide (accelerated by oxalate, which is inhibited by AA) to produce iodine 2. Iodine reacts with Rhodamine 6G to form a colorless compound Sample throughout = 100/h	UV 528 nm	Fan et al. (1999)
AA	Soft drinks and beer	Degasification by ultrasonication	1. Fe(III) is reduced to Fe(II) by AA 2. Fe(II) is complexed by 1,2-phenanthroline to colored complex Sample throughout = 60/h	UV 512 nm	Luque-Pérez et al. (2000)
AA	Orange juice	Trichloroacetic acid (1 M) addition and filtration	AA inhibited the reaction between pyrogallol red (2.2×10^{-4} M) and iodate (30 μM) in acidic media (sulfuric acid, 0.070 M) producing colorless products Samples throughput = 20/h	470 nm	Ensafi et al. (2002)
AA	Orange, tomato, lime, grapefruit, and lemon juice	Dilution with water and addition of acetic buffer containing 2-mercaptoethanol (6 mg/mL)	1. Acetic buffer (6 mg/mL) with 2-mercaptoethanol to protect AA 2. Iodine destroys AA Sample throughout = 110/h	UV 267 nm	Noroozifar and Khorasani-Motlagh (2003)

the interaction with another chemiluminescent reagent such as luminol. Compared with other techniques, the main pros of chemiluminescence for analytical applications are excellent sensitivity over a wide linear dynamic range and low limits of detection than can be achieved in comparison with fluorescence, because no external excitation source is required and, therefore, the background signal is very low. Agater and Jewsbury (1997) determined the AA content in juices by measurement of the chemiluminescence

TABLE 11.6

Flow Injection Analysis Methods with Fluorescence Detector for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Flow Injection Conditions	Detection	References
AA	Fruit juices and soft drinks	Mixing with 0.2 mol/L phosphate buffer of pH 3.0	Photooxidation of AA by Thionine Blue to form fluorescent Leucothionine Blue Samples throughput = 80/h	Fluorescence: ex. 340 nm em. 464 nm	Pérez-Ruíz et al. (1997)
AA	Fruit juice	Mixing with trichloroacetic acid (1 M) and filter	1. Tl(III) is reduced to Tl(I) by AA 2. Tl(I) reacts with potassium chloride to form fluorescent $TlCl_3^{2-}$ Samples throughput = 45 h	Fluorescence: ex. 227 nm em. 419 nm	Ensafi and Rezaei (1998)

TABLE 11.7

Flow Injection Analysis Methods with Chemiluminescence for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Flow Injection Conditions	Detection Conditions	References
AA	Fruit juices	Mixing with *m*-phosphoric acid solution (1%)	1. Fe(III) is reduced to Fe(II) by AA 2. Fe(II) reacted with luminol in the presence of hydrogen peroxide to provide a chemiluminescent product Sample throughout = 2–56 h	Chemiluminescence: Thorn EMI, 9789QB photomultiplier tube	Alwarthan (1993)
AA	Fruit juices and soft drinks	Directly inject	Sample and Toluidine Blue solution were injected simultaneously into two phosphate buffer (pH 3.0) streams and mixed with a Lucigenin solution in alkaline medium to provide chemiluminescent product Sample throughout = 80/h	Chemiluminescence: Bio Orbit luminometer or SLM-Aminco Model JD490 photomultiplier tube	Pérez-Ruíz et al. (1995)
AA	Tomatoes, mung bean sprouts, and whilte gourd	Homogenization with acetic acid buffer solution (pH 4.0), filtration, and dilution with water	Luminol and hexacyanoferrate (III) were immobilized on an Amberlyst A-27 anion-exchange resin. These reagents were eluted from the resin through sodium phosphate injection. Reduction of chemiluminescence is proportional to AA in the sample Sample throughout = 60/h	Chemiluminescence: R456 photomultiplier tube	Zhang and Qin (1996)
AA	Cucumbers, tomatoes, Chinese cabbage, mung beans sprouts	Homogenization with acetic acid buffer solution (pH 4.0), filtration, and dilution with water	Luminol and Fe(II) were immobilized on an anion-exchange resin and cation-exchange resins, and can be eluted from the resin through sodium sulfate injection. Reduction of chemiluminescence was proportional to AA in the sample Sample throughout = 60/h	Chemiluminescence: R456 photomultiplier tube	Qin et al. (1997)
AA	Fruit drinks	Dilution with a solution containing *m*-phosphoric acid solution (3%) and acetic acid (8%)	AA reacted with permanganate in the presence of sulfuric acid. Enhancement of chemiluminescence was proportional to AA in the sample	Chemiluminescence: R928F photomultiplier detector	Agater and Jewsbury (1997)

continued

TABLE 11.7 (continued)

Flow Injection Analysis Methods with Chemiluminescence for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Flow Injection Conditions	Detection Conditions	References
AA	Lemon, orange, and kiwi juices	Centrifugation, dilution with phosphate buffer (pH = 6.5)	*Sample*: Sample extracts were mixed with luminol and hexacyanoferrate (III) *Blank*: Sample passed through the enzymatic reactor containing ascorbate oxidase where AA is decomposed. Sample extracts were mixed with luminol and ferricyanide For reactivation of the enzyme, a 0.1 mol/L phosphate buffer, pH = 5.6, was passed through the enzymatic reactor for 5 min after each sample AA is the difference in chemiluminescence between sample and blank Sample throughout = 4 h	Chemiluminescence: 24°C	Dãnet et al. (2000)
AA	Celery, cabbage, cole, and long crooked squash	Crushing, centrifugation with calcium chloride solution (0.2%) to decrease interference of oxalic acid in vegetables	The reaction between cerium(IV) with Rhodamine B in sulfuric acid produces chemiluminescence products Enhancement of chemiluminescence is proportional to AA in the sample	Chemiluminescence: sensitive photomultiplier tube	Ma et al. (2002)
AA	Soft drink powder	Dilution with water	The reaction between iron–chlorophyllin complex and a solution of acetonitrile/water produces chemiluminescence products. Reduction of chemiluminescence is proportional to AA in the sample	Chemiluminescence: 600-type lumicounter	Kato et al. (2005)

TABLE 11.8

Flow Injection Analysis Methods with Electrochemical Detection for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Flow Injection Conditions	Detection Conditions	References
AA	Fruit juices, sport drinks, carbonated beverages	Dilution with phosphate (50 mM) containing potassium chloride (50 mM), pH 6.5	AA is oxidized by 2,6-dichloroindophenol immobilized in a cellulose acetate film on a glassy carbon electrode	Electrochemically: 100 mV vs. Ag/AgCl (3 M KCl at pH 6.5) reference electrode, commercial working electrode of glassy carbon Sample throughout = 25/h	Florou et al. (2000)
AA and sulfur diode simultaneously	Red and white wines and various fruit juices	Dilution with degassed sulfuric acid (0.01M) with mannitol (0.01 M), pH adjust to 5.9, pass throughout a quaternary amine SAX cartridges	AA is oxidized on glassy carbon electrodes Sulfur diode on platinum electrode	Electrochemically: *AA*: 420 mV vs. Ag/AgCl reference electrode *Sulfur diode* 900 mV vs. Ag/AgCl reference electrode Sample throughout = 30/h	Cardwell and Christophersen (2000)

from direct oxidation with permanganate in an acidic medium. The determination of L-AA in a soft drink powder based on the chemiluminescence of the quenched iron–chlorophyllin complex has been described (Kato et al., 2005). Regarding AA determination in vegetables, a high-sensitive flow injection method with chemiluminescence detection based on the reaction of Rhodamine B with cerium(IV) in sulfuric acid media has been proposed by Ma et al. (2002). The chemiluminometric determination of AA based on its photooxidation sensitized by Toluidine Blue has also been described by Pérez-Ruíz et al. (1995).

11.5.5 Capillary Electrophoresis

Analytical procedures based on the relatively new technique of capillary electrophoresis are rapidly gaining acceptance as viable analytical procedures. The advantages of capillary electrophoresis over HPLC were reported to be short analysis time, small consuming sample (nanoliter levels), high separation efficiency, and simple experiment separation (Tang and Wu, 2005). Capillary zone electrophoresis and micellar electrokinetic capillarity have been used for the determination of AA in different matrices. These methods have been developed using both uncoated (Marshall et al., 1995; Schiewe et al., 1995; Thompson and Trenerry, 1995; Davey et al., 1996; Choi and Jo, 1997; Cancalon, 2001; Galiana-Balaguer et al., 2001; Liao et al., 2001; Versari et al., 2004; Tang and Wu, 2005; Wu et al., 2007; Peng et al., 2008) and coated columns (Fukuski et al., 1997) (Tables 11.9 and 11.10) . In micellar electrokinetic capillary chromatography, an ionic surfactant is added to the buffer to provide a phase for chromatographic separation. Quantification of AA in foods has been performed by micellar electrokinetic capillary chromatography using sodium deoxycholate as surfactant (Marshall et al., 1995; Thompson and Trenerry, 1995). Capillary zone electrophoresis is based on difference of electrophoretic mobility that results from the varied charge numbers and particle sizes between electrolytes under applied voltage. Capillary zone electrophoresis has become a powerful and popular separation technique because of its fast separation and the high resolution achieved. Choi and Jo (1997) demonstrated that capillary zone electrophoresis was effective in determining L-AA within 2 min using 0.1 M sodium borate (pH 8.0) as carrier electrode. Their method was faster, more sensitive, and more reproducible than the dinitrophenylhydrazine spectrophotometric determination and in-house HPLC method. Total AA was estimated by Cancalon (2001) in citrus juices after reduction with dithiothreitol (0.2%) using capillary zone electrophoresis with 35 mM sodium borate buffer (pH 9.3) containing 5% acetonitrile at 21 kV and 23°C. L-Cysteine (10 mM) was used to determine vitamin C using capillary zone electrophoresis with 20 mM phosphate buffer (pH = 8) in citrus juices (Schiewe et al., 1995). Simultaneous quantification of L-AA

TABLE 11.9

Capillary Electrophoresis Methods with UV-Detector for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Stationary Phase	Mobile Phase	Detection	References
Vitamin C (AA + DHAA)	Citrus fruits and fruit beverages	Dilution with water, reduction with L-cysteine (10 mM); filter	Uncoated fused silica (60 cm total length) $T = 25°C$	Capillary zone electrophoresis: buffer of phosphate pH = 8.0 +25 kV	UV 266 nm	Schiewe et al. (1995)
Vitamin C (AA + DHAA)	Beer, wine, fruits, and vegetables	Extraction with *m*-phosphoric acid (1%) and reduction with dithiothreitol (0.2%); cleaning up with C18 SPE cartridge	Uncoated fused silica (40 cm total length) $T = 28°C$	Micellar electrokinetic capillary chromatography using buffer consisting of sodium deoxycholate (0.05 M), sodium borate (0.01 M), and potassium dihydrogen orthophosphate (0.01 M) pH = 8.6 +25 kV	UV 254 nm	Thompson and Trenerry (1995); Marshall et al. (1995)
AA, IAA	Parsley and mushroom	Extraction with *m*-phosphoric acid (3%) containing EDTA, centrifugation and cleaning up with C18 SPE cartridge	Uncoated fused silica (57 cm total length) $T = 25°C$	Capillary zone electrophoresis: buffer of borate (50 mM) pH 8.8 +25 kV	UV 260 nm	Davey et al. (1996)
AA	Spinach, chingentsui, komatsuna, turnip, perilla, parsley	Homogenization with hydrochloric acid (10 mM) containing thiourea (2%), dilution with hydrochloric acid (5 mM), centrifugation and filtration	Polyamide-coated fused silica (72 cm total length) $T = 35°C$	Capillary zone electrophoresis: buffer of sodium tetraborate pH 9.2 +20 kV	UV 270 nm	Fukuski et al. (1997)
AA + DHAA	Fruit, vegetables, juices, biscuits, candy, and chocolate	Extraction with *m*-phosphoric acid (5%) containing cysteine, centrifugation, and filtration	Uncoated fused silica (27 cm total length) $T = 25°C$	Capillary zone electrophoresis: buffer of sodium borate (100 mM) pH 8, containing L-cysteine (10 mM) +15 kV	UV 270 nm	Choi and Jo (1997)

AA	Tomatoes	Extraction with *m*-phosphoric acid (2%) and centrifugation	Uncoated fused silica (27 cm total length) $T = 25°C$	Capillary zone electrophoresis: buffer of borate, pH 8, containing hexadimethrine bromide (0.01%) with or without 20% acetonitrile −15 kV	UV 254 nm	Galiana-Balaguer et al. (2001)
AA, vitamin C (AA + DHAA)	Citrus juices	Dilution with EDTA (1000 M), addition ferulic acid as standard and *AA*: filtration *AA + DHAA*: reduction with dithiothreitol (0.2 %) and filtration	Uncoated fused silica (70 cm total length) $T = 23°C$	Capillary zone electrophoresis: buffer of 35 mM sodium borate buffer (pH 9.3) containing acetonitrile (5%) +21 kV	UV 270 nm	Cancalon (2001)
AA, IAA, AA + DHAA	Lemon, Sunkist, pineapple, and spinach	*Lemon, Sunkist, and pineapple*: homogenization with a solution containing EDTA (1 mM) and *m*-phosphoric acid (3%), and centrifugation *Spinach*: homogenization with a solution containing EDTA (1 mM), potassium dihydrogen phosphate (0.2 M), and phosphoric acid (0.474 M) (pH 2.0), mixing with EDTA (1 mM) and *m*-phosphoric acid (3%) and centrifugation: *AA*: filtration *AA + DHAA*: reduction with homocysteine solution ($T = 28°C$; for 15 min) and filtration	Uncoated fused silica (57 cm total length) $T = 25°C$	Capillary zone electrophoresis: buffer of borate (0.2 M) pH 9.0 +30 kV	UV 265 nm	Liau et al. (2001)
AA, IAA simultaneously	Apricot juice	Dilution with *m*-phosphoric acid (10%), centrifugation and filtration	An uncoated fused-silica (50 cm total length) $T = 30°C$	Capillary zone electrophoresis: buffer of 50 mM tricine (pH 8.8) +11 kV	UV 265 nm	Versari et al. (2004)
AA and sorbic acid	Orange juice, orange, apple, and orange drink	Dilution with water, centrifugation and filtration	An uncoated fused-silica (55 cm total length) $T = 20°C$	Capillary zone electrophoresis: buffer of boric acid (80 mmol/L) and borax (5 mmol/L) (pH = 8.0) +30 kV	UV 270 nm	Tang and Wu (2005)

TABLE 11.10

Capillary Electrophoresis Methods with Electrochemical Detector for Vitamin C Analysis in Food Products

Compounds Determination	Type of Sample	Sample Preparation	Stationary Phase	Mobile Phase	Detection Conditions	References
AA and phenolic compounds, simultaneously	Grapefruit juice	Treating the pulp with a juice extractor and the juices undergo centrifugation and filtration	Uncoated fused silica (75 cm of length) T = ambient	Capillary zone electrophoresis: buffer of borate buffer (60 M) pH 9.0 +30 kV	Electrochemically: +950 mV vs. saturated calomel electrode reference electrode, commercial working electrode of glassy carbon, and a platinum auxiliary electrode Sample throughout = 2/h	Wu et al. (2007)
AA and phenolic compounds, simultaneously	Tomato	Freezing in liquid nitrogen, freeze-drying, extraction with methanol (80%) in a ultrasonic bath filtration, neutralization with NaOH and dilution using the running buffer	Uncoated fused silica (80 cm of length) T = ambient	Capillary zone electrophoresis: buffer consisting of borate (50 mmol/L) at pH 8.7 +16 kV	Electrochemically: +950 mV vs. saturated calomel electrode reference electrode, commercial working electrode of glassy carbon, and a platinum auxiliary electrode Sample throughout = 7/h	Peng et al. (2008)

and D-IAA in food systems, usually added for nonvitamin purposes, has been reported. Capillary zone electrophoresis with migration time lower than 7 min was found effective in epimeric separation of L-AA and D-IAA in parsley and mushroom (Davey et al., 1996); lemon, Sunkist, pineapple, and spinach (Liao et al., 2001); and apricot juice (Versari et al., 2004). On the other hand, a quick, facile, sensitive, and selective method for the determination of AA and sorbic acid in fruit juices by capillary zone electrophoresis was developed (Tang and Wu, 2005). Phenolic acids and AA in grapefruit juices were well separated in 25 min with a stable baseline in 50–60 mmol/L borate buffer (pH = 8.9–9.0) (Wu et al., 2007; Peng et al., 2008).

11.6 Final Remarks

This chapter discussed the significant developments regarding the application of HPLC, flow injection, and capillary electrophoresis methods to the determination of vitamin C in functional foods. The growing interest toward these techniques is due to its versatility and simplicity, the possibility of reducing analysis time, and the improvement in reliability and sensitivity. These advantages, though, have led to a proliferation of methods and thus it has become difficult to choose a proper method for any specific food. The analyst has a wide selection of methods to choose from those that have been applied to many food matrices. However, because of the variability that exists in the application of these methods, considerable dispersion in the analysis results can occur when different laboratories assay similar samples using proven methods of their choice.

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12

Vitamin D

Jette Jakobsen and Rie Bak Jäpelt

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One of the great scientists Sir Lord Kelvin said: "When you can measure what you are speaking about and express it in numbers, you know something about it, and when you cannot measure it, when you cannot express it in numbers, your knowledge is of a meager and unsatisfactory kind." This statement somehow makes sense for vitamin D. The discovery and elucidation of the understanding of the physiological mechanism of vitamin D is linked to the history of analytical chemistry, and it explains why it took 50 years to discover the active vitamin D metabolite and why we still have much to learn about vitamin D.

12.1 Introduction

Vitamin D is related to compounds that possess antirachitic activity. If vitamin D is not present, humans will develop a deficiency disease; rickets in children or osteomalaci in the elderly.

Vitamin D deficiency was already a problem for our ancestors, the Neanderthal, more than 50,000 years ago. However, the first scientific description of rickets was not until 1645. In the early nineteenth century cod liver oil was described as a curing agent for rickets, and later the probable beneficial effect of sunlight was also described. However, the evidence that supported the benefits of cod liver oil came from the successful studies of Sir Edward Mellanby, who in 1919 prevented puppies from developing rickets by feeding them cod liver oil or butter.

In contrast to other vitamins, the human body is able to produce vitamin D in the skin when exposed to UV-light below 315 nm. However, the sun only emits these rays all year round in places that lie below 35° latitude. In the northern hemisphere, this is, for example, Northern Africa. Hence, in wintertime, when the vitamin D needs of human beings cannot be met by endogenous production, the dietary intake of vitamin D becomes essential to cover the requirement for vitamin D.

12.2 Description of Vitamin D

Compounds possessing vitamin D activity are fat-soluble organic substances belonging to the group of seco-steroids. The parent compounds are cholecalciferol (vitamin D3) and ergocalciferol (vitamin D2), which originate from their precursors 7-dehydrocholesterol and ergosterol, respectively.

Breakage of the B-ring of the sterol moiety requires UV-light below 315 nm. The compounds produced in this photochemical reaction are shown in Figure 12.1. The difference in absorption for these

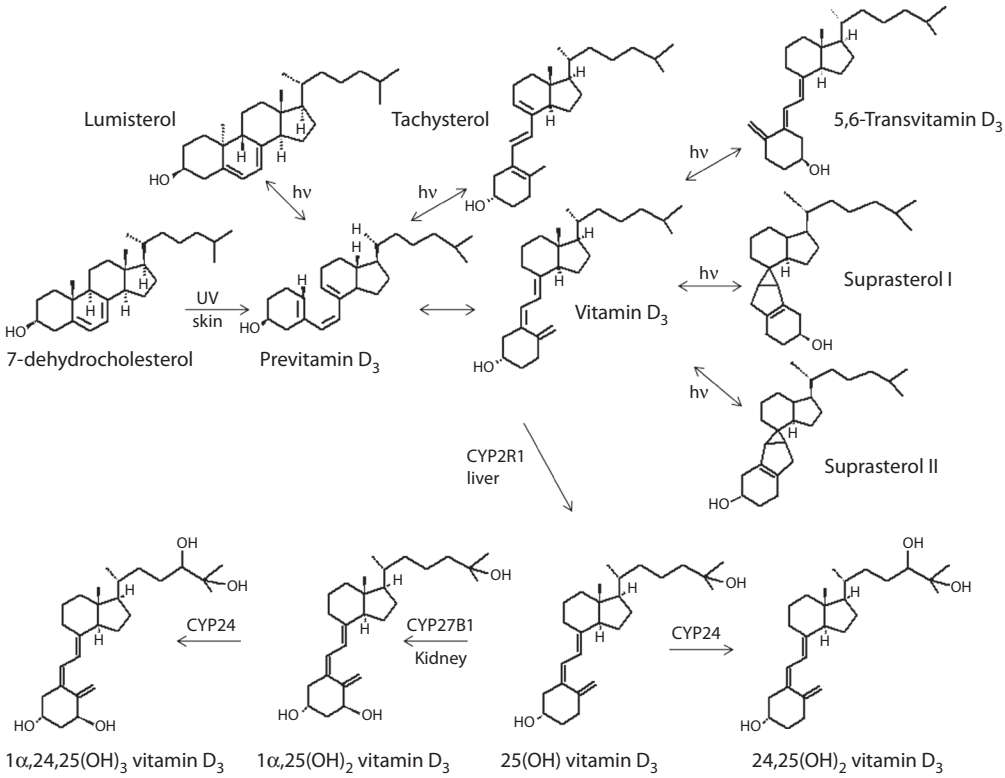

FIGURE 12.1 Vitamin D metabolism. (From Ebert et al. *Mol cell Endocrinol*. 2006; 248(1–2): 149–159. With permission.)

metabolites for the corresponding vitamin D2 metabolites is shown in Figure 12.2. The strongest band to absorb is 280 nm, but these rays do not appear on earth due to absorption by the ozone layer, which leaves only the band 290–315 nm for natural active production. The amount of vitamin D synthesized in skin depends on the energy of the light source, whereas the wavelengths influence the distribution of the synthesized metabolites tachysterol and lumisterol. If the light source emits rays between 290 and 300 nm, there will be four times as much tachysterol as lumisterol, whereas the catabolism in the solar spectrum will enhance the synthesis of lumisterol compared to tachysterol (MacLaughlin et al., 1982).

How vitamin D2 is produced when ergosterol in yeast is exposed to light has been known since 1920s (Hess et al., 1931).

Solutions of cholecalciferol as well as ergocalciferol undergo thermal, photochemical, and oxidative reactions as illustrated in Figure 12.1.

A study of the degradation at increased humidity and temperature showed a difference in the degradation pattern, that is, stability between the powder preparations of the two compounds (Grady and Thakker, 1980). In the dark, at 25°C, both compounds were stable in a desiccator, and in 85%, relative humidity (RH) for 7 days. In the desiccators, cholecalciferol was even stable for up to 56 days, after which only 2–5% ergocalciferol was present, while at 85% RH, both compounds did not degrade until after 25 days. A solution of the vitamers in *n*-heptane is stable for up to 2 years at –20°C, but in these solutions cholecalciferol is the first to degrade.

The molecular weight and the molecular absorption coefficient for the vitamin D metabolites are given in Table 12.1.

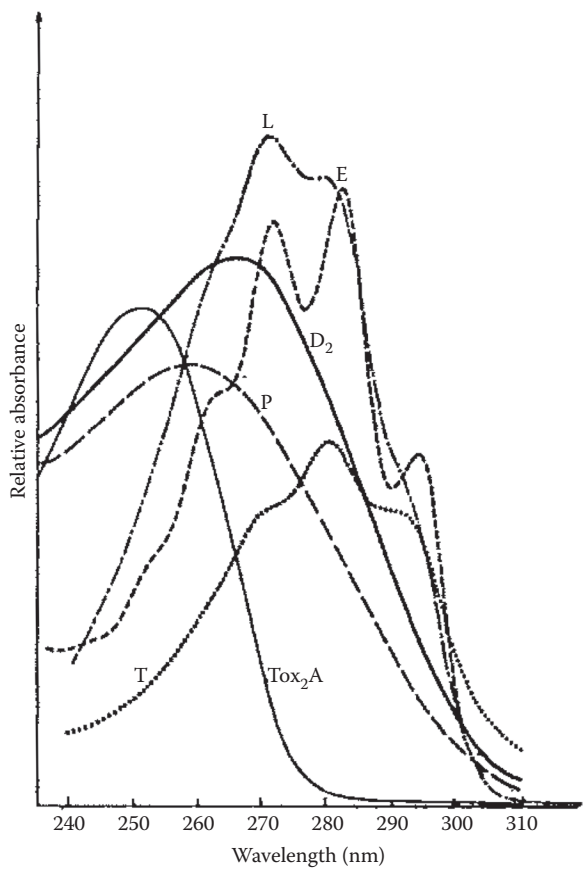

FIGURE 12.2 UV-absorption graphs. E: ergosterol, L: lumisterol2, P: previtamin D2, T: Tachysterol2, D: vitamin D2 and Tox_2A: Toxisterol2A. (Abillon E, Mermet-Bouvier R. Effect of wavelength on production of previtamin D2. *J Pharm Sci*. 1973; 62(19): 1688–1691. Copyright Wiley-VCH Verlag GmbH & Co. KgaA. Reproduced with permission.)

TABLE 12.1

Vitamin D Metabolites: Chemical Data

Vitamin D Metabolite	Formula	Molecular Weight (g/mol)	Molecular Absorption Coefficient m^2/mol	Reference
Vitamin D3	$C_{27}H_{44}O$	384.64	18,461	Pham Codex (1979)
Vitamin D2	$C_{28}H_{44}O$	396.65	18,843	Pham Codex (1979)
25OHD3	$C_{27}H_{44}O_2$	400.64	18,584	Mattila et al. (1995)
25OHD2	$C_{28}H_{44}O_2$	412.65	19,400	Mattila et al. (1995)
1α,25(OH)2D3	$C_{27}H_{44}O_3$	416.64	—	—
1α,25(OH)2D2	$C_{28}H_{44}O_3$	428.65	—	—

12.3 Role of Vitamin D in Food

In mammals, the production of vitamin D from the provitamin D, 7-dehydrocholesterol, mainly occurs in the two inner layers of the skin, the stratum basale, and the stratum spinosum. The breakage of the B-ring of the sterol moiety to produce previtamin D3 is activated by UV-light lower than 315 nm. The previtamin D3 undergoes thermally induced transformation to vitamin D3.

Endogenous vitamin D3 produced in the skin and dietary vitamin D are absorbed in the lymph system. Vitamin D binds to vitamin D-binding protein (DBP) and is transported to the liver through chylomicrons. The nonvitamin D active metabolites, lumisterol and tachysterol, have no affinity for DBP (Holick et al., 1981).

In the liver, vitamin D3 is hydroxylated at position 25 by vitamin D 25-hydroylase in a nonrestricted reaction. Whereas hydroxylation in the kidney by 25-hydroxyvitamin D3-1α-hydroxylase is regulated by a variety of factors including serum calcium and phosphorous, 1,25-dihydroxyvitamin D, and parathyroid hormone (PTH). In addition to the kidney, many other tissues also express the 1,25-dihydroxylase activity, for example, skin, placenta, colon, brain, osteoclasts, macrophages, and parathyroid glands (Brannon et al., 2008). 1,25-Dihydroxyvitamin D mediates its biological effect by binding to the vitamin D receptor (VDR). The binding of vitamin D allows VDR to act as a transcription factor in the expression of the transport proteins involved in the transport of calcium in the intestine; see Figure 12.1.

As mentioned, the known and documented nutritional deficiency diseases are rickets in children and osteomalacia in the elderly. The evidence for links to other diseases such as cancer, diabetes, immune diseases, and cardiovascular diseases is subject to heavy debate worldwide; however, this will not be dealt with in this chapter.

12.4 Content in Food

12.4.1 Natural Content

Generally, only foods of animal origin contain vitamin D. The main compound in food is vitamin D3, but the metabolites that are part of the metabolic pathway in vertebrates, that is, 25-hydroxyvitamin D3, 1,25-dihydroxyvitamin D3, and other dihydroxyvitamin compounds, may also be present. The content of vitamin D in foods of animal origin depends on what the animal has been fed during its upbringing.

The solar effect on the content of vitamin D translates into different amounts of vitamin D in milk depending on the cow's access to grazing. The vitamin D content in milk is higher in summer compared to that in winter (Kurmann and Indyk, 1994; Jakobsen and Saxholt, 2009).

Milk from dairy cows contains a significant, although low, amount of vitamin D2, which is expected to derive from grass and hay. Due to the metabolism in cows, dairy products also contain 25-hydroxyvitamin D2 (Jakobsen and Saxholt, 2009).

Usually, the source of vitamin D in feed for pigs is vitamin D3. The meat of these pigs contains vitamin D3 and 25-hydroxyvitamin D3. If the source of vitamin D is 25-hydroxyvitamin D3, the meat of these pigs

only contains 25-hydroxyvitamin D3 (Jakobsen et al., 2007). Similarly, the effect of varying concentration of vitamin D in feed has been tested in pigs, hens, and fish. Pigs fed 2–3 mg vitamin D3/kg feed for 24 days had 4.6–17.2 µg vitamin D3/100 g meat (14–18% fat) 3 weeks before slaughtering (Thompson and Plouffe, 1993). Furthermore, eggs from hens that were fed 266 µg vitamin D3/kg had approximately 10 times higher content of vitamin D3 than the eggs from hens fed 26.6 µg vitamin D3/kg. No difference was observed in the content of 25-hydroxyvitamin D3 (Mattila et al., 2004). Generally, fish have the highest amount of natural vitamin D. In fat-rich fish, for example, salmon, vitamin D is present in the flesh, while in fatless fish, for example, cod, vitamin D is stored in the liver. Studies of the content of fat and vitamin D in fish species show that the determinants of vitamin D in fish are rather related to species than the content of fat (NFAD, 1990). Furthermore, fish species differ as herring do show a correlation between vitamin D and fat, whereas the vitamin D content in mackerel is not dependent on the fat content; see Figure 12.3.

Feeding trials in trout weighing 500 g and more and in salmon weighing 170 mg showed contradictory results. There was no effect on the content of vitamin D in the final trout product, whereas the content of vitamin D in salmon was positively related to the vitamin D content in the feed (Mattila et al., 1999; Graff et al., 2002).

The only known food of nonanimal origin containing vitamin D is wild mushrooms (Mattila, et al., 1994, 2002; Teichmann et al, 2007). Due to the high content of ergosterol and the possibility to increase the vitamin D content by irradiation with lower wavelength than 315 nm, cultivated mushrooms have been in focus as a functional food. The formation of vitamin D2 depends on the intensity of UV-light, distance and duration (Koyyalamudi et al., 2009) as well as the moisture content of the mushrooms (Perera et al., 2003). The content may increase to as much as 23 µg/g dry weight, corresponding to ~141 µg per serving (Koyyalamudi et al., 2009). Table 12.2 shows the variability in the natural content in food.

For a population which depends on nonfortified food, the dietary intake of fish is essential. An extract of results from studies performed to assess the content of vitamin D in fish show that generally there is a lower content of vitamin D3 in aqua-cultured fish than in wild fish, see Table 12.3.

12.4.2 Enriched Food and Supplements

In the 1920s, irradiation of yeast was shown to produce vitamin D2-enriched yeast, and vitamin D3 was derived by irradiation of lanolin in sheep wool (Hume et al., 1927; Hess et al., 1931). Today, the synthetic production of vitamin D3 utilizes the content of cholesterol in lanolin from sheep wool. There is a common market for supplements and vitamin D-enriched foods; however, this area is regulated very differently in each country. Milk and margarine are the primary products that are enriched with vitamin D, and orange juice, bread, cheese, and yogurt also contain this vitamin (Coulter and Thomas, 1968; Upreti et al., 2002; Tangpricha et al., 2003; Natri et al., 2006).

Fortification may either be voluntary or mandatory, and the levels added vary accordingly. An exhaustive description of fortification worldwide is impossible; however, a few examples are presented here to

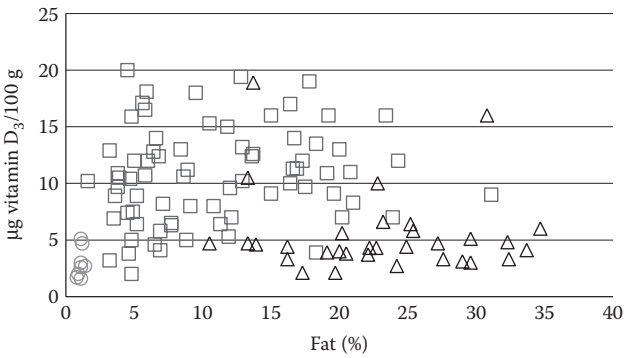

FIGURE 12.3 Content of vitamin D in fish species related to fat. Δ: Mackarel, □: Herring, and ○: Tuna. (Based on data from NFAD, 1990: *Food Monitoring in Denmark—Nutrients and Contaminants 1983–1987.* The National Food Agency of Denmark: Levnedsmiddelstyrelsen, Denmark.)

TABLE 12.2

Natural Content of Vitamin D in Selected Nonfortified Food: Exclusive Fish

Food	Remarks	μg/100 g Minimum	μg/100 g Maximum	Metabolite	Reference
Cod liver oil		250		VitD3	DFCD (2010)
Pork, fillet, 6%–7% fat	Autumn–summer	0.09	0.13	VitD3	Mattila et al. (1995)
Pork, loin, 1.9% fat		<0.03	0.06	VitD3	Clausen et al. (2003)
Pork, loin, lard, 80% fat		0.16	0.59	VitD3	Clausen et al. (2003)
Pork, loin, 1.9% fat		<0.05	0.10	25OHD3	Clausen et al. (2003)
Pork, loin, lard, 81% fat		0.08	0.23	25OHD3	Clausen et al. (2003)
Egg yolk	Extra vitD3 in feed	1.2	23	VitD3	Mattila et al. (1999)
Egg yolk	Extra vitD3 in feed	0.5	1.5	25OHD3	Mattila et al. (1999)
Mushroom, wild		10.7	58.7	VitD2	Teichmann et al. (2007)
Mushroom, cultivated		0.3	1.2	vitD2	Teichmann et al. (2007)
Cow milk		0.005		VitD3	Kunz et al. (1984)
Cow milk	Winter–summer	0.006	0.026	VitD3	Kurmann and Indyk (1994)
Cow milk, 3.5% fat	Winter–summer	0.007	0.014	VitD3	Jakobsen and Saxholt (2009)
Cow milk		0.05		25OHD3	Kunz et al. (1984)
Cow milk, 3.5% fat	Winter–summer	0.006	0.009	25OHD3	Jakobsen and Saxholt (2009)
Cow milk		0.001		1,25(OH)2D3	Kunz et al. 1984

illustrate the levels. In the United States, fortification is voluntary in the range 10–15 μg vitamin D3 per quart (= 0.95 L) milk, corresponding to 1–2 μg per serving of milk products. In Sweden, the mandatory fortification level depends on the fat content of the milk, but is approximately 4 μg/L (Murphy et al., 2001; Calvo et al., 2004; SLV, 2010).

As is the case for all vitamins, the challenge for the supplier of vitamin D premixes is the stability; hence different types of coating are used, for example, microencapsulation, which makes it possible to transform oily vitamin D3 into a dry product form. Dry products may be more easily redispersed in aqueous solutions.

12.4.3 Stability in Food

Vitamin D is sensitive to light, air, and acidic conditions in general, but is not sensitive to heat. Similar consequences are seen in food in heat treatment and stability studies performed in fish, eggs, mushrooms, milk, cheese, and meat.

TABLE 12.3

Content of Vitamin D3 in Wild versus Aqua-Cultured Salmon

Food	Country	Wild (μg/100 g)	Aquacultured (μg/100 g)	Metabolite	Reference
Salmon, fresh	Ireland/Norway	9.6	7.6	VitD3	Ostermeyer and Schmidt (2006)
Salmon	United States	24.9	6.0	VitD3	Lu et al. (2007)
Salmon, smoked	Poland/Norway	11.0	4.1	VitD3	Usydus et al. (2009)
Salmon	Denmark	13.7	6.7	VitD3	Jakobsen, unpublished

The stability of vitamin D may be divided into industrial and household treatment. In the industrial production of milk, both regular and ultra-high temperature (UHT), the high-pressure evaporation process did not cause any loss of added vitamin D (Indyk et al., 1996; Wagner et al., 2008; Hanson and Metzger, 2010). Similar stability was observed in vitamin D fortified cheddar, but a loss of 25–30% was observed in processed cheese due to heating (Upreti et al., 2002; Wagner et al., 2008). Furthermore, in UHT-milk and skimmed-milk, the vitamin D level was stable when stored for 60 days at 4°C (Renken et al., 1993; Upreti et al., 2002). A significant decrease was only observed when the milk was exposed to light (Renken et al., 1993).

Household cooking of fish, eggs, mushrooms, and pork indicated no degradation of vitamin D3. Furthermore, in pork, a 100% retention of vitamin D was observed for 25-hydroxyvitamin D3 (Mattila et al., 1999; Clausen et al., 2003).

However, solar drying of mackerel and saury showed a decrease in the content of vitamin D3 to <1.4 μg/100 g from a content of 5.1–7.4 μg/100 g in fresh samples (Suzuki et al., 1988).

12.5 Critical Review on the Available Methodologies for Analysis

The determination of vitamin D in natural foods has always been a challenge due to the low amount of vitamin D present in food. Even in sources rich in vitamin D, such as cod liver oil, the content of vitamin D is only 2–3 mg/kg.

12.5.1 Official Methods: In the Past

For many years, the accepted official method was the line test using animals. Either a rat or a chicken was put on a vitamin D-deficient diet until the animal developed rickets. The animal was subsequently fed different amounts of vitamin D3s for 7–14 days to establish a calibration curve for the sample based on to what degree the animal was cured of rickets. This was done by staining with silvernitrate or radiographic pictures. Rat is able to utilize vitamin D2 and vitamin D3 equally, while chicken utilizes vitamin D2 only 10% as efficiently as vitamin D3. In this way, the method is able to distinguish between the two families of metabolites if the rat and chicken assay are combined (The Danish Pharmacopoeia Commission, 1964). For a more thorough explanation of the animal test system, see Parrish (1979).

The biological method is time consuming as it takes approximately 5 weeks and it is run with low precision. The accuracy of the method may be discussed. The fact is that the amount of quantified vitamin D corresponds to the total vitamin D activity independent of the specific chemical compounds and their possible difference in activity. This is true if we presume that vitamin D-deficient rats are similar to well-nourished or vitamin D-deficient human beings.

However, the biological assay has not been used for analyzing the vitamin D level in food since around the 1980s.

12.5.2 Official Methods Used Today (AOAC/NMKL/CEN)

For different food sources, the suppliers of official methods are the European Committee for Standardisation (CEN), the Nordic Committee on Food Analysis (NMKL), and the Association of Official Analytical Chemists International (AOAC). CEN and NMKL have one method each for analyzing vitamin D3 and vitamin D2 in food matrices, namely EN 12821 and NMKL 167, respecitvely (NMKL, 2000; CEN, 2008), whereas AOAC currently have three different methods for analyzing the food and fortified food (AOAC, 2005a–c). Standardization of analytical methods is a lengthy process, which include development, validation, and collaborative testing. The NMKL method and the AOAC method 2002 9.05 (AOAC, 2005a–c) are identical and they are very similar to the CEN method. These three methods include quantification by an internal standard (IS), alkaline saponification, extraction with an organic solvent, and different cleanup steps, for example, solid-phase extraction and preparative high-performance liquid chromatography (HPLC) for final quantification by UV at 265 nm. The other two AOAC methods use an external standard, which should be used with caution; see Section 12.5.5.

12.5.3 Sample Preparation and Extraction

12.5.3.1 Natural Food

12.5.3.1.1 Vitamin D

Like in any other analyses of natural components, the extraction process is essential. The extraction should optimally release all active vitamin compounds to be quantified.

If total vitamin activity of a food is required, the sum of vitamin D and any other metabolites that may present vitamin D activity must be measured, for example, vitamin D_3, vitamin D_2, $25OHD_3$, $25OHD_2$, and the dihydroxyvitamin D metabolites.

Only few studies have investigated the amount of all the different vitamin D active compounds using specific chemical methods. This chapter mainly describes the assessment of vitamin D3/D2 and 25-hydroxyvitamin D3/D2 and sum up what is known about 1,25 dihydroxyvitamin D metabolites and the relative activity of the metabolites compared to vitamin D3.

12.5.3.1.2 Vitamin D3/Vitamin D2

Not much is known about the free and bound forms of vitamin D in the food. A study in fish liver oil used molecular distillation to extract the vitamin. The results showed that vitamin D was present as free vitamin D as well as in the form of esters. The free form is distilled at temperatures below 190°, while the esters of vitamin D need a temperature higher than 190°C (Hickman, 1937). Furthermore, the amount and form of vitamin D metabolites in rat tissues were included in the elucidation of the metabolism of vitamin D in the 1960s (Lund et al., 1967; Fraser and Kodicek, 1968a–c; 1969). These studies show that in contrast to vitamin A esters, which are the storage form of vitamin A, vitamin D esters in food are apparently present in a smaller amount. Two hours after administration of vitamin D3 67% of vitamin D in liver was found as esters while only 10% in the kidney (Fraser and Kodicek, 1968b). The vitamin D esters identified were vitamin D palmitate, stearate, oleate, and linoleate (Lund et al., 1967; Fraser and Kodicek, 1968b). However, it is not necessary to assess the different ester forms as their activity is similar to that of the free form (Fraser and Kodicek, 1969).

Extraction with hexane without prior saponification has been reported for cod liver oil and showed a recovery of 85%. This may indicate content of nonquantified vitamin D esters, which however was not part of the discussion in the paper (Pask-Hughes and Calam, 1982).

By alkaline saponification, the vitamin D ester bond is cleaved, vitamin D is released, and the lipids are hydrolyzed into glycerol and fatty acids thereby leaving the free vitamin D in the nonsaponified matter. After saponification, the analyst may observe an oily extract before proceeding with extraction. This is due to the content of diacylglycerols; however, their presence does not cause any interference in the next step (Phillips et al., 2008).

In the analysis of vitamin D, precaution has to be taken to prevent decomposition due to exposure to light and oxidation. The laboratory environment must have the UV absorbance film on the windows or similar precautions must be taken (CEN, 2008). Precaution during the saponification step includes addition of an antioxidant, for example, ascorbic acid, and bubbling through with nitrogen (Jackson et al., 1982). The next step in the analytical procedure is the extraction of the free vitamin D present in the nonsaponifiable matter usually performed by liquid–liquid extraction using a rather nonpolar organic solvent for example, *n*-hexane, *n*-heptane, diethylether, diethylether:petroleumehter (50:50), and ethylacetat:pentane (20:80). See Table 12.4 for appropriate choice. Alternatively, a disposable kieselguhr cartridge may be used, but the choice of cartridge is critical (Heudi et al., 2004; Ostermeyer and Scmidt, 2006).

12.5.3.1.3 25-Hydroxyvitamin D3/25-Hydroxyvitamin D2

The hydroxylated form of vitamin D, 25-hydroxyvitamin D, has a higher polarity than vitamin D. The initial successful attempts to quantify 25-hydroxy vitamin D_3 were performed in beef and egg, but did not include quantification of vitamin D3 (Koshy and Vanderslik, 1977, 1979). Though different in polarity, vitamin D and the 25-hydroxyvitamin D metabolite may be extracted in the same run (Takeuchi et al., 1986; Mattila et al., 1995; Jakobsen et al., 2004).

TABLE 12.4

Extraction and Cleanup Steps for Quantification of Vitamin D Metabolites: Examples

Saponification[a]	Solvent Extraction	Cleanup	Food	Metabolite	Detection Limit, μg/100 g	Reference
10 g (min. 1 μg vitD3), 10 mL ethanol, 2 mL 50% KOH + hydroquinone Boiling water-10 min	Ethylether (1 × 100 mL + 2 × 40 mL)	Methanol (freezer) and preparative HPC (Silica–Partisil)	Fish	VitD3		Egaas and Lambertsen (1979)
50 g, 150 mL ethanol: KOH. Nitrogen, boiling water-30 min	Petroleumether:ether (1 + 1) 500 mL × 2.	Methanol (freezer) and TLC	Egg	VitD3		Jackson et al. (1982)
20–100 g, 50 ml 50% KOH + 100 mL ethanol. Room over night	Petroleumether:ether (1:1) 230 mL × 2	Prep 1: μPorasil Prep 2: 201TP54 Methanol:water (93:7)	Meat, milk	VitD3	0.02–0.05	Mattila et al. (1995)
		Prep 1: μPorasil Prep 2: 201TP54 Methanol:water (83:17)	Meat, milk	25OHD3	0.02–0.05	Mattila et al. (1995)
10–50 g, 30 mL 60% KOH + 90 mL ethanol. Nitrogen 45 min boiling water or room over night	Petroleumether:ether (1:1)150 mL × 1 + 75 mL × 2	SPE Si (2 g) Prep: Silica + amino (gradient *n*-heptane: 2-propanol)	Meat, milk Meat, milk	VitD3/vitD2 25OHD3/25OHD2	0.004–0.02 0.004–0.05	Jakobsen et al. (2004, 2009)
9–30 g, 135 mL 1 M–7.5 g KOH + 15 mL ethanol. 30 min 75°C.	Ethylether 130 mL × 2	Prep: Silica (2-propanol: methyl-*t*-butyl-ether:cyclohexane:*n*-heptane) Silica/gradient eluent[a]	Salmon and fortified milk, cereal, cheese, orange juice	VitD3	0.03 (UV) 0.10 (MS)	Byrdwell (2009)

[a] 0.4–2 g ascorbic acid added as antioxidant.

The extraction procedure exclusively with methanol for kidney, liver, muscle, and spleen has also been reported, but validation of the method did not include quantification of the esters (Höller et al., 2010).

12.5.3.1.4 Dihydroxy Vitamin D Metabolites

As all vertebrates have the same vitamin D metabolism as humans, the active hormone, 1,25-dihydroxyvitamin D, as well as other dihydroxyvitamin D metabolites, for example, 24,25-dihydroxyvitamin D, may be present in meat. Only few studies have included quantification of 1,25-dihydroxyvitamin D in food. The extraction process used has differed from the standard procedure for vitamin D and 25-hydroxyvitamin D as no saponification has been included. For milk, direct extraction with tetrahydrofuran was used, and for beef, homogenization in saline was done followed by extraction of the dihydroxymetabolites with chloroform or dichloromethane combined with methanol after addition of methanol (Kunz et al., 1984; Takeuchi et al., 1988; Montgomery et al., 2000). The possibility that these extraction principles will extract total 1,25-dihydroxyvitamin D is questionable as long as no data are available for the level of protein or other conjugated forms of 1,25-dihydroxyvitamin D in food.

12.5.3.2 Fortified Food

Only vitamin D2 and vitamin D3 are legally allowed for fortification purposes. Usually the amount is many times higher than the natural content, which implies that the analysis of fortified food only includes the parent vitamin D2 and/or D3 and the analytical method applied may be less sensitive regarding the detection and quantification limit. However, the extraction procedure has to be able to extract the vitamin D from the coatings, which easily implies the inclusion of saponification.

12.5.3.3 Supplements

In the analysis of vitamin D in supplements, only parent vitamin D metabolites may be included. Even though similar extraction techniques may be applied as for natural food, extraction with dimethyl sulfoxid has been applied to microencapsulated vitamin D (vanHaelen-Fastré and vanHaelen, 1978; Pastore et al., 1997). As for fortified food, the extraction step must safely extract the vitamin from coating materials, and is therefore usually included a saponification step.

12.5.4 Cleanup

Procedures for removing interfering compounds, especially vitamins A and E, and sterols prior to analytical chromatography are necessary. Sterols were originally precipitated with methanol:water (Egaas and Lambertsen, 1979; Jackson et al., 1982). Further cleanup steps included column chromatography with alumina and magnesia, or/and thin-layer chromatography (TLC) (Jackson et al., 1982; Thompson and Plouffe, 1993). Development in chromatographic systems replaced these tools with preparative adsorption HPLC systems on silica and/or cyano columns if UV/diode array detector (DAD) detection is used (Mattila et al., 1995, 1999; NMKL, 2000; Jakobsen et al., 2004, 2009; CEN, 2008). In addition to the detection system, the cleanup steps depend on the sample matrix. Additional cleanup steps may be needed for fortified products such as margarine (CEN, 2008). In the latest version of the European Standard for vitamin D (CEN, 2008), TLC, solid-phase extraction, as well as column chromatography on a polyethylene-celite column were amended as being optional especially for fortified margarine. However, if liquid chromatography-mass spectrometry (LC-MS) techniques are used, the cleanup steps are limited to solid-phase extraction (Heudi et al., 2004; Byrdwell, 2009).

12.5.5 Quantification

In official methods, an IS technique is used as the quantification principle. In a preliminary ring test of the European project that aimed at certifying reference materials in the beginning of the 1990s, it was reckoned that the huge variability between laboratories was due to the use of external standards (unpublished). The reason for this very essential part of the analytical method is the equilibration between

vitamin D3 and previtamin D3. The rate of isomerization of the conjugated trienes is not influenced by the solvent, pH, or UV-exposure (Verloop et al., 1957; Schlatmann et al., 1964). However, the process is a reversible and a temperature-dependent process, and it is similar for vitamin D3 and vitamin D2 (Buisman et al., 1968; Hanewald et al., 1968). Duration to achieve equilibration at 80°C is 145 min which will generate 78% vitamin D3 and 22% vitamin D3 (Buisman et al., 1968). However, during saponificaction at 80°C for 30 min, vitamin D will be present as 86.4% vitamin D3 and 13.6% previtamin D3. These equilibration rates are in accordance with the recovery at 63% after hot saponification (Mattila et al., 1995) and at 95% for cold saponification (J. Jakobsen, unpublished). Due to the temperature-dependent isomerization, the use of an IS is necessary to secure the highest precision and accuracy. The selection of the IS is an important issue. The IS chosen should preferably closely resemble the structure of the vitamin D analytes. In addition, it should preferably be commercially available. The two forms of vitamin D, vitamin D2 and vitamin D3, are chemically very similar and because it is rare to encounter both forms in a sample, they have been utilized as an IS: vitamin D2 as the IS when determining vitamin D3 and vice versa. This was originally utilized in gas chromatographic methods (Nair et al., 1965; Bell and Christie, 1974). Similar experience, separation of the two vitamers, was obtained by way of HPLC, and thereby continued the use of vitamin D metabolites as an IS for each other (Wiggins, 1976; Egaas and Lambertsen, 1979; Jackson et al., 1982; NMKL, 2000; Staffas and Nyman, 2003; CEN 2008). But alternative standards such as dihydrotachysterol are applicable (Byrdwell et al., 2008), but caution should be taken concerning the equilibrium between vitamin D and previtamin D.

Similar to vitamin D, the use of an IS method for quantification is essential as 25-hydroxyvitamin D metabolites isomerize to pre-25-hydroxyvitamin D like vitamin D in a temperature-dependent manner. Consequently, 25-hydroxyvitamin D2 and 25-hydroxyvitamin D3 have been used as IS identical to vitamin D2 and vitamin D3 (Mattila et al., 1995; Jakobsen et al., 2009).

If samples contain vitamin D2 and vitamin D3, quantification of vitamin D3 using vitamin D2 as an IS requires more analyses and quantification of vitamin D2 and vitamin D3 is less precise. Using MS-techniques makes it possible to quantify vitamin D2 and vitamin D3 as well as 25OHD2 and 25OHD3 in a single run if labeled compounds are used as ISs. This technique is called isotope dilution and it minimizes or even eliminates signal variation during ionization in the mass spectrometry (MS) system (Dimartino, 2007). Labeled, for example, deuterated standards are not commercially available for all compounds, but a compound with a structure similar to the target analyte can be used as a surrogate. However, deuterated standards for both vitamin D2 and vitamin D3 and for 25OHD2 and 25OHD3 and 1,25(OH)2D3 are now commercially available, for example, http://www.isosciences.com, http://www.synthetical.no.

12.5.6 Separation and Detection

12.5.6.1 Gas Chromatography

Gas chromatography (GC) was the first chromatography principle used to replace the biological assay. In contrast to the biological assay, GC could provide discrimination between vitamin D_2 and vitamin D_3 in one run, and the cost, labor, and time required were advantages as well. Due to the poor volatility of vitamin D, the vitamin D metabolites need to be derivatized into, for example, trimethylsilylation (TMS) ethers with NO-bis(trimethylsilyl)acetamide (Bell and Christie, 1974). Vitamin D undergoes thermal cyclization in the flash heater zone (>125°C) resulting in the formation of the corresponding pyro and isopyro compounds. As a result of this transformation, both types of vitamin D give rise to double peaks. An improvement was made using hydrochloric acid and dichlorethane to form isotachysterol derivatives that elute as one peak for isotachysterol2 and isotachysterol3, respectively (Seamark et al., 1980). TMS derivatization also produces analytes that not only possess sufficient volatility for analysis, but also yield a good variety of structurally informative fragments if mass spectrometric detection is used (Yeung and Vouros, 1995). Because the molecular ions of these derivates tend to be very weak in intensity as compared to their fragment ions in the low mass range, selected ion monitoring (SIM) of the fragments is in general used for detection purposes (DeLeenheer and Cryul, 1978; Coldwell et al., 1995; Yeung and Vouros, 1995). Since the SIM sensitivity for vitamin D is

decreased by isomerization and GC-MS methods necessitate a derivatization step, they did not gain use for routine assays.

12.5.6.2 High-Performance Liquid Chromatography

Original HPLC was used in the study of the photoisomers of vitamin D2 or vitamin D3 and vitamin D metabolites using adsorption HPLC (Ikekawa and Koizumi, 1976; Tartivita et al., 1976). Simultaneously, the separation of vitamin D2 and vitamin D3 were performed using partitioning HPLC by use of a 3-m column of silica with an octadecyltrichlorosilane layer, and a mobile phase of methanol:water (78 + 22) (Wiggins, 1976).

The next versions of a methods for determination of vitamin D3 performed in fish products and in eggs, separated vitamin D2 and vitamin D3 on a 25-cm partitioning column—C_{18} combined with mobile phase methanol:water (95 + 5) and C_{22}-column and a mobile phase of methanol:water (90 + 10), respectively. 265 nm and UV-scanning between 210–280 nm, was applied for detection and quantification (Egaas and Lambertsen, 1979; Jackson et al., 1982). This method has continuously been improved with different cleanup depending on the type of food being analyzed (Mattila et al., 1992, 1993, 1995; CEN, 2000; Staffas and Nyman 2003; Jakobsen et al., 2004); see Table 12.5 for selected specific information. Due to interference problems, quantification of the two pairs of vitamin D metabolites, D2/D3 and 25OHD2/25OHD3, has been performed on two separate HPLC systems (Takeuchi et al., 1986; Mattila et al., 1995; Jakobsen et al., 2004). Recent developments in liquid chromatographic equipment, especially column design, have made it possible to run the four vitamin D metabolites for 10 min on a 10 cm reversed-phase column. However, injection of the extracted samples of fortified flour showed severe interference problems when UV-detection is used as a final detection principle. The interference could only be seen by obtaining the purity spectrum by DAD 220–320 nm. See Figure 12.4.

Compared to UV-detection, electrochemical detection is a more selective and sensitive principle that has been used for fish samples. However, extraction and cleanup step was not easier compared to methods using UV-detection (Ostermeyer and Schmidt, 2006).

TABLE 12.5

Operation Parameters for Selected HPLC Systems Used for Separation and Detection of Vitamin D Metabolites

Stationary Phase	Mobile Phase	Detection	Food	Metabolite	Precision	Reference
LiChrosorb $10RP_{18}$	Methanol:water (95:5)	265 nm	Fish	VitD3	2.9%	Egaas and Lambertsen (1979)
Sperisorb S5 ODS2	Methanol:water (95 + 5/90 + 10)	265 (210–280 nm)	Egg	VitD3	13%	Jackson et al. (1982)
Zorbax ODS+Vydac 201TP54	Methanol:water (96:4)	265 nm	Meat, milk	VitD3	9 ± 7%	Mattila et al. (1995)
Sperisorb S5NH2+μPorasil	*n*-Hexane:2-propanol (97:3)		Meat, milk	25OHD3	13 ± 10%	Mattila et al. (1995)
VYDAC201TP54	Methanol:Acetonitril (20:80)	265 (220–320 nm)	Meat, milk	VitD3/vitD2	7–9%14%	Jakobsen et al. (2004, 2009)
C18 (LUNA)	Acetonitril:water (90:10)	265 (220–320 nm)	Meat, milk	25OHD3/D2	9–10%14%	Jakobsen et al. (2004, 2009)
Inertil ODS-2	Methanol:Acetonitrol (20:80)	265 nm (190–400 nm)/ MS (SIM-APCI+)	Salmon and fortified milk, cereal, cheese, orange juice	VitD3	1.8–9.3% 6.1–9.5%	Byrdwell (2009)

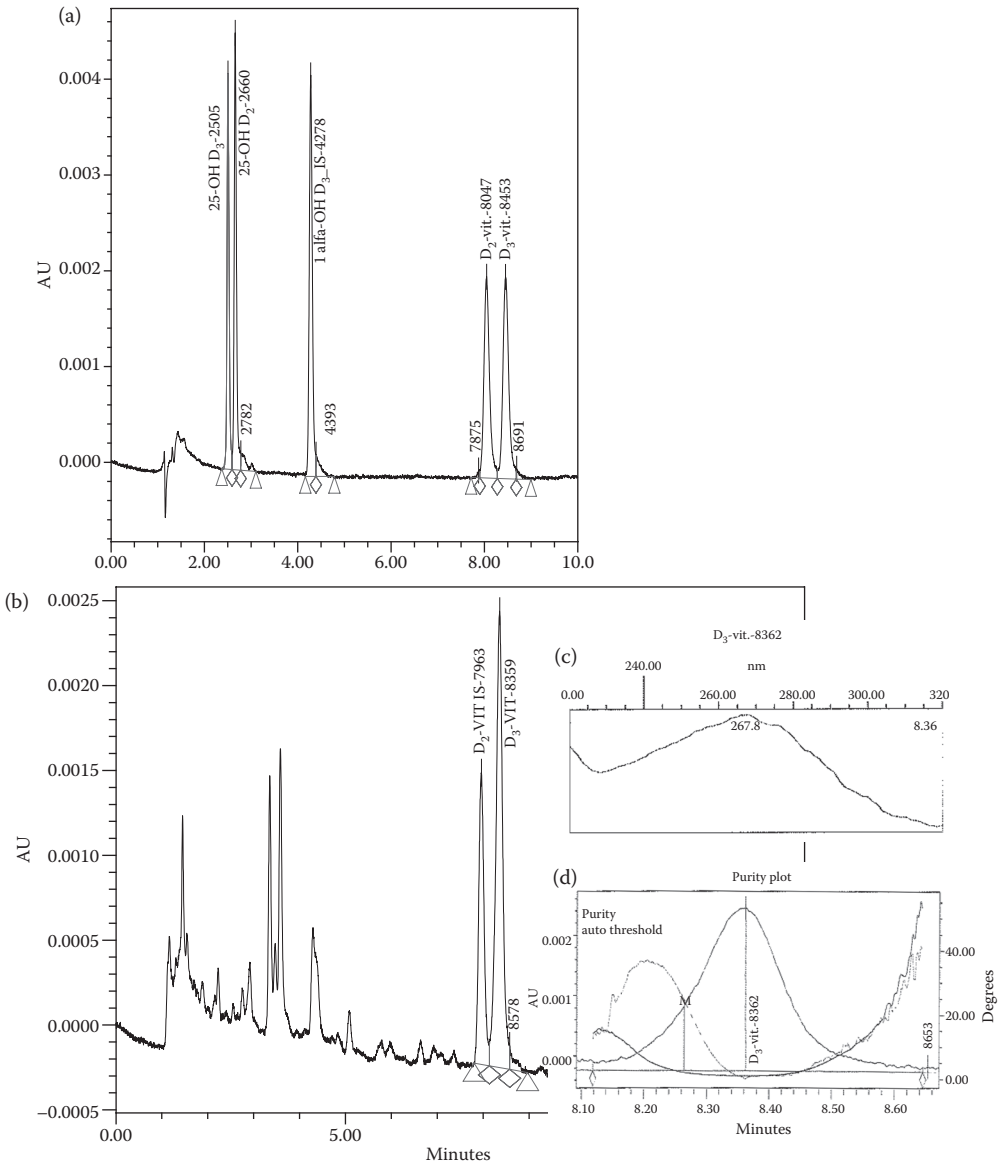

FIGURE 12.4 HPLC system: Column: Acquity UPLC HSS C18 1.8 μm; 2.1 × 150 mm; Mobile phase: Acetonitril:methanol (96:4), flowrate 0.3 mL/min. (a) Chromatogram 265 nm: 0.1 μg/mL vitD2/vitD3/25OHD2/25OHD3 and 1α-hydroxyvitamin. (b) Chromatogram 265 nm: extract of fortified flour, that is, saponified, silica SPE, silica preparative HPLC. (c, d) Spectrum 220–320 nm and purity plot, respectively of extract of fortified flour.

An alternative detection principle is the MS combined with liquid chromatography, and in contrast to GC-MS, the LC-MS do not necessarily include a derivatization step (Gao et al., 2005). Since 2004, various methods have been published which utilize LC-MS. As shown, vitamin D metabolites may be separated by reversed-phase chromatography, and this is also preferably used for LC-MS because it displays higher reproducibility than normal phase and because the polar mobile phase favors ionization.

The ionization efficiency of vitamin D is low in conventional electrospray ionization (ESI) methods because there are few polar functional groups (Dimartino, 2007). Poor sensitivity using ESI MS has in general been observed for compounds that lack an ionic, functional group or which cannot transfer ions

from solution into the gas phase efficiently (Gao et al., 2005). This is because preformation of ions is very important in the ESI detection mode. Atmospheric pressure chemical ionization (APCI) is most widely used for sterol and vitamin D analysis. Under APCI conditions, gas-phase electrons are provided by the corona discharge and solvent molecules form reagent ions first, followed by gas-phase ion–molecule reactions of reagent ions with analytes. The analyte molecule can then be protonated as long as its proton affinity exceeds that of the protonated solvent molecule. Hence, APCI is a much better ionization technique for neutral and apolar substances as vitamin D. Only effective results are obtained in the positive mode. Atmospheric pressure photoionization (APPI) is also suitable for lipophilic compounds (Soldin et al., 2009). In the APPI ion source, UV-light initiates the ionization process, as opposed to corona discharge in APCI. The principle utilized for the ionization is based on the irradiation of the vaporized effluent of the HPLC column inside the ion source with UV-light, which initiates a cascade of gas-phase reactions leading to ionization of the targeted analyte (Kushnir et al., 2010). In the future, APPI may be the method of choice due to the efficacy of nonpolar analytes for this detector. For the determination of vitamin D metabolites in food, LC-APCI-MS has been used to measure vitamin D3 along with other fat-soluble vitamins in infant formula (Heudi et al., 2004). Dimartino et al. (2007) used APCI-LC-MS for the analysis of vitamin D_3 in fortified cheese. In Figure 12.5, an LC-MS spectrum of a fortified cereal sample can be observed. It is obvious that both vitamin D2 and vitamin D3 have high baselines in LC-MS due to its low selectivity.

As a consequence of developing methods for more complex sample matrices as nonfortified food, tandem mass spectrometry (MS/MS) is the preferred choice. MS/MS improves the selectivity and, therefore, diminishes matrix interferences. MS/MS is typically performed on a triple quadropole instrument. The sensitivity of the triple quadropole is low in the full scan mode. The sensitivity is better in the SIM mode, but the highest sensitivity is achieved by using selected reaction monitoring (SRM). The difference

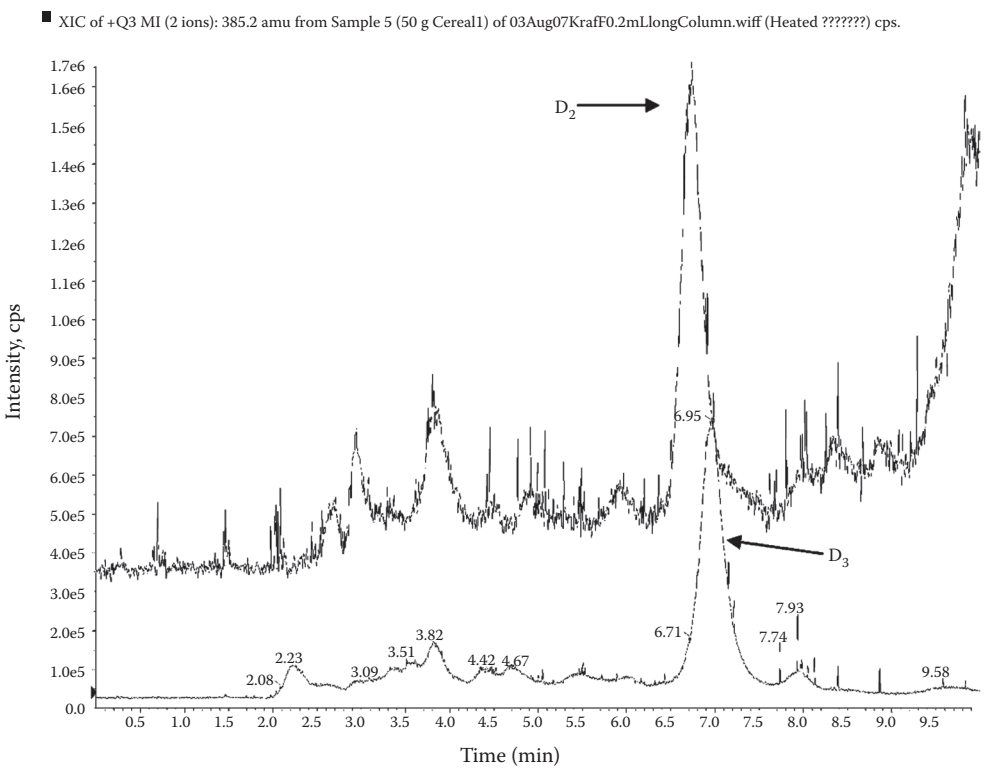

FIGURE 12.5 LC-MS spectra of vitamin D in cereals. (From Huang et al. *J AOAC Int.* 2009;92(5):1327–1335. With permission.)

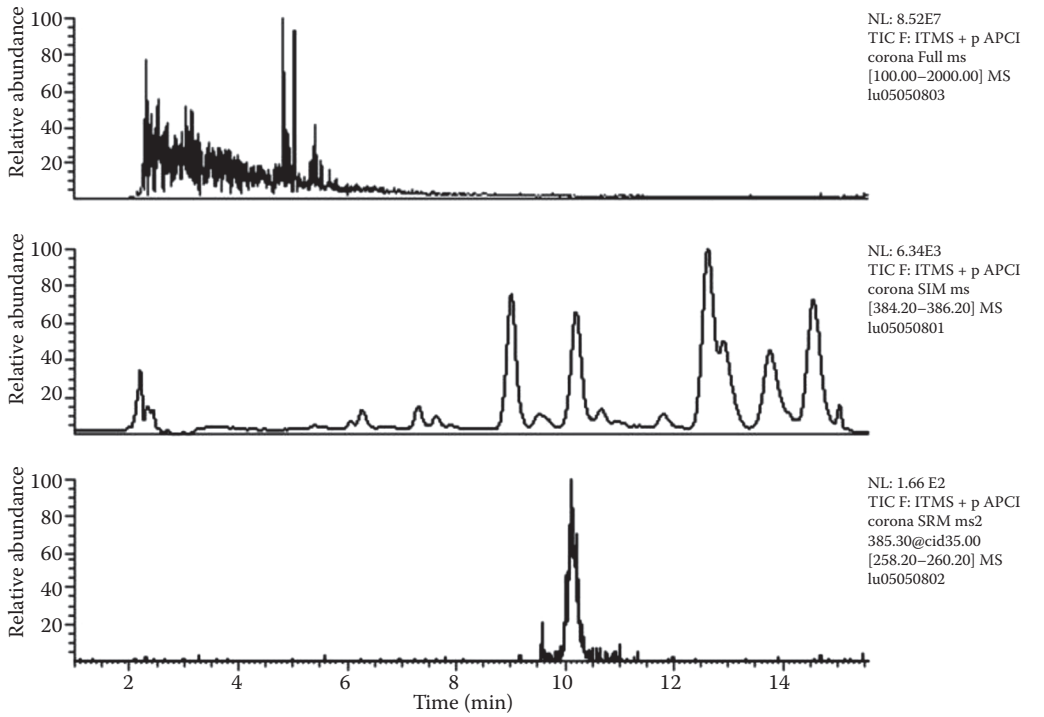

FIGURE 12.6 Total-ion chromatogram of a processed cheese sample analytical test with scanning at the top, selected ion monitoring (SIM) in the middle, and selected reaction monitoring (SRM) at the bottom. (From Dimartino, *J AOAC Int*. 2009; 92(2): 511–517. With permission.)

between these modes was demonstrated by Dimartino (2009) in a processed cheese sample as seen in the Figure 12.6.

It is obvious from Figure 12.7 that the selectivity is improved when using SRM. Consequently, the use of MS/MS is justified by the need to achieve low detection and quantification limits without interferences, for which SRM is really useful (Capote et al., 2007).

The specific method for the dihydroxyvitamin D (24,25-dihydroxyvitamin D3 and 1,25-dihydroxyvitamin D2/D3) has only been introduced in serum. Detection and quantification were performed with electrospray/MS-MS following Diels–Alder derivatization to achieve a sufficiently low detection limit of 25 pg/mL when 500 μL serum was taken for analyses (Aronov et al., 2008). This method also included 25-hydroxyvitamin D2/D3, but has not been applied on food samples.

12.5.6.3 Immunoassays

In food samples, the dihydroxyvitamin D, 1,25-dihydroxyvitamin D and 24,25-dihydroxyvitamin D, has been detected and quantified in milk and beef by radioimmunoassay and competitive binding assay (Takeuchi et al., 1988; Montgomery et al., 2000). These methods are based on detection principles used for quantification of the metabolites in plasma and serum (Eisman et al., 1976; Hollis et al., 1996).

12.5.7 Method Specification

12.5.7.1 Selectivity and Specificity

Chemical analysis of vitamin D and its hydroxylated metabolites is difficult because it coexists naturally with other compounds that have similar physical and chemical properties, hence good selectivity is

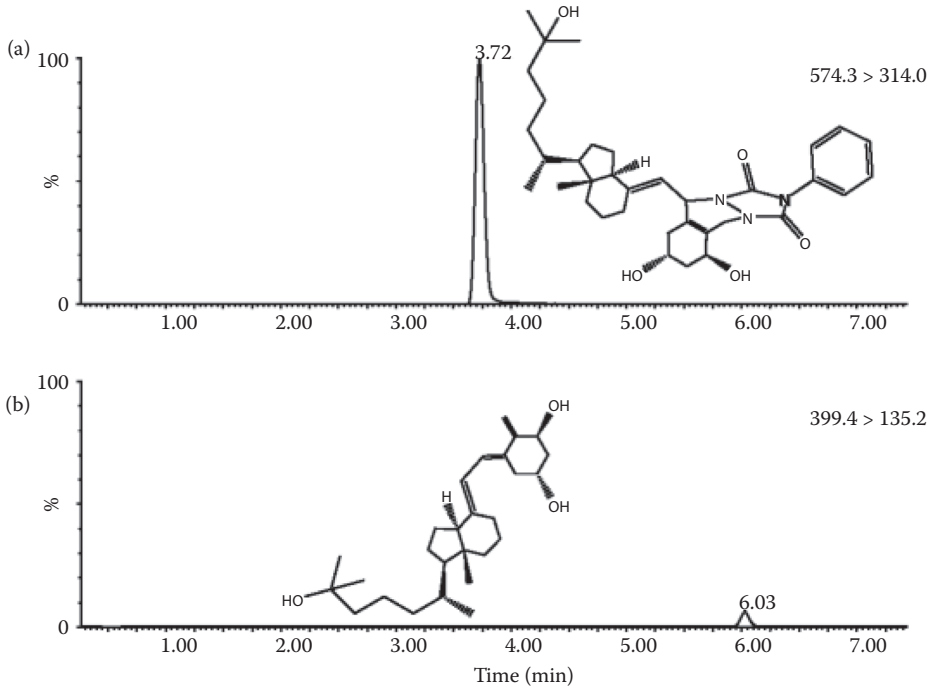

FIGURE 12.7 Improvement in the sensitivity of MS-MS analysis of 1α,25(OH)2D3 by derivatization with PTAD. Analysis of 1α,25(OH)2D3-PTAD, 1 ng injected on column (574.3 > 314.0 reaction trace) is shown in panel (a), and that of native 1α,25(OH)2D3, 10 ng injected on a column (399.4 > 135.2 reaction trace), is shown in panel (b). Both chromatograms were scaled the same way, indicating a 100-fold increase in signal intensity for the derivatized 1α,25(OH)2D3. Standards were injected into 10 μL acetonitrile and separated using gradient elution on a 5 cm UPLC BEH C18 column. (Adapted from Aronov PA et al. *Anal Bioanal Chem.* 2008;391(5):1917–1930.)

mandatory. MS is a selective technique and limitations caused by incomplete resolution of the different molecular species in a complex mixture can be overcome. Currently, most vitamin D analysis is based on HPLC with UV-detection. These methods rely on multiple chromatographic purification steps. These methods are generally both sensitive and highly repeatable. But it takes a long time to perform such an analysis and therefore the cost of analysis is high, especially for complex matrices (Dimartino, 2007). Byrdwell (2009) showed the necessity to include DAD or MS, as the UV-chromatogram showed a clean peak, with no indication of overlapping peaks. However, the MS data on the same sample indicated that there were interfering species present that produced ions homologous with vitamin D. MS/MS can eliminate interfering peaks with different precursor to product ion fragmentations. But even though SRM are used, you need to monitor multiple SRM pairs for a single analyte and combine these data with other evidences such as relative intensities of product ions in the mass spectra, retention time, and peak shape to positively identify the compound as vitamin D.

12.5.7.2 Limit of Detection

As described in Section 12.4, very few foods naturally contain a substantial amount of vitamin D; natural sources include fish, cod liver oil, and certain mushrooms. Other kinds of foods such as meat and milk products contain very small amounts of vitamin D and its hydroxylated metabolites. Therefore, there is a need for a low limit of detection. The limit of detection depends on the sample amount taken for analyses and detection principles. The lowest detection limit has been obtained in milk. By using freeze-dried samples, 0.004 μg/100 g was detectable with UV/DAD (Jakobsen and Saxholt, 2009). One of the problems when using MS detection is the relatively low sensitivity. Derivatization may be utilized to

enhance the detection response of vitamin D by ESI-MS or APCI-MS (Higashi and Shimada, 2004; Gao et al., 2005; Aronov et al., 2008). In LC-MS experiments, derivatization has an advantage in that the molecular weight of the analyte is shifted to a higher mass range, where the background noise is relatively low. In addition, by introducing polar groups, the derivatization procedure would typically result in a 100–1000-fold increase in sensitivity over nonderivatized compounds (Aronov et al., 2008); see Figure 12.7. Several reagents have been used to enhance the detection of vitamin D by LC-MS; however, several of them have a practical problem; they are not commercially available. The conjugated diene group of vitamin D metabolites makes them a specific target for Diels–Alder derivatization. A cookson-type reagent (4-substituted 1,2,4-triazoline-3,5-dione) rapidly and quantitatively reacts with s-*cis*-diene in the vitamin D molecule by Diels–Alder cycloaddition in a structurally selective fashion, allowing their facile identification and isolation from interferences in the analysis (Weiskopf et al., 2001). This has been examined in order to enhance the sensitivity of vitamin D compounds in various modes of ionization (Higashi et al. 2002). Derivatization with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) has been used for characterization of vitamin D analogs and metabolites in positive ESI-MS-MS (Weiskopf et al., 2001; Higashi et al., 2002; Yeung and Vouros, 2005). PTAD can be purchased from several reagent-manufacturing companies (Aronov et al., 2008), which is of great significance when developing a method for routine purposes.

Though derivatization may seem attractive, it is time consuming and furthermore derivatization may lead to reduction in recovery and formation of artifacts. For food matrixes, derivatization has only been used for milk samples, which provided a detection of 1 pg in an injection (Kamao et al., 2007). Compared to derivatization, adjustments of the mobile phase are in many cases easier. Solvent composition has proved to be a vital factor for achievement of high sensitivity (Gao et al., 2005).

12.5.7.3 Precision

Precision depends on the sample type. Relative standard deviation within lab (repeatability) is 2.2–7.6%, while between labs (reproducibility) show a relative standard deviation of 6.8–24.1% in margarine, cooking oil and fish oil, and milk (liquid/powder/infant formular) (CEN, 2008). Although MS methods are very selective, in general they do not provide an improved precision. Byrdwell (2009) observed higher relative standard deviations for MS detection than for UV-detection for skim milk, salmon, spiked peanut butter, and cheese that is, 6.9–9.5% and 1.8–8.7%, respectively. While for cereals, the variation was 6.1% for MS and 9.3% for UV-detection. This imprecision in the MS detection is a result of instabilities in the ionization process and can be corrected by the use of isotope dilution. This technique showed a precision at 6.0–11.2% for quantification of 25-hydroxy vitamin D_3 in swine tissue (Höller et al., 2010).

12.5.7.4 Accuracy

The accuracy of each of the vitamin D metabolites quantified has to be assessed. However, in food, the only vitamin D compound for which comparison between laboratories have been tested for is vitamin D3. Certified reference materials may be obtained from the National Institute of Standards and Technology in the United States (www.nist.gov) and the Institute of Reference Materials and Measurements in Belgium (www.irmm.jrc.be).

Even though the first results for 25-hydroxyvitamin D in food were published in 1980, no certified reference materials have been implemented.

12.5.8 Quality Control

The performance of an analytical method should partly include analysis of a certified reference material and preferably be compared with other similar methods in proficiency testing systems such as those offered by the National Institute of Standards and Technology in the United States (www.nist.gov), the National Food Administration in Sweden (www.slv.se), and FAPAS in the UK (www.fapas.com).

12.6 Vitamin D in Food: Transformation of Specific Data to Vitamin D Activity

The essential factor for estimating the dietary intake of vitamin D is to have valid data for the vitamin D content of the foods tested. The determination of each specific vitamin D active compound in food is a challenge. However, the next challenge for the analyst and biochemist is to assess the total vitamin D activity in food. In 2010, the consensus of the similar activity of vitamin D2 and vitamin D3 is being debated (Trang et al., 1998; Armas et al., 2004; Holick et al., 2008; Houghton and Vieth, 2006).

The potency of 25OHD3 has often been attributed to possess five times the potency of vitamin D3. The literature and recent research in this area argue that this may not be the case (Ovesen et al. 2003; Jakobsen, 2007; Jakobsen et al., 2007; Jetter et al., 2009).

Furthermore, specific quantification of the vitamin D metabolites by analytical methods that are less time consuming will be a challenge in the future, and so will the transformation of these data to total vitamin D activity derived from food.

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Section III

Terpenes

13

Hydrocarbon Carotenoids

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The earliest studies on carotenes date back to the beginning of the nineteenth century. In 1831, the German Wackenroder, an analytical chemist, isolated the pigment yellow/orange from carrots and named it "carotin." After almost one century, in 1907, Willstatter and Mieg established the empirical formula of carotene as composed by 40 carbon atoms and 56 hydrogen atoms. Twelve years later, a relationship between the yellow pigments found in plants and vitamin A was suggested by Steenbock. The structure of the molecule of β-carotene was first established in 1931 by the Suisse organic chemist Paul Karrer, who received the Nobel Prize for Chemistry in 1937 for work concerning β-carotene and vitamin A.

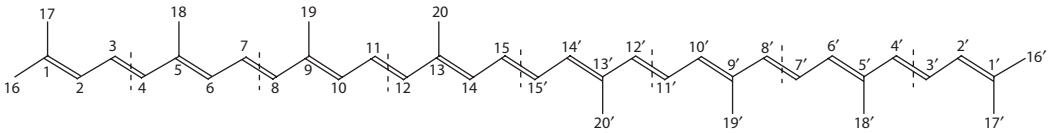

FIGURE 13.1 General formula of carotenes with the numeration of the carbon skeletal; the pointed lines pointed out the formal division in isoprenoid units.

13.1 Classification and Structure

Carotenes are a group of hydrocarbons which in combination with their oxygenated derivatives (xanthophylls) constitute the carotenoid class. They consist of eight isoprenoid units arranged in a manner such that the arrangement of isoprenoid units is reversed at the center of the molecule so that the two central methyl groups are in a 1,6-positional relationship and the remaining nonterminal methyl groups are in a 1,5-positional relationship (IUPAC-IUB, 1974). All carotenes or hydrocarbon carotenoids may be formally derived from the acyclic structure $C_{40}H_{56}$ (Figure 13.1), having a long central chain of conjugated double bonds, by hydrogenation, dehydrogenation, or cyclization, or any combination of these processes.

The modification of this skeleton by cyclization could be done in one or two terminals of the molecule, and it would be possible to obtain seven different terminal groups denominated by Greek letters (Figure 13.2), which named the prefix of a hydrocarbon carotenoid.

Each carotene could be named using a semisystematic form, defining it and describing their structure. Basically, all the specific names of the hydrocarbon carotenoids are based on the root name "carotene," preceded by prefixes, Greek letters, which designate the two terminal groups. Changes in hydrogenation/

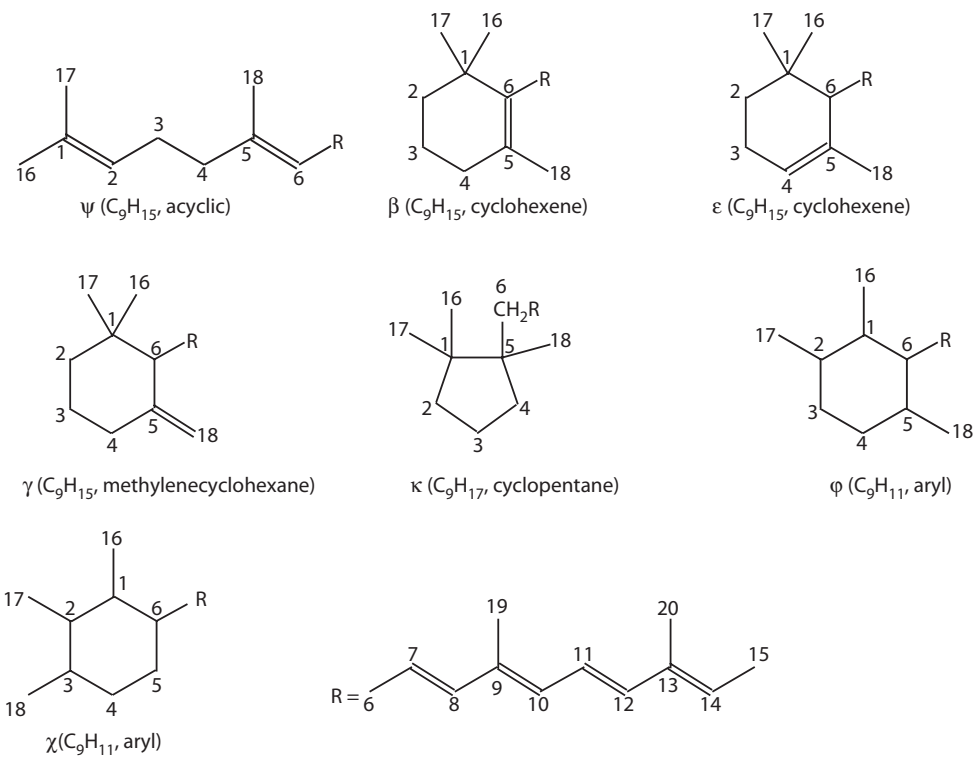

FIGURE 13.2 Terminal groups of hydrocarbon carotenoids and respective prefixes.

dehydrogenation are indicated by the prefixes and suffixes utilized in organic chemistry. Nevertheless, traditionally, carotenes have trivial names derived from the biological source from which they were isolated. For simplicity and because they are named in scientific literature by their trivial names, this nomenclature will be used.

13.2 Photochemical Properties

One of the main characteristics of carotene structure is the extensive conjugated double-bond system of the molecule central part, where π-electrons are highly delocalized. This characteristic confers to this group of compounds, their molecular shape, chemical reactivity, and distinctive properties of light absorption. As a consequence of the absorption of light energy by carotene molecule, a higher-energy excited state is produced, the relevant transition being between π-orbitals, $\pi \rightarrow \pi^*$. One of the bonding π-electrons of the conjugated double-bond system is promoted to a formerly unoccupied π* antibonding orbital. In a double bond, a $\pi \rightarrow \pi^*$ transition is obtained at the expense of the absorption in the far ultraviolet (UV), but when conjugated double bonds are present, the high delocalization of π-electrons in the chromofore lowers the energy of the excited state of the molecule, consequently lowering the energy necessary for this transition. So, absorption is displaced to high wavelengths, and if the number of conjugated double bonds is enough, absorption is in the visible region, in the range of 400–500 nm, and the compound will have color. The pigment, β-carotene, with 11 conjugated double bonds shows yellow color because it absorbs at the violet extremity of the visible spectrum. Hydrocarbon carotenoids with at least seven conjugated double bonds have a perceptible color, the great majority being intensely colored with colors from yellow to red.

13.3 Stereochemistry

Geometric isomerism around the double bonds C=C originates different configurations, diastereoisomers in the case of carotenes. In principle, each double bond in the poliene chain can be in two configurations, *E* or *Z*, from the German "entegegen" and "zusammen," respectively, depending on the arrangement of the substituent groups of that double bond. The precedence order of the atoms or groups of atoms bonded to the two carbons of the double bond is attributed using the Cahn–Ingold–Prelog rule. Considering the great number of double bonds in the carotene molecules, in theory, the formation of an enormous number of mono-Z- and poli-Z-isomers is possible. Nevertheless, the steric hindrance gives rise to an all-*E* form thermodynamically more stable than the *Z*-isomers. In hydrocarbon carotenoids with cyclic terminal groups, 9*Z*-, 13*Z*-, and 15*Z*-isomers are relatively stable but 7*Z*- and 11*Z*-isomers are energetically hard to form. In nature, most carotenes occur predominantly or entirely in the all-*E* form.

The terms all-*E*- and *Z*- correspond, respectively, to the terms all-*trans*- and *cis*-, commonly referred in carotenoid literature. *Trans*- and *cis*- are not the appropriate nomenclature for in-chain geometrical isomers of carotenes, according to the International Union of Pure and Applied Chemistry (IUPAC).

13.4 Solubility and Absorptivity

Carotenes are lipophilic compounds insoluble in water and readily soluble in solvents such as acetone, ethyl ether, chloroform, ethyl acetate, petroleum ether, hexane, toluene, tetrahydrofuran (THF), and dichloromethane. As far as chloroform is concerned, it could contain traces of hydrochloric acid difficult to remove and it is generally stabilized with 1% ethanol, which can affect its properties as a solvent. Chloroform can be replaced by dichloromethane. Crystalline carotenes may be difficult to dissolve in some organic solvents but do dissolve easily in dichloromethane (Schiedt and Liaaen-Jensen, 1995). The relative solubility and absorptivity of β-carotene, in common organic solvents, are presented in Table 13.1. The absorptivity in hexane for α-carotene and lycopene are, respectively, 2800 (444 nm) and 3450 (472 nm) (Hart and Scott, 1995).

TABLE 13.1

Relative Solubility and Absorptivity of β-Carotene in Organic Solvents

Solvent	Solubility (mg/L)	λ_{max} (nm)	Absorptivity $E^{\%}_{1cm}$
Acetone	200	452	2559
Acetonitrile	10	452	2540
Chloroform	2000	462	2330
Cyclohexane	2000	454	2508
Cyclohexanone	2000	462	2359
Dichloromethane	6000	460	2369
Dimethylformamide	200	460	2389
Dimethylsulfoxide	30	466	2259
Ethanol	30	450	2529
Ethyl acetate	500	452	2520
Ethyl ether	1000	448	2659
Hexane	600	448	2592
2-Propanol	40	450	2508
Methanol	10	450	2540
Methyl *tert*-butyl ether	1000	450	2588
Tetrahydrofuran	10,000	456	2399
Toluene	4000	462	2270

Source: Adapted from Craft, N.E., Wise A.S., and Soares Jr, J.H. 1992. *Journal of Agricultural and Food Chemistry* 41: 208–213.

13.5 Hydrocarbon Carotenoids in Functional Food

The primary role of the diet is to provide sufficient nutrients to meet the nutritional requirements of an individual. There is much greater recognition today that people can help themselves and their families to reduce the risk of disease and to maintain their state of health and well-being through a healthy lifestyle, including the diet. Increasing scientific evidence supports the hypothesis that some foods and food components have beneficial physiological and psychological effects over and above the provision of the basic nutrients. According to the International Food Information Council Foundation (IFIC, Foundation, 2011), functional foods are "foods or dietary components that may provide a health benefit beyond basic nutrition."

Nowadays, one of the main recommendations of dietary guidelines is to increase the consumption of plant-based foods, namely fruits and vegetables that are good sources of carotenoids and other biologically active phytochemicals. Several *in vitro*, *ex viv*o, and *in vivo* studies in animals demonstrated numerous carotene biological actions such as provitamin A activity, antioxidant function, participation in the metabolism of the xenobiotics, and improvement of the immune response and cell–cell communication (Rao and Rao, 2007). Based on these biological actions and on extensive epidemiological observation, fruits and vegetables that are rich in carotenoids are considered to improve the state of health and well-being, or even reduce disease, by prevention of chronic degenerative diseases particularly some cancers, cardiovascular disease, skin diseases, age-related macular degeneration, and cataracts. In this context, fruits, vegetables, whole grains, and fortified or enhanced foods and beverages containing carotenoids, and, particularly, hydrocarbon carotenoids can act as functional foods.

In spite of the theoretical possibility of many combinations of the terminal groups, 11 hydrocarbon carotenoids are reported in foodstuffs, two bicyclic, α- and β-carotene, four monocyclic, δ- and γ-carotene, and α- and β-zeacarotene, and five acyclic, ζ-carotene, lycopene, neurosporene, phytoene, and phytofluene. Chemical structures and trivial and semisystematic names of these carotenes are presented in Figure 13.3.

Although 11 hydrocarbon carotenoids have been isolated from dietary fruits and vegetables and characterized, only a selected group of carotenes, α-carotene, β-carotene, and lycopene are largely predominant and routinely found in food. β-carotene is the most widespread of all carotenoids in human food,

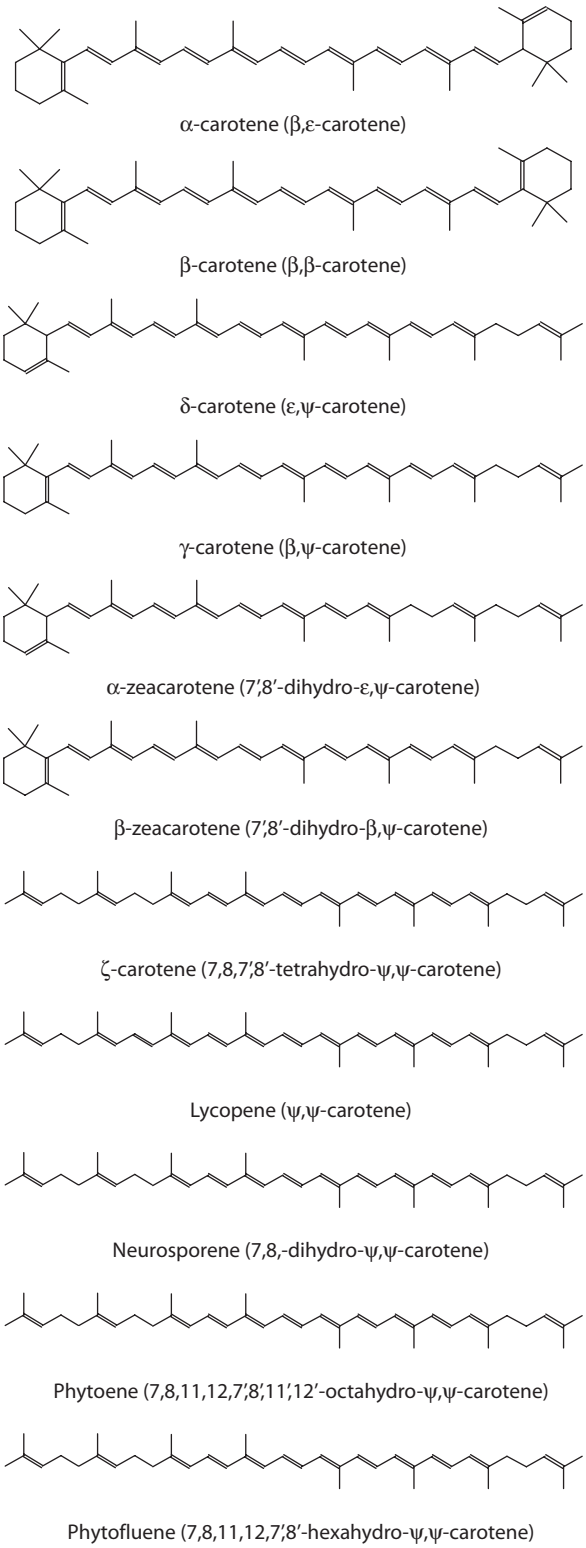

FIGURE 13.3 Chemical structures and trivial and semisystematic names of carotenes.

sometimes as the major carotenoid. As far as the bicyclic compounds are concerned, α-carotene often accompanies β-carotene, generally at much lower concentrations, with some exceptions as in certain varieties of pumpkin and carrot, where it was found in substantial amounts. γ-carotene occasionally accompanies β-carotene, generally at much lower concentrations but the other monocyclic carotenes are practically never reported. Lycopene is the most abundant of the acyclic carotenes, being the main component of red-fleshed fruits such as tomato and watermelon, usually in considerable quantities. ζ-carotene sometimes accompanies lycopene in minor levels but it is more ubiquitous. Neurosporene is rarely reported and always in very low levels. Phytoene and phytofluene are colorless carotenes and their presence may probably be more widely distributed than reported.

Foods of plant origin, namely fruits and vegetables, are the main source of hydrocarbon carotenoids, and because plants are able to synthesize carotenes *de novo*, the composition of foods of plant origin is variable and complex. Incapable of biosynthesizing hydrocarbon carotenoids, animals depend on dietary carotenes, which are selectively or unselectively absorbed, converted to vitamin A, deposited as such, or slightly altered to form hydrocarbon carotenoid typical of animal species. The main sources of carotenes from animal origin, derived from their diet, are dairy products and egg yolk.

13.6 Provitamin A Hydrocarbon Carotenoids

Provitamins are vitamin precursors, which are not themselves vitamins, but which can be converted by the human metabolism into vitamins. All sources of vitamin A are derived ultimately from provitamin A carotenoids. Humans obtain preformed vitamin A exclusively from animal sources and provitamin A is obtained from plant and certain animal sources. Hsieh and Karel (1983) estimated that approximately half of the human dietary vitamin A intake is derived from red, yellow, and green fruits and vegetables containing the hydrocarbon carotenoids, α-, β-, and γ-carotene.

The unique specific nutrient function that could be attributed to carotenoids is a source of vitamin A (Cooper, 2004). Humans need vitamin A for their normal growth and development and in many underdeveloped countries throughout the world, vitamin A deficiency is a major nutritional problem. According to the World Health Organization 250,000–500,000 children become blind every year due to vitamin A deficiency (WHO, 2009). The primary sources of vitamin A in these populations are provitamin A carotenoids from fruits and vegetables, and nowadays intense research is done and steps are taken to supply to these populations golden rice, a genetically modified rice that produces β-carotene in the grain's endosperm. All carotenoids with a nonsubstituted β-ring and a poliene chain with 11 carbon atoms, which include the hydrocarbon carotenoids, β-, α-, and γ-carotene, exhibit vitamin A activity due to their possible conversion to retinol (vitamin A), one part being converted to vitamin A and the other part absorbed intact. As retinol is approximately half of a β-carotene molecule, this carotene has the highest provitamin A activity. However, this bioconversion is highly variable, depending on the reaction yield, the amount of carotenoid ingested, source of carotenoid, other dietary ingredients, current nutritional status, individual, and so on.

In 1967, an FAO/WHO group recommended "that in the absence of more specific data for foods, the availability of β-carotene be taken as one-third (i.e., approximately 33% of the β-carotene from the diet was available for absorption) and that the utilization efficiency in human beings be taken as one-sixth (assuming 50% conversion to vitamin A)" (FAO/WHO, 1967, p. 30). Summarizing, 1 mg of β-carotene in the diet is taken to have the same biological activity as 0.167 mg of retinol.

In 1974, the NAS/NRC *Recommended dietary allowances* proposed "that in addition to any expression as international unit activity, vitamin A should also be given in terms of retinol equivalents defined as follows: 1 retinol equivalent (RE) = 1 μg retinol = 6 μg β-carotene = 12 μg other carotenoid vitamin A precursors" (NAS/NRC, 1974, p.80), equivalences that are generally still in use.

Different approaches about potential absorption of carotenoids from various foods were adopted but no consensus was established. Large variations in the potential and actual absorption of β-carotene highlight the fact that a single conversion factor is inappropriate. Scott and Rodriguez-Amaya (2000) suggested that the use of current data on retinol equivalents should be abandoned, and tables of food composition should quote only retinol and the individual carotenoids as amount per 100 g edible portion.

Most of the published reports on the qualitative and quantitative distributions of carotenes in food have primarily focused on provitamin A carotenes because of the perception that provitamin A activity may have been a contributing factor in the observed health benefit of carotenoids in epidemiological studies. With the recognition of other health-promoting effects, such as the prevention of cancer, heart disease, and age-related macular degeneration, independent of the provitamin A activity, the nonvitamin A active carotenes have also been realized.

13.7 Hydrocarbon Carotenoids Isomers

Lower vitamin A activity has long been attributed to *Z*-isomers from provitamins A than to the corresponding (all-*E*)-isomers (Zechmeister, 1962). More recently, it was verified that (all-*E*)-β-carotene is preferentially absorbed than (9*Z*)-β-carotene by humans (Ben-Amotz and Levy, 1996; Graziano et al., 1995; Stahl et al., 1993). As far as nonprovitamin A carotenes are concerned, Unlu et al. in 2007 suggested that the *Z*-lycopene isomers could be better absorbed than all-*E*-isomers. Regarding the health attributes of carotenoids related to its antioxidant function, lycopene has been demonstrated to be the most potent *in vitro* antioxidant. In a recent review (Erdman et al., 2009) of human and animal trials with lycopene, they concluded that there is limited support for the *in vivo* antioxidant function as licopene's major mechanism of action. Investigation of *in vitro* antioxidant of prominent carotenoid geometrical isomers (Böhm et al., 2002) conclude that three unidentified (*Z*)-isomers of lycopene showed the highest antioxidant activity, being significantly higher than the result for (all-*E*)-lycopene, which had approximately two times the activity of (all-*E*)-β-carotene. (13′*Z*)-α-carotene also showed a significantly higher antioxidant activity than their corresponding (all-*E*)-isomer. On the other hand, (9*Z*)-zeaxanthin had less than half the antioxidant activity compared with that of (all-*E*)-zeaxanthin, and the four isomers, (all-*E*)-, (9*Z*)-, (13*Z*)-, and (15*Z*)-β-carotene, demonstrated comparable antioxidant activity.

This field needs more investigation, but as geometric configuration apparently affects their biological relevance, the separation, identification, and quantification of stereoisomers should be considered in the analytical method selection for carotenes determination in food analysis.

13.8 Hydrocarbon Carotenoids Profile in Functional Foods

The green fruits and vegetables usually consist of the same carotenes, mainly β-carotene accompanied by α-carotene in very lower quantities. However, in some green vegetables, such as green beans and green peas, the α-carotene content is much higher than the content of this compound in other greens. Since there is a correlation between the green color, the chlorophyll concentration and the carotenoid concentration, the darker green the fruits and vegetables are, the higher is the carotene content (Khachik, 2006).

The yellow/red fruits and vegetables are very rich in β-carotene, α-carotene, and lycopene. The more common yellow fruits are carrot, pumpkin, and sweet potato, very rich in β-carotene and α-carotene. The red fruits such as tomatoes and watermelons are the main sources of lycopene in the occidental diet and have minor quantities of γ-carotene and β-carotene (Khachick, 2006). Consumer demand for healthy food products provides an opportunity to develop lycopene-rich foods as new functional foods.

The yellow/orange fruits and vegetables (e.g., orange, peach) have their own unique and complex carotenoid profile. As far as carotenes are concerned, β-carotene and α-carotene are the predominant.

Even though cereal grains contain far fewer carotenoids than most vegetables and fruits (Table 13.2), they are consumed frequently in considerable amounts. Besides their nutritional and health benefits, they are responsible for the attractive bright yellow color of durum wheat. This color is an important factor in the use of cereal grains in food manufacture, especially for durum wheat and the quality of pasta. As far as carotenes are concerned, according to more recent publications wheat (whole grain and semolina) do not contain carotenes (Burkhardt and Böhm, 2007) or the presence of α- and β-carotene reached maximally 4.5% of the total carotenoids (Konopka et al., 2006; Abdel-Aal et al., 2007) as well as in sorghum (Kean et al., 2007). Corn (whole grain, semolina, and flour) usually contains α- and β-carotenes in higher quantities than wheat, but in much minor quantities than xanthophylls. As far as other cereals

TABLE 13.2

Approximate Carotene Content of Foods (Natural Occurrence) Based on Analyses of Different Laboratories (mg/100 g Wet Weight)

	Carotene Content (mg/100 g)		
Food	**All-*E*-α-Carotene**	**All-*E*-β-Carotene**	**All-*E*-Lycopene**
Tomato		0.3–3	1–9
Pumpkin	0.01–8	1–8	
Milk		0.007–0.02	
Carrot	2–5	4–11	
Corn	0.02–0.2	0.07–0.7	
Olive oil		0.2–0.2	
Cabbage		0.05–8	
Purslane	0.009–0.01	2–4	

are concerned, oat, spelt, dicoccon, and barley, α + β-carotene account from 2.5% to 4.1% (barley) of the total carotenoid content (Panfili et al., 2004).

In milk, carotenoids are stored in fat globules (Jensen, 1995) and hence are present in a highly bioavailable form (Castenmiller and West, 1998). β-Carotene is the main carotenoid present in dairy, comprising approximately 90% of total carotenoid content. Two approaches to analyze β-carotene in milk and milk products were done in The Netherlands (Hulshof et al., 2006) and Finland (Ollilainen et al., 1989). According to the first approach, retention in dairy after processing the corresponding raw milk varies between 30% and 50% for different ripened cheeses and above 90% for liquid and semiliquid dairy products such as buttermilk, vanilla custard, and yogurt.

Among the functional foods containing β-carotene, vitaminized drinks, so-called ATBC drinks, have experienced growing popularity. For the formulation of ATBC drinks, gelatine formulations of β-carotene are usually employed to enhance water solubility. The provitamin A moiety of ATBC drinks may also originate from carrot juice as a natural source of β-carotene.

13.9 Determination of Hydrocarbon Carotenoids in Foods

The possible relationship between the consumption of fruits and vegetables containing carotenoids and the human health (Cooper, 2004), the fact that carotenoids vary qualitative and quantitatively from food to food and that, within each species, their content generally varies largely with variety, maturity, soil, and light intensity, determines the need for their analysis. Also, the constantly changing and expanding of markets, altering of consumer tastes, improvement of analytical instrumentation and methodology, and new knowledge on the biological functions of food components make the task of food composition data generation a continuous endeavor.

The selection of the method for carotene determination in foods depends on the kind of information intended. Nevertheless, the physicochemical properties of these molecules, the long chain of unsaturated double bonds conferring instability and facilitating their reaction, namely with acids, oxygen, and active radicals, these reactions being potentiated by light, should always be taken into account. To avoid artifacts formation and quantitative losses, the analysis should be completed within the shortest possible time, at relatively low temperatures, in exclusion of oxygen, using protecting atmosphere, under protection from light, excluding the light wavelengths absorbed by carotenes, avoiding contact with acids, using high-purity solvents free from harmful impurities, as chlorinated compounds and peroxides, and using material that does not adsorb carotenes (e.g., glass) (Britton, 1991; Davies, 1976; Schiedt and Liaaen-Jensen, 1995).

High-performance liquid chromatography (HPLC), after a careful extraction of samples, has become the method of choice for the determination of hydrocarbon carotenoids. Their separation and quantification is carried out by a combination of HPLC and UV–vis photodiode array detection sometimes associated with mass spectrometry (MS) or nuclear magnetic resonance spectroscopy, and com-

parison of the HPLC–UV–vis profiles obtained in a chromatogram form of unknown samples with those of pure substances.

Other techniques such as gas chromatography (GC), mass spectroscopy coupled with GC or HPLC, immunoassays, supercritical fluid chromatography, and capillary electrophoresis have proven to be useful in specific applications.

13.9.1 Chromatographic Methods

13.9.1.1 Food Identification

Data generation on food components is complex and expensive. Since carotene variability is high, namely with species, variety, part of the item analyzed, maturation state, processing form, year, and place of production (Dias et al., 2009), to allow an effective management, updating and comparability of the produced data, a complete description of the food items analyzed is fundamental. With regard to biological classification, at least species and variety identification is needed, even if it presents difficulties, especially for some of the native or wild plants; variety is a taxonomic rank below subspecies, which is usually the result of selective breeding and diverges from the parent in relatively minor ways (IFIS, 2005).

13.9.1.2 Sampling

Natural variations in carotene composition of foods as well as in analytical methods determine the need of a sampling plane definition, based on statistical methods to obtain representative and homogeneous samples. With regard to heterogeneous materials, the only procedure which can be relied upon to give a truly representative sample is to grind the entire lot to such a degree of fineness that, after through mixing, the homogeneity is approached. Although such a treatment is usually impractical, it must be recognized that any other procedure represents a compromise between practicality and accuracy. In each case, the technique employed must be based on consideration of the accuracy acceptable or attainable, the degree of homogeneity in the lot, the amount of time, labor, and economic resources available, and the purpose of the analysis. Depending on the food under investigation, and on the weight of each unit, 200 g to 5 kg per lot are usually carried to the laboratory taking into account proportional representativity of all parts that are intended to be analyzed in the homogenized sample.

Laboratory work should be planned to analyze samples soon after collection because it is difficult to avoid changes in carotene composition during sample storage, even at low temperature. Depending on matrices, freezing under an inert gas such as nitrogen could be an appropriate mean to preserve samples. In relation to lyophilization, some studies evidenced carotene degradation during this process (Craft, 1993; Park, 1987; Ramos and Rodriguez-Amaya, 1993) possibly due to the increase in sample porosity enlarging the contact surface with oxygen during storage. Moreover, because carotene content is expressed per unit weight of sample "as consumed," it is necessary to determine moisture, a proximate analysis, introducing an increase of uncertainty in carotene measurement results.

13.9.1.3 Extraction

Applicability of an analytical method depends on the matrix, the analytes present, and their levels. For extracting hydrocarbon carotenoids, there is no universally accepted method, mainly due to the great number of combinations carotene/matrix that could be the object of study. In practice, several organic solvents or solvent mixtures are in use. The system of conjugated double bonds of the carotenes is the main source of problems associated with carotene manipulation, conferring to these molecules instability, especially related to light, oxygen, and heat. Antioxidant's use is one of the common strategies, butylhydroxytoluene (BHT) being the most employed in a concentration between 0.01% and 0.1% (m/v); complementary, manipulations should be done under the cover of light, evaporations should be made under inert atmosphere at temperatures below 40°C, and prepared samples should be stored in the dark, under nitrogen or other inert gas, at temperatures between −20°C and −80°C.

Common extraction solvents are mixtures including methanol associated with other more apolar solvents such as THF, diethylic ether, chloroform, hexane, petroleum ether, and sometimes ternaries

TABLE 13.3

HPLC Methods for Carotene Analysis in Food

Column Stationary Phase	Mobile Phase (Eluent)[a]	Extraction Solvent[a]	Samples/Hydrocarbon Carotenoids	Reference
5 μm C18 Shimpack CLC (M) 250 × 4.6 mm	Acetone:MeOH:AcOEt (99:1:0–60:10:30, gradient) 0.7 mL/min	Acetone	Orange/α-, β-, and ζ-carotene	Gama and Sylos (2007)
5 μm Nucleosil C18 250 × 4 mm	Acetone:water (75:25–95:5, gradient) 1.5 mL/min	Acetone	Pepper/β-carotene	Topuz and Ozdenir (2007)
5 μm LiChrocart C18 250 × 4 mm	Acetone:water (85:15–100:0, gradient) 1.5 mL/min	MeOH:hexane (1:1)	Apricot/β- and γ-carotene	Ruiz et al. (2005)
3 μm LiChrosphere C18 150 × 4.6 mm or 3 μm ODS-2 150 × 4.6 mm	ACN and MeOH:EtOAc (1:1) (gradient)	MeOH:THF (1:1) Hot EtOH Acetone	Banana and plantain (*Musa)*/α- and β-carotene	Davey and Ozdemir (2006)
5 μm Zorbax ODS 250 × 4.6 mm	ACN:DCM:MeOH 70:10:20 1 mL/min	–	Cereals, pulses, vegetables, spices, condiments, and unconventional sources/β-carotene	Kandlakunta et al. (2008)
5–6 μm Zorbax ODS 250 × 4.6 mm	ACN:DCM:MeOH (70:20:10) 2.0 mL/min	Acetone	Fruits and vegetables/α- and β-carotene, and lycopene	Heinonen et al. (1989)
5 μm Microsorb C18 Rainin 250 × 4.6 mm	ACN:DCM:MeOH (55:23:22) 1 mL/min	THF or acetone	Yellow/orange fruits and vegetables/α-, β-, γ-, and ζ-carotene, and lycopene	Khachick et al. (1992)
10 μm C18 YWG 200 × 4.6 mm	ACN:DCM:MeOH (7:1:1) 1 mL/min	Petroleum ether:acetone (1:1)	Pink- and red-fleshed citrus/β-carotene and lycopene	Xu et al. (2006)
5 μm Spheri-5-RP-18 or Spheri-5-ODS 220 × 4.6 mm	ACN:DCM:MeOH (70:20:10) 1.8 ml/min	THF	Vegetables/α- and β-carotene, and lycopene	Granado et al. (1992)
5 μm Prevail C18 RP 150 × 4.6 mm	ACN:MeOH (95:5) and ACN:MeOH:EtOAc (60:20:20) (gradient)	MeOH:THF (1:1)	Berries/α- and β-carotene	Marinova and Ribarova (2007)
5 μm C18 Microsorb 250 × 4.6 mm	ACN:MeOH:DCM 75:20:5 2.0 mL/min	Acetone	Vegetables, fruits, staples, miscellaneous foods and beverages/α- and β-carotene, lycopene	Lako et al. (2007)
5 μm SGE C-18 ODS 120A0 250 × 4.6 mm	ACN:MeOH:DCM (60:20:20) 1 mL/min	Acetone	Medicinally important green leafy vegetables/α- and β-carotene	Raju et al. (2007)
5 μm ODS2 100 × 4.6 mm connected to a Vydac 201TP54 250 × 4.6 mm	ACN:MeOH:DCM (75:20:5) 1.5 ml/min	MeOH:THF (1:1)	Fruits and vegetables/α- and β-carotene, and lycopene	Dias et al. (2009)
5 μm ODS2 100 × 4.6 mm connected to a Vydac 201TP54 250 × 4.6 mm	ACN:MeOH:DCM (75:20:5) 1.5 ml/min	MeOH:THF (1:1)	Fruits and vegetables/α- and β-carotene, and lycopene	Hart et al. (1995)

5 µm ODS2 100 × 4.6 mm connected to a Vydac 201TP54 250 × 4.6 mm or 5 µm Vydac 201TP54 250 × 4 6 mm	ACN:MeOH:DCM (75:20:5) 1.5 mL/min or MeOH:THF (92.5:7.5) 1.0 mL/min or ACN and MeOH:DCM (80:20) (67:33–73-27, gradient)	MeOH:THF (1:1) or THF	Fruits and vegetables, and baby food/α- and β-carotene, and lycopene	Dias et al. (2010)
5 µm Microsorb C18 Rainin 250 × 4.6 mm	ACN:MeOH:DCM:hexane (85:10:2.5:2.5–45:10:22.5:22.5, gradient) 0.7 mL/min	THF or acetone	vegetables and fruits/α-, β-, γ-, and ζ-carotene, and lycopene	Khachick et al. (1992)
5 µm C18 RP 201 TP 54, Vydac 250 × 4.6 mm	ACN:MeOH:DCM:hexane (85:10:2.5:2.5) 0.7 mL/min	THF or acetone	Fruits and vegetables/Geometric isomers α-, β-, γ-, and ζ-carotene, and lycopene	Khachick et al. (1992)
5 µm C18 Hypersil ODS 250 × 4.6 mm	ACN:MeOH:DCM:hexane (85:10:2.5:2.5-45:10:22.5:22.5, gradient) 0.8 mL/min	THF	Tomato juices/β- and γ- carotene, and lycopene	Sánchez-Moreno et al. (2006)
3 µm Sherisorb monomeric C18 ODS2 150 × 4.6 mm	ACN:MeOH:EtOAc (95:5:0–60:20:20, gradient), with variations for some produts 0.5 mL/min	Acetone	Different fruits and vegetables/α- and β-carotene, and lycopene	Rodriguez-Amaya et al. (2008)
3 µm monomeric C18 RP Waters Spherisorb ODS 2, 4.6 × 150 mm	ACN:MeOH:EtOAc, 80:10:10 0.7 mL/min and 95:5:0–20:40:40 (gradient, maize) 0.5 mL/min	Acetone MeOH:THF (1:1)	Sweet potato, cassava, and maize/β-carotene	Kimura et al. (2007)
Supelcosil C18 250 × 4.6 mm	ACN:MeOH:hexane:DCM (82:6:6:6–42:14:14:14, gradient) 0.8 mL/min	THF	Tomato/β- and γ- carotene, and lycopene	Raffo et al. (2006)
L-column ODS 150 or 250 × 4.6 mm	ACN:MeOH:THF (50:40:5) 1.5 mL/min	HAET in ethanol	Carrot/α- and β-carotene	Tsukakoshi et al. (2009)
3 µm C18 Gemini 150 × 4.6 mm	ACN:MeOH:water (100:10:5) and Acetone:EtOAC (2:1) (90:10–30:70, gradient) 1 mL/min	Acetone	Persimmon/α- and β-carotene	Veberic et al. (2010)
5 µm C18 RP 201 TP 54, Vydac 250 × 4.6 mm	ACN:THF:MeOH:water, 80:10:6:4 0.6–1.0 mL/min	MeOH:THF (1:1)	Fruits, vegetables, and roots/α- and β-carotene, lycopene	Kim et al. (2007)
4 µm C18 Nova-Pak ODS 300 × 3.9 mm	ACN:Water:EtOAc (88:10:2–85:0:15, gradient) 1 mL/min	Acetone	Camu-Camu tropical fruit/β-carotene	Zanatta and Mercande (2007)
5 µm C30 RP YMC 250 × 4.6 mm	MeOH, MeOH:water (80:20) and MTBE (gradient) 1 mL/min	Chloroform:MeOH:50 mM Tris Buffer (2:1:1)	Loquat fruits/β-carotene	Zhou et al. (2007)
5 µm C30 RP YMC 250 × 4.6 mm	MeOH, MeOH:water (80:20) and MTBE (gradient) 1 mL/min	Chloroform:MeOH:50 mM Tris Buffer (2:1:1)	Orange, pummelo, and grapefruit/β-carotene, and lycopene	Xu et al. (2007)
C18 Bonadapack 300 × 2 mm	MeOH:ACN (90:10) 0.9 mL/min	Hexane:acetone:ethanol (50:25:25)	Tomato, carrot, pepper, watermelon, persimmon, medlar/β-carotene, and lycopene	Barba et al. (2006)
3.5 µm Sunfire C18 RP 100 × 4.6 mm	MeOH:ACN:chloroform (42.5:42.5:15) 1.2 mL/min	Acetone:hexane (1:1)	Sweet potato/β-carotene	Teow et al. (2007)

continued

TABLE 13.3 (continued)

HPLC Methods for Carotene Analysis in Food

Column Stationary Phase	Mobile Phase (eluent)[a]	Extraction Solvent[a]	Samples/Hydrocarbon Carotenoids	Reference
5 μm C30 RP–YMC 250 × 4.6 mm	MEOH:ACN:water (84:14:2) 1 mL/min	Hexane:acetone:EtOH (2:1:1)	Alga/α- and β-carotene	Hu et al. (2008)
5 μm C30 Carotenoid 250 × 4.6 mm	MeOH:MTBE (100:0–0:100, gradient) 1.0 mL/min	MeOH	Maize/α- and β-carotene	Menkir et al. (2008)
5 μm C30 YMC 250 × 4.6 mm	MeOH:MTBE (89:11) 1 mL/min	HAET in ethanol	Wheat/α- and β-carotene	Konopka et al. (2006)
3 μm C30 RP YMC 250 × 4.6 mm	MeOH:MTBE (95:5–50:50, gradient) 0.9 mL/min	Acetone	Dovyalis and tamarillo/α- and β-carotene	Rosso and Mercande (2007)
3 μm polymeric C30, YMC RP 4.6 × 250 mm	MeOH:MTBE, 80:20 and 90:10–40:60 (gradient, maize) 0.8 mL/min	Acetone MeOH:THF (1:1)	Sweet potato, cassava, and maize/β-carotene	Kimura et al. (2007)
5 μm C30 YMC 250 × 4.6 mm	MeOH:MTBE:water (90:5:5–75:25:0, gradient) 1 mL/min	MeOH:acetone:hexane	Orange juice/α-, β-, and ζ-carotene	Meléndez-Martínez et al. (2008)
5 μm C30 YMC 250 × 4.6 mm	MeOH:MTBE:water (81:15:4)–MTBE:MeOH:water (90:6:4), gradient 1 mL/min	Acetone:hexane (1:1)	Carrot juices and vitamin supplemented (ATBC) drinks/ α- and β-carotene	Marx et al. (2000)
5 μm C18 Vydac 201TP54, RP 250 × 3.2 mm	MeOH:MTBE:water (95:0:5–100:0:0, gradient)	EtOH:hexane (4:3)	Orange and carrot juices/α-, β-, and ζ-carotene	Cortés et al. (2004)
5 μm C30 RP YMC 244 × 4 mm	MeOH:THF (95:5)–water:EtOH:THF (90:5:5) (gradient) 1.5 mL/min	Water:MeOH/acetone: *n*-hexane	Fruit vegetables and tubers/ β-carotene	Kidmose et al. (2006)
5 μm LiChrospher 100 RP-18 244 × 4 mm	MeOH:water (80:20) –EtOAc (gradient) 1 mL/min	Acetone	Beans, broccoli, chive, green bell pepper, lettuce, parsley, peas, spinach/ β-carotene	Larsen and Christensen (2005)
5 μm LiChrospher 100 RP-18 244 × 4 mm	MeOH:water (80:20) –EtOAc (gradient)	Acetone	Leafy vegetables/β-carotene	Kidmose et al. (2006)
5 μm C30 RP 250 × 4.6 mm	MeOH:water (99:1) METBE:MeOH (10:90–60:40, gradient, for corn) 1.3 mL/min	MeOH:THF (1:1)	Cereals/β-carotene	Burkhardt et al. (2007)
5 μm Vydac 201TP53 250 × 3.2 mm	MeOH:water:THF (88:10:2–92.5:7.5, gradient) 0.7 mL/min	Diethyl ether and petroleum ether THF	Milk and milk products/α- and β-carotene	Hulshof et al. (2006)
5 μm C30 YMC 250 × 4.6 mm	Water:MeOH:MTBE (5:90:5–0:5:95, gradient) 1 mL/min	MeOH:water (40:60)	Citrus fruits/α-, β-, and ζ-carotene	Matsumoto et al. (2007)
5 μm LiChrospher 100 RP-18 244 × 4 mm	Water:MeOH:THF (67:27:6) 2.0 mL/ min	Hexane:acetone:ethanol (50:25:25)	Tomato/β-carotene	Kidmose et al. (2006)

Note: ACN—acetonitrile, DCM—dichloromethane, EtOAc—ethyl acetate, EtOH—ethanol, HAET—hexane:acetone:EtOH:toluene (10:7:6:7), MeOH—methanol, MTBE—methyl *tert*-butyl ether, and THF-tetrahydrofuran.

[a] Modifiers and antioxidants were not included.

mixtures or even, methanol, THF or acetone, individually (Table 13.3). When acetone is chosen, caution should be taken to avoid aldol condensations. Since THF is known to promote peroxide formation that may contribute to production of artifacts, it is recommended to add BHT. Scott (1992) tested various solvents for their effectiveness in extracting carotenoids from vegetables. Methanol was the best solvent for dry materials, while for liquid materials, THF was most effective. Thus, a combination of both solvents appeared to result in efficient extraction and solubilization of carotenoids tested on a wide range of vegetable materials (Hart and Scott, 1995). In an interlaboratory study conducted by 17 European laboratories, this extraction procedure using methanol/THF (1:1, v/v) containing 0.1% BHT for stabilization was tested and tended to result in higher concentrations of carotenoids in the extracts (Scott et al., 1996). The same was observed by Dias et al. (2010) for different fruit and vegetable matrices.

Extraction with supercritical fluids has been suggested as an alternative method which, associated with supercritical fluid chromatography, could be a one-step method, where the mobile phase and the equipments are shared, avoiding pigment degradation (Pfander and Niggli, 1995). As a drawback, the elution force of pure CO_2 for carotenoids is very low, the addition of cosolvents being necessary to increase the solubility in CO_2. Limitations due to pressure, temperature, and solubility render these methods not entirely adequate to an exhaustive separation of a complex mixture of pigments and this technique has received relatively little attention.

Supercritical carbon dioxide extraction of β-carotene and lycopene was applied to dried tomato by-products rich in lycopene, the best conditions being obtained for 460 bar and 80°C (Vági et al., 2007). Vasapollo et al. (2004) applied this technique using a vegetable oil as the cosolvent and the maximum amount of the extractable lycopene from dried tomato, at 450 bar and 66°C, was 60%. Carotenes are not quantitatively extractable from some cereal grains using common solvent extraction methods. Burkhardt and Böhm (2007) observed a positive impact of soaking in water on the extraction of carotenoids for different corn samples (whole grain, semolina, and flour). Five minutes of soaking in water at room temperature prior to organic solvent extraction resulted in hydrocarbon carotenoid contents in the extracts about 1.5–2.5-fold higher than without soaking.

13.9.1.4 Saponification

Hydrocarbon carotenoids analysis in matrices with significant content of lipids needs a saponification step. Taking into account that carotenes are easily destroyed in the presence of acids, saponification should be conducted in mild basic medium, usually in potassium hydroxide (KOH) at 5% (m/v), during 1–12 h. Degradation increases with temperature and base concentration (Kimura et al., 1990).

Taking into account the fat content of dairy products, carotene analysis claims a saponification step. The fat extract is saponified in mild conditions (KOH, 5%, m/v) at room temperature to avoid β-carotene degradation. Besides, a nonwatery environment is recommended, in order to avoid conversion of β-carotene into monoepoxy carotenes and *E/Z* isomerization (Kimura et al., 1990). For high-lipid samples, such as red palm oil, a better procedure for eliminating lipids is being pursued. Using the nonspecific *Candida cylindracea* lipase, complete hydrolysis of red palm oil was achieved after 4 h at 35°C under a nitrogen atmosphere (Lietz and Henry, 1997). This mild hydrolysis allowed quantitative analysis of carotenes without isomerization and degradation. In our laboratory, olive oil samples are injected in the HPLC system without fat elimination. The sample is first dissolved in dichloromethane and then in acetonitrile-based mobile phase, but the chromatographic column must be solvent cleaned between injections. Stöggl et al. (2005) for crude rice oil suggested the oil, dispersion in a mixture of acetonitrile, methanol and 2-propanol, 5 min mixture centrifugation at 2300 rpm, and injection of the upper layer.

13.9.1.5 Separation, Identification, and Quantification by HPLC

13.9.1.5.1 Stationary Phase

With the advent of HPLC, carotenoids quantification underwent great development. Considering that these compounds have hydrophobic character, a separation using reversed-phase (RP) C_{18} columns is preferred. However, there is some literature referring to normal-phase columns, which are better in separating the xanthophylls, lutein and zeaxanthin, and their geometric isomers. Also, an RP-C_{30} column was

specially, conceived for carotenoid separation taking into account that the pigments and the stationary phase interactions are maximized by similar dimension. This column is efficient in discriminating different carotene *Z*-isomers and (*all-E*)-isomers (Emenhiser et al., 1999), but the time of elution is much higher than with C_{18} columns. Separation of carotene *E/Z*-isomers is also promoted by RP-C_{18} columns of low carbon charge/large pore size. The use, if possible, of metal-free columns and "biocompatible" column frits is also recommended by Hart and Scott (1995).

To improve pigments separation, column heating is sometimes proposed in the literature. In the case of carotenes, this practice is not recommended because isomerization is activated, and at temperatures higher than 40°C, carotene degradation is significant (Huck et al., 2000). According to Scott and Hart (1993), the optimal temperature is in the range 20–22.5°C; the use of a temperature-controlled system is recommended to obtain repeatable time elution profiles (Huck et al., 2000; Scott and Hart, 1993).

13.9.1.5.2 Mobile Phase and Solvent Modifiers

Mobile phase is ordinary composed of organic solvents. Mobile phases used in HPLC methods for the determination of carotenes in different foods are summarized in Table 13.3. Carotenoids may undergo losses or degradation in the chromatographic column. Different studies indicated that solvent modifiers improve the recovery of carotenoids from the column and reduce or eliminate on-column degradation. When 0.1% triethylamine was added to acetonitrile-based mobile phases, the recovery of α- and β-carotenes increases 30% and 42%, respectively (Hart and Scott, 1995). Epler et al. (1993) tested the effect of ammonium acetate (0.05 M in methanol used in the mobile phase), and addition of 0.05% triethylamine to the mobile phase further increased the recovery to around 100% for all studied columns. The possible action of these solvent modifiers is related to buffering of the acidity of the free silanols and preventing of reactions with free metal ions of the stationary phase.

13.9.1.5.3 Identification and Quantification

The molecule conjugated double-bond system constitutes the light-absorbing chromophore that gives carotenes their attractive color and provides the visible absorption spectrum that serves as a basis for their identification and quantification. The conjugated polyene structures of these compounds give them unique light absorption spectra and at the same time high absorptivities, and hence outstanding lower limits of detection. The ultraviolet and visible spectrum is the first diagnostic tool for the identification of hydrocarbon carotenoids. The most commonly used detectors are UV–vis light absorbance detectors. Recently, UV–vis diode array detectors (DADs) combined with actual computer capacity that enables the acquisition of huge amounts of data and the implementation of complex software increased drastically the HPLC analytical potency. With these detectors, it is nowadays possible to follow the elution of a compound in the complete UV–vis range (190–800 nm) and to minimize erroneous identifications due to eventual matrix components with the same retention time (RT) in the column. When a high sensibility is required, electrochemical detection is a good alternative (Ferruzzi et al., 1998; Rozzi et al., 2002). With regard to fluorescence detection, in spite of greater specificity than absorbance spectroscopy, only phytofluene shows appreciable fluorescence, and this technique is not widely used for routine analysis.

The wavelength of maximum absorption (λ_{max}) and the shape of the spectrum (spectral fine structure) are characteristic of the chromophore. Most carotenoids absorb maximally at three wavelengths, resulting in three-peak spectra (Figure 13.4), the absorption spectra being markedly solvent dependent.

The greater the number of conjugated double bonds, the higher the λ_{max} values. Thus, the most unsaturated acyclic carotene lycopene, with 11 conjugated double bonds, is red and absorbs at the longest wavelengths (λ_{max} at 448, 474, and 506 nm, in the solvent mixture presented in Figure 13.4).

Z-isomerization of a chromophore's double bond causes a slight loss in color, small displacement of λ_{max} to lower wavelength (usually 2–6 nm for mono-*Z*), and decrease in absorbance, accompanied by the appearance of a peak in or near the UV region (at around 360 nm, depending on the solvent). The intensity of the *Z*-peak is greater as the *Z*-double bond is nearer to the center of the molecule.

Carotene identification is made using the HPLC system software by comparing their RT and visible absorption spectra, λ_{max} and spectral fine structure ($\lambda_{max,III}$ and ratio of the heights of the peaks III and II, Figure 13.4c) with those of carotene standards, according to the recommendations of Britton (1991).

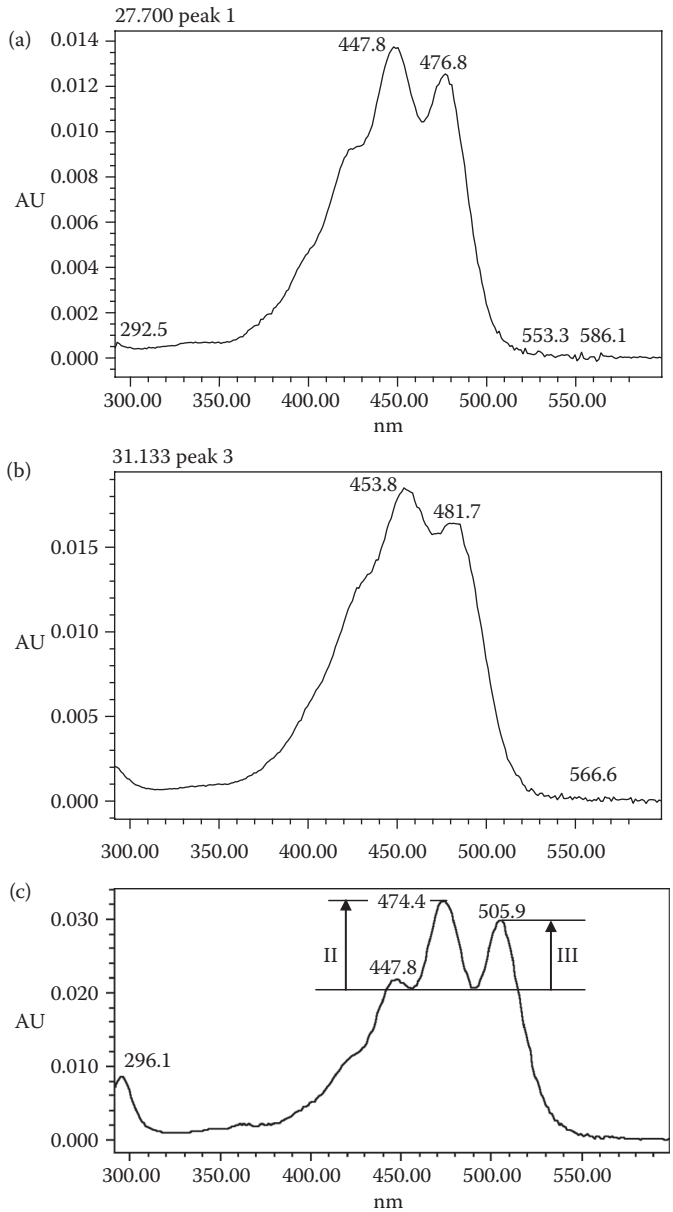

FIGURE 13.4 UV/vis spectra of (a) all-*E*-α- and (b) all-*E*-β-carotene and (c) all-*E*-lycopene in the HPLC mobile phase acetonitrile:methanol (containing 0.05 M ammonium acetate):dichloromethane, 75:20:5, v/v/v, containing 0.1% BHT and 0.05% triethylamine.

Analyte quantification is done by external calibration, based on peak areas (or least frequent peak heights) resulting from the HPLC software treatment of the detector response. Standards should be acquired with the maximum purity, and after dissolution in an appropriate solvent to obtain stock solutions, suitable solutions should be prepared to access concentration through absorbance measurement at the maximum absorbance wavelength (peak II, Figure 13.4c) using a spectrophotometer, the appropriate absorptivities, and the Beer–Lambert law.

Trivial and semisystematic names of hydrocarbon carotenoids present in foods, the respective main absorption maxima, and the relation between them (Khachik et al., 1992) are presented in Table 13.4.

TABLE 13.4

Hydrocarbon Carotenoids Found in Foods and Absorption Maxima

	Trivial Name	Semisystematic Name	Absorption Maxima (wavelength, nm)	%III/II[a]
Bicyclic	α-Carotene	β,ε-Carotene	422 445 473	55
	β-Carotene	β,β-Carotene	(425) 450 477	25
	δ-Carotene	ε,ψ-Carotene	431 456 489[b]	85
Monocyclic	γ-Carotene	β,ψ-Carotene	437 462 494	40
	α-Zeacarotene	7′,8′-Dihydro-ε,ψ-carotene	398 421 449	
	β-Zeacarotene	7′,8′-Dihydro-β,ψ-carotene	406 428 454	
Acyclic	Z-carotene	7,8,7′,8′-Tetrahydro-ψ,ψ-carotene	378 400 425	103
	Lycopene	ψ,ψ-Carotene	444 470 502[b]	65
	Neurosporene	7,8-Dihydro-ψ,ψ-carotene	414 439 467[b]	100
	Phytoene	7,8,11,12,7′,8′,11′,12′-Octahydro-ψ,ψ-carotene	(276) 286 (297)	10
	Phytofluene	7,8,11,12,7′,8′-Hexahydro-ψ,ψ-carotene	331 348 367	90

Note: Values for all-*E* forms in hexane; parentheses indicate a shoulder.

[a] Ratio of the height of the longest-wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline, multiplied by 100.

[b] In petroleum ether.

It is also important to check carotene impurities within individual solutions and correct concentrations accordingly. For example, α-carotene is frequently a contaminant in β-carotene and if a mixed standard is used for quantification of a sample containing the two analytes, the standard carotene concentration must be recalculated. Usually, five or six levels of working calibration solutions are injected in the HPLC system and calibration curves for each carotene are constructed by the least-squares method by plotting HPLC response at various concentrations. Regression analysis of the data for the standard curve for each carotene provides several parameters that should be evaluated. The coefficient of determination should be >0.995, and the confidence limits of the intercept should contain zero. For carotenes, the detector response is in general linear in the concentration range, 0.05–5.0 μg/mL, but for each system in the scope of the method validation, these values must be confirmed. From our experience, individual or mixed standard hydrocarbon carotenoid solutions are relatively stable at very low temperatures (at least 6 months at –70°C, data not published), but a control system to monitor the stability should be implemented.

In cases in which the reference standards of *Z*-isomers are not available, the *Z*-carotenes content in food extracts may be determined from the standard curve of their all-*E* counterparts (Khachick, 1992).

To access the losses during the analytical process, the use of an internal standard is recommended. The most common is the β-apo-8′-carotenal. Depending on coelution with matrix components and/or market availability, echinenone is usually an alternative. For example, β-apo-8′-carotenal in green vegetables extracts, in the majority of chromatographic systems, coelutes with chlorophyll and it is replaced by echinenone. Some other internal standards are referred in some applications such as Sudan I, methyl- or ethyl-β-apo-8′-carotenoate, and nonapreno-β-carotene. Particular attention must be paid to food extracts that need saponification, since a carotenoid ester could not be employed as internal standard. Besides, the internal standard peak interference with the HPLC peaks of the carotenes under study and/or with other components of the matrix should always be excluded.

Recently, chromatographic equipments of ultra high-performance liquid chromatography (UHPLC) that enable the use of columns with microparticles (1.7 μm) could at least contribute to reduce the volume of solvents used, reducing costs and saving the environment.

13.9.1.6 High-Performance Liquid Chromatography/Mass Spectrometry

In complex matrices, namely at low concentrations, when DAD is not enough, MS associated with liquid chromatography has been applied successfully to the complete characterization of molecular structure,

enabling the analysis of subnanogram quantities. Nevertheless, carotene molecules similarity, poliene chain, and the combination of terminal groups could easily conduct to erroneous identifications claiming trained experts. The mass spectrometer generally used in this field is the atmospheric pressure chemical ionization (APCI) in the positive-ion mode.

13.9.1.7 Comprehensive Bidimensional Liquid Chromatography

Carotenes are in the great majority of applications analyzed along with xanthophylls, this group being found either in its free form or in a more stable fatty acid esterified. For this reason, to simplify the analysis, the process includes a saponification step, to release carotenoids bound to the fatty acids and acts as a cleanup step, eliminating interfering compounds such as lipids and chlorophylls. However, this step has drawbacks, such as carotenoid degradation and artifact formation. Recently, Dugo et al. (2008) for the first time applied the comprehensive two-dimensional liquid chromatography (LC × LC) for the determination of the orange essential oil carotenoid composition. This is a novel technique coupling two independent LC separation processes with orthogonal selectivities (Dugo et al., 2008). In LC × LC, the whole sample is analyzed in the two dimensions independently by using a switch valve as a transfer system between them, and it was already demonstrated as an effective technique for the analysis of complex matrices (Dugo et al., 2008). This group demonstrated the ability of an LC × LC-DAD/APCI-MS method to analyze and identify the native carotenoid composition of an extremely complex matrix without any kind of sample pretreatment.

13.9.1.8 Open Column Chromatography

The first chromatographic separation method applied to carotenes determination in food was the adsorption chromatography in an open column, at atmospheric pressure (Almeida and Penteado, 1988). Different chromatographic beds were used namely powder sucrose, DEAE-sepharose, cellulose, or MgO/Hiflosupercel (Omata and Murata, 1983; Rodriguez-Amaya et al., 1976; Strain et al., 1971; Wasley et al., 1970). This technique has the advantages of using common laboratory equipment (UV–vis spectrophotometer), without the continual use of the expensive carotene standards, and it enables a good separation for food samples with a complex carotenoid profile, allowing the discrimination of carotenes from xanthophylls and providing separation within these classes (Almeida-Muradian et al., 1997; Rodriguez-Amaya et al., 1988; Rodriguez-Amaya, 1996). However, notwithstanding the low cost, this technique requires appreciable quantities of sample (Mercande, 1999; Su et al., 2002), the productivity is very low and data quality depends largely on the competence/capacity of the analyst.

13.9.1.9 Thin-Layer Chromatography

Thin-layer chromatography and high-performance thin-layer the chromatography is often used for the separation and the isolation of individual classes of molecules, because it is fast, effective, and relatively cheap. In case of carotenes especially care is necessary, silica acidity should be previous neutralized to avoid the carotenoid epoxid–furanoxid rearrangement (Schiedt and Liaaen-Jensen, 1995). Unfortunately, with this technique the separation of compounds with a similar structure is very difficult and it has never been used extensively for carotene analysis.

13.9.2 Spectroscopic Methods

Absorbance spectroscopy is the simplest form to identify and quantify the carotenes since the absorbance spectrum is the fingerprint of each carotene molecule. However, the samples are complexes and the overlapping of the carotenoid absorbance bands complicates the estimation of individual carotene concentrations, resulting in a much less efficient method, especially if several carotenoids are present. Spectroscopic methods usually allow a crude identification of the compounds present in a sample extract, but the specific composition remains unknown.

13.9.3 Nondestructive Techniques

The methods described above are destructive techniques that destroyed the sample, are expensive, and are time consuming. Alternatively, analytical noninvasive procedures have been developed for specific applications, namely to monitor pigments content along time and to estimate its impact in product perception by the consumer.

13.9.3.1 Color Perception and Evaluation

The pigments and the surface topography absorb, reflect, and refract incident visible light perceived by the eye. Color could be defined through the coordinates of a Cartesian referential, L* (luminosity, black to white), a* (green to red), and b* (blue to yellow). Color measurement was successfully used to characterize the color change of fish, related to some diets including different carotenoid compositions (Akhtar et al., 1999; Bjerkens et al., 1997). However, this method has several limitations because many mathematical combinations could explain the color change during, for example, food processing or maturation (Gomez et al., 1998; Ma and Shimokawa, 1998; Steet and Tong, 1996).

13.9.3.2 Raman Resonance Spectroscopy

Raman resonance spectroscopy has enabled fast detection and quantification of carotenes, namely in fruits, without sample destruction. Typically, the product under study is illuminated with a laser beam, and the large and intense absorption bands of carotene molecules are used for resonance excitation. A high-specific Raman answer to these molecules is obtained. Nevertheless, the fast test does not distinguish different carotenoids and the Raman answer is highly dependent on matrix. It is very useful to compare the same-class members (e.g., different tomato varieties) in the same maturation state, monitor juice production, combine different fruits to obtain a higher carotenoid content, or to select field products more rich in carotenoids (Bhosale et al., 2004). Schulz et al. (2006) also applied this technique for the determination of lycopene in tomato and tomato products.

13.9.3.3 Near-Infrared Reflectance Spectroscopy

The Near-Infrared Reflectance Spectroscopy (NIRS) technique may greatly simplify the analysis of carotenes, namely in cereals, when it is necessary to screen a large number of samples, for example, in breeding programs for the development of maize hybrids with grain enriched in carotenes. Berardo et al. (2004) successfully applied this technique to 64 samples of corn grains. They drew comparisons between two analytical methods, HPLC and NIRS, and the values of the determination coefficient, r^2, ranged from 0.82 to 0.94 for different carotenoids, including α- and β-carotenes. Halim et al. (2006) also applied the infrared technique for direct determination of lycopene in tomato.

13.9.3.4 Portable Devices

More recently, application of a portable near-infrared reflectance (NIR) was reported by Kusumiyati et al. (2008) to evaluate the lycopene content of tomato. Although portable NIR spectrometers based on InGaAs sensors are available in market, their prices are prohibitive for filed application. In 2009, Choudhary et al. proposed an economical, fast, and miniature-sensing device to predict the lycopene content in watermelon and tomato puree from their visible reflectance spectra acquired by a fiber-optic diffuse reflectance sensor. The high linear correlations between spectral parameters and lycopene content (1–8 mg/100 g) suggest that this method can be reliably used for fast and safe quantification of this carotene in the studied products.

13.9.4 Method Validation

The validation of an assay method is a process that has an objective to evidence that the method is able to obtain results which quality is adequate to the intentions it was designed. The validation process

includes systematic laboratory studies to know and evaluate the assay method characteristics. Implementation of an assay method includes steps such as selection, validation, personal qualification, and the establishment of a protocol to control the method execution. It is a basic principle from good laboratory practices that only validated methods should be implemented. The validation concept has been defined by different groups, namely the EURACHEM, the International Standard Organization (ISO) (with the 17025 standard), the United States Pharmacopeia (USP), and the World Health Organization (WHO) in a convergent form. Carotene analysis needs careful evaluation of analytical procedures and validation of carotene responses in order to avoid factors that cause variation and inaccuracies in the quantitative determination of carotenes. Quality control systems should be implemented to monitor the system through the definition of the control criteria in routine applications. Although the theme is consensual in generic terms, it is controversial specially concerning the definition and the form of determination of the validation parameters. The parameters usually evaluated are selectivity/specificity, work range, linearity, sensibility, detection and quantification limits, robustness, and accuracy (trueness and precision). Examples of the evaluation of these parameters in the context of in-house method validation applied to carotenoids analysis by HPLC were published (Hard and Scott, 1995; Dias et al., 2008).

13.9.5 Measurement Uncertainty

For consistent interpretation of an analytical method result, it is necessary to evaluate the confidence that can be placed in it; this can be provided by the quantification of its accuracy (trueness and precision) combining bias with the measurement uncertainty estimate.

The *Guide to the Expression of Uncertainty in Measurement* issued by the International Organization for Standardization (ISO, 1995) establishes the rules for evaluating and expressing uncertainty. Although it is a very powerful tool, it may be extremely complex when analytical methods include mass transfer steps, as in the present case (e.g., extraction, evaporation), that lack descriptive models of the behavior of the analyte in the analytical system. The guide was interpreted for analytical chemistry by EURACHEM (EURACHEM/CITAC, 2000), which presents several examples that involve the treatment of interlaboratory information and also the use of information obtained from in-house validation of the analytical methods, namely the precision studies.

The analytical method performance criterion, trueness, can be assessed by recovery tests, analyses of standard reference materials, and results from interlaboratory/proficiency tests. It is widely recognized that spiked analytes cannot behave in the same way as the endogenous compounds, which are frequently in fat globules or naturally well protected by membranes and cell walls, and can be linked to other components in matrices. The added internal standard cannot enter the fat globule or protection structures and equilibrate with endogenous carotenes to provide useful information about the efficiency of carotenoid extraction. Nevertheless, because the preparation of carotene samples for HPLC analysis requires extensive work-up procedures with many steps of mass transfer, the use of an internal standard is essential.

Analysis of standard reference materials is indispensable to assure traceability and comparability of laboratory results as well as to evaluate trueness of laboratory results. Availability of standard reference materials that resemble the food samples of interest, in terms of matrix and carotene composition/content is limited, however. Additionally, due to perishability of samples and stability of the analytes, standard reference materials of raw foods are not available and from our experience processed foods are easier to extract than raw foods. Two studies were published referring the work to carotene values assignment, through interlaboratory comparison exercises, of two certified reference materials, the lyophilized mixed vegetable material (Scott et al., 1996) and the baby food composite (Sharpless et al., 1999).

Approaches for the measurement uncertainty estimate in HPLC analysis of carotenoids were undertaken by Dias et al. (2008, 2009). The authors conclude that the relative expanded measurement uncertainty was mainly between 0.10 and 0.31 and higher values were found for measurements near instrumental quantification limits or when sample chromatograms present interferences with the analyte peak making peak integration difficult.

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14

Oxycarotenoids (Xanthophylls)

Daniele Giuffrida, Paola Dugo, Paola Donato, Giovanni Dugo, and Luigi Mondello

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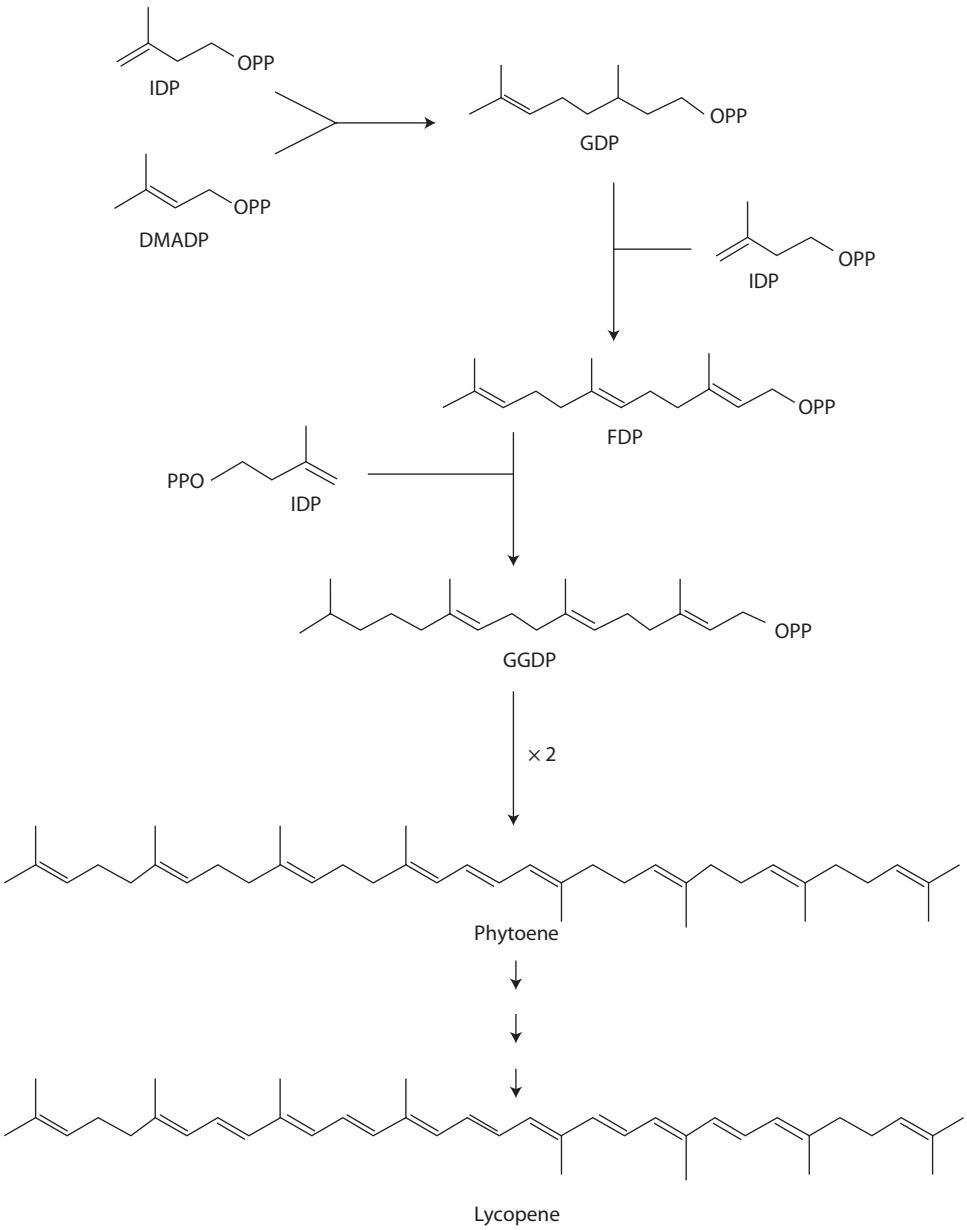
14.1 Oxycarotenoids: Structure and Functions

The chemical structure of carotenoids is usually based on a C_{40}-tetraterpenoid structure with a centrally located, extended conjugated double-bond system that acts as the light-absorbing chromophore and is related to the color shown. Taking into account their chemical structure, these compounds can be divided into two different groups: hydrocarbon carotenoids, generally named carotenes, and oxygenated carotenoids, commonly known as xanthophylls. Carotenoids are biosynthesized in plants, algae, fungi, and bacteria, whereas animals cannot biosynthesize carotenoids (Poulter and Rilling, 1981). More than 700 different carotenoids have been isolated from nature and their biosynthesis occurs in the so-called acetate–mevalonate pathway (Britton, 1998). Initially, the "head-to-tail" condensation, through the action of the enzyme prenyl transferase, of the C_5-isoprenoid precursors IDP (isopentenyl diphosphate) and DMADP (dimethylallyl diphosphate) leads to the formation of the C_{10}-monoterpene GDP (geranyl diphosphate). A second addition of the IDP unit to the GDP leads to the formation of the C_{15}-sesquiterpene FDP (farnesyl diphosphate). A further addition of the IDP to the FDP leads to the formation of the C_{20}-diterpene GGDD (geranylgeranyl diphosphate). Successively, the phytoene synthase complex regulates the "tail-to-tail" condensation of two molecules of the C_{20}-diterpene GGDD to form the C_{40}-tetraterpenoid structure, which is a characteristic of the symmetrical carotenoid skeleton. This last condensation forms the phytoene molecule, which has a short triene chromophore and which undergoes a series of sequential desaturation (dehydrogenation) reactions, regulated by the phytoene desaturase enzymes, with the final formation of the lycopene molecule, which has an acyclic structure with an extended conjugated double-bond system, usually with the (all-*E*)-configuration. The polyene chain may possibly have up to 15 conjugated double bonds in the chromophore of the C_{40}-carotenoid, although 7–11 conjugated double bonds

are more commonly found. A general stepwise formation, using "skeletal formulas," of the lycopene molecule from the C_5-isoprenoid precursors IDP and DMADP is shown in Scheme 14.1.

Acyclic xanthophylls are carotenoids with oxygen functions in the acyclic end groups. These are found more frequently in phototropic bacteria, but also in some fungi and plants and usually contain tertiary hydroxyl or methoxy groups at the C(1) and C(1′) positions. Moreover, the 1,2-epoxide of lycopene has been isolated from tomatoes. As an example, the chemical structures of some acyclic xanthophylls commonly found are shown in Figure 14.1.

Usually, however, the formation of lycopene is followed by cyclization at one or both terminal ends of the basic C_{40}-carotenoid skeleton leading to the formation of a wide variety of natural carotenoids.



SCHEME 14.1 A general stepwise formation, using "skeletal formulas," of the lycopene molecule from the C_5-isoprenoid precursors IDP and DMADP.

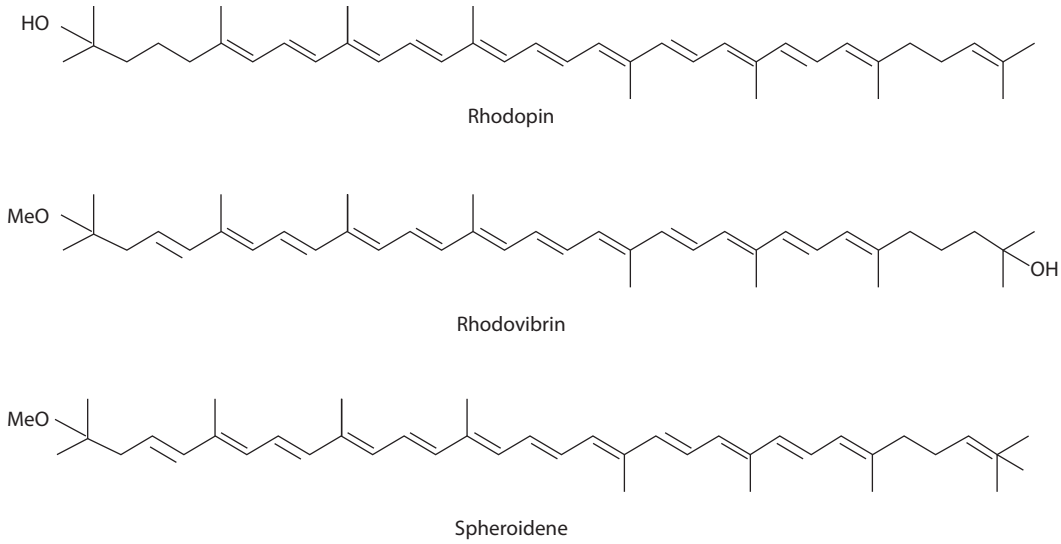
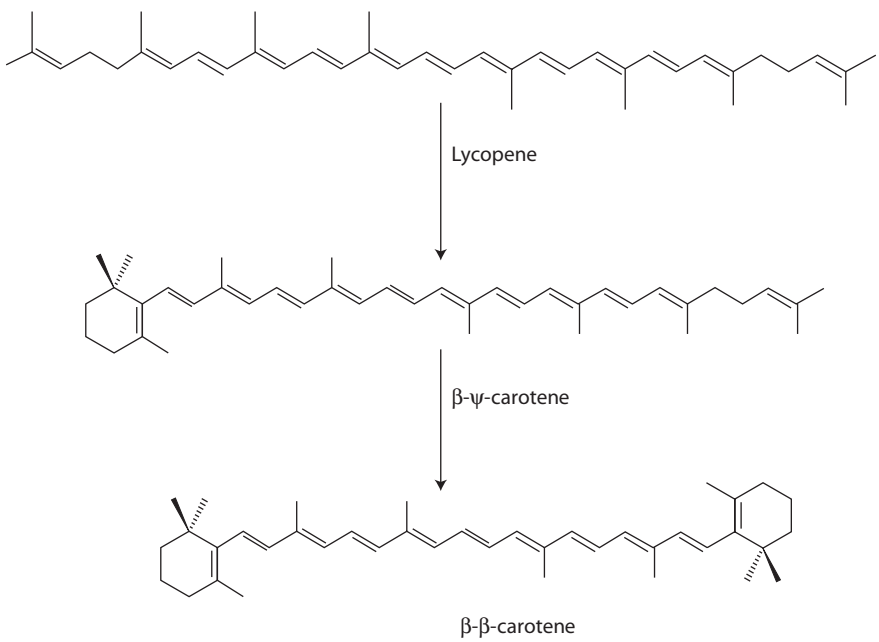


FIGURE 14.1 Chemical structures of some acyclic xanthophylls commonly found in food matrices.

Cyclization, regulated by the cyclase enzymes, is considered as a rearrangement of an acyclic end group to give monocyclic and dicyclic carotenoids with the usual formation of six-membered rings, which are commonly named as cyclic β, γ, or ε end groups and which are independently formed. Alternative pathways have been presented for the formation of cyclic carotenes, and as an example, Scheme 14.2 illustrates the formation of carotenoids with the β type of end group.

Among all the naturally occurring carotenoids, the most common are those that contain cyclic end groups with at least one oxygen function, which are known as cyclic xanthophylls.



SCHEME 14.2 General formation of carotenoids with the β type of end group.

The most common oxygen function observed in the cyclic xanthophyll moieties is the hydroxyl group, particularly at the 3-position or 4-position. Other common oxygen functions found in xanthophylls are the epoxy groups usually in the 5,6-position or 5,8-position, and the keto (C = O) group. Also encountered are methoxy (OMe), aldehyde (CHO), carboxy (CO_2H), carbomethoxy (CO_2Me), acetates (OCOMe), and lactones. As an example, Figure 14.2 shows the chemical structures of some very widespread xanthophylls.

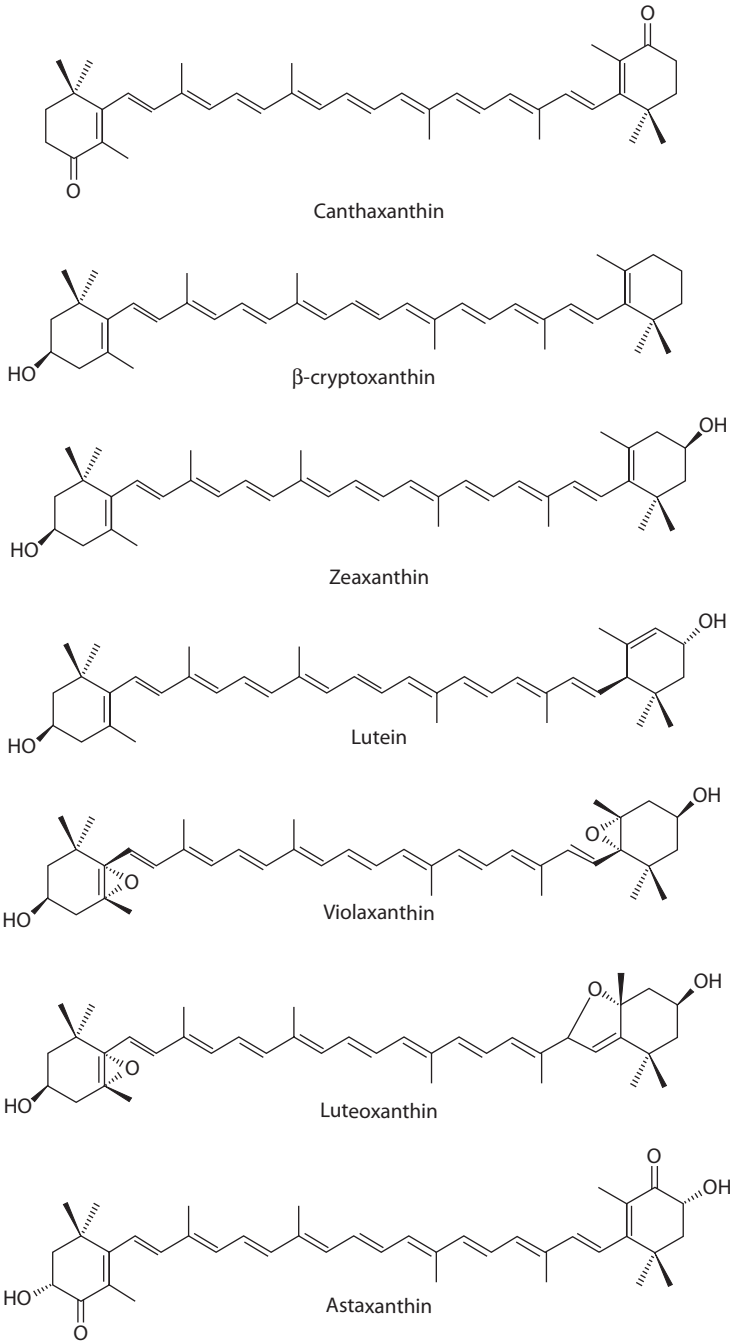
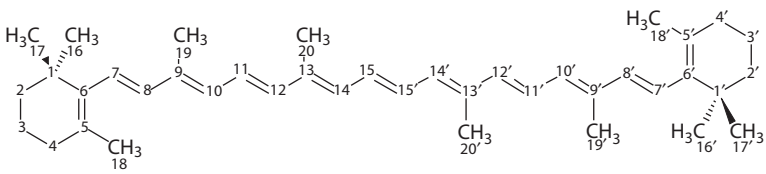


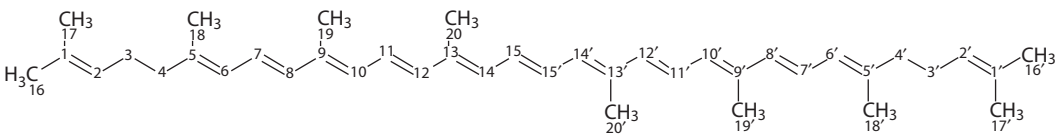
FIGURE 14.2 Chemical structures of some very widespread xanthophylls.

The nomenclature of carotenoids is based on the carotene backbone, and because of the symmetrical structure, one half of the molecule is numbered 1–15 from the end to the center, with additional methyl groups numbered 16–20. The other half is numbered in the same manner, only 1′–15′ and 16′–20′. Carotenoids undergo geometrical changes about the double bonds, and using the IUPAC nomenclature rules of carotenoid, the stem name implies *trans* (*E*); thus, *cis* (*Z*) configurations are specified by citing the specific bond as being *cis*. Figure 14.3 shows the conventional numbering of the positions of the atoms in the carbon skeleton in acyclic and cyclic carotenoid moieties.

Frequently, the hydroxyl groups present in the various xanthophylls are esterified with fatty acids or are glycosylated. In fact, in the chromoplast of fruits, the xanthophylls are usually esterified with mixtures of long-chain fatty acids, most frequently C_{12}–C_{18} saturated and unsaturated. Polyhydroxylated xanthophylls could therefore form different esters with different fatty acids. Although esterification does not change the visible light absorption properties, esterification increases the solubility of xanthophylls in lipids with which they are associated in nature and this is related to specific objectives for the plants and may also be related to an improvement of the carotenoid bioavailability. Moreover, although esterification does not change the chromophore properties of the carotenoid molecule, it does modify the immediate molecular environment and therefore the chemical activities might be altered depending on the kind of fatty acid bound to the xanthophylls. In some fruits during ripening, the esterification degree of carotenoids increases and this has been directly linked to the transformation of the chloroplast into the chromoplast. In addition, a high stability against possible thermo-, photo-, and enzymatic oxidation reactions has been related to the degree of esterification (Biacs et al., 1989). Carotenoids have structures that often contain at least one chiral center or axis and can therefore exist as different optical isomers and in many cases biological actions may be specific for one enantiomer. The extended delocalized π-electron system that characterizes the central part of the carotenoid structure is the key to many important properties of carotenoids. The light absorption properties in the visible wavelength range of 400–500 nm are responsible for the observed carotenoid colors and are related to the conjugated polyene chain. In plants, xanthophylls are located in the light-harvesting antenna complex, and the light absorbed by the carotenoids can be used as an energy source for photosynthesis; moreover, the carotenoids can protect the plants from possible oxidative damaging conditions, such as at high light intensity, by quenching potentially destructive species such as singlet oxygen. Moreover, they also contribute to the color of fruits and vegetables. Carotenoids serve a number of functions in the animal kingdom, including actions such as plumage coloration in birds, and are widely used in poultry feeding and in aquaculture. Carotenoids naturally occur in many foods, particularly fruits and vegetables, and are therefore normal component of our diet. A healthy diet should have additional attributes, contributing to protection against diseases. Such protection is achieved by the presence of bioactive



β-carotene



Lycopene

FIGURE 14.3 The conventional numbering of the positions of the atoms in the carbon skeleton in acyclic and cyclic carotenoid moieties.

compounds contained in "functional food," which are defined as "a food that may provide a health benefit beyond the traditional nutrients it contains" (Scrinis, 2008). Micronutrients like the carotenoids are considered secondary metabolite phytochemicals that play an active role in human health protection. In fact, carotenoids have been described to possess several important functional properties; the most important one is their antioxidant activity (Beutner et al., 2001; El-Agamey et al., 2004; de Quiros and Costa, 2006). Carotenoids react with virtually any radical species, mainly reactive oxygen species (singlet oxygen, superoxide anions, hydroxyl radicals, and hydrogen peroxide) likely to be encountered in biological systems where they are continuously generated by normal metabolism in the body, and this reaction involves the extended polyene chain by at least three possible mechanisms: (1) radical addition, (2) electron transfer to the radical, and (3) allylic hydrogen abstraction. Carotenoids showed antioxidant activity *in vitro* at physiological oxygen tension (Krinsky and Yeum, 2003), but their antioxidant activity remains controversial *in vivo* (Young and Lowe, 2001) and in higher oxygen partial pressure where they may act as prooxidants (Palozza, 1998). The effects of carotenoids on cardiovascular diseases proved their role as preventing agents (Arab and Steck, 2000; Rao and Rao, 2007); because of their ability to act as antioxidants, β-carotene, lycopene, lutein, zeaxanthin, and β-cryptoxanthin have been proposed as protective agents against coronary heart diseases (Mayne, 1996; Krinsky and Johnson, 2005). Many evidences suggest a beneficial effect of the carotenoids against the progression of cancer, although for some cancers more than others, for example, for lung, breast, ovarian, and prostate cancers (Nishino et al., 1999; Omoni and Aluko, 2005; Willett, 2005). Apart from the carotenoid-derived vitamin A molecule and its importance in vision, the role of lutein and zeaxanthin in eye health has recently received great attention; in fact those xanthophylls are associated with protection of the retina and retinal pigment epithelium from damage by light and oxygen, thus providing protection against age-related macular degeneration (AMD) and cataract (Snodderly, 1995; Landrum and Bone, 2001; Billsten et al., 2003). Moreover, it has been proposed that dietary carotenoids help protect the immune system from oxidative damage (Chew and Park, 2004), and that they also provide skin photoprotection because of their reactive oxygen species (ROS) scavenging and quenching properties, which are structurally related to their conjugated polyene chain (Sies and Stahl, 2004); astaxanthin, for example, exhibited significant antioxidant action and its ability to inhibit the lipid peroxidation process could greatly contribute to preserve membrane structures. Generally, a synergistic/cooperative interaction between carotenoids and other antioxidants, such as tocopherols, ascorbic acid, and flavonoids, appears to play an important role in the biological antioxidant network.

14.2 Oxycarotenoids in Food

Foods that provide health benefits (in addition to their nutritive value) or have a role in disease risk prevention are termed *functional foods*. Xanthophylls are bioactive components in food and updated and new databases on the levels of these phytonutrients are important so that researchers may accurately assess their dietary intake and therefore their true bioavailability, investigate their physiological functions, and determine their relationships with health and disease. Information about food xanthophyll composition of different cultivars, regions, and countries is extremely important and needs to be more widely disseminated in order to guarantee the preservation and sustainable use of biodiversity in food security and human nutrition programs. Although some carotenoids are used as food colorants and supplements, the majority of the 40 dietary carotenoids assumed in the typical human diet are obtained from natural fruits and vegetables (Bendich, 1993). Animal-derived xanthophylls assumed with our diet are more limited and are mainly ingested from some fish and crustaceans, eggs, and seafood. Several factors like cultivars, species, cultivation practices, time and method of harvest, degree of ripening, geographical areas, environmental conditions, year of production, processing, storage, and also different ways of cooking can affect the food carotenoid composition. Reports on the carotenoid composition of various foods and food products are available in the literature (Gross, 1987; Gross, 1991; Khachik et al., 1991; Oliver and Palou, 2000; Breithaupt and Bamedi, 2001; Pennington, 2002; Humphries and Khachik, 2003; Breithaupt, 2007; Rodriguez-Amaya et al., 2008; Britton and Khachik, 2009; Perry et al., 2009). Among the xanthophylls, lutein, violaxanthin, neoxanthin are usually present in green vegetables and

fruits; capsanthin and capsorubin are mainly present in yellow, orange, and red fruits and vegetables; β-cryptoxanthin and zeaxanthin are also widely distributed in fruits and vegetables; astaxanthin and canthaxanthin are typical animal xanthophylls that are often applied in animal feed so that the resulting food products are also more appealing to the consumers. Geometrical isomers can be found in food; the main *cis*-isomers detected in foods are 13-*cis* and 9-*cis* forms, although other forms have also been found (Constance et al., 1992; Sander at al., 1994; Sander et al., 2000). Because of their unsaturated nature, carotenoids are subjected to changes, mainly to oxidation; however, other factors such as temperature, light, and pH can also produce alterations that can influence the color of foods as well as their nutritional value (Melendez-Martinez et al., 2004). Although several nutrient databases provide estimations of the daily intake of carotenoids by humans, these values vary considerably due to the sensitivity and specificity of different analytical methods that are used in the detection of these phytochemicals. Although some human clinical trials are beginning to be undertaken, there is a great need for well-designed human intervention studies that take into consideration study designs, including subject selection, end point measurements, and the levels of carotenoids being tested, so that the important role played by carotenoids will be enhanced and used to develop complementary strategies for the prevention, treatment, and management of diseases (Rao and Rao, 2007). Currently, there is no formal diet recommendation for xanthophylls.

14.3 Oxycarotenoid Analysis

Guides to carotenoid analyses in foods are available in the literature (Schiedt and Liaaen-Jensen, 1995b; Rodriguez-Amaya, 2001; Rodriguez-Amaya and Kimura, 2004; Khachik, 2009). In this chapter, the different analytical steps used in carotenoid analysis will be overviewed with special attention to oxycarotenoid analysis and to novel analytical procedure and techniques applied to carotenoid analysis.

14.3.1 General Precautions

Xanthophylls are sensitive to oxidation and temperature- or light-induced decomposition and isomerization. General sample handling should be carried out with the use of amber glassware or at reduced light. Storage of samples and crystalline carotenoids should be at –20°C or even at –70°C under nitrogen or argon. Lyophilization is considered an appropriate way of preserving biological samples that have to be stored for carotenoid analysis because no enzymatic reactions occur in the dry state. Contact with acids should be avoided and contaminant-free pure solvents should be used for their extraction. Chloroform and dichloromethane often contain traces of hydrochloric acids, which should be removed prior to use, and diethyl ether and tetrahydrofuran may contain peroxides that react with carotenoids, which should also be removed prior to use. The evaporation of the solvents used for carotenoid extraction should be carried out under reduced pressure and at not more than 40°C. Addition of lipophilic antioxidant to samples and/or extraction solvent, such as BHT (butylated hydroxytoluene), is recommended to stabilize the original carotenoid profile. Sample homogenization is often necessary prior to carotenoid extraction followed by filtration, and addition of $MgCO_3$ or $CaCO_3$ to the extraction solvent to neutralize organic acids that are often present in food is advisable. Whenever possible, fresh material should be used and laboratory work should start as soon as samples are collected in order to minimize carotenoid degradation and losses.

14.3.2 Extraction

Differences in structural characteristics influence the solubility of carotenoids and therefore their extractability from the different matrices. Carotenes are soluble in solvents of low polarity, whereas oxycarotenoids (xanthophylls) are soluble in more polar solvents. For food samples that contain large amount of water, it is advisable to use organic solvents that are miscible with water, to allow a better release of the carotenoids from the matrix. Dried materials should be remoistened before extraction. Xanthophylls are well extracted with acetone, methanol, or a mixture of acetone/methanol (7:3) or a mixture of methanol/ethyl acetate/petroleum ether (1:1:1). Xanthophyll extraction from fatty food matrices like olive oil can

be achieved by partitioning of the carotenes into hexane and xanthophylls into DMF. Extraction should be repeated until the residue and the filtrate are colorless. In most cases, the acetone extract, which contains water, is transferred to a separatory funnel and the xanthophylls are transferred to an ether phase. If emulsions form, these may be resolved by the addition of a saturated NaCl solution. The ether phase is pooled and washed with water to remove any trace of acetone and unwanted water-soluble substances; it is then dried over $MgSO_4$ and evaporated in a rotary evaporator. The residue is then redissolved in a suitable solvent prior to the successive chromatographic separation.

14.3.3 Saponification

Saponification (alkaline hydrolysis) has been widely used to facilitate carotenoid analysis because it is an effective method to remove chlorophylls and unwanted lipids that may interfere with the chromatographic separation, or to hydrolyze the carotenoid esters, therefore simplifying the chromatographic profiles. Saponification is usually carried out by the treatment of the carotenoid extract with methanolic potassium hydroxide (5–10% w/v) under nitrogen or argon at room temperature for a few hours in the dark. After this, the carotenoid solution is transferred to a separatory funnel and a mixture of ether/hexane (1:1) is added and then the solution is washed with water (five times) to remove the alkali and evaporated. For high-fat food that contains a high concentration of lipids and sterol esters, it is necessary to carry out the saponification at a higher temperature or for longer times, or with the use of lipase enzymes. However, saponification is not free from drawbacks; in fact it may provoke artifact formation and degradation of the carotenoids; therefore, the information regarding the native carotenoid composition might be lost.

14.3.4 Chromatography

14.3.4.1 Liquid Chromatography

Liquid chromatography is widely used in carotenoid separations, both in preparative and in analytical works. Open column chromatography (OCC) and thin layer chromatography (TLC) are still used in preliminary fractionation of complex carotenoid extracts. Details of OCC and TLC of carotenoids were reported in Bernhard (1995) and Schiedt (1995a), respectively. Hydroxycarotenoids and their epoxide are best separated on silica gel, whereas C_{18} reversed-phase plates are most appropriate for the separation of xanthophyll esters. Special care should be taken when the separation is done on silica gel; in fact it is necessary to neutralize the acidity of silica in advance in order to avoid epoxide–furanoxide rearrangement in the carotenoids.

14.3.4.2 High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is today the method of choice for carotenoid analysis (Craft et al., 1992b; Pfander and Riensen, 1995, Khachik, 2009). A number of different stationary phases have been applied, including normal-phase (NP) and reversed-phase (RP) materials, and thousands of methods have been published. Normal-phase HPLC of xanthophylls is usually carried out using silica, and the mobile phase usually consists of an apolar hydrocarbon solvent such as hexane to which a more polar solvent such as methyl-*tert*-butyl ether (MTBE), acetone, isopropanol, or methanol is added as modifier. Absorption affinities and hence retention times increase in the order monohydroxy < dihydroxy < trihydroxy. Xanthophyll epoxides are retained more strongly than the corresponding simple hydroxycarotenoids. Normal-phase HPLC, employing a silica-based nitrile-bonded column, has been applied for the separation of lutein, zeaxanthin, and their *trans/cis*-isomers in extracts from selected fruits, vegetables, and pasta products (Humphries and Khachik, 2003). Reversed-phase separation on C_{18} column has been widely used for xanthophyll separation (Craft, 1992a) because of the weak hydrophobic interactions with the analytes and compatibility with most carotenoid solvents and the polarity range of carotenoids. The majority of carotenoid separations on C_{18} column have been carried out on spherical particles of 3 or 5 microns in diameter. Monomeric nonendcapped column provided

better resolution for xanthophylls; polymeric columns showed better selectivity for geometrical isomer separation. The most important properties in selecting the mobile phase are polarity, viscosity, volatility, and toxicity; the most used solvents for carotenoid separation on reversed-phase C_{18} columns are acetonitrile and methanol, and frequently a stronger less polar solvent is added as a modifier, such as tetrahydrofuran (THF) or MTBE. In reversed-phase separations, xanthophylls are expected to elute in the order of decreasing polarity, with generally a running order reversed with respect to normal phases, although, interestingly, the elution order of lutein and zeaxanthin is generally the same in NP and RP, with lutein eluting before zeaxanthin; xanthophyll *Z* isomers usually elute later than all-*E* isomers in both NP and RP systems as well. Lutein and violaxanthin esters (fatty acids esters), together with free lutein and violaxanthin, have been separated in acorn squash (Khachik and Beecher, 1988), using a C_{18} column and acetonitrile/methanol (9:1, solvent A) and hexane/dichloromethane/methanol (4.5:4.5:1, solvent B) as the mobile phase, within 50 min, as shown in Figure 14.4; the order of elution for the different carotenoid classes was: free xanthophylls < xanthophyll monoesters < carotenes < xanthophyll diesters.

Since its first application to the separation of carotenoid isomers (Sander et al., 1994), reversed-phase C_{30} columns are now the most popular choice for carotenoid separation (Oliver et al., 2000; Sander et al., 2000). In comparison with classical C_{18} stationary phase, the use of the much more hydrophobic C_{30} phase has shown a better resolving power for carotenoids; in fact, the C_{30} stationary phase provides sufficient phase thickness to enhance the interaction with long-chain molecules. The order of elution cannot be always correlated between the C_{18} and C_{30} stationary phases. Generally, selectivity is strongly influenced by certain stationary phase properties, including bond density, stationary phase chemistry (i.e., monomeric vs. polymeric surface modification), and alkyl chain length; moreover, the separation of the relatively polar xanthophylls is also influenced by xylanol activity. Molecular shape also seems to greatly influence the selectivity, and geometrical isomers are well resolved. Selectivity is strongly dependent on the choice of the mobile phase modifier, and the separation of polar carotenoids is also influenced by the presence of water. The relatively less polar carotenoid esters of β-cryptoxanthin were separated in mandarin essential oil using a polymeric C_{30} column and a simple gradient of nonaqueous mobile phase, consisting of methanol and MTBE (Giuffrida et al., 2006a); the same simple binary gradient was applied in xanthophyll separation, together with other pigments, in pistachio kernels (Giuffrida et al., 2006b). Various hydroxylated xanthophylls, together with other pigments, were separated in a

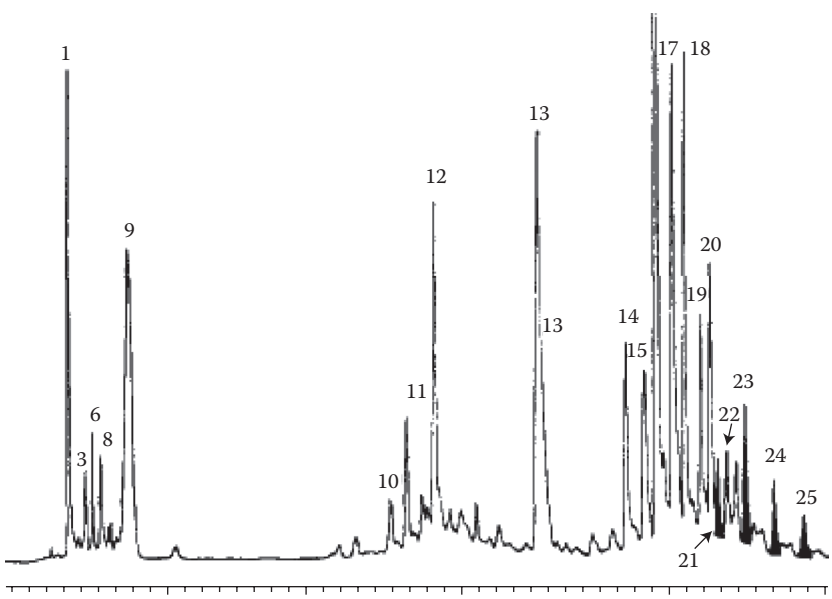


FIGURE 14.4 The HPLC profile of the native carotenoids present in raw acorn squash analyzed using a C_{18} column and acetonitrile/methanol (9:1) as the mobile phase. (Reprinted from Khachik, F. and Beecher, G. R. 1988. *Journal of Agricultural and Food Chemistry* 36: 929–937. With permission.)

nonaqueous matrix such as olive oil, by the use of a C_{30} column and a binary gradient consisting of (a) MeOH/MTBE/H_2O (90:7:3) and (b) MeOH/MTBE/H_2O (7:90:3) (Giuffrida et al., 2007). Orange juice is considered one of the most complex natural matrix for carotenoid composition and therefore carotenoid separation; a non-endcapped polymeric C_{30} column was applied to carotenoid separation in saponified orange juice using a water, methanol, and MBTE gradient elution (Rouseff et al., 1996) and the analysis of carotenoids in orange juice has also been reviewed (Melendez-Martinez et al., 2007). Astaxanthin esters were identified in extracts of commercial shrimps and dried microalgae samples (Breithaupt, 2004). Triacontyl-bonded (C_{30}) stationary phases have also recently been applied in the separation of a standard mixture of epoxycarotenoid isomers (Melendez-Martinez et al., 2009), by using a gradient elution of methanol, MTBE, and water. Sometimes, due to the high complexity of some samples containing carotenoids, conventional LC cannot have enough separation power; serial connection of several columns has been proposed as an alternative to LC for the first time in the separation of carotenoids in saponified red orange essential oil (Herrero et al., 2008). The applicability of connecting two C_{18} columns and also of connecting two C_{30} columns for this separation was demonstrated. The serial coupling of two C_{30} columns significantly increased the separation power, resolution, and peak capacity; in fact a peak capacity of 79 was obtained when using two C_{30} serial-coupled columns, compared to 61 achieved using a single column. This novel analytical approach based on using serial-coupled conventional LC column was applied to the study of both the native and the saponified carotenoid composition of orange juice (Dugo et al., 2008a). The increase in the resolution and separation power obtained when using two serial-coupled C_{30} columns was demonstrated, and significant increases in peak capacity have been achieved. By using this new methodology, 45 different carotenoids have been tentatively identified. Among them, several violaxanthin diesters have been directly identified in orange juice for the first time. The main xanthophylls in orange juice were violaxanthin, lutein, luteoxanthin, antheraxanthin, and β-cryptoxanthin. In Figure 14.5, the HPLC profiles of the saponified orange juice, analyzed using (a) one and (b) two C_{30} serial-coupled columns, are shown.

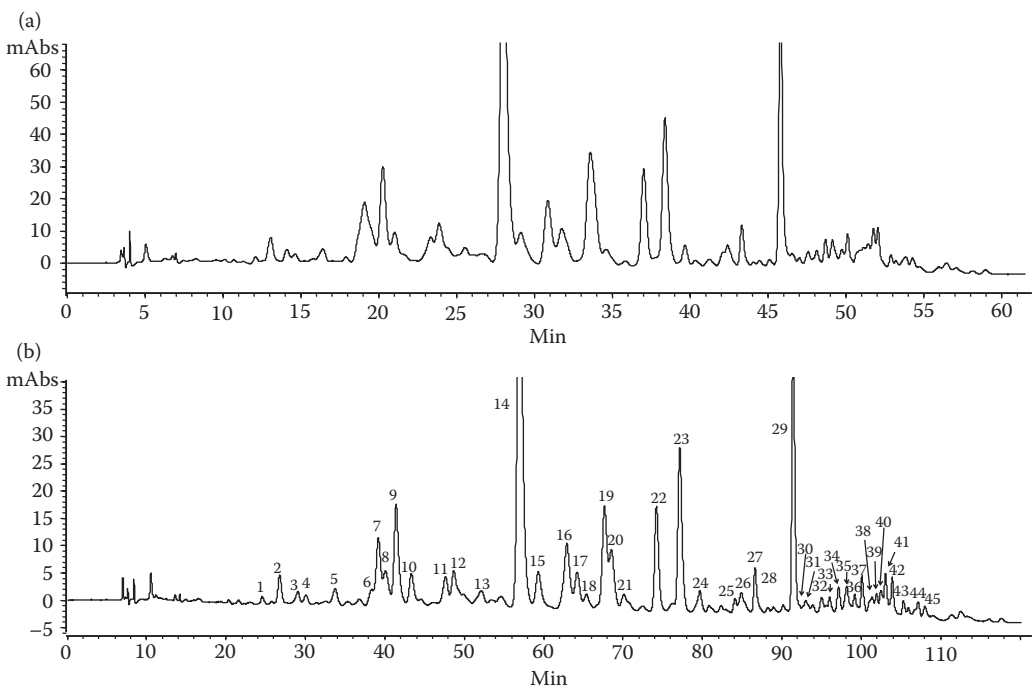


FIGURE 14.5 The HPLC carotenoid profiles of the saponified orange juice analyzed using (a) one and (b) two C_{30} serial-coupled columns. (Reprinted from Dugo, P. et al. 2008a. *Journal of Separation Science* 31: 2151–2160. With permission.)

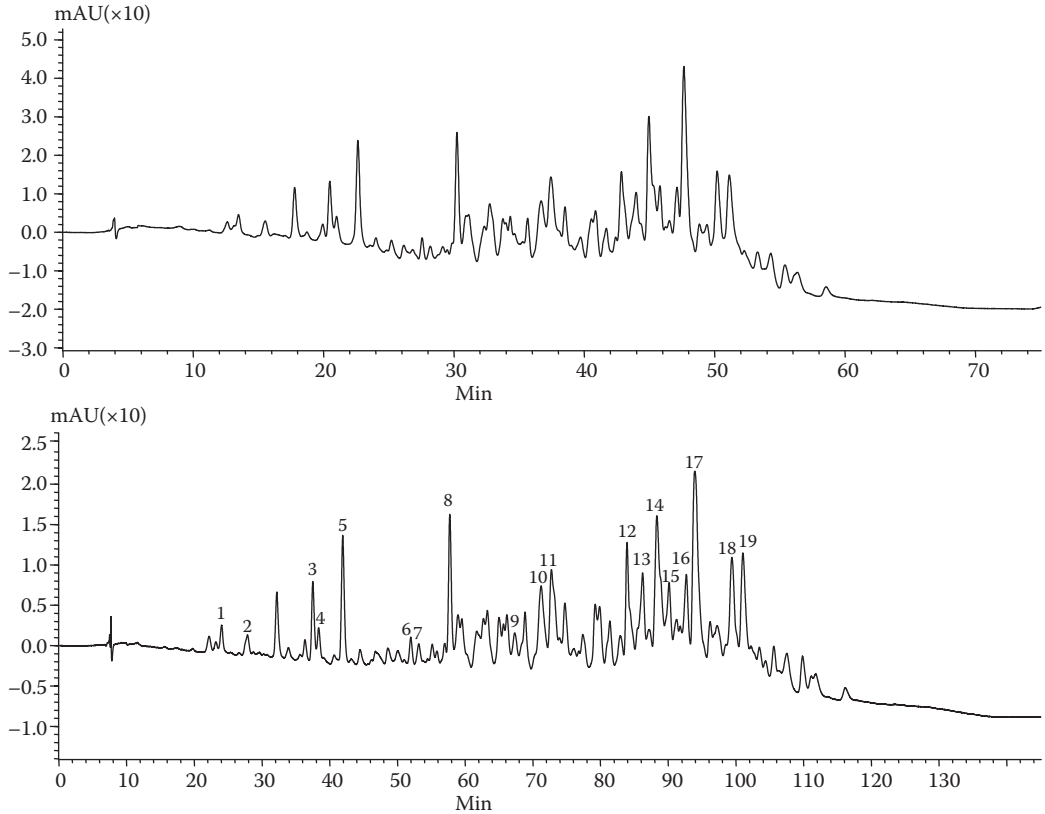


FIGURE 14.6 The HPLC carotenoid profiles of native orange juice analyzed using (a) one and (b) two C_{30} serial-coupled columns. (Reprinted from Dugo, P. et al. 2008a. *Journal of Separation Science* 31: 2151–2160. With permission.)

In Figure 14.6, the HPLC profiles of native orange juice, analyzed using (a) one and (b) two C_{30} serial-coupled columns, are shown.

In nature, normally one optical isomer of a carotenoid occurs specifically; however, in some cases, mixtures have been observed. The separation of the enantiomers can be carried out in two ways: after the conversion into diastereoisomers and their subsequent separation or by the use of chiral stationary phases. The knowledge of the stereoisomeric ratios may help identify the source of the aquaculture product (whether wild or farmed). The chiral separation of the enantiomeric forms of astaxanthin produced by microalgae and yeast was reported (Grewe et al., 2007) and in commercial supplements (Wang et al., 2008). The separation of astaxanthin enantiomers is of great interest since astaxanthin is widely used as a functional food additive in human nutrition.

14.3.4.3 Comprehensive (LC × LC) Liquid Chromatography

One-dimensional liquid chromatography is widely applied to the analysis of real-world samples in several fields. However, such monodimensional analytical method does not provide sufficient resolving power in those cases where the sample matrix is very complex. A possible solution to this problem can be the use of multidimensional separation mechanisms (Dugo et al., 2008b; Francois et al., 2009). The most important feature of comprehensive multidimensional separation is that the entire injected sample is subjected to two distinct separation steps, thus greatly enhancing the resolving power. In the comprehensive 2D mode, all sample compounds eluting from the first dimension are subjected to separation in the second dimension. The whole effluent from the first dimension is transferred into the second dimension separation system in subsequent aliquot fractions collected in multiple repeated cycles; the columns

of two HPLC systems are connected via a transfer device (called interface, modulator, or switching valve) located between them. The interface cuts the fractions of the primary column effluent and releases them onto the secondary column by continuously and alternatively switching between the sample collection and sample elution mode using two different sample loops of identical volume, one of which collects the effluent from the first dimension column, while the previous effluent fraction contained in the second loop is transferred onto the second dimension column and is subjected to separation; the multiport switching valve (interface) is operated at a precise frequency called the modulation time. The fraction injected onto the secondary column should be completely analyzed before the successive transfer occurs. The second dimension analysis time should be at least equal or less than the duration of the modulation period. A microcolumn and a low mobile phase flow rate in the first dimension are frequently used to transfer small sample fraction volumes to the second dimension, where a short column is operated at a high flow rate for fast separation. Selectivity of the columns used in the two dimensions must be different to attain maximum gain in peak capacity, and therefore reduction of components overlaps. The best results are achieved in the so-called orthogonal systems with noncorrelated retention times in both dimensions. Ideally, the total peak capacity is equal to the product of the peak capacities in the first and in the second dimensions in fully orthogonal 2D systems with noncorrelated selectivity in the first and in the second dimensions. The record of the detector at the outlet from the second dimension column is transformed into a 2D-chromatogram, which is usually represented as a contour plot showing the separation time in the second dimension versus the separation time in the first dimension. In the bidimensional contour plot, each component is represented as an ellipse-shaped peak, defined by double-axis retention time coordinates. The formation of a chemical class pattern on the 2D space plane offers great potential for the identification of "unknown" peaks. The first application of comprehensive (LC × LC) to elucidate the carotenoid pattern in saponified sweet orange essential oils was reported by (Dugo et al., 2006). In this study, a silica micro-HPLC column was used in the first dimension and a monolithic C_{18} column was used in the second dimension, with photodiode array detection. Under normal-phase conditions, the components were separated according to their increasing polarity and under reversed-phase conditions, carotenoids were eluted according to their increasing hydrophobicity and decreasing polarity. Figure 14.7 shows the comprehensive LC × LC chromatogram, plotted at 450 nm, of the carotenoid fraction of saponified sweet orange essential oil.

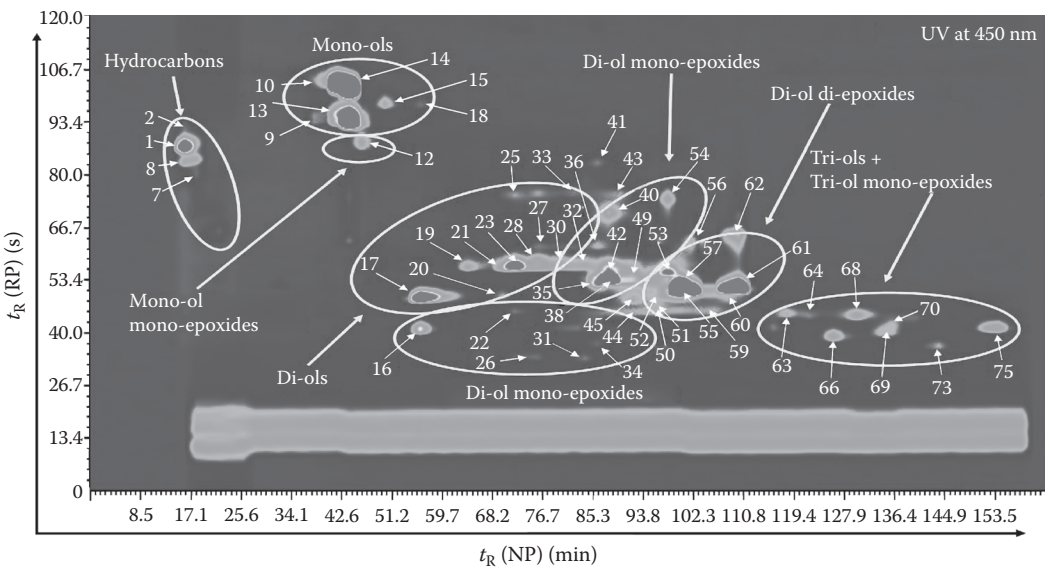


FIGURE 14.7 The comprehensive (LC × LC) 2D contour plot, at 450 nm, of the carotenoid fraction of saponified sweet orange essential oil. (Reprinted from Dugo, P. et al. 2006. *Analytical Chemistry* 78: 7743–7750. With permission.)

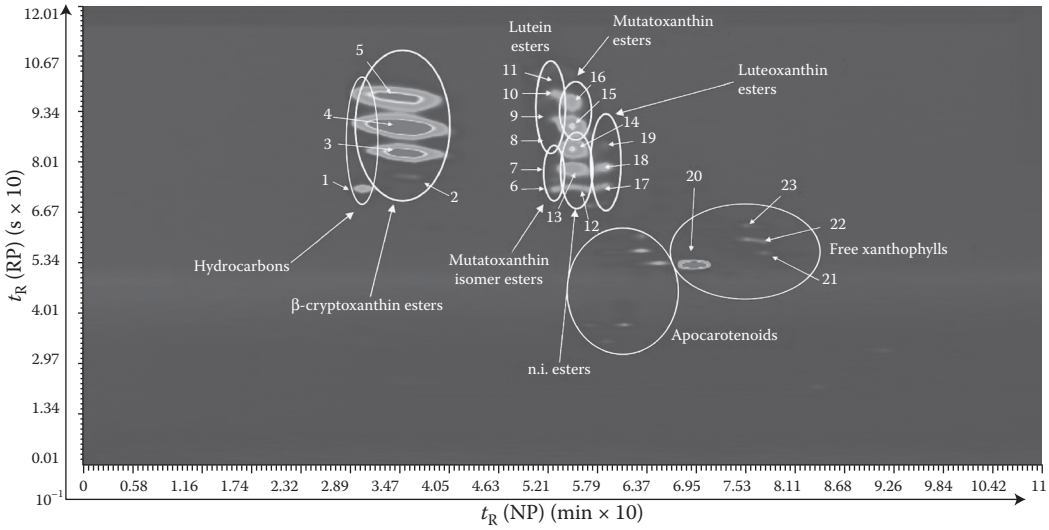


FIGURE 14.8 The comprehensive (LC × LC) 2D contour plot, at 450 nm, of the native carotenoid composition in mandarin essential oil. (Reprinted from Dugo, P. et al. 2008c. *Journal of Chromatography A* 1189: 196–206. With permission.)

Fifty-seven peaks have been detected in the two-dimensional space (2D plot), which also clearly shows the separation of carotenoids in different chemical classes, thus identifying a bidimensional pattern where the different groups are marked. A completely new comprehensive LC × LC method was developed by Dugo et al. (2008c) to determine both the free carotenoids and the carotenoid esters present in intact mandarin essential oil by NPLC × RPLC coupled to DAD and mass spectrometry (MS) detectors. In this case, the first separation was performed with a microbore cyano column, while the second dimension separation was performed using a C_{18} monolithic column. In Figure 14.8, the 2D plot corresponding to the carotenoid ester analysis is shown. In this figure, the compounds separated as well as the different groups are marked. β-Cryptoxanthin and carotenoid monoesters were detected in the native composition of mandarin essential oil.

A further step in the carotenoid analyses of citrus products was reported by Dugo et al. (2008d). In this study, the native carotenoid composition in a very complex matrix such as red orange essential oil was reported. Both the free carotenoids and the carotenoid monoesters and diesters were determined. To achieve this goal, a comprehensive LC × LC-DAD/APCI-MS method was developed based on a cyano microbore column in the first dimension and a monolithic C_{18} column in the second dimension. By using this novel analytical technique together with the use of DAD and APCI-MS detectors, it was possible to identify in the sample, without the need of any pretreatment, 40 different carotenoids. Among them, 16 carotenoid monoesters were identified, mainly β-cryptoxanthin palmitate ($C_{16:0}$), myristate ($C_{14:0}$), and laureate ($C_{12:0}$) as well as several lutein, violaxanthin, antheraxanthin, and luteoxanthin monoesters. Moreover, 21 carotenoid diesters composed of several antheraxanthin, luteoxanthin, violaxanthin, and auroxanthin diesters were found in the native carotenoid composition of the orange oil. The main carotenoid diesters were the laureate–palmitate ($C_{12:0}$, $C_{16:0}$), myristate–palmitate ($C_{14:0}$, $C_{16:0}$), and di-palmitate ($C_{16:0}$, $C_{16:0}$) diesters, although other diesters were also identified. Besides, two different free carotenes, ζ-carotene and phytofluene, and a xanthophyll, lutein, were also determined. The typical 2D plot of the intact red orange essential oil is shown in Figure 14.9. In this figure, the different peaks are numbered and their assignments can be found in Tables 14.1 and 14.2.

The elution order observed in the second dimension separation corresponded to a typical behavior in which the more hydrophobic compounds are retained more and elute later. On the other hand, from the identification carried out and the 2D plot showed in Figure 14.9, it is possible to deduce the elution order in the normal-phase separation carried out in the first dimension. It can be appreciated that the first

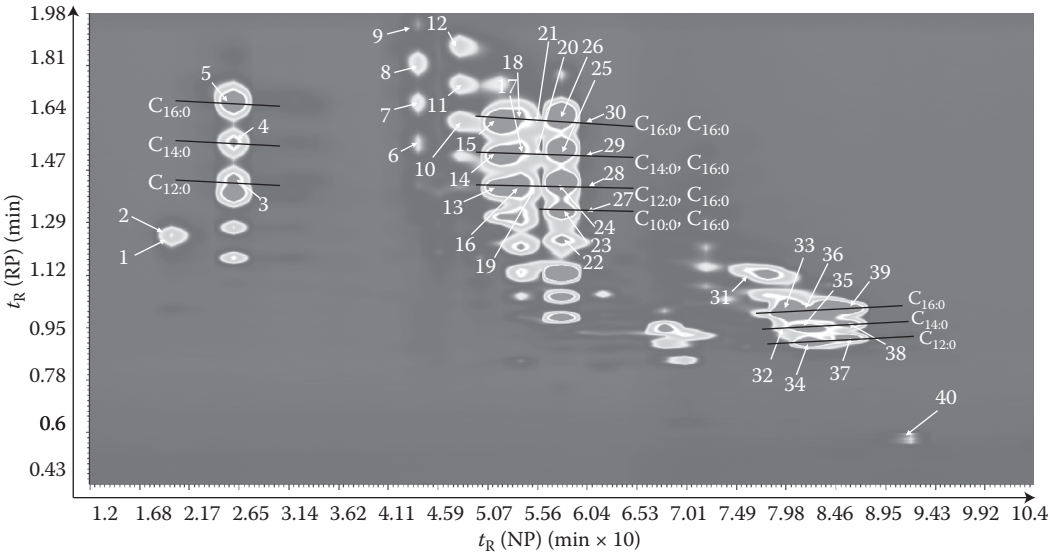


FIGURE 14.9 The comprehensive (LC × LC) 2D contour plot, at 450 nm, of the native carotenoid composition in red orange essential oil. (Reprinted from Dugo, P. et al. 2008d. *Journal of Agricultural and Food Chemistry* 56: 3478–3485. With permission.)

TABLE 14.1

UV–Vis and MS Information and Identification of the Free Carotenoids and Carotenoid Monoesters Found in Red Orange Essential Oil

ID	Retention Time (min)	Identification	UV/Vis Maxima	$[M + H]^+$	$[M + H\text{-}FA]^+$
1	21.2	ζ-Carotene	381, 401, 426	541	
2	21.2	Phytofluene	334, 350, 369	543	
3	27.4	β-Cryptoxanthin-laureate ($C_{12:0}$)	426s, 452, 480	735	535
4	27.6	β-Cryptoxanthin-myristate ($C_{14:0}$)	426s, 452, 480	763	535
5	27.7	β-Cryptoxanthin-palmitate ($C_{16:0}$)	426s, 452, 480	791	535
6	45.6	Lutein-laureate ($C_{12:0}$)	420, 448, 474	733	533
7	45.7	Lutein-myristate ($C_{14:0}$)	420, 448, 474	761	533
8	45.8	Lutein-palmitate ($C_{16:0}$)	420, 448, 474	789	533
9	45.9	Lutein-stearate ($C_{18:0}$)	420, 448, 474	835	533
31	77.1	Antheraxanthin-palmitate ($C_{16:0}$)	422, 443, 472	823	567
32	80.9	Luteoxanthin (b)-myristate ($C_{14:0}$)	401, 420, 446	811	583
33	81.0	Luteoxanthin (b)-palmitate ($C_{16:0}$)	401, 420, 446	839	583
34	82.9	Luteoxanthin (a)-laureate ($C_{12:0}$)	401, 423, 448	783	583
35	82.9	Luteoxanthin (a)-myristate ($C_{14:0}$)	401, 423, 448	811	583
36	83.0	Luteoxanthin (a)-palmitate ($C_{16:0}$)	401, 423, 448	839	583
37	86.9	Violaxanthin-laureate ($C_{12:0}$)	419, 440, 468	783	583
38	87.0	Violaxanthin-myristate ($C_{14:0}$)	419, 440, 468	812	583
39	87.0	Violaxanthin-palmitate ($C_{16:0}$)	419, 440, 468	839	583
40	92.6	Lutein	422, 446, 474	551[a]	

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[a] $[M + H\text{-}H_2O]^+$.

TABLE 14.2

UV–Vis and MS Information and Identification of the Carotenoid Diesters Found in Red Orange Essential Oil

ID	Retention Time (min)	Identification	UV/Vis Maxima	$[M + H]^+$	$[M + H\text{-}FA_1\text{-}FA_2]^+$ and Other Main Ions
10	49.6	Antheraxanthin-laureate-palmitate ($C_{12:0}$, $C_{16:0}$)	422, 444, 469	1005	531[b], 805 ($[M + H\text{-}C_{12:0}]^+$)
11	49.8	Antheraxanthin-myristate-palmitate ($C_{14:0}$, $C_{16:0}$)	422, 444, 469	1033	531[b], 777 ($[M + H\text{-}C_{16:0}]^+$)
12	49.9	Antheraxanthin-di-palmitate ($C_{16:0}$, $C_{16:0}$)	422, 444, 469	1061	531[b], 805 ($[M + H\text{-}C_{16:0}]^+$)
13	53.4	Luteoxanthin (a)-laureate-palmitate ($C_{12:0}$, $C_{16:0}$)	397, 420, 446	1021	565, 821 ($[M + H\text{-}C_{12:0}]^+$)
14	53.5	Luteoxanthin (a)-myristate-palmitate ($C_{14:0}$, $C_{16:0}$)	397, 420, 446	1049	565, 803 ($[M + H\text{-}C_{14:0}]^+$)
15	53.6	Luteoxanthin (a)-di-palmitate ($C_{16:0}$, $C_{16:0}$)	397, 419, 446	1059[a]	565, 803 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$)
16	55.4	Violaxanthin-laureate-palmitate ($C_{12:0}$, $C_{16:0}$)	415, 437, 466	1003[a]	565, 765 ($[M + H\text{-}C_{16:0}]^+$), 821($[M + H\text{-}C_{12:0}]^+$)
17	55.5	Violaxanthin-myristate-palmitate ($C_{14:0}$, $C_{16:0}$)	415, 437, 466	1049	565, 775 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$), 803 ($[M + H\text{-}C_{14:0}\text{-}H_2O]^+$)
18	55.7	Violaxanthin-di-palmitate ($C_{16:0}$, $C_{16:0}$)	415, 437, 466	1059[a]	565, 803 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$)
19	57.4	Auroxanthin-laureate-palmitate ($C_{12:0}$, $C_{16:0}$)	381, 401, 425	1021	565, 1003 ($[M + H\text{-}H_2O]^+$), 803 ($[M + H\text{-}C_{12:0}\text{-}H_2O]^+$), 747 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$)
20	57.5	Auroxanthin-myristate-palmitate ($C_{14:0}$, $C_{16:0}$)	381, 401, 425	1049	565, 1031 ($[M + H\text{-}H_2O]^+$), 803 ($[M + H\text{-}C_{14:0}\text{-}H_2O]^+$), 775 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$)
21	57.6	Auroxanthin-di-palmitate ($C_{16:0}$, $C_{16:0}$)	381, 402, 426	1078	565, 821 ($[M + H\text{-}C_{16:0}]^+$)
22	59.2	Luteoxanthin (b)-di-laureate ($C_{12:0}$, $C_{12:0}$)	401, 420, 447	965	565, 765 ($[M + H\text{-}C_{12:0}]^+$)
23	59.3	Luteoxanthin (b)-caproate-palmitate ($C_{10:0}$, $C_{16:0}$)	400, 420, 446	993	565, 975 ($[M + H\text{-}H_2O]^+$)
24	59.4	Luteoxanthin (b)-laureate-palmitate ($C_{12:0}$, $C_{16:0}$)	398, 420, 446	1003[a]	565, 747 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$), 803 ($[M + H\text{-}C_{12:0}\text{-}H_2O]^+$)
25	59.5	Luteoxanthin (b)-myristate-palmitate ($C_{14:0}$, $C_{16:0}$)	398, 420, 446	1049	565, 1031 ($[M + H\text{-}H_2O]^+$), 803 ($[M + H\text{-}C_{14:0}\text{-}H_2O]^+$), 775 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$)
26	59.6	Luteoxanthin (b)-di-palmitate ($C_{16:0}$, $C_{16:0}$)	400, 420, 446	1077	565, 803 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$)
27	61.3	Auroxanthin isomer-caproate-palmitate ($C_{10:0}$, $C_{16:0}$)	382, 403, 429	993	565, 975 ($[M + H\text{-}H_2O]^+$)
28	61.4	Auroxanthin isomer-laureate-palmitate ($C_{12:0}$, $C_{16:0}$)	382, 403, 429	1021	565, 1003 ($[M + H\text{-}H_2O]^+$), 747 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$), 803 ($[M + H\text{-}C_{12:0}\text{-}H_2O]^+$)
29	61.5	Auroxanthin isomer-myristate-palmitate ($C_{14:0}$, $C_{16:0}$)	382, 403, 429	1049	565, 1033 ($[M + H\text{-}H_2O]^+$), 775 ($[M + H\text{-}C_{16:0}\text{-}H_2O]^+$)
30	61.6	Auroxanthin isomer-di-palmitate ($C_{16:0}$, $C_{16:0}$)	382, 403, 429	1077	565, 1059 ($[M + H\text{-}H_2O]^+$), 821 ($[M + H\text{-}C_{16:0}]^+$)

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[a] $[M + H\text{-}H_2O]^+$.

[b] $[M + H\text{-}FA_1\text{-}FA_2\text{-}H_2O]^+$.

eluted compounds were the hydrocarbons. Regarding the rest of the compounds, it could be thought that according to their polarity, diesters would elute faster than monoesters. However, this was only the case when considering a particular carotenoid. Thus, the carotenoid structure was found to be of great importance since the presence of free polar groups had a stronger influence than the presence of two fatty acids. Therefore, the elution order of the several mono- and diesters found corresponded to a combination between their esterification degree and the polarity of the carotenoid bound to their structure. An investigation on the native carotenoid pattern of orange juices of different varieties studied by LC × LC-DAD/APCI-IT-TOF-MS was reported by Dugo et al. (2009). As an example of these separations, Figure 14.10 shows the contour plot (450 nm) of the comprehensive HPLC analyses of the carotenoid esters present in native orange juice of the (a) Valencia and (b) Moro varieties.

In this work, the ability of the comprehensive LC method to separate intact epoxycarotenoid esters in real matrices such as orange juices and its usefulness to estimate some juice quality-related parameters were demonstrated for the first time.

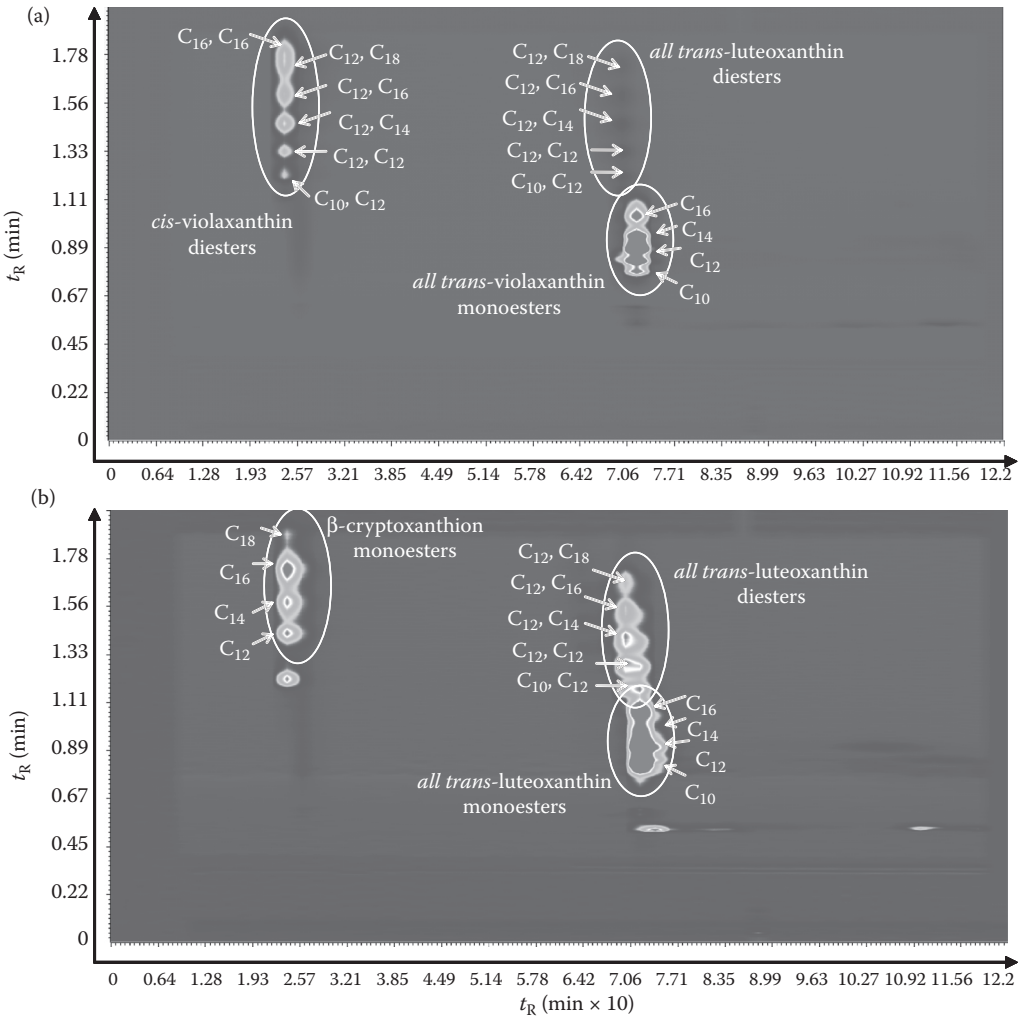

FIGURE 14.10 The comprehensive (LC × LC) 2D contour plot, at 450 nm, of the carotenoid esters present in native orange juice of the (a) Valencia and (b) Moro varieties. (Reprinted from Dugo, P. et al. 2009. *Journal of Separation Science* 32: 973–980. With permission.)

14.3.5 Identification and Quantification

14.3.5.1 Identification

The carotenoid structure elucidation has been performed by the application of several spectroscopic techniques such as UV/Visible spectroscopy, circular dichroism, infrared spectroscopy, resonance Raman spectroscopy, NMR spectroscopy, mass spectrometry, and x-ray crystallography (Britton et al., 1995). Carotenoids separated by liquid chromatography such as HPLC can be identified online based on a combination of the congruence of their retention times with known pure carotenoids, their spectroscopic characteristics provided by a UV–Vis detector such as a photodiode array detector (PDA) and their mass spectra recorded by a mass spectrometry detection system. Comprehensive tables listing the absorption maxima, absorption coefficients, mass spectra data, circular dichroism data, and NMR references of carotenoids are available in the literature (Britton et al., 2004). The absorption and spectroscopic data are characteristic of each carotenoid separated and measured under similar conditions. The typical UV–Vis spectra present three main bands (I, II, III) at three different wavelengths. The spectroscopic data can be used for determining various spectral fine structural parameters such as the *Q* ratio, %III/II, and $\%D_B/D_{II}$. The *Q* ratio is defined as the quotient between band II (λ_{max}) and the *cis* peak band, and it is used to identify different *cis*-isomers of carotenoids; the appearance of the new *cis*-band usually occurs around 142 nm below the longest-wavelength absorption maximum (III) in the spectrum of the all-*trans* compound, when measured in hexane. The intensity of the *cis*-band is greater as the *cis* double bond is nearer to the center of the molecule. The %III/II is the percentage of the quotient between band III and band II (λ_{max}), taking the through line between the two bands as the baseline, and it is used to identify carotenoids. The $\%D_B/D_{II}$ is the percentage of the quotient between the *cis* peak band and band II (λ_{max}), and it is used to help in the identification of the *cis*-isomers of carotenoids. Mass spectrometry provides accurate mass measurements for carotenoids and important fragment ions useful for structure elucidation. Electron impact (EI), chemical impact (CI), and fast atom bombardment (FAB) are useful ionization techniques, and the use of HPLC-MS has greatly increased the use of atmospheric pressure chemical ionization (APCI) mode. Typical carotenoid fragments occur at $[M\text{-}92]^+$ and $[M\text{-}106]^+$, corresponding to the loss of toluene or xylene. Other possible important fragments are $[M\text{-}18]^+$ for loss of water, $[M\text{-}133]^+$ for arylcarotenoids, $[M\text{-}73]^+$ for methoxycarotenoids, $[M\text{-}165]^+$, $[M\text{-}205]^+$ ($[M\text{-}181]^+$ and $[M\text{-}221]^+$ if a C-3 hydroxy group is also present), $[M\text{-}80]^+$ for 5,6-epoxides and 5,8-epoxides, and $[M\text{-}56]^+$ for ε-ring carotenoids.

14.3.5.2 Quantification

Because carotenoids absorb visible or, in a few cases, UV light strongly, and obey to the Beer–Lambert law, they are normally determined quantitatively by spectrophotometry. Accurate and reproducible quantitative analyses of carotenoid content are obtained by HPLC. For well-resolved peaks in a chromatogram, the peak areas, and the peaks heights, are to a good approximation proportional to the amount of the respective compounds. Both the external-standard calibration method and the internal-standard calibration method are used in the quantitative HPLC carotenoid quantification.

14.4 Conclusions

There is a general growing interest in carotenoids because of their role in human health and nutrition.

In addition to the role of vitamin A precursors of many carotenoids, many important biological activities have been associated with these molecules, such as their singlet oxygen radical scavenging capability. The importance of reliable analytical techniques for their determination is of great interest. High-performance liquid chromatography is now considered the best method for carotenoid separation and quantification. Some recent advances in analytical separations applied to the carotenoids for the first time, such as the use of serial connected columns, and the use of comprehensive liquid chromatography have been described in this chapter. The use of combined techniques such as HPLC-PDA-MS, and HPLC-NMR, is also becoming of great interest for both carotenoid separation and identification.

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Section IV

Phenolic Compounds

15

Flavonoids: Flavonols, Flavones, and Flavanones

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15.1 Introduction

Flavones (including 3-hydroxyl flavones, also known as flavonols) and flavanones are important phytochemicals in food products. They normally occur as *O*-glycosidic combinations with mono- and disaccharides, although combinations with a higher number of sugars are also frequent. *C*-Glycosidic combinations have also been reported in different food products (cereals, grains, Swiss chard, sweet peppers, etc.). Glucose, galactose, rhamnose, arabinose, and xylose are the main monosaccharides that conjugate with flavonoid aglycones. In addition, uronic acids, mainly glucuronic acid, are often found in naturally occurring flavonoids (strawberries, grapes, spinach, etc.). The more relevant disaccharides are sophorosides [glucosyl(1 → 2)glucosides], gentiobiosides [glucosyl(1 → 6)glucosides], rutinosides [rhamnosyl(1 → 6)glucosides], and neohesperidosides [rhamnosyl(1 → 2)glucosides], although sambubiosides, glucosyl-galactosides, etc. are also found. Acylation of the flavonoid glycosides with aliphatic monocarboxylic (acetic, malic) or dicarboxylic (malonic, tartaric, etc.) acids, or aromatic acids (mainly hydroxycinnamic acids, such as caffeic, *p*-coumaric, ferulic, and sinapic) is also frequent. In some cases, they can also be conjugated with sulfate (as in the case of palm dates). The conjugation with glucuronides and/or sulfates is particularly relevant in the metabolism occurring *in vivo*, as a general way of detoxification using the Phase II enzyme system. Flavone and flavanone aglycones have also been found in different food products. For instance, they are present as polymethoxyflavones in citrus fruits (sinensetin, nobiletin, tangeretin, etc.), and as different flavone and flavanone combinations in propolis (pinocembrin, pinobanksin, chrysin, and galangin), a resinous by-product collected by bees from trees.

Flavonols are the most common flavonoids in plant-derived food products, and they include combinations of myricetin, quercetin, and kaempferol and their methyl ethers (isorhamnetin). Flavonols are relevant in onions, apples, green tea, grapes, and wine, although they are also present in many different food products. Flavones are less frequent in foods, but they occur in sweet peppers, celery, and many herbs. Flavanones are relevant in citrus fruits, where they occur in large amounts. Some food products produce large amounts of specific flavones. This is the case of the methylated flavonol glycosides in spinach (i.e., spinacetin glycosides). These are relevant as they can be ingested in the diet in relevant amounts, and they have relevant roles in the sensory properties of food products as they can be bitter.

15.2 Extraction of Flavones and Flavanones

15.2.1 Solvent Choice

Hydroalcoholic mixtures are generally used for the extraction of flavones and flavonols from different plants and food products. Both ethanol and methanol can be used. The water percentage in the mixture depends on the material to be extracted. In general, mixtures methanol/water 7:3, v:v are recommended for dried materials, and 8:2, v:v for fresh products (as they already provide some water to the extraction mixture). In general, a solvent/plant ratio between 5/1 and 10/1 is recommended, and the recovery with the first extraction can reach 90–95% of the plant flavonoid content. In some cases, specific solvents are needed for the complete extraction of flavonoids. The extraction can be carried out by maceration at room temperature, with or without stirring, at low temperatures (in an ice bath) when using fresh material that can have polyphenol oxidases or other enzymes that can degrade flavonoids, and at high temperatures (60–80°C) to speed up and enhance the extraction and inactivate the degradative enzymes. In some cases, vacuum or an inert atmosphere (N_2) can be used to increase the stability and avoid the degradation. For preparation of flavonoid extracts from fresh plant materials (leaves, fruits, etc.), the

extraction can be achieved using an Ultra-Turrax with the sample in an ice bath, using pure methanol (or methanol–water 8:2) or acetonitrile added of 0.01M NaF to prevent the action of polyphenol oxidases and degradation producing brown polymers (Gil et al., 1999; Marín et al., 2004). Freeze-drying generally enhances flavonoid extraction, as the plant membranous structures are destroyed during the freezing process. Ultrasound or microwave systems can be used to enhance the flavonoid extraction.

In the case of citrus flavanones, particularly hesperidin, these have very low solubility in hydroalcoholic mixtures, and even in pure methanol or acetonitrile. In this case, the extraction with dimethylsulfoxide (DMSO) or dimethyl formamide (DMF) is necessary to solubilize the flavanones leading to a 95% recovery. There is one limitation, however, as DMSO has a high boiling point and therefore the concentration of the obtained extracts is often very difficult.

15.2.2 Extraction of Plant Surface Lypophylic Flavones

This requires specific conditions that can lead to quite purified extracts, recovering only those compounds that are present on the plant surfaces. For instance, some flavones occurring in aromatic and medicinal plants (cirsimaritin, xanthomicrol, nobiletin, etc.) are lypophylic aglycones that are excreted to the plant surface together with waxes, resins, and essential oils. These flavonoids can be extracted with apolar solvents using the Soxhlet as the extraction system. In some cases, the whole plant material (preferably fresh, but also dry unground plant material) can be washed with an apolar solvent for a short period of time (30–50 s) to wash out exclusively the surface compounds without extraction of the intracellular constituents (Wollenweber, pers. comm.). This method is useful for the extraction of highly methylated flavonoid aglycones from Labiatae and Compositae herbs, among others, that have also been introduced in the nutraceuticals and botanicals market.

15.2.3 Large-Scale Preparative Extractions

For subsequent isolation of fractions and isolation of flavonoid compounds, it is advisable to extract using a Soxhlet equipment that combines high temperature and solvent recirculation for a quantitative extraction with smaller solvent volumes. In this case, a series of solvents can be used sequentially in an order of increasing polarity. A good strategy for flavonoids is a first extraction with apolar solvents (*n*-hexane, petrol, etc.) to extract fats, chlorophylls, and lypophylic compounds. In this extract, it is possible to find lypophylic flavones (isoprenylated or fully methylated flavones), although these are quite uncommon flavonoids. A second extraction with methanol will extract most flavonoids (Ferreres et al., 1997).

15.2.4 Liquid–Liquid Fractionation

The hydroalcoholic extracts can then be fractionated by liquid–liquid extraction systems. The first step would be alcohol removal using vacuum rotary evaporators at low temperatures (40°C) that allow the removal of methanol or ethanol, leaving the water extract. The remaining water extract can then be successively extracted using ethylic ether (to extract flavonoid aglycones), ethyl acetate (to recover flavone monoglycosides and the remaining highly hydrolylated aglycones), and *n*-butanol (to extract flavone *C*-glycosides and poly-*O*-glycosylated flavones). This sequential extraction is quite useful as it renders extracts with different flavonoid types. Acylated flavonoid glycosides with hydroxycinnamic acids linked to the flavonoid-*O*-glycoside structure often behave as aglycones in terms of the extracting behavior, and could be present in the lypophylic extracts.

15.3 Purification of Flavones and Flavanones

15.3.1 Extract Purification and Fractionation

The crude extracts obtained can be used for the extraction and purification of flavonoids. In this case, the extracts must be submitted to different fractionation processes. The first process can be a liquid–liquid

extraction, described above, that consists of a partition of the extract components into two immiscible solvents. The different flavonoids are extracted depending on their polarity. The isolation and purification of flavonoids from some sugar-rich matrices (honey, fruit jams, etc.) that also contain other polar compounds is very complicated due to the formation of solvent interphases during the liquid–liquid extraction process. In these cases, several nonionic polymeric resins (Amberlite XAD) can be used to overcome this problem. In a comparative study of different polymeric resins for flavonoid extraction, polystyrene resins (XAD-2, XAD-4, and XAD-16) were found to be more useful for fractionation than the polyacrylic resins (XAD-7 and XAD-8) (Tomás-Barberán et al., 1992). The results suggested that Amberlite XAD-2 was the most appropriate for flavonoid extraction in honey (Tomás-Barberán et al., 1992). Amberlite XAD-2 has been successfully used for the recovery of flavonoids from honey and fruit jams (Ferreres et al., 1991; Tomás-Lorente et al., 1992). The different honey or jam samples were dissolved in five parts of water acidified with HCl (pH 2 in order to favor the flavonoid adsorption in the stationary phase) until completely fluid and stirred with a magnetic stirrer in contact of the resin with the dissolved honey sample. The Amberlite resin with the flavonoid adsorbed was packed into the column and washed with deionized water. Flavonoids and other phenolics were then eluted with methanol and concentrated with a rotary evaporator (40°C).

15.3.1.1 Protocol for Honey Flavonoid Extraction

Honey samples (100 g) were thoroughly mixed with 500 mL of distilled water (adjusted to pH 2 with concentrated HCl) until completely fluid, by stirring with a magnetic stirrer at room temperature. The solution was then filtered through cotton to remove the solid particles. The filtrate was mixed with Amberlite XAD-2 (100 g) and stirred with a magnetic stirrer to retain the phenolic compounds on the surface of the nonionic Amberlite particles. The Amberlite particles were then packed in a glass column (25 × 2 cm). The column was washed with acidified water (200 mL) and distilled water (300 mL) successively to remove all sugars and other polar constituents of honey. The phenolic compounds remained adsorbed in the column and were then eluted with methanol (400 mL); this extract was then concentrated under reduced pressure (at 40°C) (Martos et al., 1997).

15.3.2 Sephadex Chromatography

Other classical methods for the fractionation and purification of flavonoid extracts are the filtrations through different Sephadex gels that separate by a combination of adsorption, partition, and size exclusion using organic solvents (Sephadex LH-20) or aqueous solvents (Sephadex G-10, G-20, etc.).

15.3.3 Preparative Column Chromatography

On the other hand, the classical separation by column chromatography using different solid phases (silica gel, reversed-phase C-18 or C-8, polyamide, cellulose) and elution with appropriate solvent mixtures for each case are also used for flavonoid fractionation and purification. Different column systems can be used. The classical open column chromatography uses relatively large particle size, with poor resolution, and the solvent filtration through the column proceeds by the pressure of the solvent column placed on top of the stationary phase. In other cases, smaller particle sizes can be used for an enhanced resolution and flavonoid purification. In these cases, different systems have to be applied in order to force a solvent flow through the stationary phase. Vacuum can be applied at the bottom of the column, or pressure on the top of the column. Thus, there are lobar column chromatography and flash chromatography (both low-pressure chromatography), and vacuum chromatography. Different stationary phases can be used for the isolation and separation of different flavonoids. The stationary phase to be used depends on the type of flavonoid to be purified. The different flavonoid fractions can be followed using a UV detector coupled to the outlet of the column, or using a UV lamp (360 nm) to follow the development of the fractionation directly in the stationary phase. (This is useful in cellulose and polyamide or Sephadex chromatography, but has some limitations in silica gel and reversed-phase chromatography.)

15.3.3.1 Silica Gel Chromatography

This is used for the separation of apolar flavonoids (aglycones), as the polar compounds can be irreversibly adsorbed to the stationary phase. The separation is carried out using solvent mixtures starting with highly apolar solvents such as *n*-hexane, petrol, etc. and increasing the polarity of the solvent by increasing the proportion of ethyl acetate or methanol. The separation takes place following the principles of adsorption chromatography, initially eluting the more polar compounds, and then eluting flavonoids with increasing polarity. A typical elution in the separation of a mixture of quercetin methyl ethers would be the following: quercetin 3,7,4′-trimethyl ether, quercetin 3,4′-dimethyl ether, quercetin 3-methyl ether, quercetin 4′-methyl ether, quercetin. A key step in silica gel chromatography is the deposit of the extract to be separated on top of the stationary phase of the column. It is recommended to dissolve the extract in the mobile phase to be used, but the solubility of the extract is often low in the stationary phase leading to precipitations at the top of the column and decrease of the solvent flow and poor resolution of the flavonoid bands. An alternative to this is the solution of the extract in an appropriate solvent (no water should be present), and the concentration in a rotary evaporator after the addition of a small amount (a few grams) of the silica gel stationary phase to the extract. This will produce a dried stationary phase mixed with the extract, which can be placed dried on top of the stationary phase of the column. This will help in the preparation of solvent mixtures with increasing polarity for silica gel chromatography. In this type of chromatography, the stationary phase is used for only one separation and then is discarded (this is not a problem as silica gel is quite inexpensive).

15.3.3.2 Reversed-Phase Chromatography

The hydroxyls present in the silica gel stationary phase are blocked by 18 (C-18) or 8 (C-8) carbon hydrocarbon chains. The principle of separation is adsorption as in the case of silica gel, but in this case, the polarity of the stationary phase has been reversed, and the polar compounds elute first and the apolar compounds are more retained in the column. In this case, the mobile phase consists of mixtures of methanol or acetonitrile with water. Elution starts with water or mixtures with a small percentage of the organic solvent (5% methanol) and ends with 100% organic solvent. Gradient elution is a common strategy although isocratic separations are more appropriate for preparative chromatography. Addition of an organic acid to the solvent mixture (acetic acid) can increase the efficacy of the separation, but the isolated compounds have to be stable under these acidic conditions, particularly during the concentration process of the isolated fractions that usually increases the temperature to remove the solvents.

15.3.3.3 Cellulose Chromatography

This separates flavonoids by partition chromatography. It can be particularly useful for the separation of flavonoid glucuronides from the rest of the flavonoids in complex mixtures. The separation with water as mobile phase takes glucuronides with the solvent front, while the other flavonoids are separated by the degree of glycosylation, the more polar eluting first.

15.3.3.4 Polyamide Chromatography

This chromatographic method often produces very good separations of flavonoids that are difficult to separate by other methods. The main inconvenience is the elevated cost of the stationary phase, and this prevents its use for large-scale preparations, but it is useful for laboratory-scale preparations. Mobile phases like chloroform–methanol–acetone (1:1:1) for flavonoid aglycones or chloroform–methanol–ethylmethylketone–water (11:8:4:2) for flavonoid glycosides are useful for the fractionation and purification of flavonoids. In some cases, the samples can be contaminated with stationary phase and a further purification (filtration through a Sephadex LH-20 microcolumn) can be recommended particularly prior to nuclear magnetic resonance (NMR) analysis.

15.3.4 Thin Layer Chromatography and Paper Chromatography

For partially purified extracts, thin layer chromatography (TLC) and paper chromatography (PC) have been used for analytical and preparative purposes. TLC using silica gel, polyamide, cellulose, or reversed-phase C-18 can be used successfully for the separation of specific flavonoids. The type of flavonoid to be separated suggests the type of TLC plate to be used. Thus, reversed-phase, cellulose, or polyamide are recommended for polar flavonoids (flavonoid glycosides, polyhydroxylated aglycones, etc.), while silica gel with organic solvents is recommended for lypophylic flavonoids (polymethoxylated flavones, flavone methyl ether, isoprenylated flavones, etc.).

15.3.5 Preparative HPLC

Finally, a more precise purification obtaining fractions with a higher purity can be obtained using preparative or semipreparative high-performance liquid chromatography (HPLC) (even analytical HPLC columns can be repeatedly used for the preparation of small amounts of a highly purified compound). In the case of flavonols and flavones, both aglycones and glycosides, reversed-phase columns are often used (C-18).

15.4 Chemical and Biochemical Treatments

Once isolated, the flavonoids can be subjected to a number of chemical and biochemical processes in order to help with the identification of complex molecules through the analysis of more simple molecules. Thus, many flavonoid glycosides that are acylated with aliphatic (acetic, malonic, etc.) or aromatic acids (hydroxycinnamics, etc.) can be studied by alkaline hydrolysis that will lead to the desacylated flavonoid glycosides. Acid hydrolysis would lead to the corresponding aglycones. Enzymatic hydrolysis can also be useful for the identification of glucuronide, sulfate, and glucoside conjugates.

15.4.1 Alkaline Hydrolysis

Alkaline hydrolysis is applied for removing organic acid esters from the original flavonoid molecules. After this hydrolysis, both the released organic acid and the flavonoid glycoside can be chemically characterized. This can be applied to the isolated flavonoids or even to crude extracts. In this case, HPLC-Photodiodearray detector/mass spectrometry (PAD-MS/MS) comparison of the original acylated extract with the desacylated product is very useful. This is achieved by adding 1 mL of 4 N NaOH to the freeze-dried purified flavonoids (<1 mg) or 1 mL of the hydroalcoholic extract, or flavonoid fraction, and keeping the solutions for 16 h in a stoppered test tube under an N_2 atmosphere (to prevent the oxidation of the released compounds). The alkaline hydrolysis products are then acidified with concentrated HCl (color change from yellowish to white, pH ~1) and directly analyzed by HPLC-PAD-MS/MS (Llorach et al., 2003; Ferreres et al., 2006) or other chromatographic or spectrophotometric methods to identify the acylating organic acids and the remaining flavonoid glycoside.

15.4.2 Acid Hydrolysis

Acid hydrolysis of the naturally occurring flavonoids is used to release the linked sugar residues and the flavonoid aglycone. This is applied to the hydrolysis of flavonoid-*O*-glycosides. Flavone-*C*-glycosides. are not hydrolyzed with acid, but they are isomerized to convert 8-*C*-glycosides partially into 6-*C*-glycosides. (It seems that the 6-*C* configuration is more favored.) Acid hydrolysis is achieved by dissolving 1 mg of the isolated flavonoid in 1 mL of MeOH (or 1 mL of the crude extract) and adding 1 mL of 4 N HCl, and the solution is heated at 90°C for 45 min. In the case of flavonoid glucuronides, stronger conditions can be necessary to complete the hydrolysis [higher temperatures (100°C) and longer hydrolysis times (4 h)]. The aglycones are then extracted from the aqueous solution by liquid–liquid fractionation using diethyl ether, and the unhydrolyzed glycosides with ethyl acetate after removal of the MeOH, while the released sugars remain in the aqueous phase.

Partial acid hydrolysis can be used for characterization purposes, for sequential release of sugar residues. In this case, mild acidic conditions (0.1–0.5 N HCl at 75°C) are used, and the time is controlled by taking samples after different times of hydrolysis. Samples are taken after different times and then monitored using TLC or HPLC to select the conditions more appropriate for the isolation of the intermediates of interest.

15.4.3 Enzyme Hydrolysis

Enzymatic hydrolysis can be used for two purposes: to identify specific sugars and their linkage position and to preserve the naturally occurring structure of the flavonoid, when it is sensitive to the acidic conditions of a classical acid hydrolysis (i.e., 8-hydroxyflavonoids are converted to 6-hydroxyflavonoids through acid-catalyzed isomerization). As an example, the UV study of the flavonoid-*O*-glycosides will show the free phenolic hydroxyls in the naturally occurring molecule. The UV analysis of the enzyme-hydrolyzed aglycone will show any additional free hydroxyls released after the removal of the sugar residue by the action of the enzyme, thus indicating the position of the sugar linkage. In addition, a positive hydrolysis with β-D-glucosidase will show that the original flavonoid is a flavonoid-β-D-*O*-glucoside.

15.4.3.1 β-Glucosidase Hydrolysis

This is achieved by adding 0.5 mg of flavonoid to 3 mg of β-D-glucosidase in 0.5 mL of 0.1 M citrate phosphate buffer, pH 5 (37°C, 24 h). The hydrolysis products are extracted with ethyl acetate, taken to dryness under reduced pressure (at 40°C), and redissolved in methanol for HPLC analysis (Gil et al., 1998).

15.4.3.2 Glucuronidase and Sulfatase Hydrolysis

This is useful for the quantification of the phase II conjugate metabolites of flavonoids present in biological fluids such as plasma and urine. Flavonoid glucuronide and sulfate conjugates can be hydrolyzed by incubation of 100 μL of the sample (urine or plasma) in 50 μL of 0.1 M sodium acetate buffer (pH 5.2) containing an aqueous solution of *Helix pomatia* enzyme extract H-2, G-0876 (EC 3.2.1.31), containing glucuronidase and sulfatase enzymes, at 37°C for 18 h. The reaction mixture is then extracted with 300 μL of ethyl acetate, vortexed for 3 min and centrifuged at $1000 \times g$ for 1 min. The supernatant fraction can be dried under N_2 and the residue dissolved in 100 μL of methanol for HPLC analysis (Vallejo et al., 2010). To evaluate if the enzyme hydrolysis is complete, it is always recommended to analyze the remaining water phase by HPLC-PAD-MS/MS. The hydrolysis of glucuronides is generally complete while the hydrolysis of sulfates with this enzyme preparation is not so efficient and a percentage of the original sulfates (5–30%) can remain unhydrolyzed and this needs to be considered in quantitative studies (Crozier et al., 2009).

15.5 Analysis of Complex Extracts

15.5.1 Paper Chromatography

Two-dimensional paper chromatography (2D PC) has been traditionally used for a direct and complete qualitative analysis of complex flavonoid extracts. This is usually completed with UV detection and following the changes after exposure to NH_3 vapors (Mabry et al., 1970). The paper used for this purpose is Whatman no. 1. The sample is applied with the help of a glass capillary, and submitted to a first chromatographic run using *n*-butanol–acetic acid–water (4:1:5, v:v:v, upper phase) (BAW). After drying at room temperature, a second run with 15% or 30% acetic acid is completed (depending on the nature of the glycosidic combinations of the flavonoids present in the extract; 15% is recommended for more polar flavonoids) (Figure 15.1). In the first dimension, the flavonoid aglycones elute first, and the more polar glycosides run

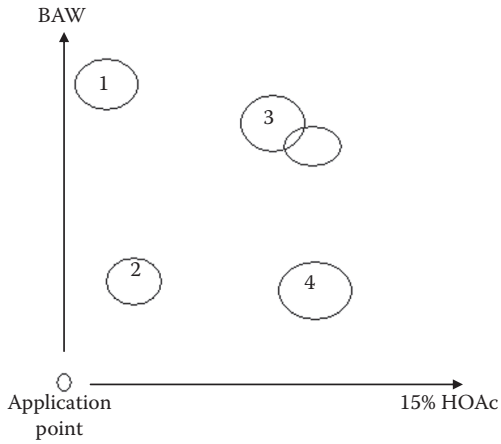

FIGURE 15.1 Bidimensional paper chromatography of flavonoid extracts. The extract is deposited in the application point, dried, and developed with BAW and 15% HOAc. Spot 1 corresponds to flavonoid aglycones, and 2, 3, and 4 flavonoid glycosides with different glycosylation patterns.

with shorter retention factor (Rf) values. In the second dimension, the more polar compounds run with higher Rf values. This method separates flavonoids mainly by partition chromatography, and is complementary to the reversed-phase HPLC analyses that separate mainly by adsorption chromatography interactions. Both methods are therefore complementary, and could be used in combination for complex extracts.

15.5.2 Paper Electrophoresis

This method is used for the detection of flavonoid glucuronides and flavonoid sulfates in complex extracts. Both flavonoid glucuronides and sulfates migrate toward the anode when the extracts are applied for paper electrophoresis using acetate buffer pH 4.5. The nonglucuronidated or sulfated flavonoids remain at the application point, while glucuronides and sulfates migrate (Figure 15.2). The migration of the

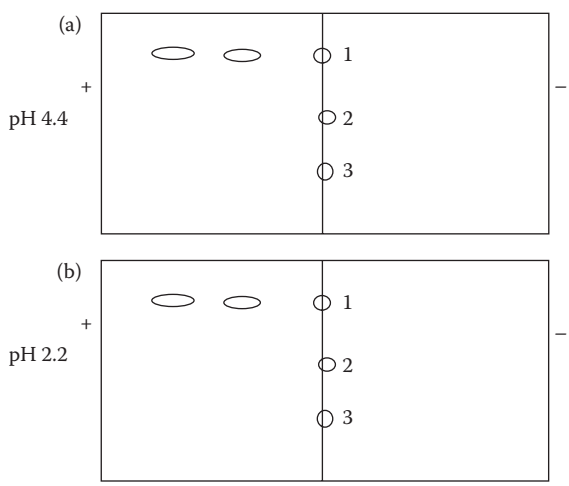

FIGURE 15.2 Paper electrophoresis used for the detection of flavonoid glucuronides (pH 4.4) and sulfates (pH 2.2). The glucuronides migrate toward the anode at pH 4.4 (sample 1). Sulfates migrate toward the anode at pH 2.2, but not glucuronides.

flavonoid spots can be followed using a UV lamp (360 nm). After 30 min migration, the glucuronides and sulfates are easily spotted and the migration toward the anode is evaluated. A formic acid solution (pH 2.2) can also be used for the detection of flavonoid sulfates, as under these conditions, only the flavonoid sulfates migrate. (Only the sulfate residue remains ionized at this pH while the glucuronic acid is not ionized.) This methodology could also be used as a preparative method for the analysis of biological samples (plasma and urine).

15.5.2.1 Methodology for Paper Electrophoresis Using Acetate Buffer pH 4.4

The buffer used is acetate buffer 0.1 M pH 4.4 on paper Whatman no. 3. A small amount of the hydroalcoholic extract is inoculated using a glass capillary (or a Pasteur pipette) in the middle of the paper (Figure 15.2). After drying, the paper is placed in the electrophoresis cuvette and the anode and cathode are marked with a pencil on the paper, and then moistened with the buffer using a Pasteur pipette. Electrophoresis was developed at 400 V and after 30 min the paper was removed and dried and the flavonoid spots visualized under UV light (360 nm).

For the analysis of sulfates, this was achieved using a formic acid solution (pH 2.2) prepared by adding 12.5 mL formic acid to 38 mL acetic acid in 1 L water. This solution was used in place of acetate buffer.

15.5.3 High-Performance Liquid Chromatography

15.5.3.1 Sample Preparation

In the case of extracts or samples that are available in very small amounts, in which the isolation is not intended, but the study of the crude extract by HPLC is the objective, it is sometimes recommended to filter the extract through a solid-phase extraction cartridge (i.e., C-18). In this sense, the aqueous fraction obtained from the hydroalcoholic crude extract after alcohol removal using a rotary evaporator can be passed through a C-18 Sep Pack cartridge previously activated with a volume of methanol (i.e., 5 mL) and then water (5 mL) and the same volume of air. The aqueous extract, filtered, is adsorbed on the C-18 cartridge, and then washed with water. The water extract should be kept for analysis to make sure that no flavonoids are eluted with water. (Some polar flavonoids, as is the case of flavonoid glucuronides and sulfates, can be only poorly adsorbed, and in these cases acidification of the water is recommended to enhance the adsorption on the stationary phase.) The adsorbed flavonoids are then eluted with pure methanol and the extract recovered.

15.5.3.2 Reversed-Phase High-Performance Liquid Chromatography

Reversed-phase HPLC is considered the method of choice for the analysis of flavones and flavanones due to the high resolution of the chromatographic separations and the sensitivity of the detection methods that include UV, fluorescence, electrochemical, and MS detectors. The best combination would be the detection system that links UV detection with a diode array detector (DAD) that allows the registration of the UV spectra of the eluting compounds, with an MS/MS detector, which with an ion trap allows the isolation and fractionation of specific ions, even if they coelute under the same chromatographic peak.

In the market, there are different HPLC chromatographic columns with different particles sizes (generally 5 or 3 μm particle size) for regular HPLC. They may offer different resolutions and may need to be assayed for the specific extract that is being analyzed. The solvents used as mobile phases include mixtures of methanol and water, or acetonitrile and water (normally in gradient elution with different solvent rates depending on the extract to be analyzed and the compounds that need to be resolved). Addition of an organic acid (acetic acid or formic acid) to the solvent mixture is necessary to increase the peak resolution. The acidic conditions prevent phenolic hydroxyls from being ionized and therefore lead to a better interaction with the stationary phase in turn leading to less “tailing” and a better peak height.

Ultra performance liquid chromatography (UPLC) (which uses smaller columns with smaller particle size and higher resolution and pressure) has recently been introduced and allows better resolution of chromatographic peaks, a shorter analysis time, and less solvent consumption.

15.5.4 Capillary Electrophoresis

Capillary electrophoresis (CE) was first applied for the analysis of flavones in complex extracts in the 1990s. There was much expectation on the possibilities of this analytical method due to the inexpensive columns used (fused silica capillary columns), the development in buffer solutions that do not consume organic solvents, and the short times of analysis. CE can be coupled to detectors, including UV (with PAD detectors that allow recording of the UV spectra of the different compounds) and MS detectors. In spite of these expectations, CE has not replaced HPLC from being the method of choice for the analysis of flavones. This is mainly due to the lack of reproducibility of the CE conditions for a given analysis. CE has been applied for both flavonoid glycosides, as capillary zone electrophoresis (CZE), and flavonoid aglycones, by the addition of micelles to the buffer and organic solvents, separating by micellar electrokinetic capillary chromatography (MEKCC).

15.5.4.1 Capillary Zone Electrophoresis

CZE is applied for the separation of flavonoid glycosides. It separates the flavonoid molecules following the ratio charge/mass of the different flavonoids. The higher this ratio, the faster the compound migrates in the column. In addition, when borate buffers are used, borate can complex with the sugar hydroxyls depending on their stereochemical distribution, allowing the separation of different flavonoid hexosides, which seldom happens in HPLC separations.

15.5.4.2 Micellar Electrokinetic Capillary Chromatography

Micelles that migrate countercurrent in the capillary can be used for the separation of apolar compounds, particularly when organic solvents such as methanol are added to the buffer solution. Using this method, flavone aglycones present in honey were separated, although no specific advantage with the HPLC separation using reversed-phase columns was observed.

15.6 Structural Analysis by Spectroscopic Methods

The main spectroscopic methods used for the structural characterization of isolated flavonoids are ultraviolet spectrophotometry (UV), mass spectrometry (MS), and NMR spectroscopy. UV and NMR methods (both ^{1}H NMR and ^{13}C NMR) have been extensively covered in previous publications, and therefore will only be summarized in the present chapter. MS, particularly HPLC-MS/MS, has been applied extensively to flavonoid characterization in the past few years, and in this chapter, we will review the state of the art of this powerful method, which is particularly useful in the analysis of flavonoid glycosides present in complex extracts.

15.6.1 Ultraviolet–Visible Spectrophotometry

The flavonoid compounds may be easily identified by their spectra in methanol since the majority exhibit a maximum in the long UV range [band I (BI)] between 325 and 400 nm and a second maximum at shorter wavelength [band II (BII)] between 240 and 295 nm. These two bands can be split into two maxima or a maximum and a shoulder or an inflection (BIa, BIb, BIIa, and BIIb). In some cases, it is possible to distinguish a supplementary maximum, shoulder, or inflection (band III) between 295 and

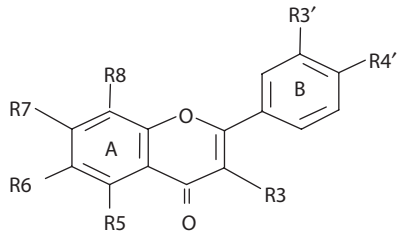

FIGURE 15.3 General structure of flavonols (3-hydroxy-flavones).

325 nm (Barberán et al., 1985). The BI is associated with the absorption of the flavonoid ring B, while the BII is associated with ring A (Mabry et al., 1970) (Figure 15.3).

Thus, the UV spectra of flavones and flavonols (3-hydroxyflavones) disubstituted in ring B (luteolin, quercetin, etc.) exhibit two maxima in BII (usually at ~255 and 266 sh nm) (Figure 15.4).

However, the B-ring monosubstituted flavones (apigenin, kaempferol, etc.) show only a single response for this band (~266 nm) (Figure 15.5).

On the other hand, while the BI of flavones and 3-methoxyflavones shows an absorption maximum between 325 and 355 nm, the maximum of this band in flavonols with free hydroxyl at 3 position is shifted to larger wavelengths (~360–380 nm) (Figure 15.6).

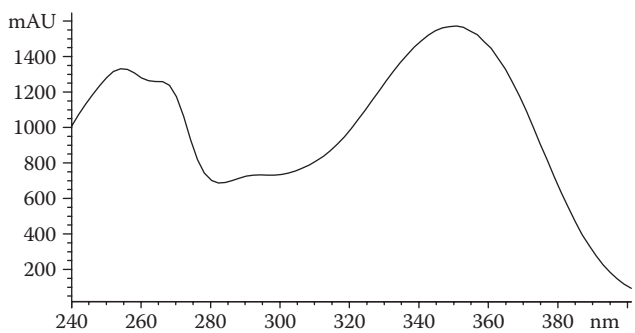

FIGURE 15.4 UV spectrum of luteolin (5,7,3′,4′-tetrahydroxyflavone).

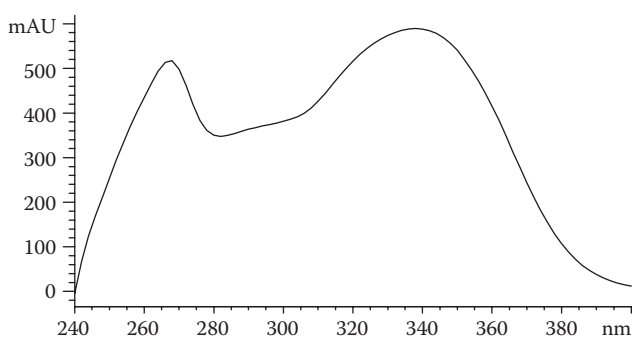

FIGURE 15.5 UV spectra of apigenin (5,7,4′-trihydroxyflavone).

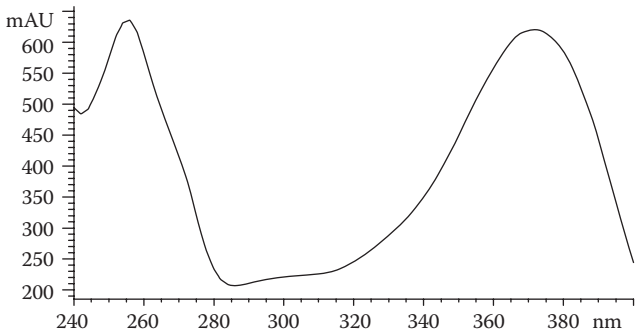

FIGURE 15.6 UV spectrum of quercetin (3,5,7,3′,4′-pentahydroxyflavone).

In flavanones (2–3 dihydroflavones), the lack of a double bond between 2 and 3 positions avoids the resonance between rings A and B and this reflects in a BI with very low absorption and at a short wavelength (~320–330 nm), while BII shows a maximum between ~280 and 290 nm (Figure 15.7).

The position of the BII maximum, in flavones, is affected by the introduction of additional substituent(s) at position(s) 6 and/or 8 that, in general, produces a bathochromic shift placing this maximum above 270 nm (Voirin 1983) (Figure 15.8).

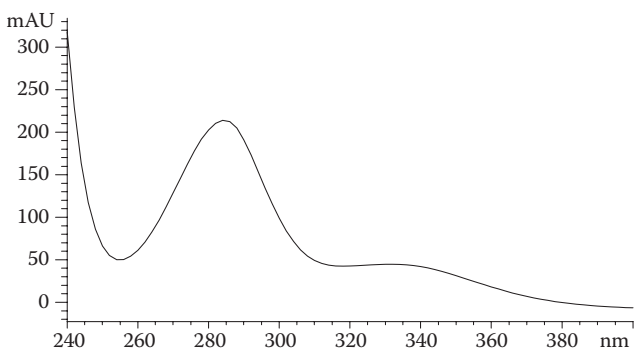

FIGURE 15.7 UV spectrum of hesperetin (5,7,3′-trihydroxy-4′-methoxyflavanone).

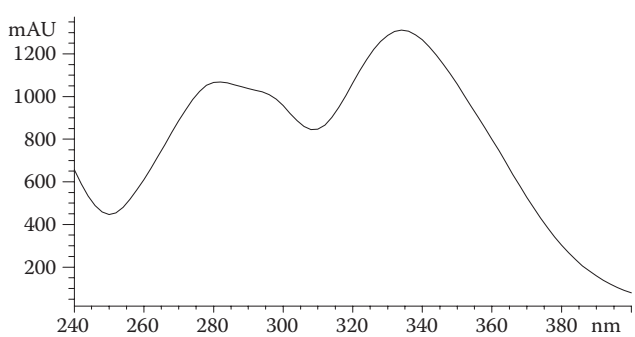

FIGURE 15.8 UV spectrum of xanthomicrol (5,4´-dihydroxy-6,7,8-trimethoxyflavone).

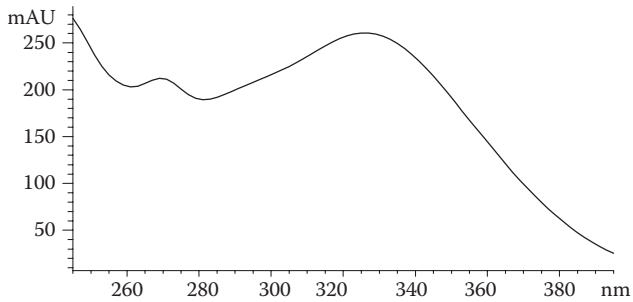

FIGURE 15.9 UV spectrum of kaempferol-3-*O*-(feruloyl)triglucoside-7-*O*-glucoside.

Hydroxylation at C-6 produces a higher bathochromic shift than hydroxylation at C-8. Moreover, the ratio of Abs band I/Abs band II (Abs, absorbance) constitutes a criterion for distinguishing the substituted position on A-ring. Thus, 8-substitution particularly decreases this value whereas 6-substitution increases it (Barberán et al., 1985). Useful information to differentiate among 5,6-dihydroxy- and 5,8-dihydroxyflavones, 3-methoxyflavones and flavonols trisubstituted on A-ring, as well as 5,6-dihydroxy-7,8-dimethoxy- and 5,8-dihydroxy-6,7-dimethoxyflavones can be gained by comparison of their UV spectra in methanol (Barberán et al., 1985).

Flavonoids acylated with hydroxycinnamic acids show a UV spectrum of the flavonoid overlapped with that of the acid, this last one being the main one. They are characterized by a maximum with a high absorption ~330 nm (310–335 nm), and eventually show a small maximum that coincides with the flavonoid band II (~255–268 nm) and a shoulder at higher wavelengths that reflects the flavonoid band I (Vallejo et al., 2004) (Figure 15.9).

The addition of alkaline (NaOMe, NaOAc, and NaOAc/H_3BO_3) or methal reagents ($AlCl_3$ and $AlCl_3$/HCl) to the methanol solution of the isolated flavonoids produces shifts in the UV spectra absorption bands, allowing the detection of the presence or absence of specific hydroxyls in different characteristic positions 5, 7, 3′, and 4′ (Mabry et al., 1970). They studied the effect of these reagents on a large number of flavonoids. Addition of NaOMe induces in flavonoids with a free hydroxyl in 4′ position a bathochromic shift of BI between 40 and 65 nm without a decrease in the absorbance intensity. Addition of NaOAc, however, is useful for the determination of the presence of a free hydroxyl in 7 position as it produces a bathochromic shift between 5 and20 nm in the maximum of BII. Alkaline reagents can also induce a flavonoid decomposition that is reflected in the disappearance of the UV spectrum when the flavonoid structure presents a 3,4′-dihydroxylic or a trihydroxylation (5,6,7-, 5,7,8-, or 3′,4′,5′-), this decrease being more marked when adding NaOMe than NaOAc, which is related to the more intense alkaline character of the first reagent. Addition of NaOAc plus H_3BO_3 can be used for the detection of *o*-dihydroxylic groupings due to the effect of the complex produced between the boric acid and the dihydroxylic grouping that reflects in the UV spectrum as a bathochromic shift in BI between 12 and 30 nm. The $AlCl_3$ reagent forms complexes in the presence of *o*-dihydroxylic groupings (3′,4′-, 5,6-, or 6,8), and between the hydroxyl in 5 position and the carbonyl in 4 or the hydroxyl in 3 and the carbonyl in 4, that induce a bathochromic shift in BI. The addition of HCl to the solution that already contained $AlCl_3$ provokes the destruction of the complex formed with the *o*-dihydroxylic groupings, but preserves those complexes involving the carbonyl group, producing a hypsochromic shift of 30–40 nm, and keeping the bathochromic shift with respect to the UV spectrum in MeOH due to the stable complex between the carbonyl and the hydroxyls at 5 and/or 3 positions.

Voirin (1983) reported a complete study of the UV spectra of 151 flavonoids in methanol alone and with added $AlCl_3$ and $AlCl_3$/HCl for the differentiation of 5-hydroxy- and 5-hydroxy-3-methoxyflavones with mono-(4′)-, di-(3′,4′)-, or tri-(3′,4′,5′)-substituted B-rings, and he distinguished 20 groups of compounds depending on the UV spectra shapes and position of BI.

15.6.2 Nuclear Magnetic Resonance Spectroscopy

NMR spectroscopy is a powerful analytical method for the determination of flavone structures. It has, however, some limitations as the sensitivity is rather low, and compounds have to be isolated. The assignments of the different proton and carbon signals in ^{1}H and ^{13}C NMR can be based on the chemical shifts (δ) and coupling constants (*J*), and correlations observed in homo- and heteronuclear 2D NMR. NMR spectra of flavones have been extensively published previously (Markham and Chari, 1982; Agrawal, 1989; Markham and Geiger, 1993; Fossen and Andersen, 2006).

The solvent most frequently used for flavones is hexadeuterodimethylsulfoxide (DMSO-δ6) and tetradeuteromethanol (CD_3OD). For lypophylic flavone aglycones, solvents like carbon tetrachloride (CCl_4) are recommended.

NMR experiments include correlation spectroscopy (COSY), total correlation spectroscopy (TOCSY), ^{1}H-^{13}C heteronuclear NMR experiments, NOESY (nuclear Overhauser enhancement spectroscopy), and ROESY (rotating frame Overhauser effect spectroscopy) as well as other two- and three-dimensional methodologies (Fossen and Andersen, 2006).

Comprehensive lists of NMR data for different flavonoids are published in the above-mentioned books and book chapters, and therefore they will not be repeated here.

15.6.3 Mass Spectrometry

The use of mass spectrometry as a tool for the structural analysis of flavonoids has constituted a fundamental step forward, particularly altering the development of emerging techniques that allow working at atmospheric pressure without the need for compound derivatization before analysis for its volatilization and ionization. On the other hand, coupling these mass detectors to separation systems, particularly HPLC, has allowed the screening and characterization of flavonoids in complex matrices, and the tentative structural identification of complex flavonoids without the need for isolation and purification. This is of great importance for research studies in which only very small amounts of samples are available, and with compounds that are present in trace amounts in the extracts as is the case of the study of the flavonoid metabolites present in biological fluids and tissues.

Among the different ionization sources actually used, APCI (atmospheric pressure chemical ionization) and ESI (electrospray ionization) are the ones more generally used for flavonoid analyses, APCI being more appropriate for apolar low-molecular-weight molecules. ESI is the main system used for the study of flavonoids, and particularly for flavonoid glycosides, and the negative mode is the method of choice. The mass detectors with better applications for flavonoid analysis include the quadrupole-time of flight (Q-ToF), triple quadrupole (3Q), and ion-trap, the first two being more used for target analyses in which the flavonoid to be quantified and detected in a complex matrix is already known, and the first one providing the exact mass that is useful for flavonoid identification. The mass transitions and fragmentation also provide information for flavonoid characterization and identification. The ion-trap detector is more valuable for the structural analysis of unknown compounds, in which a specific ion present in a complex chromatographic peak can be isolated in the ion trap and fragmented, and this can be related to the structural analysis.

Stobiecki (2000) and Cuyckens and Claeys (2004) reported exhaustive mass spectrometry studies of the use of mass spectrometry in the structural analysis of flavonoids with a review of the currently available mass spectrometric methodology used in the structure elucidation of flavonoid glycosides.

15.6.3.1 Flavonoid Aglycones

In general, the flavonoid aglycone fragmentation can lead to the loss of small fragments (18 mass units, H_2O; 28 m.u., CO; 42 m.u., C_2H_2O; etc.). A particular characteristic is the loss of 15 m.u. (CH_3) in the fragmentation of methoxylated flavonoids, and the relative abundance of the resulting ions can indicate the position of the methoxyl residue on the flavonoid nucleus. In this sense, a study to differentiate 5,6-dihydroxy-7,8-dimethoxyflavones from 5,8-dihydroxy-6,7-dimethoxyflavones by the relative abundance of the ions produced during the fragmentation was published (Barberán et al., 1986). Other degradation

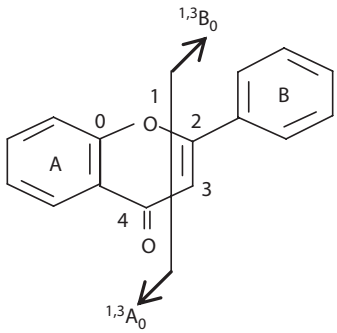

FIGURE 15.10 RDA cleavage of the flavonoid nucleus.

pathways can lead to fragments providing information of the substituents present in the A- and B-rings. The main one is the retro-Diels–Alder (RDA) fragmentation of the flavone nucleus that results in the cleavage of the C–C bonds at positions 1/3, 0/2, 0/3, 0/4, or 2/4 of the C-ring. The fragmentation pathways depend on the substitution pattern and the class of flavonoid studied, as well as on the collision energy applied (Cuyckens and Claeys, 2004). Figure 15.10 presents one of these possible ring cleavages.

15.6.3.2 Flavonoid Glycosides

Most flavonoids are present in food products and biological fluids linked to different sugars by *O*-glycosidic bonds (flavonoid-*O*-glycosides), C–C bonds (flavonoid-*C*-glycosides) or both glycosylation types in a single flavonoid molecule (*O*-glycosyl-*C*-glycosyl flavonoids). This last case involves *C*-glycosyl flavonoids in which an additional *O*-glycosylation takes place either on a phenolic hydroxyl or on a hydroxyl of the *C*-glycosidic sugar or both. The MS fragmentation of these two types of glycosidic bonds is different due to the difficulty in breaking the C–C bond. The different theoretical fragmentation patterns of a flavonoid-*O*-glycoside are shown in Figure 15.11.

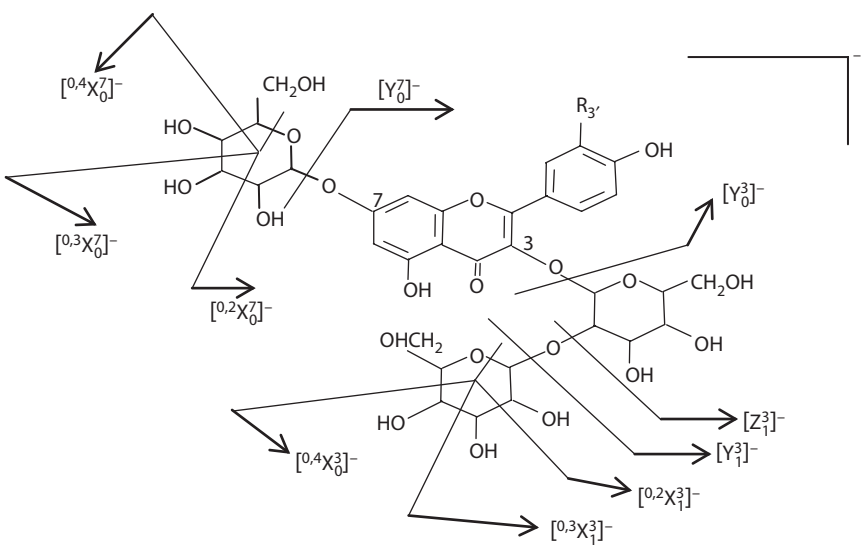

FIGURE 15.11 General scheme of the MS fragmentations of flavonoid glycosides.

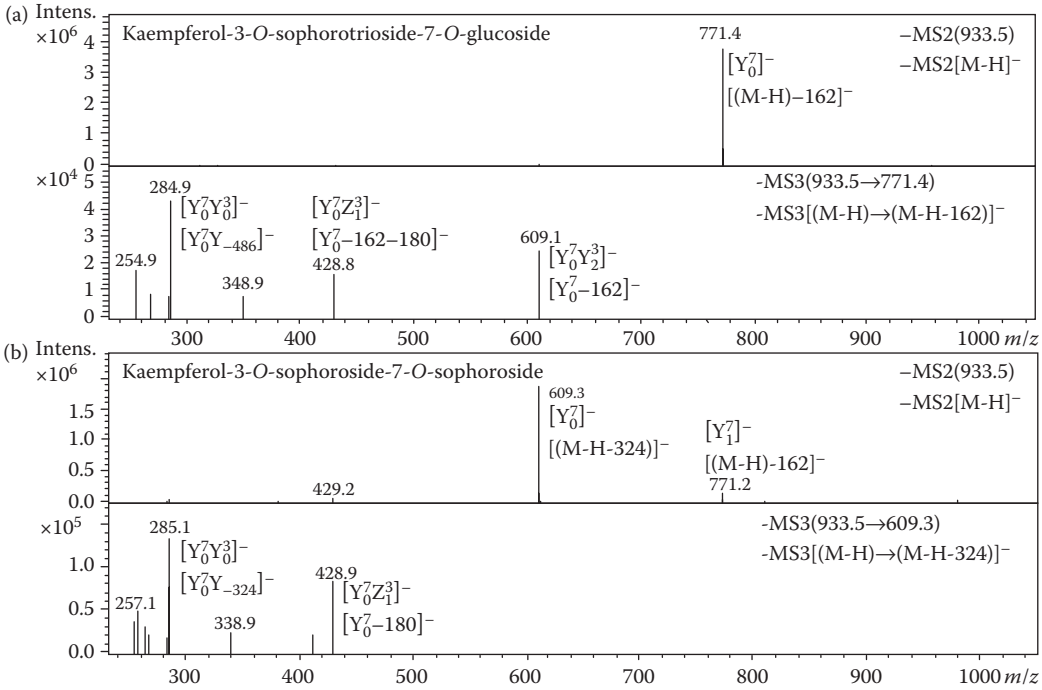

FIGURE 15.12 MS2[M–H]⁻ and MS3[(M–H) → Y_0^7]⁻ of isomer flavonoids tetraglucosides: (a) kaempferol-3-*O*-sophorotrioside-7-*O*-glucoside; (b) kaempferol-3-*O*-sophoroside-7-*O*-sophoroside. (From Ferreres, F., Llorach, R., and Gil-Izquierdo, A. 2004. *J. Mass Spectrom.* 39: 312–321. With permission.)

The classical nomenclature (Domon and Costello, 1988) for glycoconjugates was adopted to designate the fragment ions. Ions ${}^{k,l}X_j$, Y_j^n, and Z_j^n represent those fragments still containing the flavonoid aglycone, where *j* is the number of the interglycosidic bond broken, counted from the aglycone; *n* represents the position of the phenolic hydroxyl where the oligosaccharide is attached; and *k* and *l* denote the cleavage within the carbohydrate rings. In other MS spectra (Figure 15.12), the ions obtained as a consequence of a second oligosaccharide fragmentation have been labeled according to previous reports (Ferreres et al., 2004). Thus, ions obtained from the ion Y_0^{7-} (–MS3[(M–H) → Y_0^7]⁻) have been labeled starting with the ion Y_0^{7-} and followed by the resultant MS3 ion; for example, the ion $[Y_0^7Y_2^3]^-$ (Figure 15.12a) denotes the loss of the terminal sugar of the triglucoside in the 3 position $[Y_2^3]^-$ from the fragmentation of ion $[Y_0^7]^-$ (total loss of a glycosylation in the 7 position). The losses indicated in the MS3 scan show that the fragment came from the trapped and fragmented ion $[Y_0^7]^-$ and not from the deprotonated molecule. This terminology completes the nomenclature provided by Domon and Costello (1988) by addition of specific ways of the ions obtained from the MS3 event.

In general, it is posible to differentiate the type of sugar by means of the MS losses and the relative abundance of the ions formed, and in some cases the glycosylation position and the interglycosidic linkage. In the following discussion, the results have been obtained using ESI-ion-trap mass spectrometry in the negative mode.

15.6.3.3 O-*Glycosyl Flavonoids*

In the MS spectra of flavonoid-*O*-glycosides, the deprotonated aglycone ion is always observed as a consequence of the fragmentation of the glycosidic linkage. This aglycone ion is generally the base peak (the most abundant peak) when the glycosylation is only on one phenolic hydroxyl of the flavone molecule (Figure 15.13a, b, ion $[Y_0^3]^-$). In the monoglycosylated flavonoids, the observed loss can be 162/146/132 m.u.

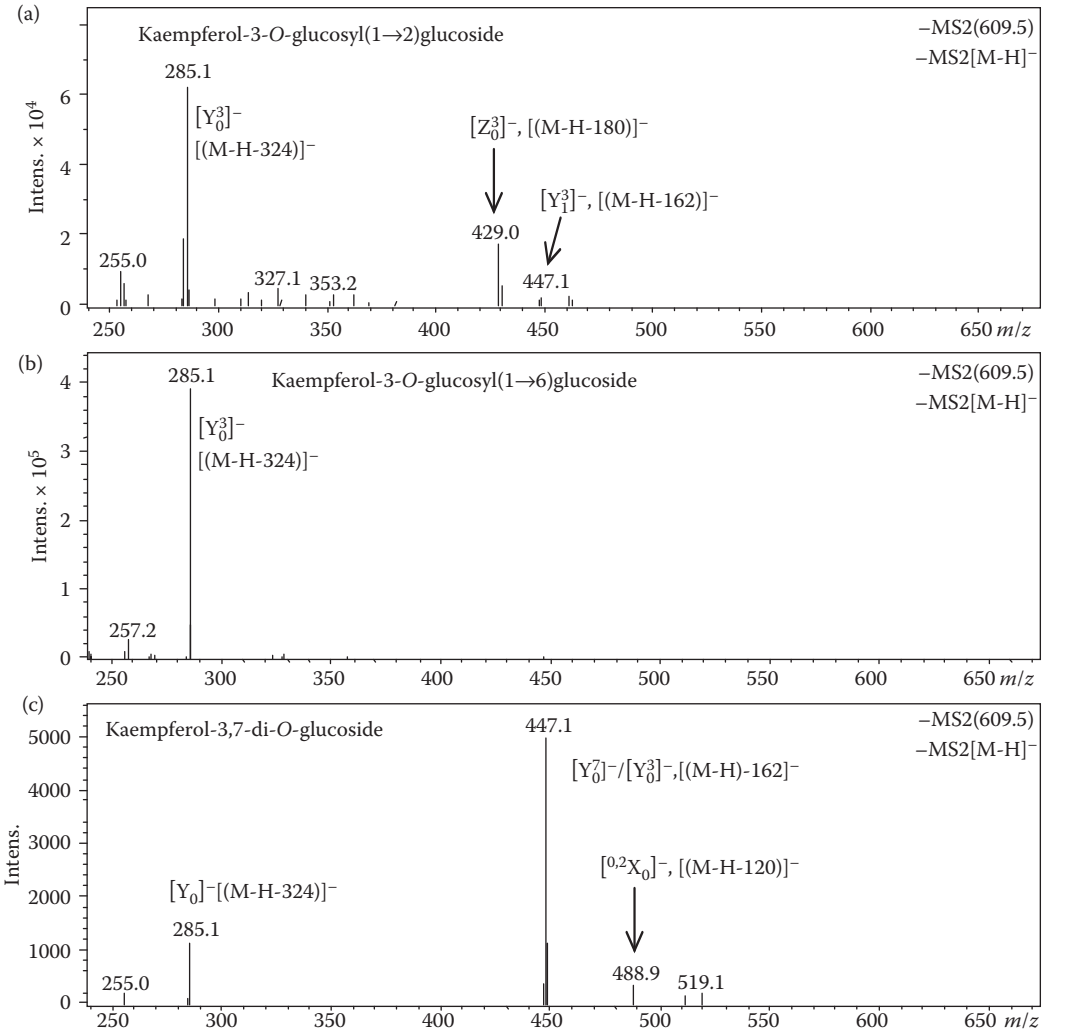

FIGURE 15.13 MS2[M–H]⁻ of isomeric flavonoid diglucosides: (a) kaempferol-3-*O*-sophoroside (glucosyl(1–2)glucoside); (b) kaempferol-3-*O*-gentiobioside (glucosyl(1–6)glucoside); (c) kaempferol-3,7-di-*O*-glucoside. (From Ferreres, F., Llorach, R., and Gil-Izquierdo, A. 2004. *J. Mass Spectrom.* 39: 312–321. With permission.)

for hexose/desoxyhexose/pentose, respectively. In the flavonoid oligosaccharide fragmentation, fragments due to the rupture of the interglycosidic linkage with the losses mentioned above can be observed (Figure 15.13a, ion $[Y_1^3]^-$) and/or these losses plus 18 m.u. (180/164/150 m.u. for hexose/desoxyhexose/pentose, respectively) (Figure 15.13a, ion $[Z_1^3]^-$). This loss of a glycosidic fraction plus water is not observed in the losses of the sugars directly linked to the phenolic hydroxyls and are associated with interglycosidic linkages.

In flavonoids glycosylated over more than one phenolic hydroxyl, the base peak is originated by the loss of one of the glycosidic fractions over one of the hydroxyls, the aglycone ion being less abundant or unexisting, and the ion $[Z]^-$ that characterizes the interglycosidic linkages is not observed (Figure 15.13c).

Claeys and coworkers (Ma et al., 2001; Cuyckens et al., 2001; Cuyckens and Claeys, 2002) carried out an exhaustive study under different conditions of the interglycosidic linkage such as in the case of the rhamnoglucosides, *O*-neohesperidosides [rhamnosyl(1 → 2)glucosides], and *O*-rutinosides

[rhamnosyl(1 → 6)glucosides], reaching the conlusion that it is possible to differentiate them in the negative mode, by a comparative study of the relative abundance of the ions produced by the fragmentation of the interglycosidic linkage. In the same way, a considerable number of flavonoid diglucosides, including flavonoid sophorosides [glucosyl(1 → 2)glucosides] and gentiobiosides [glucosyl(1 → 6)glucosides], were studied reaching similar conclusions (Ferreres et al., 2004). Thus, in the MS spectra of flavonoid sophorosides, ions produced by the fragmentation of the interglycosidic linkage are observed (Figure 15.13a), while this fragment ions are not observed in the case of gentiobiosides or they are less abundant, showing that the gentiobioside linkage is more stable in the conditions of the MS analysis (Figure 15.13b). In the same study, it was also demonstrated that for flavonoid di-*O*-glycosylated in 3,7-positions, the fragmentation of the sugar linked to 7 position is produced preferably, and therefore, it is possible to determine the type of oligosaccharide linked in each position. Thus, in the MS2[M–H]$^-$ fragmentations

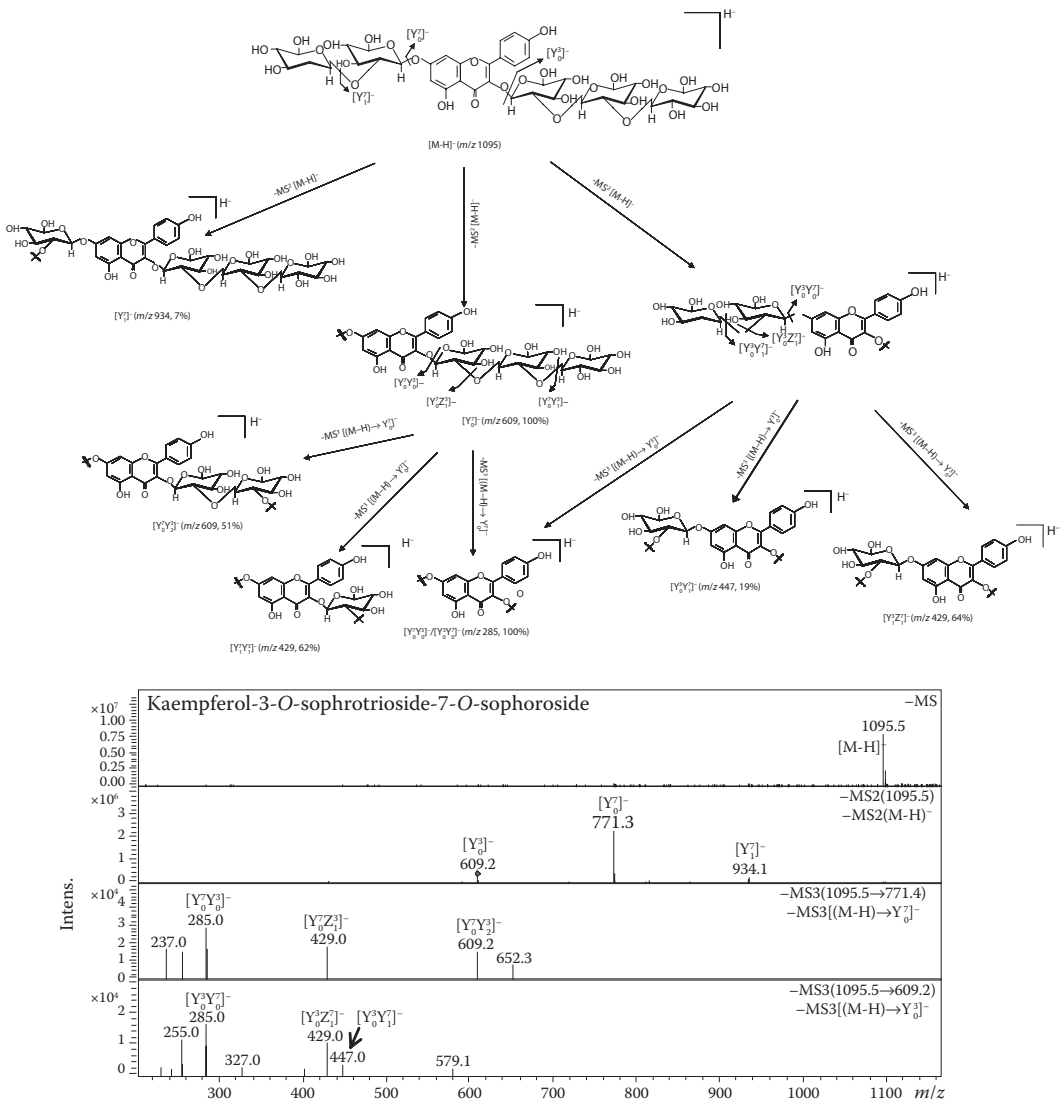

FIGURE 15.14 MS fragmentation pathway of kaempferol 3-*O*-sophorotrioside-7-*O*-sophoroside; MS2[M–H]$^-$; MS3[(M–H)→Y_0^7]$^-$; MS3[M–H)→Y_0^3]$^-$. (From Ferreres, F., Llorach, R., and Gil-Izquierdo, A. 2004. *J. Mass Spectrom.* 39: 312–321. With permission.)

of flavonoid isomers with three glucoses (-3-*O*-sophorotriosides and -3-*O*-sophoroside-7-*O*-glucoside) or four glucoses (-3-*O*-sophorotriosides-7-*O*-glucoside and -3-*O*-sophoroside-7-*O*-sophoroside) (Figure 15.12), the base peak indicated the complete loss of the sugar moieties in position 7.

The MS3[(M–H) → Y_0^7]$^-$ event (Figure 15.12a and b) revealed the ions resultant from the fragmentation of the glycoside in 3 position.

Figure 15.14 presents the fragmentation scheme of a kaempferol penta-glucoside (Ferreres et al., 2004) in which the main ions produced in the MS2 and MS3 events are observed.

The preferential fragmentation MS2[M–H]$^-$ observed is due to the loss from the 7 position ([Y_0^7]$^-$ base pick), and other ions due to the fragmentation of the diglucoside in 7 position are also observed ([Y_1^7]$^-$), as well as the combined loss of the complete tri-glucoside linked in 3 position ([Y_0^3]$^-$). In the MS3 events, [(M–H)→Y_0^7]$^-$ and MS3[M–H)→Y_0^3]$^-$ ions are produced by the fragmentation of the glycosidic fractions in 3 and 7 positions, respectively. Thus, ions at *m/z* 429 ([$Y_0^7Z_1^3$]$^-$ and [$Y_0^3Z_1^7$]$^-$) are not the same as they are originated by different fragmentation pathways.

15.6.3.4 C-Glycosyl Flavonoids

The flavonoids, as we have indicated above, can also be present combined with sugars through a C–C bond. In most cases, the *C*-glycosylation takes place in the 6 and/or 8 positions of the flavonoid nucleus. They are often found as *O*-glycosylated *C*-glycosyl flavonoids. In a way different to flavonoid-*O*-glycosides, the breakdown of the linkage between the sugar and the flavonoid nucleus is not possible, and an internal fragmentation of the sugar is observed. From the analysis of the MS data available, it is possible to know the type of sugar directly linked to the flavonoid, and from the comparative study of the relative abundance of the ions of these fragments it is possible to differentiate the *C*-glycosylation position (6 and 8 positions of the flavonoid). It is also possible to indicate the *O*-glycosylation position, either on a phenolic hydroxyl or on the *C*-glycosyl sugar, as well to differentiate tentatively the substitution position on the 2 or 6 position of the *C*-glycosidic sugar.

In general, the absence of an abundant [aglycone–H]$^-$ ion in the –MS2 and/or –MS3 events, and the presence of [aglycone + 41/42]$^-$ and/or [aglycone + 71/72]$^-$ are indicative of mono-*C*-glycosyl flavones, while for di-*C*-glycosyl flavones [aglycone + 83/84]$^-$ and/or [aglycone + 113/114]$^-$ are important ions.

In mono-*C*-glycosyl flavonoids, the internal fragmentation of the sugar in positions 0/2 and 0/3 are the most frequent leaving the ions [^{0,2}X]$^-$ and [^{0,3}X]$^-$, respectively (Figure 15.15), which is translated into 120 and 90 m.u. losses for hexoses and 90 and 60 for pentoses, and allows the characterization of sugar involved in the *C*-glycosylation. In addition, the abundant ions corresponding to the aglycone + 41/42 and the aglycone + 71/72, respectively, allow the characterization of aglycones, and their relative abundance indicates the *C*-glycosylation position. In mono-*C*-deoxyhexosyl derivatives, the ions aglycone + 41 {^{0,2}X$^-$, [(M–H)–104]$^-$} and aglycone + 83 {[^{0,4}X–18]$^-$, [(M–H)–62]$^-$} were observed (Ferreres et al., 2007a).

Thus, in mono-*C*-glycosyl flavones, the presence of ion [(M–H)–120]$^-$ and the simultaneous absence of the [(M–H)–60]$^-$ indicate a hexose as the sugar of *C*-glycosylation; in these cases, the ion [(M–H)–90]$^-$ can also be present, which is more relevant in the 6-*C*-hexosyl derivatives (being, sometimes, the base peak) than in the 8-*C*-hexosyl derivatives (being, sometimes, very small). The ion [(M–H)–18]$^-$, more frequent in 6-*C*-hexosyl derivatives than in 8-*C*-hexosyl derivatives, can also be observed (Ferreres et al., 2003) (Figure 15.15).

In the mono-*C*-glycosyl flavone, the absence of [(M–H)–120]$^-$ and the presence of ion [(M–H)–60]$^-$ ([^{0,3}X]$^-$) indicate the presence of a pentose as the sugar of *C*-glycosylation, this ion being also higher than the [(M–H)–90]$^-$ ([^{0,2}X]$^-$) ion in the 6-*C*-pentosyl derivative than in the 8-*C*-pentosyl derivative. Both isomers exhibit the ion [(M–H)–90]$^-$, which can be the base peak.

In asymmetric di-*C*-glycosyl flavones (di-*C*-substitued flavonoids with different sugars), the preferential fragmentation is that of the sugar linked to *C*-6 relatively to the one linked to *C*-8 and there is a production of partial fragments of both sugars that lead to losses of mass from the molecular ion. The relative abundance of these ions indicates the *C*-glycosylation position for both sugars. In all cases, the ions A + 83/84 and A + 113/114 can be observed, which characterize the aglycone (Figure 15.16).

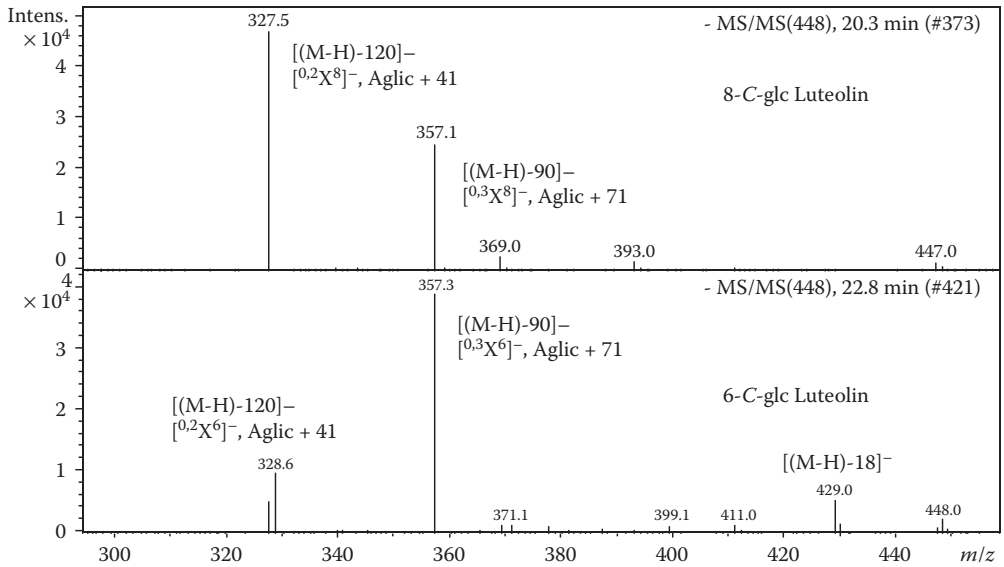

FIGURE 15.15 MS2[M–H]⁻ of mono-*C*-glycosyl flavonoid isomers: 8-*C*-glucosyl luteolin and 6-*C*-glucosyl luteolin. (From Ferreres, F. et al. 2003. *Phytochem. Anal.* 14: 352–359. With permission.)

In *O*-glycosyl-*C*-glycosyl flavones, the study of the relative abundance of the main ions from the MS preferential fragmentation on –MS2 and/or –MS3 events allows the differentiation of the position of the *O*-glycosylation, either on the phenolic hydroxyl or on the sugar moiety of *C*-glycosylation. In addition, it is possible to discriminate between *O*-glycosylation at 2″ and at 6″ positions (Figure 15.17).

The occurrence of an abundant ion Y_0^- ([(M–H)–132/–146/–162]⁻, mono-*O*-pentosyl/rhamnosyl/hexosyl-*C*-glycosyl derivatives) after –MS2 fragmentation characterizes the *O*-glycosylation on phenolic hydroxyls (Figures 15.17a and 15.18).

The preferential fragmentation leading to a relevant Z_1^- ([Y_1–18]⁻) fragment is characteristic of 2″-*O*-glycosyl-*C*-glycosyl derivatives (Figures 15.17b and 15.19a). The 6″-*O*-glycosyl-*C*-glycosyl derivatives

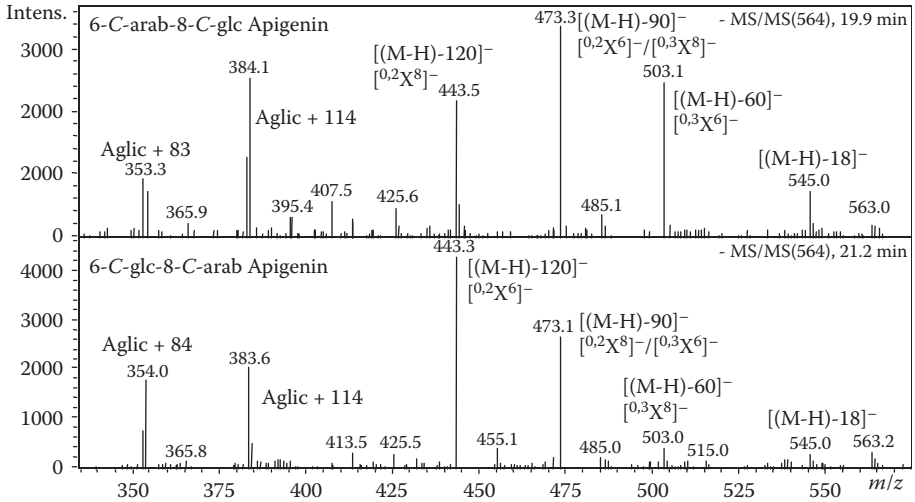

FIGURE 15.16 MS2[M–H]⁻ of asymmetric di-*C*-glycosyl flavone isomers: 6-*C*-arabinosyl-8-*C*-glucosyl apigenin and 6-*C*-glucosyl-8-*C*-arabinosyl apigenin. (From Ferreres, F. et al. 2003. *Phytochem. Anal.* 14: 352–359. With permision.)

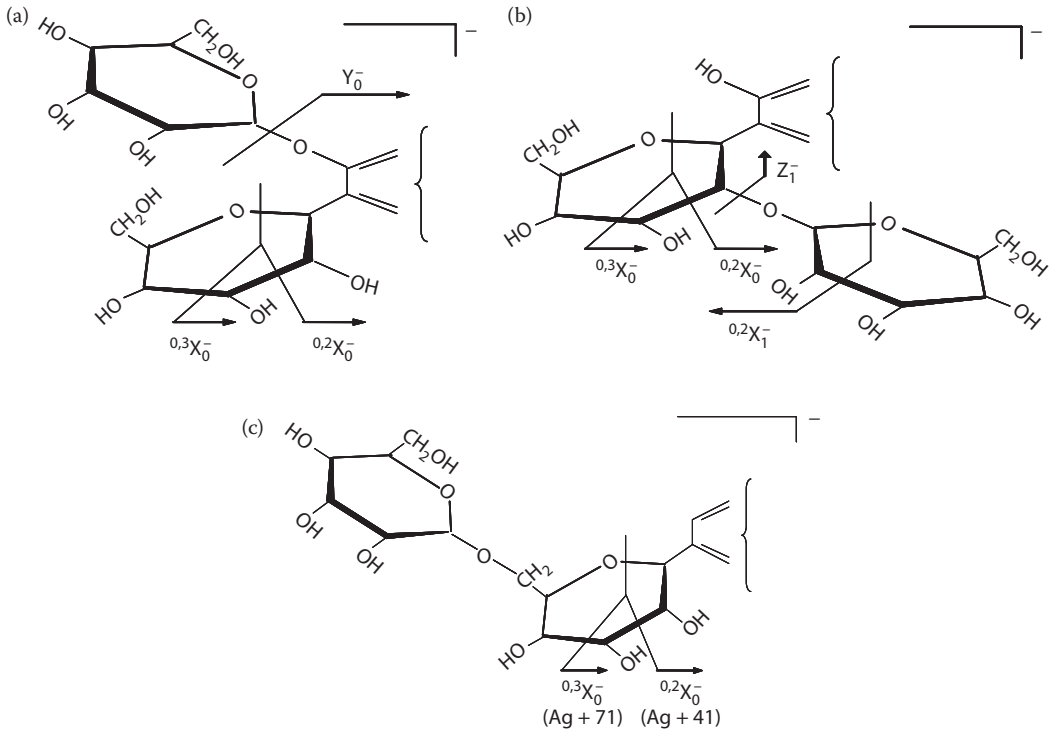

FIGURE 15.17 General fragmentation of *O*-glycosyl-*C*-glycosyl flavones: (a) *O*-glycosylation on phenolic hydroxyls; (b) 2″-*O*-glycosyl-*C*-glycosyl derivatives; (c) 6″-*O*-glycosyl-*C*-glycosyl derivatives. (From Ferreres, F. et al. 2007b. *J. Chromatogrphy* A 1161: 214–223. With permission.)

are characterized by $^{0,2}X_0^-$ and $^{0,3}X_0^-$, which is generated by a global loss of the sugar moiety from the *O*-glycosylation at 6″ and the glycosidic fraction that involves the carbons 6″-3″ and 6″-4″ of the *C*-glycosyl residue ([(M–H)–162–120]⁻/[(M–H)–162–90]⁻, in the case of 6″-*O*-hexosyl-*C*-hexosyl derivatives) (Figures 15.17c and 15.19b).

The simultaneous occurrence of sugars on the hydroxyls in 2″ and 6″ of the *C*-glycosylation sugar produces a fragmentation in which the ions Z_1^- ([(M–H)–180]⁻, loss of the sugar in 2″ with water) and $^{0,2}X_0^-$ ([(M–H)-120–162]⁻, internal fragmentation of the *C*-glycosylation sugar together with the sugar in 6″) are observed in addition to the ions that characterize the aglycone. In the case of *C*-glycosylation in 6 position the ion Z_1^- is the base peak, while the ions that characterize the aglycone are more abundant in the 8-*C* isomers (Figure 15.20) (Ferreres et al., 2007b).

Regarding the combined *O*-glycosylated compounds (both on phenolic hydroxyl and on sugar moiety at *C*-glycosylation), the main fragmentation on –MS2 events produces a Y_0^- characterizing the *O*-glycosylation on the phenolic hydroxyl, and the –MS3[(M–H) → Y_0]⁻ fragmentation of the *O*-glycosylation on the *C*-glycosyl residue.

In Figure 15.21, it can be observed that both in 6-*C* as in 8-*C* flavonoids, as well as if the substitution is in 2″ or 6″ in MS2, the base peak is due to the fragmentation of the sugar linked to the phenolic hydroxyl, and in MS3 the behavior is similar to that previously mentioned for X″-*O*-glycosyl-*C*-glycosyl flavones.

15.6.3.5 Acyl Flavonoids

Flavonoid glycosides can occur as acylated derivatives either with aliphatic or aromatic acids. Cinnamic acid derivatives are the most frequent in nature and in the MS fragmentations of this type of compounds,

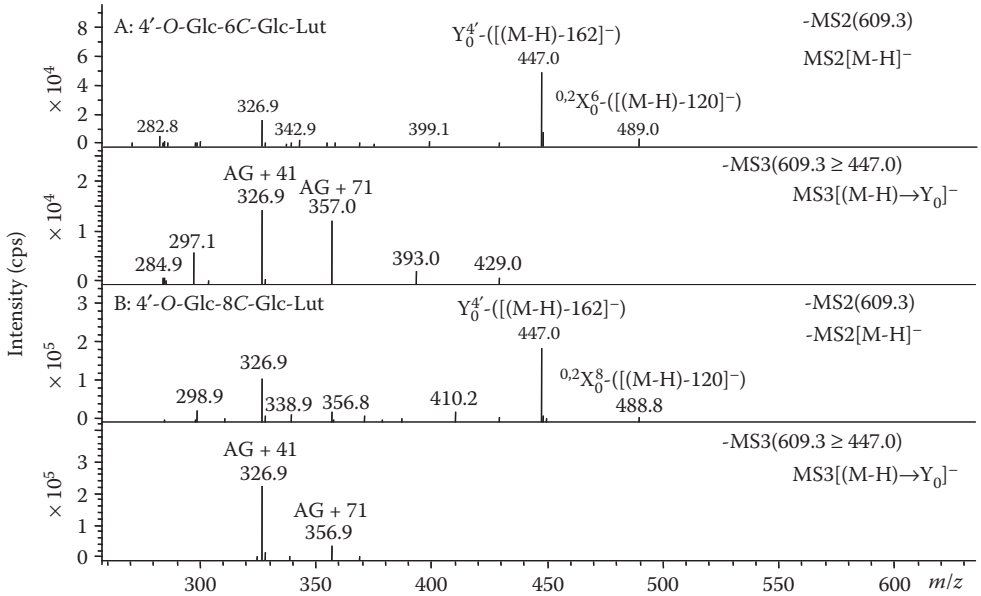

FIGURE 15.18 MS2[M–H]⁻ and MS3[(M–H) → Y_0]⁻ of *C*-glycosyl flavones *O*-glycosylated on phenolic hydroxyl: (a) 4′-*O*-glucosyl-6-*C*-glucosyl luteolin; (b) 4′-*O*-glucosyl-8-*C*-glucosyl luteolin. (From Ferreres, F. et al. 2007b. *J. Chromatography A* 1161: 214–223. With permission.)

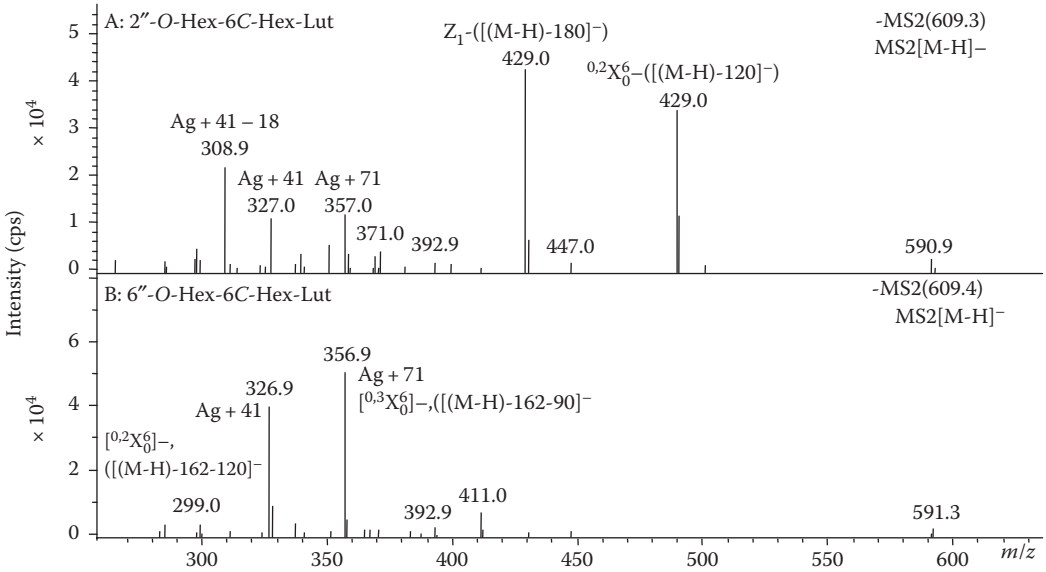

FIGURE 15.19 Fragmentation and MS2[M–H]⁻ of X′′-*O*-glycosyl-*C*-glycosyl flavones: (a) 2″-*O*-hexosyl-6-*C*-hexosyl luteolin; (b) 6″-*O*-hexosyl-6-*C*-hexosyl luteolin. (From Ferreres, F. et al. 2007b. *J. Chromatography A* 1161: 214–223. With permission.)

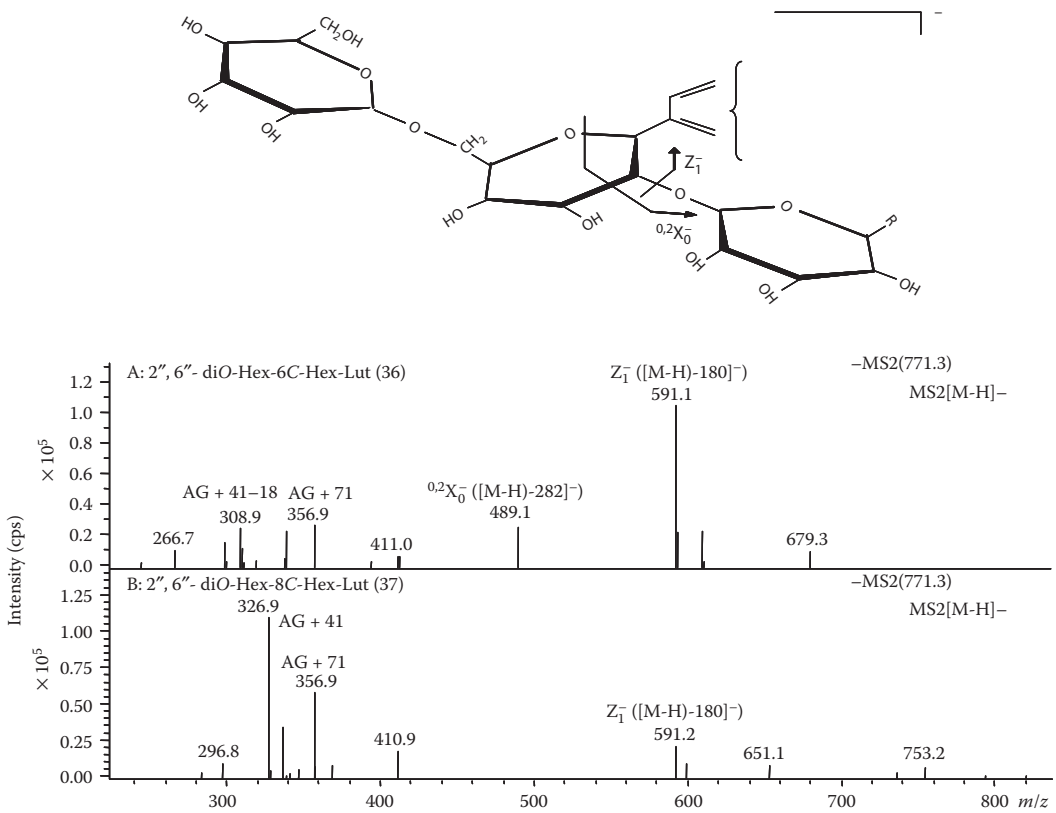

FIGURE 15.20 Fragmentation and MS2[M – H]⁻ of 2″,6″-di-*O*-glycosyl-*C*-glycosyl flavones: (a) 2″,6″-di-*O*-hexosyl-6-*C*-hexosyl luteolin; (b) 2″,6″-di-*O*-hexosyl-8-*C*-hexosyl luteolin. (From Ferreres, F. et al. 2007b. *J. Chromatography A* 1161: 214–223. With permission.)

ions produced by the loss of 146, 162, 176, or 206 m.u. are observed and they are related to the acyl residues *p*-coumaroyl, caffeoyl, feruloyl, or sinapoyl, respectively. Brassicaceae species are rich in cinnamoyl flavonoids, and most of them show the general structure of a flavonol-3-*O*-(hydroxycinnamoyl)-glycoside-7-*O*-glycoside. In their MS2[M–H]⁻, the main fragmentation produces the loss of the glycosidic residue in 7 position, and ions produced by the loss of the acyl residue can also be observed. In the MS3[(M–H)→Y_0^7]⁻ event, representative of the fragmentation of the acylated glycosidic residue on position 3, the fragments due to the loss of the acyl residue are preferentially observed (Vallejo et al., 2004). *Diplotaxis tenuifolia* (Brassicaceae) shows a glycosylation pattern with conjugations on the hydroxyls at 3, 3′, and 4′ positions (flavonol-3,3′,4′-tri-*O*-glucosides) and acylation on the glucosyl residue at 3′ (flavonol-3,4′-di-*O*-glucoside-3′-*O*-(hydroxycinnamoyl)-glucoside) or on the sugar residues at 3,3′-positions (flavonol-3-*O*-(acyl)-glucoside)-3′-*O*-(acyl)-glucoside-4′-*O*-glucoside). The MS*n* (*n*, 2–3) fragmentation behavior of the monoacylated derivatives (flavonol-3,4′-di-*O*-glucoside-3′-*O*-(hydroxycinnamoyl)-glucoside) showed sequential losses of hexosyl residues from the [M–H]⁻ and [(M–H)–Glc]⁻ ions to give a peak base. In the MS4 (fragmentation of the ion corresponding to the flavonol-3′-*O*-(acyl)-glucoside), the loss of the acyl radical produces an abundant ion that often is the base peak.

In compounds diacylated on the sugar residues at 3 and 3′ positions, the MS2 fragmentation showed a first loss of 162 units from the deprotonated molecular ion that must be due to the loss of the glucose that is not linked at the 4′ position (Figure 15.22a).

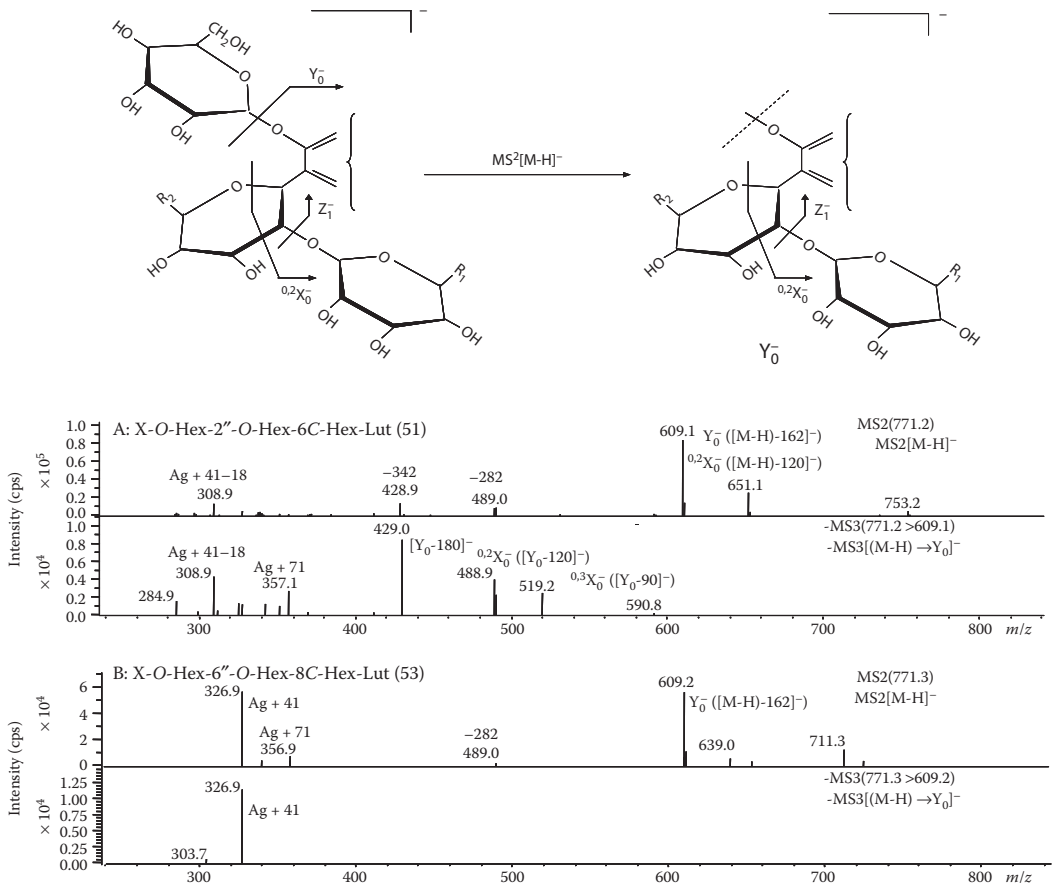

FIGURE 15.21 Fragmentation, MS2[M–H]⁻, and MS3[(M – H) → Y⁰]⁻ of X-*O*-glycosyl-X′′-*O*-glycosyl-*C*-glycosyl flavones: (a) X-*O*-hexosyl-2″-*O*-hexosyl-6-*C*-hexosyl luteolin; (b) X-*O*-hexosyl-6″-*O*-hexosyl-8-*C*-hexosyl luteolin. (From Ferreres, F. et al. 2007b. *J. Chromatography A* 1161: 214–223. With permission.)

In the MS3 event, it was possible to observe a loss of one or both acids, and the loss of glucose with its acyl moiety (Figure 15.22b), where the loss of the acyl-glucosyl radical at position 3 was the base peak in the majority of the studied ions. Finally, the MS4 events of ions that still have both glucose and acid moieties gave the deprotonated aglycone (base peak) (Figure 15.22c) or the ion coming from the corresponding desacylation (Figure 15.22d) (Martínez-Sánchez et al., 2007). This means, that in the same way as happened in the fragmentation of rutinosides/neohesperidosides and gentiobiosides/sophorosides, if the acyl substitution is on 6 position of the sugar moiety, the linkage is more stable than if the linkage is at 2 position, and produces the loss of an acyl-glucosyl residue (Figure 15.22c), while in the 2 substitution there is a breakdown of this bond with subsequent desacylation (Figure 15.22d).

In flavonoids acylated with aliphatic acids, the most common are acetic or malonic derivatives. In the MS fragmentation of the dicarboxylic acids (malonic acid), a first loss of 44 mass units (loss of the carboxylic radical, CO_2) is due to decarboxylation (Figure 15.23).

In both these and the acetyl derivatives, it is posible to know the acylation position, particulary when they are linked at position 6 of the sugar, due to the fragments produced by the internal fragmentation of the sugar, that produces fragments containing the acyl residue. Thus, in *Capsicum annuum* (Marín et al., 2004), it was possible that the characterization of the acylation position on a

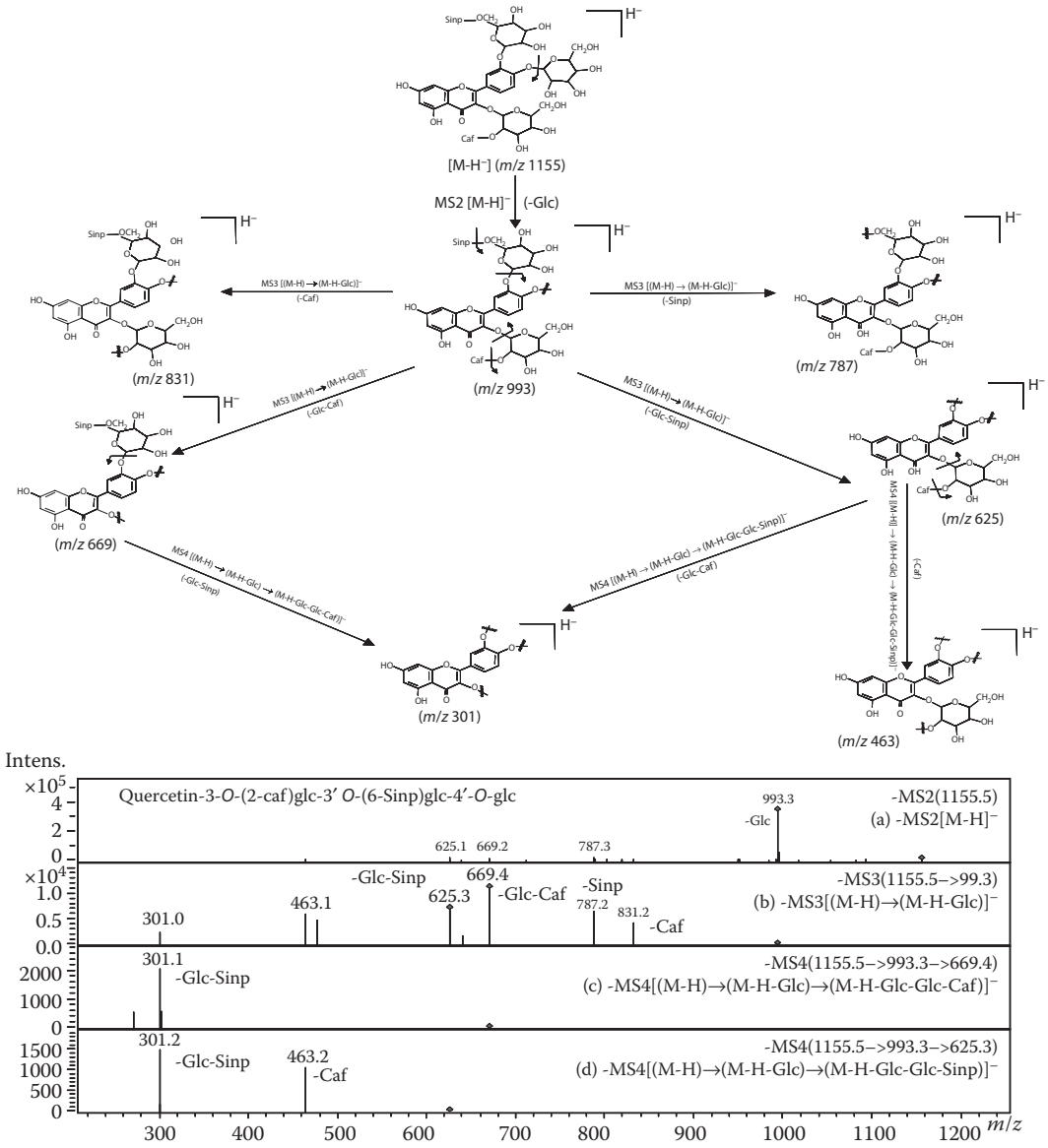

FIGURE 15.22 Fragmentation pathway and MS of quercetin-3-*O*-(2-caffeoyl)glucoside-3′-*O*-(6-sinapoyl)glucoside-4′-*O*-glucoside: (a) MS2[M–H]⁻; (b) MS3[(M–H)→(M–H–Glc)]⁻; (c) MS4[(M–H)→(M–H–Glc)→(M–H–Glc–Glc–Caf)]⁻; (d) MS4[(M–H)→(M–H–Glc)→(M–H–Glc–Glc–Sinp)]⁻. (From Martínez-Sánchez, A. et al. 2007. *J. Agric. Food Chem.* 55: 1356–1363. With permission.)

di-*C*-glycosyl-luteolin acylated with malonic acid was due to the presence in the MS3 event of ions at *m/z* 489 ($[^{0,2}X^6]^-$, –(120 + 42)) and at *m/z* 519 ($[^{0,3}X^6]^-$, –(90 + 42)) showing that the 42 residue was not linked to positions 2 or 3 of the hexose, as these carbons are not involved in these losses; therefore, the acylation should be at either positions 4 or 6 in the sugar residue. Position 6 is much more frequent in nature, and we tentatively identified this compound as luteolin 6-*C*-(6-malonyl)-hexoside-8-*C*-pentoside (Marín et al., 2004).

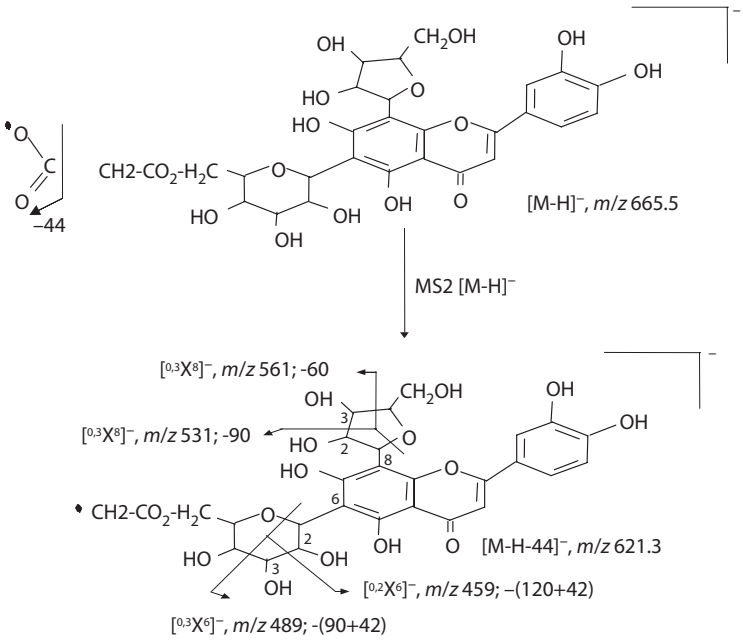

FIGURE 15.23 Fragmentation pathway of luteolin-6-*C*-(6-malonyl)hexoside-8-*C*-pentoside. (From Marín, A. et al. 2004. *J. Agric. Food Chem*. 52: 3861–3869. With permission.)

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16

Flavan-3-Ols and Proanthocyanidins

Sylvain Guyot

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16.1 Introduction

Flavan-3-ol monomers, commonly named catechins, and their oligomers and polymers (i.e., proanthocyanidins and condensed tannins) are polyphenolic compounds widespread in edible plants. Several health benefits such as antioxidant, cancer preventive, cardiopreventive, antimicrobial, antiviral, and neuroprotective agents have been reported for this polyphenol class (Scalbert 1991; Santos-Buelga and Scalbert 2000; Aron and Kennedy 2008; De Pascual-Teresa et al. 2010). They are important constituents of food for the majority of people around the world and may be highly concentrated in some plant tissues. For instance, catechins may account for more than 25% of the dry weight of tea leaves (Balentine et al. 1997). Catechins and proanthocyanidins are found in common foods such as fruits (apples, pears, plums, strawberry, kiwi, dates, many red fruits, etc.), cereals (sorghum, barley, etc.), seeds and nuts (beans, peas, almonds, etc.), spices, aromatic plants, and more scarcely in vegetables. Flavanols are also found in various foodstuffs of plant origin (wines, tea, ciders, beers, chocolates, jams, puree, etc.) (Gu et al. 2004). Noticeably, in these processed foods, catechins and proanthocyanidins are not only present in their native form, but they have sometimes undergone structural changes especially related to their susceptibility to oxidation with a significant impact on their properties. The most obvious example is probably that of black tea catechins that are enzymatically oxidized, forming theaflavins and thearubigins responsible for the color of the infusions (Tanaka et al. 2010).

Flavanols are also a remarkable class of polyphenols that exhibit a great diversity of structural features on the basis of their number and position of the hydroxyl groups; the presence of esterification by gallic acid; and the nature, the number, and the position of the interflavan linkages (IFL) in a very wide range of molecular sizes. Furthermore, by definition, proanthocyanidins are polyphenols belonging more specifically to the class of tannins (they are thus distinguished by the appellation of "condensed tannins"), which means they have the property to associate with proteins, resulting in their precipitation. In addition, they have a huge capacity to associate with cell wall polysaccharides (McManus et al. 1985; Renard et al. 2001).

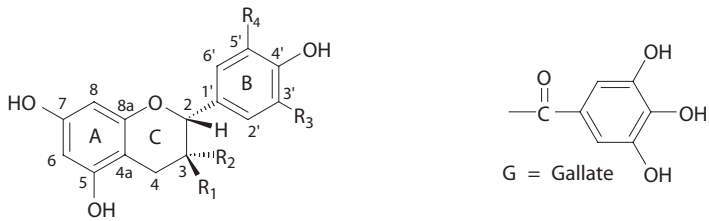
Therefore, we understand that the extraction, separation, and analysis of catechins and procyanidins are often hampered by several properties related to their structural diversity, their tanning properties, and also their sensitivity toward certain reactions such as oxidation.

Along with the presentation of some aspects of the structure, nomenclature, and distribution of catechins and proanthocyanidins in foods, the main objective of this chapter is to provide an overview of the methods currently available for their extraction, fractionation, purification, and structural characterization. We will focus particularly on techniques for quantifying these compounds in complex food matrices. The application of these methods on various plant-derived foods has often demonstrated that among the plant secondary metabolites, catechins and proanthocyanidins were often the major polyphenolic species in terms of concentration and are therefore the main polyphenol class in terms of food intake.

16.2 Nomenclature and Structures

Among phenolic compounds that are present in our everyday foods (fruits, vegetables, beans, grains, cereals, etc.), flavanols are distinguishable from other flavonoids (flavonols, anthocyanins, flavones, etc.) since they are the sole compounds that exist essentially as free forms, not substituted by sugars or by nonphenolic groups. Some glycosylated forms of flavanols have been described (Nonaka et al. 1983; Friedrich and Galensa 2002; Raab et al. 2010). However, these particular flavanols are generally absent or present in very low concentration in common foods. As another particularity, flavan-3-ols are the only flavonoid molecules exhibiting a very wide range of molecular sizes. Actually, flavanols exist from the simple monomeric form to the highly polymerized proanthocyanidins with molecular weight globally ranging from 300 to 55,000 Da (Souquet et al. 1996; Guyot et al. 2001a; Takahata et al. 2001), this upper value being probably not a limit.

The chemical structure of flavanol monomers is built on the 2-phenyl chromane nucleus (Figure 16.1) having a hydroxyl group at the 3 position. This group can be substituted by gallic acid (Figure 16.1).



	Configuration		Substitution pattern			
Flavanol name	C_2	C_3	R_1	R_2	R_3	R_4
(+)-Catechin	R	S	OH	H	OH	H
(+)-Gallocatechin	R	S	OH	H	OH	OH
(+)-Catechin gallate	R	S	OG	H	OH	H
(+)-Gallocatechin gallate	R	S	OG	H	OH	OH
(–)-Epicatechin	R	R	H	OH	OH	H
(–)-Epigallocatechin	R	R	H	OH	OH	OH
(–)-Epicatechin-3-*O*-gallate	R	R	H	OG	OH	H
(–)-Epigallocatechin-3-*O*-gallate	R	R	H	OG	OH	OH
(–)-Epiafzelechin	R	R	H	OH	H	H

FIGURE 16.1 Structure and nomenclature of catechins commonly found in foodstuffs.

Particular names are given to catechins as a function of the hydroxylation pattern of the B ring (epiafzelechin, catechin, gallocatechin), stereochemistry of the C ring [(2R, 3R), (–)-epi-form or (2R, 3S) (+)-form], and esterification by gallic acid at the 3 position (gallate forms). The structures and names of the most commonly found catechins in foods are given in Figure 16.1.

Noticeably, in the particular case of tea, the term "catechin" also includes theaflavins, theaflavic acids, and thearubigins, resulting from the enzymatic oxidative coupling of native catechins present in fresh tea leaves (Balentine et al. 1997). These molecules are characterized by the presence of a benzotropolone group in their structure (Figure 16.2).

Proanthocyanidins are flavan-3-ol oligomers and polymers. The term "condensed tannins" is commonly used to designate these molecules when they are no longer considered as individual molecules but rather as a mixture of polydispersed and polymerized species capable of precipitating proteins. The term "proanthocyanidins" is generally reserved for oligomers (dimers, trimers, tetramers, etc.), but, in practice, the rule is not very strict about this terminology. The term "proanthocyanidins" derives from a chemical property of these molecules: when subjected to a strong acid treatment in hot organic medium,

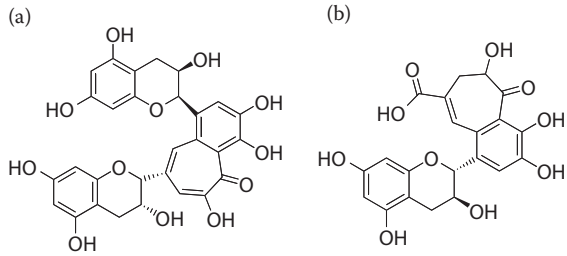


FIGURE 16.2 Structure of (a) theaflavin and (b) theaflavic acid.

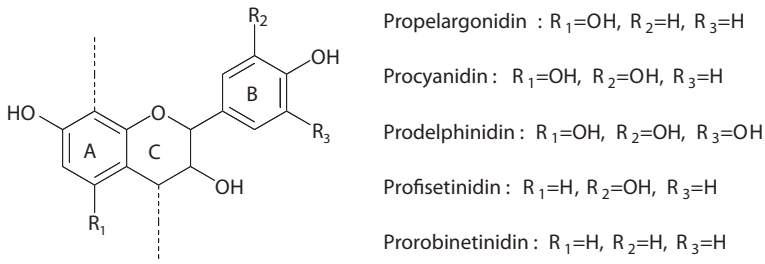


FIGURE 16.3 Basic structure of the main proanthocyanidin classes.

they produce anthocyanidins (anthocyanin aglycones) leading to a red coloration of the reaction medium. Several reviews have been published describing in detail the structural diversity, nomenclature, and properties of proanthocyanidins (Porter 1988; Ferreira et al. 2006). Proanthocyanidins are subdivided into several classes, whose names are a function of the nature of the anthocyanidins that are released in the medium after hot acid treatment (Figure 16.3). The class most frequently found in food is the procyanidins that are constituted with (epi)catechin units. Prodelphinidins and propelargonidins are less commonly found (Foo and Porter 1981; Gu et al. 2003; Phenol-Explorer 2009).

In addition, some flavanol units of the proanthocyanidin chain can be partly substituted by gallic acid (galloylated) and more rarely by a sugar moiety.

Proanthocyanidins are also divided into two categories according to the number and the position of the IFL (Weinges et al. 1968). For B-type proanthocyanidins, which are the most widespread in edible plants, flavanol units are linked by C4–C8 or C4–C6 bonds whereas A-type proanthocyanidins are characterized by an additional C2–O–C7 or C2–O–C5 IFL (Figure 16.4).

In addition, an informal nomenclature based on the numbering of molecules of the B- and A-type, initially proposed by Weinges et al. (1968), is still used to name the commonly found proanthocyanidin dimers (Table 16.1).

Lastly, proanthocyanidins are characterized by their degree of polymerization (DP) that corresponds to the number of flavanol units in a given proanthocyanidin molecule. This structural feature is essential since it is strongly linked to the physicochemical, biochemical, biological, and nutritional properties of proanthocyanidins (Santos-Buelga and Scalbert 2000). For instance, astringency and also the balance between the sensory perception of bitterness and astringency are related to the DP (Lea and Arnold 1978; Vidal et al. 2003).

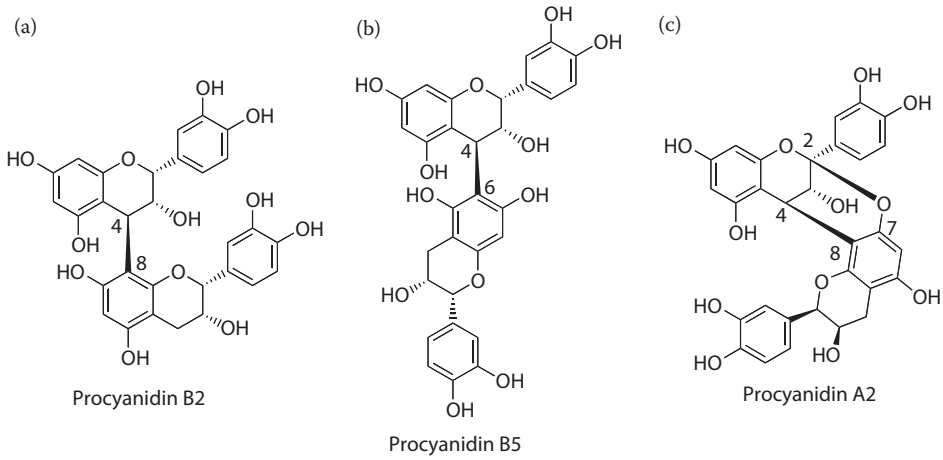


FIGURE 16.4 Examples of A- or B-type procyanidins dimers with C4–C8 or C4–C6 interflavan linkage.

TABLE 16.1

Structure of Proanthocyanidin Dimers Found in Common Foods

Name	Extension Unit	Interflavan Linkage	Terminal Unit	CAS Number
Procyanidin B1	(–)-Epicatechin	(4β → 8)	(+)-Catechin	20315-25-7
Procyanidin B2	(–)-Epicatechin	(4β → 8)	(–)-Epicatechin	29106-49-8
Procyanidin B3	(+)-Catechin	(4α → 8)	(+)-Catechin	23567-23-9
Procyanidin B5	(–)-Epicatechin	(4β → 6)	(–)-Epicatechin	12798-57-1
Procyanidin B6	(+)-Catechin	(4α → 6)	(+)-Catechin	12798-58-2
Procyanidin B7	(–)-Epicatechin	(4β → 6)	(+)-Catechin	12798-59-3
Prodelphinidin B1	(–)-Epigallocatechin	(4β → 8)	(+)-Gallocatechin	78362-04-6
Prodelphinidin B2	(–)-Epigallocatechin	(4β → 8)	(–)-Epigallocatechin	87392-61-8
Prodephinidin B3	(+)-Gallocatechin	(4α → 8)	(+)-Gallocatechin	78362-05-7
Procyanidin A2	(–)-Epicatechin	(4β → 8 and 2β-O-7)	(–)-Epicatechin	41743-41-3

Bitterness is associated with procyanidins reaching a maximum with the tetrameric forms whereas astringency is a characteristic of higher oligomers (Lea and Arnold 1978). As far as we know, oligomers up to pentamers have been individually isolated and characterized (Abe et al. 2008). However, above DP3, purified proanthocyanidins are generally obtained as a more or less polydispersed fraction that are characterized by their mean degree of polymerization (mDP). Thus, procyanidin fractions with mDP up to 190 have been purified from a cider apple variety (Guyot et al. 2001a).

16.3 Presence and Role in Food

16.3.1 Occurrence in Foods

Several reviews have been published on the nature, occurrence, and nutritional properties of flavanols in foods (Santos-Buelga and Scalbert 2000; Auger et al. 2004; Yilmaz 2006; Aron and Kennedy 2008). Thus, catechins and proanthocyanidins are very widespread and sometimes highly concentrated in edible and nonedible plants. Consequently, they are among the main phenolic compounds found in plant-derived foods (De Pascual-Teresa et al. 2000; Gu et al. 2003, 2004; Hellstrom et al. 2009) (Table 16.2). A new interesting compilation of quantitative and qualitative data is now freely available on the Internet (Phenol-Explorer 2009; Perez-Jimenez et al. 2010).

Flavanols and particularly catechins are highly concentrated in tea. Green teas contain native catechins mainly in galloylated forms whereas oolong and black teas also contain a high level of oxidation products of native catechins, including theaflavins and thearubigins (Balentine et al. 1997). Then, consumption of just 200 mL of brewed tea contributes ~20–70 mg of total catechins to the diet (Bronner and Beecher 1998). Flavanols are also widely represented in commonly consumed fruits. Thus, catechins and proanthocyanidins are present in apples (Guyot et al. 2002), plums (Tomas-Barberan et al. 2001; Nunes et al. 2008), peach (Tomas-Barberan et al. 2001), grape (Ricardo-Da-Silva et al. 1991a, b; Souquet et al. 1996), strawberry (Almeida et al. 2007), and in most edible berries (Gu et al. 2004; Hellstrom et al. 2009). They are also the major polyphenols found in some grains and cereals, such as barley and sorghum, nuts, beans, cocoa, peas, and some spices (Gu et al. 2004). In contrast, flavanols are generally not found in green vegetables.

Catechins and more particularly proanthocyanidins are also concentrated in transformed foods such as black chocolates (Adamson et al. 1999), red wines (Goldberg et al. 1998; Harbertson et al. 2008), apple juices (Oszmianski et al. 2007), apple purees (Oszmianski et al. 2008), and ciders (Alonso-Salces et al. 2004; Marks et al. 2007). However, a significant part more likely corresponds to tannin-like compounds that are formed during processing or aging (Santos-Buelga and Scalbert 2000; Cheynier 2005).

The proanthocyanidins most represented in nature are B-type procyanidins and prodelphinidins in a lesser proportion (Foo and Porter 1980). Usually, (–)-epicatechin is dominating as extension units and is

TABLE 16.2

Examples of Catechins and Proanthocyanidins Concentrations in Some Selected Foods

	Catechins	Proanthocyanidins	Reference
	mg/100 g FW or mg/100 mL		
Green tea	12		Bronner (1998)
Black tea	33		Bronner (1998)
Black chocolate	31.4	246	Gu et al. (2004)
Black chocolate	47.7	257	Hellstrom et al. (2009)
Apple (red delicious)	9.6	126	Gu et al. (2004)
Apple (red delicious)	9.5	162	Hellstrom et al. (2009)
Strawberry	4.2	145	Gu et al. (2004)
Sorghum whole grain	7.6	447	Gu et al. (2004)
Red grape	1.8	32.6	Hellstrom et al. (2009)
Green grape	2.6	54.0	Hellstrom et al. (2009)
Red wine	5.7	34.4	Hellstrom et al. (2009)
Red wine	20	313	Gu et al. (2004)
Clear apple juice	2.6	6.5	Oszmianski et al. (2007)
Cloudy apple juice	3.8	26.2	Oszmianski et al. (2007)
French cider	8	39	Alonso-salces et al. (2004)
English cider	1–22	1–72	Marks et al. (2007)

also largely present as terminal units although (+)-catechin or (–)-epigallocatechin is also commonly found (Foo and Porter 1980). Homogeneous procyanidins essentially constituted with (–)-epicatechin units as extension units are found in apples with polymerization degrees ranging from 3 to 50 depending on the variety (Sanoner et al. 1999; Wojdylo et al. 2008). These homogenous procyanidin structures are also found in high concentration in cocoa (Hammerstone et al. 1999). In contrast, in other sources, a great structural diversity can be observed depending on the plant organs or plant tissues. For example, grape seeds mainly contain galloylated and nongalloylated procyanidin oligomers whereas a significant proportion of highly polymerized prodelphinidins is found in the skin (Cheynier et al. 1997).

A-type procyanidins are not as widespread as the B-type. However, they have been found in various plants, including cranberries (Foo et al. 2000), plums (Tomas-Barberan et al. 2001), litchi (Le Roux et al. 1998), and peanut (Gu et al. 2003). Their presence in cranberries was particularly studied in relation with their potential as inhibitors of uropathogenic bacteria (Foo et al. 2000).

16.3.2 Use and Role in Foods

Although the mechanisms are far from being completely understood, catechins and proanthocyanidins are known to play a protective role in plants against bioaggressors (Treutter 2006). In foods, the primary role of catechins and proanthocyanidins is undoubtedly their contribution to the flavor and color, although more recent studies have focused more on their nutritional effect. Flavanols contribute significantly to the bitter and astringent tastes. This was verified for catechin monomers (Kallithraka et al. 1997) and more particularly for procyanidins (Lea and Arnold 1978; Peleg et al. 1999; Vidal et al. 2003) in studies dealing with cocoa products (Bonvehi and Coll 1997) or beverages such as tea, wines, and ciders, products for which these sensory properties play a key role in the overall quality. Although their native forms are colorless, flavanols, and more particularly catechins, are largely responsible for the final color of foods produced by methods that favor their oxidation. Then, these biochemical and chemical reactions have been particularly well studied to explain the formation of theaflavin, thearubigins, and others colored or colorless molecules resulting from the oxidation of tea catechins (Tanaka et al. 2010). Yellowish catechin oxidation products corresponding to dehydrocatechin A (Guyot et al. 1996) or xantylium salt (Es-Safi et al. 2000) were also characterized in cider and wine model solutions.

The bioavailability and the nutritional properties of catechins and proanthocyanidins have been extensively studied in the past two decades. Experiments have been conducted on many models, varying from simple evaluations of the *in vitro* antioxidant capacities to more complex nutritional studies on animals or humans. These experiments have shown that flavanols exhibit various activities as antioxidant, cardiopreventive, anticarcinogen, antimicrobial, antiviral, and neuroprotective agents (Santos-Buelga and Scalbert 2000; Aron and Kennedy 2008). Most of the bioavailability studies indicated that monomeric catechins are only weakly absorbed in plasma as their free forms but more as sulfated, methylated, and glucuronide derivatives (Donovan et al. 1999). Bioavailability of procyanidins seems less clear. The initial papers indicated they were not absorbed in plasma (Donovan et al. 2002) whereas Shoji et al. were able to detect apple procyanidins up to pentamers in rat plasma (Shoji et al. 2006a). However, the general idea about procyanidin bioavailability is that they are not extensively degraded in the acidic conditions of the stomach *in vivo*; a very minor part is absorbed and found in plasma and the main part exhibits only local activity in the gastrointestinal tract or activity mediated by phenolic acids produced through microbial degradation (Manach et al. 2004). Interestingly, the tannin properties of proanthocyanidins and their ability to associate with cell wall polysaccharides (fibers) may have some consequences on their bioavailability and nutritional properties (Aprikian et al. 2003). This underlines the essential role of the food matrix on the overall health effect of food micronutrients.

The aim of this chapter is not to propose an exhaustive review of the nutritional properties and possible health effect of catechins and proanthocyanidins. Several review articles have been published on this general topic (Chung et al. 1998; Santos-Buelga and Scalbert 2000; Aron and Kennedy 2008) or, more recently, focusing on a particular beneficial health effect such as cardiovascular health (De Pascual-Teresa et al. 2010) and cancer prevention (Nandakumar et al. 2008) or dealing with a particular food category such as cocoa products (Seimi et al. 2008) or green tea (Thielecke and Boschmann 2009). It should be noted that, increasingly, publications indicate that health effects of flavanols and procyanidins appear to be related to mechanisms more complex than simply involving the antioxidant activity. In addition, in recent years, catechins have been increasingly used as natural ingredients in foodstuffs and feedstuffs for various purposes such as protection of animal products, antimicrobial action in foodstuffs, and use as functional ingredient in various foods and dietary supplements (Yilmaz 2006).

To conclude, further studies are needed to really understand the roles and mechanisms of action of flavanols in foods. A hurdle that still remains in the progress of these studies is undoubtedly the difficulty to obtain standards of procyanidins in a highly purified state and in sufficient quantity to carry out studies on animal models and humans.

16.4 Solubility, Stability, Extraction, and Fractionation

16.4.1 Solubility and Stability

Catechins are water soluble but not as much as procyanidins as was observed through the measure of the octanol/water partition coefficients (Yanagida et al. 2007; Zanchi et al. 2009). Moreover, contrary to popular belief, proanthocyanidins are highly soluble in water even in a polymerized state. For instance, ultracentrifugation of highly polymerized apple procyanidin fractions with mDP up to 220 dissolved in water revealed that they were almost wholly soluble even at 5 g/L (Zanchi et al. 2009) and their solubility increased in water/alcohol mixtures. This misconception probably stems from the fact that procyanidins are very sensitive to the presence of traces of impurities such as protein or polysaccharide in the aqueous medium, often resulting in their precipitation.

Catechins are stable in weak acidic media whereas they are sensitive to alkaline treatments resulting in epimerization reactions and opening of the C-ring with the formation of catechinic acids (Kiatgrajai et al. 1982). Proanthocyanidins are sensitive to both strong acidic and alkaline media leading to the disruption of the IFL (Hemingway and Mcgraw 1983; Laks Hemingway 1987). Losses of titrable procyanidins and changes in their polymerization degree are also observed when procyanidins are kept for a long time in mild acidic model solutions that mimic wine aging (Vidal et al. 2002).

Catechins and proanthocyanidins are also very sensitive to autoxidation (Hathway and Seakins 1957). The autoxidation rate is strongly enhanced in alkaline media (Mochizuki et al. 2002) and in the presence

of metal ions (Oszmianski et al. 1996; Mochizuki et al. 2002). These reactions generate phenolic radicals leading to the formation of complex mixtures of polyphenolic products that are more or less polymerized.

The catechins and procyanidins can be modified by heat treatment, which may increase the rates of reactions described above. However, they seem to be partly resistant to boiling when they stand in food matrices, as it has been shown with procyanidins in pears during cooking (Renard 2005).

16.4.2 Extraction

Extraction of flavanols is generally done from fresh or dehydrated plant material, preferentially from freeze-dried tissues that are reduced to homogeneous fine powder. Then, solid/liquid extraction of the powder is achieved using a series of organic solvents of increasing polarity. For instance, the first extraction step using hexane allows removing lipophilic components that can be in high concentration in some samples (cocoa, grains, etc.). Low-molecular-weight flavanols (catechins and proanthocyanidin oligomers) are polar compounds that can be efficiently extracted with pure alcoholic solvents (methanol or ethanol) or alcohol/water mixtures. In most cases, these alcohol or aqueous alcohol extracts also contain other simple phenolic compounds (flavonols, anthocyanins, flavones, etc.) and an important part of nonphenolic constituents of the raw material (sugars, organic acids, salts, etc.).

Alcohol does not allow the extraction of the polymerized proanthocyanidins. In general, in fresh and intact plant tissues, procyanidins are soluble and located in the vacuoles. However, after crushing and cellular disruption, the main polymerized fraction of the proanthocyanidins is strongly associated with the insoluble cell wall material by hydrogen bonds and hydrophobic interactions (Renard et al. 2001). Water–acetone mixtures can disrupt these weak energy linkages. Thus, it seems that 60–70% of acetone in water corresponds to the suitable solvent for proanthocyanidin polymer extraction (Jones et al. 1976; Guyot et al. 1997), allowing an efficient disruption of the weak energy interactions with the cell wall material. Ternary mixtures of methanol, acetone, and water are also well adapted for the extraction of condensed tannins (Mane et al. 2007; Auger et al. 2010). Noticeably, acetone alone is a very bad solvent for proanthocyanin extraction and solubilization (Chavan et al. 2001).

The acidification of the extraction solvents is strongly recommended for two main reasons. First, this allows a more efficient extraction of proanthocyanidins by decreasing the polar interactions with the cell wall matrix. Second, it is an efficient way to prevent catechins and procyanidins from autoxidation by shifting the acidic equilibrium of procyanidins toward their protonated forms and thus limiting the presence of easy oxidizable phenolate forms. Thus, aqueous acetone extraction of pea proanthocyanidins was more efficient when 1% of hydrochloric acid was added (Chavan et al. 2001). However, the use of a strong acid at too high concentration is not recommended since it can be responsible for the structural alteration of the proanthocyanidins by causing acidic disruption of the IFLs or the formation of phlobaphenes (Foo and Karchesy 1988). Therefore, the use of diluted weak acids (i.e., acetic or formic acid in the 1–2% v/v range or 0.1–0.05% trifluoroacetic acid) is preferable.

More recently, some reports were published on the use of innovative technologies for flavanol extraction. Thus, microwave-assisted extraction in water allows good recovery of green tea catechins (Li et al. 2010). However, partial alterations of the molecules such as epimerization were observed in some particular conditions as a consequence of temperature elevation (Wang et al. 2006). Besides, when it was applied on dry grape pomaces, subcritic aqueous alcoholic extraction (with pressure close to 6 MPa) allowed an increase of the procyanidin extraction yield by a two- or threefold factor compared to the more conventional aqueous acetone extraction (Monrad et al. 2010).

Importantly, in most cases, exhaustive extraction of proanthocyanins from crude materials is not possible since a part, generally corresponding to the more polymerized forms, is strongly associated with the insoluble matrix. This may lead to the underestimation of the concentration in plant foods (Arranz et al. 2009). However, the overall quantification of condensed tannins can be obtained using methods such as chemical depolymerization by thiolysis directly applied on raw materials or insoluble residues (Matthews et al. 1997a; Guyot et al. 2001b). These methods are discussed in Section 16.5.4.

16.4.3 Liquid/Liquid Fractionation, Solid-Phase Adsorption on Resins and Filtration

16.4.3.1 Liquid–Liquid Extraction

Using water–hexane, water–chloroform, or water–petroleum ether, liquid–liquid extraction is sometimes used as the first step to remove hydrophobic components of a crude extract containing polyphenols. However, it is not selective enough for a specific fractionation of the flavanols. Thus, in a water–ethyl acetate system, flavanols are distributed in the two phases. Catechins and procyanidin oligomers are mainly present in the organic phase whereas the more polymerized forms remain in the aqueous phase (Cheynier and Fulcrand 2003). The measurement of the octanol–water partitioning coefficients for (–)-epicatechin and for a series of purified apple procyanidin oligomers from DP2 to DP6 also revealed that apple procyanidins have much higher affinity for the water phase compared to the monomer and it increases with the DP (Zanchi et al. 2009).

16.4.3.2 Solid-Phase Adsorption

Clearly, solid-phase extraction on a cartridge is the method of choice to clean up crude extracts containing flavanols and, more widely, polyphenols. This method is used to remove nonphenolic coextracted components such as sugars, organic acids, and salts. Reversed-phase C18 cartridges are efficient for trapping catechins and procyanidins in a wide range of polymerization states (Guyot et al. 2001a) and are widely used today for cleaning up samples before high performance liquid chromatography (HPLC) or ultra high performance liquid chromatography (UHPLC) analysis. In addition, using different elution solvent systems with diethyl ether, ethyl acetate, and methanol, C18 cartridges can be used for the fractionation of flavanols into monomers, oligomers, and polymers (Sun et al. 1998a). Other adsorbents are available and have been tested for their retention capacity toward flavanols. This is, for example, the case of copolymer resins of the styrene divinylbenzene type (amberlite XAD2, XAD7, XAD16) that seem well adapted for polyphenol adsorption with good yields of recovery. However, huge differences of affinity exist depending on the considered polyphenol class (Bretag et al. 2009; Lai et al. 2009).

16.4.3.3 Filtration

Sample filtration before HPLC analysis is an operation that may affect catechin assay. When they are dissolved in aqueous buffers, some catechins are absorbed by certain types of filtration membranes that caused errors in their analysis (Yoshida et al. 1999). This retention on filters was also observed for apple procyanidins in aqueous solution after filtration on nylon, polyvinylidene fluoride, or cellulose acetate filters. Only, poly tetrafluoroethylene (PTFE) filters appear inert to the filtration of procyanidin oligomers and polymers (Guyot, S., unpublished results). In this case, a prewetting of the PTFE filter with methanol is necessary prior to the filtration of the aqueous solution. No significant retention was observed when catechins and procyanidins were diluted in methanol or aqueous acetone. In practice, we recommend the checking of the retention of proanthocyanidins when new filters are used or when samples from new origins are studied.

16.4.4 Fractionation and Chromatographic Purification

The chromatographic fractionation is undoubtedly the most used and probably the most efficient method for obtaining catechins and oligomeric procyanidins in a highly purified state. Apparently, the purification of a proanthocyanidin molecule having a DP >5 is virtually impossible because of the multiplicity of isomers and conformers invariably present in the extracts. However, it is possible to fractionate proanthocyanidins according to their DP.

Polymeric materials such as LH20 (Lea and Timberlake 1974) or Fractogel TSK HW (Derdelinckx and Jerumanis 1984) gels are often used as a first step of fractionation of a crude extract by low-pressure chromatography. Usually, the column is equilibrated in acidified water. Small phenolic molecules are eluted first using alcohol–aqueous mixtures. Then, procyanidin oligomers are recovered by pure methanol or by a solvent that contains a higher proportion of alcohol. Finally, more polymerized proanthocyanidins are

eluted using aqueous acetone. Usually, all solvents are acidified by acetic, formic, or trifluoroacetic acids used very diluted. Many variations in terms of proportion of solvents, stationary phases, and acid used have been described for applications relating to extracts of different origin such as apples (Yanagida et al. 1999; Xiao et al. 2008), apple pomace (Foo and Lu 1999), cider (Lea and Timberlake 1974), malt and hop (Derdelinckx and Jerumanis 1984), cocoa (Adamson et al. 1999), or grape seeds (Ricardo da Silva et al. 1991a). Note that when they are used with a solvent system based on concentrated urea, acetone, or tetrahydrofuran, these stationary phases can really behave as a size-exclusion gel properly adapted for the fractionation of procyanidins according to their polymerization state (Yanagida et al. 1999; Nonier et al. 2004; Le Bourvellec et al. 2006). Other polymeric chromatographic materials (i.e., polystyrene-divinylbenzene) used with acidified dimethylformamide also gave comparable results for grape and hop proanthocyanidins (Kennedy and Taylor 2003).

The DP is of course a crucial structural feature of proanthocyanidins since it is directly linked to their capacity to associate proteins and polysaccharides (Haslam 1974; Cheynier et al. 1992; Renard et al. 2001). Therefore, it is strongly correlated to their bioavailability, and their nutritional and bioactive properties (Santos-Buelga and Scalbert 2000; Aron and Kennedy 2008). Thus, many methods have been developed for fractionating procyanidins into mono-dispersed fractions according to their DP. Among them, normal phase chromatography is probably one of the more efficient. Originally, the separation of cider procyanidin oligomers was achieved by thin layer chromatography (TLC) on silica (Lea 1978). Then, the method was adapted for analytical HPLC allowing a separation until decamers although the chromatographic peaks corresponding to procyanidins above pentamers were overlapped (Rigaud et al. 1993; Hammerstone et al. 1999). The method was adapted at the semipreparative scale for the HPLC fractionation of apple procyanidins (Guyot et al. 2001a). As shown in Figure 16.5, efficient separation of oligomers was achieved up to DP6, overlapping being observed for larger procyanidins.

Apple procyanidins were also efficiently separated on silica column up to pentamers with a gradient composed of hexane/acetone mixture (Yanagida et al. 2000) and greater than hexamers using a hexane/methanol/ethyl acetate solvent mixture (Shoji et al. 2006b).

Interestingly, efficient separation of cocoa procyanidins was obtained using a diol phase with a more polar solvent system consisting of acidified acetonitrile, water, and methanol (Kelm et al. 2006). The

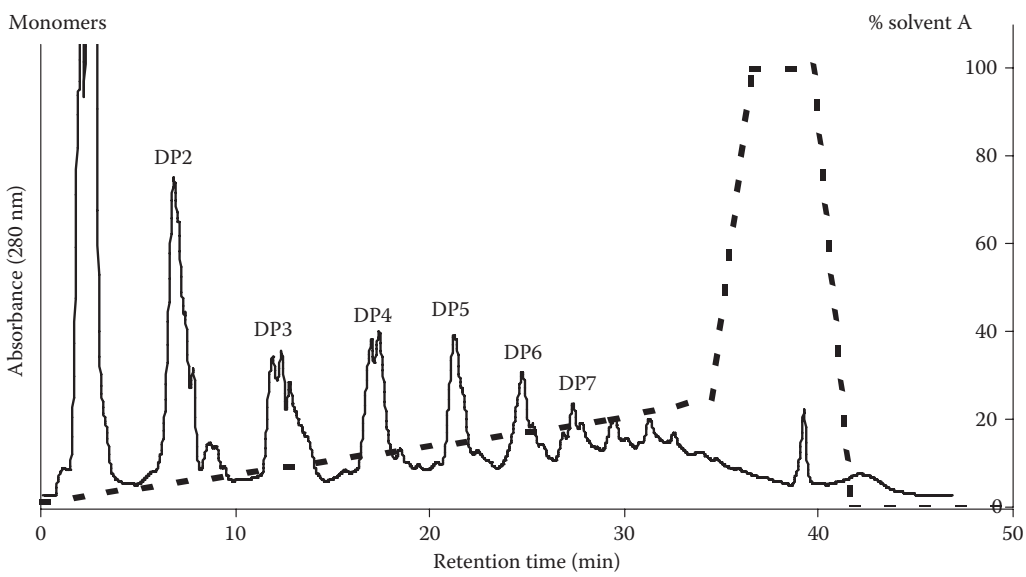


FIGURE 16.5 Normal phase HPLC fractionation of cider apple procyanidins at the semipreparative scale (according to Guyot et al. 2001a). Waters column PrepNovaPak HR silica cartridge 6 µm, 60 Å, 25 × 100 mm + guard pak cartridge 25 × 10 mm; flow rate 40 mL/min; gradient system:methanol/dichloromethane/formic acid/water, solvent A: 43/5/1/1 and solvent B: 7/41/1/1; dashed line = % of solvent A.

scaling up at the preparative scale allowed obtaining fractions up to heptamers. Very recently, the technique was applied to condensed tannins of hawthorn leaves for obtaining purified fractions from DP2 to DP13 at the preparative scale (Zumdick et al. 2009). Recently, hydrophilic interaction chromatography using an amide–silica column was used at the analytical scale to separate apple procyanidins according to the DP. Separation up to decamers was achieved with a linear gradient elution of acetonitrile–water at ratios of 9:1 to 5:5 (v/v) for 60 min at a flow rate of 1.0 mL/min (Yanagida et al. 2007). This method could be probably adapted at the preparative scale.

It is worth noting that, in many cases, a normal phase chromatography method that was developed for a particular extract cannot be readily transposable to plant extracts from other origins and of unknown composition. This is particularly true for extracts containing heterogeneous proanthocyanidins. Thus, the correspondence between retention times and the molecular weight should be established in each particular case (Cheynier and Fulcrand 2003).

Countercurrent chromatography is now currently used for fractionation of catechins and proanthocyanidins. As far as we know, the technique was first used by Lea and Timberlake for fractionation of cider procyanidins (Lea and Timberlake 1974). Then, improvement of the partition efficiency and reduction of the time of analysis were obtained by high-speed countercurrent chromatography (HSCCC), which was used, for example, in purified tea catechins (Degenhardt et al. 2000; Wang et al. 2008), and apple (Shibusawa et al. 2000) or grape seed (Kohler et al. 2008) procyanidin oligomers. In the latter case, polymers were first removed by precipitation. Then, successive solvent systems involving water, ethyl acetate, 2-propanol, and butanol allowed a clean fractionation from monomers to tetramers. Recently, a very efficient fractionation of five procyanidin dimers and two catechin digallates was also achieved by HSCCC of a tea leaves extract (Kumar et al. 2009). Most of the more classical catechin monomers were first removed on LH20 column. Then, HSCCC was performed using a mixture of hexane, ethyl acetate, methanol, and water.

16.5 Analysis

16.5.1 Colorimetric Methods

Several colorimetric methods, more or less selective, are available for the detection of catechins and proanthocyanidins in plant tissues and extracts. They may be also used for quantification. However, their reliability for quantitative purpose is limited particularly for samples containing highly polymerized proanthocyanidins.

16.5.1.1 Nonspecific Reactions of the Phenolic Groups

Flavanols react positively to the permanganate titration (Williams 1953) and Folin–Ciocalteu assays (Singleton and Rossi 1965), common methods used for detection and quantification of total phenolic compounds. These methods are not specific for flavanols; moreover, huge differences exist between molar absorptivities of flavanols depending on their chemical structures (Singleton et al. 1999).

16.5.1.2 Reaction with Aromatic Aldehydes

Phenolic compounds react with aromatic aldehydes in acidic conditions if they contain meta-oriented di- or tri-hydroxy-substituted benzene rings (resorcinol or phloroglucinol) to yield colored products (Swain and Hillis 1959; Goldstein and Swain 1963; Delcour et al. 1985). However, regarding flavonoids, the reaction requires that they have a single bond at the 2,3 position. Thus, the response is positive with flavanols (i.e., catechins and proanthocyanidins) but not with the majority of other natural phenolic compounds such as flavonols that contain a deactivated phloroglucinol A-ring (Swain and Hillis 1959; Delcour and Janssens De Varebeke 1985). Most of the methods based on this reaction were developed using vanillin or dimethylaminocinnamaldehyde (DMACA) as aldehydic reagent leading to reddish and bluish colors, respectively.

16.5.1.2.1 Red Color after Reaction with Vanillin

The reaction is wholly specific for flavanols but was also shown to give significant color development with dihydrochalcones (Sarkar and Howarth 1976). Absorbencies can be measured at 510 nm. When the reaction was applied on purified procyanidin oligomers, the best yields were obtained using 9 N sulfuric acid in pure methanol (Sun et al. 1998b). However, the color of the reaction products is not very stable and may be affected by many factors, including the solvent, the light, the temperature, the nature of the acid, and the presence of water (Broadhurst and Jones 1978; Butler et al. 1982; Sun et al. 1998b). Important differences of molar absorptivities are observed depending on the considered flavanol molecule (Goldstein and Swain 1963). In addition, it was presumed that copigmentation occurs between the residual vanillin in its cationic form and the colored catechin–vanillin complex leading to huge variations in color development and intensity (Mitsunaga et al. 1998).

16.5.1.2.2 Blue Color after Reaction with DMACA

The reaction with DMACA is reported to be more specific and sensitive compared to the vanillin reaction. Reaction products are blue and absorbances are measured at 640 nm. For instance, the molar extinction coefficient of (+)-catechin is 74,141 at 640 nm for DMACA assay whereas it is only 8500 for vanillin assay with measurement at 510 nm (Cheynier et al. 2001). In addition, since optical density measurements are performed in the 610–660 nm regions, the DMACA method allows to avoid interferences with anthocyanins in "red" matrix such as wines, red fruit juices, and so on. Thus, using catechin as a standard, the methods were successfully applied for the quantification of very low concentrations of flavanol in beers (Delcour and Janssens De Varebeke 1985). Coupled to reversed-phase HPLC (RP-HPLC) (Treutter et al. 1994; De Pascual-Teresa et al. 2000) or to TLC on cellulose plates (Glavnik et al. 2009), the method is also used as a postcolumn derivatization reaction for specific detection and quantification of flavanols (Treutter 1989). The reaction can also be applied directly on fresh tissues. For example, it was applied to specifically detect flavanols in grape berries (Cadot et al. 2006), strawberry fruits (Almeida et al. 2007), or rapeseed seeds (Auger et al. 2010) during their growth.

16.5.1.3 Depolymerization of Proanthocyanidins in Alcoholic and Acidic Media

This method is based on the disruption of the C4–C8 or C4–C6 IFL. Therefore, it is specific for proanthocyanidins and does not give positive response with monomeric catechins. As a consequence, catechin extension units are released as C4-flavanyl carbocations that are quickly oxidized to anthocyanidins structures (Figure 16.6) leading to the red coloration of the reaction media.

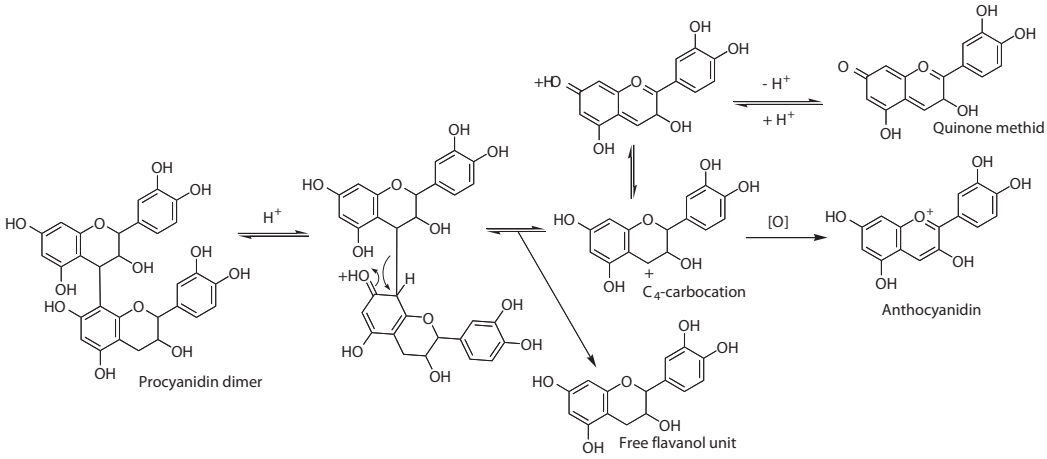


FIGURE 16.6 Acidic degradation of proanthocyanidins leading to red-colored anthocyanidins.

By comparison with anthocyanidin or proanthocyanidin used as standards, measurement of the color can be correlated to the proanthocyanidin concentration. This method, initially achieved by boiling the ground tissues in a mineral acid solution (Bate-Smith 1954), was improved by using butanol/hydrochloric acid (Swain and Hillis 1959). It allowed to obtain better yields by limiting the formation of phlobaphenes, which are complex side products resulting from heterocycle opening, rearrangements, and polymerization of flavanols when they are treated with aqueous and strongly acidic media (Swain and Hillis 1959; Foo and Karchesy 1988). Then, yields were again increased and the method was more reproducible by adding ferric salts in the medium (Porter et al. 1986). The latter procedure (Porter's reagent) is still largely used and can be summarized as follows: a few milligrams of dried grounded materials are suspended in 2.5 mL of butanol:HCl 95:5 v/v and 100 μL of $(NH_4)Fe(SO_4)_2$, $12H_2O$ (2% in 2 M HCl) are added and the mixture is heated for 40 min (Porter et al. 1986). Measurement of the absorbance is generally performed at 550 nm and converted into molar concentration according to a standard curve of cyanidin.

Recently, a procedure based on the depolymerization reaction in butanol/HCl was proposed to quantify the unextractable procyanidin fraction, which was usually not considered for quantification in many food samples (Arranz et al. 2009; Perez-Jimenez et al. 2009). However, the yield of conversion using the Porters's reagent is generally poor. The presence of a small quantity of water in the medium (close to 6–8%) gave the best yield of cyanidin formation from a procyanidin polymer. Then, a linear and strong decrease of color was observed when the water concentration increased (Porter et al. 1986). The yield was also strongly influenced by the structure of the proanthocyanidin molecules. Thus, yield was lower for the C4–C6 epicatechin–catechin dimer (procyanidin B5) compared to the C4–C8 equivalent (procyanidin B1) (Porter et al. 1986). Yield is also highly influenced by the average DP of the tannins that are present in the sample. For instance, the calculated yield for trimer C1 was only 48% (Cheynier et al. 2001).

16.5.2 Chromatographic Methods

16.5.2.1 Gas Chromatography

On the whole, gas chromatography (GC) is not really adapted for the analysis of flavanols that are very polar compounds. Derivatization or pyrolysis is necessary prior to GC analysis. In the latter case, catechin and epicatechin were shown to be converted into catechol and 4-methyl catechol, respectively, and pyrolysis coupled to GC was used to characterize proanthocyanidins in *Salix* and *Acacia* sp. bark extracts (Ohara et al. 2003). More recently, a method consisting of thermally assisted hydrolysis and methylation coupled to GC/MS (gas chromatography/mass spectrometry) was developed (Shadkami et al. 2009). Methylated dimeric products that retain the C–C interflavonoid linkage of condensed tannins were characterized.

16.5.2.2 TLC and High-Performance Thin Layer Chromatography

As already described in a previous section dealing with fractionation of condensed tannins, TLC on silica is known for a long time as a method that is able to separate procyanidin oligomers according to the DP (Lea 1978). Since then, no outstanding result has been obtained with this technique. However, high-performance thin layer chromatography (HPTLC) on silica was recently shown to be adapted for the assay of procyanidin B1 in willow bark samples (Poblocka-Olech and Krauze-Baranowska 2008).

16.5.2.3 Reversed-Phase HPLC

RP-HPLC is the most commonly used method to separate plant phenolics. Thus, it is widely employed in the separation of catechins and oligomeric procyanidins. However, in most cases, it does not allow a complete analysis of flavanols present in complex samples containing a great diversity of proanthocyanidin structures (i.e., isomers with various hydroxylation and galloylation patterns) or containing polymerized tannins. Indeed, the RP-HPLC separation of oligomers larger than tetramers is very difficult.

Figure 16.7 shows a typical C18 chromatogram of a mixture of catechins and procyanidin oligomers. No relation between the DP and the retention time is observed: for instance, monomeric (–)-epicatechin is eluted after dimer B2 and before trimer C1. Noticeably, small structural differences can be responsible for large differences of retention times. For instance, this can be observed for stereochemistry at C3 [(+)-catechin/(–)-epicatechin, ΔRt = 3.4 min], for the position C4–C8 (procyanidin B2) or C4–C8 (procyanidin B5) of the IFL (ΔRt = 7.8 min), or for the number of IFLs (procyanidin B2/A2, ΔRt = 8.7 min).

Interestingly, these important differences of chromatographic behavior on reversed phase are not simply explainable by the primary structures of these molecules since they all have almost the same ratio of hydroxyl/aromatic groups usually related to the polarity/hydrophobicity. This behavior seems to be more likely linked to their conformation in solution. Thus, although they have strong structural similarities, we can expect that these molecules may have very different bioavailability and may also exhibit very different biological and nutraceutical activities.

In contrast, a different behavior was observed on RP-HPLC for homogeneous apple procyanidin polymers based on (–)-epicatechin units. In this case, apple procyanidins fractions having homogeneous epicatechin-based structures and ranging from DP50 to DP190 were eluted in an increasing retention time order as a large and unresolved chromatographic hump with a gradient composed of acidified water and an increasing proportion of acetonitrile (Guyot et al. 2001a).

Numerous papers dealing with RP-HPLC separations of catechins and proanthocyanidins oligomers present in various plant or food extracts generally also containing many other phenolics have been published. The overall tendency is the use of C18 columns and acidified waters and acetonitrile or methanol binary gradients. Although it is not an absolute rule, the elution order of flavanols does not change greatly from one column to another.

Recently, UHPLC has been used for the analysis of flavanol in tea (Pongsuwan et al. 2008), in apple (Ceymann et al. 2011), and in cocoa (Ortega et al. 2008, 2010). This pumping system supports very high pressures and thus renders possible the use of stationary phase of very low particle size (1.7 μm) providing significantly more resolution, reducing run times, and improving sensitivity for many compound types. The technique is suitable for fingerprinting analysis, considering its speed, robustness, and high sample throughput (Pongsuwan et al. 2008).

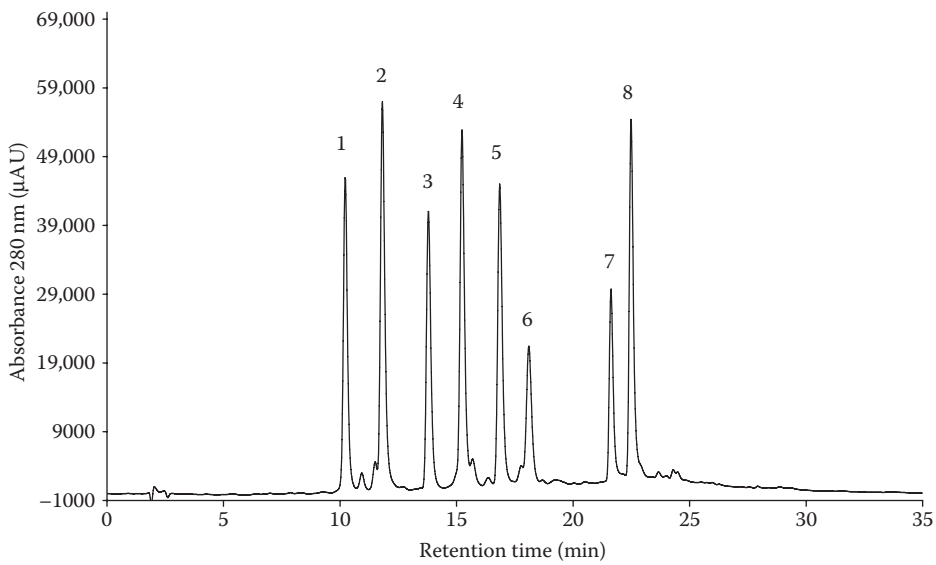


FIGURE 16.7 UV 280 nm RP18-HPLC chromatogram of a mixture of catechins and procyanidin oligomers from the B- and A-type. 1, Procyanidin B1; 2, (+)-catechin; 3, procyanidin B2; 4, (–)-epicatechin; 5, procyanidin trimer C1; 6, procyanidin tetramer D1; 7, procyanidin B5; 8, procyanidin A2; column, XDB-C18 Eclipse 2.1 × 150 mm, 3.5 μm; Agilent Technologies; flow rate, 0.2 mL/min; oven temperature, 30°C; linear gradient of mobilephase (a) water, 0.1% formic acid and (b) acetronitrile 0.1% formic acid.

16.5.2.4 Normal Phase HPLC

Normal phase HPLC chromatography on silica column was discussed in Section 16.4.4. Of course, it is also a very suitable technique for analytical purpose, aiming to precisely separate and quantitate procyanidin oligomers according to their polymerization degree (Adamson et al. 1999; Cheynier et al. 1999). More recently, cocoa oligomers were separated according to their polymerization state up to mDP14 at the analytical scale using a diol stationary phase and mixture of methanol–water and acetonitrile as mobile phase (Kelm et al. 2006). The technique is well adapted for homogeneous oligomers such as (–)-epicatechin-based procyanidins found in cocoa or apple. However, the normal phase separation appears not so resolutive when it is applied to complex samples containing mixtures of galloylated and nongalloylated procyanidins and prodelphinidins such as those found in grape seeds and skins (Cheynier et al. 1999). Nevertheless, the technique was used to characterize the distribution of proanthocyanidins in a large panel of food products of plant origins (Gu et al. 2004; Hellstrom et al. 2009).

16.5.2.5 Capillary Electrophoresis

Electromigration techniques are relatively new and their application to catechins and proanthocyanidins is still in progress (Valls et al. 2009). Capillary electrophoresis (CE) is becoming an attractive alternative, mainly due to its high separation efficiency, small sample and solvent consumption, and speed. It refers to different operating techniques having all in common that the separation is based on differences in electromigration between analytes in a given electric field (Valls et al. 2009). Thus, CE can be divided into several techniques; the more common ones are micellar electrokinetic chromatography (MEKC), capillary electrochromatography, capillary gel electrophoresis, capillary zone electrophoresis, capillary isoelectric focusing, and capillary isotachophoresis. Most of the published results concern small catechins and dimeric procyanidins in various food samples, including cocoa (Gotti et al. 2004), tea (Uysal et al. 2009), wine (Sun et al. 2008), lentils, white beans, and black beans (Cifuentes et al. 2001). However, to our knowledge, the separation of more polymerized flavanol molecules has not yet been reported. Interestingly, CE methods have been developed by adding cyclodextrin in the buffer as chiral selector for a better separation of isomers. In the case of flavanols, it allows the separation of enantiomeric catechins. It was applied to chocolate samples showing that significant epimerization of (–)-epicatechin into (–)-catechin occurs during the manufacture of chocolate (Gotti et al. 2006).

16.5.3 Detection Coupled to HPLC, UHPLC or CE

Different modes are adapted for the detection of flavanols after liquid chromatography (LC) or CE separation. The most common ones are UV, fluorescence, electrochemistry, and MS. MS is discussed in detail in Section 16.5.5.

16.5.3.1 UV Detection

All catechins and proanthocyanidins absorb UV light and can be easily detected on mono-, dual-, or diode-array UV detectors after chromatographic separation. However, this detection mode is not very selective for flavanols all of which exhibit two maxima of absorbance in the 200–220 nm and in the 260–280 nm regions. In general, the detection of flavanols by UV detectors is performed at 280 nm. For example, (–)-epicatechin and (+)-catechin have exactly the same UV spectra showing a symmetric band with a maximum at 278 nm and the spectra are similar for procyanidin oligomers and polymers homogeneously constituted with these flavanol units. However, variations are observed in the UV spectra according to the substitution patterns. For instance, esterification by gallic acid leads to an enlargement of the absorbance band with a maximum shifted to 275 nm and a more or less asymmetric band centered at 280 nm is observed for condensed tannins partly constituted with epigallocatechin units (i.e., prodelphinidins). As a whole, all phenolic compounds absorb UV light in the 280 nm region. For this reason, quantification is often rendered difficult for LC-UV quantification of flavanols in complex samples containing various interfering compounds that can be coeluted with flavanols. Interestingly,

DMACA has been used as a postcolumn reagent that reacts only with catechins and procyanidins forming blue derivatives that are selectively detected at 640 nm (Treutter et al. 1994).

16.5.3.2 Fluorescence Detection

Fluorescence is more sensitive and more selective than UV detection and has been used, coupled to normal phase chromatography, for the detection of catechins and procyanidins in food samples (Adamson et al. 1999; Carando et al. 1999; Gu et al. 2002; Gu et al. 2004; Hellstrom et al. 2009). Excitation and emission wavelengths were usually set at 276 and 317 nm, respectively. Recent improvement of the chromatographic conditions using the diol phase allowed to avoid the use of methylene chloride as eluant (Kelm et al. 2006). These changes also permit the use of new detection conditions (excitation 230 nm and emission 321 nm), which considerably increased the sensitivity for procyanidins in cocoa and chocolate-containing samples (Robbins et al. 2009). However, according to Lazarus et al. (2003), fluorescence detection is insensitive to gallocatechins, catechin gallate, and proanthocyanidins containing these flavanol units.

16.5.3.3 Electrochemical Detection

Electrochemical detection (ECD) coupled to HPLC separation (HPLC–ECD) is a sensitive and selective technique for the determination of oxidizable compounds such as flavonoids in food samples (Achilli et al. 1993; Mattila et al. 2000). It was used to detect and quantify catechins and procyanidins in lager beers and barley (Madigan et al. 1994). According to these authors, it showed higher sensitivity and selectivity than UV detection. Rohr et al. (2000) confirmed that ECD detection was 20–40 times more sensitive than UV 280 nm for the analysis of hawthorn proanthocyanidins. However, these authors recommended the use of diode-array UV detection because ECD presented a poor repeatability and was not an easy-to-use technology. Nevertheless, the new coulometric array detectors seem to have easier handling and enable increased selectivity and sensitivity for the HPLC analysis of phenolic and flavonoid compounds based on differences of their voltammetric properties (Mattila et al. 2000). In addition, they allow the use of gradient elution (Chu et al. 2004). For example, sensitive methods have been developed more recently for the analysis of catechins in biological sample (Chu et al. 2004).

16.5.4 Acidic Depolymerization of Proanthocyanidins in the Presence of Nucleophiles

This reaction, based on the lability of the IFLs, is very helpful for both characterization and quantification of proanthocyanidins, particularly when they are present in complex matrices.

We remember that, when no nucleophiles are present, the oxidative depolymerization of proanthocyanidins in acidic and alcoholic medium leads to the formation of anthocyanidin structures (see Section 16.5.1.3). The measurement of the red color allows an easy but very approximate quantification of proanthocyanidins because of color instability and formation of side reaction products. The presence of a high excess of nucleophile in the medium allows avoiding these drawbacks. In those conditions, C4-carbocations deriving from extension units of proanthocyanidins (Figure 16.8) are immediately trapped by the nucleophile to give relatively stable derivatives, whose structure is directly representative of the flavanol unit from which they come (Thompson et al. 1972).

Simultaneously, terminal units of procyanidins are released in the medium in their free flavanol form. Ideally, fine analysis of the reaction media allows the individual identification and quantification of all reaction products corresponding to the constitutive flavanol units of the native proanthocyanidin molecules. Thus, this information allows the characterization of procyanidin structures, including the nature and the proportion of the constitutive flavanol units and making the difference between extension and terminal units. As a consequence, it is possible to calculate the mDP. The method was successfully applied on individually purified proanthocyanidin oligomers (Ricardo da Silva et al. 1991a; Rigaud et al. 1991). In addition, the method can be used for the estimation of unextractable proanthocyanidins in various conifer

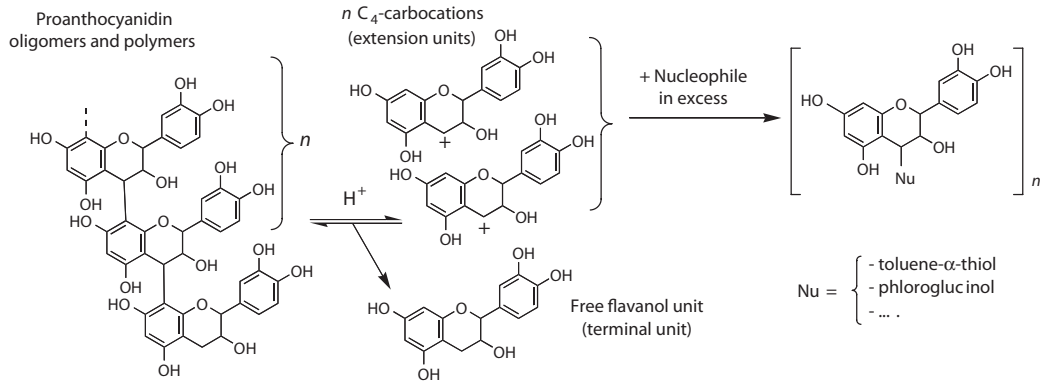


FIGURE 16.8 Acidolysis of proanthocyanidins in the presence of nucleophiles.

barks (Matthews et al. 1997b). Good yields were obtained for apple procyanidin fraction covering a wide range of polymerization state (Guyot et al. 2001a) and acidolysis in the presence of nucleophile was also applied on crude extracts from various apple varieties (Sanoner et al. 1999). It was also successfully applied directly on crude apple powders with no significant influence of the matrix on the thiolysis yields (Guyot et al. 2001b). Recently, the method was applied on insoluble residues after solvent extraction for a large number of food samples giving access to the quantification of the proanthocyanidin fraction (Hellstrom et al. 2009).

The most currently used nucleophile is toluene-α-thiol (benzylmercaptan or phenylmethanethiol), which gave rise to the name "thiolysis" to the reaction (Thompson et al. 1972). Before this work, a first thioflavanyl derivative obtained from the degradation of heather tannins had been characterized (Betts et al. 1967). Mercaptoethanol (2-sulfanylethanol) was also used as a nucleophile for the thiolytic characterization of persimmon tannins during the maturation of the fruits (Tanaka et al. 1994). However, thiol reagents have the major drawback of being highly nauseating. Therefore, recent studies tend to substitute these reagents with odorless nucleophiles. Then, phloroglucinol was used for the characterization and quantification of procyanidins in some fruits and plant extracts (Matsuo et al. 1984; Ricardo-Da-Silva et al. 1991a, b; Matthews et al. 1997b; Kennedy and Jones 2001; Buendia et al. 2010) or directly in fruit powders (Marnet et al. 2002; Buendia et al. 2010). Recently, depolymerization of procyanidins in the presence of phloroglucinol was used to follow the accumulation of the tannins in the seed coats during the development of the rapeseed seeds (Auger et al. 2010).

Thiolysis of proanthocyanidins was sometimes carried out for several hours at high temperature in ethanol or methanol with a high concentration of acetic acid (Hemingway and Mcgraw 1983). However, these conditions generate side reactions that may lead to errors and misinterpretation of the results. For instance, prolonged reaction times at elevated temperature may be responsible for the epimerization of (–)-epicatechin into (–)-catechin, which may be erroneously interpreted as (+)-catechin terminal unit of procyanidin structures (Hemingway and Mcgraw 1983). The alternative method consists of using very diluted hydrochloric or sulfuric acid in methanol, short incubation times (from 2 to 40 min), and temperature varying from 40°C to 95°C (Rigaud et al. 1991; Cheynier et al. 1992; Guyot et al. 2001b). Those milder conditions gave good, homogeneous, and repeatable yields for a series of purified apple procyanidin fractions in a wide range of polymerization state (Guyot et al. 2001a). Thiolysis yields are also decreased by the presence of water (Matthews et al. 1997a) and the kinetics of the thiolysis reaction may vary according to the structures of proanthocyanidins. For instance, the formation of the thiolether derivatives is more rapid for C4–C8 linkages compared to C4–C6 linkages (Hemingway and Mcgraw 1983). Therefore, an optimization work is needed to find the conditions that will be suitable for a considered sample.

As far as we know, the best method for analyzing the reaction media after acidolysis of proanthocyanidins in the presence of nucleophiles is RP-HPLC (Shen et al. 1986; Rigaud et al. 1991; Souquet et al.

1996; Guyot et al. 1998) even if some interesting examples of separation by GC barks (Matthews et al. 1997a) or by MEKC have been published (Herrero-Martinez et al. 2003).

Our results concerning the use of thiolysis for the analysis of apple procyanidins in purified or crude samples allow us to propose the following conditions (Guyot et al. 2001a,b):

For purified procyanidin molecules or purified procyanidin fractions: Fifty microliters of 4 g/L methanol solution of procyanidins is placed in a 250 µL HPLC glass vial. Then, 100 µL of toluene-α-thiol (5% in methanol) and 50 µL of diluted HCl (0.4 N in methanol) are added. The vial is closed by an inert Teflon cap and incubated for 30 min at 40°C. The reaction is stopped by placing the vial in an ice bath. The sample is then ready for HPLC analysis.

For procyanidins in solid crude samples (plant and fruit powders, extraction residues): Imperatively, samples must be first transformed into dried, fine, and homogeneous powder. Then, powders are precisely weighed in 1.5 mL Eppendorf vials. The convenient quantity will depend on the concentration of procyanidins that is supposed to be present in the sample. For instance, aliquots in the 50–100 mg are adapted for crude freeze-dried apple powders. Then, 800 µL of toluene-α-thiol solution (5% in dry methanol) and 400 mL of HCl solution (0.4 N in methanol) are added. Vials are closed, vortexed, and incubated at 40°C for 30 min. Additional agitations are performed every 10 min during incubation. The reaction is stopped by cooling the vials in an ice bath. Then, the reaction media is filtrated on PTFE 0.45 µm filters and transferred into HPLC vials closed with a Teflon cap and stored at 4°C until HPLC analysis. Note that samples are stable for 12 h at 4°C. Prolonged storage leads to the formation of secondary reaction products that may influence the results.

As shown in Figure 16.9, a clear improvement of the chromatographic resolution is obtained when direct thiolysis is applied. Without thiolysis (chromatogram A), numerous apple procyanidin oligomers and polymers are eluted as a broad and unresolved hump on chromatograms. With thiolysis (chromatogram B), this hump disappears almost completely since procyanidins are converted into monomeric derivatives. Peak 9 corresponds to (–)-epicatechin benzylthioether deriving from the extension unit of apple procyanidin structures. We note that hydroxycinnamic acids are partly converted into their methyl esters. However, this reaction does not influence their quantification (Guyot et al. 1998).

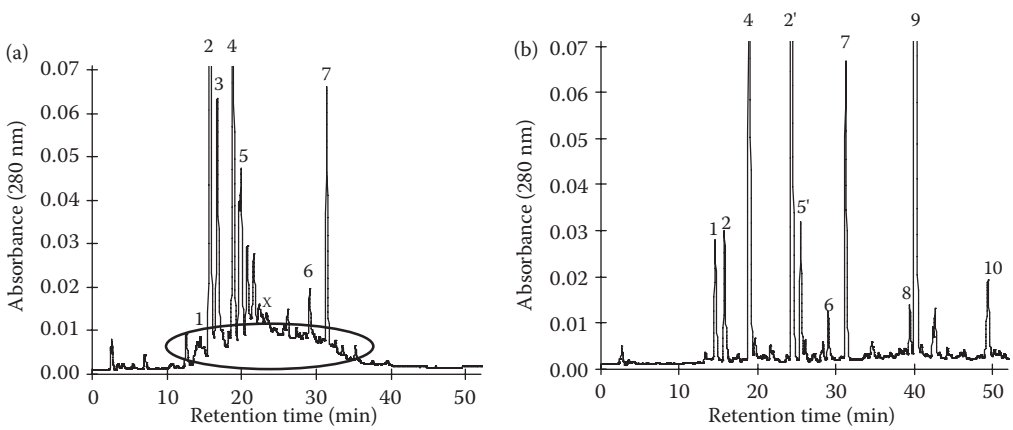


FIGURE 16.9 RP HPLC chromatograms (UV 280 nm) of a cider apple powder with (b) and without (a) thiolysis. Peaks are numbered as follows: 1, (+)-catechin; 2, chlorogenic acid; 2′, methylester of chlorogenic acid; 3, procyanidin B2; 4, (–)-epicatechin; 5, *p*-coumaroyl-quinic acid; 5′, methylester of *p*-coumaroylquinic acid; 6, phloretin xyloglucoside; 7, phloridzin; 8, phloretin; 9, (–)-epicatechin benzylthioether; 10, toluene-α-thiol. Circled zone corresponds to unresolved procyanidins. (From Guyot et al. 2001b.)

For procyanidins present in liquid complex matrix (fruit juices, ciders, etc.), liquid aliquots (500–1000 μL, depending on procyanidin concentration) are first freeze-dried in 5 mL polyethylene hemolysis vials. Then, the procedure is the same as described for solid samples (see above).

Depending on the samples, the weighted quantity and incubation time must be adapted. For instance, for cider apple pomace powders, we obtained higher yields for (–)-epicatechin benzylthioether formation by carrying out the incubation for 1 h at 50°C (Guyot, unpublished results).

16.5.5 Mass Spectrometry of Flavanols

The first developed ionization sources such as electronic impact and chemical ionization were poorly adapted for the MS analysis of catechins and procyanidins and more widely for polyphenol molecules because of their thermosensitivity and low volatility. Only derivatized molecules such as methylated, peracetylated, or trimethylsylilated derivatives were stable and volatile enough and gave limited fragmentations allowing structural characterization. Thus, the first MS analyses allowing structural characterization of procyanidin dimers of the B- and A-types were achieved by the Weinges's group (Weinges et al. 1968) with electronic impact on octamethylether derivatives. Then, the development of the fast atom bombardment sources corresponding to a less energetic ionization method allowed the observation of tetrameric procyanidin molecular ions in the positive mode (Karchesy et al. 1986; Self et al. 1986). In the same period, hexameric procyanidin molecular ions were observed as methylether derivatives by the field desorption technique (Morimoto et al. 1986).

A real development in the analysis of proanthocyanidins appeared in the late 1990s with the emergence of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) (Guyot et al. 1997; Ohnishi-Kameyama et al. 1997; Poon 1998). Since then, these techniques have been applied for the characterization of proanthocyanidins in samples from various plant origins.

16.5.5.1 MALDI-TOF Analysis

MALDI-time of flight (MALDI-TOF) analysis of proanthocyanidins was recently reviewed (Monagas et al. 2010). This ionization method presents several advantages. It is a soft ionization energy method with a high ion transmission yield and with a great tolerance to the presence of impurities in the samples. In contrast, the technique is not really adapted for the analysis of small catechin molecules of MW < 500 since this region of the spectrum is generally saturated with matrix signals. MALDI-TOF was used to detect procyanidin series in apple up to DP15 (Ohnishi-Kameyama et al. 1997) and up to DP30 in soybean seed coat (Takahata et al. 2001). In general, the matrices used to disperse the samples are trans-3-indoleacrylic acid or 2,5-dihydroxybenzoic acid. The oligomers are detected in positive reflectron mode in the form of adducts with sodium or potassium cations in the medium. Reflectron mode allows higher resolution compared to the linear mode. However, it is not well suited to high-molecular-weight ions that do not support the process of postacceleration (Ohnishi-Kameyama et al. 1997). The addition of a cationization agent (cesium trifluoroacetate, or silver trifluoroacetate) is sometimes performed to favor the formation of a particular adduct allowing improvement of the detection (Ohnishi-Kameyama et al. 1997; Xiang et al. 2006). MALDI-TOF analysis was successfully applied to characterize the diversity of proanthocyanidin structures in various fruits or plant extracts. For example, B- or A-type procyanidins or prodelphinidins were clearly and specifically detected in cranberry extracts (Porter et al. 2001; Neto et al. 2006). More recently, propelargonidin, procyanidin, and prodelphinidin oligomers were clearly distinguished in acacia extracts (Wei et al. 2010). MALDI-TOF was also used to characterize pine bark procyanidins (Jerez et al. 2009) and grape tannins of different tissue zones (skin, seeds, and stems) and to determine the origin of oenological commercial tannins (Vivas et al. 2004). Very recently, it was also used to detect and characterize products resulting from enzymatic oxidation of catechins from green tea leaves (Itoh et al. 2007). MALDI-TOF is not well adapted for the accurate estimation of the polydispersity of high-molecular-weight procyanidin polymers: suppression of ionization, problems of matrix desorption, and in-source fragmentation are major phenomena that strongly influence their detection. Recently, a new MALDI method, based on complexation of highly polymerized procyanidins with bovine serum albumin, was proposed (Mané et al. 2007). The protein serves as a charge carrier that also

protects against in-source fragmentations. The mathematical processing of the signal (i.e., subtraction of the protein signal from the protein–tannin complex) allows the determination of the number-average molecular weight that was in good agreement with the thiolysis–HPLC method. In addition, calculation of the weight-average molecular weight and the polydispersity index is also possible.

16.5.5.2 Electrospray Ionization

The first applications of this ionization mode to flavanols concerned apple and grape procyanidins (Cheynier et al. 1997; Guyot et al. 1997) and tea catechins (Poon 1998). Later, it was used extensively for the characterization of catechins and proanthocyanidins with different structures and from various sources such as, for example, litchi (Le Roux et al. 1998), hops (Li and Deinzer 2008), barley (Dvorakova et al. 2008), and cocoa (Hammerstone et al. 1999). ESI-MS analyses of catechins and proanthocyanidins are generally performed in the negative mode, although detection in the positive mode is also possible (Gabetta et al. 2000; Li and Deinzer 2007). Pseudomolecular ions are mainly detected in their mono-charged form $(M–H)^–$ for oligomers up to DP5–6 and in their doubly $[(M–2H)^{2–}]$ or triply $[(M–3H)^{3–}]$ charged forms for more polymerized proanthocyanidins (Figure 16.10). However, it seems very difficult to observe signals corresponding to polymers with DP > 20–25. Condensed tannins up to DP17 in apples

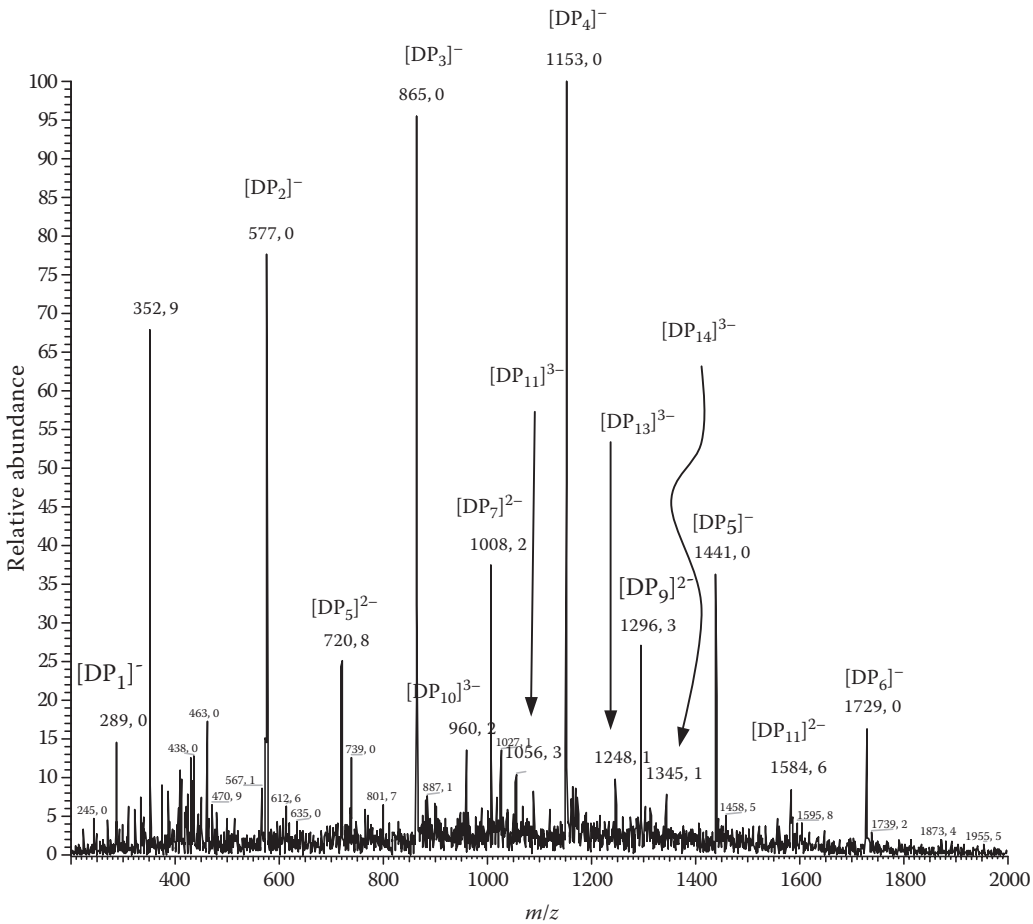


FIGURE 16.10 Negative mode ESI-MS profile of a mixture of oligomers of cider apple procyanidins (LCQ Deca ion trap mass spectrometer; direct flow infusion at 5 μL/min of a 50 mg/L solution prepared in MeOH:diluted formic acid 0.1% 50/50).

(Guyot et al. 1997) and up to DP22 in litchi pericarp (Le Roux et al. 1998) have been unambiguously detected in their triply charged molecular ions. The *m/z* values of the pseudomolecular ions allow the determination of several structural features of proanthocyanidins in complex samples, including the DP, the presence of galloyl groups, and the number of A- or B-type linkages (Fulcrand et al. 1999; Fulcrand et al. 2008).

In highly polydispersed mixtures of procyanidins, mass signals arise from both simply charged and doubly or triply charged species (Figure 16.10). In some cases, particularly for molecules corresponding to an even DP, overlapping signals are observed on mass spectrometer with poor resolution. For example, *m/z* of $(DP3)^{-}$ and $(DP6)^{2-}$ procyanidin pseudomolecular ions are 865 and 864, respectively. The charge state of the molecular species can be estimated by observing the isotopic distribution of the mass signals: one atomic mass unit (amu) for a mono-charged species; 0.5 and 0.33 amu for a doubly charged and triply charged species, respectively (Figure 16.11).

In addition, the fragmentation patterns of the molecular ions are used to provide important information concerning the type of linkages, the hydroxylation scheme, and the sequence of catechin units in the proanthocyanidin structures (Li and Deinzer 2007).

Over the past 10 years, new methodological development showed that ESI-MS detection of procyanidins using several types of mass detectors (ion traps, quadripoles, time of flight) could be successfully coupled to HPLC analysis in various food and plant samples (Gu et al. 2003; Tourino et al. 2008) and more recently to UHPLC for the analysis of flavanols in cocoa (Ortega et al. 2010) or tea (Pongsuwan et al. 2008) samples. Normal phase separation of cocoa procyanidin oligomers according to their size was achieved and coupled with online detection by ESI-MS in both the positive and negative modes (Hammerstone et al. 1999). The poor sensitivity due to the nature of the HPLC solvents was compensated by a postcolumn addition of a diluted sodium chloride or ammonium hydroxide aqueous solution to favor ionization of flavanol structures in the electrospray source. However, most of the applications of LC/ESI/MS coupling have been developed using reversed-phase columns.

16.5.6 Nuclear Magnetic Resonance Analysis

Flavanols, in both their monomeric and polymeric forms, have been exhaustively studied by mono- and bidimensional ^{1}H and ^{13}C nuclear magnetic resonance (NMR) techniques. Many published papers concern elucidation of the complete structures of molecules that have been previously prepared in a highly purified state. In this area, it is important to remember the work of Weinges and his colleagues, which, thanks to proton NMR and MS analyses, were able to propose the distinction between A- and B-type procyanidins (Weinges et al. 1968). Then, NMR was able to answer most of the questions dealing with the structure of catechins and proanthocyanidins, including the C2–C3 stereochemistry of the flavanol nucleus and the C4–C8 or C4–C6 location of the IFL (Lazarus et al. 2003). However, difficulties are often encountered when spectra are registered on native compounds at room temperature because of the coexistence of atropisomers that are rotational conformers due to stearic hindrance around the interflavan links. The proportion of each atropisomer is highly influenced by the solvent (Hatano and Hemingway 1997), the nature of the flavanol constitutive units (Tarascou et al. 2006), and the temperature (Tarascou et al. 2006, 2007). This problem can be partly solved either by methylation or acetylation of the hydroxyl groups or by recording the spectra at very low temperature (Shoji et al. 2003; Abe et al. 2008). On the whole, the distinction between epicatechin (2,3-*cis*) and catechin (2,3-*trans*) as constitutive flavanol units can be achieved on the basis of the ^{3}J coupling constants of H-2 and H-4. However, when sometimes H-2 proton appears as a broad signals, analysis of the nuclear Overhauser effect obtained from a rotational nuclear overhauser effect spectroscopy spectrum may be necessary to assess the 2,3 configuration (Balas et al. 1995). NMR is also probably the only method that allows obtaining information on the C4–C8 or C4–C6 location of the IFL of proanthocyanidins. For a long time, this structural parameter was deduced from the ^{13}C and ^{1}H chemical shift values: for example, the H-8 of a 6-substituted catechin moiety is considered to resonate at higher field than the H-6 of an 8-substituted moiety. However, according to Hemingway and coworkers, depending on the derivatization method and particularly for methylether or methylether acetate derivatives, incorrect assignment of the C-6 and C-8 signals have been reported in the literature for monomeric flavanols (Hemingway et al. 1996). The unambiguous assignment of the IFL

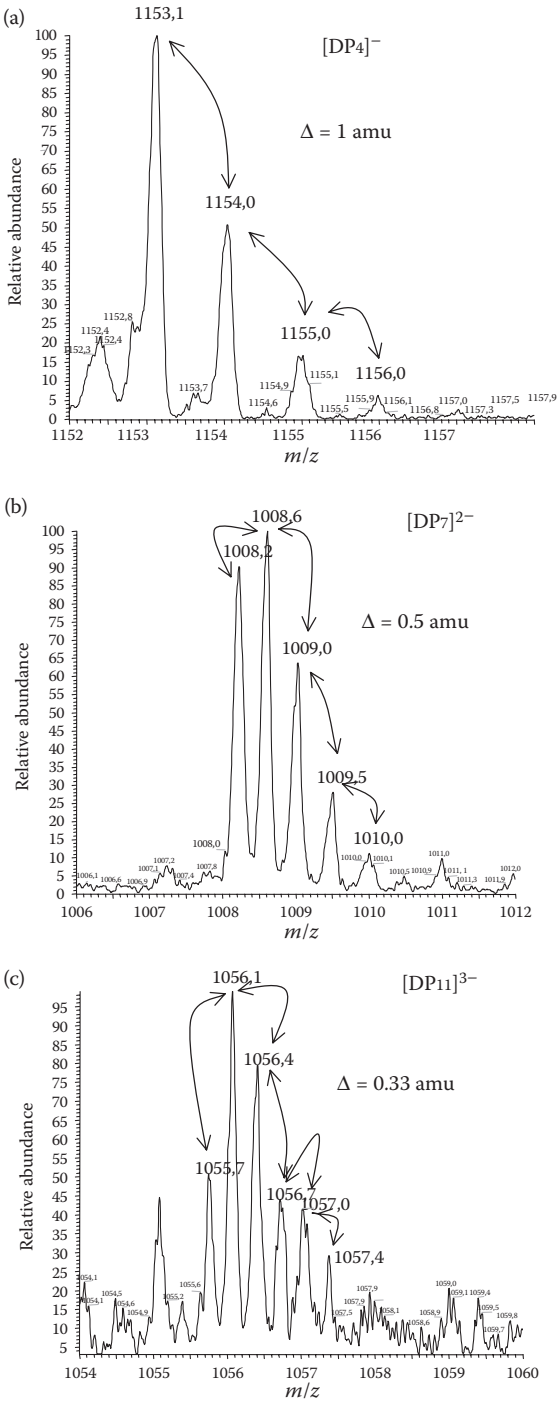


FIGURE 16.11 Isotopic MS distribution of apple procyanidins with different charge state: (a) singly charged tetramers; (b) doubly charged heptamer; (c) triply charged undecamer. Data obtained by direct infusion of an apple procyanidin solution on the LCQ Deca ion trap spectrometer using the ZoomScan option.

was obtained with the use of ^{1}H-^{13}C 2D NMR in peracetylated catechin dimers (Balas and Vercauleven 1994) and this approach is also applicable to oligomeric procyanidins in their native forms (De Bruyne et al. 1996; Khan et al. 1997; Shoji et al. 2003; Abe et al. 2008). Considering more polymerized proanthocyanidins, NMR is also useful for the estimation of the average DP. One method proposed by Czochanska and coworkers was based on the ratio of ^{13}C signal area corresponding to the carbons C-3 of the extension units to the signal area of the C-3 corresponding to the terminal unit (Czochanska et al. 1980). More recently, we have proposed a ^{1}H NMR method that considers the signal area corresponding to all H-6 (or H-8 protons) and the area of the signal attributed to the H-4 protons of the terminal unit (Guyot et al. 1999). The former area increases according to the DP whereas the latter remains unchanged. Good correlation was obtained with mDP calculated using the thiolysis–HPLC method. However, both ^{13}C and ^{1}H methods are not adapted to polymerized proanthocyanidins that show insufficient signal intensity for the signals of the terminal units.

16.6 Conclusion

Although considerable progress has been made over the past 20 years, the analysis of flavanols and especially proanthocyanidin oligomers and polymers is still difficult in complex matrices.

The recent separative methodologies that combine on the one hand the separation of oligomeric forms by normal phase HPLC and on the other the thiolysis (or phloroglucinolysis) coupled to RP-HPLC seem well adapted to a relatively precise definition of the polyphenolic profiles. Thus, it is possible to collect information, both qualitative and quantitative, of these compounds in food matrices without neglecting the contribution of procyanidins that are not easily accessible to solvent extraction. This approach was used to characterize the flavanol profile of many kinds of foods (Gu et al. 2002, 2004; Hellstrom et al. 2009; Buendia et al. 2010). UV or fluorescence detections are suitable for the determination of flavanols separated by HPLC. However, additional MS or MS/MS detection is often preferable to complete the identification. For example, this allows more easy identification of A-type procyanidins and more precise characterization of the nature of the constitutive units.

Finally, it should be remembered that many of our foodstuffs come from a more or less elaborate processing of the fresh plant material (crushing, pressing, cooking, fermentation, aging, etc.). These processes also result in undoubtedly important structural changes of native flavanols. In the future, we need to develop methodologies better adapted for the analysis of these newly formed molecules that also contribute to the sensory and nutritional quality (Cheynier 2005). MS, by its selectivity and sensitivity, is *a priori* among the methods well suited to this research in perspective. For example, we have shown that a series of new phenolic compounds resulting from the oxidative dimerization between epicatechin, procyanidins, and caffeoylquinic acid were present in significant amounts in apple juice and cider (Bernillon et al. 2004; Guyot et al. 2008). More generally, these neoformed polyphenols significantly contribute to the composition of commonly consumed food such as black teas, wines, chocolates, and many other plant-derived foods.

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17

Stilbenes and Resveratrol

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Stilbenes and resveratrol are functional components of some food products, but they are present in other matrices as plants and in a much higher concentration. Some of the different methodologies discussed in this chapter are developed for plant extracts analysis but, although sample preparation must be adequate and optimized, the analytical technique used for the food product analysis may be the same, if it is selective and sensitive enough.

The concentrations of these compounds in plants being much higher make the identification of these compounds and new ones in those matrices an easier task. However, as compounds have been already identified, their detection and identification in food matrices become somehow easier as important data for identification are already available.

17.1 Stilbenes and Distribution in Nature

Stilbenes belong to the nonflavonoid class of polyphenols and they are 1,2-diarylethenes (Figure 17.1) presenting a *trans*-ethene double bond substituted with a phenyl group on both carbon atoms of the double bond. The ring A of a stilbene has two hydroxyl groups in the *m*-position, while ring B is substituted by hydroxy and methoxy groups in the *o*-, *m*-, and/or *p*-position. These compounds are synthesized from cinnamic acid derivatives and the substitution pattern of the cinnamic acid determines that of ring B of the stilbene (Table 17.1) (Cassidy et al. 2000).

Stilbenes act against biotic and abiotic factors, defending plants from cold, heat, fungal infections, and the growth of molds. They are synthesized by a wide range of plant species (Dipterocarpaceae, Cyperaceae, Gnetaceae, Pinaceae, Leguminosae, Myrtaceae, Moraceae, Fagaceae, Liliaceae, and Vitaceae) and are commonly found in the roots, barks, rhizomes, and leaves. These compounds are often present in plants that are not routinely consumed as food, or in the nonedible tissue. Nevertheless, Vitaceae, and within this family, *Vitis vinifera* L. is an exception as it produces stilbenes and is the most important specie grown worldwide for grape and wine production. Stilbenes are present in grapevine as constitutive compounds of the woody organs (roots, canes, and stems) and as induced substances (in leaves and fruits) acting as phytoalexins in the mechanisms of grape resistance against certain pathogens.

Stilbenes can be present in the monomeric form or as dimeric, trimeric, and polymeric stilbenes (Figure 17.2).

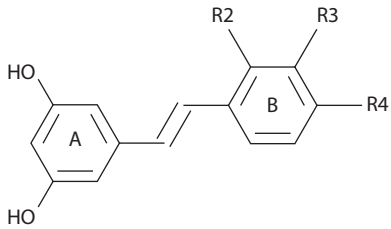

FIGURE 17.1 Representative structure of stilbenes.

TABLE 17.1

Structural Characteristics of Some Stilbenes

Stilbene	IUPAC Name	Phenolic Acid	MM	R2	R3	R4
Pinosylvin	5-[(*E*)-2-Phenylethenyl]benzene-1,3-diol	Cinnamic acid	212	H	H	H
Resveratrol	5-[(*E*)-2-(4-Hydroxyphenyl) ethenyl] benzene-1,3-diol	p-Coumaric acid	228	H	H	OH
Hydroxyresveratrol	5-[(*E*)-2-(2,4-Dihydroxyphenyl) ethenyl] benzene-1,3-diol	2′,4′-Dihydroxycinnamic acid	244	OH	H	OH
Piceatannol	4-[(*E*)-2-(3,5-Dihydroxyphenyl) ethenyl] benzene-1,2-diol	Caffeic acid	244	H	OH	OH
Rhapontigenin	5-[(*Z*)-2-(3-Hydroxy-4-methoxyphenyl)ethenyl]-1,3-benzenediol	Isoferulic acid	258	H	OH	OCH3

Note: MM: molecular mass.

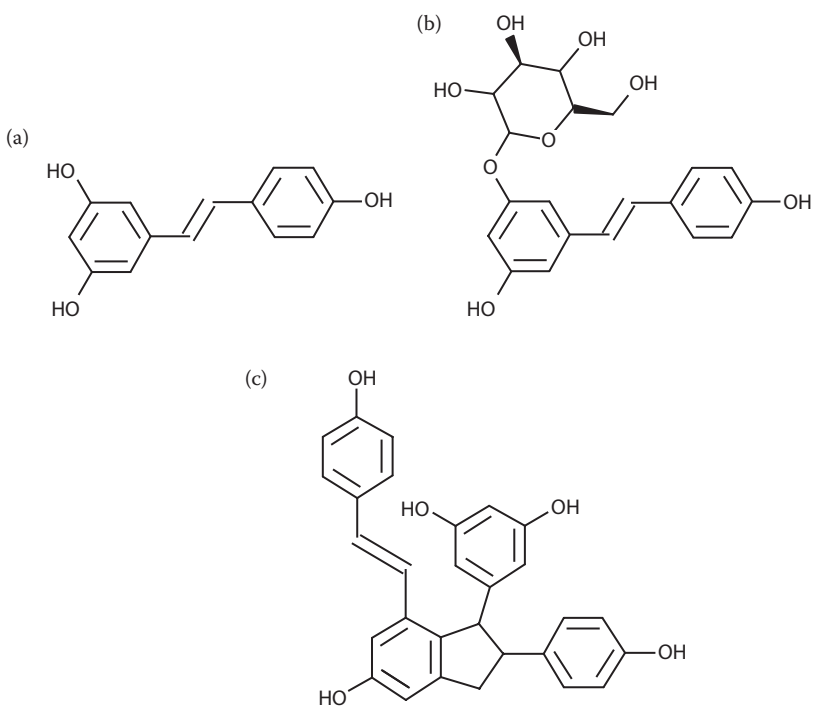

FIGURE 17.2 Chemical structures of stilbenes: (a) resveratrol, (b) piceid, and (c) ε-viniferin.

Among monomeric stilbenes, *trans*-resveratrol has been identifed as the major active compound and most of the studies in the literature about the physiological activity of this family of compounds are focused on it. There are also some studies about the 3-β-glucoside of *trans*-resveratrol known as piceid or polydatin, and polymeric stilbenes as viniferins.

17.1.1 The Importance of Resveratrol

Resveratrol also known as 3,5,4′-trihydroxystilbene is produced by a variety of plants, at least 72 species of plants distributed among 31 genera and 12 families (Jang 1997), in response to stress, injury, fungal infection (Adrian 2000, Gerogiannaki-Christopoulou 2006), and ultraviolet (UV) irradiation (Darias-Martín 2000, Tomás-Barberán 2001, Sánchez 2005, Piñeiro et al. 2006, Sun 2006). Resveratrol occurs in two isomeric forms (*trans* and *cis*) and *trans*-resveratrol was first isolated from the roots of white hellebore (*Veratrum grandiflorum* O. Loes) in 1940, and later, in 1963, from the roots of *Polygonum cuspidatum*, a plant used in Chinese and Japanese traditional medicine. These roots are one of the richest sources of resveratrol as concentrations from 2960 to 3770 ppm were determined (Vastano 2000). High contents of resveratrol have also been detected in leaves of *V. grandiflorum* and in roots and rhizomes of *V. formosanum*.

Resveratrol attracted little interest until 1992, when it was described that its presence could explain some of the cardioprotective effects of red wine, the so-called "French Paradox" (Kopp 1998). Since then, a high number of papers (Figure 17.3) have shown that resveratrol can interfere with all three steps of carcinogenesis: initiation, promotion, and progression (Kopp 1998, Bhat 2002). Resveratrol was found to have a cancer-preventive function (Jang 1997) in several animal models of cancer: inhibits proliferation of human breast epithelial cells (Mgbonyebi 1998), oral cancer cells (ElAttar 1999), HL60 leukemia cells (Clement 1998), and prostate cancer cell lines (Hsieh 1999).

The stilbene structure of resveratrol is also a promising chemical scaffold for the synthesis of potential multidrug resistance (MDR) modulators (Wesolowska 2010, Duarte 2010).

Although the anticarcinogenic function of resveratrol has been well established, the mechanisms by which resveratrol exerts its chemopreventive effects somehow remain unknown. The effects of resveratrol in other diseases prevention and progression have been reviewed (Pervaiz 2003, Xia 2010) and several biological activities are attributed to this compound: antioxidant (Kensler 1995), antiplatelet

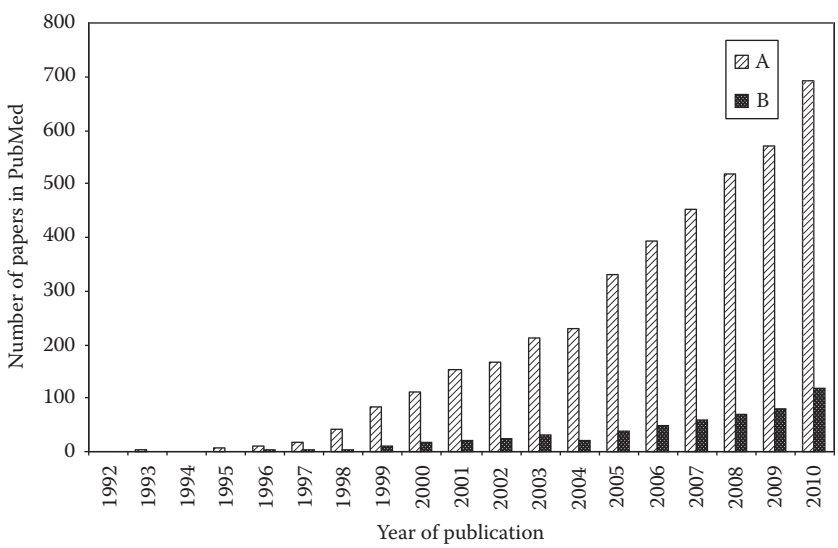

FIGURE 17.3 Resveratrol citations appearing on PubMed as a function of year, since 1992–2010. (A) key words: year [pdat] AND ("resveratrol"[Substance] OR "resveratrol"[All Fields]) (B) key words: year [pdat] AND (("resveratrol"[Substance] OR "resveratrol"[All Fields]) AND ("health"[MeSH Terms] OR "health"[All Fields])).

(Bertelli 1995, Wu 2007), neuroprotective (Bureau 2008, Lu 2008), anti-inflammatory (Khanduja 2004, Murias 2004, Kang 2009), estrogenic (Gehma 2004), cardioprotective (Ray 1999, Fremont 2000, Shigematsu 2003, Wang 2003, Dudley 2008, Seya 2009), antiaging, (Howitz 2003, Valenzano 2006, Milne 2007) antiobesity, (Rayalam 2008), antiviral (Kapadia 2002), and antidiabetic (Sharma 2011). It seems that resveratrol can elicit these effects even though serum concentrations are low. Although resveratrol is efficiently absorbed on oral administration, rapid metabolism leads to the production of sulfates and glucuronides (Dyke 1971, De Santi 2000, De Santi 2000a,b, Kuhnle 2000, Yu 2002, Vitrac 2003, Meng 2004, Walle 2004, Wenzel 2005). These facts cast doubt on the physiological relevance of the high resveratrol concentrations typically used for *in vitro* studies and suggest that at least some, if not most, of the biological effects elicited by resveratrol may be attributed to resveratrol metabolites. Resveratrol sulfate metabolites were synthesized, isolated, and the biological effects of each metabolite were studied to show their contribution to cancer chemopreventive activity. Overall, the metabolites were less active than resveratrol but as their concentration in serum is much higher than the one from resveratrol their activity may be of relevance (Hoshino 2010).

Based on the potential of health effects of resveratrol, other stilbenes as amino derivative (*Z*)-4-(3,5-dimethoxystyryl) aniline, (*Z*)-methyl 4-(3,5-dimethoxystyryl) benzoate, and (*Z*)-1,3-dimethoxy-5-(4-methoxystyryl) benzene were synthesized, tested, and showed activities in the low micromolar range (Paul 2010). Several polyhydroxylated resveratrol derivatives were prepared with the aim of discovering new leading compounds with clinical potential for human colon cancer chemotherapy. Among these compounds, 3,3′,4,5,5′-pentahydroxy-*trans*-stilbene displayed the most potent cytotoxicity in HT-29 cells (Li 2010). As 2,3′,4,5″-tetramethoxy-*trans*-stilbene (TMS) and 3,4,4″,5,-tetramethoxy-*trans*-stilbene (MR-4) are both derivatives of resveratrol and potent apoptosis-inducing agents with clinical potential, 2,3′,4,4′,5′-pentamethoxy-*trans*-stilbene, the hybrid molecule of TMS and MR-4, was synthesized and shown to be a potent inducer of apoptosis in colon cancer cells too (Li 2009).

In the future, these compounds may be used as medicines with properties similar to resveratrol.

Novel resveratrol forms have been identified in different plants as vateriaphenol F, a resveratrol dimeric form and two *O*-glucosides of resveratrol oligomers, vateriosides A (resveratrol dimmer) and B (resveratrol tetramer) were isolated from leaves of *Vateria indica* (Ito 2010), cajyphenol A and cajyphenol B together with resveratrol dimers quadrangularin A and pallidol were isolated from the stems of *Cayratia japonica* (Bao 2010), five new stilbenoids, vatalbinosides A–E (1–5), and resveratrol tetramers, (–)-hopeaphenol, vaticanol C, and stenophyllol C, were identified from *Vatica albiramis* (Abe 2010), tetramers unaphenols O and P were isolated from an acetone extract of *Upuna borneensis* (Ito 2009), dihydrostilbenes, scorzodihydrostilbenes A–E (1–5) from a crude extract obtained from aerial parts of *Scorzonera radiata* (Wang 2009). Three new resveratrol derivatives, vitisinols E–G (1–3), were isolated from the roots of *V. thunbergii* Sieb. & Zucc (Chiou 2009).

The study of the chemical composition of plant extracts has attracted recent attention as their components can be used as food additives. Plants from the *Carex* genus (Family: Cyperaceae) contain high levels of bioactive polyphenols commonly found in plant foods. Seven compounds, which included two resveratrol oligomers were isolated from the seeds of *Carex folliculata* L. (northern long sedge), a forage prevalent in the northern United States. The resveratrol oligomers were pallidol (1), a resveratrol dimer reported to be present in levels equivalent to those of resveratrol in red wine, and kobophenol A (2), a resveratrol tetramer with a unique 2,3,4,5-tetraaryltetrahydrofuran skeleton (Li 2009).

17.1.2 Stilbenes and Resveratrol in Foods and Related Food Products

Based on the quantitative data currently available, the major dietary sources of resveratrol identified are grapes, wines, grape juices, cranberries, cranberry juice, mulberry, peanuts, and peanut products (roasted peanuts, boiled peanuts, peanut butter), pistachios, cocoa, and chocolate. More recently, resveratrol and β-glucoside isomers were detected in hop, suggesting that these compounds might also be found in beer. In Table 17.2, are presented data collected on the dietary sources where resveratrol and piceid were detected and quantified.

For people who do not consume alcohol, Itadori tea, or grape juices (Romero-Pérez 1999) may be a suitable substitute for red wine concerning resveratrol intake (Burns 2002).

TABLE 17.2

Resveratrol Content in Food Products

Food Product		Compound	Content (ppm)	Reference
Grape skin		*trans*-Piceid	126.4 ± 21.8	Roldrán (2010)
		cis-Piceid	391.6 ± 61.6	
		trans-Resveratrol	10.1 ± 1.8	
		cis-Resvetratrol	100.9 ± 15.4	
Red grape juices		*trans*-Piceid	3.38	Romero-Pérez (1999)
		cis-Piceid	0.79	
		trans-Resveratrol	0.5	
		cis-Resveratrol	0.06	
		trans-Resveratrol	0.5	
		cis-Resveratrol	0.06	
Red wines		*trans*-Resveratrol	1–11	Adrian (2000); Goldberg (1995); Romero-Pérez (1995); Ribeiro de Lima (1999); Rodríguez-Delgado (2002)
	France	*trans*-Resveratrol	0.4–2 2.88 3.89	Jeandet et al. (1993); Goldberg (1995b)
		cis-Resveratrol	0.19–1.30	Jeandet et al. (1993)
	Italy	*trans*-Resveratrol	1.76–7.17	Mattivi (1993b); Moraglio (1997); Goldberg (1995b)
	California	*trans*-Resveratrol	0.99, 1.47	McMurtrey (1994); Goldberg (1995b)
	Spain	*trans*-Resveratrol	0.32–8.00	Lamuela-Ravento's (1995); Abril (2005)
		cis-Resveratrol	0.11–5.84	Lamuela-Ravento (1995); Gonzalo (1997); Abril (2005)
	Canada		3.16	Goldberg (1995b)
	Australia		1.47	Goldberg (1995b)
	South Africa		1.21	Goldberg (1995b)
	Spain and Portugal		1.64	Goldberg (1995b)
	Portugal (mainland) Azores Island		1.56–3.36	Baptista (2001)
	Portugal	*cis*-Resveratrol	2.6	Ribeiro de Lima (1999)
	Japan		0.08–0.244	Okuda (1996)
	Algeria	*cis*- and *trans*-ε-Viniferin	0.10–1.12	Amira-Guebailia (2009)
	Argentina (Malbec)	*trans*-Resveratrol glucoside	0.6–1.3	Fanzone (2010)
	Greece	*trans*-Resveratrol	0.550–2.534	Kallithrak (2001)
		cis-Resveratrol	0.54–6.3	Adrian (2000); Goldberg (1995); Lamuela-Ravento (1993); Romero-Pérez (1995); McMurtrey (1994); Ribeiro de Lima (1999); Rodríguez-Delgado (2002)
White wines		*trans*-Resveratrol	0.1–2	Cantos (2000); Lamuela-Ravento (1993); Romero-Pérez (1996)

continued

TABLE 17.2 (continued)

Resveratrol Content in Food Products

Food Product		Compound	Content (ppm)	Reference
		cis-Resveratrol	0.06	Cantos (2000); Lamuela-Ravento (1993); Romero-Pérez (1996)
Rosé wines		*trans*-Resveratrol	0.05–1.19	Cantos (2000); Romero-Pérez (1996)
	Spain	*trans*-Resveratrol	0.07–2.80	Romero-Pérez (1996); Abril (2005)
		cis-Resveratrol	0.02–3.17	Romero-Pérez (1996); Abril (2005)
	Italy	*trans*-Resveratrol	0.05–1.19	Mattivi (1993b)
Serbian wines (red, white, and rosé)		*trans*-Resveratrol	0.11–1.69 mg/L	Cvejic (2010)
		cis-Resveratrol	0.12–1.49	Cvejic (2010)
Sherry wine	Spain	*trans*-Piceid	0.10 ± 0.04	Roldrán (2010)
		cis-Piceid	<0.02	Roldrán (2010)
		trans-Resveratrol	<0.02	Roldrán (2010)
		cis-Resveratrol	<0.02 mg/L	Roldrán (2010)
Malbec red wines	Argentina	*trans*-Resveratrol glucoside	0.6–1.3	Fanzone (2010)
Red grape juices		*trans*-Resveratrol	0.003–0.23	Romero-Pérez (1999)
White grape juices		*trans*-Piceid	0.08 ± 0.06	Roldrán (2010)
		cis-Piceid	0.98 ± 0.15	Roldrán (2010)
		trans-Resveratrol	<0.02	Roldrán (2010)
		cis-Resveratrol	0.37 ± 0.12	Roldrán (2010)
		trans-Resveratrol	0.05	Romero-Pérez (1999)
Cranberries		Total resveratrol	0.278	Wang (2002)
Roasted peanuts		*trans*-Resveratrol	0.03–0.147 μg/g	Sanders (2000); Sobolev (1999)
Boiled peanuts		*trans*-Resveratrol	5.14–2.85 μg/g	Sobolev (1999)
Peanut butter		*trans*-Resveratrol	0.324 ± 0.129 μg/g	Sobolev (1999)
Pistachios		*trans*-Resveratrol	0.09–1.67 mg/kg	Counet (2006)
		trans-Resveratrol	1.2–1.7 mg/100g	Ballistreri (2009)
Mulberry	Fruit juice	Resveratrol	0.0021–0.0053	Song (2009)
		Oxyresveratrol	0.0024–0.0295	Song (2009)
	Lyophilized	Resveratrol	0.0010–0.0068	Song (2009)
		Oxyresveratrol	0.0030–0.0373	Song (2009)
Cocoa		*trans*-Resveratrol	0.4	Jerkovic (2010)
		trans-Piceid	2.6	Jerkovic (2010)
Dark chocolate		*trans*-Resveratrol	0.4	Counet (2006)
		trans-Piceid	1	Counet (2006)
Cocoa liquor		*trans*-Piceid hexoside	0.8	Jerkovic (2010)
		trans-Resveratrol	0.4	Jerkovic (2010)
			0.5	Counet (2006)
Cocoa liquor		*trans*-Piceid	2.6	Jerkovic (2010)
			1.2	Counet (2006)
Hop		*trans*-Stilbene	4.8–9	Jerkovic (2007)
		trans-Piceid	4–8.8	Jerkovic (2007)
		cis-Piceid	2–6	Callemien (2004)
		trans-Resveratrol	1	Callemien (2004)
Beer		Total resveratrol	0.5–11.7 mg/kg	Molina-García (2011)

As described, resveratrol is not widely distributed in common food sources. Some sources are not well known and are typical from some cultures. An example are fruits and seeds of an arboreal plant widely cultivated in Southeast Asia, Melinjo (*Gnetum gnmon* L.) tilbenoid where gnetin L and five known stilbenoids, gnetin C, gnemonosides A, C, and D, and resveratrol, were quantified. These fruits and seeds are used as an ordinary vegetable in Indonesia, and popular dishes include soup from melinjo (Kato 2009).

As the pathway for resveratrol biosynthesis is well characterized, metabolic engineering of this compound has been achieved in tomato plants (*Lycopersicon esculentum* Mill.) to improve their nutritional value (D'Introno 2009).

The industrial food processing used in the preparation of food products may reduce the amount of resveratrol present in the original raw material. A reduction of 84% was detected for pistachios after the sun-drying process (Ballistreri 2009). However, by-products from the food industries can be used as natural sources for resveratrol, which can be incorporated in food products to produce functional foods. Applications using peanut skins from peanut blanching have been described. The best conditions of extraction (solvent type, concentration, temperature, and time) were studied, and resveratrol was identified in MeOH extracts but was not found in samples extracted with EtOH or water (Ballard 2009). Resveratrol was also identified in an extract from the winemaking industry and antiadenovirus activity was confirmed in the extract (Matias 2010).

In addition to the *trans*-isomer, piceid a 3-β-glucoside of *trans*-resveratrol was detected in wine (Jeandet et al. 1994, Roggero 1994, Goldberg 1995) and also in grape skins (Lamuela-Ravento's 1995). This compound was isolated and structurally characterized in the roots of the medicinal plant *P. cuspidatum*, used in Asia in the treatment of atherosclerosis (Kimura 1985, Shan 1990).

The *trans*-piceid seems to exhibit very similar biological properties to *trans*-resveratrol (Romero-Pérez 1999, Aggarwal 2004): inhibiting platelet aggregation (Shan 1990, Chung 1992, Orsini 1997), inhibiting oxidation of human low-density lipoproteins (Mérillon 1996), and *in vitro* assays show it can act in Alzeihmer desease (Rivière 2009). In a less active manner than *trans*-resveratrol, *trans*-piceid reduces the elevation of lipid levels (Arichi 1982) and inhibits eicosanoid synthesis (Kimura 1985). The content of *trans*-piceid, together with its isomer *cis*-, is important and seems to confer a protective effect against cardiovascular disease after red wine ingestion (Waterhouse and Lamuela-Raventós, 1994, Adrian 2000, Martínez-Ortega 2000). As there is glycosidase activity in the human digestive tract (Hackett 1986), the glucoside of resveratrol (piceid) can release the aglycone on ingestion, increasing the amount of resveratrol.

The concentration of *cis*- and *trans*-piceid can be of the same order of magnitude or even higher than that of the free forms (Romero-Pérez 1996, Ribeiro de Lima 1999), and their health effects on humans can be very important (Kimura and Okuda, 2000).

The main stilbenes in grapevine are resveratrol and its derivatives, and, among these, pterostilbene has recently attracted much attention due to its antifungal and pharmacological properties. Indeed, pterostilbene is 5–10 times more fungitoxic than resveratrol. *In vitro* and recent studies have shown that pterostilbene exhibits anticancer, hypolipidemic, and antidiabetic properties (Schmidlin 2008). Pterostilbene (3,5-dimetoxy-4′hydroxy-*trans*-stilbene), a phenolic compound originally isolated from heartwood of red sandalwood, was reported in grapevines and blueberries (Langcake et al. 1979, Langcake 1981, Adrian 2000, Rimando 2004). This compound was identified in a traditional ayurvedic medicinal drink from India used to treat cardiovascular diseases. Structurally, pterostilbene is a dimethgylether analog of resveratrol (Lin 2009).

A paper by Bavaresco (1999) reviewed data on aspects of stilbene physiology in grapevine and on their relation to resveratrol wine levels.

17.1.2.1 Resveratrol in Grapes and Wines

Wines are the most important dietary source of resveratrol and the most studied.

In grapevine, resveratrol is synthesized in leaves, roots, and grape skins (Jeandet et al. 1991), stem, axillary bud, shoot tip, and petiole (Wang 2010) in response to microbial infection or stress. Stem phloems presented the most abundant of resveratrol, and the leaves presented the lowest (Wang 2010).

Resveratrol synthesis may be induced by chemical treatments, such as herbicide and fungicide application, following the application of the inductors carbohydrates and galacturonic acid and by UV light exposure (Threlfall 1999).

In grape berries, these compounds are primarily located in the skin and are absent or low in the fruit flesh and in grape seeds. Its maximum concentration occurs just before the grapes reach maturity and depends on grape variety (Gurbuz 2007) and abiotic factors such as hydric stress (Moreno-Labanda 2004, Abril 2005) or UV irradiation (Sánchez 2005, Sun 2006). A large variation was observed in the concentration of *trans*-resveratrol in the same cultivars analyzed in different years (Kammerer 2004). In winegrape, the concentration of resveratrol can also be influenced by postharvest dehydration: the postharvest dehydration response of Aleatico grape in terms of phenolic compounds as *trans*-resveratrol and *trans*-piceid was strongly dependent on dehydration temperature and the amount of weight loss (Mencarelli 2010).

In wines, it has been reported (Okuda 1996) that the levels of *trans*-resveratrol in the skins are similar in case of red and white grape varieties. The content of *trans*-resveratrol in wines was found to be higher for the ones obtained from grapes infected by Botrytis infestation (Jeandet et al. 1995a). The concentration of free *trans*- and *cis*-resveratrol in musts and wines is also influenced by some practices. The use of β-glucosidases causes hydrolysis of resveratrol glucosides (Goldberg 1995, Mattivi 1995, Soleas 1995). Polyvinylpolypyrrolidone decreases the concentration of all resveratrol forms (up to 90%) (Vrhovsek 1997, Castellari 1998). Some membranes can adsorb more than 60% of the resveratrol (Lamuela-Ravento's 1997). Treatment of grape juice with commercial pectolytic enzymes can increase resveratrol levels by 50%, as the enzymes break down the hypodermis of the skins, releasing phenolic compounds. However, the extent of maceration with skins and seeds during fermentation seems to be the main factor that determines the concentration of stilbenes in wines (Creasy and Coffee 1988). Wines produced with skin contact contain higher amounts of resveratrol and piceid than wines made without skin contact (Mattivi 1995, Ector 1996) and so, lower contents of *trans*- and *cis*-resveratrol are found in white wines due to little contact with the skins during maceration.

Biological aging resulting from oxidative phenomena and a combination with acetaldehyde and "flor" biofilm growth diminished the contents of resveratrol and piceid isomers by 80% (Roldán 2010) during sherry wine production. Resveratrol content in Palomino fino grapes used in the production of this type of wine was similar to other white grape varieties, but the fermentation, clarification, cold stabilization, and filtration processes considerably affected the resveratrol and piceid content in Sherry wine. The concentration of *trans*-resveratrol in Moscatel fortified wines decreased during its winemaking process (Bravo 2008).

The time of aging in oak is also important, as a slow hydrolysis can take place during wine aging in oak barrels, changing the ratio resveratrol glucoside:free resveratrol.

cis-Resveratrol is not a natural constituent of grape berries (Jeandet et al. 1991, 1995b). However, this compound and its glucoside have been detected in almost all wines, regardless of the origin and the technology applied. Some authors say that *cis*-resveratrol derives from its *trans*-isomer during winemaking, although there is no clear evidence about factors that could facilitate such conversion (Jeandet et al. 1995c).

Other stilbenes such as the biologically active dihydrodimer of resveratrol, *cis*-ε-viniferin, was isolated from a red Algerian wine and its structure was ilucidated (Amira-Guebailia 2009). Hopeaphenol (0.3–3.8 mg/L), *trans*-piceid (4.6–45 mg/L), *trans*-resveratrol (0.66–3.45 mg/L), *trans*-ε-viniferin (0.2–1.2 mg/L), and pallidol a resveratrol dimmer (0.2–9.2 mg/L) have already been quantified in North African wines in such a high level of pallidol never described in wines (Amira Guebailia 2006).

Wines from different wineries, vintages, and appellations were grouped within varieties and the amount of resveratrol and piceid present contributed to their discrimination (Romero-Pérez 1996). These compounds were used as chemotaxonomic wine markers for this purpose. Chemometric applications principal component analysis, linear and canonical discriminant analysis, probabilistic neural network, K-nearest neighbors, cluster analysis, multiregression analysis, and partial least squares have been widely used lately as the basis for discrimination of samples according to geographical origin, type, variety, and vinification technology, as a great number of data must be compared (Kallithraka 2001, Andreu-Navarro 2011).

17.2 Sample Preparation for Stilbenes Analysis

Sample preparation is the process of transforming a sample into a form that is suitable for analysis. This is always an important issue and limiting step to consider in the analytical procedure, not only because of the need for costly and time-consuming operations, but because of the error sources introduced during this analytical step. Usually, sample preparation consists of multiple steps that may include drying, homogenization, sieving, extraction, and eventually preconcentration and cleaning.

The most adequate method for sample preparation must be chosen according to the type of analysis that are going to be performed. If the analytical methodology that is going to be used is specific and sensitive, no special treatment is necessary for sample preparation. On the contrary, if the method is not specific, the sample must be treated to eliminate interferences from the matrix and simplify the sample. If the analytical methodology has a high quantification limit, often the sample must be concentrated. The main goals are to isolate/analyze analytes and improve the characteristics of the method of analysis (selectivity, sensitivity, accuracy, and repeatibility).

Sample preparation techniques may be used to prepare the sample for analysis, at an analytical scale, or to extract a compound or group of compounds in a preparative scale. At analytical scale, different tools may be used to characterize, identify, and quantify the sample components, and small amounts of samples are usually necessary. At preparative scale, higher amounts of sample are used to isolate a compound or compounds in a sufficient amount. Usually, compounds are purified, characterized by mass spectrometry (MS) and nuclear magnetic resonance, and, if purity is confirmed, they are used as standards. Most of the stilbene compounds are not communly sold, and for identification and quantification purposes they must be extracted from their raw material and purified. Also, in order to study the bioactivity in *in vitro* or *in vivo* (animals or humans) studies using samples as plasma, blood, or urine, it is necessary to have the compounds as pure as possible.

An important issue to consider is that stilbenes, and among them resveratrol and their derivatives, are usually present in very complex matrices (plants, foods, and biological fluids) and their content is usually very low. If in plant extracts the concentration of these compounds can be rather high, in food products their content is much lower, and in biological fluids, the concentration is even lower. Preparation techniques must be used to remove or mask interfering species and to get a concentration of the compound high enough to measure. Sometimes the analyte must be chemically transformed to be analyzed and a derivatization process must occur.

The preparation techniques must be chosen carefully, otherwise the results of the analysis will be compromised, and the conclusions from the work performed are not real.

Some controversy has been generated among the sample preparation procedures used in different laboratories for the analysis of stilbens in general, as there is a huge variability in the results obtained from the analysis of *trans*-resveratrol. These variations have been attributed to a possible isomerization during the process of derivatization in diffused daylight, isomerization or hydrolysis during the extraction and separation processes, losses due to oxidation, and the presence of some resveratrol derivatives that could interfere in the results, namely when less selective detection modes are used to quantify these compounds.

Sometimes, even at the analytical scale, direct analysis is performed. The advantages of this methodology, accepted by most authors (Roggero 1994, Lamuela-Ravento's 1995, Goldberg 1996, Martínez-Ortega 2000), are that the method is more reproducible and faster, since problems due to extraction are avoided, but its use depends on the type of matrix and concentration of the analyte. For wine analysis, samples were just filtered through cellulose filters and directly analyzed (Roggero 1991, Roggero 1994, Lamuela-Ravento's 1995, Goldberg 1996, Burns 2002, Vitrac 2002, Vian 2005), but some authors preferred to use liquid–liquid extraction for concentration (Merás 2008). The effect of daylight and temperature (30–60°C) in the extraction of resveratrol and their derivatives has been investigated (Kolouchová-Hanzlíková 2004) and from the results obtained, it was possible to conclude that the extraction process must be conducted with protection against daylight and, if possible, under yellow light. Extracts prepared must be stored at –20º C or –80°C in amber material, in the dark, until use and they must be filtered through 0.45 μm filters before chromatography or capillary electrophoresis (CE) analysis.

In order to get a huge amount of compounds (preparative scale) from a plant component, as leaves, roots, fruits (Sochor 2010), and seeds (Li 2009), a more complex work must be performed. The first step is the extraction process starting with the preparation of a powder, and for that purpose liquid nitrogen is often used. Sometimes the sample is previously lyophilized (Li 2009, Song 2009) or just dried and grounded (Li 2009). For samples that have a lipid matrix as chocolate and cocoa liquor, a delipidation process with toluene, cyclohexene (Jerkovic 2010), and CH_2Cl_2 (Fan 2009) must occur before extraction. The remaining sample is then extracted with solvents that are able to solubilize the important components under study and methanol (Ballistreri 2009, Song 2009, Bao 2010) water, ethanol (Kato 2009) acetone, and ethyl acetate are among the ones that have been currently used in parallel with sonication. More polar extracts such as methanolic and ethanolic are usually the ones preferred for the extraction of stilbenes (Li 2009). If concentration is required, solvent evaporation is performed followed by redissolution of the residue.

Sometimes an additional stage involving chromatographic techniques such as cation-exchange chromatography, medium pressure liquid chromatography (MPLC) with various stationary phases (XAD-16 Amberlite resin, silica gel, C18, and Sephadex LH-20), semipreparative high-speed countercurrent chromatography (Fan 2009), and preparative scale (Li 2009, Kato 2009, Royer 2010) such as centrifugal partition chromatography (CPC) (Abbott 2010, Zga 2009) can be used to simplify the extract. The purification of stilbenes (E-resveratrol, E-piceatannol, (+) E-(ε)-viniferin, ampelopsin A, and vitisin C) from methyl tert-butyl ether stem extract of *V. vinifera* (Chardonnay cv) was performed (Zga 2009) by CPC. This is a particular kind of liquid–liquid chromatography technique in which a biphasic solvent system is used to partition analytes between two immiscible liquid phases according to the partition coefficient.

Solid-phase extraction (SPE) is a common sample treatment prior to the analytical method that has been widely used in the preparation of samples. Different cartridges have been used and are usually conditioned with methanol, followed by water or a mixture of water with buffer as ammonium acetate (20 mM, pH 5.5, 5 mL) (Gamoh 1999, Rodríguez-Delgado 2002). The most common stationary phases are C18-bonded silica. Sometimes an internal standard as trihydroxyflavanone, TMS, (Lin 2009) is added to the sample and is introduced in the column at the same time to control this analytical step. The cartridge is dried with a nitrogen gas stream and finally the compounds are eluted with an eluent as an organic solvent (methanol, acetonitrile, and ethanol), water, or mixtures with water or buffer solution, in order to prevent ionization of the compounds. The solution obtained is then analyzed after filtering through a 0.45 μm membrane or can be evaporated under nitrogen, redissolved and analyzed (Juan 1999, Rodríguez-Delgado 2002).

An ultrafast analytical protocol based on online SPE/high-performance liquid chromatography-tandem mass spectrometry for the determination of resveratrol in red wines was developed (Lu 2011) using multiwalled carbon nanotubes as SPE sorbents for the analytes' online extraction and cleanup. The results indicated that this system significantly increased sample throughput and decreased solvent consumption, exhibiting great potential to be applied for analyzing the resveratrol in red wines.

In order to clean up interfering compounds that coelute with resveratrol and piceid, a peanut extract was drained (Sales 2009) through a cleanup column and the extracts obtained were evaporated and reconstituted with ethanol (15%).

Sample preparation techniques for the analysis of resveratrol and their metabolites in biological fluids as plasma (Boocock 2007, Lin 2009), whole blood (Biasutto 2010), urine (Wang 2005), brain, testes, liver, lungs, and kidney (Juan et al. 2010b) are important, as the presence of these compounds must be accurately determined to understand the mechanisms of absorption, distribution, metabolism, and excretion in humans. An accurate evaluation of the corresponding levels in the bloodstream is important to assess the delivery strategies, as well as to verify claims of efficacy based on *in vitro* results. Methods used, including sample preparation, must be validated, confirming that they can be used with confidence for the intended purposes. These biological matrices are very complex and proteins are precipitated with acetonitrile or methanol before analysis of samples (Lin 2009, Juan 2010a). Compounds may be extracted with methanol for concentration purposes, or samples can be simplified using SPE, and a major improvement in selectivity can be obtained using the adequate method for sample preparation. SPE with a C18 cartridge was used in the extraction of dihydroresveratrol from rat acidified plasma. Dihydroresveratrol was eluted with methanol, and concentrated prior to HPLC analysis (Juan et al. 2010a) and the same

methodology was used for wines and grape berries. Results obtained by MS of the chromatographic peak of resveratrol showed that there was a peak impurity that had not been removed from the resveratrol fraction using the single C18 cartridge column and a second column, a Sep-Pak PS-1 column, was used in tandem. The contamination was successfully removed.

Also, metabolites of resveratrol (*trans*-resveratrol-3-*O*-glucoronide, *cis*-4′-resveratrol-*O*-glucoronide, *cis*-resveratrol-3-*O*-glucoronide, *trans*-resveratrol-4′-*O*-sulfate, *trans*-resveratrol-3-*O*-sulfate, and *cis*-resveratrol-4′-*O*-sulfate) were isolated from urine samples using the SPE column for the cleanup procedure. Compounds were eluted with acidified methanol and ethyl acetate (Zamora-Ros 2009).

Recently (Benová 2010), supercritical fluid extraction was optimized for piceid and resveratrol extraction from Japanese knotweed (*P. cuspidatum* Siebold & Zucc.). Conditions such as type of modifier, pressure, temperature, and time of extraction were studied. SFE results for piceid were compared with those obtained by conventional Soxhlet extraction carried out for 4 h, and results were 10 times lower for the SFE method. The advantage of SFE over the Soxhlet extraction method is that it has an extraction time period more than five times shorter.

Directly suspended droplet microextraction (DSDME), a miniaturized liquid-phase extraction procedure, was used to determine *cis*- and *trans*-resveratrol isomers, piceatannol, and other phenolic compounds in herbal infusions, fruits, juices, and functional foods. A derivatization reaction by means of an injection-port reaction with bis(trimethylsilyl)trifluoroacetamide (BSTFA) was carried out to convert the polar nonvolatile compounds into volatile derivatives. The sensitivity and detection limits of the method studied using the DSDME sample pretreatment method were very low and the recoveries obtained for spiked samples were satisfactory for all the compounds (Viñas 2011).

Another sample treatment technique based on solid-phase microextraction (SPME) was used for the determination of polyphenols in wine (Viñas 2009, Cai 2009). Again a derivatization process was necessary to convert the polar nonvolatile compounds into volatile derivatives. Direct immersion (DI) SPME was used for the adsorption of polyphenols, and then the fiber was placed in the headspace of the derivatizing reagent previously referred, BSTFA. The effects of SPME fiber selection, extraction time, temperature, and desorption time were investigated. The derivatization conditions, time, temperature, and the volume of derivatization reagent were also optimized (Cai 2009).

17.3 Identification and Quantification of Stilbenes

Different methodologies can be used in the analysis of stilbenes in food. The methodologies described for plants can be adapted to food analysis; the difference must be in the sample preparation methodology used.

Sample analysis may be performed in order to chemically characterize, discriminate, or define samples authenticity—sample component or a family of compounds are identified and quantified, also compounds resulting from degradation or contamination are analyzed.

For the selection of the sample treatment method, it was necessary to previously define the analytical method that was going to be used. The implementation of the analytical method must take place before the sample preparation method optimization. In order to confirm the results obtained, the whole method, including sample preparation, and method of analysis must be validated.

Different methods have been described for the analysis of resveratrol and piceid, namely in wines samples. The most widely used ones are based on separation techniques as these compounds are usually present in complex matrices, but nonseparative methods have also been described for some applications. Validated methods are necessary and are used for the quantification of stilbenes in different matrices.

17.3.1 Separative Techniques

The *trans*-resveratrol concentrations in wines are very low; this is a polar compound with very low volatility, which makes it difficult to separate on a gas chromatography (GC) column. Although gas chromatographic techniques using flame ionization detection (Lamikanra 1996) or MS detection (Viñas 2009) (Goldberg 1994, Goldberg 1995, Soleas 1995) have been proposed for *trans*-resveratrol

analysis, the technique has a major inconvenience: usually extraction, cleanup, or a derivatization step is required prior to GC analysis of the substance and this handling can enhance the *trans*- to *cis*-isomerization of resveratrol.

Resveratrol was analyzed as a trimethylsilyl derivative by capillary GC and the *cis*- and *trans*-resveratrol peaks were resolved with a detection limit about 50 ng/mL (Blache 1997). *Trans*-resveratrol in red and white wines (Montes 2010) was analyzed using SPE, acetylation of the analyte in aqueous media, and further determination by GC with MS detection. The use of a mixed-mode SPE sorbent provided an improvement in the selectivity of the extraction step and the presence of characteristic mass spectra for the acetyl derivative, obtained by electron impact MS, guarantees the unambiguous identification of *trans*-resveratrol. Limit of quantification (LOQ) of the method was 0.8 ngm/L. The reduced form of resveratrol, dihydro-resveratrol, was also identified in red wines. A review about techniques for resveratrol silylation has been published (Antonelli 1996).

Although as referred GC techniques can be used, nowadays the resveratrol content is mostly determined using liquid chromatography (LC) with UV detection and gradient elution of the mobile phase. The two analytical techniques, GC and high-performance liquid chromatography (HPLC), were compared using wine samples. The results obtained were according to those reported elsewhere in the literature but higher results were obtained by GC than by HPLC analysis (Barbanti 1996).

In HPLC analysis, conventional reversed-phase columns, constituted by packed microparticulate bonded silica, have been used and good separations are obtained even in complex matrices. C18 are the most commonly used, and column lengths as well as the internal diameter vary between 100–250 mm and 2.1–4.6 mm, respectively. For HPLC-MS applications, narrow-bore columns are usually recommended and the flow rate of the mobile phase, in some cases, must be lower or equal to 0.3 ml/min. The particle size in the column ranges from 3 to 10 μm. Reducing the particle size (2 μm) and the internal diameter of the column decreases the time of analysis, and this fact is important as the time required for these samples analysis is long (40–90 min), due to the complexity of the samples, which makes routine analysis of these compounds very tedious. Using columns with low particle size can be a problem due to the high pressure in the LC system. Sometimes a good choice is to use particle sizes of 3.5 μm, which can operate with conventional systems.

Other materials such as mesoporous silica have been used to prepare monolithic columns (Figure 17.4), which operate at higher flow rates with lower backpressures than conventional columns allowing the analysis of samples using direct injection or low sample dilutions. By using these types of columns, the time of analysis is decreased.

Gradient elution is usually performed although some applications describe the use of isocratic conditions, but the choice must depend on the sample preparation methodology used: if the sample to be analyzed has been cleaned from possible interferences, an isocratic elution can be used.

For gradient elution, an aqueous phase (water or water acidified with acetic, formic, or phosphoric acid at pH about 2–2.5) and a less polar organic solvent (methanol and/or acetonitrile) are used. The use of acetonitrile has some adavantages because the time of analysis is decreased, peaks usually elute sharper, and the pressure in the equipment is lower.

Reverse-phase high-performance liquid chromatography (RP-HPLC), either with spectrophotometric UV detection (Ratola 2004), diode array detection (DAD) (Ribeiro de Lima et al. 1999, López 2001, Burns 2002, Vitrac 2005, Vian 2005), fluorimetric detection (Rodríguez-Delgado 2001, Stecher 2001, Rodríguez-Delgado 2002, Vitrac 2002), DAD–fluorimetric detection (Jeandet et al. 1997, Sánchez 2005), DAD–fluorimetric–electrochemical detection (Kolouchová-Hanzlíková 2004) or with MS DAD-MS (Domínguez 2001, Pozo-Bayón 2003, Bravo 2006), has been widely used.

In Figure 17.5, the UV spectra of *trans*- and *cis*-resveratrol and piceid (Vian 2005) are presented.

As a result of the lack of peak identity in many biological matrices, a UV detector cannot be used for the identification and quantitation and a photodiode-array detector has been used to obtain a UV spectrum of the analyte detected. In this way, it is possible to confirm the interference of other compounds in resveratrol or piceid quantification (Figure 17.6).

Higher concentration values reported in the literature for resveratrol content may be due to interferences from other components in samples and nonselectivity of the detector.

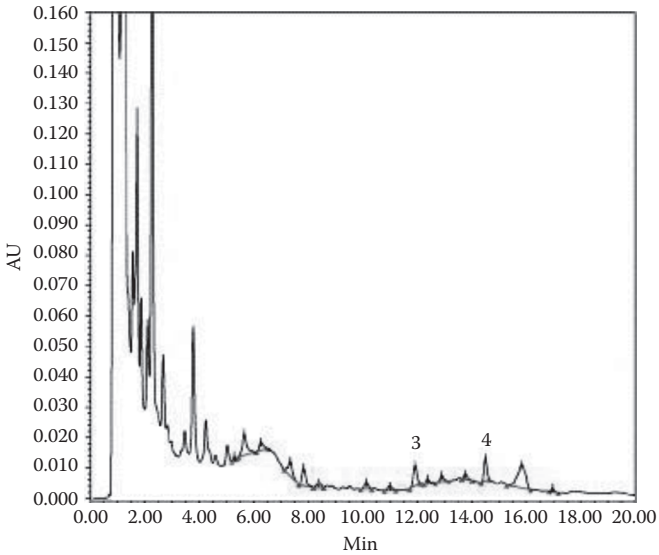

FIGURE 17.4 Chromatogram of a Grenache wine at 285 nm (3: *cis*-piceid; 4: *cis*-resveratrol) analyzed by LC using a chromolith column. (From Vian, M.A. et al. 2005. *Journal of Chromatography A*, 1085:224–229. With permission.)

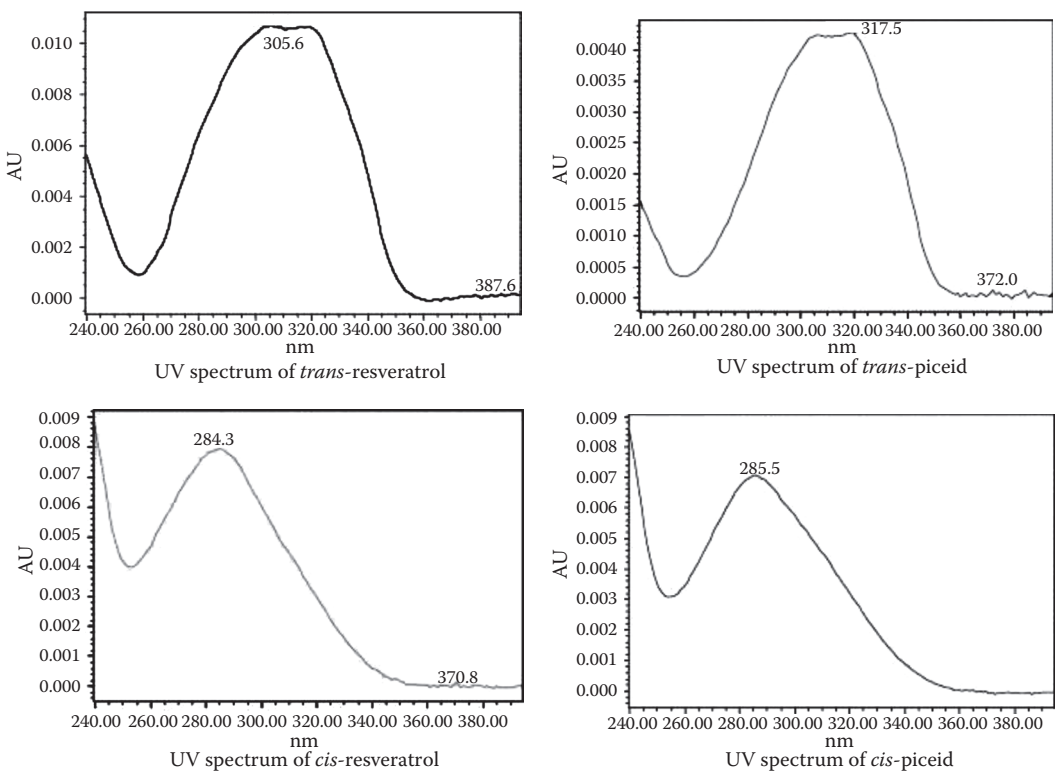

FIGURE 17.5 UV spectrum of *trans*-resveratrol, *trans*-piceid, *cis*-resveratrol, and *cis*-piceid. (From Vian, M.A. et al. 2005. *Journal of Chromatography A*, 1085:224–229. With permission.)

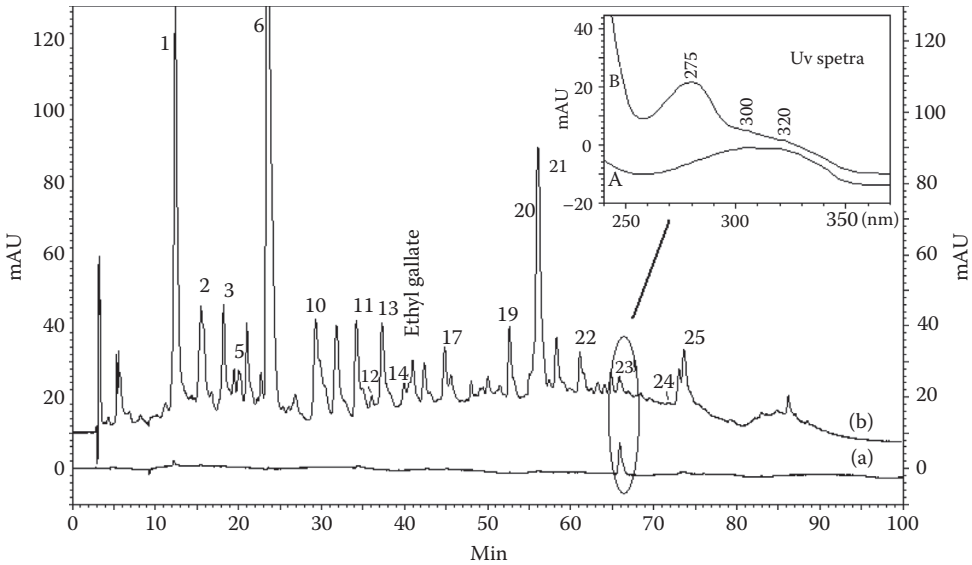

FIGURE 17.6 Chromatograms at 300 nm and UV spectra of *trans*-resveratrol solution at 0.45 mg L^{-1} (a) and muscatel Setúbal wine B_3 (b). Identification: 1—gallic acid; 2—5-HMF; 3—protocatechuic acid; 5— furfural; 6—caftaric acid; 10—catechin; 11—caffeic acid; 12—fertaric acid; 14—epicatechin; 17—ferulic acid; 19—*trans*-piceid; 20—quercetin-3-glucuronide; 21—quercetin-3-glucoside; 22—*cis*-piceid, kaempferol-3-glucoside; quercetin-3-rutinoside; 23—*trans*-resveratrol; 24—*cis*-resveratrol, ethyl caffeate; 25—quercetin. (From Bravo, M.N. et al. 2008. *J Food Compos Anal* 21(8): 634–643. With permission.)

The highly sensitive and selective fluorimetric detector has also been used to determine the content of resveratrol in grapes and wine based on the intrinsic fluorescence of some phenolics, but the number of compounds determined by measuring the native fluorescence is low. The detection limits in real samples was 0.02 and 0.003 mg/L for absorbance and fluorescence detection, respectively (Rodríguez-Delgado 2002). Results obtained from the analysis by LC with photometric and fluorimetric detection were used for wine classification based on the phenolic compound content of *trans*- and *cis*-resveratrol. The chemometric treatment of the data provided an appropriate way to classify and characterize the wine samples (Andreu-Navarro 2011).

A derivatization reaction was described to produce chelates between analyte and terbium (III). The method involves the formation of luminescent chelates between phenolics and the lanthanide ion in an alkaline medium in the presence of ethylenediaminetetraacetic acid to prevent terbium precipitation. Detection limits achieved are lower than or comparable to those reported by other methods (Russo 2008).

Electrochemical detection has proved to be at least as selective and as sensitive as detections based on the fluorescence detection.

The use of hyphenated techniques as LC/MS for the routine analysis of resveratrol is becoming increasingly important. Interfaces with LC using atmospheric pressure ionization, including electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) (Counet 2006, Bravo 2008), have been developed for polar compounds (Figure 17.7). The soft ionization with ESI-MS provides the molecular masses of compounds and when a triple quadrupole (MS/MS) analyzer or an ion trap (MS^n) are used, experiments performed can give much information about the fragmentation pattern of the compounds under study. In Figure 17.8, the mass spectra obtained for resveratrol standard and the corresponding peak in a wine sample are presented.

HPLC in tandem with MS using an ESI ion source (HPLC/(-)ESI-MS^n) and FTICR-MS^n was used for the analysis of polyphenol compounds in the root and stems of *Parthenocissus laetevirens*, and the fragment C_2H_2O (42 Da) from the resorcinol ring of resveratrol, (E)-5-styrylbenzene-1,3-diol,

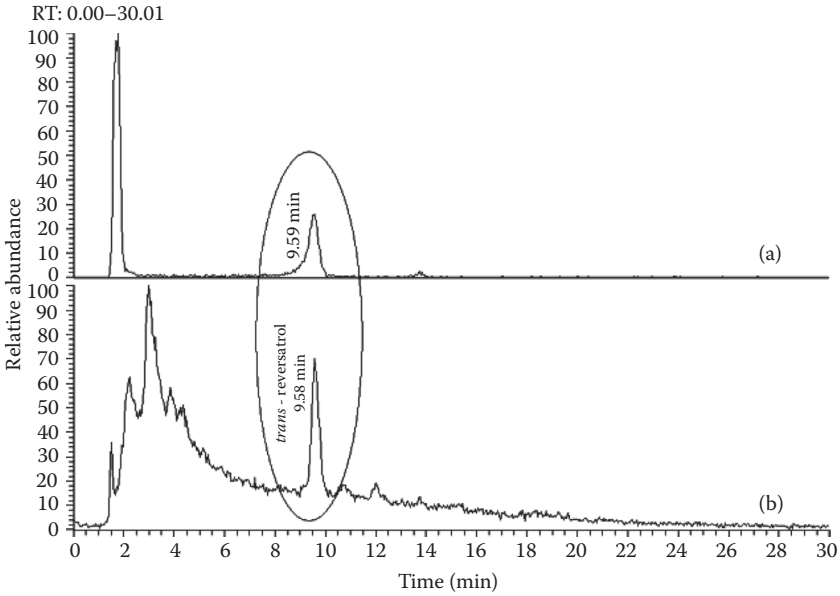

FIGURE 17.7 SIM at *m/z* 229 for a (a) standard solution of *trans*-resveratrol at 120 mg L^{-1} and (b) Muscatel Setúbal wine extract, C_1. (From Bravo, M.N. et al. 2008. *J Food Compos Anal* 21(8): 634–643. With permission.)

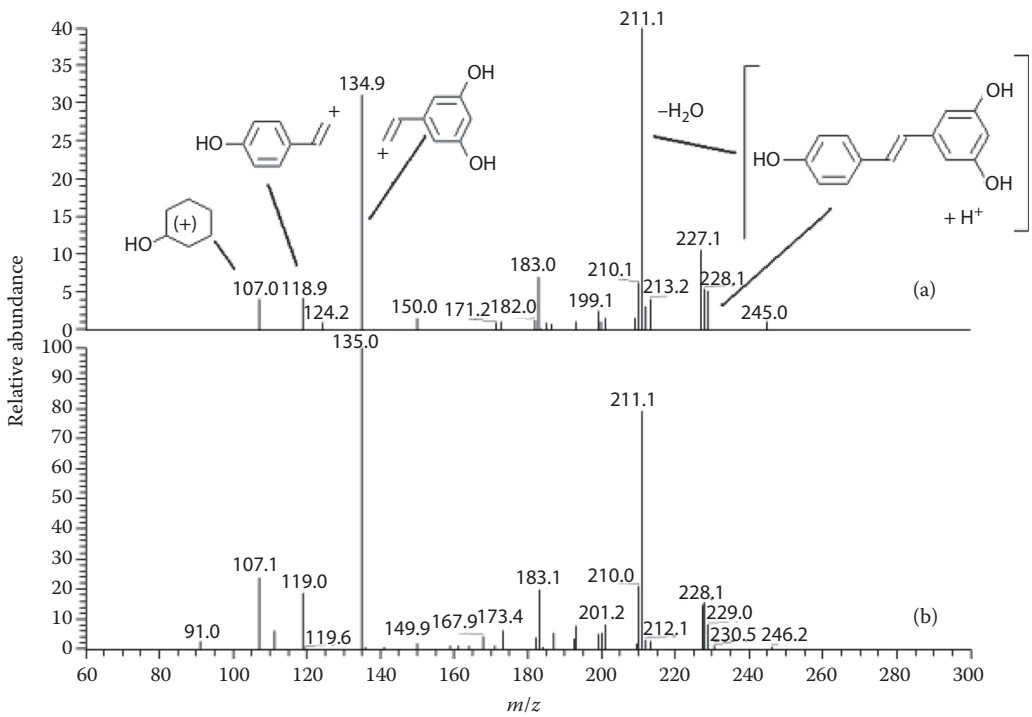

FIGURE 17.8 MS^2 from the precursor ion *m/z* 229 and the proposed fragments for values at *m/z* 107.0, 118.9, 134.9, 211.1, and 229.0 (a) standard solution of *trans*-resveratrol at 120 mg L^{-1} and (b) Muscatel Setúbal wine extract C. (From Bravo, M.N. et al., 2008. *J Food Compos Anal* 21(8): 634–643. With permission.)

(E)-4-styrylphenol, and (E)-4-(3,4,5-trimethoxystyryl)phenol was used for the structural identification of the compounds (Chen 2009). HPLC/ESI-MS*n* features high sensitivity, low levels of sample consumption, relatively short analysis times, and important structural data information. A simple method based on HPLC/(–)ESI-MS^n was established for the analysis of seven isomers of resveratrol dimers and three of their analogs in Xinjiang wine grapes (Kong 2011). However, this analytical methodology cannot provide accurate masses of precursor and fragments obtained in the mass spectra analysis. Fourier transform ion cyclotron ion resonance tandem mass spectrometry (FTICR-MS/MS) can be used to identify the compounds and provide information on the molecular formulas and accurate molecular weight (Kong 2011).

The analysis of piceid detection has been performed using fluorescence detector (Pezet 1994), electrochemical detector (McMurtrey 1994, McMurtrey 1997), or photodiode array detector (Lamuela-Ravento's 1995).

In Table 17.3, data on the analytical conditions used by different authors in the analysis by HPLC of stilbenes are summarized.

Capillary zone electrophoresis has also been described as a separation methodology to be used and presents some advantages over LC, as time of analysis is usually shorter than the ones obtained for chromatographic techniques. An 8-min CE-DAD method was developed for quality evaluation, differentiation, and authentication of *Rhizoma Smilacis Glabrae* from two confusable plant species, *R. Smilacis Chinae* and *R. heterosmilacis*. The optimal CE separation conditions were developed with a background electrolyte of 20 mM borax and 30 mM β-cyclodextrin at pH 9.4. *trans*-Resveratrol was one of the peaks identified in the electropherogram (Zhang 2011). The content of *trans*-resveratrol and other phenolic compounds was measured in bilberry, cowberry, cranberry, strawberry, blackcurrant, and redcurrant using sodium tetraborate 35 mM at pH 9.3 as the background electrolyte (Ehala 2005). In grape skins, resveratrol was determined using a 50 mM sodium tetraborate with 10% MeOH (v/v) at pH 8.4 (Priego Capote 2007) and sodium tetraborate with 30% MeOH (v/v) at pH 9.0 (Berli 2008). In red wine, a 25 mM borate solution at pH 9.4 was used to determine *cis*- and *trans*-resveratrol (Pazourek 2005), sodium borate + sodium phosphate + SDS at pH 9.0 for *trans*-resveratrol (Gu et al. 2000), boric acid + dibasic phosphate + SDS + ACN at pH 9.2 for *cis*- and *trans*-resveratrol (Prasongsidh 1998) as well as sodium deoxycholate + disodium hydrogen phosphate + disodium tetraborate+ phosphoric acid at pH 9.3. DAD (Du 2010) and amperometric detection were used as the detection mode for the detection of these compounds (Yang 2010).

A new dual opposite carbon-fiber microdisk electrode detector was fabricated and tested for hyphenation with CE in the polyphenol determination. Under optimized conditions, CE-dual opposite carbon-fiber microdisk electrode was adequate to baseline separate and determine five important polyphenols namely, *trans*-resveratrol in red wine (Du 2010).

Micellar electrokinetic CE has also been used (Chu 1998) in wine samples analysis with a clear lack of sensitivity attributed to the need for preconcentration techniques.

Other techniques have been described that are able to detect resveratrol. An electronic tongue (ET) (Rudnitskaya 2010) was capable of detecting *trans*-resveratrol and other phenolic compounds when a set of 14 Madeira wines comprising wines produced from four *V. vinifera* L. varieties (Bual, Malvasia, Verdelho, and Tinta Negra Mole) that were 3, 6, 10, and 17 years old were analyzed using HPLC and an ET multisensor system. The ET consisting of 26 potentiometric chemical sensors with plasticized PVC and chalcogenide glass membranes. Results obtained show that effects of age grape variety, and their interaction were significant for the HPLC data set and only the effect of age was significant for the ET data and had the capability of predicting Madeira wine age with good accuracy (1.8 years).

17.3.2 Nonseparative Techniques

For some specific applications, nonseparative techniques have been described.

A simple method for the determination of *trans*/*cis*-resveratrol ratio in water, using a simple and reliable UV–vis spectrophotometric method was developed using accurate values for UV absorbance in water $\varepsilon(286\ \text{nm}) = 23{,}400\ M^{-1}\ cm^{-1}$ for *trans*-resveratrol and $\varepsilon(304\ \text{nm}) = 9515\ M^{-1}\ cm^{-1}$ for *cis*-resveratrol.

TABLE 17.3

Sample Preparation and Liquid Chromatography Methodologies Used in the Analysis of Stibenes

Compound and Sample	Sample Preparation	LC Column	Delection Mode	Eluent	Linearity	LOQ	Reference
Hydroxyresveratrol in plasma from rat	SPE C18	Synergi Fusion-RP (250 mm × 4.6 mm; 4 µm)	D0AD-ESI(−)-MS	Gradient elution: A. 3% acetic acid (v/v) B. 20% acetonitrile (v/v)	5–100 µM	170 nM	Juan (2010)
trans-Resveratrol in wine	Direct injection	Spher ODS C18, (250 mm × 4 mm, 5 µm)	DAD (*trans*-308 nm, *cis*-resveratrol 288 nm).	Gradient elution: A. Water:acetonitrile (7:3) B. Water:methanol (5:5)	0.5–16 mg/L	0.015 mg/L	Dourtoglou (1999)
Piceid and resveratrol in plant Japanese knotweed	Supercritical fluid extraction	LiChrospher100, RP-18 column (125 mm × 4 mm, 5 µm)	UV (306 nm)	Gradient elution: A. Water and 0.1% formic acid B. Acetonitrile	0.5–10 mg/L	—	Benová (2010)
Free resveratrol isomers in wine	Direct injection	C18 reverse-phase nucleosil (250 mm × 4 mm, 5 µm)	Electrochemical detection	Isocratic elution: 25% Acetonitrile, 0.1% H_3PO_4 and NaCl (5 mmol/L)	0.01–10 mg/L	*trans*-Resveratrol :3 µg/L *cis*-Resveratrol 15 µg/L	Kolouchová-Hanzlíková (2004)
Resveratrol and piceid in peanuts	Solid/liquid extraction with ethanol:water	C18 reverse column (250 mm × 4.6 mm, 5 µm)	DAD (240–400 nm)	Gradient elution: A: 100% double-deionized water B: 100% acetonitrile	—	—	Sales (2009)
Resveratrol and piceid in wine	Direct injection	Monolithic columns: RP-18e (100 mm × 4.6 mm)	DAD (200–310 nm)	Gradient elution: A: Water–acetic acid (94:6) (v/v) B: Water acetonitrile–acetic acid (65:30:5) (v/v/v)	0.1–10 mg/L	*trans*-Resveratrol 0.10 mg/L *cis*-resveratrol: – 0.11 mg/L	Vian (2005a)
Resveratrol in chocolate and cocoa	1. Delipidation 2. Solid–liquid extraction	C18 prevail column (150 mm × 2.1 mm, 2 µm)	APCI(+) MS/MS	Gradient elution: A: Water with 0.1% formic acid B: Acetonitrile.	—	—	Counet (2006)
Hop cone samples	1. Solid/liquid extraction 2. Evaporation	C18 column (150 mm × 2.1 mm, 2 µm)	APCI (+)-MS/MS	Gradient elution: A: Water/acetonitrile/formic acid (98.9:1:0.1, v/v) B: Acetonitrile	—	—	Jerkovic (2007)

continued

TABLE 17.3 (continued)

Sample Preparation and Liquid Chromatography Methodologies Used in the Analysis of Stibenes

Compound and Sample	Sample Preparation	LC Column	Delection Mode	Eluent	Linearity	LOQ	Reference
trans- and *cis*-Resveratrol in wines	Direct injection	C18 column (150 mm × 2.1 mm, 5 μm)	ESI(−)MS	Gradient elution: A: Formic acid in water (pH 3) B: Formic acid in acetonitrile (pH 3)	—	—	La Torre (2006)
trans- Resveratrol and piceid in ultrasound treated peanuts	Extraction with ethanol 80% Cleanup column of aluminum oxide	C18 column (250 × 4.6 mm, 5 μm)	DAD (240–400 nm).	Gradient elution: A: 100% double-deionized water B: 100% acetonitrile	0.5–10 μg/mL	—	Sales (2009)
Resveratrol in wines	Direct injection	C18 column (25 × 0.4 cm), 5 μm	DAD diode array	Gradient elution: A: Glacial acetic in water (pH 2.4) B: Phase A/acetonitrile (20:80).	—	—	Abril (2005)
trans-Resveratrol in wines	SPE C-18 cartridges	C18 column (150 mm × 3.9 mm, 4 μm)	Fluorimetic (λex = 360 nm λem = 374 nm) and UV (280 nm)	Gradient elution: A: Methanol, acetic acetic acid, water (10:2:88) B: Methanol, acetic acetic acid, water (90:2:8)	0.4–4.0 mg/L	—	Rodríguez-Delgado (2002)
trans- Resveratrol and *cis*-resveratrol in wines	Dilution	Inertsil ODS-2 column (250 × 4.0 mm)	UV–VIS (280 nm for *trans*-resveratrol and 306 nm for *cis*-resveratrol)	Gradient elution: A: Water:CH_3COOH:CH_3CN, 90:5:5 B: Water:CH_3COOH:CH_3CN, 70:5:25	—	—	Castellari (1998)
trans-Resveratrol and piceid in celll culture medium	Direct injection	C18 column (250 × 4 mm) (5 μm)	Fluorescence detector *trans*-resveratrol and *trans*-piceid (λexc 300 nm; λem 390 nm)	Gradient elution: A: H_2O:TFA (97.5:2.5, v/v) B: ACN:A (80:20, v/v). T.	—	—	Henry-Vitrac (2006)

Resveratrol in fruits (apricots)	1. Deep-frozen 2. Solubilization	C18 column (150 × 4.6) 5 µm	UV (260 nm)	Isocratic A: Acetic acid 50 mM B: Acetic acid 50 mM in can	—	—	Sochor (2010)
Resveratrol, dihydroresveratrol monosulfate, piceid monosulfate, piceid monoglucoronide in physiological saline solution and urine	Polyamide SPE cartridges for cleaning	C18 column (150 mm × 4.6 mm; particle size, 5 µm)	ESI(−)MS/MS	Gradient elution: A: Acetic acid–methanol–water (2:10:90, v/v) B: 100% Methanol	—	—	Wang (2011)
Stilbenes in cocoa liquors	1. Lipid removal, 2. Liquid/liquid extraction 3. SPE	Normal phase: (250 mm × 2.1 mm, 2 µm)	APCI(+)-MS/MS	Gradient elution: A: Water + 1%ACN + 0.1% formic acid; B: ACN			Jerkovic (2010)
Resveratrol in wine and plasma		C18 column (7.5 cm × 2.1 mm)	ESI(−)MS/MS	Gradient elution: A: Water + 0.1% formic acid; B: Methanol			Di Donna (2009)
Resveratrol in wine	SPE (C18 and PS-1)	C18 column (250 × 1.5 mm, 5 µm)	ESI(−)MS	Isocratic elution Methanol, 20 mM ammonium acetate (pH 5.5) (55:45)	400 ng–80 pg/ injection	200 pg Resveratrol	Gamoh (1999)
Resveratrol in wine	SPE	C18 column (50 mm × 2.1 mm, 2.7 µm)	Triple–quadrupole linear ion trap mass spectrometry in negative	Gradient elution: A: Water (0.1% formic acid) B: Acetonitrile		*trans*-Resveratrol: 0.05 ng/mL *cis*-Resveratrol: 0.06 ng/mL	Lu (2011)
Resveratrol in *Pistacia vera* L.	Methanol extraction	C18 (250 mm × 4.6 mm, 5 µm)	DAD (200–450 nm)	Gradient elution: A: 0.1% (v/v) Acetic acid in water B: Methanol	—	—	Ballistreri (2009)
Pterostilbene in plasma samples	Precipitation of proteins	C18 (250 × 4.6 mm, 5 µm)	UV (320 nm)	Gradient elution ACN + 0.1% formic acid		20–2000 ng/mL	Lin (2009)

continued

TABLE 17.3 (continued)

Sample Preparation and Liquid Chromatography Methodologies Used in the Analysis of Stibenes

Compound and Sample	Sample Preparation	LC Column	Delection Mode	Eluent	Linearity	LOQ	Reference
Resveratrol and oxyresveratrol in mulberry	Extraction with MeOH	SB-C18 (4.6 × 250 mm, 5 µm)	UV (303 nm)	Gradient elution: A: Methanol; B: Water	Resv 0.022–1.376 mg/L Oxyresv 0.035–14.00 mg/L	Resveratrol: 0.11 ng/mL Oxyresveratrol: 0.17 ng/mL	Song (2009)
Red, white, rosé, and fortified wines	Direct Injection	dC18 column (250 mm × 4.6 mm, 5 µm)	UV (315 nm)	Gradient elution: Buffered solution (pH2.7) and acetonitrile	0.77–15.45 mg/L	LOQ 0.465 mg/L	Pereira (2010)
Serbian wines: *trans*- and *cis*-rseveratrol		RP-C18 (250 × 4.6 mm, 5 µm)	UV (306 and 286 nm)	Gradient elution: A: ACN:acetic acid: water (20:2:78) B: ACN:acetic acid: water (90:2.8)	—	—	Cvejic (2010)
Resveratrol in Moscatel fortified wines	Direct Injection	Merck RP-18 (250 × 4 mmm) 5 µm	DAD FD ED MS (m/z 229)	Gradient elution: A: Formic acid 0.1% B: ACN	0.075–5 0.075–5 1.35–8		Bravo (2008)

Note: SPE, solid-phase extraction; DAD, diode array detection; ESI, electrospray ionization; and APCI, atmospheric pressure chemical ionization.

These data can be used in 150 mM NaCl and 10 mM phosphate-buffered solutions and are useful for cell culture, and *ex vivo* and *in vivo* studies (Camont 2008).

Among MS techniques, different ionization methodologies were described. Matrix-assisted laser desorption/ionization of small molecules in most cases is impossible due to interferences from matrix ions on molecules <300–500 Da. As ferulic acid, a phenolic compound (phenolic acid) can be used as a common matrix, the direct laser desorption/ionization was tested for other polyphenols. Minimal sample pretreatment made the technique potentially appropriate for fingerprinting, screening, and quality control of wine samples and it was used for the analysis of *trans*-resveratrol (Spácil 2009).

A resonance light-scattering (RLS) method was developed for the determination of resveratrol in biological and pharmaceutical field based on the interaction between resveratrol and methylene blue (MB). At pH 8.69, the weak RLS intensity of MB was remarkably enhanced by the addition of trace amount of resveratrol with the maximum peak located at 385.0 nm. The detection limit for resveratrol was 0.63 μg/mL (Xiang 2011).

A spectrofluorimetric–chemometric method was developed for the direct determination of rseveratrol in human plasma. For each measurement, excitation–emission matrices were obtained from 280 to 360 nm (excitation) and from 380 to 550 nm (emission). In this work, data treatment was performed by parallel factor analysis (PARAFAC), for extracting the pure analyte signal, using the standard addition method, which permits determinations in the presence of a strong matrix effect caused by plasma analyte–protein binding. The proposed method was validated (Bernardes 2010).

A spectroscopic method, based on photochemically induced second-derivative fluorescence, has been reported for the resveratrol analysis in wine (Díaz 2007).

Flow injection–solid-phase spectroscopy (FI-SPS) or flow-through optosensor combines advantages of FI with the analyte preconcentration on a small amount of a solid support as Sephadex QAE A-25, such as high sensitivity and selectivity, rapidity, and simplicity and was used to develop a sensitive automatic fluorimetric method alternative to the chromatographic determination of resveratrol in beer (Molina–Gárcia 2011).

Piceid was determined in cocoa-containing products (cocoa powder, dark chocolate, and milk chocolate) using photo-induced fluorescence and a multicommutated continuous-flow assembly that was provided with an online photoreactor. A strongly fluorescent photoproduct was generated from piceid when it was irradiated under UV light for 30 s, the product was retained on Sephadex QAE A-25 and directly monitored on this active solid support at 257/382 nm (λ (exc)/λ (em), respectively). The quantification limits were 1.4, 1.1, and 0.09 mg/kg, respectively (Molina-García 2011).

Two methodologies, laser desorption followed by resonance-enhanced multiphoton ionization coupled with time-of-flight mass spectrometry (LD-REMPI-TOFMS) and RP-HPLC with fluorescence detection, were compared using vine leaf as the sample and *trans*-resveratrol as the target compound. Both techniques showed a similar range of linearity, but there were important differences in sensitivity: detection, and quantification limit were 2.1 and 6.7 g/L for REMPI and 20 and 67 g/L for HPLC. However, the chromatographic method required less time (30 min) than the REMPI method to implement the analysis (Sánchez 2005). When choosing the best methodology to use these two topics, sensitivity and time of analysis must be considered.

Analysis of grapevine phytoalexins was performed at the surface of *V. vinifera* leaves by laser desorption/ionization time-of-flight mass spectrometry (LDI-TOFMS) without matrix deposition. This simple and rapid sampling method was successfully applied to map small organic compounds as resveratrol and other stilbene compounds (Hamm 2010).

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18

Flavonoids: Anthocyanins

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18.1 Introduction

The anthocyanins, named after the Greek words *anthos*, flower, and *kyano*s, blue, constitute a major flavonoid group, widespread in plants and foods made from them. They are responsible for the pigmentation of numerous plant organs, play a role in plants as visual attracters for pollinators and seed disseminators, and may be involved in protection against biotic and nonbiotic stress. Anthocyanins contribute to the color of foods and beverages, an essential component in quality assessment, determination of consumer liking, and eventually food choice. In addition to giving visual clues for freshness and ripeness of fruits and vegetables, color influences taste thresholds and interferes with flavor identification and perception of its intensity (Clydesdale, 1993). Anthocyanins are widely used as natural food colorants (Francis, 1989; Mateus and de Freitas, 2009). The most traditional source is grape pomace, a by-product of the wine industry, but other extracts showing better stability toward heat, light, or pH variations can

be more suitable for some applications. Anthocyanins are listed by the European Union legislation as natural colorants E163, defined as any extract from edible fruits and vegetables such as strawberry, blackberry, cherry, plum, raspberry, blackcurrant, elder fruit, grape, red cabbage, red onion, eggplant, and black carrot. According to the US Food and Drug Administration regulation, anthocyanins extracted from grape, fruit juices, and vegetable juices are "natural colors" that do not require certification. The list of foods that can be added with anthocyanins is defined by each country. More recently, the role of anthocyanins in the prevention of chronic diseases has been emphasized, as reviewed by Clifford (2000) and Scalbert and Williamson (2000), justifying their increasing use as nutritional supplements.

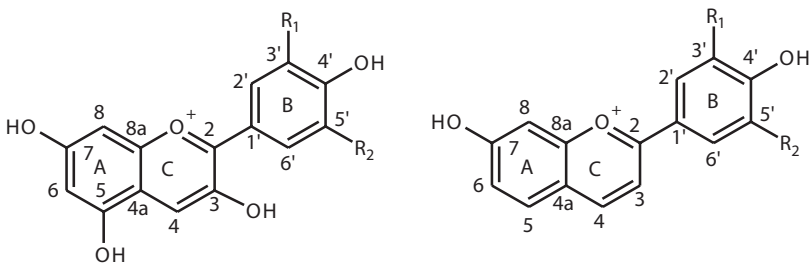
This chapter first presents anthocyanin structures, their biosynthetic pathways, and reactions in plant transformation processes, and then describes methods used to analyze them, with special emphasis on food anthocyanins.

18.2 Structures and Properties of Anthocyanins

18.2.1 Structural Diversity of Plant Anthocyanins

A recent update of the list of formally identified anthocyanins has reported 539 structures, including about half established since 1992 (Andersen and Jordheim, 2006). Their general structure is based on an aglycone, called anthocyanidin, which is glycosylated, by one or more sugars, some of which can be acylated. Although 31 anthocyanidins have been identified, the large majority of anthocyanins (about 90%) are based on six of them (pelargonidin, cyanidin, delphinidin, peonidin, petunidin, and malvidin), which differ by their B-ring substitution pattern (Figure 18.1, 1–6). Rare anthocyanidins are 3-deoxyanthocyanidins that lack the OH group at the 3-position (Figure 18.1, 7–9), and derivatives of all these structures, showing an additional hydroxyl at the 6-position and/or a methyl at the 5-, 7-, or 4′ position. Complex anthocyanidins, for example, riccionidins (Kunz et al., 1994) and rosacyanins (Fukui et al., 2002, 2006), have been described. Finally, anthocyanin derived structures, such as pyranoanthocyanins and flavanol–anthocyanin adducts, have been recently identified in plants. As they are also formed during food transformation processes, they are described in Section 18.2.3.3.

Anthocyanins are normally found as glycosylated derivatives, except 3-deoxyanthocyanins, which can occur in the aglycone form. However, pelargonidin has been detected in acerola (Vendramini and Trugo, 2004) and cyanidin in beans (Macz-Pop et al., 2006). Sugars can be monosaccharides, disaccharides, or



$R_1 = R_2 =$ H: pelargonidin (1)

$R_1 =$ OH, $R_2 =$ H: cyanidin (2)

$R_1 = R_2 =$ OH: delphinidin (3)

$R_1 = OCH_3$, $R_2 =$ H: peonidin (4)

$R_1 =$ OH, $R_2 = OCH_3$: petunidin (5)

$R_1 = R_2 = OCH_3$: malvidin (6)

$R_1 = R_2 =$ H: apigeninidin (7)

$R_1 =$ OH, $R_2 =$ H: luteolinidin (8)

$R_1 = R_2 =$ OH: tricetinidin (9)

FIGURE 18.1 Structure of common anthocyanidins and 3-deoxyanthocyanidins.

trisaccharides. The 3-position is nearly always glycosylated, the only known exceptions being cyanidin 4′-*O*-glucoside and 7-*O*-(3″-*O*-glucoside-6″-*O*-malonyl-glucoside)-4′-*O*-glucoside, identified in red onion (Fossen et al., 2003). The 5, 7, 3′, 5′, and rarely 4′ position can also be *O*-glycosylated. Moreover, a *C*-glycosylated anthocyanin has recently been identified (Saito et al., 2003).

Sugar acylation (found in 65% of anthocyanins), with phenolic (279 structures) and aliphatic (178 structures) acids, increases anthocyanin diversity and can lead to very complex structures. Anthocyanins containing up to four acyl substituents on four different glycosides have been identified (Kondo et al., 1989). Moreover, the acyl groups can be substituted by other glycosides or acylglycoside groups (Goto and Kondo, 1991). Phenolic acids are mostly represented by *p*-coumaric and caffeic acids, but ferulic, sinapic, *p*-hydoxybenzoic, and gallic acids also occur. They are generally linked to a glucose in its 6-position but other substitutions are also encountered, for example, to the rhamnose 4-hydroxyl group of 3-rutinosides in solanacea (Fossen and Andersen, 2000). Hydroxycinnamoyl substituents are in the *trans*- (E) configuration in all formally identified anthocyanins but malvidin 3-*(cis-p*-coumaroyl-glucoside) has been tentatively identified on the basis of its UV–visible and mass spectral properties (Monagas and Bartolome, 2009). The most common aliphatic acyl substituent is malonic acid, followed by acetic acid, while malic, succinic, tartaric, and oxalic acids are rarely encountered. Finally, dicarboxylic acids can be linked on one end to an anthocyanin and on the other to a flavone or a flavonol glycoside (Andersen and Jordheim, 2006).

18.2.2 Anthocyanin Biosynthesis

Anthocyanin profiles depend on the expression of genes involved in the biosynthetic pathway and are characteristic of particular plant families and species. Anthocyanins derive from a precursor common to all flavonoids: flavanone 2*S*-naringenin (Figure 18.2). Hydroxylation of its C3, catalyzed by flavanone 3-hydroxylase (F3H), yields dihydrokampferol, which is reduced by dihydroflavonol 4-reductase (DFR) to a flavan 3,4 diol (leucoanthocyanidin), in this case leucopelargonidin, converted to pelargonidin by anthocyanidin synthase (ANS), also called leucoanthocyanidin deoxygenase (LDOX). Biosynthesis of 3-deoxyanthocyanidins involves reduction of 2*S*-naringenin by flavanone 4-reductase (FNR) to the corresponding flavan 4-ol, which serves as a substrate for ANS (Stich and Forkman, 1988). DFR also has FNR activity, so that the orientation toward anthocyanins rather than 3-deoxyanthocyanins appears driven by F3H activity (Halbwirth et al., 2003).

B-ring hydroxylation is catalyzed by flavonoid 3′-hydroxylase (F3′H) and flavonoid 3′5′-hydroxylase (F3′5′H) that are active on naringenin and dihydrokampferol (Menting et al., 1994) and possibly on other substrates further down the biosynthetic route. Successive activities of DFR and ANS lead to cyanidin and delphinidin. Further reactions include glycosylation, catalyzed by glycosyltranferases, methylation, catalyzed by *O*-methyltransferases, and acylation, catalyzed by acyltransferases. In contrast to the well-conserved main pathway, these steps are family-, species-, or even variety-dependent. Glycosylation, that is, transfer of a monosaccharide from a uridine diphosphate-monosaccharide (UDP-monosaccharide) to an anthocyanidin acceptor, is specific both to the type of sugar and to the substitution position. The sequence of methylation, glycosylations, and acylations is variable but the first glycosylation usually takes place at the 3-position.

18.2.3 Anthocyanins in Plant-Based Foods

18.2.3.1 Anthocyanin Composition of Common Fruits and Vegetables

Anthocyanin food composition tables are still rather limited. In that established by USDA (USDA database for flavonoid content of selected food), using representative samples of 59 fruits, vegetables, and nuts, anthocyanin values have been converted to the corresponding aglycones, thus overlooking the nature of substituents and their impact on bioavailability and other properties. An extensive overview of the anthocyanin composition of fruits, vegetables, and grains, including information on varietal differences and other factors affecting anthocyanin levels, has been provided by Mazza and Miniati (1993). The list of dietary anthocyanins has since been expanded. An update of anthocyanin composition of

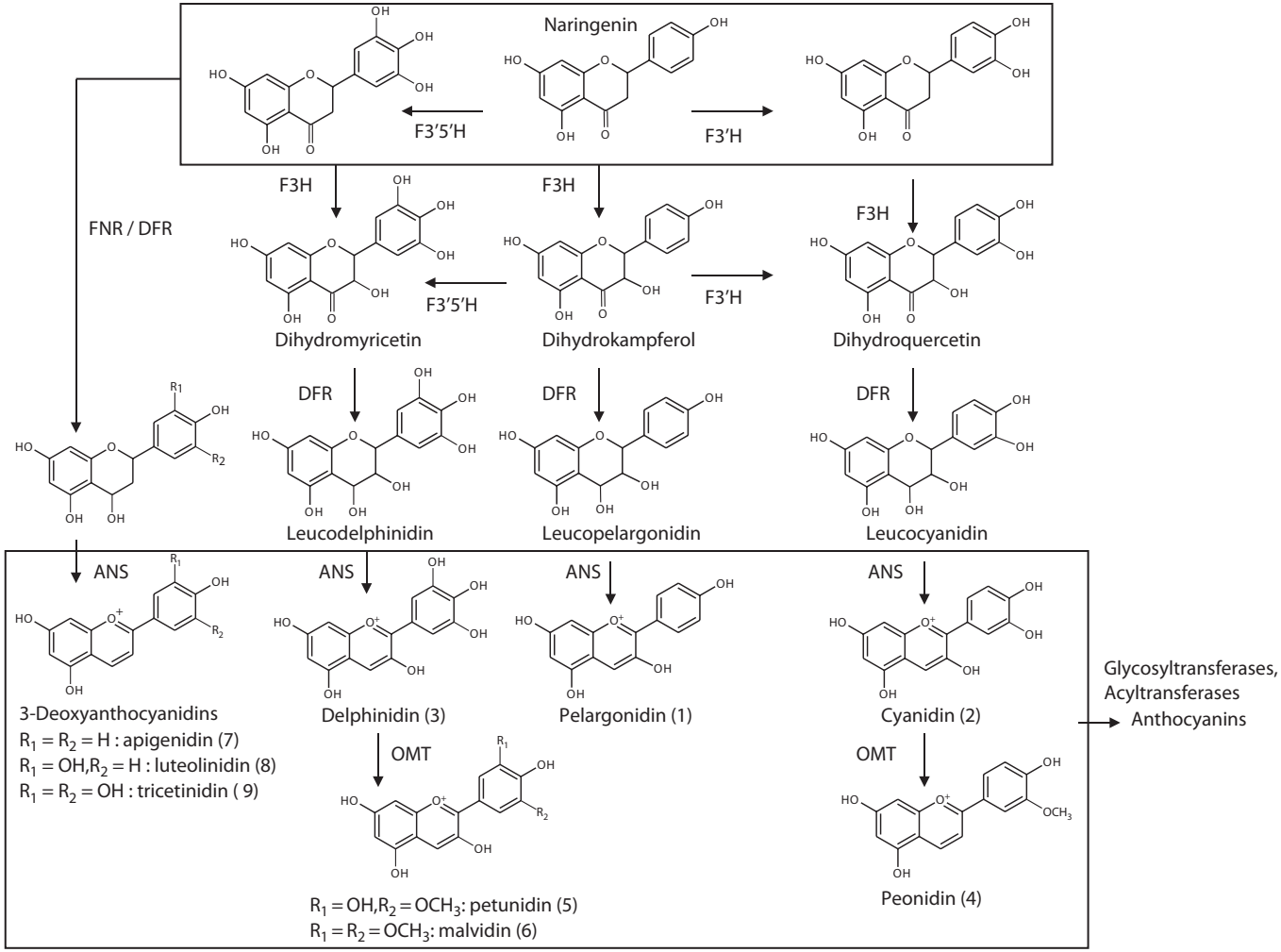


FIGURE 18.2 Scheme of anthocyanin biosynthesis.

foods consumed in the United States, based on high-performance liquid chromatography (HPLC) mass spectrometry (MS) analysis, has been recently provided (Wu et Prior, 2005a,b). They are particularly abundant in red and blue fruits and berries but some vegetables can also be major dietary sources.

Food anthocyanins are based on the six common anthocyanidins, except for sorghum anthocyanins, which are 3-deoxyanthocyanins. Pelargonidin is often missing while cyanidin is almost ubiquitous, except in eggplant and black bean where it is replaced by delphinidin. Methylated anthocyanins are rather common and found in both the cyanidin and delphinidin series. Each species is characterized by specific substitution patterns. The 3-position is often glucosylated. Three other monosaccharides (galactose, rhamnose, and xylose) are also found, as well as four disaccharide substitutents, rutinose (6-*O*-α-L-rhamnosyl-D-glucose), sambubiose (2-*O*-β-D-xylosyl-D-glucose), laminaribiose (3-*O*-β-D-glucosyl-D-glucose), sophorose (6-*O*-β-D-glucosyl-D-glucose), formed by glycosylation of the glucose. A second substitution by a simple sugar in the 5-position is encountered in raspberry (5-rhamnoside), cranberry (5-galactoside), grape (5-glucoside), eggplant (5-glucoside, 5-galactoside), and black bean (5-galactoside). Trace amounts of anthocyanins with 4′- and/or 7-*O*-glucosylation have also been detected in red onion. Eight acylating groups, acetyl, malonyl, oxalyl, coumaryl, caffeyl, ferulyl, sinapyl, *p*-hydroxybenzyl, have been reported in common foods. Anthocyanins of brasicacea such as red cabbage and red radish are highly conjugated with sugars and acyl groups. Sorghum anthocyanins are 3-deoxyanthocyanins (luteolinidin, apigenidin, 5-methoxyluteolinidin, and 7-methoxyapigenidin), found as aglycones and as 5- or 7-glucosides.

While the anthocyanin profiles appear mostly like varietal characters, the amounts of anthocyanins depend on cultivation conditions and fruit ripeness. Moreover, anthocyanins are often more abundant in the fruit skins, in relation to their role in UV protection, so that consumption habits (with or without the peel) and transformation processes (e.g., crushing, pressing, maceration, etc.) have an influence on their dietary intake.

18.2.3.2 Anthocyanin Equilibria in Aqueous Solutions

Anthocyanins are commonly represented under their flavylium cation form. However, this form is prevalent only under highly acidic conditions (pH < 2). As the pH increases, it is converted, on the one hand, through acid–base equilibrium, to the blue quinonoidal base (A) which can be further deprotonated to its anionic form, on the other hand, through deprotonation and hydration, to the colorless hemiketal (Figure 18.3, reaction A), which is in equilibrium with the tautomeric yellow chalcone forms (Brouillard and Dubois, 1977; Brouillard and Delaporte, 1977). The hydration equilibrium relating the flavylium and the mixture of hemiketal and chalcone forms and the proton transfer equilibrium between the flavylium and the quinonoidal bases are characterized by thermodynamic constants, K_h and K_a, which can be determined by UV–visible spectrophotometry. Usual anthocyanins have hydration constants ($pK_h = -\log K_h$) in the range 2–3. The hydration constant and acidity constant ($pK_a = -\log K_a$) of malvidin 3-glucoside have been calculated at 2.8 and 4.2, respectively, so that the hydrated forms predominate in the pH range 2–7 (Brouillard et al., 1989). 3-Deoxyanthocyanidins are much more resistant to hydration than anthocyanins (Mazza and Brouillard, 1987). Luteolinidin and apigenidin at pH 3 retain 80% and 60%, respectively, of their absorbance at pH 1.0, while cyanidin, peonidin, and pelargonidin retain only 16%, 1%, and 5% (Awika et al., 2004). Flavylium cations also form colorless adducts by reaction with ascorbic acid (Markakis, 1982) and with sulfite (Jurd, 1963), commonly used in the food industry as antimicrobial and antioxidant agent. 3-Deoxyanthocyanidins are also bleached by sulfites but the resulting adducts are less stable than those formed by anthocyanins (Timberlake and Bridle, 1967). Sulfite addition, like hydration, is reversed upon acidification.

18.2.3.3 Anthocyanin Reactions and Structures of Resulting Pigments in Foods

Both the flavylium and the hemiketal undergo various reactions in food processing. These reactions and the resulting structures have been particularly explored in the case of red wines, as recently reviewed (Monagas and Bartolome, 2009).

The positive charge, which can be delocalized in C2 or C4, confers the flavylium a strong electrophilic character (i.e., capacity to attract electron rich species) while the hemiketal shows a nucleophilic character

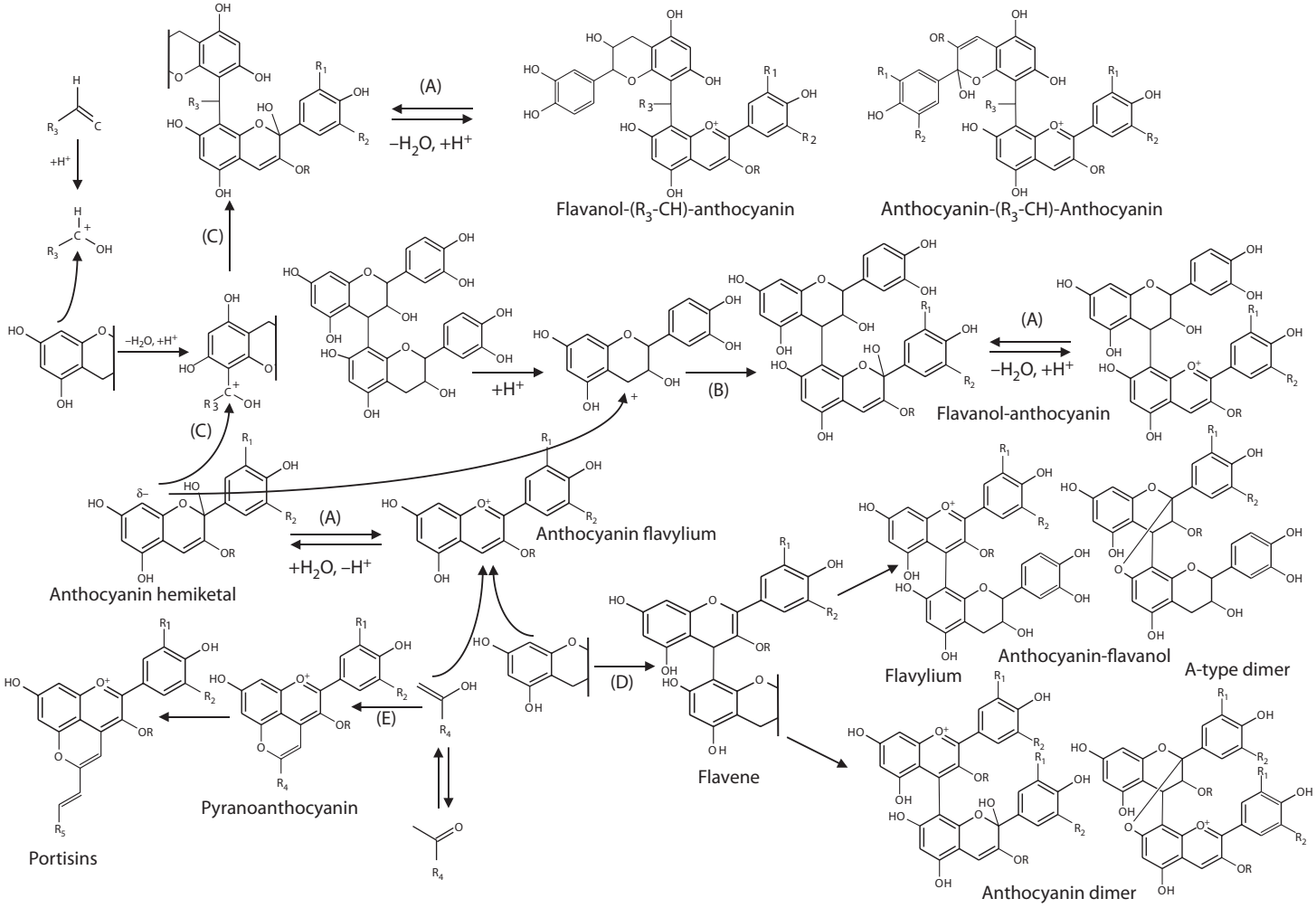


FIGURE 18.3 Formation of anthocyanin derived pigments from flavylium and hemiketal forms of anthocyanins.

(i.e., electron excess) in its C6 and C8 positions, like other uncharged flavonoids. This ambivalence enables a variety of reactions. The nucleophilic hemiketal adds to the electrophilic carbocations resulting from acid-catalyzed cleavage of proanthocyanidins (flavanol oligomers, syn. condensed tannins), to form flavanol–anthocyanin adducts (Figure 18.3, reaction B) (Salas et al., 2004a). These pigments have been detected in wine but also in fruits and vegetables, including strawberry (Fossen et al., 2004), black currants (McDougall et al., 2005), black beans, grapes (Gonzalez-Paramas et al., 2006), and purple corn (Gonzalez-Manzano et al., 2008), suggesting that they can form *in planta*. The hemiketal is also involved in condensation with aldehydes (RCHO), such as acetaldehyde (Timberlake and Bridle, 1976), glyoxylic acid, furfural, hydroxymethyl furfural (Es-Safi et al., 2002), or vanillin (Sousa et al., 2007). In this reaction (Figure 18.3, reaction C), a first flavonoid adds to a protonated aldehyde which acts as an electrophile. The resulting adduct, through loss of a water molecule and protonation, yields a carbocation onto which a second flavonoid adds in the same way. *R*-methine flavanol–anthocyanin copolymers and *R*-methine anthocyanin oligomers can thus be generated. In all these products, the anthocyanin units are in the hemiketal form but can dehydrate to the flavylium.

The flavylium cations suffer nucleophilic addition of species such as water and sulfites, but also flavonoids (Figure 18.3, reaction D), yielding colorless flavenes. The anthocyanin–flavanol flavene can be oxidized to the flavylium (Jurd, 1969; Somers, 1971; Salas et al., 2003) or rearrange to another colorless compound showing an A-type bond (4–8, 2-*O*-7) (Bishop and Nagel, 1984; Remy-Tanneau et al., 2003). The flavylium may dehydrate to the yellow xanthylium (Jurd, 1969; Somers, 1971) but none of the xanthylium salts identified thus far showed the expected structure. Anthocyanin oligomers presumably based on similar structures have been detected in grape (Vidal et al., 2004a) and wine (Salas et al., 2005a). Another group of pigments, called pyranoanthocyanins, result from nucleophilic addition of a compound with a polarizable double bond onto the flavylium cation (Figure 18.3, reaction E). The first pyranoanthocyanins have been detected in red wine (Cameira dos Santos et al., 1996) and identified to phenylpyranomalvidin 3-glucoside (Figure 18.3, R_1, $R_2 = OCH_3$, R = glc, R_4 = 4-hydroxyphenol) and its *p*-coumaroyl derivative, formed by addition of vinylphenol onto the anthocyanin (Fulcrand et al., 1996). Similar cyanidin and delphinidin derivatives were isolated from black current seeds (Lu et al., 2002). The vinylcatechol, vinylsyringol, and vinylguaiacol adducts have also been described (Hayasaka and Asenstorfer, 2002) and an alternative formation pathway, starting from the hydroxycinnamic acids, has been proposed (Schwarz et al., 2003a). Analogous products formed by reaction with acetaldehyde (pyranoanthocyanins: $R_4 = H$) and with pyruvic acid (carboxypyranoanthocyanins: $R_4 = COOH$) (Cheynier et al., 1997; Fulcrand et al., 1998) have been referred to as vitisins B and A, respectively, when first isolated from red wine (Bakker and Timberlake, 1997). Carboxypyranoanthocyanins were also detected in red onion (Fossen and Andersen, 2003) and in strawberry (Andersen et al., 2004). Methylpyranoanthocyanins ($R_4 = CH_3$) have been first found in black current extracts (Lu et al., 2000) and in fermentation media (BenAbdeljalil et al., 2000). They can be extraction artifacts formed by the reaction of anthocyanins with acetone (Lu and Foo, 2001) or result from reaction with acetoacetic acid. (He et al., 2006). The list of pyranoanthocyanins is constantly expanding. Pyranoanthocyanin-flavanols (R_4 = flavanol) (Francia-Aricha et al., 1997; Asenstorfer et al., 2001) are formed by the reaction of anthocyanins with vinylflavanols (Cheynier et al., 1999; Cruz et al., 2008). Portisins, which are vinylpyranoanthocyanin derivatives found in Port wines, result from reactions of carboxypyranoanthocyanins with vinylflavanols (Mateus et al., 2004), vinylphenols (Mateus et al., 2006), or hydroxycinnamic acids (Oliveira et al., 2007). Finally, structures in which two pyranoanthocyanin units are linked by a methine bridge have been recently identified (Oliveira et al., 2010).

18.2.4 Color Properties of Anthocyanins

Absorption maxima of the flavylium cations in the visible range are characteristic of the different aglycones, shifted toward larger wavelengths as the B-ring hydroxylation increases (from 520 nm for pelargonidin to 546 nm for delphinidin, petunidin, and malvidin), and hypsochromically of about 10–15 nm for the corresponding 3-monoglycosides (Strack and Wray, 1989). The flavylium ions of 3-deoxyanthocyanidins are more yellow-orange (λ_{max} 464–468 nm) than those of anthocyanins (Mazza and Brouillard,

1987; Awika et al., 2004). Since anthocyanins should normally be present mostly as colorless hemiketals under mildly acidic conditions, the red-to-purple color of foods containing anthocyanin depends on color stabilization mechanisms, including conversion to new pigments and interaction processes.

18.2.4.1 Color Properties of Derived Anthocyanins

Anthocyanin derivatives show large color diversity, maximum absorbance wavelengths of flavylium cations in the pyranoanthocyanin series ranging from 480 nm for methylpyranoanthocyanin to 730 nm for the dimers (Oliveira et al., 2010). Besides, some derived pigments are more resistant to hydration and sulfite bleaching than their anthocyanin precursors. Maximum absorption wavelength (λ_{max}) in the visible range, extinction coefficients (ε), and pK_h and pK_a values of some anthocyanin pigments are listed in Table 18.1.

The UV–visible spectra and pK_h value of catechin-malvidin 3-glucoside are similar to those of malvidin 3-glucoside (Salas et al., 2004b). In contrast, methylmethine–flavanol–anthocyanins formed by condensation with acetaldehyde show lower pK_a and higher pK_h values than those of malvidin 3-glucoside (Dueñas et al., 2006). This implies that flavanol–anthocyanin adducts are mostly colorless in mildly acidic conditions while ethyl-linked pigments remain colored and appear bluer in a wider pH range. pK_h values of methylmethine-malvidin 3-glucoside dimer are at 1.8 and 4.6, meaning that one of the anthocyanin units is hydrated and the other is not, over a large pH range (Atanasova et al., 2002a). Pyranoanthocyanins are resistant to sulfite bleaching (Sarni-Manchado et al., 1996; Bakker and Timberlake, 1997). This was attributed to the nonavailability of the C-4 position, which is the major site for nucleophilic attack by sulfites (Berké et al., 1998). Nuclear magnetic resonance (NMR) analysis of carboxypyranomalvidin 3-glucoside (vitisin A) showed the presence of five forms (flavylium cation, quinonoidal base, two hemiketal, and one chalcone), suggesting that hydration and ring opening can occur at C-2 and C-5 (Bakker et al., 1997). Protonation and hydration constants determined by spectrophotometry and high-voltage paper electrophoresis (Asenstorfer and Jones, 2007) indicated that vitisin A exists at wine pH as a complex mixture of hydrated and nonhydrated forms, the predominant species being the quinonoidal base. In contrast, the absorption spectra of carboxypyranopelargonidin 3-glucoside varied little with pH, implying limited hydration. Its molar absorptivity (ε) was about half that of pelargonidin 3-glucoside at pH 1.1 but nearly four times higher at pH 5.1 (Andersen et al., 2004). Finally, NMR and spectroscopy studies showed that vitisins B do not undergo hydration and their equilibria involve only protonation/deprotonation reactions (Oliveira et al., 2009). Portisins are more intensely colored and more resistant to hydration and sulfite bleaching than anthocyanins (Oliveira et al., 2006).

18.2.4.2 Copigmentation

Anthocyanin color also depends on their interactions with other molecules present in the medium. The occurrence of this phenomenon has been demonstrated *in vitro* and its role in flower pigmentation has been suggested in 1931 (Robinson and Robinson, 1931). Its major driving force is hydrophobic vertical stacking (a combination of van der Waals interactions and hydrophobic effect) between the planar anthocyanin flavylium or quinonoidal base chromophore and another planar molecule, to form π–π complexes from which water is excluded (Brouillard et al., 1989; Goto and Kondo, 1991). Such associations can involve another identical anthocyanin molecule (self-association), one of its aromatic substituents (intramolecular copigmentation) or another molecule (intermolecular copigmentation). They result in a strong color enhancement, due to shift of the hydration equilibrium toward the flavylium cation, and usually a slight bathochromic effect (shift of the maximum absorbance toward higher wavelengths, i.e., from red to purple). These effects are observed under conditions where hydrated forms predominate, that is, in mildly acidic aqueous solutions. Copigments produced no significant effect on 3-deoxyanthocyanins (Awika, 2008) or methylmethine-linked anthocyanin derivatives (Dueñas et al., 2006), which are mostly present as colored species in such media. Acylation with phenolic acids provides the possibility for intramolecular sandwich-type stacking, increasing pigment stability (Yoshida et al., 1991). This phenomenon is particularly important in the case of polyacylated anthocyanins, and responsible for the remarkable

TABLE 18.1

Color Properties of Some Anthocyanidins, Anthocyanins, and Derivatives

Compound	λ_{max} (εmol) nm (M^{-1} cm^{-1})	Medium	pK_h and pK_a Values (Determined by Spectrophotometry)	Reference
Aglycones				
Peonidin	516 (27,200)	0.2 M KCl, pH 1.0, buffer		Awika et al. (2004)
Cyanidin	516 (24,800)	0.2 M KCl, pH 1.0, buffer		Awika et al. (2004)
Luteolinidin	482 (31,700)	0.2 M KCl, pH 1.0, buffer		Awika et al. (2004)
Apigeninidin	468 (30,400)	0.2 M KCl, pH 1.0, buffer		Awika et al. (2004)
Glycosides				
Pg 3-glc	496 (17,000)	Buffer solution pH 1.1		Andersen et al. (2004)
	502.5	Buffer solution pH 3.0		
	510 (1720)	Buffer solution pH 5.1		
	521.5	Buffer solution pH 6.0		
	540.0	Buffer solution pH 7.3		
	549.5	Buffer solution pH 8.0		
	553.0	Buffer solution pH 8.9		
	497 (21,000)	Aqueous buffer (pH 1.0)		Jordheim et al. (2007)
	502 (24,000)	HCl/MeOH (0.01:99.99)		
Pg 3,5-diglucoside	498 (28,800)	0.2 M KCl, pH 1.0, buffer		Awika et al. (2004)
Cy 3-glucoside	512 (28,600)	0.2 M KCl, pH 1.0, buffer		Awika et al. (2004)
	510 (20,000)	Aqueous buffer (pH 1.0)		Jordheim et al. (2007)
	515 (21,000)	HCl/MeOH (0.01:99.99)		
Cy 3-galactoside	508 (23,000)	Aqueous buffer (pH 1.0)		Jordheim et al. (2007)
	519 (22,000)	HCl/MeOH (0.01:99.99)		
Cy 3-rutinoside	514 (26,100)	0.2 M KCl, pH 1.0, buffer		Awika et al. (2004)
	510 (33,900)			
Cy 3,5-diglucoside				
Cy 3-(3″-glucosylglucoside)	520	Online after HPLC		Fossen et al. (2003)
Cy 3-(6″-malonylglucoside)	520			
Cy 3-(3″-glc-6″-malonylglc)	522			
Cy 4′-glc, Cy 3,4′-diglc	508			
Pn 3-(6″-malonylglc)-5-glc	519	Online after HPLC		Fossen et al. (2003)
Pn 3-(6″-malonylglc)	523	Online after HPLC		

continued

TABLE 18.1 (continued)

Color Properties of Some Anthocyanidins, Anthocyanins, and Derivatives

Compound	λ_{max} (εmol) nm (M^{-1} cm^{-1})	Medium	pK_h and pK_a Values (Determined by Spectrophotometry)	Reference
Pt 3-glc	515 (21,000)	Aqueous buffer (pH 1.0)		Jordheim et al. (2007)
	527 (23,000)	HCl/MeOH (0.01:99.99)		
Mv 3-glc	515 (23,050)	0.1M citric acid/HCl pH1	pK_h= 2.83 pK_a= 3.78	Dueñas et al. (2006)
	538 (16,000)	Methanol/HCl 0.01%		Mateus and de Freitas (2001)
	517 (23,000)	Aqueous buffer (pH 1.0)		Jordheim et al. (2007)
	529 (25,000)	HCl/MeOH (0.01:99.99)		
Derivatives				
Flavanol-mv 3glc	526		pK_h = 2.6	Salas et al. (2004)
Aldehyde Condensation Products: Mv 3-glc-(R-methine)catechin/Mv 3-glc-(R-methine)Mv 3-glc				
R = CH3	535 (12,120)	0.1 M citric acid/HCl pH 1	pK_h = 4.17 pK_{a1} = 3.53 pK_{a2} = 5.04	Dueñas et al. (2006)
(2nd isomer)	535 (10,870)		pK_{a1} = 3.29 pK_{a2} = 5.38	
	535 (17,100)	10% ethanol/HCl pH 0.5		Escribano et al. (2001)
	560	Citric acid buffer pH 5.5		
R = vanillyl	549 (12,247)	90% methanol pH 1		Sousa et al. (2007)
Mv 3-glc-(CH-CH_3)-mv 3-glc	528	Online after HPLC	pK_{h1} = 1.8 pK_{h2} = 4.6	Atanasova et al. (2002a,b)
Pyranoanthocyanins				
Pyranomv 3-glc	490	Online after HPLC		Benabdeljalil et al. (2000)
	480	pH 0.8	pK_{a1} < 0.68 pK_{a2} = 4.3 pK_{a3} = 7.3	Oliveira et al. (2009)
Pyranomv 3-*p*-coumglc	485	pH 0.8	pK_{a1} < 0.75 pK_{a2} = 4.7 pK_{a3} = 6.8	Oliveira et al. (2009)
COOH-pyrano pg 3-glc	484–356	Buffer solution pH 1.1		Andersen et al. (2004)
	480	Buffer solution pH 3.0		
	490 (6250)	Buffer solution pH 5.1		
	503.5	Buffer solution pH 7.3		
	533.0	Buffer solution pH 8.0		
	549.5	Buffer solution pH 8.9		
	495 (22,000)	HCl/MeOH (0.01:99.99)		Jordheim et al. (2007)

COOH-pyranopy 3-gal	506 (21,000)	HCl/MeOH (0.01:99.99)		
COOH-pyranomv 3-glc	508	Online after HPLC		Benabdeljalil et al. (2000)
	532 (12,900)	Methanol/HCl 0.01%		Mateus and de Freitas (2001)
	516	pH 0	$pK_{a1} = 0.98$ $pK_{h1} = 4.51$ $pK_{h2} = 7.57$	Asenstorfer and Jones (2007)
	599	pH > 9		
CH3-pyranomv 3-glc	480	Online after HPLC		Benabdeljalil et al. (2000)
CH3-pyranocy 3-glc, 3-rut	480			Lu et al. (2000)
CH3-pyranodp 3-glc, 3-rut	480			
Phenyl-pyrano mv 3-glc	508	Online after HPLC		Cameira et al. (1996)
Phenyl-pyranomv 3-coumglc	508–sh310			
Phenyl-pyranocy 3-glc, 3-rut	503	Online after HPLC		Lu et al. (2002)
Phenyl-pyranodp 3-glc, 3-rut	503			
Pyranomv 3glc-dimer B3	512	Online after HPLC		He et al. (2006)
Pyranomv3-coumglc-dimer B1	512	Online after HPLC		He et al. (2006)
Pyranomv 3glc-(epi)cat	503	Online after HPLC		He et al. (2006)
Pyranomv 3-coumglc-(epi)cat	503	Online after HPLC		He et al. (2006)
Vinylpyranoanthocyanins (Portisins)				
Vinylpyranomv 3-glc-phenol	538	Online after HPLC		Mateus et al. (2006)
	533	Aqueous buffer (pH 1.0)		Oliveira et al. (2007)
Vinylpyranomv 3-glc-catechol	537	Aqueous buffer (pH 1.0)		Oliveira et al. (2007)
Vinylpyranomv-glc-gaiacol	537			
Vinylpyranomv-glc-syringol	540			
Vinylpyranomv 3-glc-catechin	572	Online after HPLC		Mateus et al. (2004)
	587 (82,933)	90% methanol pH 1		Oliveira et al. (2006)
Vinylpyranomv 3-coumglc-catechin	590			
Vinylpyranocy 3-glc-catechin	583 (62,136)			

Note: glc, glucoside; coum, coumaroyl.

stability of heavenly blue anthocyanins (Goto and Kondo, 1991). The increased resistance of *R*-methine flavanol-anthocyanins toward hydration (Escribano-Bailon et al., 1996; Dueñas et al., 2006) and sulfite bleaching (Sousa et al., 2007) is also attributed to self-association and intramolecular copigmentation between the flavanol and the chromophore group, limiting nucleophilic attack and formation of colorless adducts.

Some metal ions, such as iron and aluminum, can chelate with *o*-diphenolic anthocyanins to form highly colored complexes that are stable over a large range of pH values (Bayer et al., 1966) and contribute to the blue pigmentation of flowers. Spectrophotometric and NMR investigations showed that the flavylium cation is converted to the blue quinonoidal base upon complexation with aluminum (Dangles et al., 1994). Complex supramolecular structures, involving six anthocyanins, six flavones, two magnesium, or one magnesium and one ferric ion, have been described. (Kondo et al., 1992, 1994).

18.3 Extraction

Extraction of anthocyanins can be performed for analytical purposes or industrially for the preparation of colorants. Classical laboratory extraction processes use soaking, with shaking or gentle agitation on a magnetic stirrer, in acidified polar organic solvents such as methanol or ethanol. Methanol is more effective than ethanol and much more than water in recovering anthocyanins (Metivier et al., 1980). Acidification, often performed using 1% HCl (Bate-Smith, 1948; Harborne, 1958), is believed to help denaturate the plant cell membranes, and maintains the anthocyanins under their flavylium form. However, acidic media may induce hydrolysis of acyl and glycosidic bonds, especially during concentration steps. To avoid such bias, the use of lower HCl concentration or neutral solvents (Markham, 1982), or the replacement of HCl with weaker acids (Strack and Wray, 1989) has been proposed. A recent study (Downey et al., 2007) comparing the efficacy of different methanol–water mixtures and of methanol acidified with HCl, formic acid, acetic acid, citric acid, or maleic acid (1%, v/v) concluded that the best solvents for extracting anthocyanins from grape berry skins are methanol–water (50/50, v/v) and 1% HCl in methanol. However, substantial hydrolysis of malvidin 3-acetylglucoside to malvidin 3-glucoside was observed in the latter solvent, confirming earlier results (Revilla et al., 1998). Acetone enables an efficient and more reproducible extraction and the use of lower temperature for sample concentration (Garcia-Viguera et al., 1998), but it should be removed rapidly after extraction to avoid the formation of methylpyranoanthocyanins (Benabdeljalil et al., 2000; Lu and Foo, 2001).

Other parameters such as the solvent volume to plant material ratio, the duration and number of extractions, and the temperature are also important. Some authors use the exhaustion method consisting in successive extractions with the same solvent, while others prefer single-step extraction. Identical yields were obtained for grape skin anthocyanins when comparing four successive extractions (each with 0.5 mL solvent) with a single extraction (2 mL) (Mane et al., 2007). When studying simultaneously the effects of extraction duration, solvent to plant material ratio, and percentage of methanol in methanol–water–acetone with 0.05% trifluoroacetic acid, the anthocyanin extraction yield decreased with longer times and higher methanol rate and slightly increased with larger solvent volumes.

Extraction of anthocyanins as natural food colorants (E163) is achieved with those generally recognized as safe (GRAS) solvents (i.e., water and ethanol). The simplest and most cost-effective procedure is aqueous extraction, but aqueous sulfite solutions are more efficient, because of the high solubility of anthocyanin bisulfite adducts, and widely used industrially. Moreover, sulfites may facilitate the release of anthocyanins associated with macromolecules such as polysaccharides and proteins (Shrikhande, 1984) and increase their diffusion through the plant cell walls (Gao and Mazza, 1996), while protecting them from oxidation. Processes increasing the permeability of the cell membranes and the contact between the sample and solvent improve extraction of anthocyanins. Physical processes include ultrasounds, high hydrostatic pressure, pulsed electric fields (Corrales et al., 2008; Puértolas et al., 2010), pressurized liquid extraction (Monrad et al., 2010), high temperature, must freezing, and flash release that consists in heating the fruit quickly at high temperature (>80°C) and placing them

under a strong vacuum (Moutounet and Escudier, 2000). Increasing the temperature enhances anthocyanin extraction but also their subsequent degradation. Pectolytic enzymes, widely used in the juice industry to degrade plant cell walls and improve filterability, can also increase anthocyanin extraction (Bakker et al., 1999; Bautista-Ortin et al., 2005; Ducasse et al., 2010). However, side activities such as anthocyanase or esterases hydrolysing the glycosidic and ester bonds, respectively, may result in color instability.

18.4 Purification and Separation

18.4.1 Cleanup and Fractionation

Extraction procedures are not selective of anthocyanins, so that cleanup and/or fractionation is often necessary to remove other material present in the extract. Cross-flow ultrafiltration is used industrially to eliminate sugars, usually present at much higher concentration than the pigments in natural extracts, and concentrate the coloring material (Lin and Hilton, 1980). Ultrafiltration on cellulose acetate membranes removes proteins and polysaccharides but some of the oligomeric pigments form aggregates that may be retained by the membrane (Bridle and Timberlake, 1997). Adsorbents resins are used to remove the contaminating material and concentrate the pigments. Thus, red cabbage extracts have been treated with polymeric resin and anion exchange to remove unpleasant odor (Bridle and Timberlake, 1997).

For laboratory applications, less polar phenolic compounds can be removed by solvent partition using ethyl acetate, the anthocyanins being recovered in the aqueous phase, except for those acylated with hydroxycinnamic acids which remain in the organic phase. Liquid–liquid extraction has been developed further in countercurrent chromatography (CCC) procedures. The main advantage of such methods is that irreversible adsorption onto a solid stationary phase is excluded. Recent refinements of the technique, based on the introduction of a centrifugal force to retain one of the liquid phases in a coil, while the other one continuously passes through it, are available in different types of equipments, such as centrifugal partition chromatography, multilayer coil countercurrent chromatography, and high-speed countercurrent chromatography.

Preparative fractionation of anthocyanins from several sources (Renault et al., 1997; Degenhardt et al., 2000; Schwarz et al., 2003b; Vidal et al., 2004b; Salas et al., 2005a) was achieved using tert-butyl methyl ether/*n*-butanol/acetonitrile/water (2/2/1/5), acidified with 0.1% TFA or ethyl-actetate/*n*-butanol/water (0.2% TFA) as solvent systems. Separation can be performed isocratically but step gradient elution improves the resolution (Renault et al., 1997; Vidal et al., 2004b). The organic phase is often used as stationary phase ("head to tail") but better retention has been obtained in the "tail to head" mode, using the organic phase as the mobile phase (Vidal et al., 2004b).

Alternative cleanup and concentration procedures involve adsorption on solid phases, for example, insoluble polyvinylpyrrolidone, polystyrene–divinylbenzene such as Amberlite XAD-7 or XAD-2 resins, Sephadex LH-20, or octadecylsilyl (C-18). After a comparison of 16 solid-phase materials, Amberlite XAD7, especially for industrial applications, and C-18 were selected as the most suitable for anthocyanin concentration (Kraemer-Schafhalter et al., 1998). After washing with acidified water to remove polar contaminating material (sugars, organic acid, minerals, etc.), anthocyanins are recovered, by stepwise elution with solvents of decreasing polarity, often increasing proportions of methanol in acidified water. Depending on the sample size and objective (cleanup of small samples for analytical applications/large-scale purification), this can be achieved on solid-phase extraction cartridges, in open column or by medium pressure chromatography. Cation exchange chromatography has seldom been applied for separation of anthocyanins. However, an interesting approach, based on cation exchange chromatography in the absence and presence of excess bisulfite, has been developed to separate pyranoanthocyanins, which remain under their cationic form in the presence of sulfites, from other anthocyanins, which form neutral bisulfite adducts (Asenstorfer et al., 2001). Anthocyanins have also been purified, as their negatively charged bisulfite adducts, by high-voltage paper electrophoresis (Asenstorfer et al., 2003).

18.4.2 Chromatographic Separation

Further purification and separation are carried out by chromatography. Although they rely upon identical technologies, analytical methods, which do not require recovery of the compounds, should be distinguished from preparative methods, aiming at isolating pure substances from a mixture. Purification is usually achieved by successive chromatographic steps, involving low or medium pressure liquid chromatography, using the same phases as described above, preparative thin-layer chromatography (TLC), CCC, and/or (semi)-preparative HPLC. For example, a procedure involving ethyl acetate partition, followed by chromatography on XAD7, then LH20, and finally preparative HPLC on a C-18 column has proven efficient for purification of anthocyanins from onion (Fossen and Andersen, 2003) and from strawberry (Andersen et al., 2004) while a combination of column chromatography on Toyopearl HW-40 and polyamide resin has enabled isolation of flavanol–pyranoanthocyanins from wine (He et al., 2006). Analytical separations are performed in a single step, usually by HPLC.

18.4.2.1 Paper and Thin-Layer Chromatography

Earlier methods involved paper chromatography using different developing solvents, depending on the nature of the original sample (Harborne, 1967). It has been replaced by TLC using cellulose, silica, diol, and C-18 plates and a variety of solvents. TLC is widely employed, as it is fast, cheap, and resolutive. It enables simultaneous analysis of numerous samples and can be used for preparative purposes. Anthocyanins, including those that fail to migrate, appear as colored spots on the plate. The use of reversed-phase TLC with scanning densitometry for quantitative anthocyanin analysis has recently been reviewed (Oka et al., 2007). High-performance TLC shows increased speed and resolution and has found some applications in the separation, quantitative analysis, and purification of anthocyanins.

18.4.2.2 High-Performance Liquid Chromatography

HPLC has become the most popular technique for separation of anthocyanins, due to its high resolution compared to other available techniques. It is generally performed on reversed-phase C-18 columns, using gradients of methanol or acetonitrile in water. Elution solvents are acidified with rather high concentrations of formic or acetic acid (5–10%) to ensure that all anthocyanins are present as flavylium cations and improve the detection and the chromatographic resolution. Anthocyanins are separated in decreasing polarity order: glycosylation and hydroxylation increase mobility, while methylation or acylation, especially with phenolic acids, decreases it. No irreversible adsorption is observed in the case of anthocyanins, with the possible exception of some polymeric pigments. A major advantage of HPLC is the possibility to optimize the separation at the analytical scale and transfer it to preparative scale. However, solvents classically used in analytical methods such as acetonitrile that is rather difficult to evaporate, and solvents containing high concentrations of acids should be avoided at the preparative scale, because of the risk of compound degradation during concentration. Ultraperformance liquid chromatography (UPLC), showing increased speed and resolution, is gradually replacing HPLC for analytical purposes.

18.4.2.3 Capillary Electrophoresis

Capillary electrophoresis (CE) offers several advantages for analytical separations, including low sample and solvent consumption, and short run times. However, it has been seldom applied for anthocyanin analysis. The first method described used capillary zone electrophoresis with standard silica capillaries and borate running buffers at pH 8, resulting in anthocyanin degradation and very poor sensitivity (Bridle and Garcia Viguera, 1997). Better resolution was obtained with capillary zone electrophoresis using acidic (apparent pH of 1.5) phosphate buffer containing 30% acetonitrile (da Costa et al., 1998) but the limit of detection remained rather high (25 mg/L). More recently, the potential of CE coupled with MS for analyzing must and wine anthocyanins was evaluated, using 200 mM chloroacetate-ammonium, pH 2.0 or 200 mM borate-ammonium, pH 9.0 (Bednar et al., 2005). Better separation was

obtained under the latter conditions but the limit of detection was higher (4 mg/L for mv 3-glc, versus 0.9 mg/L at pH 2). HPLC was more sensitive than high performance capillary electrophoresis (HPCE) (Pg 3-glc limit of detection of 0.04 mg/L in HPLC versus 2.06 mg/L in HPCE, due to the different injection volumes and detection conditions used) and more resolutive, while HPCE was faster and consumed less solvent and sample (Comandini et al., 2008).

18.5 Identification and Quantification

Simple tests are available to detect anthocyanins, which are water soluble, red under acidic conditions, turn blue in alkaline media, and are bleached by sulfites. These general properties can be used as a first approach to distinguish them from other red pigments.

Identification classically relies on comparison of retention times or Rf in chromatography with those of reference compounds. However, given the risk of coelution, this should be restricted to plant material with established anthocyanin composition. Formal identification of anthocyanins requires a combination of spectrometric methods, including UV–visible spectrophotometry, MS, and NMR, all of which can be hyphenated with HPLC separation.

Quantification relies on spectrometric measurements, usually measurement of absorbance values in the visible range, which can be performed on isolated compounds, fractions, or extracts, online after HPLC separation or by image analysis on TLC plates. Moreover, noninvasive spectroscopic methods have been developed to estimate anthocyanin content directly on plants and foods.

18.5.1 Identification of Substituents after Hydrolysis

Although seldom used nowadays, chemical hydrolysis under acidic (to cleave the glycosidic linkages) or alkaline (to hydrolyze the ester bonds) conditions, followed by analysis of the released aglycone, sugar, and acyl groups, can give relevant structural information. Identification of sugars is classically achieved, after acidic hydrolysis and extraction of the aglycone with iosoamylalcohol or ethyl ether, by comparison with reference sugars in paper chromatography (Mabry et al., 1970). Paper chromatography can be replaced with TLC chromatography on silica plates developed with an ethylacetate:isopropanol:water (65:30:5, v/v/v) solution. Sugars are visualized by spraying with appropriate staining reagents (e.g., naphthoresorcinol/sulfuric acid in ethanol) and heating at 110°C for 10 min. Quantitative data can be obtained by cutting the chromatographic spots, extracting them and measuring the absorbance of the resulting solutions (Mabry et al., 1970) or by measuring spot intensity. Sugars have also been identified and individually quantified by gas chromatography analysis of the alditol acetate derivatives, after hydrolysis by heating at 120°C for 75 min in 2 M trifluoroacetic acid (Cameira dos Santos et al., 1996). The proportions of anthocyanins present under colorless forms in wine fractions was estimated using this technique, assuming that linked glucose originated from anthocyanins (Salas et al., 2005a). Further information on the linkage position on the sugar can be obtained by methylation of the sugar-free hydroxyl groups, followed by acid hydrolysis, conversion of the partly methylated sugars to alditol acetates, and GC-MS analysis (Saulnier et al., 1988). The sugar sequence in oligosaccharides can also be inferred from analysis of the intermediate heterosides released by enzymatic hydrolysis, using specific glycosidases, or by mild acidic hydrolysis.

18.5.2 UV–Visible Spectrophotometry

UV–visible spectrophotometry is essential for anthocyanin identification since the different structures show characteristic λ_{max} (Table 18.1). The nature of the sugar substituent does not modify the absorption spectrum while 3,5-diglycosides can be distinguished from 3-glycosides by their much lower absorbance in the 400–460 region (Hong and Wroslstad, 1990). Information of the anthocyanin substitution pattern can be obtained by recording its UV–visible spectrum in the presence of $AlCl_3$. A shift in the λ_{max} in the visible region, reversible after addition of HCl to the anthocyanin/$AlCl_3$ solution, indicates the presence

of a free *o*-diphenolic group on the B-ring (Mabry et al., 1970). Anthocyanins with sugar units on the B-ring also have their visible absorption maximum at shorter wavelengths than the 3-glycosides. Acylation with cinnamic acids can be detected by the presence of a characteristic shoulder at the maximum absorbance wavelength of the acyl moitey (310 nm for *p*-coumaric acid, 320 nm for caffeic acid). The ratio between absorbances at this wavelength and in the visible range indicates the hydroxycinnamic acid to anthocyanidin molar ratio (Harborne, 1967). Similarly, the ratio of absorbance at 280 nm to absorbance at 520 nm increases with the number of flavanol units in flavanol–anthocyanin adducts (Salas et al., 2005b).

Quantification of anthocyanins is based on measurement of their absorbance at their maximum absorbance wavelength in the visible range. This can be performed directly on isolated anthocyanins or anthocyanin mixtures by spectrophotometry, usually in acidified methanol, or on each individual compounds after chromatographic separation. Quantification requires calibration curves (or extinction coefficient) to be established, using relevant standard compounds, under the conditions used for the analysis. The number of anthocyanins available as standards is rather limited. Thus, calibrations are often established with the major anthocyanin present in a given extract (e.g., malvidin 3-glucoside in grape and wine, cyanidin 3-glucoside in strawberry), quantities being then expressed in molar equivalent.

Additional measurements have been developed to characterize anthocyanin composition of complex pigment mixtures like wine. Absorbance values are measured directly, with 1 mm path-length cells, to determine pigment concentration in wine. Comparison of this value with the absorbance measured after addition of acetaldehyde, which combines sulfite, gives access to bisulfite adducts. Total anthocyanins are measured after acidification (pH 1). Two methods are commonly used to measure and distinguish anthocyanins from derived pigments. The first one (Ribereau-Gayon and Stonestreet, 1965) calculates anthocyanin concentration from the difference of absorbance values in the visible range at pH 4.5 and at pH 1:

$A = (A_{\lambda max} - A_{700})_{pH1} - (A_{\lambda max} - A_{700})_{pH4.5}$; [anthocyanin] (mol L^{-1}) $= A/\varepsilon$, where ε is the molar extinction coefficient of the major pigment in the extract.

The second method measures pigments before and after sulfite bleaching and calculates the anthocyanin concentration from a calibration curve established using the same protocole on a standard anthocyanin (Somers, 1971). Both methods are based on the assumption that the color of genuine (monomeric) anthocyanins is modified by pH and sulfites while that of derived (polymeric) anthocyanins is not. However, this assumption proves false in the case of flavanol–anthocyanin adducts (Salas et al., 2004b). Polymeric anthocyanins, assumed to behave like tannins, have also been estimated by comparing absorbance values before and after protein precipitation (Haberston et al., 2003).

18.5.3 Mass Spectrometry

MS separates ionized molecules on the basis of their mass-to-charge ratio (*m/z*). This implies that the molecular species of interest are charged and transferred into the gas phase by the MS ionization source, and then separated according to their *m/z* by the mass analyzer. Several MS instruments based on different combinations of sources and analyzers are available. Chemical ionization or electron impact sources are poorly suited for the analysis of anthocyanins, which have to be derivatized to render them volatile. Anthocyanins are analyzed without derivatization by sources based on desorption and spray ionization techniques, associated to quadrupole (Q), time-of-flight (TOF), ion trap (IT), or Fourier-transform ion cyclotron resonance (FT-ICR) analyzers. Fast atom bombardment mass spectrometry (FAB-MS) uses xenon or argon atoms to bombard the sample dissolved in a matrix (often glycerol). Matrix-assisted laser desorption ionization (MALDI), mostly associated with the TOF analyzer, is a soft ionization technique, in which the analyte, mixed with a matrix, is desorbed and ionized on laser irradiation. MALDI-TOF-MS is sensitive, fast, and relatively tolerant to contaminants. As it forms only single-charged ions, with limited fragmentation and large available mass range, it is well suited for the analysis of large molecules and also has the ability to analyze the complex mixtures. Other mild ionization sources, namely atmospheric pressure ionization (API), including atmospheric pressure chemical ionization (APCI) and atmospheric pressure photoionization (APPI), and electrospray ionization (ESI), have now become the most popular techniques for anthocyanin analysis, as they can be coupled with

HPLC. ESI and API are associated to Q, TOF, FT-ICR, which provide the highest mass resolution, enabling unambiguous determination of molecular formula, and IT, designed to perform MSn experiments. This consists in isolating specific ions and then inducing their dissociation by collision with inert gas molecules so as to generate MS/MS (MS2) fragments. The process can be repeated on selected daughter ions, in consecutive fragmentation steps (MS3, MS4, etc.). Tandem mass spectrometry experiments (MS/MS) can also be performed using equipments combining two analyzers in series (e.g., triple quadupole, Q-TOF).

Anthocyanins are preferably analyzed in the positive-ion mode, as the flavylium cation (A^+). In the negative-ion mode, anions corresponding to the deprotonated hemiketal forms or quinonoidal base ($[M\text{-}H]^-$) can be detected. In both modes, cleavage of the glycosidic linkages generates characteristic fragment ions, providing information on the molecular weight of the aglycone and of its substituents (Table 18.2). Isobaric compounds based on different aglycones but not those based on the same aglycone can be distinguished. Malvidin 3,5-diglucoside was differentiated from malvidin 3-caffeoylglucoside after deuterium exchange experiments, different numbers of exchangeable protons in both molecules leading to different mass shifts (Favretto et Flamini, 2000). Positions of substituents on the anthocyanin hydroxyl groups cannot be inferred from the MS data. In contrast, successive fragmentations enable determination of the linkage positions in anthocyanin-tannin adducts (Salas et al., 2003).

HPLC coupled to MS, and particularly ESI-MS, which is more sensitive than APCI-MS (Revilla et al., 1999), is extensively used for anthocyanin analysis. It enabled detection of minor anthocyanins and of numerous anthocyanin derivatives and led to propose structures and formation mechanisms for these compounds (cf. 18.2.3). MS can also establish anthocyanin profiles, without prior HPLC separation. MALDI-TOF-MS has been used to identify anthocyanins in plant extracts (Sporns and Wang, 1998; Sugui et al., 1998). Screening by MS/MS neutral loss scanning of precursor ions with elimination mass of 162 (glucoside), 204 (acetylglucoside), or 308 (*p*-coumaroylglucoside), with ESI-triple quadrupole, has detected anthocyanins based on 14 different aglycones in red wine (Hayasaka and Asenstorfer, 2002). ESI-FT-ICR MS (Cooper and Marshall, 2001), ESI- and APCI-TOF (Fulcrand et al., 2008), and MALDI-TOF (Carpentieri et al., 2007) have been applied for wine fingerprinting. The major signals detected in the positive-ion mode could be attributed to anthocyanins, including derived pigments, while the negative-ion mode spectra were more complex. The signal-to-noise ratio was better with

TABLE 18.2

Molecular Weight of Anthocyanidins and Mass Differences Associated to Some Common Substituents

Aglycone (A+)		Common Anthocyanidin Substituents	
Anthocyanin	**Δ Mw in Some Derivatives**	**Sugar (Mw): Δ Mw[a]**	**Acyl Group (Mw): Δ Mw[a]**
Pelargonidin: 271	Methylmethine: +28	Hexose	Acetic acid (60): +42
Cyanidin: 287	Pyrano: +24	Glucose, galactose (180): +162	Oxalic acid (90): +72
Peonidin: 301	Methylpyrano: +38		Malonic acid (104): +86
Delphinidin: 303	Carboxypyrano: +68	Pentose	Succinic acid (118): +100
Petunidin: 317	Vinylpyrano: +50	Arabinose, xylose (150): +132	Malic acid (134): +116
Malvidin: 331	4-hydroxyphenol: +92	Rhamnose (164): +146	*p*-Hydroxybenzoic acid (138): +120
Apigenidin: 255	Catechol: +108		*p*-Coumaric acid (164): +146
Me-apigenidin: 269	Gaiacol: +122		Caffeic acid (180): +162
Luteolinidin: 271	Syringol: +164		Ferulic acid (194): +176
Me-luteolinidin: 285	Catechin: +288		Sinapic acid (224): +206

Note: Fragment ions and neutral loss corresponding to glycosidic/acylglycosidic substituents are diagnostic signals of the aglycone and substituents.

[a] As sugars and acids are linked through glycosidic and ester linkages, respectively, the associated mass gain corresponds to their Mw–18 ($-H_2O$).

APPI-Q-TOF than with ESI-Q-TOF for anthocyanin profiling but some fragmentation took place (Gomez-Ariza et al., 2006). MALDI-TOF (Wang et al., 2000) and ESI-Q-IT MS (Favretto and Flamini, 2000) have also been proposed as rapid methods for quantification of anthocyanins, using the relative abundances of the various M^+ species. Suppression effect, that is, suppression of ionization of some compounds in the presence of others, was considered negligible in the case of anthocyanins that are present as cations in solution. However, this provides only semiquantitative data. More accurate quantification can be obtained by selective ion monitoring, consisting in monitoring selected fragments corresponding to neutral loss of a particular group (e.g., 162 for hexosides), with triple quadrupole mass analyzer.

18.5.4 NMR Spectroscopy

NMR spectroscopy is essential for formal identification of anthocyanins. This involves complete assignment of all proton and carbon signals, on the basis of the chemical shifts and coupling constants in one-dimensional (1D) ^1H and ^{13}C spectra and of correlation cross-peaks in 2D experiments. NMR spectra are usually recorded in deuterated solvents, often deuterated dimethylsufoxide (DMSO-d6) or methanol (CD_3OD), to avoid overlapping of solvent signals with those of the analyte. This has become less of a problem with the development of solvent suppression and of 2D and 3D NMR techniques. Identification is normally performed on the flavylium cation, in acidified solvents, for example, with deuterated trifluoroacetic acid, but NMR can also be used to study the different anthocyanin forms.

NMR strategies start with recording of the proton spectrum (chemical shifts, coupling constants, and integration data for each proton), which gives information on the relative number of hydrogen atoms and on their environment in the molecule. (Proton chemical shifts of anthocyanin aglycones have been listed by Strack and Wray (1989).) Further information is provided by 2D NMR as detailed by Fossen and Andersen (2006). Briefly, correlations between ^1H chemical shifts are given by homonuclear ^1H–^1H experiments. COSY (correlation spectroscopy) and TOCSY (total correlation spectroscopy) show correlations between adjacent protons and between protons belonging to the same spin system, respectively. As the correlation is disrupted by the presence of heteroatoms, TOCSY proves particularly useful for the attribution of overlapping proton signals belonging to different sugars. NOESY (nuclear Overhauser enhancement spectroscopy) and ROESY (rotating frame Overhauser effect spectroscopy) give information on the proximity of protons in space within a molecule or between two molecules. NOESY has been used to study inter- and intramolecular interactions of anthocyanins, while ROESY has enabled identification of several anthocyanins (Fossen and Andersen, 2006) and elucidation of the 8,8 position of the linkage in a methylmethine-linked malvidin 3-*O*-glucoside dimer (Atanasova et al., 2002b). Heteronuclear ^1H ^{13}C 2D heteronuclear single quantum coherence (HSQC) experiments correlate each proton to the carbon to which it is linked. Long-range correlations between protons and carbons separated by more than one bond (usually two ($^2J_{CH}$) or three ($^3J_{CH}$) but sometimes up to 4 or even 5), provided by heteronuclear multiple-bond correlation spectroscopy (HMBC), are especially useful to determine substitution positions. NMR has permitted structural identification of anthocyanins, 3-deoxyanthocyanins, and anthocyanin derivatives, or degradation products but it is not always sufficient to solve a structure. For instance, the structure proposed for vitisin A (Bakker et al., 1997) and the alternative carboxypyranoanthocyanidin structure (Fulcrand et al., 1998) were both compatible with the NMR data. The final argument for the latter was provided by the loss of 44 mass units in MS fragmentation, indicating the presence of a carboxylic group, and confirmed by demonstration of its formation from pyruvic acid.

18.5.5 Infrared, Resonance Raman, and Fluorescence Absorption Spectroscopies

Infrared (IR) and resonance Raman (RR) are two types of vibrational spectroscopy, providing qualitative and quantitative information about the vibrations of the chemical bonds of the molecules. The mid-infrared, 4000–400 cm^{-1} (30–2.5 μm), based on the fundamental stretching and rotating vibrations of molecules, is only of limited use for anthocyanin identification, although methylated anthocyanins can be distinguished on the basis of a characteristic band near 1450 cm^{-1} (Strack and Wray, 1989). RR spectral

characteristics of anthocyanins in acidic medium are mostly related to the vibrational mode of the benzopyrylium ring but anthocyanidins, their 3-monoglycosides, and 3,5-diglycosides can be easily recognized (Merlin et al., 1994). Additional B-ring hydroxyl groups induce slight changes in the RR spectra, while methylation has no effect. Drastic spectral changes are associated with conversion of flavylium to the quinonoidal base, making it possible to distinguish these two forms *in vivo* by microspectrometry (Merlin et al., 1985). Autofluorescence of anthocyanins has not been explored. However, a recent paper (Poustka et al., 2007) has shown that anthocyanins exhibit autofluorescent properties when excited with the helium–neon laser at 543 nm and used this property to detect them in plants.

Rapid noninvasive spectroscopic methods (e.g., near-infrared, fluorescence) are increasingly used for cost-effective characterization of samples, in particular for food authentication and quality control. NIR spectra (around 14,000–4000 cm^{-1}; 2.5–0.8 μm) result from molecular overtone and combination bands, leading to broad bands and complex spectra that render interpretation difficult. However, the use of multivariate statistical techniques (chemometrics) enables extraction of the information hidden in the NIR spectra, and prediction of the anthocyanin content. The strategy consists in recording the spectra of a collection of samples with known anthocyanin contents, developing a calibration model using partial least square or principal component regression, and then testing its robustness and accuracy using a validation set (consisting of samples not included in the calibration set). NIR spectroscopy (often extended to the visible range) is commonly used by the wine industry to measure anthocyanin content in grape berry homogenates (Cozzolino et al., 2006).

New developments aim at increasing sample throughput by measuring the spectra directly on the fruit. Anthocyanin reflectance index (ARI), based on ratios of reflectance in the red band of chlorophyl absorptions and in the green range of spectrum, where both cholorphyll and anthocyanin absorb, have been proposed for quick and nondestructive assessment of leaf anthocyanin content (Gitelson et al., 2001). ARI, used alone or in combination with NIR, was able to predict anthocyanin content in grapevine leaves (Steele et al., 2009). Another method, relying on acquisition of chlorophyll fluorescence (ChlF) using two excitation light bands (green and red) which are differentially screened by anthocyanins, has been proposed to measure anthocyanin in intact plant organs (Kolb et al., 2003; Agati et al., 2005, 2007). The logarithm of the ratio between green- and red-excited ChlF is related to the anthocyanin concentration measured after extraction. Portable devices using this approach have been successfully tested on grape berries and on whole grape clusters (Cerovic et al., 2008).

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19

Isoflavones

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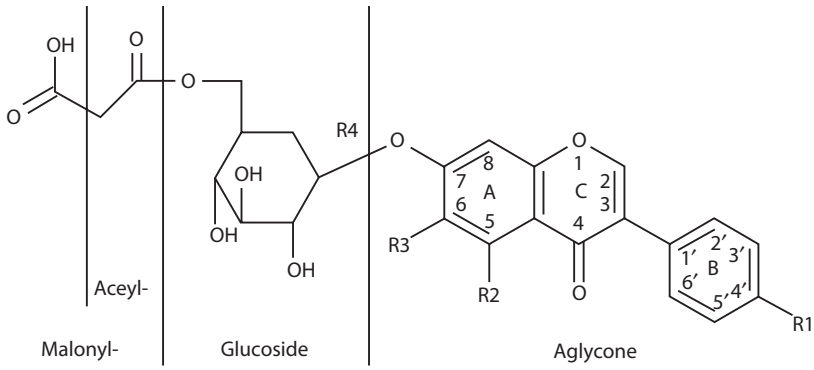
19.1 Introduction

Isoflavones are the largest group of isoflavonoids, a distinctive subclass of flavonoids (Figure 19.1). By 2007, ~1600 isoflavonoids had been characterized and described (Tahara, 2007). Depending on their substitution patterns, they can be divided into three different groups: those with simple *O*-substitutions (e.g., hydroxy or methoxy groups), prenylated forms, and glucosides (Veitch, 2007). The main structural difference between isoflavonoids and flavonoids is the position of the B-ring, which is attached at the 3-position instead of the 2-position. The 3-phenylchroman skeleton of these compounds is derived by rearrangement of 2-phenylchroman (the skeleton of flavonoids) by 1,2-aryl rearrangement (Dewick, 1993). This rearrangement is catalyzed by an enzyme commonly referred to as isoflavone synthase (Tahara and Ibrahim, 1995), although this is a term of convenience describing a sequence of reactions involving 2-hydroxyisoflavone synthase (2HIS, a microsomal cytochrome P450 of the CYP93C subfamily) and 2-hydroxyisoflavone dehydrase (HID or 2HID) (Veitch, 2009). In the first 2HIS-catalyzed step, a hydrogen is abstracted from C-3 by heme-bound oxygen in an NADPH-dependent reaction, followed



R1	*R2*	*R3*	*R4*	*Name*
OMe	OH	H	H	Pallidiflorin
OH	H	H	OH	Daidzein
OMe	H	H	OH	Formononetin
OR	H	H	OH	Nordurlettone
OMe	OH	H	OH	Biochanin A
OH	H	OMe	OH	Glycitein
OH	OH	H	OH	Genistein
OH	H	H	Glucosyl	Daidzin
OH	H	OMe	Glucosyl	Glycitin
OH	OH	H	Glucosyl	Genistin

FIGURE 19.1 Structure of selected isoflavones.

by the migration of the aryl group from C-2 to C-3, and the rebinding of the hydroxyl radical to the carbon radical at C-2 (Hashim et al., 1990). This reaction is stereoselective, using only 2S-flavanones as substrate (Dixon, 2004), and requires a 2′- or 4′-hydroxyl group (Bhandari et al., 1992). The dehydration step from the 2-hydroxyisoflavone is less well understood, although Akashi et al. (2005) suggested a mechanism in which amino acid residues of a catalytic triad and an oxyanion hole eliminate water cooperatively from the 2-position.

Most isoflavones originate from two flavanone precursors, naringenin (5,7,4′-trihydroxyflavanone) and liquiritigenin (7,4′-dihydroxyflavone), that are ubiquitous in plants (Dixon, 2004). These are converted mainly into the isoflavones genistein (5,7,4′-trihydroxyisoflavone) and daidzein (7,4′-dihydroxyisoflavone), respectively, which then undergo further modification, in particular hydroxylation at the C-2′ and C-3′ position of the B-ring, carried out by microsomal enzymes of the CYP81E subfamily (Veitch, 2009), and 4′-*O*-methylation by isoflavone 4′-*O*-methyltransferase (Wengenmayer et al., 1974) at the 2-hydroxyisoflavone level (Veitch, 2009). The precursor of isoflavones with a 6,7,4′-hydroxylation pattern such as glycitein (7,4′-dihydroxy-6-methoxyisoflavone) and afrormosin (7-hydroxy-6,4′-dimethoxyisoflavone) is 6,7,4′-trihydroxy-flavone, which is formed from liquiritigenin by flavonoid-6-hydroxylase (Latunde-Dada et al., 2001).

Further modifications that isoflavones undergo are prenylation, glucosylation, and malonylation. Isoprenoid substituents are generally added after the basic skeletons have been partly or completely constructed (Tahara and Ibrahim, 1995). Several prenyltransferases have been identified, for example, from *Lupus albus* roots, which introduces a dimethylallyl group into positions 6, 8, and 3′ of genistein and 2′-hydroxygenistein (Laflamme et al., 1993). Glucosylation is catalyzed by glycosyltransferases, which transfer nucleotide-diphosphate-activated sugars (often UDP-glucose) to a broad range of targets (Vogt and Jones, 2000; Noguchi et al., 2007). Several isoflavone glucosides are also malonylated at the glucose residue, a reaction catalyzed by isoflavonen-7-*O*-glucoside-*O*-malonyltransferase (Suzuki et al., 2007). A summary of the biosynthetic pathway is shown in Figure 19.2.

Isoflavonoids are mainly found in Leguminosae (Fabaceae), particularly in Papilionoideae (Faboideae) (Hegnauer and Gpayer-Barkmeijer, 1993; Veitch, 2009), although they are found in other plants as well (Reynaud et al., 2005). As a result of diet and gastrointestinal metabolism, isoflavones can also be found in animal products such as meat, eggs, or dairy (Kuhnle et al., 2008a). They have an important role as signal molecules in plant–microorganism interactions (Dixon and Steele, 1999): the isoflavonoid

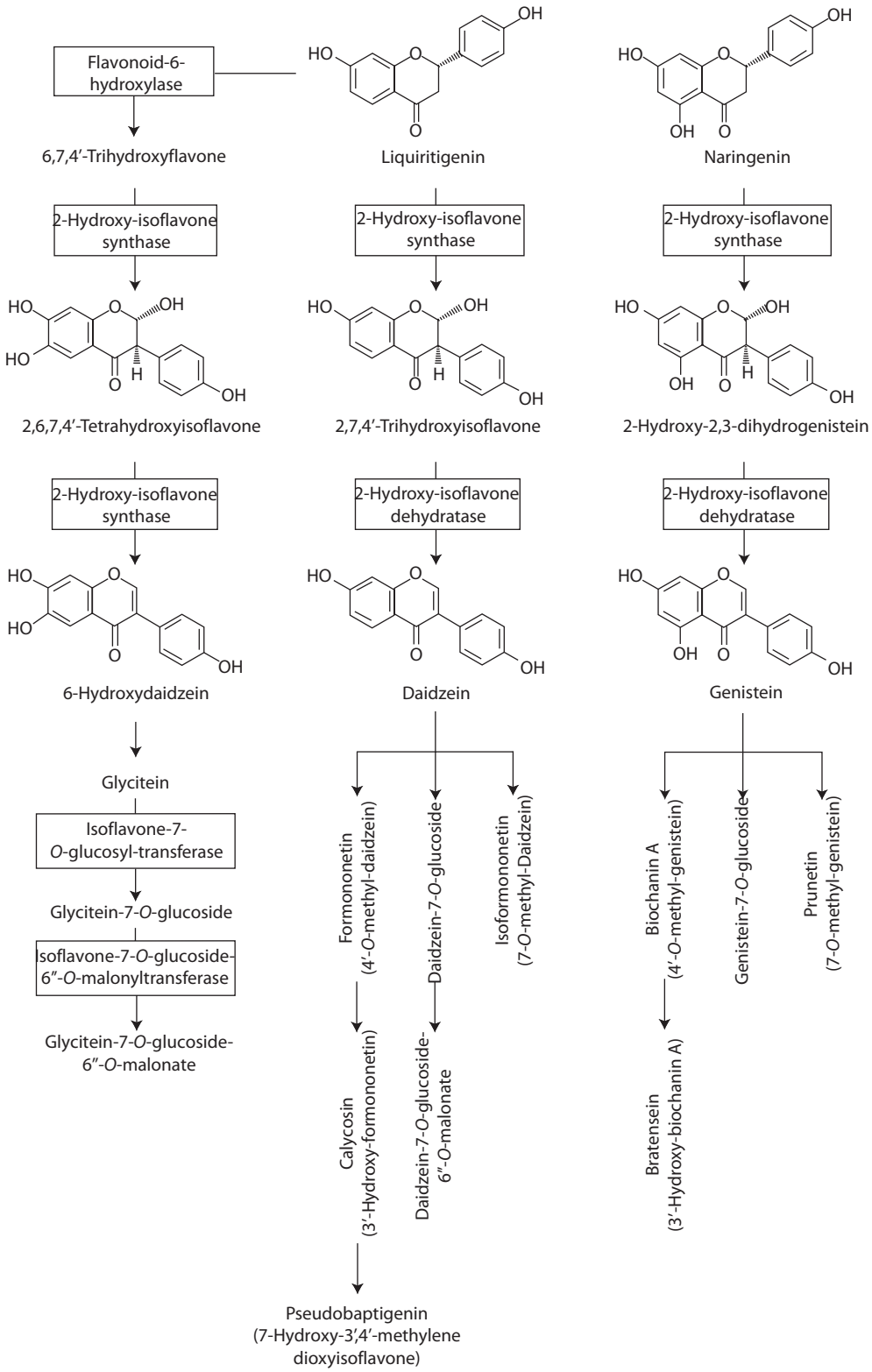


FIGURE 19.2 Biosynthetic pathway of isoflavones.

pterocarpans act as phytoalexins in the interaction between garden peas and fungal pathogens (Dixon and Steele, 1999); however, the isoflavones daidzein and genistein stimulate chemotropic behavior in *Phytophthora sojae*, suggesting that they might help the hyphal tips of zoospores to locate their host (Morris et al., 1998). Furthermore, isoflavones induce the *nod* gene in rhizobia, which is responsible for legume–rhizobial nodulation (Subramanian et al., 2007). Isoflavones are also found in nonlegumes; one of the first isoflavones obtained from a natural source was iridin from *Iris florentina* (de Laire and Tiemann, 1893), which is not a legume (Lapcik, 2007) and in contrast to legumes, a monocot. In 1910, prunetin was isolated from the bark of *Prunus* species (Finnemore, 1910). In monocotyledons, isoflavones were only found in six families, mainly in Iridaceae, in particular the genus *Iris*, but also in other monocots such as Liliaceae (Reynaud et al., 2005). In nonlegume dicotyledons, isoflavones are mainly found in five plant families, Asteraceae, Chenopodiaceae, Nyctaginaceae, Moraceae, and Ochnaceae (Reynaud et al., 2005), although they are also found in other plants. Nonleguminous isoflavones are often methylated or prenylated, or contain methylenedioxy groups (Harborne and Baxter, 1999); a more comprehensive list of isoflavones can be found, for example, in Dewick (1994).

Isoflavones show a wide range of biological activity in humans and animals. They are best known for their estrogenic activity (see Section 19.1.1), but pharmacological studies have also shown other effects; for example, the prenylated isoflavone scanderone from *Derris scandens* has mild hypertensive activity (Mahabusarakam et al., 2004) and kraussianone 1 and 2 (*Eriosema kraussianum*) have hypoglycemic activity (Ojewole et al., 2006). They can also act as inhibitors of enzymes such as tyrosine kinase (Akiyama et al., 1987) and DNA topoisomerase (Markovits et al., 1989).

19.1.1 Estrogenic Activities of Isoflavones

Isoflavones with estrogenic activity are often referred to as *phytoestrogens*. These compounds can mimic or modulate the activity of estrogen, often by binding to its receptors (Committee on Toxicity of Chemicals in Food, 2003). The bioactivity is based on their structural similarity with 17β-estradiol and their ability to bind to the α and β estrogen receptor (ERα and ERβ) (Shutt and Cox, 1972) (Martin et al., 1978; Verdeal et al., 1980; Setchell and Adlercreutz, 1988; Branham et al., 2002); important structural features to bind the receptor are an aromatic ring and a hydroxyl group (Anstead et al., 1997) and the structure of phytoestrogens with a *p*-hydroxy-substituted aromatic ring, 12 Å (1.2 nm) away from a second planar hydroxyl group that resembles the structure of estradiol. In contrast to 17β-estradiol, phytoestrogens have different affinities for the estrogen receptors (ERα and ERβ), have a much lower estrogenic activity (10^{-2}–10^{-3} compared with 17β-estradiol), and fail to stimulate a full estrogenic response (Tang and Adams, 1980; Miksicek, 1994; Santell et al., 1997; Zava and Duwe, 1997; Kuiper et al., 1998; Tham et al., 1998). In addition to their interaction with estrogen receptors, these compounds can also act as inhibitors of enzymes such as tyrosine kinase (Akiyama et al., 1987) and DNA topoisomerase (Markovits et al., 1989).

The estrogenic effect of these compounds has first been observed in Western Australia when sheep grazing on clover-rich pastures developed the so-called clover disease (Bennetts et al., 1946), which included in ewes low lambing rates, prolapse of the uterus and dystocia, and enlargement of the bulbourethral glands and death in wethers (Adams, 1995). The phytoestrogen content of fodder has also affected other animals, for example, cattle (Moule et al., 1963; Mirocha et al., 1968, 1974), guinea pigs (Bradbury and White, 1954; Moule et al., 1963), rabbits (Wright, 1960), mice (Leavitt and Wright, 1963), and cheetahs (Setchell et al., 1987), and production of phytoestrogen-low fodder has been a priority for some time (Dixon, 2004). Phytoestrogens affect the fertility of birds too, and it has been hypothesized that the California quail can control its fertility in times of food shortage by increasing the intake of legumes (Leopold et al., 1976). Despite these adverse effects in animals, similar effects have not been observed in humans on a phytoestrogen-rich diet (Petrakis et al., 1996) and it is the antiestrogenic activity that raises the possibility that they are protective in hormone-related diseases (Bingham et al., 1998), for example, breast, colorectal, or prostate cancer. Epidemiological studies conducted in populations with high habitual intake of phytoestrogens showed a protective effect for breast cancer (Wu et al., 2008), whereas studies conducted in Europe (Keinan-Boker et al., 2004; Touillaud et al., 2006; Ward et al., 2010; Ward and Kuhnle, 2010) or the United States (Horn-Ross et al., 2001) failed to find a significant effect.

19.2 Analytical Methods

19.2.1 Sampling

The isoflavone content of foods is very variable and depends on a number of different factors, for example, plant variety, growth conditions, harvesting, processing, and storage (Eldridge and Kwolek, 1983; Wang and Murphy, 1994; Kuhnle et al., 2009c); the isoflavone concentration is affected more by the environment than by the genotype of the plant (Kim et al., 2005). A previous study has shown a threefold variability in isoflavone content in foods commonly consumed in the United Kingdom (Kuhnle et al., 2009c), with up to 70-fold differences for glycitein in one type of food. For processed foods, differences in the formulation can also affect the amount of isoflavones and lignans present; in the United Kingdom, most breads are prepared using the *Chorleywood Bread Process* (Chamberlain et al., 1966), which uses soy enzymes. Therefore, U.K. nonspecialty breads contain up to 1 mg/100 g isoflavones (mean content 450 μg/100 g) (Kuhnle et al., 2009a,b), whereas the isoflavone content of U.S. white bread is significantly lower (190 μg/100 g) (U.S. Department of Agriculture, 2002). Similarly, the isoflavone content of baked beans varies up to fivefold in baked beans from different manufacturers (Kuhnle et al., 2009c). For the analysis of isoflavones and lignans in food, an appropriate sampling strategy is therefore important to ensure that the results are representative.

19.2.2 Sample Storage

The preservation of native isoflavones and lignans is important for the reliable analysis of these compounds in food. Degradation—in particular deglucosylation, decarboxylation, and deacetylation—can occur very soon after harvesting and thereby affecting the results in two ways: by altering the total concentration of individual compounds and by changing the profile of isoflavones. In a detailed study, Kim et al. (2005) investigated the effect of cultivar and storage conditions on the degradation of isoflavones in soybeans and found a significant effect of storage condition and plant variety on the stability. In most cases, the concentration of aglucones and glucosides is likely to increase whereas the concentration of malonyl-glucosides decreases. Indeed, the concentration of isoflavone aglucones and glucosides increased two- to threefold, whereas the concentration of malonyl-glucosides decreased approximately twofold; likewise, the concentration of individual isoflavones changed significantly during storage, although not in the same direction (Lee et al., 2003). In addition to enzymatic hydrolysis of acetyl, malonyl, or carbohydrate moieties, free hydroxy groups can scavenge reactive oxygen species and thereby become oxidized. For studies comparing the isoflavone and lignan content of different foods, it is, therefore, important to take the degradation of these compounds into consideration.

Immediate extraction and storage of the extracts instead of the whole food does not result in a better preservation of isoflavones. Even in 80% methanol, isoflavones can still be decarboxylated from malonyl-glucosides to glucosides (de Rijke et al., 2001). For the analysis of glucosides and in particular malonyl-glucosides, it is therefore necessary to store samples carefully, ideally frozen or at low temperatures, to reduce the amount of degradation. To preserve glucosides from hydrolysis by native β-glucosidases, *tris*-(hydroxymethyl)-aminomethane can be added as inhibitor (Delmonte and Reader, 2006).

19.2.3 Analyte Isolation

Different methods for the extraction of isoflavones from soy-containing foods have been reviewed recently in great detail by Rostagno et al. (2009); Table 19.1 gives an overview of different extraction methods used. The sample extraction is the most important step in the analysis of isoflavones from foods as it can alter the content of free and modified compounds. The choice of the most appropriate method depends on the objective of the analysis: a complete extraction for the identification of all compounds present or a targeted extraction to analyze only particular compounds. Ideally, the extraction method should remove all compounds interfering with analysis; however, in reality this is often not possible. Historically, Soxleth solvent extraction (von Soxleth, 1879) was commonly used to extract natural compounds such as isoflavones and lignans. More recently, two-phase solvent systems, counterflow chromatographic methods

TABLE 19.1

Comparison of Different Extraction Methods

Sample	Isoflavones	Compared Techniques/ Methods	Relative Yield (%)[a]	Reference
Soy flour	Gi, Ge, and De	SFE/UAE/Soxhlet	28/100/68	Rostagno et al. (2002)
Soybean cake	Di, Gi, Gly, De, Ge, Gle, MDi, MGi, MGly, ADi, AGi, and AGly	SFE/shaking	74/100	Araújo et al. (2007)
Soybean hypocotyls	De and Ge	SFE/stirring	26/100	Franke et al. (1994)
Soybean meal	Di, Gi, De, and Ge	SFE/stirring	87/100	Zuo et al. (2008)
Soybeans	Di, Gi, Gly, and MGi	UAE/stirring	100/85–100[b]	Rostagno et al. (2003)
Soybeans	Di, Gi, Gly, De, Ge, Gle, MDi, MGi, MGly, ADi, AGi, and AGly	PLE/UAE/Soxhlet/ shaker/vortex/stirring	100/93/68/71/66/70	Luthria et al. (2007)
Soy bits	Di, Gi, De, and Ge	PLE/UAE/Soxhlet/ PLE + UAE	49/14/64/100	Klejdus et al. (2004)
Soy bits	Di, Gi, Gly, Ononin, De, Gle, and Ge	UAE/Soxhlet/PLE + UAE	22/68/100	Setchell and Cole (2003)
Soy flour, meat substitute, nuts, and protein isolate	Gi, MGi, Agi, and Ge	PLE/stirring	98–100/88–100[c]	Twaddle et al. (2002)
Soy flour	De and Ge	UAE/UHOM/SFE/PLE/ Soxhlet	100/93/16/71/69	Bajer et al. (2007)
Soy flour	Di, Gi, Gly, De, Ge, Gle, MDi, MGi, MGly, ADi, AGi, and AGly	MAE/UAE	100/100	Liggins et al. (1998)

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Note: De: daidzein, Ge: genistein, Gle: glycitein, Di: daidzin, Gi: genistin, Gly: glycitin, MDi: malonyl daidzin, MGi: malonyl genistin, MGly: malonyl glycitin, ADi: acetyl daidzin, AGi: acetyl genistin, AGly: acetyl glycitin, UHOM: ultrasonic homogenizer.

[a] Relative to the technique that extracted the highest amount of total isoflavones.

[b] Depending on the solvent used.

[c] Depending on the sample used.

(Yang et al., 2001), ultrasound-assisted methods (Rostagno et al., 2003), and supercritical carbon dioxide extraction methods (Rostagno et al., 2002) have been introduced to supplement existing techniques. Optimal extraction from foods will require intimate contact between the extraction solvent and the solid food. Freeze-drying and grinding is, therefore, often the first step in the isolation process.

The freeze-drying step can be applied to liquid samples such as soy milk as well, but also milk, beverages, and oil as well. However, liquid samples can also be extracted directly, and most authors use methanol or ethanol as the extraction solvent (Rostagno et al., 2009). In some studies, liquids were used directly and only diluted with buffer to ensure optimal conditions for enzymatic hydrolysis and solid-phase extraction (Kuhnle et al., 2008b).

19.2.3.1 Solvent Extraction

The choice of the most appropriate extraction solvent depends on the solubility of the analyte; this is often difficult to achieve due to the different chemical forms isoflavones can be found in (Rostagno et al., 2009). The optimal extraction solvent will therefore depend not only on the nature of the food, but also

the expected compounds. For this reason, experiments to develop the most appropriate extraction solvent will often be necessary prior to analysis.

In an early study of isoflavones in soy, Eldridge investigated several different solvents (50%, 80% and absolute ethanol and methanol, ethyl acetate, and acetonitrile) for isoflavone extraction by refluxing and found 80% methanol to be the most suitable (Eldridge, 1982). In a similar study, Murphy found that absolute solvents result in poor extraction efficiency and investigated the effect of water and acidification on isoflavone extraction, showing that acidified acetonitrile provides the best recovery (Murphy, 1981). Since then, 80% methanol and acidified acetonitrile (83%; 10 mL acetonitrile with 2 mL 0.1 M HCl) have been commonly used as solvents for the extraction of isoflavones from plant material; the latter method can be improved by increasing the water content (Murphy et al., 1999). However, more recent results showed that acidification is often unnecessary and can result in significantly lower yields for malonyl daidzin, malonyl genistin, and malonyl glycitin; β-glucosides and aglucones were less affected by acidification, although a conversion of glucosides into aglucones could still be observed (Lin and Giusti, 2005). In contrast to this, the U.S. Department of Agriculture–Iowa State University Isoflavone Database recommends the addition of acid for isoflavone extraction (U.S. Department of Agriculture, 2002). In many cases, acidification can be omitted to simplify the extraction method, although this should be assessed for each food separately.

The choice of the extraction solvent also depends on the hydrophobicity of the target compound. For isoflavones, the order of hydrophobicity is aglucon > acetyl-β-glucoside > malonyl-β-glucoside > β-glucoside based on their behavior in a reversed-phase chromatographic system with an acidic mobile phase, capable of protonating the malonyl form (Murphy et al., 1999, 2002). In two detailed studies of isoflavone extraction yield from different soy foods (Murphy et al., 2002; Lin and Giusti, 2005), acetonitrile was found to be the most efficient solvent (Table 19.2). In a recently published method, DMSO has been added to the extraction mixture (10 mL acetonitrile, 5 mL water, 0.5 mL DMSO), which improved extraction slightly (Griffith and Collison, 2001) although the authors did not investigate this further. The optimum acetonitrile concentration for the extraction of malonyl-glucosides, glucosides, and acetyl-glucosides is 60% although an acceptable extraction yield can be achieved at lower concentrations as well. Almost complete extraction was achieved after only 5 min (Griffith and Collison, 2001). For the analysis of aglucones only, where the instability of malonyl-glucosides, acetyl-glucosides, and glucosides is not important, slightly acidic aqueous methanol (10%, pH 5) can be used as the extraction solvent (Kuhnle et al., 2007). Although in most methods water was found to improve the extraction yield, Zhang et al. found the opposite for the isolation of isoflavones from soybeans; in this study, the highest extraction yield was obtained with 99.9% ethanol (Zhang et al., 2007). The only method validated by the AOAC (Method 2001.10) uses 80% methanol as the extraction solvent to extract isoflavones in a 60°C water bath for 2 h (Delmonte and Reader, 2006).

Table 19.3 gives an overview of extraction methods used for the analysis of isoflavones from soy foods. However, it is difficult to compare these methods because the studies had different objectives and focused on different isoflavones. The selection of the most appropriate extraction solvent will depend on several factors, for example, the nature of the sample, the concentration of isoflavones, and whether the objective is to analyze total isoflavone content or individual compounds.

19.2.3.2 Ultrasound-Assisted Methods

Ultrasound-assisted extractions have been used in some studies to improve extraction of isoflavones from their food matrices (Kuhnle et al., 2007). Ultrasound assists the extraction by allowing greater penetration of the solvent into the matrix and by improving mixing. However, ultrasound can also result in the formation of reactive hydroxyl radicals, which can result in a degradation of the target compounds (Paniwnyk et al., 2001). In a recent study, an extraction with 50% aqueous solvent (ethanol, acetonitrile, or methanol) at 60°C and 10 min sonication yielded the best results (Rostagno et al., 2003). Automated extraction methods are also used, for example, sonication-assisted accelerated solvent extraction (S/ASE) with 90% methanol at 145°C and 14 MPa, which resulted in an almost quantitative extraction of isoflavones from soy (Klejdus et al., 2005).

TABLE 19.2

Relative Extraction Yields (Acetonitrile = 1; ± Standard Error) of Different Isoflavones

	83% MeCN[a]	83% MeCN (Acidified)[a]	53% MeCN[b]	53% MeCN (Acidified)[a]	53% Acetone[b]	53% EtOH[b]	80% MeOH[a]	80% MeOH[a] (Acidified)	53% MeOH[b]
Glucosides	0.8 ± 0.09	0.7 ± 0.12	1	0.9 ± 0.09	1.1 ± 0.15	1.1 ± 0.15	0.9 ± 0.04	0.9 ± 0.06	1.1 ± 0.16
Malonyl-glucosides	0.7 ± 0.04	0.6 ± 0.06	1	0.7 ± 0.04	0.9 ± 0.07	0.9 ± 0.02	0.9 ± 0.02	0.8 ± 0.02	0.9 ± 0.03
Acetyl-glucosides			1		0.8 ± 0.14	1.0 ± 0.15			0.8 ± 0.20
Aglucons			1		0.9 ± 0.07	0.9 ± 0.07			0.9 ± 0.11
Daidzein			1		1.0 ± 0.03	1.0 ± 0.02			0.9 ± 0.04
Genistein			1		1.0 ± 0.03	0.9 ± 0.03			0.9 ± 0.05
Glycitein			1		1.0 ± 0.06	0.9 ± 0.05			0.9 ± 0.07
Total			1		1.0 ± 0.03	0.9 ± 0.02			0.9 ± 0.05

Source: Data adapted from Lin, F. and Giusti, M. M. 2005. *Journal of Agricultural and Food Chemistry*, 53(10), 3795–3800; Murphy, P. A., Barua, K., and Hauck, C. C. 2002. *Journal of Chromatography* B, 777(1–2), 129–138.

Note: Solvents were acidified by adding 2.0 mL 0.1 M HCl to 10 mL solvent.

[a] From manokin soybeans using acetonitrile (MeCN), acetone, methanol, and ethanol.

[b] From soy foods [raw soy flour, tempeh, tofu, textured vegetable protein (TVP), and soy germ].

TABLE 19.3

Conventional Methods for the Extraction of Isoflavones from Soybeans and Soy Foods

Food	Isoflavones	Fixed Extraction Conditions	Evaluated Parameters	Selected Conditions	Reference
Defatted soybeans	Di, Gi, Gly, De, Ge, and Gle	Technique: refluxing Sample: 1 g Solvent: 25 mL Temperature: boiling point of solvent	Solvent: MeOH, 50% MeOH, 80% MeOH EtOH, 50% EtOH, 80% EtOH, MeCN, ethyl acetate Extraction time: 1–5 h	80% MeOH, 4 h	Barnes et al. (1994)
Toasted defatted soy flakes	Gi, Ge, Di, and De	Technique: Wrist-action shaker Sample: 5 g Solvent: 25 mL of pure solvent or: 5 mL (H_2O or HCl 0.1N) + 20 mL (solvent) Temperature: RT Extraction time: 2 h	Solvent: MeOH, 80% MeOH, 80% MeOH, chloroform–MeOH (90:10), 80% chloroform–MeOH (90:10), 80% chloroform–MeOH (90:10), MeCN, 80% MeCN, 80% MeCN (acidified), acetone, 80% acetone, 80% acetone (acidified)	80% MeCN and 80% MeCN (acidified)	Murphy (1981)
Soy isolate, tofu, soybeans, and miso	Ge, De, Gi, Di, Gly, MGi, MDi, MGly, and AGi	Technique: Stirring Sample: 2 g, Solvent: 12–22 mL (12 mL MECN + 2 mL HCl 0.1N + water) Extraction time: 2 h Temperature: RT	Solvent: Different amounts of water (0–10 mL) added to the solvent (MeCN)	The amount of water optimized depending of the sample ranged from 5 to 10 mL of water	Murphy et al. (1999)
Toasted soy flour	Di, Gi, Gly, De, Ge, Gle, MDi, ADi, MGi, Agi, and MGly	Technique: tumbling mixer Sample: 0.5 g Solvent: 4 mL	Solvent: 80% MeOH and 80% MeCN (0.1% HCl) Extraction time: 1, 2, and 24 h Temperature: RT, 60°C, and 80°C	1 h, RT	Grun et al. (2001)
Soy protein	Di, Gi, Gly, De, Ge, Gle, MDi, MGi, MGly, ADi, AGi, and AGly	Technique: Rotary mixer Sample: 1 g or amount containing 10 mg total isoflavones (always < 1 g) Solvent: ~17 mL Extraction time: 2 h Temperature: RT	Solvent: 10 mL MeCN + 6 mL water + 0.5 mL DMSO (IS), 10 mL MeCN + 2 mL HCl 0.1 M + 5 mL water, 80% MeOH water % (10–100% MeCN)	10 mL MeCN + 6 mL water + 0.5 mL DMSO (IS)	Griffith and Collison (2001)
Soy flour, tempeh, TVP, and soy germ	Di, Gi, Gly, De, Ge, Gle, MDi, MGi, MGly, ADi, AGi, and AGly	Technique: Stirring Sample: 2 g Solvent: 19 mL (10 mL solvent + 2 mL (HCl 0.1N or water) + 7 mL water) Extraction time: 2 h Temperature: RT	Solvent: 53% MeCN, 53% ACE, 53% EtOH, 53% MeOH With and without acid addition	53% MeCN without acidification	Murphy et al. (2002)

continued

TABLE 19.3 (continued)

Conventional Methods for the Extraction of Isoflavones from Soybeans and Soy Foods

Food	Isoflavones	Fixed Extraction Conditions	Evaluated Parameters	Selected Conditions	Reference
Soybeans	Di, Gi, Gly, De, Ge, Gle, MDi, MGi, MGly, ADi, AGi, and AGly	Technique: Stirring Sample: 2 g Solvent: 12 mL Extraction time: 2 h Temperature: RT	Solvent: 83% MeCN, 83% MeCN (+0.1 N HCl) 58% MeCN, 58% MeCN (+0.1 N HCl), 80% MeOH, 80% MeOH (+0.1 N HCl)	58% MeCN without acidification	Lin and Giusti (2005)
Freeze-dried soybeans	Di, Gi, Gly, and MGi	Technique: Stirring Sample: 0.5 g Solvent: 25 mL Extraction time: 10 min	Solvent: MeCN (30–70%), EtOH (30–70%), MeOH (30–70%) Temperature: 10°C and 60°C	50% EtOH, 60°C	Rostagno et al. (2003)
Defatted soybean meal, soy protein isolate	Di, Gi, Gly, De, Ge, Gle, MDia, MGia, and MGlya	Technique: Shaking Sample: 2 g Solvent: 10 mL Extraction time: 2 h Temperature: RT	Solvent: 80% MeCN–HCl 0.1 N, 80% MeOH, 80% EtOH Number of extractions: 1 and 5	80% MeCN–HCl 0.1 N, 5 sequential extractions	Achouri et al. (2005)
Soybean flour	Di, Gi, Gly, MDi, MGly, MGi, De, and Ge	Technique: Homogenization probe and hand agitation Sample: 0.1 g Solvent: 4 mL (80% MeOH) (homogenization) + 1 mL (agitation) Extraction time: 1 min (homogenization) + 30 min (agitation) Temperature: RT (homogenization) and 70°C (agitation)	Proposed method and reference method (modified Murphy method)	Proposed method	Tsai et al. (2007)
Soybean flour	Ge and De	Technique: Stirring Solvent: 4 mL (80% MeOH) (homogenization) + 1 mL (agitation) Extraction time: 1 min (homogenization) + 30 min (agitation) Temperature: RT (homogenization) and 70°C (agitation)	Solvent: 40–99.99% EtOH Volume: sample ratio: 1:1 to 10:1 (mL/g) Temperature: 40–90°C Extraction time: 2–24 h	99.99% EtOH, 3:1 mL/g, 80°C and 8 h	Zhang et al. (2007)

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Note: ADi: acetyl daidzin, AGi: acetyl genistin, AGly: acetyl glycitin, De: daidzein, Di: daidzin, EtOH: ethanol, Ge: genistein, Gi: genistin, Gle: glycitein, Gly: glycitin, MDi: malonyl daidzin, MeCN: acetonitrile, MeOH: methanol, MGi: malonyl genistin, MGly: malonyl glycitin, RT: room temperature.

19.2.3.3 Microwave-Assisted Methods

Microwave-assisted extraction (MAE) is based on the rapid localized heating of moisture in the sample by microwaves and the interaction of microwaves and polar compounds. The efficiency of MAE depends on several factors, for example, sample material, compounds to be extracted, and in particular the dielectric constant of the solvent. With increasing dielectric constant, more energy is absorbed and the temperature of the solvent increases faster. MAE can provide very good extraction yields: almost quantitative extraction with no degradation of isoflavones could be achieved using a 20 min extraction method with 50% ethanol as solvent at 50°C and 500 W microwave setting, although most isoflavones were extracted already after 10 min (Rostagno et al., 2007). Despite the successful quantitative extraction of isoflavones using this technique, its application is still limited due to the potential degradation of malonyl- and acetyl-glucosides with high temperatures or long exposure to microwave radiation. Yet this might be very useful for the development of hydrolytic method in which the total content of particular isoflavones as aglucones is to be determined.

19.2.3.4 Supercritical Fluid Extraction

Supercritical fluids are substances at a temperature and pressure above their critical point. These fluids can penetrate sample material almost as good as gases due to their viscosity and diffusion coefficients, but retain the dissolving power of liquids (Rostagno et al., 2009). Supercritical fluid extraction has become a very important technique for the analysis of environmental samples, for example, foods (Barnabas and Dean, 1994). Liquid carbon dioxide is the most commonly used liquid, in particular because of its low cost and toxicity, and its ability to dissolve nonpolar compounds. Modifiers, such as methanol, can be used to improve extraction efficiency—in particular of polar compounds—and to overcome interactions between analyte and matrix (Björklund et al., 1998). Supercritical fluid extraction is one of the most complicated methods for isoflavone extraction from foods because of the many factors affecting the outcome. In one study, the extraction of genistein and genistin was affected mainly by temperature, whereas daidzein was affected by pressure (Rostagno et al., 2002). For this reason, it is important to optimize all aspects of the extraction method using simplified model systems (Björklund et al., 1998). An overview of the different methods used for the extraction of isoflavones from soy food using supercritical fluids is shown in Table 19.4. Although supercritical fluid extraction requires high temperatures, thus far there are no studies into the stability of isoflavones under these conditions. Using high amounts of modifier (70% EtOH) and a pressure of 35 MPa, the highest extraction yield for malonyl-glucosides and glucosides was found with a temperature of 60°C, whereas the highest yield for acetyl-glucosides and aglucones was found at 80°C, suggesting that malonyl-glucosides are unstable at higher temperatures (Kao et al., 2008). Furthermore, an increase in extraction temperature does not necessarily increase extraction efficiency as higher temperatures decrease the density of supercritical CO_2 and thus its extraction power (Rostagno et al., 2009).

19.2.3.5 Pressurized Liquid Extraction

Pressurized liquid extraction (PLE), also known as accelerated solvent extraction (ASE) or pressurized solvent extraction (PSE), combines high temperatures and pressures to achieve rapid and efficient extraction. In this technique, the sample is enclosed in a cartridge with a solvent under high pressure (3–21 MPa; 500–3000 psi) and temperature (50–200°C) for short time periods (5–10 min) and the extract is purged into a collection vessel using compressed gas (Richter et al., 1996). High temperatures can improve the extraction yield by increasing analyte solubility, whereas the high pressure ensures that the solvent remains liquid. However, high temperatures may also adversely affect the stability of the samples (see below). Table 19.5 shows an overview of different studies investigating different extraction parameters.

TABLE 19.4

Supercritical Fluid Methods for the Extraction of Isoflavones from Soybeans and Soy Foods

Food	Isoflavones	Fixed Extraction Conditions	Evaluated Parameters	Selected Conditions	Reference
Standards	De and Ge	Extraction cell: n.e. Temperature: 50°C Flow rate: 950–1000 mL/min Extraction time: 60 min Restrictor temperature: 175°C Rinse solvent: none	Extraction conditions: 41 MPa and no modifier 41 MPa and 5% chloroform 41 MPa and 5% MeOH 61 MPa and 20% MeOH 61 MPa and 20% EtOH	61 MPa and 20% EtOH	Chandra and Nair (1996)
Freeze-dried soybeans	Gi, Ge, and De	Sample amount: 1 g Extraction cell: 7.0 mL (reduced to 5.46 mL) Inert material: glass stick Modifier: 70% MeOH Static cycle length: 10 min	Modifier concentration: 0.5 and 10 mol%[a]	50°C/36 MPa, 10 mol% (TIS and De) 70°C/20 MPa, 10 mol% (Gi and Ge)	Rostagno et al. (2002)
		Dynamic cycle length: 20 min CO_2 flow rate: 1.0 mL/min	Temperature: 40–70°C Pressure: 20–36 MPa		
		Extraction time: 90 min (3 × 30 min) Trap: ODS Rinse solvent: 1.5 mL MeOH Rinse flow rate: 0.5 mL/min			
Soybean cake	Di, Gi, Gly, De, Ge, Gle, MDi, ADi, MGi, AGi, and MGly	Sample amount: 1 g Extraction cell: 10 mL Modifier: 70% EtOH Modifier concentration: 10 mol%[a] Static cycle length: 10 min Dynamic cycle length: 20 min CO_2 flow rate: 1.0 mL/min Extraction time: 90 min (3× 30 min) Fluxing solvent: 5 mL 50% EtOH	Temperature: 50–80°C Pressure: 30–40 MPa	Malonyl-glucosides, glucosides, and TIS: 60°C/35 MPa Acetyl-glucosides and aglucones: 80°C/35 MPa	Araújo et al. (2007)
Soybean hypocotyls	De and Ge	Sample amount: 0.08 g Extraction cell: 7.0 mL (reduced to 5.46 mL) Inert material: glass stick Modifier: 70% MeOH Static cycle length: 15 min	Modifier: MeOH, EtOH, and MECN Modifier concentration: 0.5 and 10 mol%[a]	60°C/380 bar, 10 mol% 80% MECN	Franke et al. (1994)

		Dynamic cycle length: 15 min CO_2 flow rate: 1.5 mL/min Extraction time: 90 min (3 × 30 min) Rinse solvent: 1.5 mL 80% MeOH Rinse flow rate: 0.5 mL/min	Temperature: 50–70°C Pressure: 18–36 MPa		
Soybean flour	De and Ge	Sample amount: 0.3 g Inert material: glass beads	Pressure: 15–40 MPa Temperature: 10–100°C Extraction time: 10–50 min Restrictor diameter: 25 and 50 µm	35 MPa, 70°C, 5% MeOH, 30 min, 50 µm	Bajer et al. (2007)
Soybean meal	Di, Gi, De, and Ge	Sample amount: 100 g Extraction cell: 1 L Separator 1: 8 ± 0.3 MPa (40°C) Separator 2: 6 ± 0.3 MPa (30°C)	Temperature: 40–70°C Pressure: 30–60 MPa Modifier composition: MeOH (60–100%) Modifier concentration: 5.4, 7.8, and 10.2 mass%[a] CO_2 flow rate: 3.92–9.80 kg/h Sample particle size: 10–60 mesh Extraction time: 0–200 min	40°C, 50 MPa, 9.80 kg/h, 80% MeOH at 7.8% mass, 20–30 mesh, 200 min	Zuo et al. (2008)

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Note: ADi: acetyl daidzin, AGi: acetyl genistin, AGly: acetyl glycitin, De: daidzein, Di: daidzin, EtOH: ethanol, Ge: genistein, Gi: genistin, Gle: glycitein, Gly: glycitin, MDi: malonyl daidzin, MeCN: acetonitrile, MeOH: methanol, MGi: malonyl genistin, MGly: malonyl glycitin, n.e: not specified, TIS: total isoflavones.

[a] Mol% of the CO_2 mass passed through the system during the dynamic extraction.

TABLE 19.5

Pressurized Liquid Extraction Methods for the Extraction of Isoflavones from Soybeans and Soy Foods

Food	Isoflavones	Fixed Extraction Conditions	Evaluated Parameters	Selected Conditions	Reference
Freeze-dried soybeans	Di, Gi, Gly, and MGi	Extraction cell: 11 mL Inert material: sea sand	Solvent: EtOH (30–80%), MeOH (30–80%), water Temperature: 60 and 200°C Pressure: 10–20 MPa Sample amount: 0.5–0.05 g Static cycle length: 5–10 min Number of static cycles: 1–3 (7 min) and 1–2 (10 min)	0.1 g, 100°C, 70% EtOH, 3 × 7 min static cycles (~22 mL)	Rostagno et al. (2004)
Soy bits	Di, Gi, De, and Ge	Sample amount: 0.2 g Extraction cell: 10 mL Cell content: 5 mL of a commercial matrix (SPE-ed™ matrix) Static cycle length: 5 min	Solvent: MeCN, EtOH (50–90%), MeOH (50–90%) Pressure: 13–15 kPa Sonication time: 1–5 min Number of static cycles: 1–3	1 min sonication time, 0.1 g, 90% MeOH, 14 kPa, 2 static cycles (~20 mL)	Klejdus et al. (2004)
Soybeans	Di, Gi, Gly, De, Ge, Gle, MDi, MGi, MGly, ADi, AGi, and AGly	Sample amount: 0.5 g Extraction cell: 11 mL Inert material: Ottawa sand Pressure: 6.9 MPa Temperature: 100°C Static cycle length: 5 min Number of static cycles: 3	Solvent: 58% MeCN, 58% MeCN + 5% DMSO 70% EtOH, 70% EtOH + 5% DMSO 90% MeOH water 95% Genapol	70% EtOH + 5% DMSO	Luthria et al. (2007)

Soybean flour	De and Ge	Sample amount: 2 g Temperature: 100°C Inert material: quartz wool/ glass beds	Solvent: MeOH, acetone, MeCN Pressure: 5–15 MPa Number of static cycles: n.e. Extraction time: n.e.	MeCN, 2 static cycles of 15 min	Bajer et al. (2007)
Defatted soybean flakes	Di, Gi, Gly, De, and Ge	Sample amount: 180 g Extraction cell: 2 L Solvent: 1800 mL of water	Temperature: 60–130°C Pressure: 2.2–5.2 MPa Extraction time: 1–3 h	110°C, 4.5 MPa, 2.3 h	Eldridge and Kwolek (1983)
Defatted soybean flakes	Di, Gi, Gly, De, and Ge	Extraction cell: 2 L	Temperature: 60–120°C Pressure: 0.4–4.4 MPa Solvent flow rate: 10–25 mL/min Solvent: EtOH:water ratio (0–95%) Sample amount: 80–450 g	80% EtOH, 110°C, 0.6 MPa, 25 mL/min, 80 g	Chang and Chang (2007)

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Note: ADi: acetyl daidzin, AGi: acetyl genistin, AGly: acetyl glycitin, De: daidzein, Di: daidzin, EtOH: ethanol, Ge: genistein, Gi: genistin, Gle: glycitein, Gly: glycitin, MDi: malonyl daidzin, MeOH: methanol, MGi: malonyl genistin, MGly: malonyl glycitin, MeCN: acetonitrile, n.e: not specified.

19.2.3.6 Sample Stability during Extraction

In addition to a high—or ideally quantitative—extraction yield, the preservation of the compounds of interest is equally important, in particular for the determination of isoflavone profiles in foods. Of particular interest is the effect of long extraction processes at high temperature that can lead to the deconjugation of isoflavone glucosides. In 1991, Kudou et al. (1991) described a significant decrease in malonyl isoflavone glucosides and a concomitant increase of isoflavone glucosides during extraction with 70% alcohol at 80°C. Similarly, Barnes et al. (1994) observed ester hydrolysis of acetyl- and malonyl-glucosides during extraction at elevated temperature (60–80°C). The isoflavone profile is even more affected at higher temperatures, for example, during microwave-assisted extraction: when temperatures above 100°C were used, no malonyl-glucosides were detectable, and glucoside hydrolysis was observed at temperatures above 125°C (Rostagno et al., 2007). However, even at room temperature, conversion of acetyl- and malonyl-glucosides occurs in a time-dependent manner and has been estimated to be between 0.2 and 0.3 mol%/h (Rostagno et al., 2009). Therefore, a timely extraction method is therefore necessary to preserve the profile of compounds as much as possible. For best results, a fast extraction method at low temperatures is preferable. An additional method to retain conjugated isoflavones is the use of inhibitors of endogenous β-glucosidases. *Tris* (*tris*-(hydroxymethyl)-aminomethane) has been used as an inhibitor and at a concentration of 350 mM in 80% EtOH increased the extraction yield of malonyl conjugates by 13–24 times (Toebes et al., 2005); other inhibitors used are $AgNO_3$, $HgCl_2$, and D-glucono-δ-lactone (Matsuura and Obata, 1993).

19.2.4 Sample Hydrolysis and Deconjugation

In many studies, it is important to maintain the isoflavone profile and prevent any hydrolysis of malonyl-glucosides, acetyl-glucosides, and glucosides. However, for the determination of total isoflavone content, it is often desirable to hydrolyze isoflavones to obtain their aglucones (Kuhnle et al., 2007) as this also facilitates analysis. A standard nonenzymatic method is acid hydrolysis using 1–2 M hydrochloric acid at 100°C (Wilkinson et al., 2002), but this does not only affect glucosidic bonds but also the stability of some isoflavones, in particular of genistein (Franke et al., 1994; Garrett et al., 1999). Alkaline hydrolytic methods are also used, treating samples with NaOH for several minutes up to several hours (Delmonte et al., 2006; Rostagno et al., 2009). Enzymatic hydrolysis is generally preferred because it is milder and preserves the aglucones present in the sample. Enzymes used are β-glucuronidase and arylsulfatase from *Helix pomatia*, β-glucosidase from almonds, and cellulase from *Aspergillus niger* (Wilkinson et al., 2002) or other fungi. The highest yield of aglucones from three standard foods (asparagus, linseed, and red rice) has been achieved using a combination of β-glucosidase from almonds, β-glucuronidase and arylsulfatase from *Helix pomatia*, and cellulase from *Trichoderma reesei* (Kuhnle et al., 2007). *Helix pomatia* extract often has to be purified prior to analysis because it can contain significant amounts of isoflavones and other compounds, presumably from the diet of the snail. Grace et al. have developed a simple purification method by filtering a 10% solution of *Helix pomatia* extract in 140 mM sodium acetate buffer (pH 5) using C18-E solid-phase extraction cartridges (Grace and Teale, 2006). This method does not affect the enzyme activity but removes a significant amount of isoflavones; however, because of the residual isoflavone content it is important to adjust analysis results accordingly (Kuhnle et al., 2007).

Hydrolytic methods will provide more accurate information on the total isoflavone content because all isoflavones will be converted into their aglucone form, facilitating analysis. However, the absorption of isoflavones from the gastrointestinal tract depends on the form in which they are present in food, and therefore information on their glucosidic form is important to assess the bioavailability and bioactivity.

19.2.5 Postextraction Treatment

Following the extraction of isoflavones from their food matrix, it is often necessary to further process the extract to obtain a clean and concentrated sample (Rostagno et al., 2005), although in particular following PLE and SFE this is often unnecessary (Rostagno et al., 2009). Centrifugation or filtration are often the only clean-up steps used to remove any insoluble matter, but commonly solid-phase extraction (SPE),

sometimes directly combined with chromatographic methods for isoflavone analysis, is applied. Another method used is high-speed countercurrent chromatography (HSCCC). These procedures can remove compounds interfering with the analysis and concentrate the samples. Following these steps, samples are commonly dried and reconstituted, either in mobile phase or in 80% methanol.

19.2.5.1 Solid-Phase Extraction

Solid-phase extraction is conducted using a wide variety of different solid phases, ranging from silica-based C8 and C18 phases to new polymeric compounds. Silica-based materials have the disadvantages of having a limited pH range and poor retention of polar compounds (Franke et al., 1994). Due to their hydrophobicity, the conditioning or wetting step is extremely important with these phases. In contrast, polymeric sorbents can be used without prior conditioning (Klejdus et al., 1999). Table 19.6 shows an overview of isoflavone recovery from soy foods using different types of solid-phase extraction sorbents. For most sorbents, the recovery is better than 90% for all compounds, with two notable exceptions: the polymer-based LiChrolute EN and the Strata C18-E, which show a much lower recovery. Interestingly, the recovery of genistein and glycitein for the Strata C18-E column is close to 100%, and it is only daidzein that shows a poor recovery (67%) (Rostagno et al., 2005). In a different study, albeit using serum samples, recovery for daidzein (96%) is comparable with the recovery of genistein and glycitein (both 96%) (Grace et al., 2003). These results suggest that polymer sorbents such as Oasis HLB or Strata X provide the best recovery for both glucosides and aglucones; in particular the Strata X sorbent was found to provide the best results (Rostagno et al., 2005). Following their detailed study, Rostagno et al. recommend the following method: conditioning of the cartridge with methanol and water, washing with water, and elution with methanol (Rostagno et al., 2005); however, it might be necessary to increase the amount of organic compound in the washing step to remove impurities. To ensure quantitative recovery, the elution step is often repeated several times (Kuhnle et al., 2007).

Solid-phase microextraction (SPME) is a sample preparation technique that uses fused silica-fiber coated on the outside with a stationary phase. The sample is then extracted directly to the fiber coating

TABLE 19.6

Recovery of Different Isoflavones by Solid-Phase Extraction Using Different Sorbents

SPE Cartridge	Sorbent	Malonyl-Glucosides[a]	Acetyl-Glucosides[b]	Glucosides[c]	Agluconese[d]
Bond Elut C18	Octadecyl-silica	95	99	100	100[e]
Discovery C18	Octadecyl-silica	96	99	100	100[e]
Strata C18-E	Octadecyl-silica	36	44	77	88[e]
C18	Octadecyl-silica				89[f]
C8	Octyl-silica				90[f]
Strata X	Polymer	100	100	100	100[e]
Oasis HLB	Polymer	100	100	99	99
LiChrolut EN	Polymer	21	26	73	36[e]
Bond Elut ENV	Polymer	99	99	100	99[e]
Strata SDB-L	Polymer	96	99	100	100[e]
Amide 2	Polymer				94[f]
RP 105	Polymer				96[f]
ABN	Polymer				100[f]

Source: Data adapted from Klejdus, B., Vitamvasova, D., and Kubáň, V. 1999. *Journal of Chromatography A*, 839, 261–263; Rostagno, M. A., Palma, M., and Barroso, C. G. 2005. *Journal of Chromatography A*, 1076, 110–117..

[a] Malonyl-glucosides of daidzein, genistein, and glycitein.
[b] Acetyl-glucosides of daidzein, genistein, and glycitein.
[c] Daidzin, genistin, and glycitin.
[d] Daidzein, genistein, glycitein, biochanin A, and formonontein.
[e] Daidzein, genistein, and glycitein only.
[f] Biochanin A, daidzein, genistein, and formononetin only.

(Kataoka et al., 2000). Alternatively, in-tube solid-phase microextraction uses silica-fibers coated on the inside that can be easily coupled with analytical techniques such as HPLC and LC-MS (Mitani et al., 2003).

19.2.5.2 High-Speed Countercurrent Chromatography

Countercurrent chromatography is a chromatographic technique in which both the mobile and stationary phase are liquids (see Pauli et al., 2008 for more details). This technique is a suitable alternative for the isolation of isoflavones at large scales, in particular because of the low solubility of some specimens (Stürtz et al., 2006). The lack of active surfaces improves the isolation of isoflavones, in particular of labile species (Valls et al., 2009). Ethyl acetate:water is the most common solvent system, using an alcohol (e.g., ethanol, methanol, or *n*-butanol) that distributes between phases. By changing the system mixture, it is possible to alter the selectivity of the system and Yang et al. showed that while the less polar aglucones were best separated in one system ($CHCl_3$:MeOH:H_2O; 4:3:2 v/v/v), the addition of a small amount of *n*-butanol increased the polarity sufficient to elute glucosyl derivatives (Yang et al., 2001).

19.2.6 Analytical Techniques

A large variety of different techniques have been developed for the analysis of isoflavones from different sources, for example, plasma and urine, and also food. The development of these techniques was directed both by the technology available and by the objective of the analysis, for example, quantification of bioactive compounds or the structural elucidation of novel isoflavone derivatives. Quantification is commonly performed using internal standards, whereby for mass spectrometry isotope-labeled standards are preferred. Although deuterated standards are often used, the instability of the deuterium label, in particular under acidic conditions, can lead to overestimation (Adlercreutz et al., 1993; Wähälä et al., 1995; Wähälä and Rasku, 1997); for this reason, ^{13}C-labeled standards (Whalley et al., 1998, 2000) will provide the best results. Table 19.7 gives an overview of internal standards commonly used for isoflavone analysis.

TABLE 19.7

Analytical Parameters of Common Isoflavones

Compound	Molecular Weight (g/mol)	UV λ_{max} (nm)	Detection Potential (mV)	Tandem MS Fragment Ions (Retro Diels–Alder Products and Other Ions)
Daidzin	416	260[a]	590[a]	415 $[M-H]^-$; 253 $[M-H-Glc]^-$ [3]
Malonyl daidzin	502	258[d]	590[a]	457 $[M-COOH]^-$; 253 $[M-H-MalGlc]^-$ [3]
Glycitin	446	262[a]	590[a]	445 $[M-H]^-$; 283 $[M-H-Glc]^-$ [3]
Malonyl glycitin	532	260[d]	590[a]	487 $[M-COOH]^-$; 283 $[M-H-MalGlc]^-$ [3]
Genistin	432	262[a]	590[a]	431 $[M-H]^-$; 269 $[M-H]^-$ [3]
Malonyl genistin	518	260[d]	590[a]	473 $[M-COOH]^-$; 269 $[M-H-MalGlc]^-$ [3]
Acetyl daidzin	458	256[d]	590[a]	457 $[M-H]^-$; 253 $[M-H-AcGlc]^-$ [3]
Acetyl glycitin	482	260[d]	590[a]	487 $[M-H]^-$; 283 $[M-H-AcGlc]^-$ [3]
Daidzein	254	250, 302[b]	510[a]	133 ($^{0,3}A^-$); 117 ($^{1,3}B^-$); 197; 181[b]
Glycitein	284	262[a]	590[a]	163 ($^{0,3}A^-$); 117 ($^{1,3}B^-$)[e]
Acetyl genistin	474	261[d]	590[a]	473 $[M-H]^-$; 269 $[M-H-AcGlc]^-$ [3]
Genistein	270	260, 328[b]	510[a]	133 ($^{0,3}A^-$); 107 ($^{0,4}A^-$); 163 ($^{0,4}B^-$); 197[b]
Biochanin A	284	261, 326[b]		177 ($^{0,4}B^-$); 213 ($^{1,2}A^-$); 254 ($^{1,3}A^-$); 252; 195[b]
Formononetin	268	249, 302[b]		107 ($^{0,4}A^-$); 177 ($^{0,4}B^-$); 229 ($^{1,2}A^-$); 270 ($^{1,3}A^-$); 268; 211[b]

[a] Data from Nurmi et al. (2002).
[b] Data from de Rijke et al. (2006).
[c] Data from Barnes et al. (1994).
[d] Data from Murphy et al. (2002).
[e] Estimated data.

19.2.6.1 Chromatography

Chromatographic separation can be used either for analytical purposes or as preparative or semipreparative method. The main chromatographic methods used for isoflavone analysis are gas chromatography (GC), HPLC, and thin-layer chromatography (TLC).

19.2.6.1.1 Gas Chromatography

The first GC method for isoflavone analysis had already been described in 1974 (Mazur et al., 1996). Later on, GC methods—in general with mass spectrometric detection—have been developed to overcome the low sensitivity of HPLC methods available at the time (Naim et al., 1974). GC methods provide an excellent chromatographic resolution but they are very labor-intensive compared with other methods as they require a careful sample cleanup and derivatization prior to analysis (Wang et al., 2002). Isoflavones are normally derivatized to their trimethyl-silyl-ethers (TMS) to improve the volatility, thermal stability, and sensitivity of the method. Derivatizating agents used include *N*,*O-bis*-(trimethylsilyl)-trifluoracetamide (containing 1% trimethylchlorsilane (TMCS)) or pyridine-hexamethyldisilazane-trimethylchlorosilane (Heinonen et al., 1999; Wang et al., 2002). For some compounds, that is, genistein, it has been reported that incomplete derivatization can affect analysis (Ingram et al., 1997) and this might have to be overcome by optimizing the derivatization conditions. However, the potential of loss of compounds from samples needs to be considered when using this method (Wu et al., 2004). The derivatives can then be analyzed using a nonpolar capillary column with a linear temperature gradient (Wang et al., 2002). Using selective ion monitoring (SIM), GC-MS provides a very high sensitivity and selectivity for individual compounds; however, unknown or unexpected compounds will not be detected using this method; conversely, operating the instrument in full-scan mode will allow the identification of unknown—or unexpected—compounds, but will significantly reduce the sensitivity. Electron impact ionization of TMS derivates will often result in $[M\text{-}15]^+$ ions due to the loss of CH3•; this can be used to determine the molecular weight of unknowns (Wang et al., 2002; Tekel et al., 1999).

19.2.6.1.2 High-Performance Liquid Chromatography

In contrast to GC, which is rarely used for preparative purposes, HPLC can be used for both analytical and preparative purposes. The main advantage of HPLC over GC is that it does not normally require any derivatization and it is more versatile regarding the method of detection. Most HPLC methods currently used apply reversed-phase columns—in particular C18—with methanol, acetonitrile, and water as mobile phases, often including modifiers to adjust pH, for example, formic acid, acetic acid, trifluoracetic acid, and phosphoric acid (Wu et al., 2004); the modifier often also depends on the detection method used. In normal RP-HPLC, the order of elution would be puerarin (the 8C-glucoside of daidzein) < daidzin < glycitin < genistin with the aglucones eluting in the same order but with considerably more organic solvent in the mobile phase (Valls et al., 2009). Due to their phenolic nature, alternative stationary phases with phenyl functionality have also been used successfully (Kuhnle et al., 2007; Klejdus et al., 2008). A more recent development in HPLC technology is the emergence of UPLC (ultra-high pressure liquid chromatography) in which higher solvent pressures (41 MPa) and low particle sizes are used. This results in a much greater resolution and much-reduced analysis time; whereas a method for the analysis of isoflavones in food used recently for the UK food table required 15 min per run (Kuhnle et al., 2007), a similar UPLC method can separate the same number of analytes (and more) within 2 min (Klejdus et al., 2008).

19.2.6.1.3 Detection Methods for HPLC

- *Ultraviolet detection*: UV single-wavelength or diode array detector are among the most commonly used detection methods coupled with HPLC; the phenolic ring structure of isoflavones results in a UV absorption maximum between 230 and 280 nm. This method has been used successfully for the analysis of isoflavones from a variety of food sources (reviewed, e.g., in Wu et al., 2004). However, this method has considerable disadvantages, for example, the difficulty to distinguish between related compounds and the necessity of complete separation of peaks. Table 19.8 provides an overview of the absorption maxima of a selection of important isoflavones.

TABLE 19.8

Internal Standards Used for Isoflavone Quantification in Food and Other Matrices and Selected Literature

Internal Standard	Application	Reference
1-Methyl-3-isobutylxanthin	CZE-UV analysis of isoflavones	Shihabi et al. (1994); Mellenthin and Galensa (1999)
2,4,4′-Trihydroxydeoxybenzoin	HPLC-UV, limited commercial availability	Griffith and Collison (2001)
2′-Methoxy-flavone	HPLC-UV	Delmonte et al. (2006)
4-Methylumbelliferone sulfate		Mellenthin and Galensa (1999)
6-Methoxy-flavone	HPLC-UV	Krenn et al. (2002)
Apigenine	LC-MS analysis of isoflavones	Coward et al. (1996); Barnes et al. (1999); Griffith and Collison (2001); Mellenthin and Galensa (1999)
Flavone		Franke and Custer (1994)
Fluorescin	HPLC analysis of phytoestrogens	Coward et al. (1998)
Kaempferol	GC-MS analysis of 8-prenylnaringenin	Tekel et al. (1999)
p-hydroxybenzoic acid	HPLC analysis of isoflavones	Bednarek et al. (2001)
Phenolphtaleine β-glucuronide		Barnes et al. (1999); Mellenthin and Galensa (1999)
Deuterated isoflavones	LC-MS and GC-MS analysis of isoflavones	Adlercreutz et al. (1995); Mazur et al. (1996)
^{13}C-labeled standards	LC-MS analysis of isoflavones in food	Kuhnle et al. (2007)

Source: Adapted from Wang, C.-C., Prasain, J. K., and Barnes, S. 2002. *Journal of Chromatography B*, 777(1–2), 3–28.

- *Electrochemical detection (ECD)*: Phenolic compounds can generally become oxidized and are therefore electrochemically active. In contrast to UV detection, electrochemical detection provides a much higher sensitivity. ECD in conjugation with chromatography has been used successfully to identify and quantify different isoflavone derivatives (Nurmi et al., 2002), but baseline stability due to impurities in the mobile phase can be a problem, in particular for analyses close to the limit of detection (Wang et al., 2002).
- *Fluorescence detection*: Fluorescence detection is normally also more sensitive than UV detection; however, it requires that the analytes are fluorescent and only a small number of isoflavones are—for example, daidzein and formononetin, but not genistein and biochanin A (Wang et al., 2002). Different excitation wavelengths have been used (e.g., 365 nm, Wang et al., 1990 and 250 nm, de Rijke et al., 2001) to measure the fluorescence at 418 nm. Postcolumn chelation with aluminum can be used to allow fluorescence detection of other isoflavones by adding $Al(NO_3)_3$ to the eluent (Hollman et al., 1996).
- *Mass spectrometric detection*: Mass spectrometric detection is the most versatile detection method for the analysis of isoflavones, as it can offer high sensitivity and high selectivity. Details for LC-MS are discussed below.

19.2.6.1.4 Thin-Layer Chromatography

Isoflavones can be separated on TLC on silica gel plates using different solvent systems. Naim et al. (1974) uses a method described by Beck (1964) using Kieselgel-G plates with a chloroform–methanol eluent and detection with Folin–Ciocalteau's reagent (Folin and Denis, 1915) in an ammonia saturated chamber or under UV light to detect isoflavones in soybeans. Yokoyama and Kuzuguchi (2007) developed a method for the detection of equol and other isoflavones using normal- and reversed-phase TLC plates with toluene:acetone (2:1) and acetonitrile:water:acetic acid (60:40:1), respectively, as solvent system.

19.2.6.2 Capillary Electrophoresis

Capillary electrophoresis (CE) (Altria, 1999) is a separation technique that relies on differences in the electrophoretic mobility of charged species in an electric field in small-diameter capillaries (50–100 μm)

(Wang et al., 2002). This method offers rapid and high-resolution separation for sample volumes in the nanoliter range, providing a sensitivity in the femto- to attomole range. Isoflavones as weak acids are negatively charged at high pH and therefore possess electrophoretic mobility. The addition of borate buffer creates charged complexes by interaction with the *cis*-diol moiety of sugars. As the migration time increases with pH, the resolution of the compounds under investigation can be adjusted by using different running buffers (Peng and Ye, 2006); however, this also decreases the peak current leading to a deterioration of peak shapes. Likewise, higher buffer concentrations result in higher run times and resolution, but also decrease performance by increasing the limit of detection (Delmonte and Reader, 2006). Capillary zone electrophoresis (CZE) and micellar electrokinetic chromatography (MEKC) are both techniques that have evolved from original CE. MEKC requires the addition of surfactants such as SDS to the running buffer to achieve the concentrations above their critical micellar concentration. During analysis, the analytes partition between running buffer and micelle, which thereby acts as a pseudostationary phase, thus increasing the selectivity of the method.

CE can be combined with the same detection methods as HPLC, for example, UV, fluorescence, electrochemical, and mass spectrometric detection. Shihabi et al. used CZE with 200 mM borate buffer and a pH range of 8.5–8.8 to separate plant isoflavones and coumestrol using UV detection at 214 nm (Shihabi et al., 1994). Using laser-induced fluorescence detection, Beekman et al. achieved detection limits of 25–100 ng/mL for daidzein and formononetin (Beekman et al., 1999). Hyphenation with electrochemical detectors is more difficult since the high voltage can induce high noise levels. In contrast, mass spectrometry has been shown to be an ideal detector for CE separation, in particular because of the low flow rate that results in high sensitivity with electrospray ionization. Under optimum conditions, a limit of detection of 600 attomole could be achieved using selected ion monitoring (Beekman et al., 1999).

19.2.6.3 Immunoassays

Immunoassays can provide a sensitive high-throughput analysis at low costs; however, they require the development of suitable antibodies. Most immunoassays have been developed for the analysis of isoflavones in biological fluids such as urine or serum, for example, radioimmunoassay for formononetin (Wang et al., 1994) and daidzein (Lapčík et al., 1997). However, these methods do not seem to be used for food analysis.

19.2.7 Mass Spectrometric Methods for Isoflavone Analysis

19.2.7.1 Hyphenated Techniques

Mass spectrometry (MS)—in particular in combination with separation techniques such as GC (see above), HPLC, or CE—provides one of the most versatile tools for the detection, identification, and quantification of isoflavones. Whereas sample introduction in GC-MS is facilitated by the fact that samples are already in the gas phase, this is more difficult for liquid techniques such as HPLC or CE. In the 1980s and early 1990s, several techniques were available to couple MS with these techniques, for example, thermospray (Blakley and Vestal, 1983), continuous flow fast-atom bombardment (cfFAB) (Ashcroft et al., 1987), or moving belt (Redrup et al., 1989). However, these techniques have been superseded by the development of electrospray ionization (ESI) (Fenn and Yamashita, 1984; Aleksandrov et al., 1985) and atmospheric pressure chemical ionization (APCI).

Both ESI and APCI are the so-called soft ionization methods that result in the formation of quasi-molecular ions (chiefly $[M + H]^+$ or $[M - H]^-$—small molecules normally only carry one charge) or the formation of adducts with modifying agents in the mobile phase such as acetate, formate, or ammonia. For some applications, this—in combination with a high-resolution mass analyzer such as an OrbiTrap (Makarov, 2000)—can be sufficient for the identification of certain compounds and metabolites; however, in general, tandem MS experiments are necessary for identification and structure elucidation.

Tandem MS experiments can be performed in most instruments except for those equipped with a single-quadrupole mass analyzer, although in-source fragmentation can be used, for example, for structural elucidation work. Most commonly, triple-quadrupole mass analyzers are used that allow the

fragmentation of selected ions by collision-induced dissociation (CID) and the subsequent analysis of these fragments. Triple-quadrupole analyzers are commonly considered to be the most suitable for quantitative analyses; however, quadrupole ion traps provide a similar functionality and are also often used for quantification. In addition, they allow multiple MS experiment (MS^n) that can be very useful for structure elucidation (see below). For quantification, selected ion monitoring (SIM) and multiple reaction monitoring (MRM) are most commonly used. SIM does only require a single-quadrupole instrument but generally has a lower sensitivity and selectivity than MRM. Using MRM, a limit of detection of 1.2 fmol for daidzein and 1.6 fmol for genistein could be achieved (Vacek et al., 2008).

Although the development of MRM methods for the analysis of isoflavones does not require knowledge of the fragmentation pathway per se, as most modern instruments allow the automatic selection of the most abundant ion for a given precursor ion, it is important for structural elucidation and for the validation of the analytical method. Table 19.8 includes a list of common fragments of selected isoflavones; however, the individual fragmentation pattern depends on several factors and might therefore differ among different instruments. In glucosides, the loss of the malonyl, acetyl, and glucosyl group are common fragmentation reactions that are very valuable for the structural elucidation of isoflavone conjugates (Wu et al., 2003). Using multistage fragmentation experiments (MS^n), Kang et al. investigated the fragmentation mechanisms in negative ion mode (Kang et al., 2007) and the predominant fragments were neutral losses of CO, CO_2, C_3O_2, and C_2H_2O. Even though fragments resulting from the retro Diels–Alder reaction (Figure 19.3 shows the nomenclature commonly used to describe these fragments) were found in much lower abundance, they are in particular useful for structure elucidation as they allow not only the determination of OH groups on different rings, but also to identify the position of glucosidic bonds using fragments with intact glucosidic bonds. A detailed investigation of fragmentation processes can also be found in de Rijke et al. (2006).

19.2.7.2 Nonhyphenated Techniques

Most hyphenated techniques can easily be uncoupled from the separation device, for example, by infusing a sample directly using a syringe pump. This is in particular useful to identify unknown compounds as it provides more time to conduct MS experiments. In contrast to the methods described above, MALDI-TOF-MS is almost exclusively used separately from separation techniques. In MALDI, the sample is embedded in a matrix and ionized using laser beam. Although ion formation by laser shots was known since the 1960s (Vastola et al., 1970), MALDI ion sources have only been developed in the late 1980s (Karas and Hillenkamp, 1988). Like ESI and APCI, MALDI also produces mainly quasi-molecular ions although adducts with the matrix compound are not uncommon. 2′,4′,6′-Trihydroxyacetophenone (THAP) and 2,5-dihydroxybenzoic acid have been used successfully for the analysis of isoflavones from soy

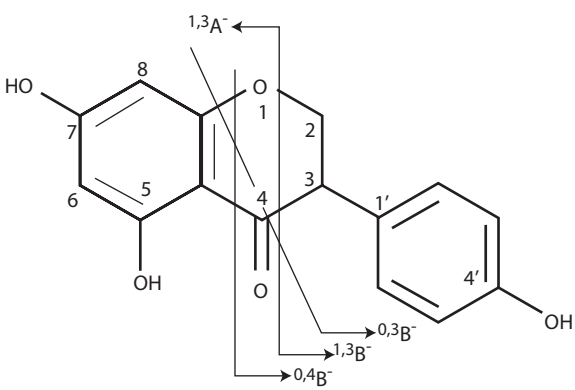

FIGURE 19.3. Nomenclature of major isoflavone pathways. (Adapted from Kang, J., Hick, L. A., and Price, W. E. 2007. *Rapid Communications in Masss Spectrometry*, 21, 875–868.)

samples (Wang and Spornes, 2000) with DHB producing better reproducibility. In this study, isoflavones only fragmented to lose their carbohydrate moiety and resulted in the formation of aglucone ions.

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20

Hydrolyzable Tannins: Gallotannins, Ellagitannins, and Ellagic Acid

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20.1 Introduction

The chemical structures of the hydrolyzable tannins are basically composed of a central sugar core, typically a glucose unit, to which gallic acid moieties are esterified. β-Glucogallin is the simplest glucosyl gallate known and serves as a galloyl unit donor in the biosynthesis of the fully galloylated β-d-glucopyranose (β-PGG), which is itself considered to be the immediate precursor of the two subclasses of hydrolyzable tannins, that is, gallotannins and ellagitannins (ETs) (Figure 20.1) (Gross 1999, 2008; Niemetz and Gross 2005; Quideau et al. 2011). Gallotannins are the result of further galloylations of β-PGG and are characterized by the presence of one or more *meta*-depsidic digalloyl moieties, as exemplified with the hexagalloylglucose 3-*O*-digalloyl-1,2,4,6-tetra-*O*-galloyl-β-D-glucopyranose (**1a** in Figure 20.1). Alternatively, β-PGG can be subjected to intra- and intermolecular

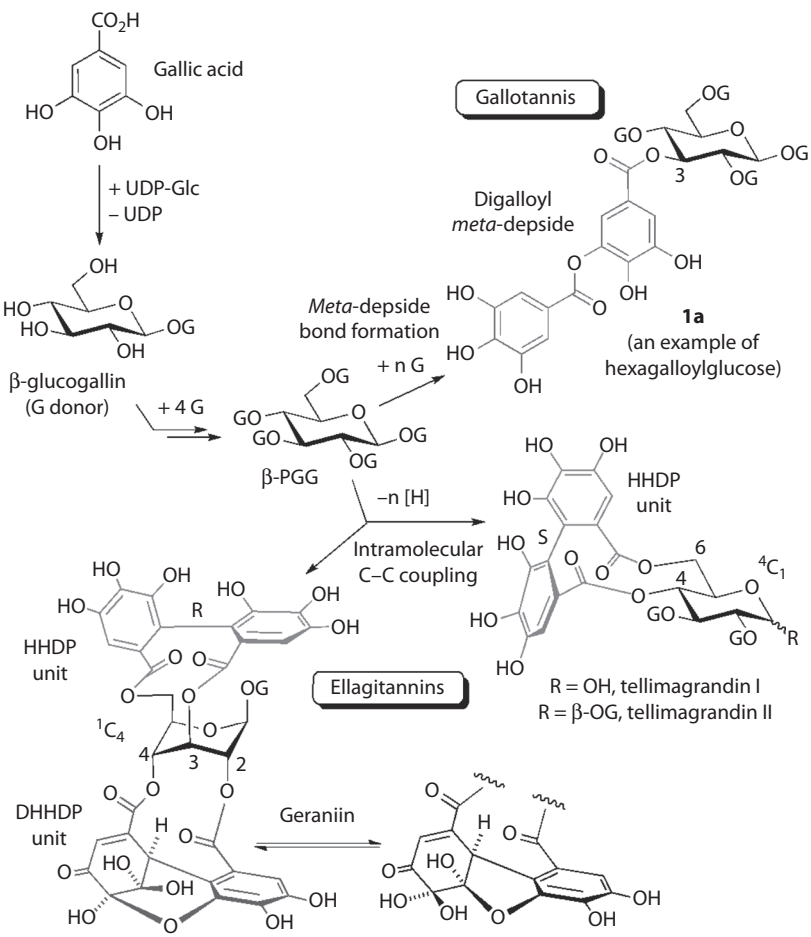

FIGURE 20.1 Biosynthesis of hydrolyzable tannins (gallotannins and ellagitannins).

phenolic coupling processes that create connections between spatially adjacent galloyl residues by forming C–C biaryl and C–O diaryl ether bonds. The so-called hexahydroxydiphenoyl (HHDP) biaryl unit generated by intramolecular coupling is the structural characteristic that defines hydrolyzable tannins as ETs. The nature of the atropisomeric form of these chiral biaryl motifs, such as the (*S*)-HHDP unit of the tellimagrandins or the (*R*)-HHDP unit of geraniin, is determined by the position of the galloyl motifs on the glucopyranose core in either its $^4C_1$- or its $^1C_4$-conformation. Besides, the HHDP motif is susceptible to many additional transformations, among which its oxidation leads to the dehydrohexahydroxydiphenoyl (DHHDP) unit characteristic of the dehydroellagitannin natural products, such as geraniin (Quideau and Feldman 1996; Khanbabaee and van Ree 2001a; Feldman 2005; Pouységu et al. 2011).

ETs release ellagic acid (EA) upon hydrolysis. This occurs spontaneously in the gastrointestinal tract under physiological conditions (Larrosa et al. 2006). In addition, free EA and its glycoconjugated derivatives with sugars are also found in most ET-containing plants.

20.1.1 Occurrence in Food and Medicinal Plants

ETs are present in significant amounts in many berries, including strawberries, red and black raspberries (Zafrilla et al. 2001), blackberries, and nuts, including walnuts (Fukuda et al. 2003), pistachios, cashew nuts, chestnuts, oak acorns (Cantos et al. 2003), and pecans (Villarreal-Lozoya et al. 2007). They are also abundant in pomegranates (Gil et al. 2000), and muscadine grapes (Lee et al. 2005), and are important constituents of wood, particularly oak wood (Glabasnia and Hofmann 2006). ETs can be incorporated into several food products such as wines, and whiskies, through migration from wood to the food matrix during different aging processes. EA has also been found in several types of honey and this phytochemical has been proposed as a honey floral marker for heather honey (Ferreres et al. 1996). Free EA and different glycoside derivatives are also present in these food products, including glucosides, rhamnosides, arabinosides, and the corresponding acetyl esters (Zafrilla et al. 2001).

In a previous review, it was documented that there were no reliable figures available on the ET dietary burden but that it would probably not exceed 5 mg/day (Clifford and Scalbert 2000). Since then, a number of studies have shown that the ET content of several food products can be quite high. A glass of pomegranate juice can provide as much as 300 mg, a raspberry serving (100 g of raspberries) around 300 mg, a strawberry serving 70 mg, and four walnuts some 400 mg of ETs. As a result, the intake of dietary ETs can be much higher than previously estimated (Clifford and Scalbert 2000), especially if some of these ET-rich foods are regularly consumed in the diet.

20.1.2 Antioxidant Activity of Hydrolyzable Tannins and EA

ET-rich foods generally show a high free-radical scavenging activity evaluated *in vitro*. Especially relevant is the antioxidant activity of pomegranate juice (Gil et al. 2000). This study shows that pomegranate juice has twice the antioxidant activity of red wine and that this is due to the extraction of ETs from the fruit husk during juice manufacturing (Gil et al. 2000). This remarkable antioxidant activity has been the driving power of research on the biological activity of these powerful antioxidants from pomegranate, and is used by the food industry to market pomegranate juice products as super-antioxidant food. ETs are also responsible for a relevant part of the antioxidant activity observed in strawberries (Hannum 2004), raspberries (Zafrilla et al. 2001), blackberries, walnuts (Blomhoff et al. 2006), and pecans (Villarreal-Lozoya et al. 2007). This antioxidant activity can probably be related to the biological activity reported for these food products.

In parallel to those studies of the antioxidant activity of ET-rich food, clinical studies have also shown relevant biological activities that have been associated with these antioxidants, although no direct evidence of the biological activity of these polyphenols has been demonstrated. Several clinical studies have reported relevant biological activity after the intake of ET-rich foods, especially regarding the protective effect against cardiovascular diseases and cancer.

20.2 HPLC Analysis

ETs can be analyzed by high-performance liquid chromatography (HPLC) using reversed-phase columns with methanol, acetonitrile, and water gradients. The addition of 1% of formic or acetic acid to the water solvent helps increasing the resolution of the chromatograms through the separation in sharper peaks avoiding peak tailing. In general, complex ET mixtures are observed in the extracts. Some effects of the ET structure on the chromatographic retention and elution order have been reported (Salminen et al. 1999; Moilanen and Salminen 2008). In general, the occurrence of free galloyl groups in the ET molecule increases the retention times, while the formation of an HHDP in the hydrolyzable tannin molecule decreases the retention time. The opening of the glucopyranose ring, as it happens in some *C*-glycosyl ETs (i.e., vescalagin, castalagin), also decreases the retention time. ETs with a cyclic sugar, and without galloylation in C-1, produce two peaks corresponding to the α- and β-anomer, while galloylated ETs at C-1 produce only one chromatographic peak. This has been reported in punicalagin and punicalin, the characteristic ETs of pomegranate, that show two peaks for each ET corresponding to both α- and β-anomer (Gil et al. 2000). Acyclic epimers having hydroxyl groups at C-1 of the glucose can be distinguished from each other since the orientation of the hydroxyl group causes vescalagin-type ETs to elute before the castalagin-type ones (Moilanen and Salminen 2008).

EA is present in nature in a free state or in combination with different sugars and forming methyl ethers. EA hexosides (glucosides), deoxyhexosides (rhamnosides), and pentosides (xylosides and arabinosides) as well as glucuronides have been reported as Phase II metabolites present in biological fluids. In addition, acetylated derivatives of EA pentosides have been reported in raspberries (Zafrilla et al. 2001). Glucuronides are the first eluting metabolites, followed by hexosides (glucosides), desoxyhexosides (rhamnosides), and pentosides (xylosides first and arabinosides). Free EA elutes after the glycosides but earlier than the acetyl pentosides. EA methyl ethers and sulfates (that are often found in biological fluids after the intake of ETs and EA) elute with longer retention times than free EA, and the retention time increases with the number of methyl ethers introduced on the EA molecule.

The chromatographic behavior of the microbial metabolites of ETs and EA, known as urolithins (metabolites related to EA in which one of the lactone rings has been removed by the colonic microbiota), follows a similar trend as EA derivatives, increasing the retention time when decreasing the number of hydroxyl groups on the urolithin nucleus, and when increasing the number of methyl ethers (González-Barrio et al. 2011). Again, the introduction of a glucuronyl conjugation decreases the retention time, while the introduction of a sulfate residue increases the retention time. In addition, the chromatographic peaks corresponding to sulfate conjugates are broader, hence decreasing the chromatographic resolution.

20.3 UV Spectrophotometry Detection

The UV spectra of the different ETs, gallotannins, and EA derivatives are easily recorded in the analysis of the extracts by HPLC-diode-array detection (DAD) analysis. The occurrence of free galloyl groups in the ET molecule produces two absorption maxima in the spectrum, one around 270–280 nm and another around 210–220 nm. The higher the number of the galloyl groups with respect to the HHDP units, the steeper is the valley between the two maxima (Salminen et al. 1999), and the maximum for BI (the band between 270 and 290 nm) appears at higher wavelengths. In HHDP-rich tannins, the valley between the two absorption maxima even disappears from the UV spectrum, as is the case of bis-HHDP-glucopyranose, and no defined maximum is observed for the absorption band around 270–280 nm (Table 20.1).

The change from cyclic to acyclic sugars also has a substantial effect on the UV spectrum.

Free EA shows a UV spectrum characterized by two absorption bands at 365–380 and 253–255 nm, with a characteristic shape that allows its easy detection and identification in the UV-DAD chromatograms. In general, substitution with pentoses, hexoses, and glucuronides produces shifts of the UV

TABLE 20.1

UV Spectra of Ellagitannins. Effect of the Occurrence of Galloyl or Hexahydroxydiphenoyl Residues

Compound	BI (nm)	BII (nm)	% of BI Respect BII Absorbance
Gallic acid	276	219	38
Pentagalloyl-glucopyranose	285	220	42
Trigalloyl-HHDP-glucopyranose	283	215	27
Digalloyl-HHDP-glucopyranose	280	211	23
Galloyl-bis-HHDP-glucopyranose	279	214	19
Bis-HHDP-glucopyranose	270i	205i	–

Source: Extracted from Salminen, J. P. et al. 1999. *J. Chromatogr. A* 864: 283–291.

maxima to shorter wavelengths. This effect is also observed when methyl ethers are introduced on the phenolic hydroxyls, and is particularly evident when a sulfate residue is introduced as the hypsochromic shifts in wavelengths are more marked. The same effects are observed for the urolithin derivatives. Urolithin A, the main ET and EA metabolite in mammals, has a UV spectrum close to that of EA, but it is clearly distinctive to allow the unambiguous differentiation of both compounds (Figure 20.2). The same effects on the UV spectrum described for the EA conjugation are valid for urolithin conjugations (González-Barrio et al. 2011). Urolithins with different hydroxylation patterns show characteristic UV spectra that can be used for the structural differentiation of the metabolites present in biological fluids using HPLC coupled to UV detection (DAD).

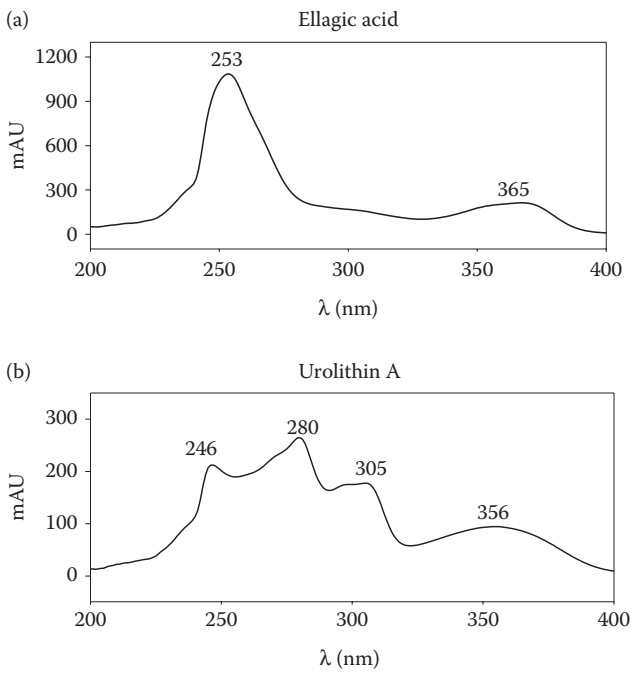

FIGURE 20.2 UV spectra of (a) ellagic acid and (b) urolithin A.

20.4 Quantitative Indirect Analysis of ETs Using HPLC-DAD after Acid Hydrolysis

Acid hydrolysis is the most practical and widely employed technique used to quantify the hydrolyzable tannins present in vegetable extracts. As an example, in the case of *Rubus*, the use of both HPLC analysis after hydrolysis (Vrhovsek et al. 2008) and direct HPLC analysis (Gasperotti et al. 2010) has demonstrated that the average structure of ETs is well conserved in the different genotypes, while differing in terms of absolute concentration. This means that for routine quantification of complex ETs, the method using analysis after hydrolysis is still appropriate and able to provide useful information.

However, care must be taken to choose the appropriate solvent for extraction and the whole essay, since these natural compounds are not easy to manage, and each step (extraction, hydrolysis, HPLC analysis, and data processing) should be carefully considered in order to provide consistent data. Moreover, EA is poorly soluble and can precipitate if not properly handled, escaping detection (Törrönen 2009). As a consequence, the ET content reported in the literature is highly variable, since different extraction and acid hydrolysis conditions significantly affect the yield of EA (Törrönen 2009). Earlier studies relied on the quantification of released EA and did not consider other phenolics formed during hydrolysis, which may provide helpful information on the chemical structure of ETs.

For this purpose we describe how to perform and interpret an optimized hydrolytic procedure (6 h with 4 M HCl), which includes quantification of all the four products of hydrolysis and provides a rationale for estimating the mean degree of polymerization (mDP) of *Rubus* ETs. The approach described below is essentially as reported by Vrhovsek et al. (2006), with some minor improvements in terms of quantification, in order to include all the products of hydrolysis in the computation of the mDP. The composition of eight blackberry samples is discussed as an example of the application of this method. Such an approach may also be extended to the analysis of hydrolyzable tannins from other botanical sources, provided that sufficient information on their structure is available (Koponen et al. 2007).

20.4.1 Procedure

20.4.1.1 Standards and Solvents

All chromatographic solvents should be HPLC grade: acetonitrile, methanol, diethyl ether, hexane, formic acid, acetic acid, and hydrochloric acid. EA standard (purity ≥ 96%) and methyl gallate standard (purity ≥ 98%) are both available from Fluka (Steinheim, Germany). Sanguisorbic acid (SA) and methyl sanguisorboate are not currently available, but their concentration can be estimated by applying the available molar extinction coefficients (see Section 20.4.3).

20.4.1.2 Sampling and Extraction of Polyphenols

Freshly collected samples of blackberries (*Rubus fruticosus*) from eight different cultivars were produced under standardized conditions in the experimental fields of the Edmund Mach Foundation (Vrhovsek et al. 2008). Polyphenols were extracted from freshly collected berries following the method of Mattivi et al. (2002) in which 60 g of fresh fruit are homogenized in a model 847-86 Osterizer blender at speed one in 250 mL of acetone/water mixture (70/30 v/v) for 1 min. Prior to extraction, the fruit and extraction solution should be cooled to 4°C to limit enzymatic and chemical reactions. The centrifuged extracts can be stored at –20°C until analysis, conditions under which the composition remains stable for a few months.

20.4.1.3 Sample Preparation

An aliquot (20 mL) of the extract is evaporated to dryness in a 100 mL pear-shaped flask by rotary evaporation under reduced pressure at 40°C. The sample is then brought back to 20 mL with methanol immediately prior to processing, due to the limited solubility of EA and its derivatives.

20.4.1.4 Acid Hydrolysis

A 6 h hydrolysis with 4 M HCl at 85°C has been shown to provide the maximal yield for the four hydrolysis products of *Rubus* ETs and has also been reported to be appropriate for strawberry extracts (Vrhovsek et al. 2006; Mertz et al. 2007). In order to carry out acid hydrolysis in 4 M HCl, 16.6 mL of 37% HCl are added to the sample prepared as above and the mixture is then diluted to 50 mL with methanol. After hydrolysis, the sample is brought back to its initial volume (50 mL) with methanol. An aliquot (10 mL) is then adjusted to pH 2.5 with 5 N NaOH and diluted to 20 mL with methanol. Finally, an aliquot (2 mL) is filtered with 0.22 µm, 13 mm polytetrafluoroethylene (PTFE) syringe-tip filters (Millipore, Bedford, MA) and transferred into LC vials for HPLC analysis.

20.4.2 HPLC-DAD-ESI-MS Analysis

20.4.2.1 HPLC-DAD Analysis

HPLC analysis before and after hydrolysis was carried out according to Vrhovsek et al. (2006) using a Waters 2690 HPLC system equipped with Waters 996 DAD (Waters Corp., Milford, MA), and Empower Software (Waters). Separation is carried out using a 250 × 2.1 mm i.d., 5 µm, endcapped reversed-phase Purospher Star column (Merck) and 4 × 4 mm, 5 µm, Purospher precolumn. The solvents are: A (1% formic acid in water) and B (acetonitrile). The gradients are as follows: from 0% to 5% B in 10 min, from 5% to 30% B in 30 min. The column is then washed with 100% of B for 2 min and equilibrated for 5 min prior to each analysis. The flow rate is 0.8 mL/min, the oven temperature set at 40°C, and the injection volume is 10 µL. EA and its derivatives are detected and quantified by UV detection at 260 nm. EA (RT = 30.8 min) is quantified following calibration with an EA standard (concentration range of 10–200 mg/L). Methyl sanguisorboate (RT = 34.6 min) and free SA (RT = 25.1 min) are quantified following calibration with the pure standard isolated according to Vrhovsek and co-workers (2006). Methyl gallate (RT = 21.8 min) is quantified following calibration with the corresponding standard compound within the concentration range 3–30 mg/L.

20.4.2.2 HPLC-ESI-MS Analysis

Detailed compound identification was carried out using the Micromass ZQ electrospray ionization-mass spectrometry (ESI-MS) system (Micromass, Manchester, UK). The mass spectrometry (MS) detector operated at capillary voltage 3000 V, extractor voltage 6 V, source temperature 105°C, desolvation temperature 200°C, cone gas flow (N_2) 30 L/h, and desolvation gas flow (N_2) 450 L/h. The outlet of the HPLC system was split (9:1) to the ESI interface of the mass analyzer. ESI-mass spectra ranging from *m/z* 100 to 1500 were taken in negative mode with a dwell time of 0.1 s. The cone voltage was set in scan mode at the values of 20, 40, and 60 V. Typical ions for the ESI-MS detection in negative mode are: EA, molecular ion at *m/z* 301; SA, molecular ion at *m/z* 469 and main fragment at *m/z* 301; methyl sanguisorboate, molecular ion at *m/z* 483 and main fragments at *m/z* 315 and *m/z* 301; and methyl gallate, molecular ion at *m/z* 183.

20.4.3 Molar Extinction Coefficients

Care must be taken in choosing the appropriate standard and comparing data obtained using different methods. Here we summarize a comprehensive list of the experimental values of the molar extinction coefficients reported in the literature (Vrhovsek et al. 2006; Gasperotti et al. 2010) and expressed as M^{-1} cm^{-1}.

20.4.3.1 Molar Extinction Coefficient at Maximal Absorption in Methanol

In methanol, at maximal absorption, EA: $\varepsilon_{254\ nm} = 40{,}704$, $\varepsilon_{365\ nm} = 8066$; methyl sanguisorboate: $\varepsilon_{254\ nm} = 58{,}543$, $\varepsilon_{371\ nm} = 13{,}022$; methyl gallate: $\varepsilon_{274\ nm} = 11{,}818$.

20.4.3.2 Molar Extinction Coefficient at 260 nm in Methanol

In methanol, at 260 nm, EA: $\varepsilon_{260\,nm} = 32{,}099$, sanguiin H-6: $\varepsilon_{260\,nm} = 72{,}070$, lambertianin C: $\varepsilon_{260\,nm} = 104{,}344$.

20.4.3.3 Molar Extinction Coefficient for HPLC Analysis

In the conditions suggested for HPLC analysis with UV detection (Section 20.4.2), the molar extinction coefficient is as follows: EA: $\varepsilon_{260\,nm} = 35{,}822$ (solvent: 21% of acetonitrile in 1% formic acid in water; v/v), methyl sanguisorboate: $\varepsilon_{260\,nm} = 45{,}114$ (23.9% of acetonitrile in 1% formic acid in water; v/v).

In the slightly different conditions suggested for HPLC analysis by Gasperotti et al. (2010), where the separation is adapted to a C18 Luna column (solvent: 88% of acetonitrile and 12% of 1% formic acid in water; v/v): EA: $\varepsilon_{260\,nm} = 28{,}266$, sanguiin H-6: $\varepsilon_{260\,nm} = 63{,}615$, lambertianin C: $\varepsilon_{260\,nm} = 95{,}744$.

20.4.4 UV Quantification

To encourage the application of this method, overcoming the lack of a methyl sanguisorboate standard, the molar absorbivity of pure standards of methyl sanguisorboate and EA, measured at the optimal wavelength for UV detection in HPLC analysis, can be exploited. The ratio of the molar absorbivity at $\lambda = 260$ nm of methyl sanguisorboate vs. EA is 1259. This value is in agreement with the presence of three and two galloyl units in methyl sanguisorboate and EA, respectively, and has been found to be consistent with the experimental response of the two compounds in the HPLC analysis conditions reported above (Vrhovsek et al. 2006).

20.4.5 Principle and Application of the Method

20.4.5.1 Presence of ETs and EACs in Rubus *Extracts*

The 13 structures of *Rubus* ETs thus far described (Gasperotti et al. 2010) are in agreement with the assumption that *Rubus* oligomeric ETs contain only the sanguisorboyl linking ester group, besides the well-known EA and gallic acid moieties. All known *Rubus* oligomeric ETs share a common structure, originating in C–O oxidative coupling. More specifically, the linking unit in *Rubus* ETs comes from the donation of galloyl hydroxyl oxygen to form an ether linkage to an HHDP group, which produces the class of GOD-type ETs (Okuda et al. 2009). Blackberries were reported to contain on average 1080 mg/kg of ETs and 200 mg/kg of ellagic acid conjugates (EACs). Lambertianin C (Figure 20.3) is the main ET in blackberries, with an average lambertianin C/sanguiin H-6 ratio of 1.7 (range 0.9–3.4). It must be underlined that besides the 13 known ETs, *Rubus* extracts contain at least five other minor compounds, whose structures are still unknown (Gasperotti et al. 2010).

20.4.5.2 Four Products Are Obtained from ETs Using Acid Hydrolysis in Methanol

The presence of EA and one or two unidentified compounds with absorbance spectra very similar to that of EA after acid hydrolysis of red raspberry and strawberry samples has been reported by some authors (Rommel and Wrolstad 1993; Mattila and Kumpulainen 2002; Määttä-Riihinen et al. 2004). More recently, Vrhovsek et al. (2006) demonstrated the formation of methyl gallate, methyl sanguisorboate, and a minor unknown EA derivative, named "derivative 1," during hydrolysis, in addition to EA. On the basis of UV and MS data already reported (Vrhovsek et al. 2006) and further confirmation by accurate MS and MS/MS, the latter was shown to be SA.

The updated scheme of the reaction is shown in Figure 20.3. Oligomeric ETs, such as in the example of lambertianin C, do not release only ellagic acid and methyl gallate. Also the sanguisorboyl linking ester groups are hydrolyzed, yielding methyl sanguisorboate as the main product, only a limited fraction

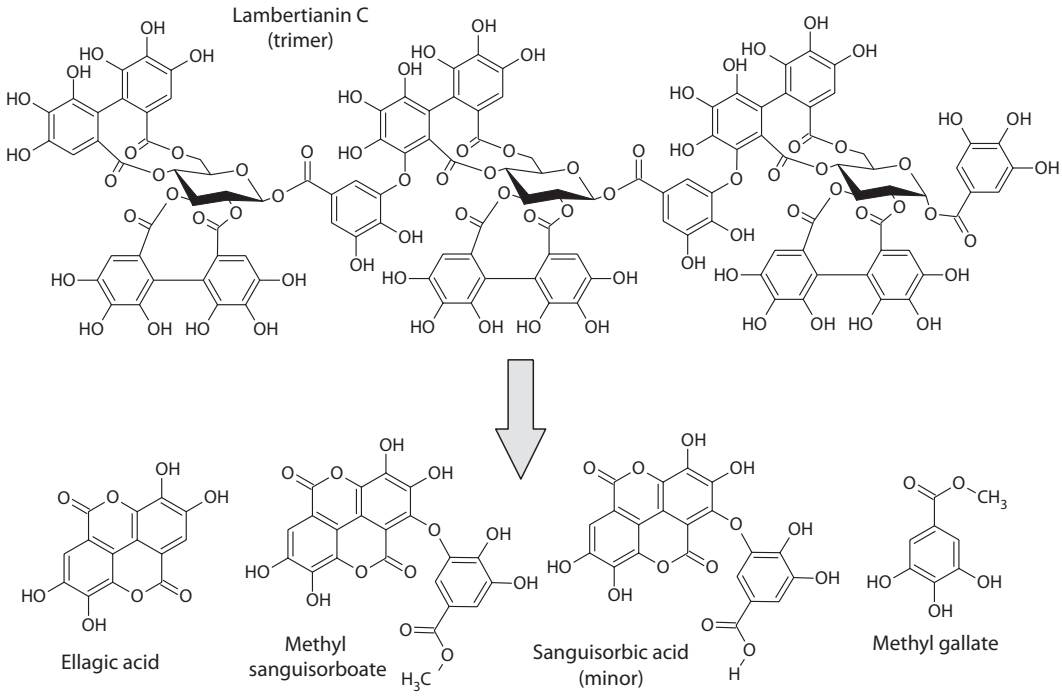

FIGURE 20.3 Updated scheme for hydrolysis, which accounts for the presence of oligomers. Besides ellagic acid and methyl gallate, the sanguisorboyl linking ester groups are released mainly as methyl sanguisorboate. A limited fraction of sanguisorbic unit escapes esterification and can be found after hydrolysis in its free form.

of sanguisorbic unit escapes esterification and can be found after hydrolysis in its free form. Four compounds are quantified by HPLC after the hydrolysis.

20.4.5.3 Detailed Composition of Blackberries

Table 20.2 gives the quantitative composition of eight blackberry samples, analyzed using the HPLC-DAD method of Gasperotti et al. (2010). EA and ETs were quantified using UV detection at 260 nm. EA and its conjugates were quantified following calibration with EA standard. Sanguiin H-6 and lambertianin C were quantified following calibration with the pure standard, and other ETs were quantified as equivalents of sanguiin H-6. For each known structure, Table 20.2 also gives the theoretical number of the three moieties (ellagic, gallic, and SAs), that should be released from complete hydrolysis.

20.4.5.4 Data Interpretation

Under these conditions, the amount of EA measured after hydrolysis not only derives from the breakdown of ETs, but also includes free EA and the product of the hydrolysis of EA glycosides, usually present in *Rubus* extracts (Gasperotti et al. 2010). The interference of free EA with the assay can be avoided by using a blank analysis of the sample before hydrolysis. However, this makes it necessary to double the number of HPLC analyses and is therefore not usually performed. Other EACs, such as the methyl-EA glycosides (i.e., peaks 25 and 26 in Table 20.2), are expected to release the different isomers of methyl-EA, which do not interfere with the ET estimate, since under the suggested conditions they elute as separate peaks (two peaks with molecular ion at *m/z* 315 in the case of blackberries) after methyl sanguisorboate.

TABLE 20.2

Quantification of Ellagitannins and Ellagic Acid Conjugates in Blackberries, Expressed in mg/kg, Quantified Using the Direct HPLC-DAD Method Suggested

Cultivar	1	2	3	4	5	6	9	10	13	14	15	16	17	18	19	Sanguiin H-2	Lambertianin C	Sanguiin H-6	EA	25	26	Total ETs	Total EACs
Apache	14.2	10.0	13.6	11.0	10.6	13.3	155.3	26.2	34.9	n.d.	n.d.	36.2	163.1	n.d.	9.5	16.1	205.1	152.5	74.1	86.3	68.4	872	229
Black satin	19.0	9.1	18.7	19.1	12.3	14.7	213.9	25.3	42.5	n.d.	9.0	16.7	222.4	10.4	12.8	18.0	353.0	324.9	45.6	75.6	n.d.	1342	121
Cacak	13.9	10.2	17.9	n.d.	n.d.	14.1	83.4	28.6	n.d.	10.2	n.d.	42.8	95.3	8.9	15.2	n.d.	671.7	256.2	61.9	96.9	53.3	1268	212
Hull tornless	29.6	10.3	27.3	n.d.	10.1	17.5	99.5	20.5	28.7	n.d.	9.3	19.5	97.9	14.5	19.2	20.5	559.2	550.5	103.5	108.7	69.0	1534	281
Kotata	16.8	9.7	24.2	n.d.	n.d.	15.4	164.3	n.d.	24.8	n.d.	n.d.	n.d.	179.5	n.d.	11.1	n.d.	671.5	257.6	85.0	93.1	90.4	1375	269
Lochness G	17.2	9.0	16.4	13.6	16.3	15.4	149.8	36.2	21.3	8.6	11.2	26.2	149.4	11.9	12.0	10.8	299.7	292.5	50.4	73.4	n.d.	1118	124
Lochtay	15.7	10.6	21.8	n.d.	n.d.	12.5	111.8	14.0	23.0	n.d.	n.d.	18.1	132.5	9.5	13.3	10.9	756.1	354.4	68.3	102.2	79.2	1504	250
Triple crown	16.8	11.4	20.9	n.d.	n.d.	11.5	81.8	30.0	n.d.	17.9	n.d.	57.1	91.2	9.2	14.8	10.9	615.7	279.4	72.7	115.4	39.3	1269	227
Molecular size	2	2	3	?	2	3	?	2	?	3	?	?	3	2	3	1	3	2	0	0	0		
n° of ellagic acid units	2	3	3		2	3		2		3			3	2	3	1	4	3	1	0	0		
n° of gallic acid units	1	0	1		1	1		1		1			1	1	1	1	1	1	0	0	0		
n° of sanguisorbic acid units	1	1	2		1	2		1		3			2	2	2	1	2	1	0	0	0		
n° of methyl–ellagic units	0	0	0		0	0		0		0			0	0	0	0	0	0	0	1	1		

Source: Adapted from Gasperotti, M. et al. 2010. *J. Agric. Food Chem.* 58: 4602–4616.

It should be highlighted that the absolute values obtained with the two independent methods, that is, indirect HPLC-DAD measurement of total ETs plus EACs after hydrolysis, externally calibrated with the building units (EA, methyl sanguisorboate, methyl gallate, and SA) are only comparable in terms of the order of magnitude, but do not overlap with the more precise HPLC data obtained separately for ETs and EACs using the direct quantification method (Gasperotti et al. 2010), where sanguiin H-6 and lambertianin C were used as external standard for the ETs, and EA for EACs. The indirect method was reported to have a much lower repeatability, with CV% around 12% for the two major compounds and 18% for the minor compounds (Vrhovsek et al. 2008), and can be considered as an acceptable and cheaper way of providing an estimate of the total quantity of EA derivatives.

20.4.5.5 Computation and Interpretation of mDP

The simultaneous quantification of the four main hydrolytic products of *Rubus* ETs provides direct measurement of the relative molar abundance of the building units of ETs. According to the method developed by Vrhovsek et al. (2006), and also including the presence of free SA in the updated method, the estimate of the mDP of *Rubus* ETs can be theoretically derived from the molar ratio between the sanguisorboyl units (methyl sanguisorboate plus SA) and the EA produced in the reaction, $R_{[MS+SA]/[EA]}$. The experimental value is highly reproducible, with a CV% of around 2% (Vrhovsek et al. 2008). Taking into consideration the structure of the major known *Rubus* ETs (Gasperotti et al. 2010), and assuming complete hydrolysis, this ratio is expected to increase from 0 for the monomers (galloyl-bis-HHDP-glucosides) up to a value of 0.60 for tetrameric lambertianin D, with intermediate values for dimeric sanguiin H-6 and trimeric lambertianin C (Vrhovsek et al. 2006). For oligomeric compounds such as sanguiin H-6 and lambertianin C, which have been shown to account for 67% (range 41–83%) of ETs in blackberries (Gasperotti et al. 2010), as well as for the other lambertianin oligomers, this ratio is expected to increase according to the equation $R_{[MS+SA]/[EA]} = (DP - 1)/(DP + 1)$. In conclusion, the value of $R_{[MS+SA]/[EA]}$ can be obtained experimentally from HPLC analysis of the hydrolytic products of raw *Rubus* extract and can be used for computation of the mDP of *Rubus* ETs, which can be derived from the following equation: $mDP = (R_{[MS+SA]/[EA]} + 1)/(1 - R_{[MS+SA]/[EA]})$.

Table 20.3 gives an example of practical workflow. From the experimental values obtained from the HPLC run after hydrolysis, expressed in mg/L, the data can be converted into mg/kg in order to give the concentration in the berries and can be converted in mmol/L, which are used for the computation of *R* and mDP.

The application of this method to the eight raspberry samples in our example gives an average mDP of ca. 1.9 (Table 20.3). This value is slightly higher than reported in a previous survey (Vrhovsek et al. 2008), also due to the inclusion of the contribution of free SA in the updated formula. The correction is not major since the latter is on average ca. 7 times less concentrated than methyl sanguisorboate (last column in Table 20.3). An mDP value close to 2 suggests that the sum of oligomers with DP > 2 (such as trimer lambertianin C), or with a lower content of EA (such as the peaks of dimers 1, 5, and 10, as well as of the trimers 3, 6, 17, and 19 in Table 20.2) or with a higher presence of SA (such as the monomer sanguiin H-2, in addition to the peaks of dimer 18 and trimer 14 in Table 20.2) roughly balance the sum of the monomers and free EA in terms of concentration. Such a result is in acceptable agreement with the detailed HPLC data of ETs and EACs reported in Table 20.2.

It should be kept in mind that mDP computed according to this method is estimated, which could lead to misleading values if directly applied to hydrolyzable tannins of a different nature (Koponen et al. 2007). However, sufficient data are available to support its application to the analysis of ETs in botanical species characterized by the presence of GOD-type ETs, as in the case of *Rubus* (e.g., raspberries, blackberries, boysenberries), Sanguisorba, and strawberry samples.

After careful characterization of hydrolysis products, it could also in principle be extended to other sources of ETs, once enough structural information on the chemical structure of ETs and the products of degradation is available.

TABLE 20.3

Quantification of Ellagitannins and Ellagic Acid Conjugates in Blackberries, Quantified Using HPLC-DAD after Acid Hydrolysis

Methylgallate (mg/L)	SA (mg/L)	EA (mg/L)	Methylsanguisorboate (mg/L)	Methylgallate (mmol/L)	SA (mmol/L)	EA (mmol/L)	Methylsanguisorboate (mmol/L)	Methylgallate (mg/kg)	SA (mg/kg)	EA (mg/kg)	Methylsanguisorboate (mg/kg)	R = [MS + SA]/[EA]	mDP = (R + 1)/(1 − R)	Sum ETs mg/kg	Ratio MS/SA
6.5	5.0	138.3	62.7	0.035	0.011	0.457	0.129	27.0	20.7	576.0	261.3	0.306	1.882	901	12.3
12.3	11.3	168.4	69.2	0.067	0.024	0.557	0.143	51.1	47.2	701.9	288.5	0.300	1.856	1130	5.9
16.9	15.8	277.7	125.4	0.092	0.034	0.919	0.259	70.2	65.9	1156.9	522.6	0.318	1.934	1875	7.7
16.6	16.4	287.6	121.1	0.090	0.035	0.952	0.250	69.3	68.5	1198.5	504.5	0.299	1.855	1903	7.2
9.8	15.8	189.9	94.7	0.053	0.034	0.628	0.196	40.9	65.7	791.3	394.6	0.364	2.147	1352	5.8
14.6	10.9	179.1	65.4	0.079	0.023	0.593	0.135	60.7	45.5	746.3	272.5	0.267	1.729	1165	5.8
18.7	15.9	261.0	97.3	0.101	0.034	0.864	0.201	77.7	66.3	1087.7	405.4	0.272	1.746	1697	5.9
20.9	26.7	295.4	135.5	0.113	0.057	0.977	0.280	87.0	111.3	1230.7	564.4	0.344	2.050	2097	4.9

Note: From the experimental values obtained from the HPLC run after hydrolysis of the extract, expressed in mg/L, the data can be converted into mmol/L, which are used for the computation of R and mDP, and into mg/kg in order to give the concentration in the berries.

20.5 HPLC-MS-MS Analysis

To obtain information of the molecular masses of ETs and EA derivatives preliminarily detected by HPLC-DAD, HPLC-ESI-MS analyses of the crude extracts, or fractions obtained from them, can be carried out. However, the chromatographic conditions have to be changed as the acid composition of the mobile phase for HPLC-MS analysis must be milder than those for HPLC-DAD analysis, and 0.1–0.4% formic acid should be used instead (Salminen et al. 1999). MS analysis in the negative mode provides more information on the ET and EA conjugate structures than those in the positive mode.

For most ETs, it is possible to obtain *m/z* values corresponding to $[M–H]^–$, $[M–2H]^{2–}$, or $[2M–H]^–$ depending on the mass of the compound. Often, both monomeric and dimeric ETs give the same *m/z* values, but the $[M–H]^–$ and the $[M–2H]^{2–}$ signals can be separated by their isotopic *m/z* values. For $[M–H]^–$, the isotopic signals differ in 1 m.u., while for the $[M–2H]^{2–}$, the isotopic differences are only 0.5 m.u.

ET fragmentation is produced by the sequential losses of galloyl residues (*m/z* 152, 169, or 170) and HHDP (hexahydroxydiphenic) residues (*m/z* 301). The galloyl units attached to phenolic hydroxyls of other galloyl molecules are more cleavable in the negative ESI-MS than the galloyl units attached directly to the glucose core (Salminen et al. 1999). This means that in the first case, losses of 152 units are found in the MS spectrum while in the last ones, losses of 169 or 170 units are observed instead. In the case of ETs containing catechin moieties, the characteristic cleavage releases an *m/z* of 289 from the flavan-3-ol residue.

For acyclic epimers having hydroxyl groups at C–1, they can be distinguished by the loss of water $[M–H_2O]^–$ from the vescalagin-type ETs, while this is hardly observed from castalagin-type ETs (Moilanen and Salminen 2008).

For ET derivatives with catechins, it is possible to show the place of substitution. When catechin is added to vescalagin-type ETs, as is the case of hippophaenin B, it is linked at C-1 of the glucose residue, as the $[M–H_2O]^–$ signal is not observed and the $[M–catechin–H]^–$ is observed instead.

In addition, it has been shown that the catechin addition did not occur at the carboxyl (–COOH) group, since the MS data showed both the cleavage of catechin and carboxyl group $[M–catechin–H–COOH]^{2–}$.

Characteristic MS fragmentation of vescalagin are: 1103 $[M–H]^–$; 1085 $[M–H_2O–H]^–$; 1041 $[M–H_2O–COOH]^–$; 529 $[M–H–COOH]^{2–}$; and 520 $[M–H_2O–H–COOH]^{2–}$.

Characteristic MS fragmentation of hippophaenin B are: 1375 $[M–H]^–$; 1085 $[M–catechin–H]^–$; 687 $[M–2H]^{2–}$; 665 $[M–H–COOH]^{2–}$; 520 $[M–catechin–H–COOH]^{2–}$; and 289 $[catechin–H]^–$.

Oligomeric ETs can be detected and characterized by LC/ESI-MS, by examination of the multicharged ions, and by looking at the isotopic ions (Karonen et al. 2010).

For EA conjugates, the molecular masses are easily observed in the negative mode of the HPLC-ESI-MS as $[M–H]^–$ pseudomolecular ions (*m/z* 463 for EA-hexosides; *m/z* 447 for EA-rhamnosides, and *m/z* 433 for EA-pentosides), and the loss of the sugar residue (M-162 for hexosides, M-146 for rhamnosides, and M-132 for pentosides) released the EA molecule (*m/z* 301).

In addition, HPLC-ESI-MS allows the detection of ET oligomers, dimers and trimers being quite common (i.e., oenothein A and B, respectively), and even hexameric and heptameric ETs have been recently evidenced (Karonen et al. 2010). For the identification of these oligomers, the use of high-resolution MS, and the analysis of the isotopic patterns are essential for the detection of the pentamers, hexamers, and heptamers (Karonen et al. 2010).

20.6 NMR Analysis

20.6.1 Gallotannins

As far as the gallotannins are concerned, most studies concern the detection and identification of these polygalloylglucoses in various natural sources (e.g., the traditional Chinese herb *Galla chinensis*) using

analytical techniques (Mueller-Harvey 2001) such as HPLC-MS (Salminen et al. 1999; Tian et al. 2009) or MALDI-TOF mass spectrometry protocols (Xiang et al. 2007), or relying on degradation studies, for example, using tannases (i.e., galloyl esterase) (Mingshu et al. 2006) or performing mild methanolysis in methanolic acetate buffer (pH 5.5) (Haslam et al. 1961), followed by mass spectrometry analysis of the residues. Several gallotannins, from hexa- up to tetradecagalloyl glucoses, were thus observed in such investigations, but no structural determination of the different isomers could be achieved. In contrast, pure gallotannins, which were obtained either through isolation from natural sources or by enzymatic or chemical synthesis, could be successfully characterized by nuclear magnetic resonance (NMR) spectroscopy.

20.6.1.1 Isolated Gallotannins

In the early 1980s, Nishioka and coworkers reported the isolation of several pure gallotannins from various medicinal plant extracts such as *Rhus semialata* (Chinese gall), *Quercus infectoria* (Turkish gall), *Paeoniae albiflora* (syn. *P. lactiflora*), and *Moutan cortex* (Nishizawa et al. 1980a,b, 1982, 1983a,b). Each component could be separated from the plant extract according to the degree of galloylation using a combination of Sephadex® LH-20 column chromatography and normal-phase HPLC. The structures of the resulting individual gallotannins were next determined mainly by ^{1}H and ^{13}C NMR spectroscopy. The position of digalloyl depside motifs onto the glucopyranose core was determined by comparison of the ^{13}C NMR chemical shifts recorded in acetone-d_6 of both the glucose carbon atoms and the carbonyl atoms of the galloyl groups with those of β-PGG. Typically, a downfield shift of 0.4–0.6 ppm is observed for a glucose carbon atom linked to a digalloyl depside, which is in accordance with the difference measured between the ester methyl carbons of methyl *meta*-digallate and methyl gallate (δ 52.2 and 51.9 ppm, respectively). In addition, the carbonyl carbon signals of the proximal depsidically linked galloyl moieties were observed at ca. 0.6 ppm upfield. These ^{13}C NMR spectroscopic analyses allowed Nishioka and coworkers to demonstrate that the digalloyl depside moiety of the hexagalloylglucose isolated from the root of *Paenioae albiflora* (i.e., *P. radix*) and *Moutan cortex* was attached to the C-6 position of the glucopyranose (i.e., **1d**), but that of *Galla chinensis* was randomly distributed among the C-2, C-3, and C-4 positions (i.e., **1b**, **1a**, and **1c**, respectively) (Nishizawa et al. 1980a). Similar ^{13}C NMR spectra analysis led to the structural determination of six heptagalloylglucoses, featuring either two digalloyl groups (i.e., **2a–e**) or one trigalloyl motif (i.e., **2f**), and even one octagalloylglucose bearing three digalloyl groups (i.e., **3**) (Figure 20.4). Moreover, the investigations carried out by Nishioka and coworkers also revealed that the previously reported *meta*-depsidic digalloyl units (Fischer 1914; Nierenstein et al. 1925) are in fact equilibrium mixtures of *meta*- and *para*-depsidically linked galloyl units resulting from intramolecular transesterification (Nishizawa et al. 1982). This conclusion was supported by the analysis and comparison of ^{13}C NMR spectra, run in acetone-d_6, of pure gallotannins with those of methyl *meta*- and *para*-digallate, notably by carefully examining both the carbonyl and the aromatic regions.

20.6.1.2 Enzymatically and Chemically Synthesized Gallotannins

Within the framework of their studies on the biosynthesis of hydrolyzable tannins, Gross and coworkers intensively investigated enzymatic synthesis of gallotannins (Gross 1999, 2008; Niemetz and Gross 2005). Experiments carried out *in vitro* with cell-free extracts from leaves of staghorn sumac (*Rhus typhina*) and β-PGG as a standard acceptor substrate led to the isolation of β-glucogallin-dependent galloyltransferases (Niemetz and Gross 2005; Gross 2008). It was found that none of these enzymes displayed high substrate specificity, but some of them preferentially acylated β-PGG to give the 2-, 3-, or 4-*O*-depsidic digalloylated hexagalloylglucoses **1a–c**, while others preferentially catalyzed the galloylation of hexa- and heptagalloylglucoses to furnish, for example, 3-*O*-trigalloyl-1,2,4,6-tetra-*O*-galloyl-β-d-glucopyranose (**2f**) and higher galloylated gallotannins (Figure 20.4). Structural determination of **1a–c** and **2f** was accomplished mainly by ^{1}H NMR spectroscopy, and further comparison with the materials isolated by the Nishioka group. The ^{1}H NMR spectra recorded

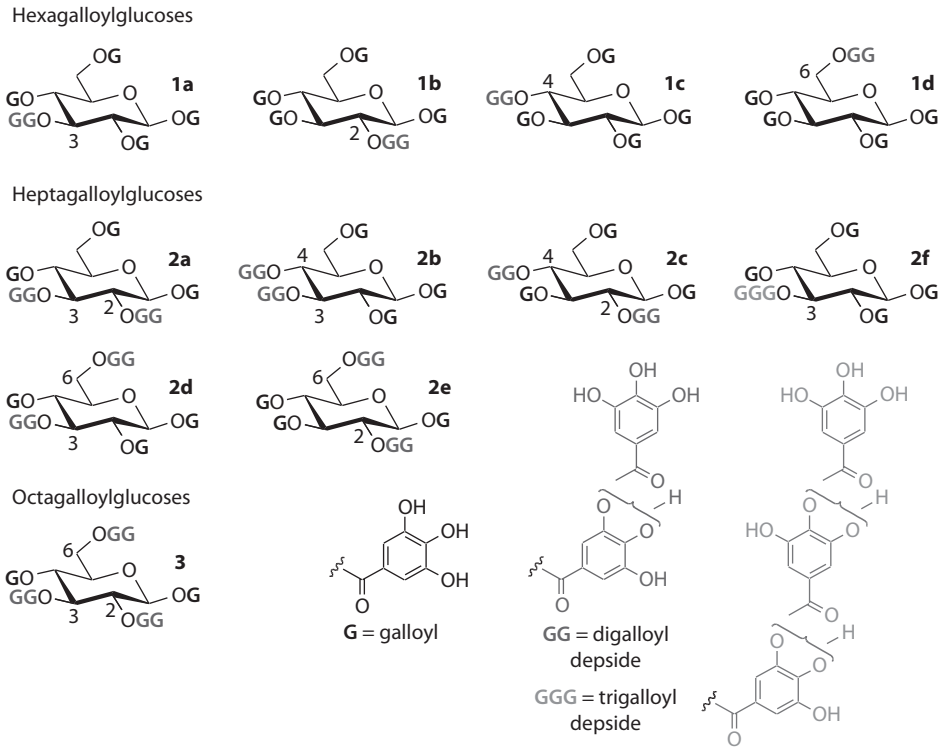

FIGURE 20.4 Selected examples of depsidic di- and trigalloyl-containing glucoses.

in acetone-d_6 were compared with that of β-PGG, and *meta*-depsidic digalloyl moieties were typically detected from the diagnostic appearance of doublets for the aromatic protons of the proximal galloyl group, instead of the initially observed set of two singlets, as the result of the disymmetry introduced by the formation of the *m*-depsidic link. In addition, these signals were shifted from the 7.00–7.10 ppm region (singlets) to significantly higher δ values of 7.25–7.55 ppm (Hofmann 1996; Gross 1999). In contrast, the aromatic hydrogens of newly introduced distal galloyl residues generally displayed the expected sharp singlets at 7.10 ppm (Hofmann 1996; Gross 1999). It is worth noting that no NMR evidence of *meta*/*para*-depsidic digalloyl equilibrium mixtures was reported by the Gross group.

To the best of our knowledge, despite a few chemical studies on depside motifs carried out in the early 1900s (Fischer 1914; Nierenstein et al. 1925), no total chemical synthesis of "complex" (or "true") gallotannins, as opposed to "simple" gallotannins, that is, their mono- to pentagalloylglucose precursors, has been reported thus far. The chemical elaboration of *meta*-depsidic digalloyl units acylating a glucose core has been reported only by Romani and coworkers in their synthesis of the 2,3-bis-*O*-digalloylglucose (Arapitsas et al. 2007). They also showed that UV–visible spectra of compounds featuring the *m*-depsidic digalloyl moiety display a characteristic shoulder at 300 nm (Arapitsas et al. 2007). In order to determine the influence of the gallotannin depsidic link on the biological activities of hydrolyzable tannins, Quideau and coworkers recently engaged efforts in the total synthesis of the hexagalloylglucose **1a**, the heptagalloylglucose **2f**, as well as the decagalloylglucose, referred to as "tannic acid" and whose commercial sample is known for not being a structurally well-defined gallotannin but rather a complex mixture of various gallotannin species and derivatives thereof (Mueller-Harvey 2001; Romani et al. 2006). Full characterization of the chemically pure synthetic gallotannins **1a**, **2f**, and tannic acid (Figure 20.5), as well as their α-anomeric analogs, was accomplished by 1D and 2D NMR experiments (i.e., ^{1}H and ^{13}C NMR, COSY H–H, heteronuclear multiple quantum coherence, and heteronuclear multiple bond

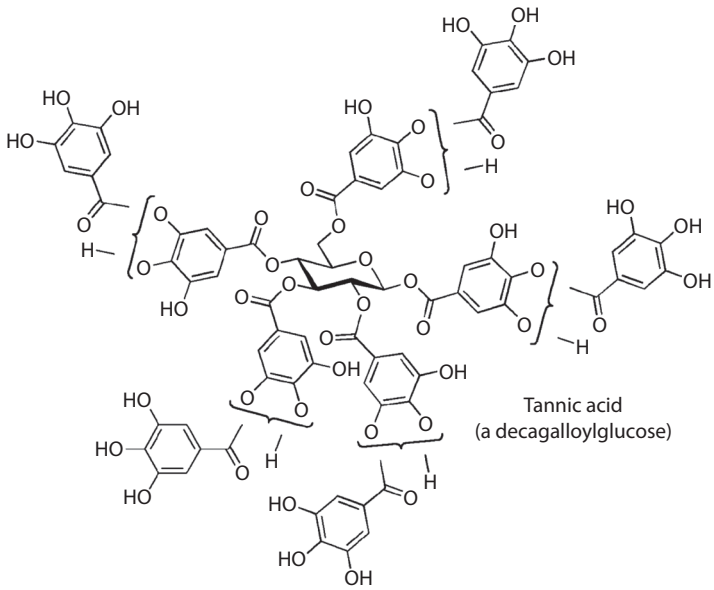

FIGURE 20.5 Structure of tannic acid.

coherence [HMBC]) and mass spectrometric analysis (Sylla 2010). The *meta*/*para*-depsidic equilibrium was confirmed by ^{1}H and ^{13}C NMR, and could even be evaluated as a ca. 2:1 ratio by ^{1}H NMR spectroscopic analysis.

20.6.2 Ellagitannins

The ETs constitute the second subclass of hydrolyzable tannins, which is today composed of nearly 1000 members that have been identified and fully characterized following their isolation from various plants (Haslam and Cai 1994; Quideau and Feldman 1996; Okuda et al. 2009; Yoshida et al. 1992, 2009). The chirality of their characteristic HHDP (i.e., 6,6′-dicarbonyl-2,2′,3,3′,4,4′-hexahydroxybiphenyl) units is a consequence of the prevention of free rotation around their biaryl axis, which is impeded by the presence of the phenolic hydroxyl groups and the glucose-esterified carboxyl groups *ortho*-positioned relatively to that carbon–carbon bond axis (Quideau and Feldman; 1996; Khanbabaee and van Ree 2001b). The determination of the configuration (*R* or *S*) of this axial chirality or atropisomerism is an essential step of the structural characterization of ETs (Figure 20.6), together with the determination of the regiochemistry of all galloyl and galloyl-derived units on the glucose core and that of the stereochemistry at the anomeric position of the latter.

20.6.2.1 Absolute Configuration of ET Axially Chiral Biaryl Groups

Until 1982, the axial chirality of ET HHDP biaryl units was determined by a chemical degradation procedure beginning with a methylation step using dimethyl sulfate and potassium carbonate (Tanaka et al. 1986; Yoshida et al. 2000). The permethylated biaryl unit was then cleaved from the glucopyranose core by methanolysis using sodium methoxide in methanol. The chirality of the hexamethoxydiphenic acid derivative thus released under its native atropisomeric form was then determined by comparison with atropisomerically pure standards, the main drawback of this procedure being the degradation of the samples.

However, since 1982, a more suitable and nondestructive procedure has been developed using circular dichroism spectroscopy to establish the absolute stereochemistry of HHDP units linked to the ET glucopyranose core. Okuda et al. (1982a,b, 1984) showed that the Cotton effects observed near 220–230 and

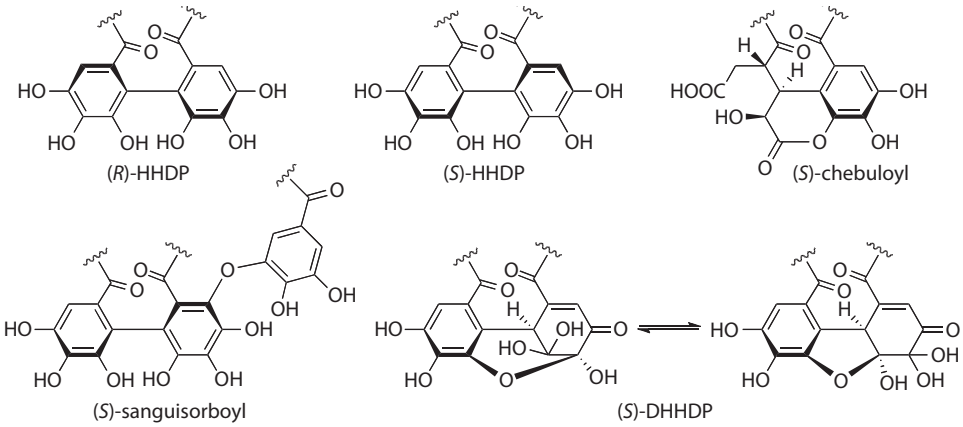

FIGURE 20.6 Structure of the (*R*)-HHPD, (*S*)-HHPD, (*S*)-chebuloyl, (*S*)-DHHPD, and (*S*)-sanguisorboyl units.

250–260 nm were correlated with the absolute configuration of the HHDP units. An (*S*)-configured HHDP group is characterized by a positive and a negative Cotton effect at 220–230 and 250–260 nm, respectively, whereas an (*R*)-configured HHDP group exhibits instead a negative and a positive effect at the same values, respectively. Similar Cotton effects are also observed in the case of other HHDP-derived groups such as the DHHDP, chebuloyl, and sanguisorboyl groups, as well as for the *C*-glucosidic ET nonahydroxyterphenoyl (NHTP) group (Yoshida et al. 2000). Moreover, this characteristic Cotton effect is not influenced by the position of the biaryl group or by the presence of galloyl groups on the glucopyranose core, making this procedure applicable to any ETs (Okuda et al. 1982a,b). Interestingly, ET HHDP units linked to the 2,3- or 4,6-positions of a $^4C_1$-glucopyranose core, or to the 1,6-positions of a $^1C_4$-glucopyranose core, are predominantly, and to a large extent, (*S*)-configured, while those linked to the 2,4- or 3,6-positions of a $^1C_4$-glucopyranose core are essentially always (*R*)-configured (Quideau and Feldman 1996; Khanbabaee and van Ree 2001b).

20.6.2.2 Determination of the Position of ET Galloyl-Derived Acyl Units

Proton and carbon NMR analyses, together with partial hydrolysis procedures under mild conditions, provide useful structural information on ETs, such as the nature and number of galloyl and galloyl-derived acyl groups esterified to the glucopyranose core (e.g., galloyl, HHDP, DHHDP, sanguisorboyl groups). However, the crucial step in the structural determination of monomeric or oligomeric ET is to establish the position of each acyl group. For this purpose, 2D long-range HMBC experiments provide a straightforward assignment by establishing two key three-bond correlations; one between the ester carbonyl carbon and an aromatic proton of the acyl unit, and another one between the same ester carbonyl carbon and the proton on the glucopyranose position at which the ester bond is connected, as shown on the punicalagin structure in Figure 20.7.

20.6.2.3 Determination of the Absolute Configuration of the Anomeric Carbon

The determination of the absolute configuration at the anomeric position of the glucopyranose core is another important point in the structural elucidation of ETs, especially since in some cases both α- and β-anomer can be in solvent-dependent equilibrium, like in the case of punicalagin (Figure 20.7) (Lu et al. 2008). Furthermore, in some dimeric ETs, for example, each glucopyranose moiety can possess an anomeric center with a different configuration. For example, in sanguiin H-6, the glucose moiety "1" displays a β-configuration at that locus (i.e., equatorial orientation of the sanguisorboyl group), whereas the glucose "2" shows an α-configuration (Figure 20.8) (Tanaka et al. 1985; Gasperotti et al., 2010; Kool et al. 2010).

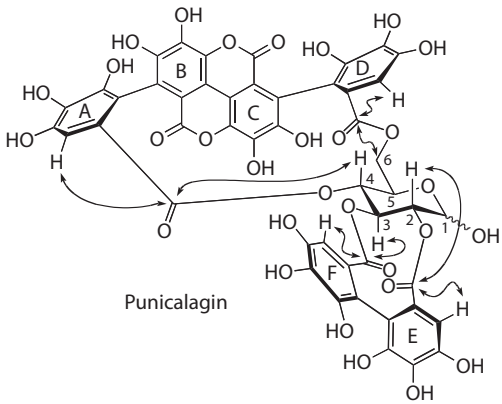

FIGURE 20.7 Structure of punicalagin.

The stereochemistry at the anomeric position can be easily determined by measuring, on the ^{1}H NMR spectrum, the coupling constant between the anomeric proton H-1 and the adjacent proton H-2. A large coupling constant ($J_{H\text{-}1,H\text{-}2} > 5$ Hz) generally indicates a β-glucosidic configuration (i.e., substituent with an equatorial orientation), whereas a small coupling constant ($J_{H\text{-}1,H\text{-}2} \approx 0$–5 Hz) indicates an α-glucosidic configuration (i.e., substituent with an axial orientation). The presence or absence of a galloyl or galloyl-derived acyl group at the anomeric position has only a minor influence on the value of this H-1, H-2 coupling constant (Table 20.4). Moreover, the chemical shift of the anomeric proton H-1 of an α-anomer usually presents a lower-field shift compared to that of the corresponding β-anomer (Hatano et al. 1988). These differences are also observed in oligomeric ellagitannins such as the dimers Sanguiin H-6 and rubusuarin B, and in the trimers lambertianin C and rubussuarin C (Gaspertotti et al., 2010). In contrast, the chemical shifts of the glucopyranosic carbons C-1, C-2, C-3, and C-5 of an α-anomer are up-fielded compared to those of its corresponding β-anomer. For example, the chemical shifts of the carbons C-1, C-2, C-3, and C-5 of the α-punicalagin (i.e., with an axially oriented hydroxyl group at the anomeric position) are shifted upfield by 4.2, 1.9, 2.4, and 4.8 ppm, respectively, compared with the corresponding signals of the β-punicalagin (Tanaka et al. 1986).

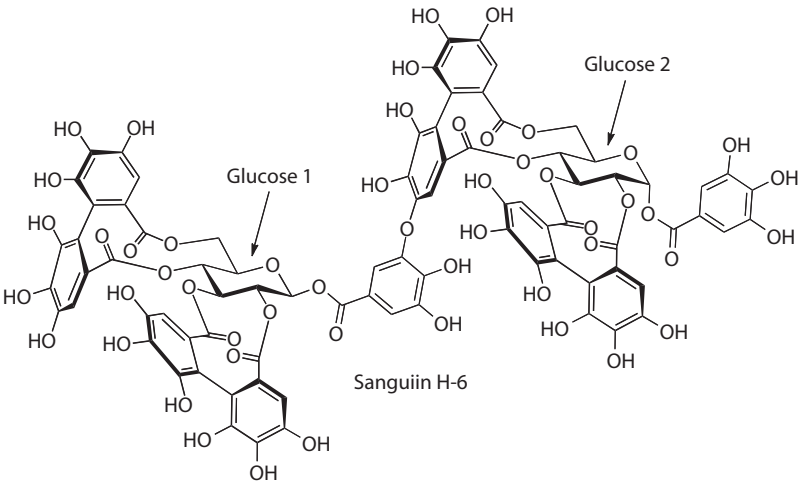

FIGURE 20.8 Structure of sanguiin H-6.

TABLE 20.4

Chemical Shift and Coupling Constant ($J_{H\text{-}1,H\text{-}2}$) of the Anomeric Proton H-1

	α-Anomer	β-Anomer
Gemin D[a]	5.31 (d, J = 4.0 Hz)	4.78(d, J = 7.5 Hz)
Tellimagrandin I[a]	5.57 (d, J = 4.0 Hz)	5.13 (d, J = 8.0 Hz)
Pedunculagin[a]	5.50 (d, J = 3.5 Hz)	5.09 (d, J = 8.0 Hz)
Punicalagin[b]	5.34 (d, J = 3.5 Hz)	5.01 (d, J = 8.0 Hz) (see casuarictin)
Potentillin[c]	6.63 (d, J = 4.0 Hz) (see potentillin)	
Casuarictin[c]		6.22 (d, J = 9.0 Hz)
Sanguiin H-6[d]	6.50 (d, J = 3.5 Hz) (glucose 2)	6.01 (d, J = 8.5 Hz) (glucose 1)

[a] Hatano et al. (1988).
[b] Tanaka et al. (1986).
[c] Okuda et al. (1984).
[d] Kool et al. (2010).

20.6.2.4 About the C-Glucosidic Ellagitannins

The *C*-glucosidic ETs constitute an important subclass of ETs with the structural particularity of having a highly characteristic C–C linkage between the carbon C-1 of an open-chain glucose core and the carbon C-2 of the O-2 galloyl-derived moiety of a 2,3,5-NHTP unit (also known as the flavogalloyl group), such as in vescalagin and castalagin. This characteristic C–C linkage on an open-chain glucose core can also occur with a 2,3-HHDP unit, such as in casuarinin and stachyurin (Figure 20.9). Vescalagin and its C-1 epimer castalagin were the first members of this ET subclass to be isolated from *Castanea* (chestnut) and *Quercus* (oak) species in 1971 (Mayer et al. 1967, 1969, 1971). However, their structures, as well as those of stachyurin and casuarinin (Okuda et al. 1981, 1982c, 1983), were fully determined much later when Nishioka's group revised the assignment of the configuration at the C-1 position of all *C*-glucosidic ETs (Nonaka et al. 1990). This structural revision was based on the observation of spatial correlations between the protons H-1 and H-3 by Nuclear Overhauser Effect Spectroscopy (NOESY) in the corresponding spectra of vescalagin and stachyurin. These correlations are not observed for castalagin and casuarinin, for which these protons H-1 and H-3 are oriented toward a different side of the molecule (Figure 20.9). Furthermore, the observation of such diagnostic cross-peaks on the 2D NOESY spectra of

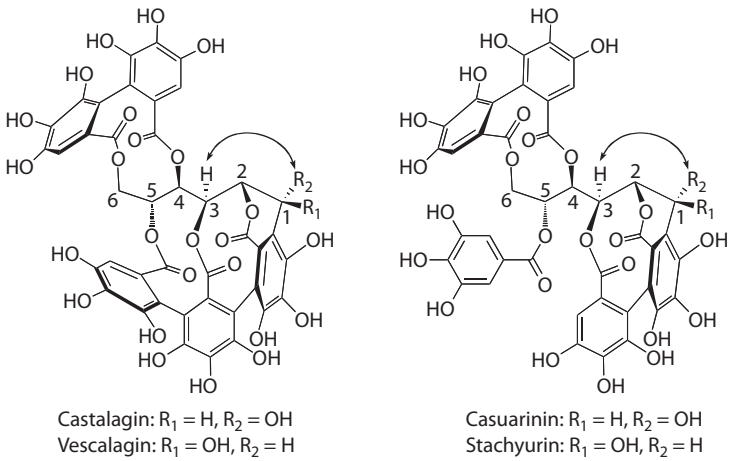

FIGURE 20.9 Structures of main monomeric *C*-glycosidic ellagitannins vescalagin, castalagin, casuarinin, and stachyurin.

vescalagin and stachyurin was confirmed by 1D Nuclear Overhauser Effect (NOE) experiments. These NMR experiments unambiguously established that both protons H-1 and H-3 are α-oriented (R_2=H); thus the C-1 hydroxyl groups of vescalagin and stachyurin are β-oriented (Figure 20.9).

However, NOESY and NOE experiments are not absolutely required for determining the stereochemistry at the C-1 position of *C*-glucosidic ETs. In fact, this stereochemistry can also be easily established from the value of the coupling constant between the proton H-1 and the proton H-2. As first reported by Nishioka's group, a rather large H-1, H-2 coupling constant ($J_{H\text{-}1,H\text{-}2} > 4$ Hz) indicates an α-orientation of the C-1 substituent, like in the case of castalagin and casuarinin, whereas a small coupling constant ($J_{H\text{-}1,H\text{-}2} \approx 0$–2 Hz) corresponds to a β-oriented substituent, like in the case of vescalagin and stachyurin (Nonaka et al. 1990).

20.6.2.5 About the Flavano-Ellagitannins

The *C*-glucosidic ETs subclass also includes the flavano-ETs (also known as complex tannins), which are hybrid entities composed of a *C*-glucosidic ET moiety derived, for example, from vescalagin or stachyurin, and a flavanoid moiety (e.g., flavan-3-ols, procyanidines, and anthocyanins). In these flavano-ET hybrids, the two moieties are connected via C–C linkage between the carbon C-1 of the *C*-glucosidic ET and the carbon C-8 or C-6 of the A ring of the flavanoid. Depending on the nature of each moiety, these complex tannins present a large diversity of structures such as the catechin-based stenophyllanins A/B (Nonaka et al. 1985) and acutissimins A/B (Ishimaru et al. 1987) or the epicatechin-based camelliatannins A, B, and F, and malabathrins A and E (Hatano et al. 1991; Okuda et al. 2009; Yoshida et al. 2009). Moreover, these complex tannins can be further transformed by oxidative means to generate different types of derivatives such as the mongolicains A/B (Nonaka et al. 1988), which contain a *o,m*-hydroxyphenylcyclopentenone motif (also known as the dihydrofuran group). Recently, new types of colored flavano-ETs, named anthocyano-ETs, were obtained by hemisynthesis in aqueous acidic media from vescalagin and the anthocyanin oenin or its malvidin aglycone (Quideau et al. 2005; Jourdes et al. 2009; Chassaing et al. 2010). All of the isolated and characterized flavano-ETs to date present a β-oriented linkage between the flavanoid unit and the *C*-glucosidic ET moiety. These β-configurations were determined by the observation of a small coupling constant between the proton H-1 and the proton H-2 ($J_{H\text{-}1,H\text{-}2} \approx 0$–2 Hz). Such a stereoselectivity was recently rationalized through molecular modeling studies by the Quideau group (Quideau et al. 2003, 2005).

After having characterized the *C*-glucosidic ET moiety using some of the strategies highlighted above, the crucial element of the structural elucidation of a flavano-ET structure is to establish by which A ring carbon (C-8′ or C-6′) the flavanoid unit is connected to the carbon C-1 of the *C*-glucosidic ET unit (Okuda et al. 2009; Yoshida et al. 2009). This connectivity can be established by the observation of two- and three-bond HMBC correlations between proton H-1 and carbons C-7′, C-8′, and C-8′a in the case of a C-8′/C-1 linkage, whereas a C-6′-linked flavanoid unit will show correlations between proton H-1 and carbons C-5′, C-6′, and C-7′ (Figure 20.10) (Jourdes et al. 2009; Quideau et al. 2003, 2005). Moreover, in order to establish this connectivity unambiguously, the HMBC data can be supported by those of Rotational Nuclear Overhauser Effect Spectroscopy (ROESY) experiments that reveal, for example, through-space connectivities between the proton H-2′ and H-6′ of the B-ring of the flavanoid unit and the proton H-1, H-2, and H-3 of the *C*-glucosidic ET unit. Such spatial correlations are only possible in the case of the C-8′/C-1 linkage between the flavanoid and *C*-glucosidic ET units (Figure 20.10). Such ROESY experiments were used to confirm the structure of the camelliatannins A and B (Hatano et al. 1991), as well as that of the hemisynthesized anthocyano-ETs (Chassaing et al. 2010).

Hatano et al. (1995) also used a different strategy to establish the connectivity between an epicatechin unit and a *C*-glucosidic ET unit (i.e., 5-*O*-desgalloylstachyurin-derived unit) in the case of the camelliatannins C and E isolated from *Camellia japonica* leaves (Figure 20.11). After methylation of all phenolic hydroxyl groups using dimethyl sulfate and potassium carbonate in acetone, NOE experiments were performed by successively irradiating the flavanoid A-ring methoxy groups at positions O-5′ and O-7′. These experiments revealed that for camelliatannin E in which the epicatechin unit is connected by its C-8′ center to the 5-*O*-desgalloylstachyurin-derived unit, the irradiation of both methoxy groups at O-5′ and O-7′ results in NOE signals with the A-ring aromatic proton H-6′ (Hatano et al. 1995). In contrast,

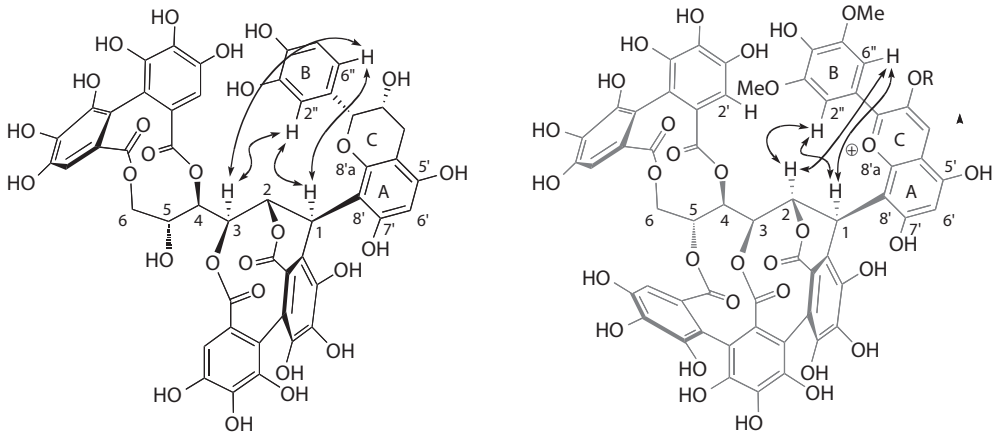

FIGURE 20.10 Structures of camelliatannin A and 1-deoxyvescalagin-(1β → 8)-oenin.

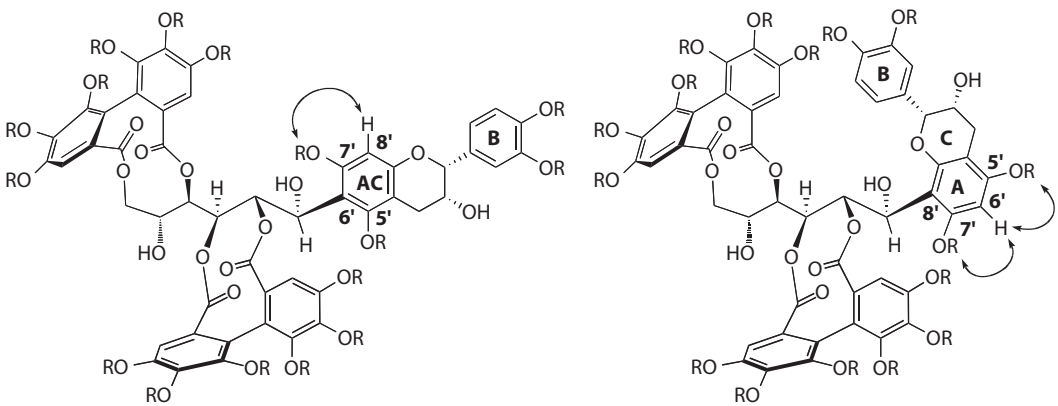

FIGURE 20.11 Structures of camelliatannins C and E, as well as their polymethylated derivatives hexadeca-*O*-methylcamelliatannins C and E.

in the case of camelliatannin C, only the irradiation of the methoxy group at O-7′ gave an NOE signal with an A-ring aromatic proton (i.e., H-8′), thus establishing that the epicatechin unit is linked to the carbon C-1 of the *C*-glucosidic ET unit by its A-ring carbon C-6′ (Figure 20.11).

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21

Analysis of Chlorogenic Acids and Other Hydroxycinnamates in Food, Plants, and Pharmacokinetic Studies

Nikolai Kuhnert, Hande Karaköse, and Rakesh Jaiswal

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21.1 Introduction

The interest in chlorogenic acids (CGAs) and other hydroxycinnamates has expanded rapidly in the past 5–10 years. Numerous epidemiological studies have frequently linked the consumption of a diet rich in CGAs with numerous beneficial health effects (Czok, 1977; Tavani and Vecchia, 2000; Dorea and Da

Costa, 2005; Hamer et al., 2006; Higdon and Frei 2006; van Dam, 2008; Crozier et al., 2009; Risken et al., 2009). Further interest in this ubiquitous class of compounds is due to their interesting sensory and organoleptic properties (Frank et al., 2007).

This chapter summarizes the current state of the art of structure elucidation, identification, and chemical analysis of CGAs and other hydroxycinnamate derivatives.

The chapter is organized as follows: We start with some general remarks on the types of compounds and their occurrence followed by descriptions of the types of analytical methods used for their quantification and structure elucidation. In each section, we highlight the scopes and limitations of the individual analytical techniques (UV–Vis, infrared (IR) spectroscopy, nuclear magnetic resonance (NMR) spectroscopy, and mass spectrometry). In each section, we present selected spectroscopic data that we have found useful in previous years, when actively working in this field. We hope that the reader can use this chapter as a helpful reference source of relevant information on all aspects of CGA characterization, which have been systematically compiled. Particular emphasis was laid on tandem mass spectrometry, where we present a newly developed tutorial style guide for CGA analysis. Finally, we describe some selected pharmacokinetic studies on CGAs in animals and humans. The chapter contains only selected references and does not aim at being comprehensive. Wherever possible, we have selected educationally instructive material from the literature, which we complement with published and unpublished data from our own research group, where adequate. We do not wish to criticize individual published work, where we believe structure assignment was frequently incorrect, but try to lead the reader to a correct and critical way, in which structure assignment of this highly complex and important type of dietary natural products should be carried out in the future. Furthermore, we hope that authors will, in the light of this chapter, critically reassess their structure assignment and correct it wherever necessary.

21.2 Classes of Compounds

Hydroxycinnamates are plant secondary metabolites, found ubiquitously in plants including most fruits and vegetables relevant to the human diet. Hydroxycinnamic acids can occur in their free form with caffeic, ferulic, sinapic acids, and *p*-coumaric acid being the most widespread derivatives (for structures, see Figure 21.1). Other minor cinnamic acid derivatives such as *m*-coumaric, dimethoxycinnamic, trihydroxy cinnamic acids, or isoferulic acid have also been reported in plants. Hydroxycinnamates may be conjugated to many other molecules, with conjugates to (+)-quinic acid being the most widespread compounds in the human diet. Other conjugates, although usually found as minor components in plants or of compounds with limited distribution, found only in a few species include:

1. Esters of hydroxyl acids such as, shikimic, tartaric, galactaric, glucaric, gluconic, malic, dehydrocaffeic, hydroxycitric, phenylpyruvic, methoxyaldaric, lactic, and tartronic acids
2. Amides of biogenic amines and amino acids including aromatic amino acids, glycine, spermidine, spermine, choline, and anthranilic acids
3. Esters of carbohydrates and polyols including monosaccharides, sugar alcohols including glycerol, inositols, and glycosides of anthocyanins, flavanols, and diterpenes
4. Glycosides
5. Esters of lipids including sterols

21.3 Chlorogenic Acids: Definition, History, and Dietary Burden

Classically, CGAs are a family of esters formed between quinic acid and certain *trans*-cinnamic acids, most commonly caffeic, *p*-coumaric, and ferulic acids (Clifford, 1999; Clifford, 2000; IUPAC Commission on the Nomenclature of Organic Chemistry (CNOC) and IUPAC-IUB Commission on Biochemical Nomenclature (CBN). Nomenclature of Cyclitols. Recommendations, 1973, 1976). Representative

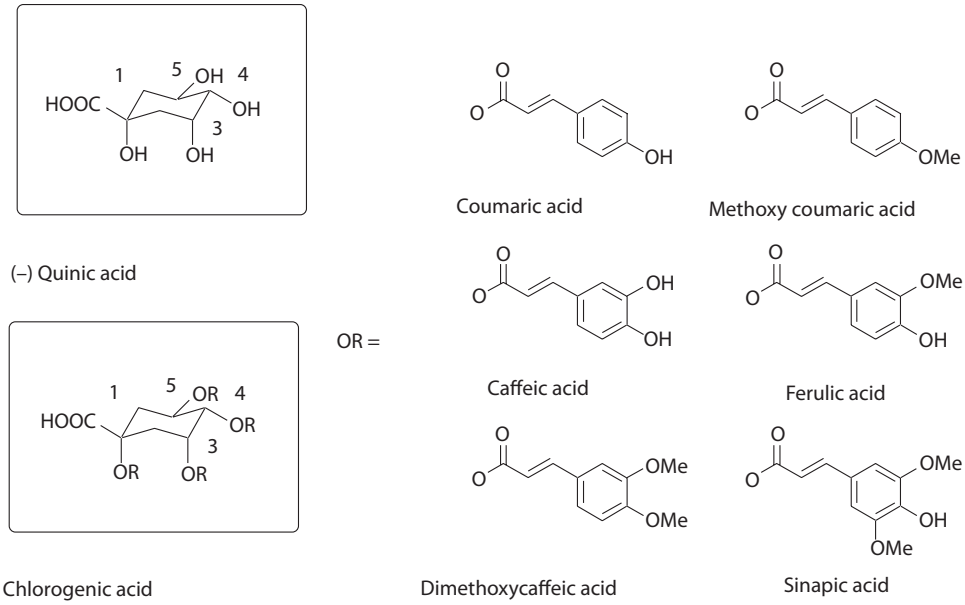


FIGURE 21.1 Basic chemical structure of quinic acid, chlorogenic acid, and typical hydroxycinnamate substituents.

structures are shown in Figure 21.1. In the IUPAC system, (–)-quinic acid is defined as 1L-1(OH),3,4/5-tetrahydroxycyclohexane carboxylic acid, but Eliel and Ramirez (1997) recommend 1α,3*R*,4α,5*R*-tetrahydroxycyclohexane carboxylic acid. In this contribution, we use the term chlorogenic acids in its broadest sense covering all esters of quinic acid containing at least one hydroxycinnamate substituent.

Using the recommended IUPAC nomenclature, the most common CGA is 5-caffeoylquinic acid (5-CQA), still often called chlorogenic acid or 3-CQA (pre IUPAC). The use of these terms should be discouraged. In this chapter, the IUPAC nomenclature is used throughout and if necessary numbering as appeared in the references has been changed for clarity and consistency. In this chapter, we use shorthand to abbreviate chlorogenic acids. The shorthand has the following format: N-XQA or NX,MY-QA, where N or M denominates the position of acyl substitution and X and Y define an abbreviation for the chemical nature of the substituent (C = caffeoyl, Co = *p*-coumaroyl, F = feruloyl, S = sinapoyl, D = dimethoxycinnamoyl, etc.). QA stands for quinic acid, whereas SA stands for shikimic acid. 3-FQA stands for 3-feruloylquinic acid and 4*S*, 5*C*-diQA stands for 4-sinapoyl-5-caffoylquinic acid.

The first report referring to CGAs was published in 1837 by Robiquet and Boutron (1837). But it was not until 1846 that the term was introduced by Payen (1846). The unusual and possibly misleading term suggesting chlorine to be present in the structure can be found in this publication, where it was observed that CGAs show a color reaction with ferric chloride to produce a green pigment. Finally, in 1942, it was shown by Fischer and Dangschat (1932) that CGAs are caffeoyl esters of quinic acid.

CGAs are widely distributed in plants and its nature and occurrence including its dietary burden has been reviewed by Clifford (1999, 2000). Also, an excellent and comprehensive review summarizing the chemistry of CGAs has been provided by Clifford (2000).

Figures for an average dietary intake vary between 1 and 2.5 g per day per human. It has to be kept in mind that these figures vary dramatically depending on the individual diets and consumer preferences. They are as well a consequence of our limited knowledge of this important class of compounds. Numbers on exact quantities of CGAs in dietary material are rare and little is known about statistical variances between the CGA content in different plant species and varieties (Table 21.1). Moreover, analytical data available are in many cases obsolete, obtained through methods based on derivatization followed by colorimetry, quantifying only one particular class of CGAs, for example, caffeoyl or feruloyl esters, resulting in a possible underestimation of real CGA content. Secondly, agricultural practice has changed dramatically over the past decade with many new varieties of fruits and vegetables introduced in the

TABLE 21.1

Occurrence of Chlorogenic Acids in Selected Fruits and Vegetables

	Source	Amount (mg kg^{-1})	Reference
Coffee	Roast coffee	20–675 mg 200 mL^{-1}	Clifford and Walker (1987)
Tea	Black tea	10–50 g kg^{-1}	Cartwright and Roberts (1954); Hara et al. (1995)
Maté	Maté	107–133 mg 200 mL^{-1}	Clifford and Ramirezmartinez (1990)
Pome fruits	Apple	62–385 mg kg^{-1}	Mosel and Herrmann (1974); Risch and Herrmann (1988); Spanos and Wrolstad (1992)
	Pear	60–280 mg kg^{-1}	Igile et al. (1994); Wald et al. (1989)
Stone fruits	Cherries, apricot	150–600 mg kg^{-1}	Risch and Herrmann (1988)
Berry fruits	Blueberries	0.5–2 g kg^{-1}	Schuster and Herrmann (1985)
	Blackcurrants	140 mg kg^{-1}	Gao and Mazza (1994)
	Blackberries	70 mg kg^{-1}	Koeppen and Herrmann (1977)
	Raspberries	20–30 mg kg^{-1}	Clifford (1999)
	Strawberries	20–30 mg kg^{-1}	Clifford (1999)
	Redcurrant	20–30 mg kg^{-1}	Clifford (1999)
	Gooseberries	20–30 mg kg^{-1}	Clifford (1999)
Citrus fruits	Oranges	170–250 mg kg^{-1}	Risch and Herrmann (1988); Winter et al. (1987)
	Grapefruit	27–62 mg kg^{-1}	Clifford (1999)
	Lemon	55–67 mg kg^{-1}	Clifford (1999)
Grapes and wines	Grape juice	10–430 mg L^{-1}	Spanos and Wrolstad (1992)
	American wine	9–116 mg L^{-1}	Okamura and Watanabe (1981)
Other fruits	Pineapple	3 mg L^{-1}	Desimon et al. (1992)
	Kiwi	11 mg L^{-1}	Bartolome et al. (1992)
Brassica vegetables	Kale	6–120 mg kg^{-1}	Brandl and Herrmann (1983); Winter et al. (1987)
	Cabbage	104 mg kg^{-1}	Clifford (1999)
	Brussells sprouts	37 mg kg^{-1}	Clifford (1999)
	Broccoli	60 mg L^{-1}	Plumb et al. (1997)
	Cauliflower	20 mg kg^{-1}	Clifford (1999)
	Radish	240–500 mg kg^{-1}	Brandl et al. (1984)
Chenopodiaceae	Spinach	200 mg kg^{-1}	Tadera and Mitsuda (1971)
Asteraceae	Lettuce	50–120 mg kg^{-1}	Winter and Herrmann (1986)
	Endive	200–500 mg kg^{-1}	Clifford (1999)
	Chicory	20 mg kg^{-1}	Clifford (1999)
Solanaceae	Potato	500–1200 mg kg^{-1}	Malmberg and Theander (1984)
	Aubergines	600 mg kg^{-1}	Clifford (1999)
	Tomato	10–80 mg kg^{-1}	Brandl and Herrmann (1984)
Apiaceae	Carrot	20–120 mg kg^{-1}	Winter et al. (1987)
Cereals	Barley bran	50 mg kg^{-1}	Hernandez et al. (1995)
	Rice	12 g kg^{-1}	Shibuya (1984)

market and older varieties consequently disappearing from the market for patent reasons. Therefore, it is likely that both CGA profiles and quantities within these new varieties have changed dramatically.

CGAs may be subdivided by the identity, number, and positions of the individual acyl residues. The following subgroups can be identified:

1. Monoesters of hydroxycinnamic acids (caffeic, ferulic, sinapic, etc.)
2. Diesters, trimesters, and a single tetraester of a single hydroxycinnamate moiety (e.g., diferuloyl, tricaffeoyl, or tetracaffeoyl quinic acid). We refer to this class of compounds as homo-diesters or homo-triesters

3. Mixed diesters, triesters of caffeic, and ferulic acid or any other hydroxycinnamate moieties (referred to as hetero-diesters or hetero-triesters)
4. Mixed esters involving various permutations of a hydroxycinnamate and other aromatic or aliphatic ester substituents (e.g., glutaric, oxalic, succinic acid characteristic for many plants of the *Asteraceae* family)
5. Other derivatives including *cis*-hydroxycinnamate esters or esters of diastereoisomers of quinic acid

Some instructive figures on quantities of CGAs typically found in selected fruits and vegetables are given in Table 21.1. All quantitative figures refer to total CGA contents with information on the major classes of compounds given.

21.4 Chlorogenic Acids: The Isomer Problem

Typically, a plant produces a large number of CGA secondary metabolites, many of them being isomers of one another. The most impressive source in terms of number of CGAs is the coffee plant, where in the green coffee beans, 70 different CGAs have been identified thus far (45 published, the remaining 25 in press). An additional 20 derivatives have been identified in the coffee leaf (Clifford et al., 2008). In total, a coffee bean biosynthesizes around 10% of its dry mass as CGAs. The second-most rich dietary plant, in terms of number of CGAs identified, is mate (*Ilex paraguarensis*), with a total of 42 different CGAs identified (Jaiswal et al., 2010).

The correct identification of CGA isomers, either as regioisomers or as stereoisomers, forms the main challenge of CGA analysis. Therefore, it is worthwhile considering first of all, all theoretically possible isomers of some selected classes of compounds.

For all monoesters of quinic acid, due to the four different OH functionalities, four regioisomers result. This number is doubled by the possibility of *trans–cis* isomerism at the cinnamoyl moiety. It was shown that in plant tissue exposed to ultraviolet (UV) light, in particular leaves, a photochemical *trans–cis* isomerization occurs, producing *cis*-cinnamoyl-monoesters. It is worth noting that in most of the current literature, *cis*-isomers have not been considered leading frequently to erroneous structure assignment (Clifford et al., 2008).

For homo-diacyl quinic acids, six regioisomers are possible (Figure 21.2). Due to *trans–cis*-isomerization, this number increases to 24 possible, isomers.

For hetero-diacyl quinic acids, the total number of regioisomers is 12 (Figure 21.3) with an increase to 48 considering *trans–cis*-isomerization.

In addition to esters of (+)-quinic acid, any ester of a diastereoisomer of quinic acid should at least theoretically be considered as well. A number of articles have reported on the presence of diastereoisomers of quinic acid and their esters as secondary plant metabolites (Ge et al., 2007; Regos et al., 2009). Furthermore, Scholz-Böttcher et al. (1991) have reported on the presence of all diastereoisomers of quinic acid in roasted coffee beans. Whether these compounds originate from CGAs after hydrolysis remains an open question. Quinic acid has a total of five diastereoisomers and one enantiomer (Figure 21.4). Depending on the symmetry of the compound in question, again a considerable number of CGA-type regioisomers result, by elaboration with acyl substituents. Considering all quinic acid diastereoisomers (excluding enantiomers), the total number of *trans*-cinnamoyl-monoesters is 21 and that of homo-diesters is 30. The total number of all hetero-diacyl compounds excluding *trans*- and *cis*-isomers at the cinnamoyl moiety and all diastereoisomers of quinic acid stands at an impressive total of 220 (including around 800). The number of possible isomers is given in Table 21.2.

21.5 Chlorogenic Acids: Food Processing and Metabolism

While the number of potential chlorogenic isomers produced by plants as genuinely secondary metabolites might be limited, food processing and metabolism have the potential to chemically or enzymatically

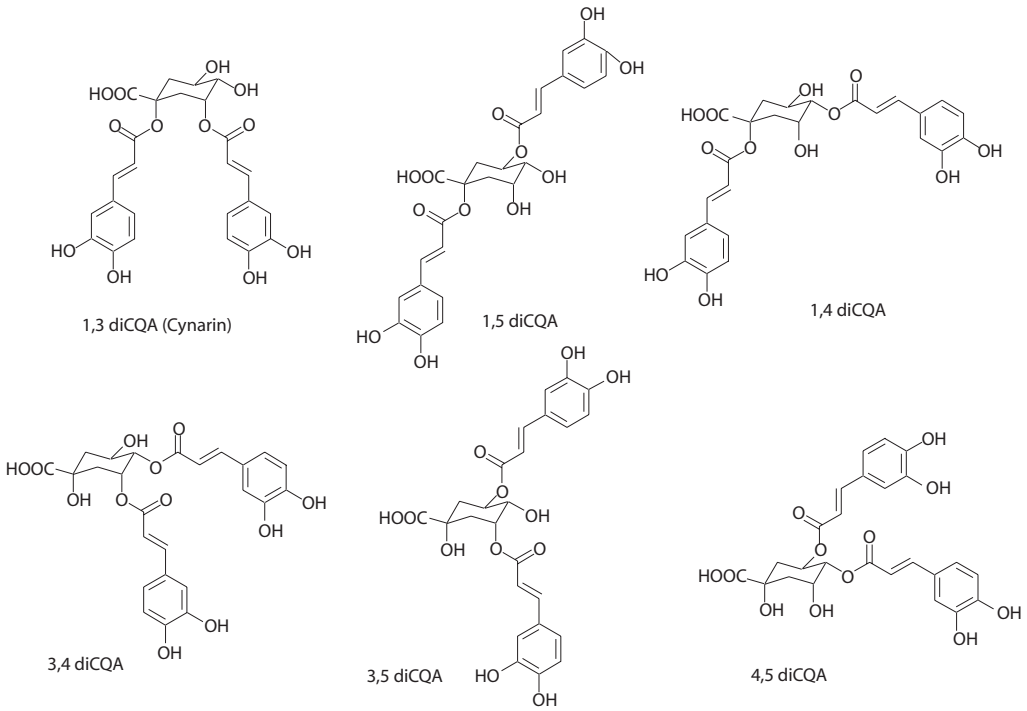


FIGURE 21.2 Structures of all six regioisomeric dicaffeoyl quinic acids.

generate a large number of additional regio- and stereoisomers. For example, on roasting, the number of CGAs detected in the roasted bean increases to over 200 if compared to the 70 derivatives observed in the green coffee bean as judged by the number of characteristic CGA fragment observed in tandem MS experiments (N. Kuhnert, unpublished results). Similarly, many other dietary plants are subjected to food processing such as cooking, baking, roasting, fermenting, microwaving, steaming, and so on, which have the potential to increase the number of CGAs in a given food, which are then available for human consumption and potentially human metabolism.

Not only food processing but also gut flora microbial metabolism and human metabolism have the chance to dramatically alter the original CGA profile of a given dietary plant after ingestion of a CGA-rich diet. This alteration can lead to either complete hydrolysis of the CGA followed by metabolism or direct chemical transformation or enzymatic functionalization. Figure 21.5 shows some possible routes of simple chemical transformations that can be expected for CGAs and in which the basic structure of CGAs is conserved. These include *trans–cis* photoisomerization, acyl-migration, lactone formation, dehydration to shikimic acid derivatives, or epimerization. While there is definite evidence for the first three mechanisms, the latter two have to our knowledge not been studied as yet.

21.6 Characterization of Chlorogenic Acids by UV–Vis Spectroscopy

CGAs exhibit a characteristic UV absorption mainly due to their cinnamate chromophore around 320 nm. Table 21.3 presents some selected λ_{max} values of selected CGAs along with some data for molar extinction coefficients log *E*. These absorptions are particularly useful in high-performance liquid chromatography (HPLC) analysis using a diode array detection (DAD) or other UV detector, thereby identifying CGAs due to their characteristic absorption at 320 nm and allowing their quantification. CGAs display a bathochromic shift in alkaline media due to the formation of phenolate anions. With increasing degree of acyl substitution, a progressive increase of log *E* is observed.

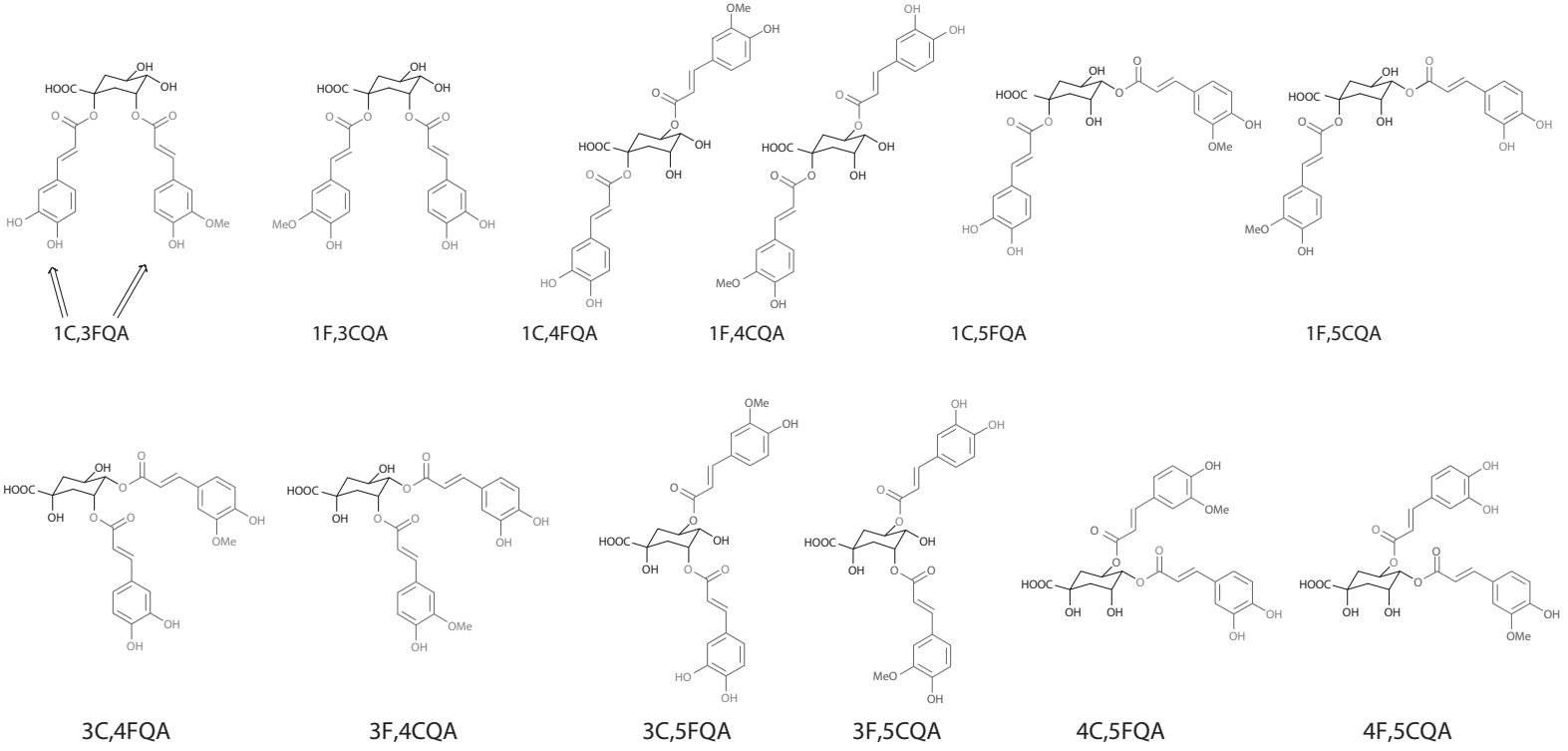


FIGURE 21.3 Structure of all 12 regioisomeric caffeoyl-feruloyl diacyl quinic acids.

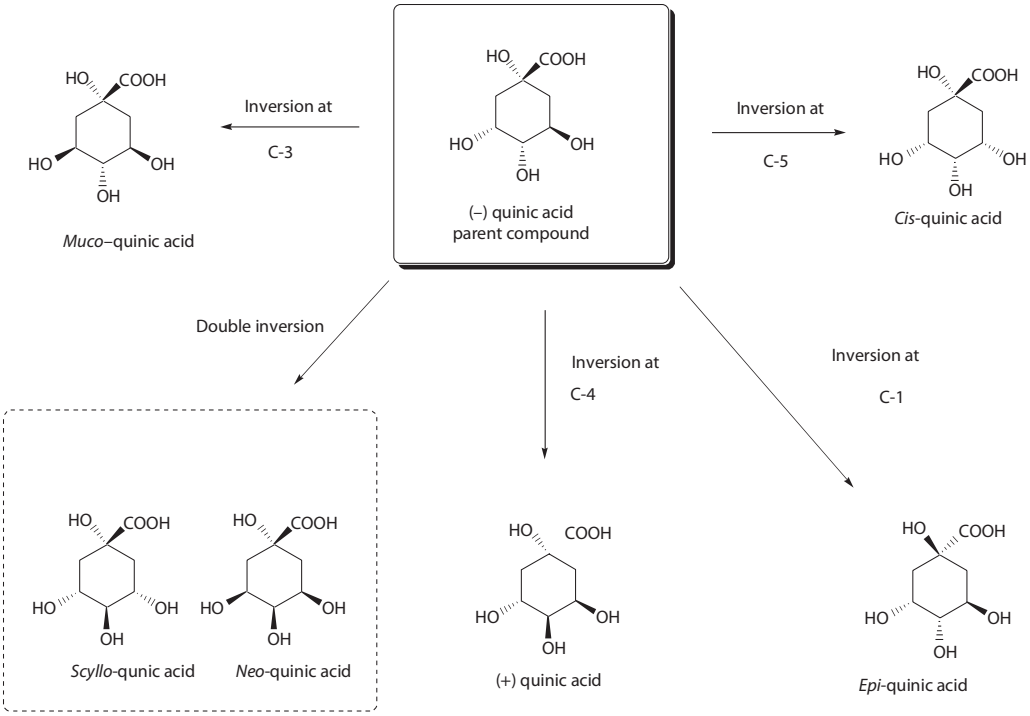


FIGURE 21.4 Structure of all diastereoisomers of (–) quinic acid.

UV–Vis spectroscopy is as well highly useful for differentiating between *trans*- and *cis*-isomers of CGAs with the *cis*-isomers showing a characteristic red-shifted shoulder in the absorption spectra (Clifford et al., 2008).

21.7 Quantification of Chlorogenic Acids by UV–Vis Spectroscopy

Most quantitative data on CGAs and hydroxycinnamates in dietary materials were obtained using UV–Vis spectroscopy. Only recently has this technique been replaced by LC-MS techniques. Two approaches have been taken to quantify CGAs:

1. Quantification of individual CGAs by HPLC-UV–VIS.

   Following chromatographic separation, individual chromatographically well-resolved CGAs can be quantified by coupling a UV–Vis detector to the HPLC instrument. Quantification is carried out at the maximum of absorption of the cinnamate chromophore typically around

TABLE 21.2

Number of Theoretically Possible Isomers of Chlorogenic Acids

	(+) Quinic Acid *trans*-Cinnamates Only	(+) Quinic Acid *trans*- and *cis*-Cinnamates	All Diastereomers of Quinic Acid *trans*-Esters Only
Monoacyl esters	4	8	21
Homo-diacyl esters	6	24	40
Hetero-diacyl esters	12	48	220
Homo-triacyl esters	4	32	21
Hetero-triacyl esters	48	384	316

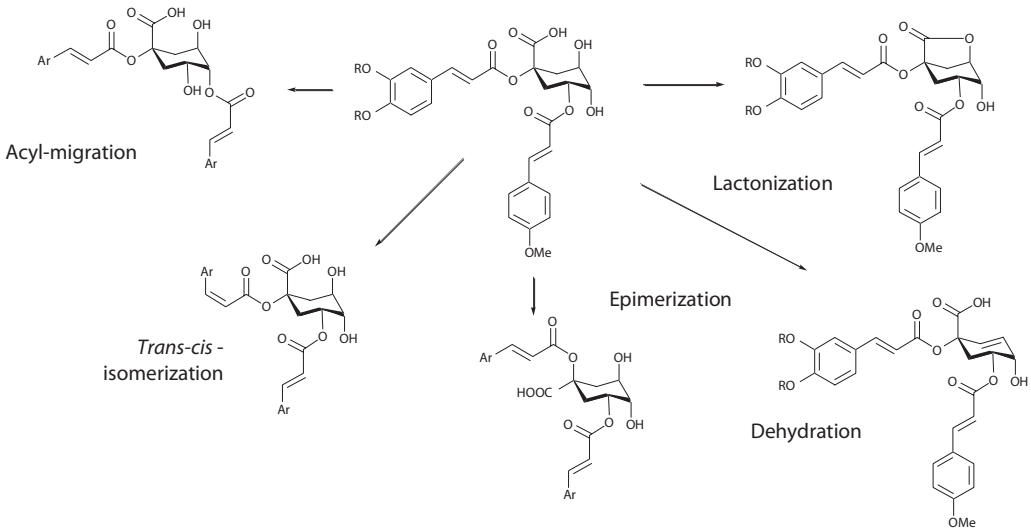


FIGURE 21.5 Basic chemical reactions of chlorogenic acids expected in food processing and metabolism.

320 nm. Calibration curves are obtained and quantification is carried out using these calibration curves (Mendonca et al., 2008).

2. Quantification of groups of CGAs by UV–Vis spectroscopy after derivatiation.

   Numbers on total hydroxycinnamate and CGA content have frequently been obtained by color derivatization reactions. Here a crude dietary extract is reacted with a reagent that specifically reacts with a selected hydroxycinnamate residue to give a colored product that is subsequently

TABLE 21.3

Characteristic UV–Vis Data of Selected Chlorogenic Acids

Compound	Abbreviation	λ_{max} (nm)[a]	Log E[a]
1-*p*-Coumaroylquinic acid	1-*p*CoQA	375	4.31
3-*p*-Coumaroylquinic acid	3-*p*CoQA	315 (310)[b]	4.30
4-*p*-Coumaroylquinic acid	4-*p*CoQA	315	4.32
5-*p*-Coumaroylquinic acid	5-*p*CoQA	315 (310)	4.31
1-Caffeoylquinic acid	1-CQA	330	4.26
3-Caffeoylquinic acid	3-CQA	330	4.22
4-Caffeoylquinic acid	4-CQA	330	4.26
5-Caffeoylquinic acid	5-CQA	327	4.29
1-Ferulyolquinic acid	1-FQA	325	4.27
3-Ferulyolquinic acid	3-FQA	325	4.28
4-Ferulyolquinic acid	4-FQA	325	4.29
5-Ferulyolquinic acid	5-FQA	325	4.29
1,3-Dicaffeoylquinic acid	1,3-diCQA	325	4.50
1,4-Dicaffeoylquinic acid	1,4-diCQA	327	4.53
1,5-Dicaffeoylquinic acid	1,5-diCQA	327	4.50
3,4-Dicaffeoylquinic acid	3,4-diCQA	330	4.53
3,5-Dicaffeoylquinic acid	3,5-diCQA	329	4.55
4,5-Dicaffeoylquinic acid	4,5-diCQA	330	4.52
3,4,5-Tricaffeoylquinic acid	3,4,5-triCQA	327	4.75

[a] All values in ethanol.
[b] Two different values in the literature.

quantified using UV–Vis spectroscopy. Representative reagents include molybdate, periodate, or thiobarbituric acid. Typically, such methods should be viewed critically, since the presence of additional polyphenolic dietary constituents might lead to an overestimation of CGA content, whereas the presence of CGAs with other substituents, other than the one targeted, leads to an underestimation of CGA content. A detailed discussion was presented by Clifford (2000).

21.8 General Comments on Liquid Chromatography of Chlorogenic Acids

While gas chromatography of derivatized CGA derivatives has never prevailed as a routine method, liquid chromatography forms an indispensable and reliable tool for CGA analysis. Possibly, the first report on the use of liquid chromatography for CGA analysis was published by Court (1977) using an unusual reversed-phase packing.

Currently, the most popular column packings for CGA analysis include reversed-phase materials in particular aromatic phases such as a phenyl–hexyl or diphenyl packing (Clifford et al., 2003, 2005). Chromatographic retention times serve as extremely helpful guidelines in compound assignment, with systematic patterns observed frequently. Calculation of log *P* values using the "free Wilson," Hansch method or other more advanced quantitative structure activity relationships (QSAR) techniques allow estimation of relative retention times.

Reasonably specific detection of CGA derivatives can be achieved using UV–Vis monitoring in the 310–330 nm region (cinnamate chromophore) with electrochemical detectors commonly used as well (Azuma et al., 2000; Nardini et al., 2002). Current practice employs mainly mass spectrometrical detection and this topic is discussed in detail in the following sections.

21.9 Characterization of Chlorogenic Acids by NMR Spectroscopy

NMR constitutes the most powerful tool for structure elucidation in organic chemistry and has been frequently applied to CGAs. CGAs isolated and purified from natural sources as well as derivatives obtained through total synthesis have been investigated in detail. The successful application of NMR spectroscopy depends on the availability of sufficient amounts of purified materials.

NMR data, for example, for all four regioisomers of monocaffeoylquinic acid, obtained by total synthesis by the Sefkow group (Sefkow, 2001; Sefkow et al., 2001) as well as complete data on dicaffeoyl quinic acids are available.

Derived from NMR studies, two parameters form the basis of CGA structure elucidation, the chemical shift in ^{1}H-NMR and ^{13}C-NMR spectra, and coupling constants in ^{1}H-NMR spectra. Tables 21.4 and 21.5 summarize ^{1}H-NMR chemical shifts for selected monoacyl and diacyl quinic acids, respectively. Atoms are numbered according to the IUPAC system (Figure 21.6). The four methylene CH_2 hydrogens appear typically between 1.9 and 2.3 ppm with unambiguous assignment possible through 2D NMR experiments or coupling constant considerations. Methine CHOH protons appear typically between 4.2 and 4.8 ppm with assignments carried out through 2D NMR techniques, in particular ^{1}H^{1}H COSY experiments. The methine proton attached to the ester group appears typically downfield around 5.3 ppm compared to the parent alcohol proton. Finally, the cinnamate substituents show characteristic signals in the olefinic and aromatic region with typical *trans*-olefinic coupling constants of 16–17 Hz. If d_6-dmso is used as an NMR solvent, the phenolic hydrogens can be observed as well, due to slow proton exchange, characteristic for this solvent.

In an ideal cyclohexyl chair confirmation, the dihedral HCCH angles in quinic acid are around 55° for H-2(ax)-C2-C-3-H-3, H-3-C-3-C-4-H-4, and H-5-C-5-C-6-H-6(ax). For H-2(eq)-C-2-C-3-H-3, the dihedral angle is 61° and for H-4-C-4-C-5-H-5 and H-5-C-5-C-6-H-6(ax), the angle is close to 180°. Accordingly, $^{3}J_{HCCH}$ coupling constants can be estimated using the Karplus relationship.

In the ^{13}C-NMR spectra, the quinic acid moiety shows two methylene carbons (C-2 and C-6) between 36 and 43 ppm and a quaternary carbon atom (C-1) appearing between 74 and 78 ppm together with a carboxyl signal (C-7) between 175 and 181 ppm. Three methine carbons bearing oxygen functionality

TABLE 21.4

^{1}H-NMR Data of Monocaffeoylquinic Acids

Number of Carbon Atoms	3-CQA		4-CQA		5-CQA	
Quinic Acid Moiety	Chemical Shift (ppm) (Islam Md. et al., 2002)	Chemical Shift (ppm) (Nakatani et al., 2000)	Chemical Shift (ppm) (Lin and Wang, 2003)	Chemical Shift (ppm) (Nakatani et al., 2000)	Chemical Shift (ppm) (Lin and Wang, 2003)	Chemical Shift (ppm) (Lin et al., 1999)
2(H_{eq})	2.03	2.13	1.6	2.06	1.8	2.04
2(H_{ax})	2.13	2.2	1.9	2.17	2.0	2.21
3	5.34	5.34	4.2	4.28	3.9	4.17
4	3.73	3.63	5.3	4.79	3.5	3.73
5	4.17	4.14	3.9	4.27	5.2	5.34
6(H_{eq})	2.28	2.13	1.9	2.06	2.0	2.21
6(H_{ax})	2.16	1.95	1.6	2	1.8	2.04
Caffeoyl moiety						
2	7.05	7.04	7.0	7.06	7.0	7.05
5	6.78	6.76	6.8	6.77	7.0	6.79
6	7.0	6.93	7.0	6.96	6.8	6.87
7	7.55	7.58	7.4	7.65	7.4	
8	6.26	6.3	6.2	6.37	6.2	6.27

can be observed for C-3, C-5, and C-4 between 65 and 75 ppm, with the carbon CHOR bearing an ester group being shifted downfield by around 3–5 ppm if compared to a CHOH carbon. Additional signals for the acyl substituent are observed in the olefinic and aromatic region. Carbons can be unambiguously assigned using ^{1}H^{13}C HSQC or HMQC techniques. ^{13}C-NMR data for the quinic acid moiety in mono- and diacyl CGAs are summarized below (Tables 21.6 and 21.7).

21.10 Limitations of NMR Spectroscopic Analysis

It has to be pointed out that CGA characterization by NMR spectroscopy is not without limitations. The first limitation concerns availability of purified compounds, which is unproblematic for most major-component CGAs. In contrast, it still forms a serious problem for minor-component CGAs produced only in small quantities by plants. These compounds frequently tend to elute closely to other CGAs or even coelute chromatographically with other CGAs, which allows analytical identification but prevents preparative separation.

The second limitation concerns spectra interpretation, in particular for hetero-diacyl and hetero-triacyl CGAs. For these classes of compounds, unambiguous structure determination by NMR spectroscopy is frequently impossible. This failure of NMR spectroscopy can be rationalized by unfavorable signal overlap mainly affecting the F2 dimension (Figure 21.7). For structure determination, 2D NMR experiments are required to establish the connectivity of the ester substituents, with ^{1}H^{13}C-HMBC techniques being the tool of choice (Figure 21.8). In the example given, the two caffeoyl substituents in cynarin (1,3-diCQA) can be identified due to their four phenolic hydrogens at 8–8.5 ppm. Two phenolic OHs interact *via* long-range couplings with the quarternary aromatic carbons and allow via identification of further long-range HC couplings the unambiguous assignment of all aromatic and olefinic carbons and hydrogens in each of the two caffeoyl groups. Again, a long-range coupling between the olefinic hydrogens and the ipso ester carbon on the quinic acid moiety allows determination of regiochemistry. After identification of signals of aromatic cinnamoyl side chain substituents, the connectivity of these substituents needs to be followed until a clear interaction with any of the four quinic acid H<u>C</u>OR carbons is established and regiochemistry defined. Typically for this class of compounds, olefinic and aromatic proton and carbon signals are very close or even overlap, resulting in failure to assign chains of interacting nuclei. The spectrum shown below for a hetero-triacyl CGA illustrates this point.

TABLE 21.5

^{1}H-NMR of Dicaffeoylquinic Acids

Number of Carbon Atom	3,4-diCQA			3,5-diCQA			4,5-diCQA		
Quinic Acid Moiety	Chemical Shift (ppm) (Purusotam and Katsumichi, 1996)	Chemical Shift (ppm) (Islam Md. et al., 2002)	Chemical Shift (ppm) (Shi et al., 2007)	Chemical Shift (ppm) (Purusotam and Katsumichi, 1996)	Chemical Shift (ppm) (Islam Md. et al., 2002)	Chemical Shift (ppm) (Shi et al., 2007)	Chemical Shift (ppm) (Shi et al., 2007)	Chemical Shift (ppm) (Islam Md. et al., 2002)	Chemical Shift (ppm) (Lin et al., 1999)
2(H_{eq})	2.02	2.3	2.03	2.3	2.4	2.16	2.26	2.2	2.21
2(H_{ax})	2.29	2.1	2.03	2.12	2.4	2.16	2.26	2.1	2.03
3	4.35	4.4	5.68	5.41	5.4	5.36	4.35	4.4	4.13
4	5.12	5.1	5.16	3.94	4.0	3.94	5.09	5.0	5.18
5	5.68	5.6	4.27	5.47	5.4	5.39	5.74	5.6	5.62
6(H_{eq})	2.2	2.3	2.16	2.18	2.4	2.29	2.26	2.4	2.21
6(H_{ax})	2.2	2.3	2.16	2.18	2.2	2.16	2.1	2.1	2.03

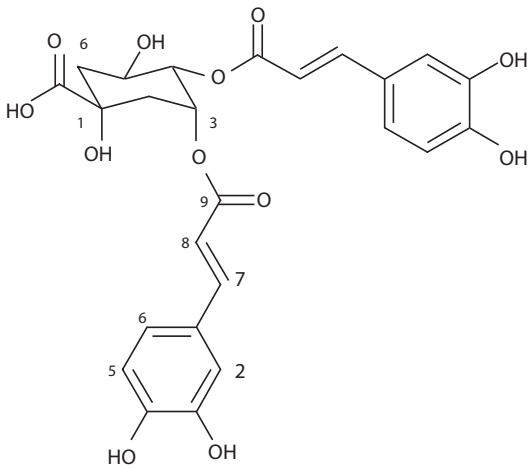


FIGURE 21.6 Representation for IUPAC numbering of 3,4-diCQA.

For unambiguous structure elucidation of these classes of compounds, only tandem mass spectrometry is able to provide a satisfactory answer.

Finally, for NMR characterization of novel CGAs, it should be noted that strong solvent effects are operating in NMR spectroscopic analysis of CGAs. These solvent effects can be traced down to two important factors. Any CGA, as a substituted cyclohexane, will exist in two chair-like minimum energy conformations, which interconvert rapidly on the NMR timescale in solution at room temperature (Scheme 21.1).

TABLE 21.6

^{13}C-NMR Data of Monocaffeoylquinic Acids

Number of Carbon Atoms	3-CQA		4-CQA		5-CQA	
Quinic Moiety	Chemical Shift (ppm) (Nakatani et al., 2000)	Chemical Shift (ppm) (Chan et al., 2009)	Chemical Shift (ppm) (Lin and Wang, 2003)	Chemical Shift (ppm) (Nakatani et al., 2000)	Chemical Shift (ppm) (Lin and Wang, 2003)	Chemical Shift (ppm) (Lin et al., 1999)
1	75.4	76.0	74.3	76.6	72.9	77.74
2	36.7	37.3	41.2	38.4	35.1	38.55
3	73.0	72.4	65.7	69.6	67.1	72.31
4	74.8	73.3	74.7	79.3	71.4	74.29
5	68.3	70.0	67.9	65.5	70.4	72.4
6	41.5	39.1	37.0	42.7	37.9	39.92
7	178.3	182.1	175.9	177.3	175.9	181.13
Caffeoyl moiety						
1	127.9	127.9	125.5	127.8	125.0	127.78
2	115.1	115.1	115.0	115.1	115.1	115.56
3	146.8	146.7	145.2	146.8	144.3	145.84
4	74.8	149.4	148.4	149.6	148.1	148.64
5	116.4	116.5	115.9	116.5	115.8	116.82
6	122.9	122.9	113.7	123	121.0	123.33
7	146.8	146.8	146.7	147.1	145.5	147.01
8	115.8	115.7	113.7	115.4	114.5	115.38
9	169.0	168.8	167.2	169	166.0	169.72

TABLE 21.7

^{13}C-NMR Data of Dicaffeoylquinic Acids

Number of Carbon Atoms	3,4-diCQA			3,5-diCQA			4,5-diCQA	
Quinic Moiety	**δ (ppm) (Purusotam and Katsumichi, 1996**	**δ (ppm) (Shi et al., 2007)**	**δ (ppm) (Shi et al., 2007)**	**δ (ppm) (Purusotam and Katsumichi, 1996)**	**δ (ppm) (Chen et al., 2007)**	**δ (ppm) (Shi et al., 2007)**	**δ (ppm) (Chen et al., 2007)**	**δ (ppm) (Shi et al., 2007)**
1	77.88	75.9	75.9	76.33	74.7	74.9	76.3	76.2
2	39.48	39.6	39.6	37.66	37.9	37.8	38.4	38.5
3	71.08	70.1	70.1	74.33	72.8	72.1	69.5	69.8
4	77.52	75.3	75.3	73.02	69.9	70.7	75.8	76.1
5	70.11	67.0	67	72.78	72.5	72.6	69.0	69.2
6	40.24	37.7	37.7	40.18	36.1	36.1	39.5	39.8
7	180.1	176.8	176.8	180.37	177.0	177.8	177.2	176.8
Caffeoyl Moiety	**4-Substituted**			**5-Substituted**			**5-Substituted**	
1	128.4	127.8	127.8	128.58	127.8	127.9	127.7	127.6
2	115.89	115.2	115.2	115.92	114.7	115.2	114.7	115.2
3	147.46	146.8	146.8	147.46	146.7	146.8	146.7	146.7
4	150.32	149.6	149.6	150.26	149.6	149.6	149.6	149.6
5	117.2	116.5	116.5	117.23	116.5	116.5	116.5	116.4
6	123.87	123.2	123.2	123.75	123.2	123.1	123.2	123.2
7	148.22	147.3	147.3	147.83	147.5	147	147.7	147.7
8	115.59	115.2	115.2	116.1	114.6	115.6	114.6	114.7
9	169.26	168.5	168.5	169.47	168.2	168.9	168.5	168.6
	3-Substituted			**3-Substituted**			**4-Substituted**	
1	128.46	127.7	127.7	128.76	127.7	127.8	127.6	127.5
2	115.89	115.2	115.2	115.92	115.2	115.1	114.7	115.1
3	147.46	146.8	146.8	147.46	146.8	146.8	146.7	146.7
4	150.32	149.6	149.6	150.13	149.6	149.5	149.6	149.6
5	117.2	116.5	116.5	117.23	116.5	116.5	116.5	116.4
6	123.87	123.2	123.2	123.75	123.2	123	123.1	123.2
7	148.37	147.3	147.3	147.68	147.3	146.8	147.5	147.5
8	115.59	115.0	115	116.59	115.0	115.1	114.6	114.7
9	169.38	168.5	168.5	169.93	168.5	168.3	168.2	168.3

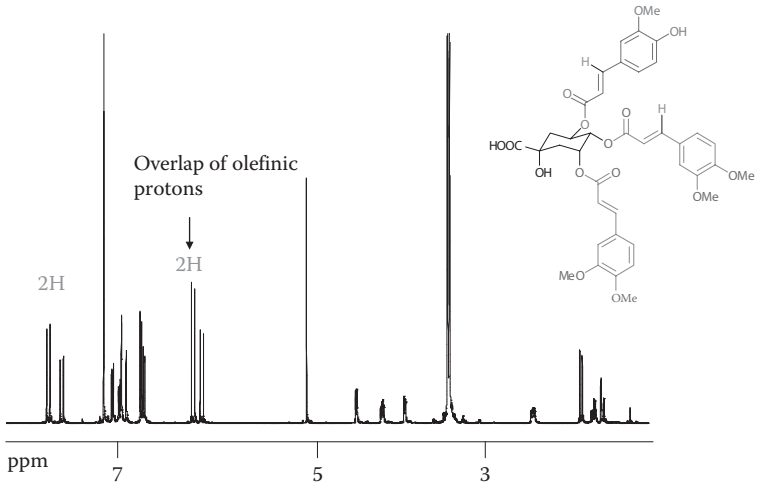


FIGURE 21.7 ^{1}H NMR (500 MHz) spectrum of a hetero-triacyl quinic acid from synthetic origin, illustrating signal overlap in aromatic and olefinic region leading to failure in signal assignments.

The result of this rapid interconversion is an averaged spectrum, averaged over two conformers. Assignment of stereochemistry based on dihedral coupling constants, according to the Karplus relationship, requires a detailed knowledge about the conformational preferences of the compounds in question. As a rule of thumb, these can be estimated using typical cyclohexane A-values. However, due to intramolecular hydrogen bonding and alignments of dipole moments, such a conformational equilibrium is strongly

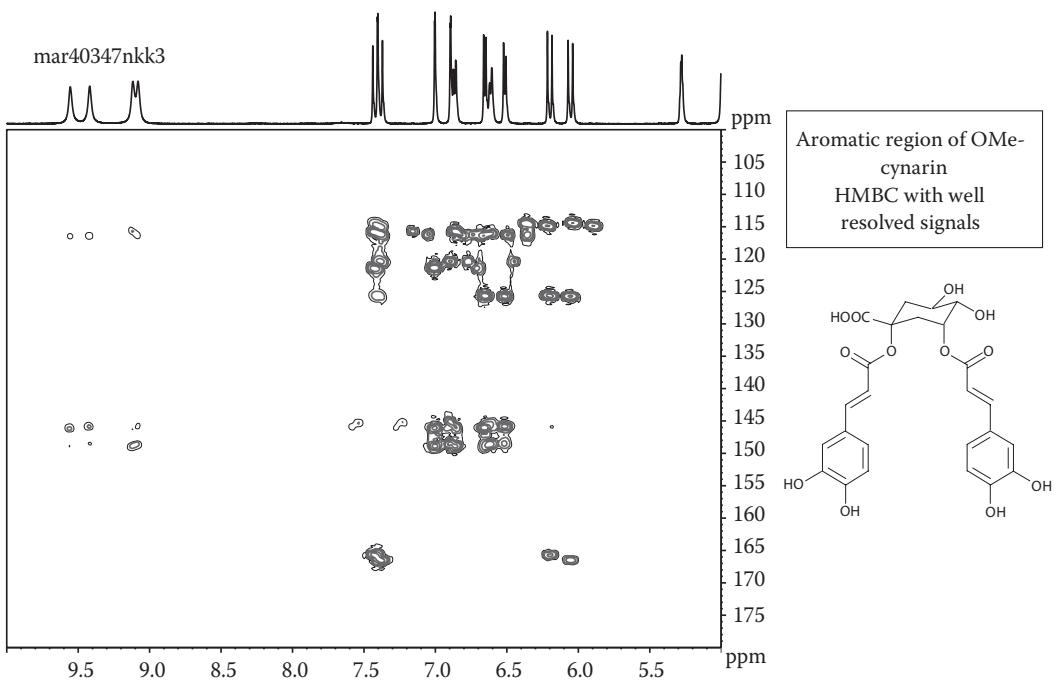
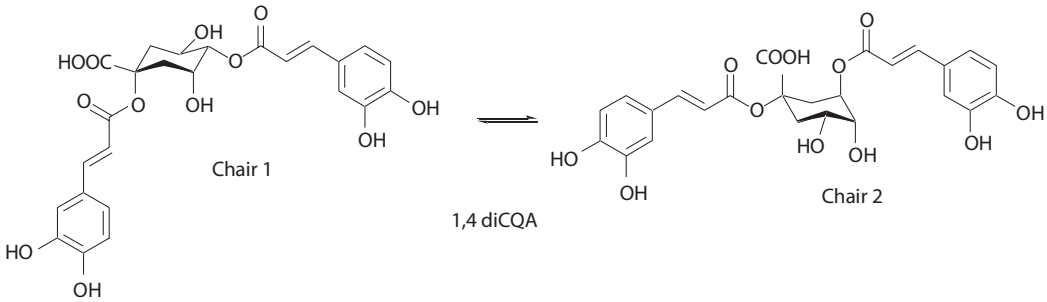


FIGURE 21.8 ^{1}H^{13}C-HMBC spectrum of cynarin (1,3-dicaffeoyl quinic acid) expanded aromatic region illustrating assignment of acyl substituents. (Adapted from Sunucu, 2011.)



SCHEME 21.1 Interconversion between two chair conformers of 1,4-dicaffeoyl quinic acid.

solvent dependent. This issue has been highlighted and investigated by Corse and Lundin (1970) and Scholz-Böttcher et al. (1991).

21.11 Characterization of Chlorogenic Acids by IR Spectroscopy

Infrared (IR) frequencies of CGAs have been reported in the literature and are summarized in Table 21.8. IR spectra of CGAs are rather complex with monoacyl derivatives possessing 130–140 and diacyl derivatives possessing 190–220 normal modes of vibration. From the IR spectra, it is seen that each individual CGA absorbs strongly in the fingerprint spectral region (900–1700 cm^{-1}). The hydroxyl group absorbs the IR frequency in the region 3400 cm^{-1}. In the region of 1600 cm^{-1} vibration in the phenyl ring (C=C), stretching of alcohol moieties (C–O) in the range of 1000–1260 cm^{-1} is observed. Stretching frequencies of carboxylic acid and ester carbonyl groups (C=O) produce peaks in the range of 1650–1800 cm^{-1}. Occasionally, intra- or intermolecular hydrogen bonding can be observed in the IR spectra (Clifford et al., 2003, 2005). Individual CGA regioisomers produce similar IR spectra, but some subtle differences can be observed that allow compound identification by direct comparison of spectra.

TABLE 21.8

IR Frequencies (cm^{-1}) of CGAs

	5-CQA		3,4-diCQA		3,5-diCQA		4,5-diCQA	
Frequency Range (cm^{-1}) (Kbr)	**Frequency (cm^{-1}) (Lin et al., 2002)**	**Frequency (cm^{-1}) (Lin et al., 1999)**	**Frequency (cm^{-1}) (Hung et al., 2006)**	**Frequency (cm^{-1}) (Lin et al., 1999)**	**Frequency (cm^{-1}) (Hung et al., 2006)**	**Frequency (cm^{-1}) (Lin et al., 1999)**	**Frequency (cm^{-1}) (Hung et al., 2006)**	**Frequency (cm^{-1}) (Lin et al., 1999)**
2000–3000	3335	3400	3400	3300	3436	3350	3440	3400
			2598					
1800–1700	1709	1705	1719	1725	1714	1725	1727	1715
				1705		1710		1705
1700–1600	1635	1610				1705		1610
1600–1500	1598		1523	1640	1518	1640	1520	
	1514			1610		1610		
1500–1400					1445	1530	1455	
1400–1300			1458					
1300–1200	1283				1277		1298	
1200–1100					1130			
					1179			

21.12 Molecular Modeling and Spectra Prediction

Computational chemistry approaches, using quantum chemical methods or classical force field methods, form a highly useful tool to assist in spectra assignment and spectral data prediction. In particular, vibrational and NMR spectral data can be accurately predicted and compared to experimental results facilitating data interpretation.

Following thorough energy minimization spectral data such as vibration frequencies, NMR chemical shifts or coupling constants can be computed. Our unpublished work in this field shows that calculated values coincide extremely well with experimental data (Karaköse, 2009).

21.13 Quantum Mechanical Calculations

Calculation of optimized structures in vacuum and ^{13}C and ^{1}H chemical shifts of the different isomers were performed with quantum mechanics methods to facilitate structure prediction from spectroscopic data. Calculated properties were compared with available experimental data and the comparison of the calculated data with experimental chemical shift showed a good agreement.

Optimized structure and bond angles of 3-CQA are shown in Figure 21.9. Cyclohexane bond angles were analyzed from the optimized structures and it was observed that none of the isomers possessed a perfect chair conformation. There were small deviations from 109.5°.

Dihedral angles of ester group and vinyl group showed that the oxygens of ester group and hydrogens of vinyl group prefer *trans*-conformation in the energy-minimized structure.

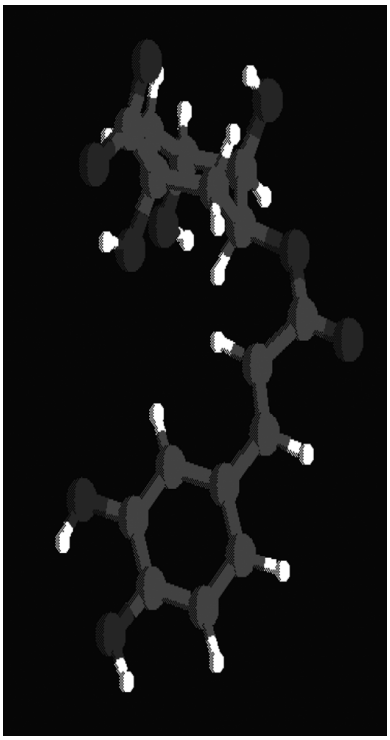


FIGURE 21.9 Optimized structures and bond angles of cyclohexane rings of 3-CQA.

21.14 Calculation of NMR Chemical Shifts of Caffeoylquinic Acids

NMR shift calculations were performed to compare computational data with experimental data to assess the reliability of the computational approach. Since the structures of all CGAs are similar, a modeling protocol if applied to several regioisomers can serve as a useful tool in structure assignment based on spectral data. The ability to calculate vibrational and NMR spectral data is in particular useful for peak assignments or the rationalization of spectra of unknown CGAs.

Calculated NMR chemical shifts for the optimized structures are compared with experimental NMR results and relative errors for each calculation have been presented. Relative error gives an indication of how good a measurement is relative to the true value of the magnitude that is measured. Thus, here, experimental values are taken as true values and relative error is calculated by the difference of calculated (x_0) and experimental data (x) divided by experimental data.

$$\delta x = \frac{\Delta x}{x} = \frac{x_0 - x}{x}$$

An example of the results for 3-CQA is given below. Chemical shifts calculations are in reasonable agreement with the experimental data. In ^{1}H-NMR of monoCQAs, the maximum error (0.7) is observed for the hydrogen at C-4 of 3-CQA and for the hydrogen at C-6 in 4-CQA (Table 21.9).

21.15 Mass Spectrometry and Chlorogenic Acids Identification

In 2003, Clifford and Kuhnert revolutionized CGA structure elucidation by introducing a method based on tandem mass spectrometry, allowing assignment of regiochemistry based exclusively on fragment spectra (Clifford et al., 2003). The method has the advantage that CGAs do not need to be isolated but can

TABLE 21.9

Calculated and Experimental Chemical Shifts for 3-CQA

Number of Carbon Atoms	3-CQA ^{1}H-NMR Chemical Shift (ppm)			3-CQA ^{13}C-NMR Chemical Shift (ppm)		
Quinic Moiety	Calculated	Experimental (Islam Md. et al., 2002)	Relative Error	Calculated	Experimental (Nakatani et al., 2000)	Relative Error
1	—	—	—	75.3	75.4	–0.001
2(H_{eq})	2.5	2.1	0.2	40.1	36.7	0.09
2(H_{ax})	1.5	2.0	–0.2	—	—	—
3	5.3	5.3	0.0	76.2	73.0	0.04
4	1.2	3.7	–0.7	79.3	74.8	0.06
5	3.9	4.1	–0.05	76.8	68.3	0.12
6(H_{eq})	1.4	2.1	–0.3	36.7	41.5	–0.11
6(H_{ax})	2.5	2.3	0.09	—	—	—
7	—	—	—	165.8	178.3	–0.07
Caffeoyl moiety						
1	—	—	—	128.9	127.9	0.008
2	8.2	7.0	0.17	110.4	115.1	–0.04
3	—	—	—	140.9	146.8	–0.04
4	—	—	—	137.3	74.8	0.83
5	6.2	6.8	–0.09	105.8	116.4	–0.09
6	6.5	7.0	–0.07	117.9	122.9	–0.04
7	7.4	7.6	–0.03	139.2	146.8	–0.05
8	7.7	6.3	0.22	113.4	115.8	–0.02
9	—	—	—	160.7	169.0	–0.05

be identified directly from analytical LC-MS runs, even if present as minor components or as close eluting compounds. Clifford and Kuhnert observed that all four regioisomeric monocaffeoylquinic acids and later all six regioisomeric dicaffeoyl quinic acids showed dramatically different tandem mass spectra in the negative ion mode, using an ion trap mass spectrometer (Clifford et al., 2005). Due to the diagnostic differences in the tandem MS fragment spectra, a consistent and predictive structure diagnostic hierarchical key for CGA structure elucidation has been established, which allows reliable determination of CGA regiochemistry even for minor-component CGAs. The basis of these differences in tandem MS spectra was rationalized in terms of different hydrogen-bonding arrays found in gas-phase ions of regioisomeric CGAs. As a rule of thumb, a 1-acyl substituent fragments easier than a 5-acyl substituent and easier than a 3-acyl substituent. A 4-acyl substituent is most difficult to fragment and fragmentation is always accompanied by loss of water. An alternative mechanism involving acyl transfer reactions has also been proposed (Clifford et al., 2005, 2006a). The basic fragmentation mechanisms for monoacyl quinic acids are shown in Scheme 21.2. Further mechanistic details can be found in the original references. In conclusion, a 1-acyl derivative loses its acyl moiety on activation by the neighboring COOH acid group readily, activation occurs as well in a 5-acyl derivative, however, in a higher-energy inverted chair conformation, leading to a more reluctant loss of the acyl group. In a 3-acyl derivative, activation occurs through a less acidic alcohol functional group leading again to a more reluctant fragmentation. Finally, in a 4-acyl derivative, loss of water by activation of the COOH group is followed by 1,2-acyl migration and final loss of the acyl group. Using this method for the first time, aspects of regiochemistry can be assessed using mass spectrometry that leads to an unambiguous and reliable structure assignment.

This tandem MS-based method has been successfully adopted in more than 30 laboratories around the world and can be viewed as the current gold standard method for CGA structure elucidation. Furthermore, the method has been adapted to other types of tandem mass spectrometers including triple quadrupole mass spectrometers (Alonso-Salces et al., 2009a).

21.16 Work Flow for Chlorogenic Acid Profiling, Identification, and Structure Elucidation by LC-Tandem MS

We would like to propose a modified version for the search for CGAs in dietary material by tandem LC-MS. A more detailed discussion on the identification of CGAs in selected dietary material, for example, green coffee beans and mate, will follow subsequently.

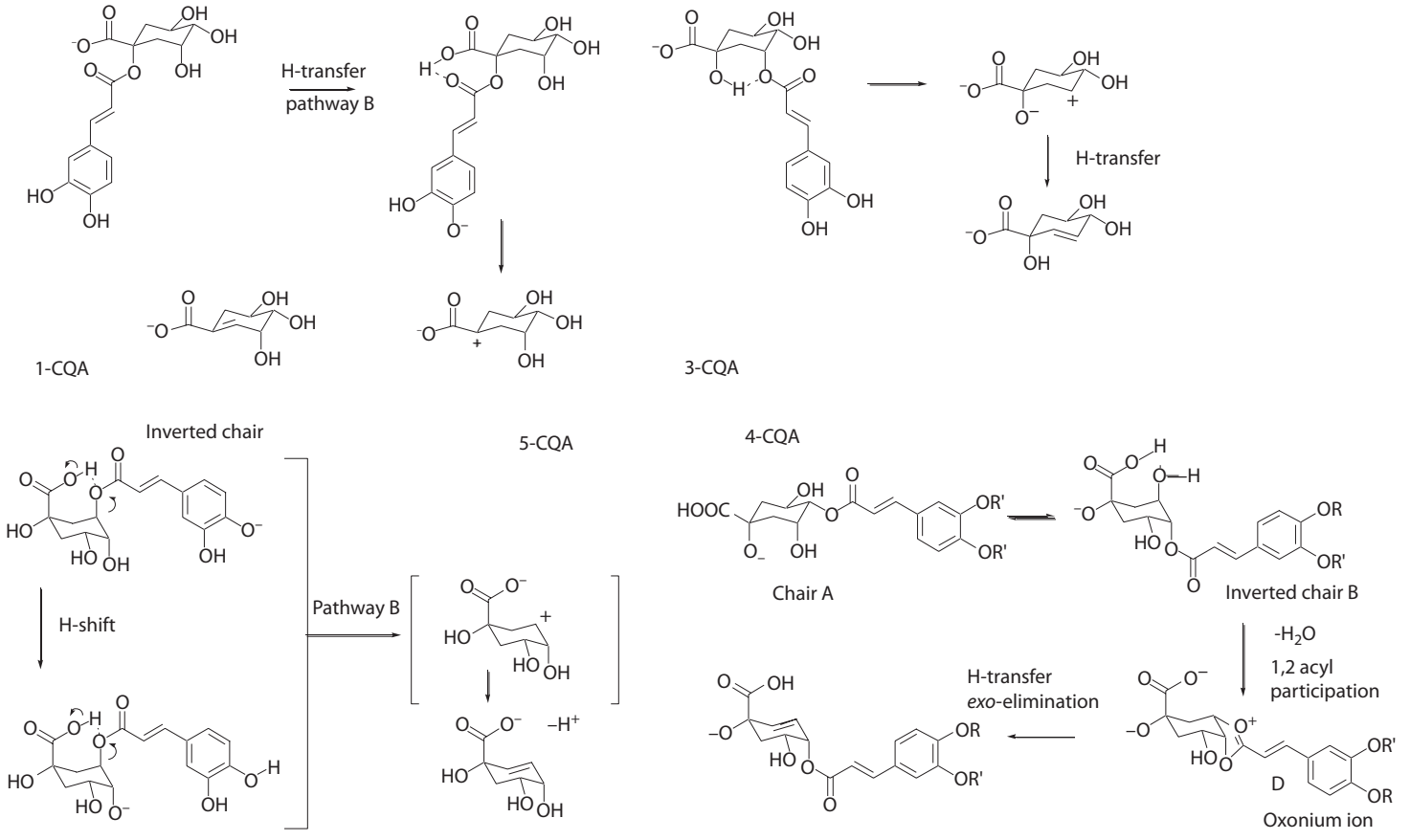
1. Sample preparation

   Crude CGA extracts are obtained by methanolic or aqueous methanolic extraction of dietary material, followed by the removal of proteins or carbohydrates where appropriate. Subsequent analysis by negative ion mode ESI-LC-MS^n using an auto MS^n mode and the published LC conditions is recommended.
2. CGA search and profiling

   CGAs are characterized by characteristic fragment ions at *m/z* 191 and 173 in MS^n fragment spectra in the negative ion mode. Hence, an all MS^n search producing extracted ion chromatograms of these two fragment ions will reveal the majority of CGAs present in any sample.
3. Assignment of compound class

   Fragment ions at *m/z* 191 and 173 in MS^2 spectra originate from monoacyl CGAs, in MS^3 from diacyl CGAs and in MS^4 from triacyl CGA derivatives. Retention times will further assist in this crude assignment. The *m/z* value of the parent ion will in most cases reveal the nature of the substituents (e.g., *m/z* 367 monoferuloyl CGA, 515 dicaffeoyl CGA, etc.).
4. Confirmation of molecular formulas

   At this stage, a high-resolution mass measurement is recommended to correctly establish the molecular formulas of the CGAs identified. For publication typically, values of 5 ppm or better should be achieved.



SCHEME 21.2 Fragmentation mechanisms for all four regioisomeric monocaffeoyl quinic acids.

5. Identification of acyl substituents

   As mentioned before, the *m/z* value of parent ion and the subsequent neutral loss in the tandem MS spectra will reveal in most cases directly, the nature of the acyl substituent. If this should not be the case, two strategies can be employed. (1) If a fragment ion in the negative ion mode of the intact acyl moiety is observed, this ion can be further studied by targeted MS^n experiments. In most cases, this is not the case. (2) For most CGAs, the intact ion of the acyl side chain can be observed in positive ion mode tandem MS spectra. The quinic acid moiety is able to stabilize a negative charge and typically can be observed in negative ion mode fragment spectra, whereas the acyl side chain is typically able to stabilize a positive charge and can be observed in positive ion mode spectra. Therefore, the use of targeted MS^n experiments in the positive ion mode usually reveals further structural information on the nature of the acyl substituent.
6. Profiling minor CGAs

   CGAs present in minor quantities are usually observable in auto MS^n runs; however, higher-order MS^n data cannot be obtained in this way. Therefore, targeted MS^n experiments, targeting the minor-component parent ions, are necessary at this stage.
7. Assignment of regiochemistry

   In a typical dietary or plant material, esters of (+)-quinic acid can be expected. After having obtained experimental tandem MS spectra, Clifford and Kuhnert´s hierarchical key can be employed to assign CGA regiochemistry (Clifford et al., 2003). All derivatives can here be sequenced in the tandem MS mass spectrometer by fragmenting selectively one acyl substituent after the other. Direct comparison of fragment spectra with those published allows unambiguous structure assignment. The majority of major-component CGAs present, in particular those already reported in the literature, should at this stage of analysis be identified and their structure assigned.
8. Remaining isomers

   In many cases, a considerable number of compounds, present in low abundance and tentatively identified as CGAs, remain unassigned at this point, in particular, if dealing with processed food, urine, or plasma. If this is the case, the compounds should be suspected to belong to any of the following groups:

   1. CGAs with variations in side-chain stereochemistry. Here, a close inspection of UV–Vis data combined with irradiation of the sample followed by LC-MS analysis will identify *cis*-cinnamoyl stereoisomers.
   2. CGAs with variations in side-chain regiochemistry. Here, targeted MS^n experiments in positive or negative ion mode are helpful in identifying variations in side-chain regiochemistry. A classical example is diagnostic MS^3 spectra for feruloyl and isoferuloyl side chains (Kuhnert et al., 2010).
   3. CGAs with variations in quinic acid stereochemistry. At this stage, assignment procedures are not available. On various occasions, stereoisomers of CGAs have been reported, but reliable structural data are not available. According to Clifford and Kuhnert´s tandem MS method, it should be possible to distinguish quinic acid stereoisomers by tandem MS, since differences in hydrogen bonding arrays are to be expected (Clifford et al., 2003). Experimental confirmation of this is, however, still outstanding. At this stage, therefore, only chemical synthesis of suspected CGA stereoisomer targets is required and direct comparison of tandem LC-MS data will reveal the chemical structure.
   4. Esters at the quinic acid COOH moiety of CGAs. In particular, methyl esters are frequently observed as artifacts of the workup procedure. Such compounds can be readily identified due to their neutral loss of *m/z* 31 (OCH_3) in MS^2 spectra. Other esters need to be carefully evaluated according to their MS^n data.
   5. Other compounds that are not CGAs. Occasionally, ions are wrongly assigned as CGAs due to their serendipitous fragmentation resembling CGA fragmentation patterns. Quite

often such compounds can be identified as non-CGAs by their high-resolution MS *m/z* values (both parent ions and fragment ions) and their MS isotope pattern. These should be carefully inspected.

Tandem LC-MS constitutes a remarkably powerful tool in food and natural product research. In our view, it is hindered by the lack of comprehensive and reliable MS spectral databases and the frequent lack of reference spectra in published literature in general. Therefore, we would like to suggest to authors and editors alike to publish or request from authors such reference spectra wherever possible. Even unassigned spectra should, in our view, be published and authors, referees, and editors alike should be brave enough to allow publication of unassigned data, which is certainly preferential to not making them publicly available or even allowing premature misassignments.

21.17 LC-MS and LC-MS^n Identification of Chlorogenic Acids in Food Materials

In this section, we present in detailed form data from the profiling of CGAs in selected dietary materials. These examples should illustrate the work flow laid out above. As examples, we have chosen green coffee beans and yerba maté *(Ilex paraguayensis)* leaves.

21.17.1 Green Coffee Beans

In green coffee beans, 48 CGAs (Table 21.10) have been reported thus far and all were characterized to their regioisomeric level based on their retention time in LC and tandem MS spectra (Clifford et al., 2003, 2005, 2006a,b; Alonso-Salces et al., 2009a). Like other food materials, in coffee no CGA derivatives are present that are acylated at C-1. A methanolic or aqueous methanolic extracts of both the varieties of coffee (*Robusta* and *Arabica*) beans have been analyzed on several occasions. A typical HPLC chromatogram (total ion chromatogram (TIC) in the negative ion mode) of a typical green coffee bean extract is shown in Figure 21.10 (Jaiswal, R., 2010, unpublished results).

Monoacyl CGAs are more polar compared to diacyl CGA; so they elute first on reverse-phase packings and appear early in the chromatogram (Clifford et al., 2003, 2005, 2006a–c, 2007, 2008; Zheng and Clifford, 2008; Alonso-Salces et al., 2009b). Generally water, acetic acid, methanol, and acetonitrile have been used as mobile phases for HPLC with reverse-phase stationary phases such as C18, C8, phenylhexyl, diphenyl, and so on.

Clifford et al. have developed an LC-MS^n hierarchical scheme for the identification of mono- and diacyl CGAs (Clifford et al., 2003). In this method, relative hydrophobicity, retention time, and fragmentation pattern in negative ion mode were considered. Based on this LC-MS^n hierarchical scheme, they have identified more than 45 CGAs in green coffee beans (Clifford et al., 2003, 2005, 2006a,b) and some mono- and diacyl CGAs containing *cis*-cinnamic acid moiety have also been identified in green coffee leaves (Clifford et al., 2008).

All monoacyl CGAs gave the expected parent ion $[CGA–H]^–$. The suggested fragment structures are presented in Figure 21.11 (Clifford et al., 2003, 2006a,b).

In the case of 3-*p*CoQA, 3-FQA, 3-DQA, and 3-SiQA (Jaiswal, R., 2010, unpublished results), the MS^2 and MS^3 base peak ions were derived from the cinnamic acid moiety [cinnamic acid $–H]^–$ (Clifford et al., 2003). For 3-*p*CoQA, 3-DQA, 3-SiQA, and 3-FQA, these ions are [cinnamic acid $–H]^–$ (A_3, A_2, A_5, and A_4, respectively) ions and their decarboxylation products [cinnamic acid $–CO_2$ $–H]^–$ (B_3, B_2, B_5, and B_4 respectively), respectively. For the remaining monoacyl CGAs, the base peak ions in MS^2 and MS^3 spectra were derived from the quinic acid moiety. Two distinct fragmentation pathways occur for the quinic acid-derived fragments. One pathway gives [quinic acid $–H]^–$ (Q_1) at MS^2 and a fragment (Q_5) at *m/z* 86 in MS^3; the other gives [quinic acid – H_2O $–H]^–$ (Q_2) at MS^2 and a fragment (Q_6) at *m/z* 93. A 4-acyl CGA can be distinguished by its "dehydrated" MS^2 base peak (Q_2) at *m/z* 173 (Figures 21.12 through 21.14), supported by an MS^3 base peak at *m/z* 93 and Q_7 at *m/z* 111 (Figures 21.12 through 21.14). All other monoacyl CGAs and 3-CQA produce an MS^2 base peak at *m/z* 191 (Q_1), supported by strong MS^3 ions at *m/z* 86 (Q_5), *m/z*

TABLE 21.10

Chlorogenic Acids Identified in Green Coffee Beans (No Refers to Labeling in Chromatograms)

No.	Name	Abbreviation	R^3	R^4	R^5
1	3-*O*-Caffeoylquinic acid	3-CQA	C	H	H
2	4-*O*-Caffeoylquinic acid	4-CQA	H	C	H
3	5-*O*-Caffeoylquinic acid	5-CQA	H	H	C
4	3-*O*-Feruloylquinic acid	3-FQA	F	H	
5	4-*O*-Feruloylquinic acid	4-FQA	H	F	H
6	5-*O*-Feruloylquinic acid	4-FQA	H	H	F
7	3-*O*-*p*-Coumaroylquinic acid	3-*p*CoQA	*p*Co	H	H
8	4-*O*-*p*-Coumaroylquinic acid	4-*p*CoQA	H	*p*Co	H
9	5-*O*-*p*-Coumaroylquinic acid	5-*p*CoQA	H	H	*p*Co
10	3-*O*-Dimethoxycinnamoylquinic acid	3-DQA	D	H	H
11	4-*O*-Dimethoxycinnamoylquinic acid	4-DQA	H	D	H
12	5-*O*-Dimethoxycinnamoylquinic acid	5-DQA	H	H	D
13	3,4-Di-*O*-caffeoylquinic acid	3,4-diCQA	C	C	H
14	3,5-Di-*O*-caffeoylquinic acid	3,5-diCQA	C	H	C
15	4,5-Di-*O*-caffeoylquinic acid	4,5-diCQA	H	C	C
16	3,4-Di-*O*-feruloylquinic acid	3,4-FQA	F	F	H
17	3,5-Di-*O*-feruloylquinic acid	3,5-FQA	F	H	F
18	4,5-Di-*O*-feruloylquinic acid	4,5-FQA	H	F	F
19	3,4-Di-*O*-*p*-coumaroylquinic acid	3,4-di*p*CoQA	*p*Co	*p*Co	H
20	3,5-Di-*O*-*p*-coumaroylquinic acid	3,5-di*p*CoQA	*p*Co	H	*p*Co
21	4,5-Di-*O*-*p*-coumaroylquinic acid	4,5-di*p*CoQA	H	*p*Co	*p*Co
22	3-*O*-Feruloyl-4-*O*-caffeoylquinic acid	3F-4CQA	F	C	H
23	3-*O*-Caffeoyl-4-*O*-feruloylquinic acid	3C-4FQA	C	F	H
24	3-*O*-Feruloyl-5-*O*-caffeoylquinic acid	3F-5CQA	F	H	C
25	3-*O*-Caffeoyl-5-*O*-feruloylquinic acid	3C-5FQA	C	H	F
26	4-*O*-Feruloyl-5-*O*-caffeoylquinic acid	4F-5CQA	H	F	C
27	4-*O*-Caffeoyl-5-*O*-feruloylquinic acid	4C-5FQA	H	C	F
28	3-*O*-Dimethoxycinnamoyl-4-*O*-caffeoylquinic acid	3D-4CQA	D	C	H
29	3-*O*-Dimethoxycinnamoyl-5-*O*-caffeoylquinic acid	3D-5CQA	D	H	C
30	4-*O*-Dimethoxycinnamoyl-5-*O*-caffeoylquinic acid	4D-5CQA	H	D	C
31	3-*O*-Caffeoyl-4-*O*-dimethoxycinnamoylquinic acid	3C-4DQA	C	D	H
32	3-*O*-Caffeoyl-5-*O*-dimethoxycinnamoylquinic acid	3C-5DQA	C	H	D
33	4-*O*-Caffeoyl-5-*O*-dimethoxycinnamoylquinic acid	4C-5DQA	H	C	D
34	3-*O*-Dimethoxycinnamoyl-4-*O*-feruloylquinic acid	3D-4FQA	D	F	H
35	3-*O*-Dimethoxycinnamoyl-5-*O*-feruloylquinic acid	3D-5FQA	D	F	H
36	4-*O*-Dimethoxycinnamoyl-5-*O*-feruloylquinic acid	4D-5FQA	H	D	F
37	3-*O*-*p*-Coumaroyl-4-*O*-caffeoylquinic acid	3*p*Co-4CQA	*p*Co	C	H
38	3-*O*-Caffeoyl-4-*O*-*p*-coumaroylquinic acid	3C-4*p*CoQA	C	*p*Co	H
39	3-*O*-*p*-Coumaroyl-5-*O*-caffeoylquinic acid	3*p*Co-5CQA	*p*Co	H	C
40	3-*O*-Caffeoyl-5-*O*-*p*-coumaroylquinic acid	3C-5*p*CoQA	C	H	*p*Co
41	4-*O*-Caffeoyl-5-*O*-*p*-coumaroylquinic acid	4C-5*p*CoQA	H	C	*p*Co
42	4-*O*-*p*-Coumaroyl-5-*O*-caffeoylquinic acid	4*p*Co-5CQA	H	*p*Co	C
43	3-*O*-*p*-Coumaroyl-4-*O*-feruloylquinic acid	3*p*Co-4FQA	*p*Co	F	H
44	3-*O*-*p*-Coumaroyl-5-*O*-feruloylquinic acid	3*p*Co-5FQA	*p*Co	H	F
45	4-*O*-*p*-Coumaroyl-5-*O*-feruloylquinic acid	4*p*Co-5FQA	H	*p*Co	F
46	4-*O*-Dimethoxycinnamoyl-5-*O*-*p*-coumaroylquinic acid	4D-5*p*CoQA	H	D	*p*Co
47	3-*O*-*p*-Coumaroyl-4-*O*-dimethoxycinnamoylquinic acid	3*p*Co-4DQA	*p*Co	D	H
48	3-*O*-*p*-Coumaroyl-5-*O*-dimethoxycinnamoylquinic acid	3*p*Co-5DQA	*p*Co	H	D

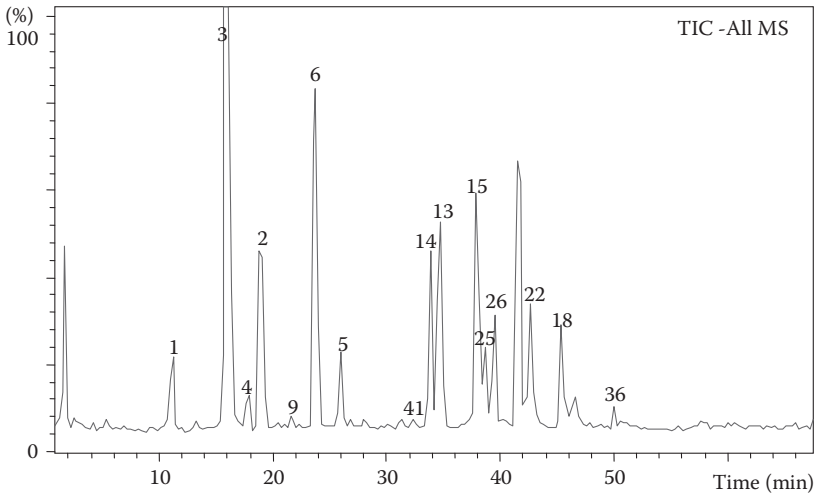


FIGURE 21.10 A total ion chromatogram of green Robusta coffee extract in negative ion mode. (Peak numbering is according to Table 21.10.)

127 (Q_7), and *m/z* 172 (Q_3) (Figures 21.15 through 21.18). 3-CQA gives the same base peak as 5-CQA but can be distinguished from 5-CQA by a comparatively intense caffeic acid-derived ion A_1 at *m/z* 179 (Figures 21.17 and 21.18). For 4-CQA and other 4-acyl caffeoylquinic acids, two characteristic secondary peaks in MS^2 or MS^3 at *m/z* 255 and 299 were reported. Similar kinds of secondary peaks at *m/z* 269 and 313 were reported for 4-FQA and other 4-acyl feruloylquinic acids (Clifford et al., 2006a,b).

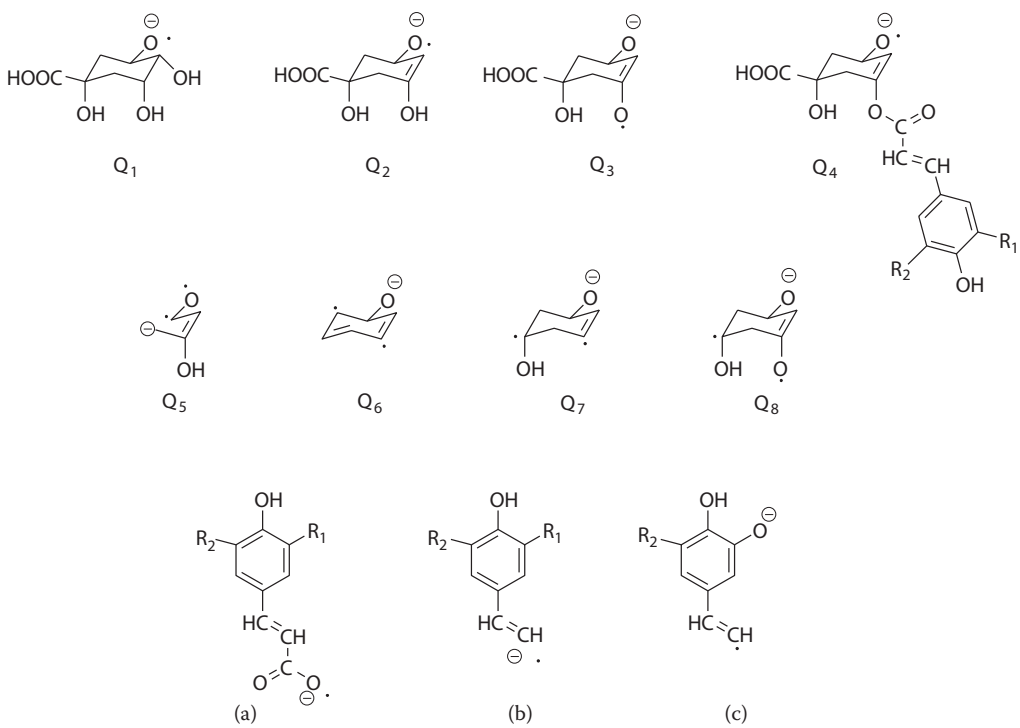


FIGURE 21.11 Structure of quinic acid-derived and cinnamic acid-derived fragments.

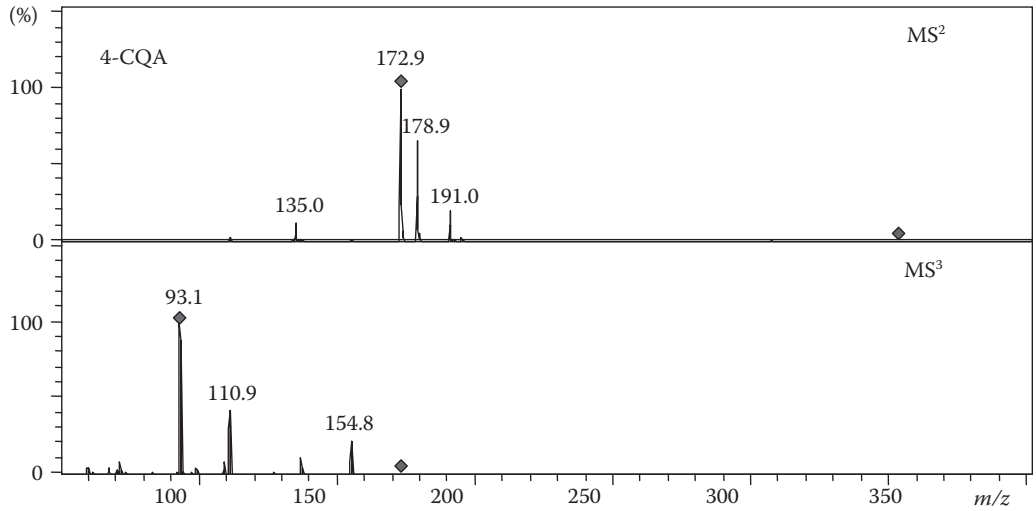


FIGURE 21.12 MS^2 and MS^3 spectra of 4-CQA in negative ion mode (*m/z* 353).

The diacyl and triacyl CGAs behave similarly, giving the equivalent parent ions [diacyl CGA–H]$^-$ and [triacyl CGA–H]$^-$, respectively (Clifford et al., 2007). All diacyl CGAs either produce [diacyl CGA–cinnamoyl–H]$^-$ (Figures 21.19 through 21.21) or [diacyl CGA–cinnamoyl–H_2O–H]$^-$ (Figures 21.22 and 21.23) (Clifford et al., 2005, 2006a,b). The *vic* diCQA (3,4-diCQA and 4,5-diCQA) give Q_2 as the MS^3 base peak at *m/z* 173 (Figures 21.19 and 21.20), supported by strong MS^4 ions at *m/z* 93 (Q_6) and Q_7 at *m/z* 111, which are consistent with the 4-acylated monoacyl CGAs. These ions were absent in 3,5-diCQA (Figure 21.21), which gives Q_1 as an MS^3 base peak at *m/z* 191, supported by strong MS^3 ions at *m/z* 86 (Q_5), *m/z* 127 (Q_8), and *m/z* 172 (Q_3), which is consistent with 3-CQA and 5-CQA. The two *vic* diCQA isomers differ (Figures 21.19 and 21.20) with regard to their intensities of Q_4 the MS^2 "dehydrated" ion [CQA–H_2O–H]–. In 3,4-diCQA, Q_4 is more intense. In contrast, in 4,5-diCQA, Q_4 is barely detectable. Similarly, the 3,4-isomer produces Q_1 in MS^3 and Q_7 in MS^4 with approximately double the intensities if compared to the 4,5-isomer (Clifford et al., 2003, 2005, 2006a,b).

MS^2 base peaks for the diCQA are identical to the parent ions for the CQA (Clifford et al., 2005). The subsequent degradation of these ions will therefore be identical, regardless of whether they have derived

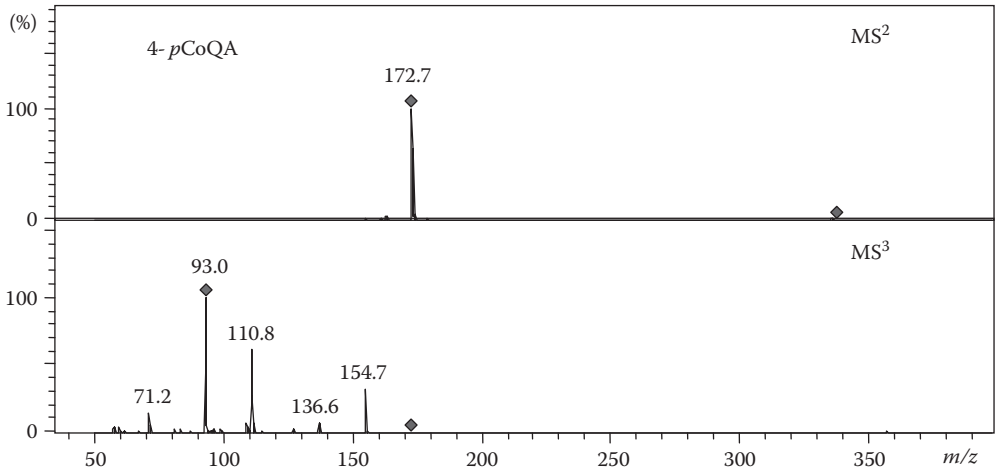


FIGURE 21.13 MS^2 and MS^3 spectra of 4-*p*CoQA in negative ion mode (*m/z* 337).

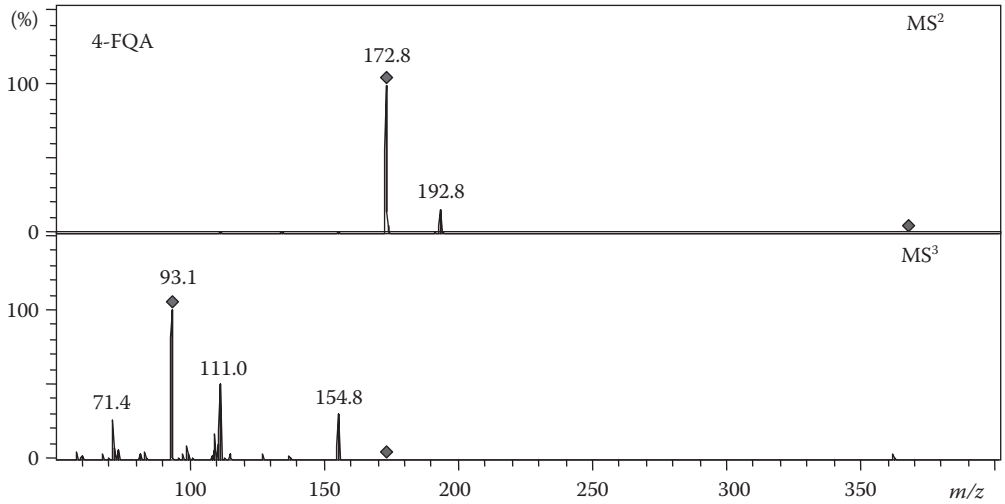


FIGURE 21.14 MS² and MS³ spectra of 4-FQA in negative ion mode (*m/z* 367).

from CQA or diCQA. By comparison with the CQA MS² data (Figures 21.12, 21.17, and 21.18 and Tables 21.11 and 21.12), it is possible to define the precise regiochemistry of the ions responsible for the diCQA MS² base peaks. Based on this information, it becomes possible to specify, which of the caffeoyl moieties are removed from the diCQA during MS² and MS³. An MS² base peak at *m/z* 173 (Q_2) is characteristic of an isomer substituted at position 4, the MS² base peak of *vic* diCQA must be [4-CQA–H]⁻ rather than [3-CQA–H]⁻ or [5-CQA–H]⁻ . The 3,4-diCQA follows the same pattern and initially loses the caffeoyl moiety at C-3, whereas 4,5-diCQA initially loses that at C-5 (Clifford et al., 2005).

Fragmentation of the MS² base peak for 3,5-diCQA yields a comparatively intense [caffeoyl–H]⁻ ion (A_1 = 50% of base peak) (Figure 21.21). This is consistent with [3-CQA–H]⁻ being the MS² base peak rather than [5-CQA–H]⁻ where only a weak [caffeoyl–H]⁻ is detected. Based on these arguments, the acyl residue at C-4 is the most difficult to remove, whereas that at C-5 is the easiest (note true in coffee in the absence of C-1 acyl derivatives). These facts are also true for CFQA and other hetero-diacyl CGAs (Figures 21.24 through 21.28 and Table 21.12).

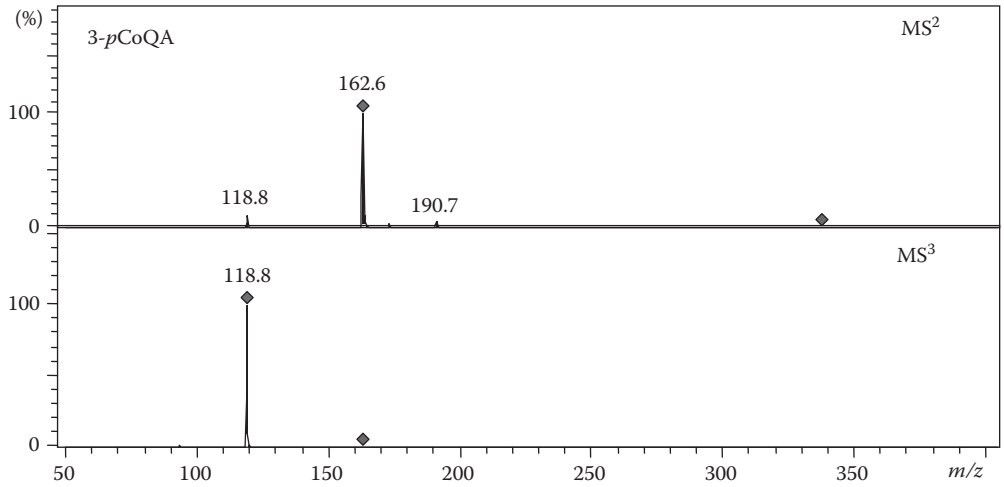


FIGURE 21.15 MS² and MS³ spectra of 3-*p*CoQA in negative ion mode (*m/z* 337).

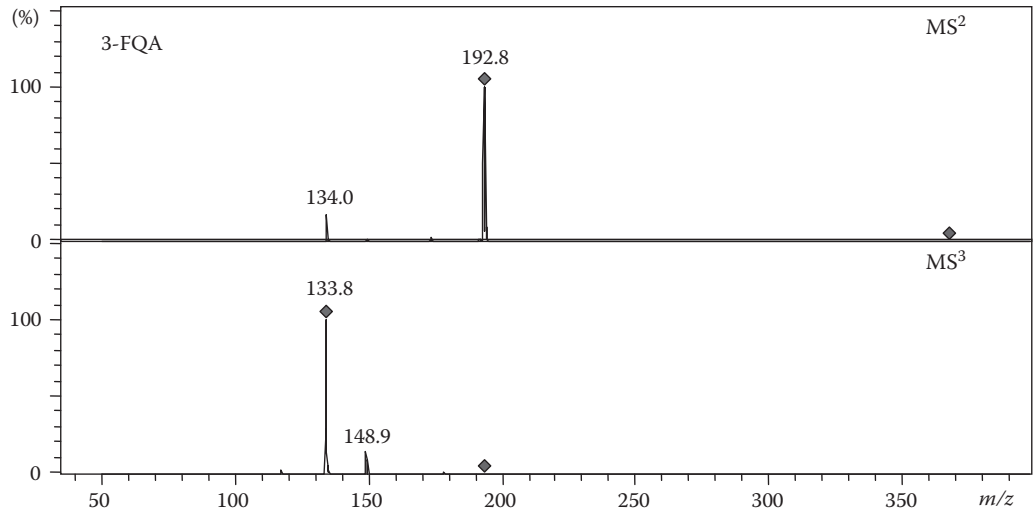


FIGURE 21.16 MS^2 and MS^3 spectra of 3-FQA in negative ion mode (*m/z* 367).

In the case of triacyl CGAs, order of removal of acyl residue is 1 > 5 > 3 > 4. 3,4,5-Tricaffeoylquinic acid produces MS^2 base peak at *m/z* 515 (Figure 21.29), MS^3 base peak at *m/z* 353 (Figure 21.29), and MS^4 base peak at *m/z* 173 (Figure 21.29). MS^3 and MS^4 data of 3,4,5-tricaffeoylquinic acid are consistent with the MS^2 and MS^3 data of 3,4-dicaffeoylquinic acid (Figure 21.19).

Generally, it has been observed that the order of elution for the monoacyl CGAs in RP columns is 1 > 3 > 5 > 4 (in green coffee there is no 1-acylated CGA) and similarly for diacyl CGAs is 1,3 > 1,4 > 1, 5 > 3,4 > 3,5 > 4,5 (Clifford et al., 2003, 2005).

A liquid chromatography coupled with a photodiode array detector, electrospray ionization, collision-induced dissociation, and tandem mass spectrometry (LC-DAD/ESI-CID-tandem MS) on a triple quadrupole has been used to detect and characterize CGAs in green coffee beans (Alonso-Salces et al., 2009). Fragmentation in the quadrupole MS stages are different compared to the ion-trap instrument,

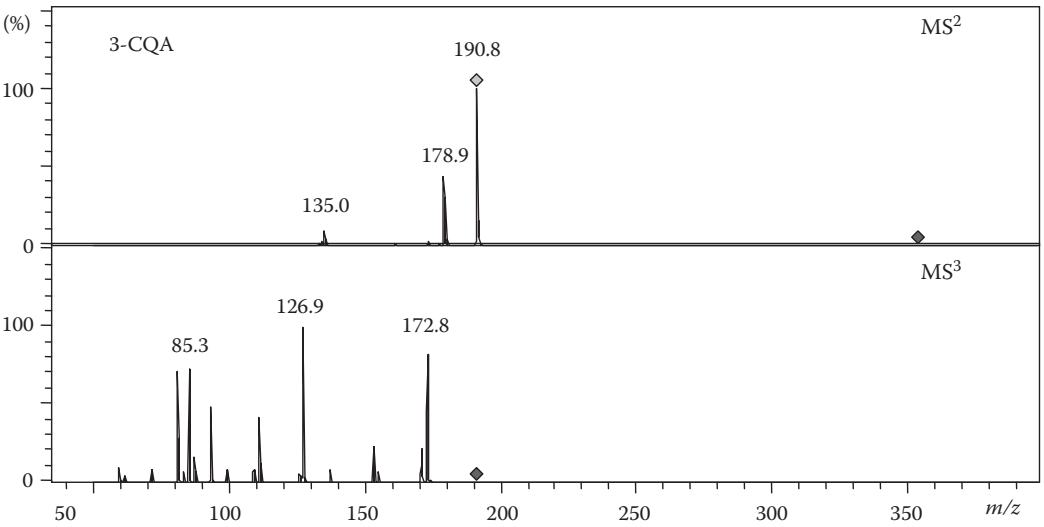


FIGURE 21.17 MS^2 and MS^3 spectra of 3-CQA in negative ion mode (*m/z* 353).

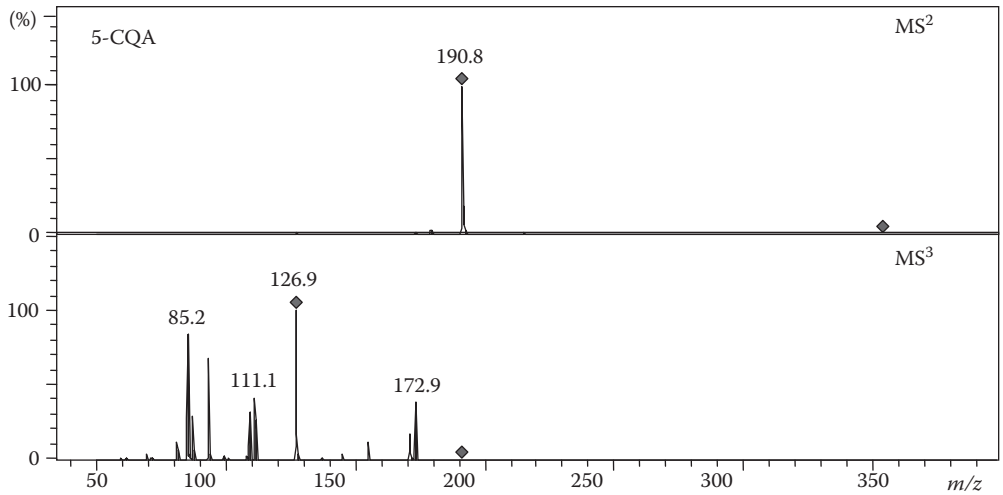


FIGURE 21.18 MS^2 and MS^3 spectra of 5-CQA in negative ion mode.

due to the excess of collision energy employed in the triple quadrupole collision cell. Therefore, the fragment ions and relative intensities of fragment ions observed in tandem MS spectra were not identical to those reported for ion trap data.

Three dimethoxycinnamoyl-caffeoylquinic acids were reported here for the first time from green coffee beans (Tables 21.13 and 21.14) (Alonso-Salces et al., 2009a). CGAs identification and structure assignment were based on their UV spectrum, retention time, and mass spectra in both positive and negative ion modes (MS^1 and MS^2). During MS experiments in the positive ion mode, CGA molecular ions were detected either as $[M + Na]^+$ or as $[2M + Na]^+$ (for monoacyl CGAs). For diacyl CGAs, a dehydrated molecular ion $[M + H–H_2O]^+$ was also detected. In negative ion mode, all CGAs showed a $[M–H]^–$ as a molecular ion and for monoacyl CGA, a deprotonated dimeric ion $[2M–H]^–$ was also detected. Based on the *m/z* value of protonated and deprotonated ions, and UV spectra, nine subclasses of CGAs containing 32 CGAs have been identified. In the positive mode of tandem MS experiments of CGAs, the [cinnamic acid + $H–H_2O]^+$ ion was a base peak for all the monoacyl CGAs. For diacyl CGAs, a dehydrated

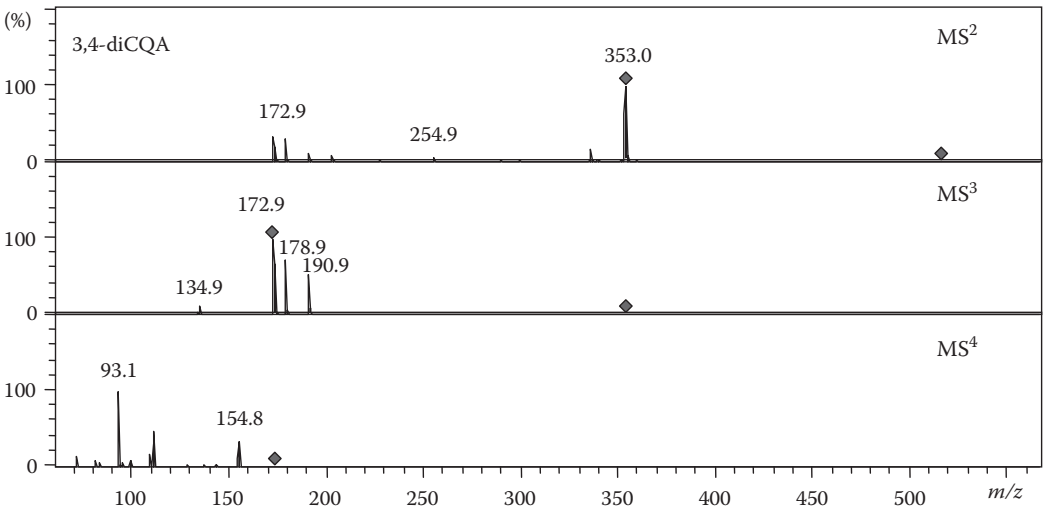


FIGURE 21.19 MS^2, MS^3, and MS^4 spectra of 3,4-diCQA in negative ion mode (*m/z* 515).

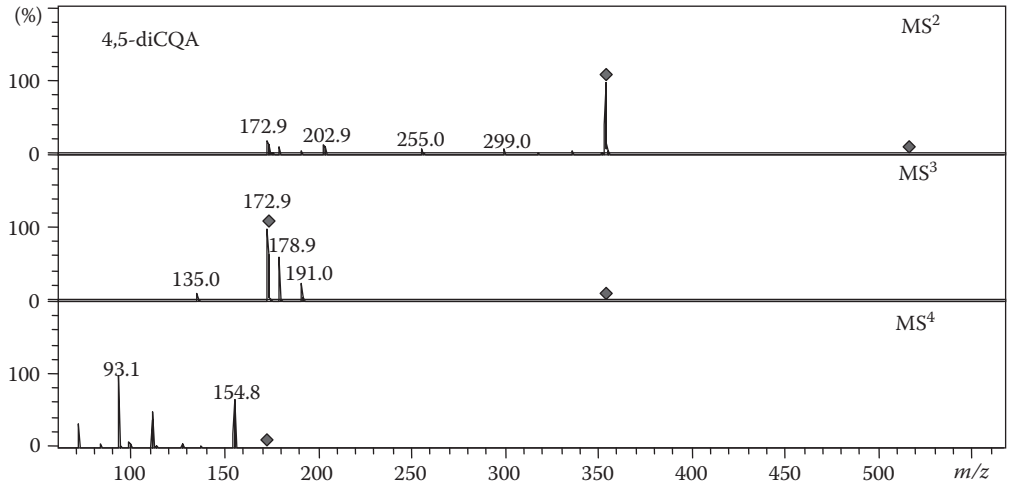


FIGURE 21.20 MS², MS³, and MS⁴ spectra of 4,5-diCQA in negative ion mode (*m/z* 515).

protonated molecular ion $[M + H–H_2O]^+$ and also a dehydrated ion due to another cinnamoyl moiety [cinnamic acid + $H–H_2O]^+$ were present. When high collision energies were applied, monoacyl CGAs gave other fragments by the neutral losses (H_2O, CO, CH_3OH) like [cinnamic acid + $H–2H_2O]^+$, [cinnamic acid + $H–H_2O–CO]^+$, [cinnamic acid + $H–2H_2O–CO]^+$, [cinnamic acid + $H–H_2O–CH_3OH]^+$, and [cinnamic acid + $H–H_2O–CH_3OH–CO]^+$ (Table 21.13). The fragmentation pattern was identical for all isomers within each class of CGAs and these data were used to confirm the assignment of subclasses of CGAs (Alonso-Salces et al., 2009a).

Mendonca et al. (2008) have been used an electrospray ionization mass spectrometry (ESI-MS) method to identify the CGA profile, which allowed the discrimination of green *Arabica* and *Robusta* coffee beans. This method also allowed discrimination between defective and nondefective coffee beans (ESI-MS positive mode). For this kind of identification and discrimination, they used principal component analysis and hierarchical cluster analysis (Mendonca et al., 2008). Alonso-Salces et al. (2009b) also used a linear discriminant analysis and a partial least-squares discriminant analysis for the number of

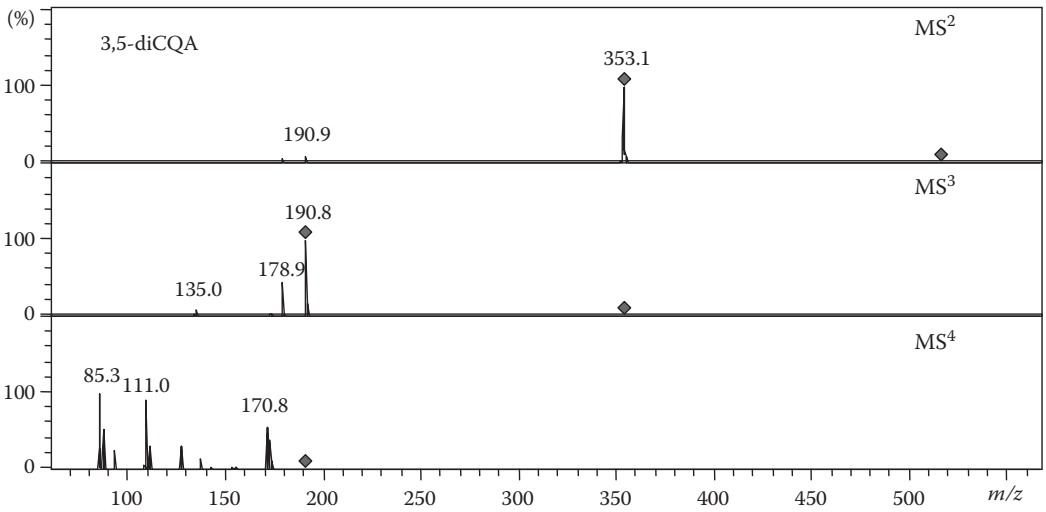


FIGURE 21.21 MS², MS³, and MS⁴ spectra of 3,5-diCQA in negative ion mode (*m/z* 515).

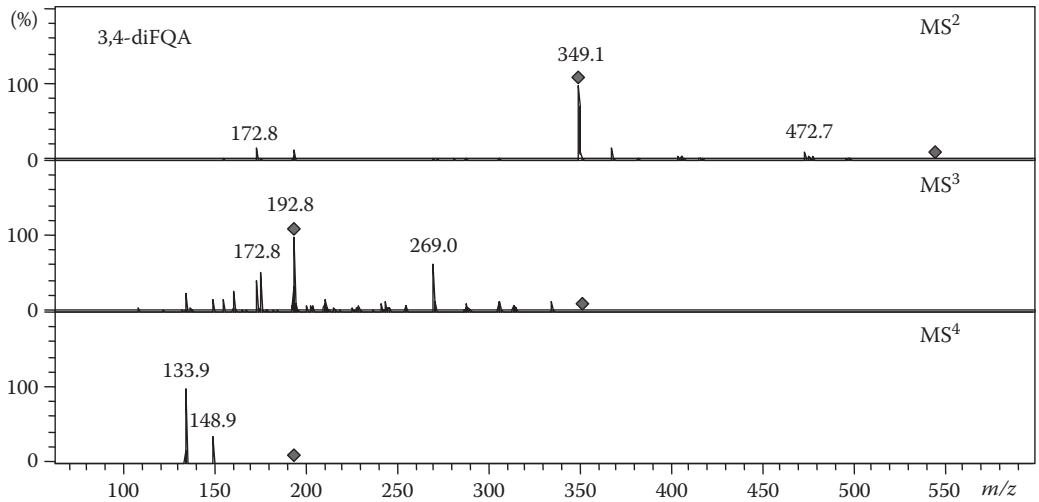


FIGURE 21.22 MS^2, MS^3, and MS^4 spectra of 3,4-diFQA in negative ion mode (*m/z* 543).

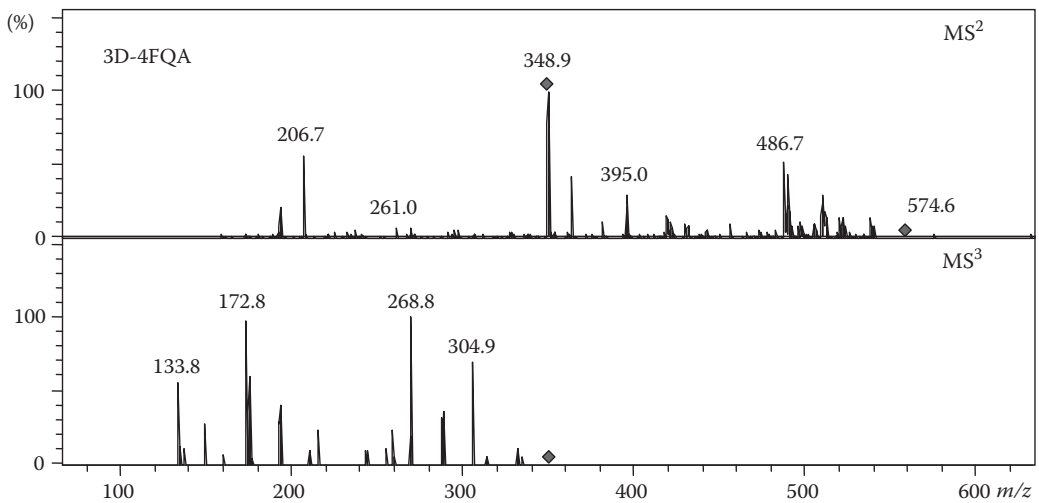


FIGURE 21.23 MS^2 and MS^3 spectra of 3D-4FQA in negative ion mode (*m/z* 557).

green *robusta* and *arabica* coffee beans from different geographical origin based on HPLC and UV spectra of phenolic (CGAs) and methylxanthin contents.

21.17.2 Roasted Coffee

Many CGAs have been reported in roasted coffee and it is well known that the CGA content decreases during the roasting process. (Bennat et al., 1994; Schrader et al., 1996). During roasting, CGAs are hydrolyzed to their corresponding cinnamic acids, quinic acid, or isomerized to their epimers and regioisomers or form CGA lactones (Bennat et al., 1994; Schrader et al., 1996; Farah et al., 2006; Duarte et al., 2010). However, these mechanisms account for only half of the CGA derivatives lost during roasting.

Schrader et al. employed an HPLC method and they reported eight CGAs (3-CQA, 4-CQA, 5-CQA, 4-FQA, 5-FQA, 3,4-diCQA, 3,5-diCQA, and 4,5-diCQA) and two CGA lactones (3-CQL and 4-CQL) (Figure 21.30) in different commercial-roasted coffee samples (Schrader et al., 1996). Further structural

TABLE 21.11

MS^2 and MS^3 Data of Monoacyl CGAs in Negative Ion Mode

	MS^1	MS^2							MS^3				
		Base Peak	Secondary Peak						Base Peak	Secondary Peak			
CGA	**Parent Ion**	***m/z***	***m/z***	**int***	***m/z***	**int**	***m/z***	**int**	***m/z***	***m/z***	**int**	***m/z***	**int**
3-CQA	353.1	190.9	178.5	50			134.9	7	85.3	127.0	71	172.9	67
4-CQA	353.1	172.9	178.9	60	190.8	20	135.0	9	93.2	111.0	48		
5-CQA	353.2	190.0	178.5	5			135.0	15	85.2	126.9	66	172.9	27
3-FQA	367.2	192.9	191.5	2	173.2	2			133.9	148.9	23		
4-FQA	367.2	172.9	192.9	16					93.1	111.5	44		
5-FQA	367.2	190.9	172.9	2					85.2	126.9	70		
3-*p*CoQA	337.1	162.9	190.0	5					118.9				
4-*p*CoQA	337.1	172.7							93.0	111.0	61		
5-*p*CoQA	337.2	190.9	162.9	5					85.2				

Note: int* = Intensity.

evidences came from their isolation by preparative HPLC, NMR, and subsequent thermospray-MS. When coffee brews were kept at an elevated temperature (80°C) for 4 h, the amount of CQA lactones reduced to 60%. During roasting, the contents of 3-CQA and 4-CQA were increased, while that of 5-CQA decreased. And overall contents of CQA were decreased during the roasting process of coffee (Schrader et al., 1996).

The degree of roasting causes a change in CGAs and CGA lactones contents. Farah et al. (2005) reported CGA lactones (Figure 21.30) in roasted coffee and the maximum amount of CGA lactones was approximately 30% of their precursor CGAs. Identification and quantification were based on the synthetic standards of CGA lactones. Relative contents of 3-CQL and 4-CQL were reverse to those of their precursor CGAs in green coffee. It was suggested that roasting causes isomerization of CGAs prior to the formation of CGA lactones (Farah et al., 2005). A series of CGA lactones were identified in roasted coffee, which were responsible for the bitter taste of coffee (Figure 21.30) (Farah et al., 2006, 2005; Frank et al., 2007).

21.17.3 Maté Leaves

Maté or yerba maté (*Ilex paraguariensis*) is a tea-like beverage consumed mainly in Southern America. On several occasions its phenolic or CGAs constituents have been studied, and structural conclusions about chemical constituents were based on their LC-MS characteristics (Carini et al., 1998; Bastos et al., 2007; Bravo et al., 2007; Dugo et al., 2009). A typical HPLC chromatogram (TIC in negative ion mode) of roasted maté is shown in Figure 21.31 (Jaiswal et al., 2010), in which some of the CGAs (peak numbering is according to Table 21.15) are highlighted, which have been reported thus far. In maté caffeoyl–sinapoylquinic acids, caffeoyl–shikimic acid esters and some unassigned CGAs have been reported and these CGAs were reported for first time in nature (Jaiswal et al., 2010). Carini et al. (1998) characterized phenolic antioxidants including CGAs based on their retention time, UV spectrum (λ_{max} 320 nm) and tandem mass spectra. Three mono- and three diacyl CGAs have been identified but only monoacyl CGAs were characterized to their regioisomeric level (Table 21.16) (Carini et al., 1998).

Bastos et al. (2007) identified CGAs in green and roasted yerba maté by their ESI-MS fingerprints of ethanolic and water extracts. Based on ESI-MS (negative ion mode) fingerprints, the following CGAs were identified: *m/z* 353—caffeoylquinic acid, *m/z* 367—feruloylquinic acid, and *m/z* 515—dicaffeoylquinic acid (Bastos et al., 2007). Similarly, Dugo et al. (2009) applied ESI-MS (both positive and negative ion modes) fingerprints for the identification and structure elucidation of CGAs to their regioisomeric level in maté extract. They have used two-dimensional LC coupled with ESI-ion trap

TABLE 21.12

MS^2, MS^3, and MS^4 Data of Diacyl CGAs in Negative Ion Mode

	MS^1	MS^2							MS^3							MS^4				
		Base Peak	Secondary Peak						Base Peak	Secondary Peak						Base Peak	Secondary Peak			
CGA	**Parent Ion**	***m/z***	***m/z***	**int***	***m/z***	**int**	***m/z***	**int**	***m/z***	***m/z***	**int**	***m/z***	**int**	***m/z***	**int**	***m/z***	***m/z***	**int**	***m/z***	**int**
1,3-diCQA	515.2	353.1	335.1	2	173.0	4			190.9	179.0	60			135.1	6	85.1	111.1	86	172.9	60
3,4-diCQA	515.2	353.1	335.1	4	172.9	20			172.9	178.9	68	191.0	32	135.1	9	93.2	111.1	30		
4,5-diCQA	515.2	353.1	335.1	2	172.9	6			172.9	178.9	76	190.9	9	135.0	19	93.1	111.0	20		
1,4-diFQA	543.2	349.1	367.1	25	172.9	17	268.8	9	192.9	172.9	31	268.8	9	133.8	22					
4,5-diFQA	543.2	367.1	349.1	35					172.9	178.9	60	190.8	20	135.0	9	93.1	111.1	40		
1C-3FQA	529.1	367.1	353.1	15					192.7	172.6	13	178.6	5	133.8	18	133.7	149.0	16	127.0	6
3F-5CQA	529.2	367.1	353.1	60	349.0	32	335.0	32	192.7	172.6	36			133.8	36	133.7	149.0	19		
4F-5CQA	529.2	367.1	335.0	4	172.7	21			172.9	192.9	71			133.8	8	93.2				
4C-5FQA	529.1	353.1	367.1	25					172.9	178.9	49	190.8	35	134.7	10	93.2			127.0	n.d.
3C-5*p*CoQA	499.0	353.1	337.0	15					190.7							85.2	93.0	70	126.9	99
4*p*Co,-5CQA	499.1	337.1	335.1	3	172.7	59			172.9	162.6	8					93.2	111.1	98		
4C-5*p*CoQA	499.3	353.0			172.8	15			172.9	178.7	66	190.6	29	134.8	12	93.2				

Note: n.d. = not detected, int*= intensity.

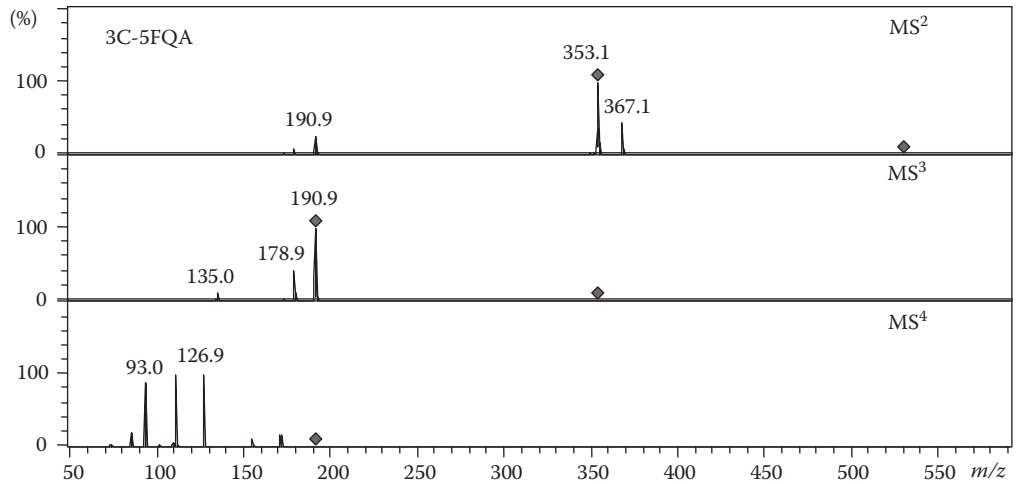


FIGURE 21.24 MS^2, MS^3, and MS^4 spectra of 3C-5FQA in negative ion mode (*m/z* 529).

MS. MS^3 spectra were recorded for all the CGAs and they were in agreement to the tandem MS data previously reported by Clifford et al. All these compounds comprise the typical UV spectra of CGAs and high-resolution mass data confirmed the molecular formulas with mass errors typically below 5 ppm. All the CGAs reported in maté and their UV spectrum and tandem MS data are represented in Table 21.17.

Recently, Vietnam bitter tea *Ilex kudingcha* has also been analyzed for its antioxidant activity and 14 CGAs were characterized to their regioisomeric level. Identification was based on their HPLC and spectroscopic analysis (Thuong et al., 2009).

21.17.4 Other Food Materials

Clifford et al. (2006d, 2007) reported a series of CGAs in herbal *Chrysanthemum* based on LC-MS^5 identification. During this study, they have also identified two tricaffeoylquinic acids that were 3,4,

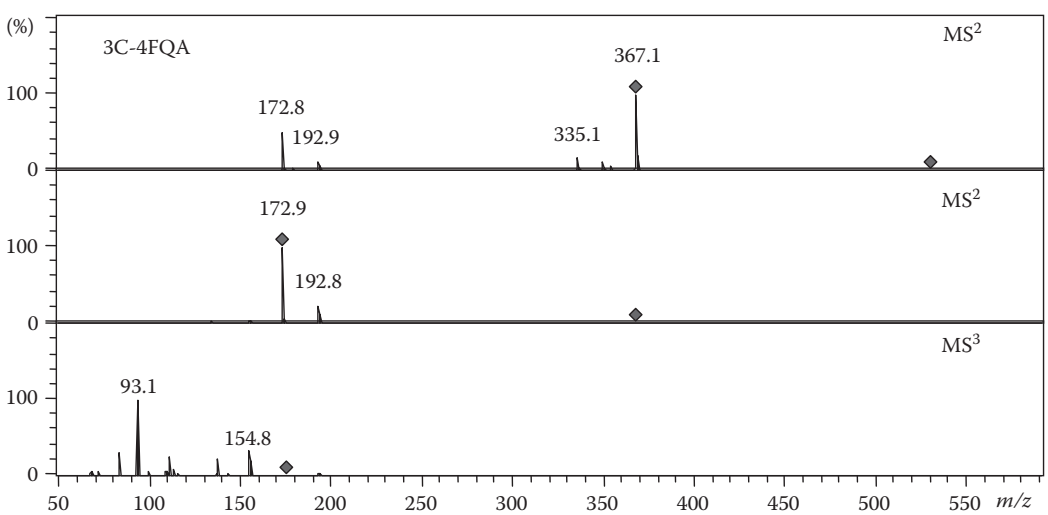


FIGURE 21.25 MS^2, MS^3, and MS^4 spectra of 5C-4FQA in negative ion mode (*m/z* 529).

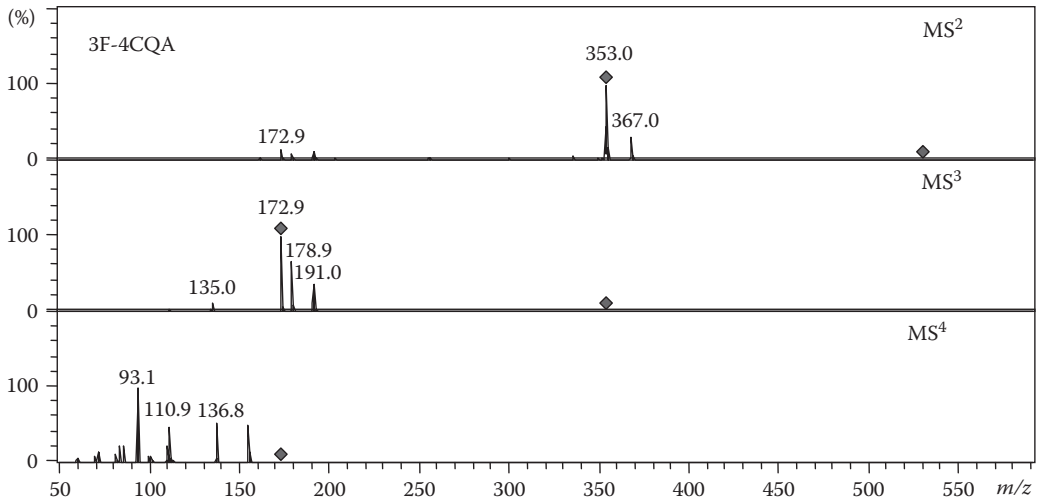


FIGURE 21.26 MS^2, MS^3, and MS^4 spectra of 3F-4CQA in negative ion mode (*m/z* 529).

5-tricaffeoylquinic acid (3,4,5-triCQA) and 1,4,5-tricaffeoylquinic acid (1,4,5-triCQA). Both these isomers progressively lost caffeoyl residues and produced a [4-caffeoylquinic acid –H]$^-$ ion at MS^3, which clearly eliminates the possibility for 1,3,5-caffeoylquinic acid that would have produced [3-caffeoylquinic acid –H]$^-$. Most hydrophobic triCQA produced only weak signals at *m/z* 299, 255, and 203 (<7% of base peak), which suggested the analogy with 1,4-diCQA that it is not 1,3,4-triCQA or 1,4,5-triCQA. Accordingly, it was tentatively assigned as 3,4,5-triCQA. Next, eluting triCQA produced an *m/z* 497 base peak accompanied by *m/z* 515 (60% of base peak). Its MS^3 base peak (*m/z* 335) was accompanied by *m/z* 299 and 255 (7% and 5% of the base peak, respectively). Targeted MS^4 (*m/z* 677 + 515 + 353) yielded [4-caffeoylquinic acid –H]$^-$, suggesting either 1,3,4-triCQA or 1,4,5-triCQA. The fragmentation pattern and retention time of latter were compared to 1,4,5-triCQA, in *Arnica* spp. and it was identical (Clifford et al., 2007).

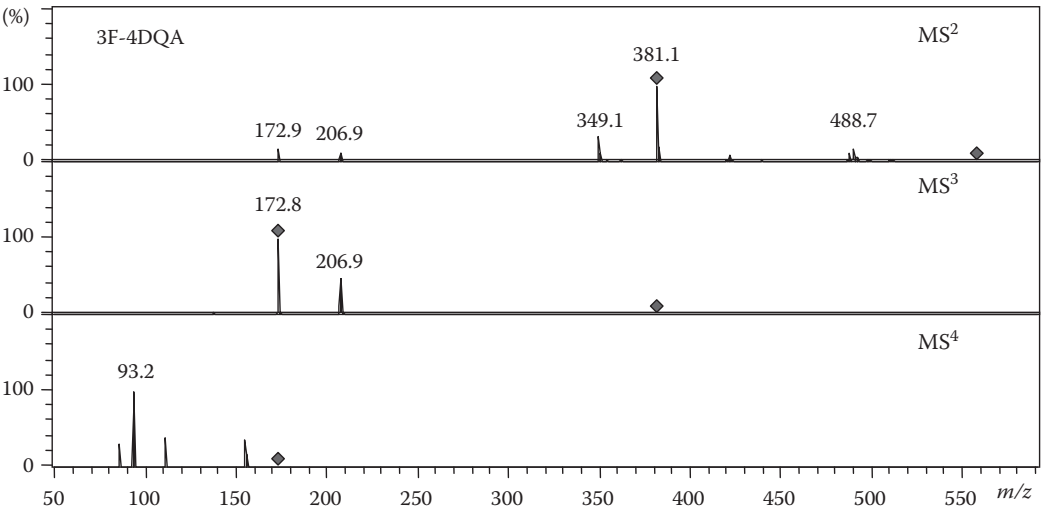


FIGURE 21.27 MS^2, MS^3, and MS^4 spectra of 3F-4DQA in negative ion mode (*m/z* 557).

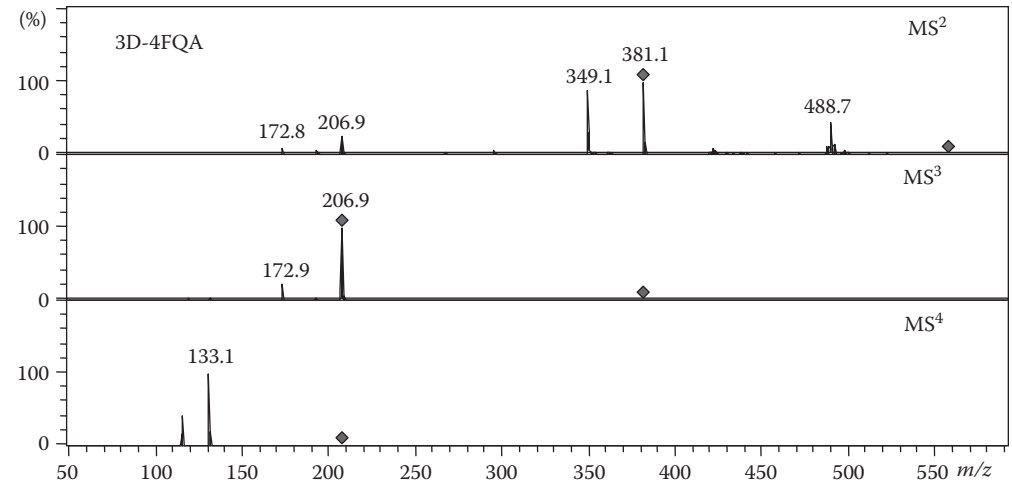


FIGURE 21.28 MS^2, MS^3, and MS^4 spectra of 3D-4FQA in negative ion mode (*m/z* 557).

Numerous CGAs have been identified in Arnica flowers (Table 21.18) and Burdock roots (Table 21.19) by using LC-DAD-ESI/MS fingerprint identification (Lin and Harnly, 2008). For the identification of CGAs, green coffee beans extract, honeysuckle flowers extract, artichoke extracts, and some synthetic and commercially available standards were used. Four different reverse-phase columns have been used and the retention time was varied only for CQAs. A general pattern of elution order is shown in Table 21.20 (Lin and Harnly, 2008).

Recently, three monocaffeoylquinic acids, one dicaffeoylquinic acid, one *p*-coumaroylquinic acid, three feruloylquinic acids, one diferuloylquinic acid, and one feruloyl-caffeoylquinic acid have been identified in black carrots (*Daucus carota sativus*) by HPLC and ESI-MS. Identification was based on the LC-MS^n hierarchical scheme developed by Clifford et al. (Kammerer et al., 2004).

Effects of three different cooking methods (boiling, steaming, and frying) on CGAs profile of Artichoke were studied by LC-tandem MS analysis (Ferracane et al., 2008). The 5-CQA and 1,5-diCQA

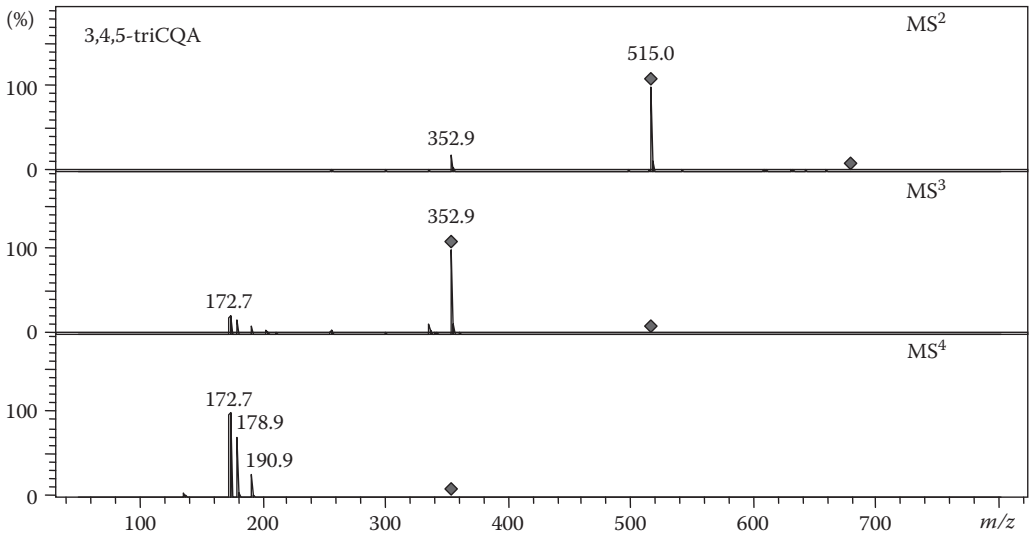


FIGURE 21.29 MS^2, MS^3, and MS^4 spectra of 3,4,5-triCQA in negative ion mode (*m/z* 677).

TABLE 21.13

Positive Ion Mode CID-MS/MS Fragmentation Data for Dimethoxycinnamoyl-Caffeoylquinic Acids

			Precursor Ion	MS² Base Peak	MS² Secondary Peak											
CGA	**RT (min)**	**CE (eV)**	***m/z***	***m/z***	***m/z***	**int.**	***m/z***	**int.**	***m/z***	**int.**	***m/z***	**int.**	***m/z***	**int.**	***m/z***	**int.**
3C,5DQA	57.2	Low	544.7	176.7			526.6	62			bp	100				
		High	544.7	177.0							bp	100			145.1	13
3C,4DQA	61.9	Low cone	544.7	544.9	bp	100	380.8	70								
		High cone	544.7	176.8	544.9	50	380.8	15			bp	100			144.7	42
4C,5DQA	63.4	Low	544.7	190.9			526.6	11	bp	100	176.9	16				
		High	544.7	190.9					bp	100	176.9	16	162.9	45	144.7	16

TABLE 21.14

Negative Ion Mode CID-MS/MS Fragmentation Data for Dimethoxycinnamoyl-Caffeoylquinic Acids

			Precursor Ion	MS² Base Peak	MS² Secondary Peak									
CGA	**RT (min)**	**CE (eV)**	***m/z***	***m/z***	***m/z***	**int.**	***m/z***	**int.**	***m/z***	**int.**	***m/z***	**int.**	***m/z***	**int.**
3C,5DQA	57.2	Low	542.8	207.0	543.2	80					bp	100		
3C,4DQA	61.9	Low	542.8	207.0	543.8	30					bp	100		
		Medium	542.8	206.9			526.3	45			bp	100		
4C,5DQA	63.4	Low	542.8	381.0	542.8	50			bp	100	207.1	40	172.9	60
		Medium	542.8	173.0					380.7	5			bp	100

were present in raw material but after cooking treatments, there was an increase in the concentration of the overall CQAs due to isomerization. Steamed and fried samples have shown same change in CGAs and boiled samples have shown comparatively less change in CGAs. All CGAs have been identified by their MS^2 and retention time (Ferracane et al., 2008). Further MS studies on artichoke were carried out by the group of Schieber (Schütz et al., 2004).

An HPLC-DAD-MS and an HPLC-DAD-tandem MS methods were developed for the online identification of CGAs in Brazilian Arnica *Lycophora ericoids* (Gobbo-Neto and Lopes, 2008). Twenty six CGAs were detected, but only 18 CGAs were identified to the regioisomeric level (Gobbo-Neto and Lopes, 2008). Recently, 3-CQA and 5-CQA were reported in Saskatoon fruits (*Amelanchier alnifolia* Nutt.) (Ozga et al., 2007) and 3-CQA, 5-CQA, 4-CQA, *Z*-CQA, one *p*-CoQA, 3,4-diCQA, 3,5-diCQA, 4,5-diCQA, one diCQA, 1,3,5-triCQA, and 3,4,5-triCQA were reported in pear skins (Lin and Harnly, 2008).

An HPLC method, using a diode array detector and ESI-tandem mass spectrometry, was used to detect 10 CGAs in *Fructus xanthii* (50% methanolic extract), a Chinese traditional medicine (Han et al., 2009). Identification was based on molecular ion peak in the negative ion mode $[M–H]^-$, their UV spectrum (λ_{max} 320 nm), and tandem MS spectra (Table 21.21). All 10 CGAs have shown characteristic tandem MS fragmentation, for example, *m/z* 191 for 1-, and 5-CQA; *m/z* 173 for 4-CQA; *m/z* 255, and 299 for 4-substituted diacyl, and triacyl CGAs (Han et al., 2009). Tandem MS data were in agreement with those previously reported by Clifford et al. (2003, 2006a,b).

21.18 Miscellaneous Characterization Techniques for Chlorogenic Acids

Although several further methods are available in the analytical and structure elucidation arsenal of an organic chemist, including techniques such as single-crystal x-ray diffractometry, circular dichroism,

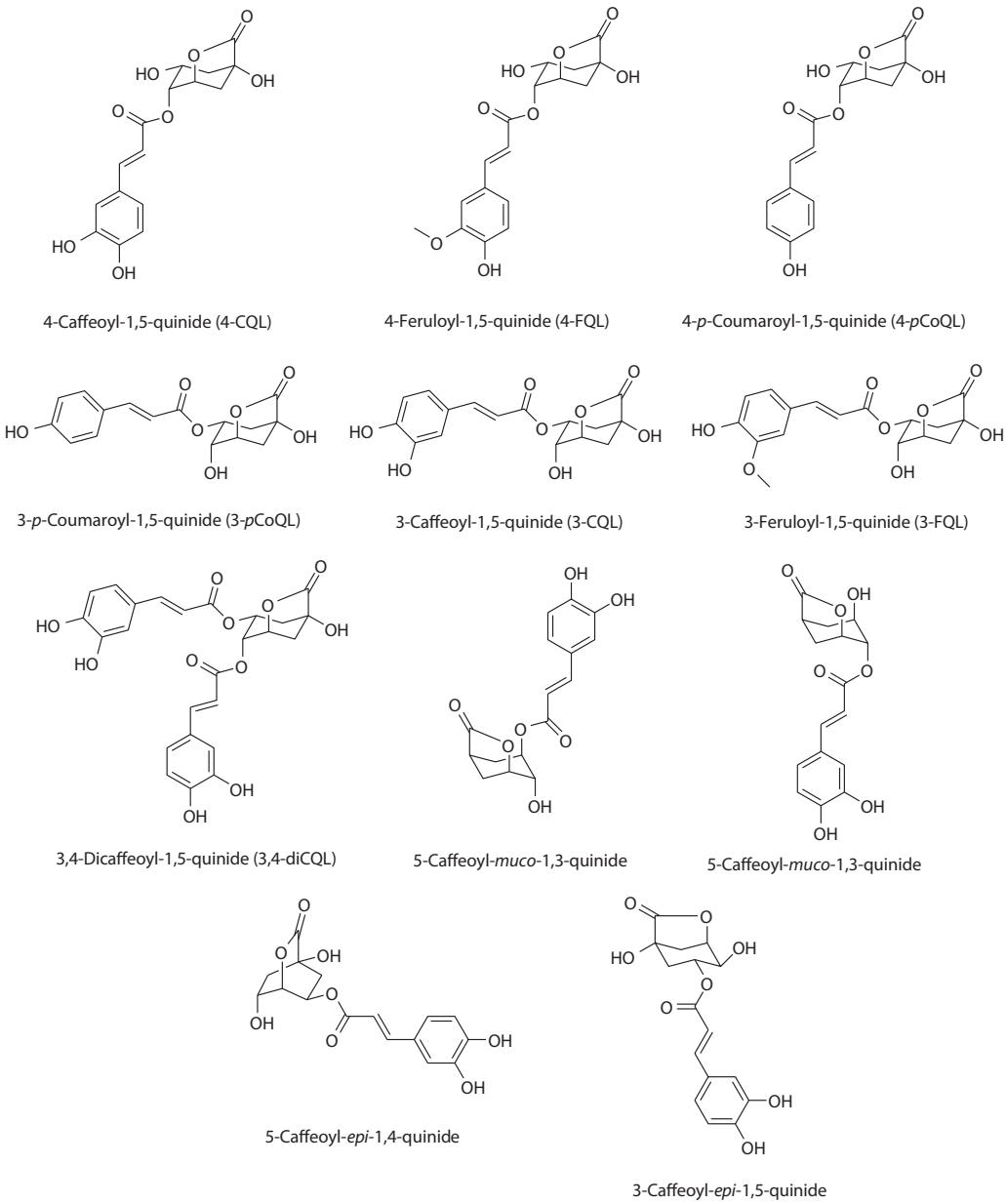


FIGURE 21.30 Structures of CGA lactones present in roasted coffee.

and fluorescent spectroscopy (Morales et al., 2005), just to name a few, such data on CGAs are extremely rare but would certainly be highly desirable and useful.

21.19 General Comments on Other Hydroxycinnamic Acid Derivatives

The chemical investigation of CGAs can now be viewed as a mature field with reliable methods, in particular tandem LC-MS, established for structure elucidation. Reference compounds and spectra are

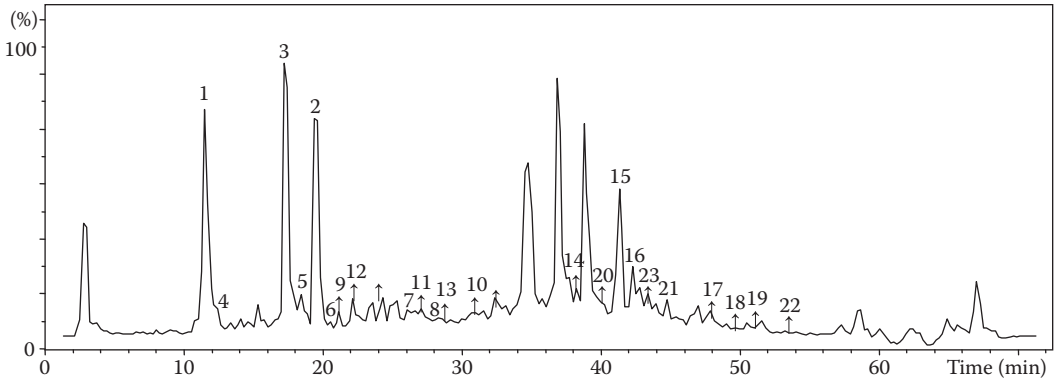


FIGURE 21.31 A total ion chromatogram of Maté extract in negative ion mode.

widely available. This is as well the case for hydroxycinnamoyl shikimic acids and CGA-lactones as described above. Similarly, reliable data are available on other esters of hydroxycinnamates such as tartrate esters, and so on.

Other classes of compounds, for example, carbohydrate derivatives of hydroxycinnamates, are less well investigated. For this class of compounds, the use of tandem LC-MS should at least in theory reveal similar spectacular results as for CGAs, since tandem LC-MS data suggest that these compounds are as widespread in nature and in our diet as CGAs. However, reference standards and a reliable hierarchical key for reliable structure assignment by tandem MS are still outstanding.

TABLE 21.15

CGAs and Their Retention Times Present in Roasted Maté

Number	Name	Abbreviation	Retention Time (min)	R^1	R^3	R^4	R^5
1	3-*O*-Caffeoylquinic acid	3-CQA	11.7	H	C	H	H
2	4-*O*-Caffeoylquinic acid	4-CQA	19.5	H	H	C	H
3	5-*O*-Caffeoylquinic acid	5-CQA	17.5	H	H	H	C
4	*cis*-3-*O*-Caffeoylquinic acid	*cis*-3-CQA	12.4	H	C	H	H
5	*cis*-4-*O*-Caffeoylquinic acid	*cis*-4-CQA	18.5	H	H	C	H
6	*cis*-5-*O*-Caffeoylquinic acid	*cis*-5-CQA	20.5	H	H	H	C
7	Caffeoyl-*epi*-quinic acid	C*e*QA*	26.2	—	—	—	—
8	Caffeoyl-*epi*-quinic acid	C*e*QA*	28.2	—	—	—	—
9	3-*O*-Feruloylquinic acid	3-FQA	20.5	H	F	H	H
10	4-*O*-Feruloylquinic acid	4-FQA	30.9	H	H	F	H
11	5-*O*-Feruloylquinic acid	5-FQA	26.9	H	H	H	F
12	*cis*-3-*O*-Feruloylquinic acid	*cis*-3-FQA	22.0	H	F	H	H
13	*cis*-4-*O*-Feruloylquinic acid	*cis*-4-FQA	28.7	H	H	F	H
14	3,4-Di-*O*-caffeoylquinic acid	3,4-diCQA	38.1	H	C	H	C
15	4,5-Di-*O*-caffeoylquinic acid	4,5-diCQA	40.7	H	H	C	C
16	A *cis*-4,5-di-*O*-caffeoylquinic acid	A *cis*-4,5-diCQA	42.2	H	H	C	C
17	A *cis*-4,5-di-*O*-caffeoylquinic acid	A *cis*-4,5-diCQA	48.0	H	H	C	C
18	1,4-Di-*O*-feruloylquinic acid	1,4-diFQA	49.6	F	H	H	F
19	4,5-Di-*O*-feruloylquinic acid	4,5-diFQA	51.2	H	H	F	F
20	Caffeoyl-feruloylquinic acid	C,FQA	40.0	—	—	—	—
21	3-*O*-Feruloyl-5-*O*-caffeoylquinic acid	3F,5CQA	44.5	H	F	H	C
22	A *cis*-3-*O*-feruloyl-5-*O*-caffeoylquinic acid	A *cis*-3F,5CQA	53.5	H	F	H	C

TABLE 21.16

APCI Data of Maté CGAs in Negative Ion Mode

CGA	t_R (min)	*m/z* (%)	Fragment Ions
3-CQA	7.39	353 (100)	$[M–H]^-$
		335 (5.2)	$[M–H–H_2O]^-$
		191 (3.2)	$[Quinic\ acid–H]^-$
		179 (6.5)	$[Caffeic\ acid]^-$
5-CQA	9.27	353 (100)	$[M–H]^-$
		335 (2.4)	$[M–H–H_2O]^-$
		191 (12.3)	$[Quinic\ acid–H]^-$
		179 (5.8)	$[Caffeic\ acid]^-$
4-CQA	12.26	353 (100)	$[M–H]^-$
		335 (2.4)	$[M–H–H_2O]^-$
		191 (12.3)	$[Quinic\ acid–H]^-$
		179 (5.8)	$[Caffeic\ acid]^-$
diCQA	35.37	515 (100)	$[M–H]^-$
		497 (13.2)	$[M–H–H_2O]^-$
		353 (25.4)	$[M–H\text{-}Caffeoyl]^-$
		191 (1.8)	$[Quinic\ acid–H]^-$
		179 (5.8)	$[Caffeic\ acid]^-$
diCQA	37.02	515 (100)	$[M–H]^-$
		497 (4.5)	$[M–H–H_2O]^-$
		353 (12.2)	$[M–H–Caffeoyl]^-$
		179 (3.7)	$[Caffeic\ acid]^-$
diCQA	38.52	515 (100)	$[M–H]^-$
		497 (3.2)	$[M–H–H_2O]^-$
		353 (7.2)	$[M–H–Caffeoyl]^-$
		179 (4.3)	$[Caffeic\ acid]^-$

TABLE 21.17

HPLC-DAD and ESI-MS^3 (Negative Ion Mode) Fingerprint of Green Yerba Maté

			MS^2		MS^3	
CGA	UV–Vis (nm)	MS^1	Base peak	Fragments	Base peak	Fragments
3-CQA	325, 298 (sh)	3.530.860	191	179, 135	85	172, 127
p-CoQA	314	3.370.924	163			
5-CQA	325, 298 (sh)	3.530.865	191	179, 161	85	172, 127
4-CQA	325, 298 (sh)	3.530.870	173	179	93	111
1-CQA	325, 298 (sh)	3.530.876	191	179, 173	123	
3-FQA	324, 298 (sh)	3.671.036	193	191	134	152
5-FQA	324, 298 (sh)	3.671.044	191	173	85	
4-FQA	324, 298 (sh)	3.671.032	173	191	93	
3,4-diCQA	327, 296 (sh)	5.151.201	353	335	173	179, 135, 93
3,5-diCQA	327, 296 (sh)	5.151.187	353		191	179, 173, 135, 85
4,5-diCQA	327, 296 (sh)	5.151.191	353		173	191, 179, 135
3F,4CQA	238, 327	5.291.343	353	367, 335	173	
1,5-diCQA	327, 296 (sh)	5.151.179	353		179	191
CSQA	238, 327	5.591.446	397		179	
3C,4FQA	238, 327	5.291.321	367	353, 335	173	193
3F,5-CQA	238, 327	5.291.334	367	335	193	173
4C, 5FQA	238, 327	5.291.341	367	393	173	

TABLE 21.18

CGAs of Arnica Flower Extracts

CGA	t_R	$[M–H]^-$ (*m/z*)	Diagnostic NI Ions (*m/z*)	UV λ_{max} (nm)
1-Caffeoylquinic acid	10.1	353	191, 179, 173, 135	240, 300sh, 328
3-Caffeoylquinic acid	10.1	353	191, 179, 173, 135	240, 300sh, 328
4-Caffeoylquinic acid	16.3	353	191, 179, 173, 135	240, 300sh, 328
5-Caffeoylquinic acid	16.9	353	191, 179	240, 300sh, 328
5-Sinapoylquinic acid	18.1	397	223, 191, 179	240, 300sh, 326
5-Feruloylquinic acid	23.7	367	193, 191, 149,	240, 324
1,3-Dicaffeoylquinic acid	25.5	515	353, 191, 179, 135	n.d.
3,4-Dicaffeoylquinic acid	39.0	515	353, 191, 173, 161, 135	240, 300sh, 328
1,4-Dicaffeoylquinic acid	40.8	515	353, 191, 173, 161, 135	240, 300sh, 328
3,5-Dicaffeoylquinic acid	41.9	515	353, 191, 179, 161, 135	240, 300sh, 330
1,5-Dicaffeoylquinic acid	42.7	515	353, 191, 179	240, 300sh, 330
3-Sinapoyl-5-caffeoylquinic acid	43.7	559	397, 223, 191, 179	240, 300sh, 328
4-Sinapoyl-5-caffeoylquinic acid	44.6	559	397, 223, 191, 179	240, 300sh, 328
4,5-Dicaffeoylquinic acid	45.5	515	353, 179	240, 300sh, 328
3-Sinapoyl-5-caffeoyl-1-methoxyoxaloylquinic acid	46.5	645	601, 397, 223, 191, 179	240, 300sh, 328
1,5-Dicaffeoyl-3-methoxyoxaloylquinic acid	47.1	601	557, 395, 353, 233, 191, 179	240, 300sh, 328
4-Sinapoyl-5-caffeoyl-1-methoxyoxaloylquinic acid	47.9	645	601, 397, 223, 191, 179	240, 300sh, 328
3,5-Dicaffeoyl-3-methoxyoxaloylquinic acid	48.2	601	557, 395, 353, 223, 191, 179	240, 300sh, 328
3-Feruloyl-5-caffeoylquinic acid	48.7	529	367, 193, 191, 179	240, 300sh, 328
4,5-Dicaffeoyl-3-methoxyoxaloylquinic acid	48.9	601	557, 395, 353, 233, 191, 179	240, 300sh, 328
3-Sinapoyl-5-caffeoyl-4-methoxyoxaloylquinic acid	49.3	645	601, 397, 223, 191, 179	240, 300sh, 328
1,5-Dicaffeoyl-3,4-dimethoxyoxaloylquinic acid	50.4	687	643, 395, 353, 191, 179, 161	240, 300sh, 328
1,4,5-Tricaffeoylquinic acid	50.9	677	515, 497, 353, 191, 179	240, 300sh, 328
Dicaffeoylsuccinyolquinic acid	51.1	615	515, 353, 191, 179	240, 300sh, 328
3,4,5-Tricaffeoylquinic acid	54.0	677	515, 353	240, 300sh, 328
1,4,5-Tricaffeoyl-3-methoxyoxalylquinic acid	54.6	763	719, 601, 557, 395, 179	240, 300sh, 328

Note: n.d. = Not detected.

21.20 Pharmacokinetic Studies of Chlorogenic Acids and Metabolites

Since CGAs form an integral part of our diet with an estimated daily intake of around 2.5 g per human per day, they must be considered as highly relevant to our diet. Numerous epidemiological studies have frequently linked the consumption of a diet rich in CGAs to a beneficial effect on human health (Dorea and De Costa, 2005; Hamer et al., 2006; Higdon and Frei, 2006). Supporting biological studies have hinted at possible mechanisms of action including antiviral activity, opioid receptor activity, glucose transport inhibition, and so on. These fascinating activities as well suggest that many CGA derivatives might represent promising lead structures in drug development.

For these reasons, detailed studies on the pharmacokinetic profiles and parameters of CGAs are highly desirable and relevant. Information must be sought on all aspects of CGA-ADME (absorption, distribution, metabolism, and excretion). Questions that need to be addressed are

TABLE 21.19

CGAs of Burdock Roots Extracts

CGA	t_R	$[M–H]^-$ *(m/z)*	Diagnostic NI Ions *(m/z)*	UV λ_{max} (nm)
1-Caffeoylquinic acid	10.1	353	191, 179, 173, 135	240, 300sh, 328
3-Caffeoylquinic acid	10.1	353	191, 179, 173, 135	240, 300sh, 328
4-Caffeoylquinic acid	16.3	353	191, 179, 173, 135	240, 300sh, 328
5-Caffeoylquinic acid	16.9	353	191, 179	240, 300sh, 328
1,3-Dicaffeoylquinic acid	25.5	515	353, 191, 179, 135	n.d.
3,4-Dicaffeoylquinic acid	40.0	515	353, 191, 173, 161, 135	240, 300sh, 328
1,4-Dicaffeoylquinic acid	40.8	515	353, 191, 173, 161, 135	240, 300sh, 328
3,5-Dicaffeoylquinic acid	41.9	515	353, 191, 179, 161, 135	240, 300sh, 330
1,5-Dicaffeoylquinic acid	42.7	515	353, 191, 179	240, 300sh, 330
4,5-Dicaffeoylquinic acid	45.5	515	353, 191, 179, 173, 135	240, 300sh, 328
1,5-Dicaffeoyl-3-succinoylquinic acid	47.6	615	497, 453, 353, 191	240, 300sh, 328
1,5-Dicaffeoyl-4-succinoylquinic acid	49.3	615	497, 453, 353, 191	240, 300sh, 328
1,3,5-Tricaffeoylquinic acid	49.9	677	515, 497, 353, 335, 191, 179, 161	240, 300sh, 328
1,5-Dicaffeoyl-3,4-disuccinoylquinic acid	51.3	715	553, 391, 291, 191	240, 300sh, 328
1,3,5-Tricaffeoyl-4-succinoylquinic acid	53.6	777	615, 453, 191, 179	240, 300sh, 328
3,4,5-Tricaffeoylquinic acid	54.0	677	515, 353	240, 300sh, 328

Note: n.d. = Not detected.

1. How are CGAs metabolized by the human gut microflora?
2. What is the chemical structure of these metabolites?
3. Are CGAs or any gut microflora metabolites absorbed into the plasma and if yes, to what extent?
4. What is the plasma concentration and plasma half-life time of absorbed CGAs or gut microflora (GMF) metabolites?
5. Which target tissues are reached by CGAs or any gut microflora metabolites?
6. What is the biological activity and mechanism of action of CGAs or any gut microflora metabolites that are sufficiently bioavailable?
7. How are CGAs or any gut microflora metabolites further metabolized, for example, by hepatic human enzymes?
8. What is the chemical structure, pharmacokinetic profile, and biological role of such metabolites?
9. How fast are all of these compounds excreted, typically renally?
10. Are there variations of any of these parameters between different study subject groups related to age, sex, dietary habits, lifestyle, geographic origin, genetic factors, and so on?

Recent work published has started to address some of these issues with many more studies urgently required to enhance our knowledge on these important dietary compounds.

Choudhury et al. (1999) studied the intake of 3,4-diCQA in animal models using a DAD-HPLC method without detecting any 5-CQA or its metabolites in urine collected from rats: In contrast to these findings, Azuma et al. (2000) could detect a range of glucoronides and sulfates after feeding of 5-CQA to rats in their plasma using a combination of HPLC with electrochemical detection and GC-MS after derivatization. A further series of metabolites including hippuric acid, 3-hydroxy phenyl propionic acid, and *meta*-coumaric acid were identified in both plasma and urine of rats by the Gonthier et al. (2003) using LC-ESI-MS techniques . These metabolites were believed to arise due to microbial degradation of 5-CQA in the colon. The presence of isoferulic acid after *in situ* perfusion of 5-CQA in the mesenteric plasma of rats was described by Lafay et al. (2006) using an LC-ESI-tandem MS method. Nardini et al. (2002) reported on the increase of ferulic acid, isoferulic acid, vanillic acid, hippuric acid, dihydroferulic acid, and 3-hydroxy hippuric acid

TABLE 21.20

Retention Times of Caffeoylquinic Acids and Some of Their Analogs

	t_R			
Group and Name of CGAs	**C1**	**C2**	**C3**	**C4**
Monocaffeoylquinic acid group II (M_r 354)				
1-Caffeoylquinic acid (1-CQA)	6.2	10.2	5.1	7.0
Monocaffeoylquinic acid group I (M_r 354)				
3-Caffeoylquinic acid (3-CQA)	7.3	10.2	6.5	8.2
5-Caffeoylquinic acid (5-CQA)	10.7	16.8	9.9	11.8
4-Caffeoylquinic acid (4-CQA)	12.1	16.2	10.6	13.0
Mono-*p*-coumaroylquinic acid group (M_r 338)				
3-*p*-Coumaroylquinic acid (3-*p*CoQA)	10.4	nd	9.7	nd
5-*p*-Coumaroylquinic acid (5-*p*CoQA)	15.6	20.6	15.0	17.2
4-*p*-Coumaroylquinic acid (4-*p*CoQA)	16.7	nd	15.3	17.9
Monoferuloylquinic acid group (M_r 368)				
3-Feruloylquinic acid (3-FQA)	14.5	15.1	11.0	nd
5-Feruloylquinic acid (5-FQA)	18.0	23.9	17.1	19.5
4-Feruloylquinic acid (4-FQA)	18.8	nd	17.8	nd
Dicaffeoylquinic acid group II (M_r 516)				
1,3-Dicaffeoylquinic acid (1,3-diCQA)	17.9	25.5	16.7	18.5
1,4-Dicaffeoylquinic acid (1,4-diCQA)	29.9	40.8	28.8	31.9
1,5-Dicaffeoylquinic acid (1,5-diCQA)	31.7	42.7	30.7	33.0
Dicaffeoylquinic acid group I (M_r 516)				
3,4-Dicaffeoylquinic acid (3,4-diCQA)	30.4	39.9	29.4	32.1
3,5-Dicaffeoylquinic acid (3,5-diCQA)	32.1	41.9	30.9	33.6
4,5-Dicaffeoylquinic acid (4,5-diCQA)	35.3	45.1	34.1	36.7
p-Coumaroyl-caffeoylquinic acid group I (M_r 500)				
3,4-*p*-Coumaroyl-caffeoylquinic acid	37.2	46.9	34.1	37.9
3-*p*-Coumaroyl-5-caffeoylquinic acid	38.5	48.3	36.0	39.9
3-Caffeoyl-5-*p*-coumaroylquinic acid	39.0	48.7	36.4	40.5
4-*p*-Coumaroyl-5-caffeoylquinic acid	42.2	50.3	39.3	43.3
4-Caffeoyl-5-*p*-coumaroylquinic acid	42.6	50.6	40.1	43.8
Caffeoyl-feruloylquinic acid group I (M_r 530)				
3-Feruloyl-4-caffeoylquinic acid	38.4	46.5	35.5	38.8
3-Caffeoyl-4-feruloylquinic acid	39.7	46.8	36.7	39.9
3-Feruloyl-5-caffeoylquinic acid	40.7	48.4	38.4	41.5
3-Caffeoyl-5-feruloylquinic acid	41.3	48.6	39.0	42.2
4-Feruloyl-5-caffeoylquinic acid	43.5	49.9	40.9	43.9
4-Caffeoyl-5-feruloylquinic acid	44.1	50.1	41.6	44.5
Tricaffeoylquinic acid group II (M_r 678)				
1,3,4-Tricaffeoylquinic acid (1,3,4-triCQA)				
1,3,5-Tricaffeoylquinic acid (1,3,5-triCQA)	41.4	49.9	39.0	42.3
1,4,5-Tricaffeoylquinic acid (1,4,5-triCQA)	42.8	50.9	40.4	43.1
Tricaffeoylquinic acid group I (M_r 678)				
3,4,5-Tricaffeoylquinic acid (3,4,5-triCQA)	48.8	53.8	47.4	49.3

Note: C1 = waters symmetry column; C2 = waters symmetry shield column; C3 = Agilent Zorbax eclipse column; C4 = Phenomenex Luna C18 column; and nd = not detected.

TABLE 21.21

CGAs of *Xanthium sibiricum* and Their LC-MS/MS Data in Negative Ion Mode

CGA	t_R (min)	$[M–H]^-$	MS/MS *m/z*
1-CQA	4.8	353	191
3-CQA	5.9	353	191, 135
5-CQA	10.4	353	191
4-CQA	11.2	353	173, 135
1,3-diCQA	15.3	515	353, 335, 179
1,4-diCQA	22.1	515	353, 299, 255, 173
1,5-diCQA	22.9	515	353, 335, 191
4,5-diCQA	24.6	515	353, 299, 255, 203, 173
1,3,5-triCQA	26.8	677	515, 497, 353, 335
3,4,5-triCQA	27.3	677	515, 497, 353, 317, 299, 273, 255

detected in humans after ingestion of two cups of coffee in urine. This study employed an HPLC technique coupled with an electrochemical detector. Finally, Zhang et al. (2010) recently reported on the application of LC-tandem MS methods in a pharmacokinetic study on CGA absorption.

In this section, we focus on three recent and thorough studies of CGA pharmacokinetics, using coffee as a CGA source, which serve as illustrative and instructive examples, one published by the group of Crozier (Stalmach et al., 2009) from Glasgow and two published by the group of Farah from Rio de Janeiro (Monteiro et al., 2007; Farah et al., 2008). Both studies have made use of tandem LC-MS as an analytical tool, whereby the group of Crozier has employed additionally a selection of authentic standards of metabolites obtained by chemical synthesis. Quantification of individual CGAs and metabolites were carried out by selected ion monitoring LC-MS. Interestingly, both studies have turned out to be quite different, at times even contradictory, results.

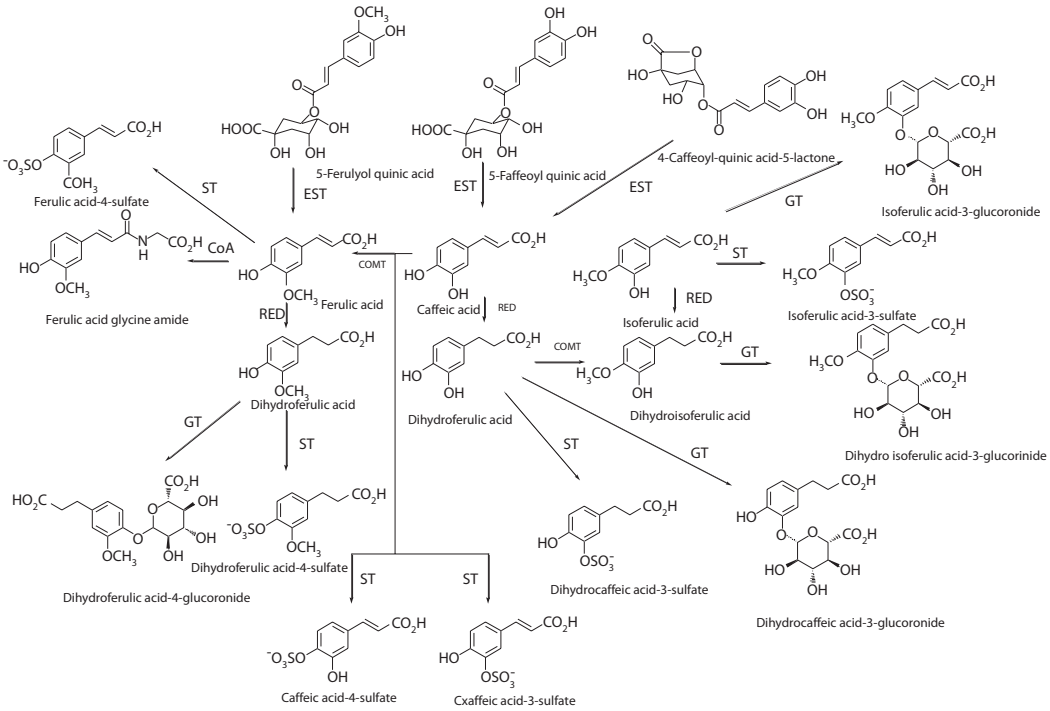
21.20.1 Crozier Study

In Crozier study (Stalmach et al., 2009), 11 healthy volunteers were subjected to instant coffee containing 412 µmol total CGAs containing 13 quantified different CGAs. CGAs and metabolites were characterized and quantified in both urine and plasma between 0 and 24 h. A total amount of CGAs and metabolites in 24 h urine was excreted accounting for 29% of the total original CGA intake. This figure clearly suggests that CGAs are to a large extent bioavailable. Out of the 13 CGAs ingested, only 3-, 4-, and 5-FQA survived the passage through the body intact and unmetabolized. A total of 21 metabolites, in majority sulfates and glucoronides, were identified in plasma and urine samples of the human volunteers. The majority of CGAs are hydrolyzed by GMF esterases and subsequently a notable portion reduced to dihydrohydroxycinnamic acid derivatives, potentially by GMF reductases. Scheme 21.3 summarizes the metabolites identified and their suggested enzymes affecting metabolism (Stalmach et al., 2009).

A total of 12 CGAs and CGA metabolites were quantified in plasma. Table 21.22 shows some selected pharmacokinetic parameters for the compounds in question.

21.20.2 Farah´s Studies

Two studies by the group of Farah (Farah et al., 2008; Monteiro et al., 2009) addressed the pharmacokinetics of CGAs. In the first study, six healthy volunteers were exposed to coffee brewed from *Coffea canephora*, whereas in the second study, 10 healthy volunteers ingested an encapsulated CGA extract. In both studies, CGAs and their metabolites were analyzed in urine and plasma using LC-MS. Eighty-five percent of the total phenolic compounds ingested were recovered in urine, suggesting a considerably higher bioavailability compared to the Crozier study (see Table 21.22). At times, CGA levels in plasma were found in the µM region in comparison with nM quantities observed by Crozier. Only selected metabolites, and not the vast majority, identified in the Crozier study were detected.



SCHEME 21.3 CGAs metabolites and their enzymes of metabolism. COMT, catechol-*O*-methyl transferase; EST, esterase; RED, reductase; GT, UDP-glucoronyl transferase; ST, sulfate-*O* transferase. (Adapted from Stalmach, A. et al. *Drug Metabolism and Disposition* 37(8), 1749–1758, 2009.)

21.20.3 Critical Comparison and Discussion

Both studies have thoroughly analyzed pharmacokinetic parameters of CGAs and their derivatives in urine and plasma. There are extreme differences with respect to the metabolite profiles and quantities identified. Crozier's study reported a significantly larger amount of sulfated and glucoronidated CGA metabolites, which are absent from the Farah study. It can be assumed that these metabolites were either not detected or not searched for in the absence of authentic standards.

The most significant differences between the two studies are the actual pharmacokinetic parameters reported by the two groups in their studies. The values, in particular maximum plasma concentration C_{max} for CGAs, are dramatically different in the two studies with values in the Crozier study in the lower nM region and values in the Farah study in the lower μM region, differing by three orders of magnitude. Table 21.22 summarizes the pharmacokinetic parameters measured in the two studies.

These differences must be considered as enormous and require some attempt of rationalization. None of the studies show any apparent flaw with respect to the methodologies and analytical techniques employed, so that the differences must be considered as real and require an explanation. It needs to be stated that both groups of volunteers are genetically different. They live in different countries using a considerably different diet, which in turn leads to a significantly different gut microflora affecting the selectivity and rate of GMF metabolism. Taking this into consideration, such big differences in ADME parameters should not come as a complete surprise, but are documented here in an extreme way for the first time for dietary natural products. In conclusion, pharmacokinetic studies require the study of many more parameters of daily life and differences between individuals (genetic, diet, lifestyle, etc.) than previously assumed and are difficult to generalize. This aspect should certainly form the topic of intense future research for further understanding of dietary polyphenols.

TABLE 21.22

Pharmacokinetic Parameters from Two Studies on Human Chlorogenic Acid Intake

References	**Monteiro et al. (2007)**	**Farah et al. (2008)**	**Stalmach et al. (2009)**	**Monteiro et al. (2007)**	**Farah et al. (2008)**	**Stalmach et al. (2009)**	**Monteiro et al. (2007)**	**Farah et al. (2008)**	**Stalmach et al. (2009)**
Name of the Compound	C_{max} (μM)	C_{max} (μM)	C_{max} (nM)	T_{max} (h)	T_{max} (h)	T_{max} (h)	AUC (μmol/h/L)	AUC (μmol/h/L)	AUC (nmol/h/L)
3-CQA	1.00 ± 0.75	0.9 ± 1.4	n.a	1.75 ± 0.99	4.0 ± 2.6	n.a	1.65 ± 0.96	3.0 ± 4.5	n.a
4-CQA	1.04 ± 0.68	1.4 ± 1.1	n.a	2.08 ± 1.20	3.6 ± 2.2	n.a	1.85 ± 1.24	4.3 ± 5.4	n.a
5-CQA	3.14 ± 1.64	5.9 ± 4.2	2.2 ± 1.0	2.33 ± 1.17	3.3 ± 2.4	1.0 ± 0.2	8.10 ± 5.05	17.9 ± 15.3	4.1 ± 2.9
Total CQA	4.89 ± 2.53	8.2 ± 6.3	n.a	2.25 ± 1.25	3.3 ± 2.4	n.a	11.48 ± 7.12	25.2 ± 24.4	n.a
3,4-diCQA	0.92 ± 0.32	1.5 ± 1.6	n.a	2.25 ± 1.25	2.6 ± 1.8	n.a	1.75 ± 0.58	5.0 ± 4.9	n.a
3,5-diCQA	1.17 ± 0.95	2.7 ± 2.7	n.a	2.33 ± 1.17	3.2 ± 2.5	n.a	1.85 ± 0.83	8.7 ± 8.3	n.a
4,5-diCQA	1.11 ± 0.36	2.5 ± 3.0	n.a	2.33 ± 1.17	3.3 ± 2.5	n.a	2.04 ± 0.67	6.8 ± 5.7	n.a
Total diCQA	3.03 ± 1.28	6.6 ± 6.9	n.a	2.33 ± 1.17	3.2 ± 2.5	n.a	5.63 ± 1.83	20.4 ± 17.5	n.a
Total CGA	7.66 ± 2.50	14.8 ± 11.7	n.a	2.25 ± 1.25	3.1 ± 2.6	n.a	17.11 ± 8.41	45.6 ± 37.1	n.a
Caffeic acid	1.56 ± 1.52	1.1 ± 0.9	n.a	1.42 ± 0.38	3.6 ± 2.1	n.a	2.81 ± 1.75	3.8 ± 3.2	n.a
Total hydroxycinnamates	n.a	—	n.a	n.a	—	n.a	19.92 ± 10.16	—	n.a
Ferulic acid	n.a	0.8 ± 0.3	n.a	n.a	2.9 ± 1.8	n.a	n.a	3.6 ± 1.5	n.a
Isoferulic acid	n.a	0.9 ± 0.2	n.a	n.a	2.9 ± 1.8	n.a	n.a	3.5 ± 1.9	n.a
p-Coumaric acid	n.a	0.4 ± 0.03	n.a	n.a	2.5 ± 1.8	n.a	n.a	0.8 ± 0.2	n.a
3-*O*-Caffeoylquinic acid lactone-*O*-sulfate	n.a	n.a	27 ± 3	n.a	n.a	0.6 ± 0.1	n.a	n.a	0.5 ± 0.1
4-*O*-Caffeoylquinic acid lactone-*O*-sulfate	n.a	n.a	21 ± 4	n.a	n.a	0.7 ± 0.1	n.a	n.a	0.4 ± 0.1
3-*O*-Feruloylquinic acid	n.a	n.a	16 ± 2	n.a	n.a	0.7 ± 0.1	n.a	n.a	0.9 ± 0.1
4-*O*-Feruloylquinic acid	n.a	n.a	14 ± 2	n.a	n.a	0.8 ± 0.1	n.a	n.a	0.9 ± 0.1
5-*O*-Feruloylquinic acid	n.a	n.a	6.0 ± 1.5	n.a	n.a	0.9 ± 0.1	n.a	n.a	0.8 ± 0.1
Caffeic acid-3-*O*-sulfate	n.a	n.a	92 ± 11	n.a	n.a	1.0 ± 0.2	n.a	n.a	1.9 ± 0.4
Ferulic acid-4-*O*-sulfate	n.a	n.a	76 ± 9	n.a	n.a	0.6 ± 0.1	n.a	n.a	4.9 ± 1.0
			46 ± 13			4.3 ± 0.3			
Dihydroferulic acid	n.a	n.a	385 ± 86	n.a	n.a	4.7 ± 0.3	n.a	n.a	1.4 ± 0.4
Dihydroferulic acid-4-*O*-sulfate	n.a	n.a	145 ± 53	n.a	n.a	4.8 ± 0.5	n.a	n.a	4.7 ± 0.8
Dihydrocaffeic acid	n.a	n.a	41 ± 10	n.a	n.a	5.2 ±0 .5	n.a	n.a	1.0 ± 0.4
Dihydrocaffeic acid-3-*O*-sulfate	n.a	n.a	325 ± 99	n.a	n.a	4.8 ± 0.6	n.a	n.a	3.1 ± 0.3

Note: n.a: Not applicable.

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Section V

Fibers and Polysaccharides

22

Analytical Methodologies of Chitosan in Functional Foods

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Chitin/chitosan-based materials have become of great interest nowadays, not only as an underutilized resource, but also as functional materials of high potential in various fields. Owing to their unique chemical, physicochemical, and biological properties, they have a broad range of applications in the food industry. In this chapter, a detailed description of the main analytical methodologies of chitosan-based materials in the food industry is given, with the recent spectroscopic techniques, imaging techniques, and chromatographic techniques highlighted.

22.1 Introduction of Chitin/Chitosan and Their Derivatives

Chitin is a linear polymer of 2-acetamido-2-deoxy-D-glucopyranose units linked together by 1,4-glycosidic bonds. The name "chitin" is derived from the Greek word "chiton," meaning a coat of mail (Lower, 1984). Being the second most abundant natural polymer in the world, it generally functions as a natural structural polysaccharide (Muzzarelli et al., 1986). It can be obtained in large quantities from the exoskeletons of crustaceans, molluscs, insects, and certain fungi, but the main commercial sources of chitin are crab and shrimp shells (Al Sagheer et al., 2009), most of which are waste products of seafood-processing industries.

Chitosan is the *N*-deacetylated product (to varying degrees) of chitin. The term "chitosan" represents a series of partially deacetylated chitin whose degree of *N*-acetylation (defined as the average number of

N-acetyl-D-glucosamine units per 100 monomers expressed as a percentage) is <50% (Kumirska et al., 2010). Another nomenclature is proposed by the European Chitin Society (EUCHIS) (Roberts, 2007): chitin and chitosan should be classified on the basis of their solubility or insolubility in 0.1 M acetic acid; the soluble material is named chitosan, whereas chitin is insoluble. Based on this, chitosan is soluble in dilute organic acids such as acetic acid, formic acid, lactic acid, and the like, which distinguishes chitosan from chitin (Kosaraju, 2005). However, a rigid nomenclature with respect to the degree of *N*-deacetylation between chitin and chitosan has not been defined (Kumar, 2000). Depending on the source and preparation procedure, commercially available chitosan has an average molecular weight ranging from 300 to over 1000 kD with a degree of deacetylation from 30% to 95%. In its crystalline form, chitosan is normally insoluble in aqueous solutions with pH above 7 due to the protonated free amino groups on glucosamine (Austin, 1988). This drawback in solubility limits its application to a large extent. Therefore, the majority of recent studies are seeking to discover new derivatives with unique properties and different potential applications (Muzzarelli et al., 2004; Tretenichenko et al., 2006; Sahoo et al., 2006; Mohamed and Mostafa, 2008; Johns and Rao, 2009; Mathew et al., 2009; Nagahama et al., 2009; Zuniga et al., 2010). The main derivatives and their proposed uses are shown in Table 22.1.

Chitin/chitosan-based materials are of commercial interest because of their special properties, including high nitrogen content (compared to synthetically substituted cellulose) and other features, such as biocompatibility, biodegradability, low-toxicity, adsorptive abilities, and intrinsic antibacterial activity compared with other present-day synthetic materials (Kumar, 2000; Muzzarelli and Muzzarelli, 2005). Consequently, a broad range of current and potential industrial applications have received considerable attention, covering biomedical products, cosmetics, waste water treatment, food, and fiber industry (Thacharodi and Rao, 1995; Shahidi et al., 1999; Di Martino et al., 2005; Crini and Badot, 2008; Dutta et al., 2009). For instance, chitosan is one of the most valued polysaccharides for drug delivery to date, mostly due to its permeability enhancer abilities (Hu et al., 2003; des Rieux et al., 2006).

Particularly, in the food industry, extensive applications of chitinous products have been developed. Much attention has been continuously attracted due to their versatile antitumor activity (Tsukada et al., 1990), immuno-enhancing effects (Tokoro et al., 1988), antifungal activity (Uchida et al., 1988; Hirano and Nagao, 1989), protective effects against some infectious pathogens in mice (Uchida et al., 1988), and antimicrobial activity (Uchida et al., 1988; Hirano and Nagao, 1989). Till now, in the areas of antimicrobial agent (Yalpani et al., 1992), edible film industry (Knorr, 1986), feed additive (Knorr, 1986), nutritional quality control (Imeri and Knorr, 1988; Chen and Li, 1996), recovery of solid materials from food-processing waste (Weist and Karel, 1992; Simpson et al., 1997), purification of water, enzyme immobilization (Synowiecki et al., 1982; Han and Shahidi, 1995), and the measurement of mold contamination in agricultural commodities and food products (Donald and Mirocha, 1977; Bishop et al., 1982), the chitin/chitosan-based materials have been playing a very important role. Table 22.2 gives a short summary of these applications.

Knowledge of the structure of chitosan products is thus a key for the understanding of the structure–property–activity relationships in it. However, for most of the commercially available chitinous materials, key parameters like weight–average molecular weight (MW), polydispersity, degree of *N*-acetylation (DA), pattern of acetylation (PA), and impurity content (protein, heavy metal) are not mentioned in detail (Aranaz et al., 2009). Besides, the quantitative determination of these parameters in food is essential for food quality control. Therefore, it is crucial to understand the benefits as well as the potential toxicity of chitinous materials in food, the characterization, detection, and quantification of them. Appropriate analytical methods have to be available to assess its quality.

22.2 Analytical Methodologies of Chitosan-Based Materials in the Food Industry

For the structural analysis and physicochemical characterization of chitinous materials, the most important techniques are spectroscopic and imaging techniques. X-ray spectroscopy, infrared (IR), ultraviolet–visible (UV–Vis) spectroscopy, and nuclear magnetic resonance spectroscopy (NMR) are among the most frequently used spectroscopic methods, while scanning electron microscopy (SEM),

TABLE 22.1

Chitin/Chitosan Derivatives and Their Proposed Uses

Derivative	Examples	Potential Uses
N-Acyl chitosans	Formyl, acetyl, propionyl, butyryl, hexanoyl, octanoyl, decanoyl, dodecanoyl, tetradecanoyl, lauroyl, myristoyl, palmitoyl, stearoyl, benzoyl, monochloroacetoyl, dichloroacetyl, trifluoroacetyl, carbamoyl, succinyl, acetoxybenzoyl	Textiles, membranes, and medical acids
N-Carboxyalkyl (aryl) chitosans	*N*-Carboxybenzyl, glycine-glucan (*N*-carboxymethyl chitosan), alanine glucan, phenylalanine glucan, tyrosine glucan, serine glucan, glutamic acid glucan, methionine glucan, leucine glucan	Chromatographic media and metal ion collection
N-Carboxyacyl chitosans	From anhydrides such as maleic, itaconic, acetylthiosuccinic, glutaric, cyclohexane 1,2-dicarboxylic, phthalic, *cis*-tetrahydrophthalic, 5-norbornene-2,3-dicarboxylic, diphenic, salicylic, trimellitic, pyromellitic anhydride	?
O-Carboxyalkyl chitosans	*O*-Carboxymethyl, crosslinked *O*-carboxymethyl	Molecular sieves, viscosity builders, and metal ion collection
Sugar derivatives	1-Deoxygalactic-1-yl-, 1-deoxyglucit-1-yl, 1-deoxymelibiit-1-yl-, 1-deoxylactit-1-yl-, 1-deoxylactit-1-yl-4(2,2,6,6,-tetramethylpiperidine-1-oxyl)-, 1-deoxy-6′-aldehydolactit-1-yl-, 1-deoxy-6′-aldehydomelibiit-1-yl-, cellobiit-1-yl-chitosans, products obtained from ascorbic acid	?
Metal ion chelates	Palladium, copper, silver, iodine	Catalyst, photography, health products, and insecticides
Semisynthetic resins of chitosan	Copolymer of chitosan with methyl methacrylate, polyurea-urethane, poly(amideester), acrylamide-maleic anhydride	Textiles
Natural polysaccharide complexes, miscellaneous	Chitosan glucans from various organisms	Flocculation and metal ion chelation
	Alkyl chitin, benzyl chitin	
	Hydroxy butyl chitin, cyanoethyl chitosan	Intermediate, serine protease purification
	Hydroxy ethyl glycol chitosan	
	Glutaraldehyde chitosan	Desalting filtration, dialysis, and insulating papers
	Linoelic acid–chitosan complex	
	Uracylchitosan, theophylline chitosan, adenine-chitosan, chitosan salts of acid polysaccharides, chitosan streptomycin, 2-amido-2,6-diaminoheptanoic acid chitosan	Enzymology, dialysis, and special papers
		Enzyme immobilization
		Food additive and anticholesterolemic

Source: Adapted from Kumar, M. 2000 *React. Funct. Polym.* 46(1): 1–27.

transmission electron microscopy (TEM), and atomic force microscopy (AFM) are popular imaging methods. In the case of the sensitive determination of chitosan, mass spectrometry (MS) is a powerful technique that provides both quantitative and structural information. Furthermore, under certain circumstances, a preseparation of chitinous products from the matrix or its derivatives is needed. Chromatographic tools are essential for these purposes. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) with good sensitivity as well as separation efficiency have been widely employed. Capillary electrophoresis (CE), characterized by high separation efficiency and absolute sensitivity, is able to provide powerful approaches for the analysis of macromolecules, and has been applied in the analysis of chitosan and its derivatives on a large scale.

Here the main analytical methodologies of chitosan-based materials in the food industry are described. In the related spectroscopic techniques, imaging techniques, and chromatographic techniques, special

TABLE 22.2

Applications of Chitin/Chitosan-Based Materials in the Food Industry

Area of Application	Examples
Antimicrobial agent	Bactericidal Fungicidal Measure of mold contamination in agricultural commodities
Edible film industry	Controlled moisture transfer between food and surrounding environment Controlled release of antimicrobial substances Controlled release of antioxidants Controlled release of nutrients, flavors, and drugs Reduction of oxygen partial pressure Controlled rate of respiration Temperature control Controlled enzymatic browning in fruits Reverse osmosis membranes
Additive	Clarification and deacidification of fruits and beverages Natural flavor extender Texture controlling agent Emulsifying agent Food mimetic Thickening and stabilizing agent Color stabilization
Nutritional quality	Dietary fiber Hypocholesterolemic effect Livestock and fish feed additive Reduction of lipid absorption Production of single cell protein Antigastritis agent Infant feed ingredient
Recovery of solid materials from food-processing wastes	Affinity flocculation Fractionation of agar
Purification of water	Recovery of metal ions, pesticides, phenols, and PCB's Removal of dyes
Other applications	Enzyme immobilization Encapsulation of nutraceuticals Chromatography Analytical reagents

Source: Adapted from Shahidi, F., Arachchi, J. K. V., and Jeon, Y.J. 1999. *Trends Food Sci. Technol.* 10(2): 37–51.

attention is paid to x-ray spectroscopy, NMR, IR, UV–Vis, MS, TEM, SEM, AFM, GC, LC, and CE. Advantages as well as drawbacks are discussed, together with the emphasis on the applications of them in the analysis of chitosan-based materials in the food industry. Their features in determining and confirming molecular structures, monitoring reactions, and controlling the purity of these compounds are discussed in detail.

22.2.1 Spectroscopic Techniques

22.2.1.1 X-Ray Spectroscopy

X-ray spectroscopy is a versatile technique to characterize the identity of crystalline solids based on their atomic structure. The structural information revealed by x-ray diffraction (XRD) includes the electronic structure and geometric structure. Apart from the traditional XRD, several spectroscopic techniques have been developed, such as x-ray absorption spectroscopy (XAS), x-ray emission spectroscopy (XES), x-ray photoelectron spectroscopy (XPS), and x-ray Auger spectroscopy. Each type of spectroscopy has its own features, and they have been widely used for characterizing the materials of all forms (Guo, 2009).

Being a nondestructive technique, x-ray crystallography (especially XRD) yields unparalleled high-resolution structures of biomolecules and complexes from the solid phase (Loo, 1997). In addition to XRD (Lima and Airoldi, 2004; Zhang et al., 2006b; Schiffman et al., 2009), various x-ray techniques can be used flexibly for different purposes. For instance, XPS reveals the occupied electronic states, thus can investigate the bonding energies of C, O, and N atoms on the surface of chitosan and its metal chelate (Minke and Blackwell, 1978; Dambies et al., 2001; Matienzo and Winnacker, 2002; Varma et al., 2004; Veleshko et al., 2008; Sun et al., 2009). It also provides information regarding the forms of species absorbed on the polymer (Dambies et al., 2001). XES analysis of the local electronic structure, the chemical bonding, and cross-linking state can be very sensitive (Kurmaev et al., 2002), while XAS is able to probe empty states and the shapes of molecules or local structures, thus suitable for determining the coordination number of metal atoms in complexes (Klepka et al., 2006; Guo, 2009).

For the crystalline structures, a great number of work have been done in an attempt to elucidate the molecular geometry (Clark and Smith, 1937) and the diffractive patterns (Samuels and Polym, 1981; Ogawa et al., 1984; Ogawa and Inukai, 1987; Cairns et al., 1992; Yui et al., Okuyama et al., 1997, 1994; Kawada et al., 1999; Muzzarelli et al., 2004) of chitin/chitosan ever since 1937 (Clark and Smith, 1937). The detailed structures of both the anhydrous form (Yui et al., 1994) and the hydrated form of chitosan were investigated by x-ray techniques (Okuyama et al., 1997). These studies showed that the chitosan molecule can adopt at least two conformations in crystals: a twofold (Clark and Smith, 1937; Samuels, 1981; Ogawa et al., 1984; Kawada et al., 1999) and an eightfold right-handed helical structure (Ogawa and Inukai, 1987; Cairns et al., 1992). Other crystalline polymorphs of chitosan have been characterized (Saito et al., 1987; Ogawa, 1991; Mazeau et al., 1994; Yui et al., 1994; Okuyama et al., 1997), such as the most abundant "tendon-chitosan" (Clark and Smith, 1937; Okuyama et al., 1997), the "annealed" (Ogawa et al., 1984), the "1–2"/"L-2" (Saito et al., 1987), the "form I"/"form II" (Samuels, 1981) and the "eightfold right-handed" forms (Ogawa and Inukai, 1987; Cairns et al., 1992).

In order to determine which parameters affect the crystalline structures of chitin and chitosan and how they work, quantities of studies have been carried out with x-ray measurements (Ogawa et al., 1992; Ogawa and Yui, 1993; Okuyama et al., 1997; Cho et al., 2000). Generally, molecular weight and DA are two major parameters significantly influencing the crystal structure of chitosan. Besides, factors such as the pH condition (Seoudi and Nada, 2007), alkalifreezing treatment (Feng et al., 2004), and temperature (Wada and Saito, 2001) have also been reported. Another popular application is to examine the behavior of chitosan–metal complex formations (Ogawa et al., 1993; Li et al., 2003; Trimukhe and Varma, 2008; El-Sherbiny, 2009; Nadavala et al., 2009; Sun et al., 2009). In this respect, two coordination modes have been postulated. One is the "bridge model" where a metal ion is coordinated with four nitrogen atoms of intra- and inter-chitosan chains (Schlick, 1986). The contrary mode is the "pendant model" in which a metal ion is attached to an amino group of the chitosan chain like a pendant (Ogawa et al., 1993).

X-ray has also been applied for the crystallized chitosan salts. Ranging from monocarboxylic through inorganic to organic acid salts, the fiber patterns ("type-I salt" or "type-II salt") were discussed based on the obtained x-ray data (Ogawa and Inukai, 1987; Saito et al., 1987; Cairns et al., 1992; Ogawa et al., 1996; Kawada et al., 1999). Results indicated that the salts forming with HNO_3, HBr, HI, and ascorbic acid took up the "type-I salt" structure, which retained the extended twofold helix of the unreacted chitosan molecule, while those with HF, HCl, and H_2SO_4 were the "type-II salt" that has an eightfold helical conformation in the crystal.

In general, the structures obtained by XRD agree with these by NMR, and it is possible to determine the position of the atoms more accurately than AFM. However, x-ray analysis has disadvantages. Apart from the long analysis time, requiring relatively large quantities of material is another great limitation. Fortunately, this drawback can be avoided by the new XRD developments. Additionally, it is an analytical method affected by the crystallinity of the sample. In another perspective, there are some reports pointing out that x-ray spectroscopy was not suitable to be a general applicable assay for degree of deacetylation (DD) (Chen and Liu, 2002; Jang et al., 2004). The intensity of the diffraction peak could be affected by physical treatments of the chitosan samples, such as reprecipitation after dissolution, air drying, and freeze drying, while the DD is not affected. Therefore, these methods are not recommended to be routinely applied for the determination of acetyl content in chitosan preparations with different approaches (Kittur et al., 2002).

22.2.1.2 Nuclear Magnetic Resonance Spectroscopy

Nuclear magnetic resonance (NMR) spectroscopy is another powerful technique for the structural and physicochemical study of organic compounds, ranging from small molecules over polymers. However, when applying this technique to the analysis of chitin and chitosan, the problem of solubility turns up. The chitinous materials are usually with poor solubility in common solvents, depending on the degree of *N*-acetylation, the degree of polymerization, the degree of crystallinity, the distribution of *N*-acetylglucosamine (GlcNAc), and glucosamine (GlcN) along the polymer chains, the ionic strength of the solvent, and the pH and concentration of chitosan in the solution (Kasaai, 2010). Therefore, considerable work must be done to make sure that the sample has been completely dissolved.

To date, different NMR techniques have been employed in this field, including ^{13}C (Pelletier et al., 1990; Raymond et al., 1993) and ^{15}N (Yu et al., 1999; Heux et al., 2000) solid-state NMR, and ^{1}H (Varum et al., 1991a; Rinaudo et al., 1992; Desbrieres et al., 1996), ^{31}P (Khan et al., 2002), and ^{13}C (Weinhold et al., 2009) liquid-state NMR. Particularly, because of the insolubility of high-DA chitin in all solvents, only solid-state NMR can be used. Very often ^{1}H and ^{13}C NMR spectroscopy are used together to study soluble compounds. When phosphorus derivatives are involved, ^{31}P NMR spectroscopy can also be applied. The application of different types of NMR in the analysis of chitin/chitosan-based materials is summarized in Table 22.3.

22.2.1.2.1 ^{1}H NMR Spectroscopy

The ^{1}H NMR spectroscopy is a kind of liquid-state NMR. Therefore, the most important step of the analysis of chitosan by ^{1}H NMR is to find a proper solvent. Quantitative analysis would not be accurate or reproducible if the sample is only partially dissolved or the residual signal of the solvent is too strong. The most common solvents for liquid-state NMR spectroscopy are D_2O/DCl, D_2O/CD_3COOD, $D_2O/DCOOD$. With proper solvents, ^{1}H NMR spectroscopy has been used to characterize chitin and chitosan derivatives (Xie et al., 2002; Rabea et al., 2005; Sun and Wan, 2007; Ma et al., 2008b; Munro et al., 2008), such as glycol chitosan (Knight et al., 2007) and acylated chitosan (Ma et al., 2009; Mine et al., 2009).

NMR techniques can also be used for the following purposes: (1) to examine different chemical and physiochemical processes by the analysis of final or intermediate products (Sorbotten et al., 2005; Einbu and Varum, 2007, 2008) such as the thermal depolymerization of chitosan chloride (Holme et al., 2001) and the enzymatic degradation of chitin (Martinou et al., 1998), (2) to determine DA of chitosan (Ogawa and Yui, 1993; Heux et al., 2000; Brugnerotto et al., 2001; Holme et al., 2001; Tommeraas et al., 2001, 2002; Chen et al., 2002; Lavertu et al., 2003; Cardenas et al., 2004; Holappa et al., 2004; Fernandez-Megia et al., 2005; Kumar et al., 2005; Vikhoreva et al., 2005; Zhang et al., 2005; Jeong et al., 2008; Ma et al., 2008a; Trombotto et al., 2008; Yang et al., 2008; Boesel et al., 2009; Kasaai, 2009; Lertwattanaseri et al., 2009; Mine et al., 2009; Weinhold et al., 2009), and (3) to analyze the degree of substitution (DS) (Holme et al., 2001; Chen et al., 2002; Fernandez-Megia et al., 2005; Kumar et al., 2005; Zhang et al., 2005; Ma et al., 2008b; Kasaai, 2009; Lertwattanaseri et al., 2009; Mine et al., 2009). It should be mentioned that ^{1}H NMR spectroscopy is the best NMR technique for determining DA of chitosan, as Lavertu et al. (2003) pointed out, ^{1}H NMR data have been routinely used as standards for calibrating alternative methods.

22.2.1.2.2 ^{13}C NMR Spectroscopy

Owing to the properties of the carbon nucleus and the only 1% abundance of the ^{13}C isotope in nature, ^{13}C NMR spectroscopy is much less sensitive than ^{1}H NMR spectroscopy. Hence, the number of scans has to be much higher to get ^{13}C NMR spectra (usually over 1000). Two kinds of ^{13}C NMR spectroscopy are involved: liquid state and solid state. Liquid-state ^{13}C NMR faces the same problem of sample solubility as that with ^{1}H NMR, while solid-state ^{13}C NMR spectroscopy does not. Chitinous materials can be analyzed in solid state and a large amount of sample can be used in solid-state ^{13}C NMR, which also enhance the sensitivity. Sometimes ^{1}H NMR and ^{13}C NMR work together to get a much deeper understanding of the structures of chitin/chitosan (Sieval et al., 1998; Aoi et al., 2000; Detchprohm et al., 2001; Rinaudo et al., 2001; Zhang et al., 2003, 2004; Vikhoreva et al., 2005).

To investigate the physicochemical properties of chitin/chitosan-based samples, ^{13}C NMR spectroscopy have been utilized ever since the 1990s (Tanner et al., 1990; Cardenas et al., 2004; Jang et al., 2004; Kameda et al., 2005; Manni et al., 2010). As mentioned above, liquid-state ^{13}C NMR spectroscopy is

TABLE 22.3

Application of Different Types of NMR in the Analysis of Chitin/Chitosan-Based Materials

NMR Techniques	Applications	References
^{1}H NMR	Structures of chitin/chitosan derivatives	Sieval et al. (1998); Aoi et al. (2000); Tokuyasu et al. (2000); Detchprohm et al. (2001); Rinaudo et al. (2001); Xie et al. (2002); Zhang et al. (2004, 2006a); Rabea et al. (2005); Sun and Wan, (2007); Knight et al. (2007); Ma et al. (2008b, 2009); Munro et al. (2008); Popa-Nita et al. (2009); Mine et al. (2009)
	Chemical and physiochemical processes	Tokuyasu et al. (1997); Martinou et al. (1998); Holme et al. (2001); Nah and Jang, (2002); Zhang et al. (2003); Sorbotten et al. (2005); Einbu and Varum, (2007); Einbu and Varum, (2008)
	Degree of *N*-acetylation (DA)	Varum et al. (1991a); Rinaudo et al. (1993); Shigemasa et al. (1996); Tan et al. (1998); Sato et al. (1998); Kawada et al. (1999); Brugnerotto et al. (2001); Tommeraas et al. (2001, 2002); Lavertu et al. (2003); Holappa et al. (2004); Fernandez-Megia et al. (2005); Zhang et al. (2005); Trombotto et al. (2008); Ma et al. (2008b); Ma et al. (2008a); Jeong et al. (2008); Yang et al. (2008); Boesel et al. (2009); Lertwattanaseri et al. (2009); Kasaai, (2009, 2010); Ma et al. (2009)
	Degree of substitution (DS)	Tommeraas et al. (2001, 2002); Holappa et al. (2004); Ma et al. (2008a,b); Jeong et al. (2008); Yang et al. (2008); Boesel et al. (2009); Ma et al. (2009)
Liquid-state ^{13}C NMR	Structures of chitin/chitosan derivatives	Sieval et al. (1998); Aoi et al. (2000); Detchprohm et al. (2001); Rinaudo et al. (2001); Chen et al. (2002); Zhang et al. (2003, 2004); Kumar et al. (2005); Vikhoreva et al. (2005)
Solid state ^{13}C NMR	Physicochemical characterization of chitin/chitosan	Cardenas et al. (2004); Kittur et al. (2003); Saito et al. (1987); Prashanth and Tharanathan (2006); Tanner et al. (1990); Kameda et al. (2005); Manni et al. (2010); Jang et al. (2004)
	Structures of chitin/chitosan derivatives	Nah and Jang (2002); Holappa et al. (2004)
	Chemical and physiochemical processes	Cardenas et al. (2004); Cervera et al. (2004); Paulino et al. (2006); Van de Velde and Kiekens (2004); Park and Park (2001); Tanodekaew et al. (2004); Fu et al. (2009); Peng et al. (2003); De Angelis et al. (1998); Capitani et al. (2001); Zhang et al. (2006a); Focher et al. (1992)
	DA	Brugnerotto et al. (2001); Duarte et al. (2001, 2002); Tolaimate et al. (2000); Guinesi and Cavalheiro (2006); Varum et al. (1991b); Ng et al. (2006); Holappa et al. (2004)
	DS	Silva et al. (2003)
^{31}P NMR	Structures of chitin/chitosan derivatives containing P atom	Zuniga et al. (2010); Lebouc et al. (2009); Jayakumar et al. (2008, 2009); Wang et al. (2001); Meng et al. (2007); Zou and Khor (2005); Palma et al. (2005); Cardenas et al. (2006)
Solid-state ^{15}N NMR spectroscopy	Structures of chitin/chitosan derivatives	Watson et al. (2009)
	DA	Watson et al. (2009); Yu et al. (1999); Heux et al. (2000)
Two-dimensional NMR	Structures of chitin/chitosan derivatives	
	Correlation spectroscopy (COSY)	Rinaudo et al. (1992); Tommeraas et al. (2002); Holappa et al. (2004); Weiss et al. (2002)
	Heteronuclear single-quantum coherence (HSQC)	Zou and Khor (2009); Holappa et al. (2004, 2006)
	Nuclear overhauser effect spectroscopy (NOESY)	Germer et al. (2003)

capable of studying the soluble derivatives of chitosan (Chen et al., 2002; Vikhoreva et al., 2005; Sun et al., 2006). In comparison, solid-state ^{13}C cross-polarization/magic angle spining NMR (^{13}C CP-MAS NMR) spectroscopy is even more popular (De Angelis et al., 1998; Capitani et al., 2001; Park and Park, 2001; Peng et al., 2003; Tanodekaew et al., 2004; Van de Velde and Kiekens, 2004; Fu et al., 2009). For instance, Jang et al. (2004) recorded solid-state ^{13}C CP-MAS NMR spectra for α-chitin, β-chitin, and γ-chitin, followed by a detailed explanation of the hydrogen bonding in α-chitin using spectroscopy (Kameda et al., 2005). Besides, quantities of work were devoted to determine the DA of chitin and chitosan products by solid-state ^{13}C NMR (Pelletier et al., 1990; Raymond et al., 1993; Ottoy et al., 1996), as well as to examine different chemical and physiochemical processes (Focher et al., 1992; De Angelis et al., 1998; Kawada et al., 1999; Tolaimate et al., 2000; Capitani et al., 2001; Duarte et al., 2001, 2002; Park and Park, 2001; Peng et al., 2003; Tanodekaew et al., 2004; Munro et al., 2008; Fu et al., 2009).

In general, solid-state ^{13}C NMR provides undisputed analysis of the structure and other properties of chitin/chitosan-based compounds. The method can be applied irrespective of DA, solubility, and crystallinity of samples. Moreover, the result of the assay is rather insensitive to the presence of trace amounts of contaminating materials. However, high cost of the instrument deters the routine use, and the low natural abundance of ^{13}C requires a long analysis time. Moreover, some chitin/chitosan materials often contain paramagnetic centers, which may distort the intensity of some ^{13}C NMR signals in the spectra (Duarte et al., 2001).

22.2.1.2.3 ^{31}P NMR Spectroscopy and ^{15}N NMR Spectroscopy

Compared to ^{13}C NMR, ^{31}P NMR spectroscopy is luckier because ^{31}P has an isotopic abundance of 100% and a relatively high magnetogyric ratio. These features make ^{31}P NMR spectroscopy a routine method to analyze chitosan derivatives containing phosphorus atoms. Both liquid-state and solid-state ^{31}P NMR spectroscopy can be used, and different phosphorus compounds usually have characteristic chemical shift ranges, making the data processing much easier. Cooperating with other NMR techniques, ^{31}P NMR can be used in the study of numerous chitin/chitosan derivatives containing phosphorus atoms (Palma et al., 2005; Zou and Khor, 2005; Lebouc et al., 2009), such as phosphorylated chitosan and chitin (Jayakumar et al., 2008, 2009; Wang et al., 2001), phosphorylcholine chitosan (Meng et al., 2007), *N*-propyl-*N*-methylene phosphonic chitosan (Zuniga et al., 2010), and diethyl phosphate chitosan (Cardenas et al., 2006).

Solid-state ^{15}N NMR spectroscopy, like solid-state ^{13}C NMR spectroscopy, is very useful for analyzing insoluble chitin. However, even applying high-field NMR spectrometers with large amounts of sample, the measurement is still time consuming owing to the low natural abundance of the ^{15}N nuclide (<0.4%). Luckily, this drawback can be avoided by ^{15}N labeling of chitosan samples to make ^{15}N NMR spectroscopy much more effective (Watson et al., 2009). One important application of solid-state ^{15}N NMR spectroscopy is to study DA of chitin and chitosan (Yu et al., 1999; Heux et al., 2000). It was found to be much more reliable and easier to analyze than ^{13}C CP-MAS spectra.

22.2.1.2.4 Two-Dimensional NMR

Two-dimensional (2D) NMR spectroscopy is much more powerful to study complex molecules than 1D NMR, owing to the high resolution it reaches. Among the many 2D NMR spectroscopic techniques, double-quantum-filtered correlation spectroscopy (COSY/DQF-COSY), nuclear overhauser effect spectroscopy (NOESY), and heteronuclear single-quantum coherence (HSQC) are most commonly applied. COSY and DQF-COSY can determine which protons are spin–spin coupled; NOESY is able to show correlations of all protons which are close enough for dipolar interaction by coupling through space (<5 Å); ^{1}H-^{13}C HSQC can reveal which protons are directly bonded with particular carbon atoms. They have been applied in the analysis of chitin/chitosan-based materials to a large extent (Weiss et al., 2002; Germer et al., 2003; Holappa et al., 2004, 2006), such as the DQF-COSY NMR study of an *N*-alkylated trimer reductively *N*-alkylated onto a fully *N*-deacetylated chitosan (Tommeraas et al., 2002), the 2D ^{1}H,^{13}C HSQC NMR study of chitins sulfated at C-3 and C-6 (Zou and Khor, 2009) and the investigation by COSY combined with ^{1}H,^{13}C HETCOR (hetero-correlation spectroscopy) for *O*,*N*-carboxymethylchitosans (Hsiao et al., 2004).

22.2.1.3 Infrared Spectroscopy

In the analysis of chitosan and its derivatives, special attention is given to IR, a very popular but neither an absolute nor a standardized technique. It is a technique based on the vibrations of the atoms of a molecule (Stuart, 2004). An infrared spectrum is commonly obtained by passing infrared electromagnetic radiation through the sample and determining what fraction of the incident radiation is absorbed at a particular energy. Fourier-transform infrared (FTIR) spectroscopy enables the recorded data to be mathematically transformed by the so-called Fourier transform process, which dramatically improves the quality of the infrared spectrum and minimizes the analysis time (Stuart, 2004). The samples for FTIR analysis are generally prepared by grinding the dry blended powder with powdered KBr and then compressed to form discs. Their FTIR spectra are usually recorded in the middle infrared (4000 cm^{-1} to 400 cm^{-1}).

One of the most important applications of IR spectroscopy is the structural analysis of chemically modified forms of chitin or chitosan, for these derivatives are of growing interest due to their improved solubility and applications (Silva et al., 2003; Jacob et al., 2007; Sajomsang et al., 2008; Zhao et al., 2009). Typical structural analysis of chitosan and its derivatives by FTIR spectroscopy involves (Kumirska et al., 2010): (1) FTIR analysis of chitin and chitosan, (2) characterization of the chemical reagent(s) used in the reaction, (3) analysis of the chitin/chitosan derivatives obtained, (4) identification of differences between the spectra, and (5) interpretation of results. A wide range of chitosan samples have been studied by FTIR as well as micro-FTIR, including chitosan derivatives, acetylated chitosan, nanoparticle–chitosan composites, and hydrogels and so on. Besides, FTIR is able to monitor and explain the interactions occurring between chitosan and the analyzed compounds (Piron and Domard, 1998; Sipos et al., 2003; Urreaga and De la Orden, 2006; Baran, 2008; Phisalaphong and Jatupaiboon, 2008; Yavuz et al., 2009) and to investigate the mechanisms of some processes (Lima and Airoldi, 2004; Niramol et al., 2005; Ma et al., 2007; Rosa et al., 2008; Kamari et al., 2009; Nadavala et al., 2009; Sundaram et al., 2009). A critical summary of these applications is presented in Table 22.4.

Among the many analytical techniques developed thus far for DA determination (Kasaai, 2009), FTIR is at the center of attention. Several procedures using different absorption ratios have been proposed (Muzzarelli et al., 1980; Domszy and Roberts, 1985; Baxter et al., 1992; Shigemasa et al., 1996; Brugnerotto et al., 2001; Kasaal, 2008; Kasaai, 2009). Generally, the amide-I bands at 1655 cm^{-1} (sometimes together with the amide-I band at 1630 cm^{-1}) or the amide-II band at 1560 cm^{-1} are used as the characteristic band(s) of *N*-acetylation. Other internal reference bands have also been proposed, such as the OH stretching band at 3450 cm^{-1} (Moore and Roberts, 1980; Domszy and Roberts, 1985), the C–H stretching band at 2870–2880 cm^{-1} (Dong et al., 2002), the $-CH_2$ bending centered at 1420 cm^{-1} (Brugnerotto et al., 2001), the antisymmetric stretching of the C–O–C bridge at around 1160 cm^{-1} (Miya et al., 1980), and the skeletal vibrations involving the C–O–C stretching bands at 1070 or 1030 cm^{-1} (Shigemasa et al., 1996).

Apart from FTIR, near-infrared (NIR) spectroscopy has also been applied (Rathke and Hudson, 1993; Varum et al., 1995), in which the spectra were recorded from 9090 to 4000 cm^{-1} and the second-derivative spectra were used to determine DA. Kasaai (2008) summarized the latest literature information on DA determination by IR spectroscopy, with critical reviews of their performances, limitations, and factors affecting the experimental results discussed.

By its popularity, the FTIR technology occupies a special position in the discussion of assays for the DA of chitosan and its derivatives, although the best way to analyze the spectrum is controversial. Hein et al. (2008) compared the data obtained from several FTIR methods using different absorbance ratios and baselines with the results of the acid hydrolysis-HPLC method (which is an absolute method). The results showed that although FTIR is powerful in qualitative examination of chitin and chitosan, it is not suitable for quantitative purposes.

22.2.1.4 Ultraviolet–Visible Spectroscopy

Unlike IR spectroscopy, UV–Vis spectroscopy involves both the absorption of electromagnetic radiation from 200 to 800 nm and the subsequent excitation of electrons to higher energy states. Due to the superimposition of rotational and vibrational transitions on the electronic transitions, the UV–Vis spectrum

TABLE 22.4

Application of Different Types of FTIR in the Analysis of Chitin/Chitosan-Based Materials

Area of Application	Examples	References
Physicochemical characterization of chitin/chitosan	Chitin and chitosan	Darmon and Rudall (1950); Pearson et al. (1960); Synytsya et al. (2008)
	Chitin-based scaffolds	Brunner et al. (2009)
	Alpha-, beta-chitin and their precipitated products	Cunha et al. (2008)
	The source of chitin	Juarez-de la Rosa et al. (2007)
	Chitin in polyplacophoransclerites	Furuhashi et al. (2009b)
	Chitosan and chitin produced from silkworm chrysalides	Paulino et al. (2006)
	Conditions for the *N*-deacetylation of chitin whiskers	Lertwattanaseri et al. (2009)
	Solid state structure of chitosan prepared under different *N*-deacetylating conditions	Prashanth et al. (2002)
	The crystallinity and polymorphic nature of chitosan	Focher et al. (1990)
	Physicochemical properties of fungal chitin and chitosan	Wu et al. (2005)
	Crosslinked chitosan	Prashanth and Tharanathan (2006)
	Low-molecular, water-soluble chitosan with free-amine group	Nah and Jang (2002)
Structural analysis of chitin/chitosan derivatives	Water-soluble derivatives	Singh et al. (2009); Xie et al. (2007); Zuniga et al. (2010); Ma et al. (2008b); Ma et al. (2008a); Jeong et al. (2008); Yang et al. (2008); Zhang et al. (2003)
	Organic-soluble derivatives	Muzzarelli et al. (2004); Ma et al. (2009); Zong et al. (2000)
	Nanoparticle-chitosan composites	Kaushika et al. (2008); Borges et al. (2005); Yang et al. (2009)
	Hydrogels	Tan et al. (2009); Madhumathi et al. (2009)
	Chitosan-graft copolymers	Xie et al. (2002); Wu (2005); Munro et al. (2009)
Interactions between chitosan and other compounds	Chitosan and cellulose	Urreaga and De la Orden (2006); Phisalaphong and Jatupaiboon (2008)
	Chitosan and uranyl ions	Piron and Domard (1998)
	Chitosan and iron(III)	Sipos et al. (2003)
	Chitosan and polyaniline	Yavuz et al. (2009)
	Chitosan and VO^{2+}	Baran (2008)
	Chitosan and kojic acid	Synytsya et al. (2008)
The mechanism of some processes	Adsorption of acid dyes onto chitosan-based adsorbents	Kamari et al. (2009); Rosa et al. (2008); Niramol et al. (2005)
	Biosorption of phenol and *O*-chlorophenol onto chitosan–calcium alginate-blended beads	Nadavala et al. (2009)
	Adsorption of fluoride on nano-hydroxyapatite/chitin composite	Sundaram et al. (2009)
	Adsorption of fluoride on magnetic-chitosan particle	Ma et al. (2007)
Degree of substitution (DS)	Chitin, chitosan, and dibutyrychitin	Van de Velde and Kiekens (2004)

TABLE 22.4 (continued)

Application of Different Types of FTIR in the Analysis of Chitin/Chitosan-Based Materials

Area of Application	Examples	References
	Acetylated alpha-chitin by acetic anhydride in ionic liquid	Mine et al. (2009)
	Chitin/chitosan and *O*,*N*-carboxymethyl derivatives	Kittur et al. (2002)
Degree of *N*-acetylation (DA)	Chitin/Chitosan	Baxter et al. (1992); Muzzarelli et al. (1980); Shigemasa et al. (1996); Brugnerotto et al. (2001); Duarte et al. (2002); Kasaal (2008); Moore and Roberts (1980); Rathke and Hudson (1993); Baskar and Kumar (2009); Domszy and Roberts (1985); Sannan et al. (1978); Domard and Rinaudo (1983); Dong et al. (2002)
	O,*N*-carboxymethyl derivatives	Kittur et al. (2002)

appears as a continuous absorption band. With UV–Vis spectroscopy, the state of electron transfers between orbitals or bands of atoms, ions, and molecules can be revealed. Samples for UV–Vis spectrophotometry are most often liquids, although the absorbance of gases and even of solids can also be measured. Analysis of solutions and crystals usually takes place in transmission, whereas powdered samples are often measured in a diffuse reflection mode (see other publications for details, Hunger and Weitkamp, 2001; Ojeda and Rojas, 2004).

In the typical conditions of UV–Vis measurement of chitin/chitosan derivatives, samples are dissolved in aqueous acid (acetic acid, phosphoric acid, perchloric acid, hydrochloric acid) solutions, while sometimes in water (Fu et al., 2008), an aqueous base (Sahoo et al., 2006) or DMSO (Singh et al., 2009). Sample solutions are commonly placed in a transparent quartz cell with an internal width of 1 cm. (This width becomes the path length in the Beer–Lambert law.) (Piron and Domard, 1998; Sipos et al., 2003; Urreaga and De la Orden, 2006; Jayakumar et al., 2009). For powdered or film samples, the Diffuse Reflectance UV–Visible (DRUV) spectroscopy is applied (Urreaga and De la Orden, 2006; Yang et al., 2006; Juarez-de la Rosa et al., 2007; Lin et al., 2009; Ramaprasad et al., 2009). Besides, analysis in the vacuum ultraviolet and the near-infrared range has also been employed (Ramaprasad et al., 2009; Nosal et al., 2005).

Chitin and chitosan have two far-UV chromophoric groups: GlcNAc and GlcN, the ratios of which are different from various samples. For chitosan in 0.1 M HCl solution, the λ_{max} is 201 nm (Piron and Domard, 1998). Its UV spectrum is quite similar to that of mixture of *N*-acetyl-glucosamine and glucosamine hydrochloride. This similarity often makes UV–Vis data insufficient for structure identification of chitinous samples.

Apart from the application in structure analysis, UV–Vis spectroscopy can be mainly used for: (1) the analysis of the physicochemical properties of chitin/chitosan-based compounds (De Souza et al., 2009), (2) the investigation of covalent and noncovalent interactions between chitosan and target compounds (Piron and Domard, 1998; Tanida et al., 1998; Liu et al., 2004; Mi, 2005; Urreaga and De la Orden, 2006) such as cellulose (Urreaga and De la Orden, 2006) and synthetic phospholipid membranes (Liu et al., 2004), (3) the qualitative identification of the presence of certain functional groups (Thanpitcha et al., 2008; Singh et al., 2009; Yavuz et al., 2009), (4) the study of the chemical reactions (Kang et al., 2007; Sun et al., 2008; Zhang et al., 2008; Ramaprasad et al., 2009), (5) the characterization of complex multilayers, nanoparticles, nanocomposite, and multilayer films (Huang and Yang, 2003, 2004; Larena and Caceres, 2004; Yang et al., 2006; Chen et al., 2009; Lin et al., 2009), (6) the optical characterization of the source of chitin (Juarez-de la Rosa et al., 2007), and (7) the determination of physiochemical parameters of chitin/chitosan and derivatives such as DA (Muzzarelli and Rocchetti, 1985; Tan et al., 1998; Pedroni et al., 2003; Hsiao et al., 2004; Liu et al., 2006; da Silva et al., 2008; Wu and Zivanovic, 2008), DS (Tommeraas et al., 2001), and the lower critical solution temperature (LCST) in different conditions (Mao et al., 2007). It has

been reported that UV techniques for DA determination are more sensitive and accurate than IR, ^{13}C NMR, and ^{15}N NMR spectroscopy (Khan et al., 2002; Kasaai, 2009).

22.2.1.5 Mass Spectrometry

Mass spectrometry (MS) is an analytical technique obtaining the structural information via measuring the mass-to-charge ratio (*m/z*) of ions (Aebersold and Mann, 2003). Different from the techniques mentioned above, MS is a destructive analytical technique. A wide mass range can be analyzed by MS, from small molecules to complicated biomolecules such as carbohydrates, proteins, and nucleic acids. Depending on the ion source, the fragmentation fingerprints of ions differs. Apart from the traditional electron impact ionization (EI), chemical ionization (CI), fast atom bombardment (FAB), electrospray ionization (ESI), and matrix-assisted laser desorption ionization (MALDI), new ionization techniques have been developed recently to simplify the sample preparation, such as desorption electrospray ionization (DESI) and direct analysis in real time (DART). While EI and CI are suitable for small molecules, FAB, ESI, and MALDI are preferable for macromolecules like polysaccharoses. In general, EI is more commonly used in GC-MS, whereas ESI in LC-MS and CE-MS technique. Besides, MS can also be classified by their mass analyzer; they are the quadrupole (Q), ion trap (IT), time-of-flight (TOF), ion cyclotron resonance (ICR), and the tandem form of them (e.g., QqQ and Q-Tof).

Being a flexible technique, different types of ion sources and mass analyzers can be chosen according to the information required (Huang and Yang, 2004). Moreover, if a preseparation of complex sample is needed, chromatographic techniques can be involved as the inlet system of MS, which will be mentioned later. In this section, the direct analysis of chitosan-based samples by MS is highlighted.

22.2.1.5.1 Matrix-Assisted Laser Desorption Ionization

Matrix-assisted laser desorption ionization (MALDI), the ion source, is usually in combination with a time-of-flight (TOF) analyzer to get a MALDI–TOF system. The so-called MALDI–TOF is a mostly used MS technique for the analysis of chitin and chitosan (Focher et al., 1992; Akiyama et al., 1995; Zhang et al., 1999; Kumar et al., 2005; Makino et al., 2006; Fernandez-Megia et al., 2007; Boesel et al., 2009; Popa-Nita et al., 2009). Generally, chitooligosaccharides are dissolved in water (Popa-Nita et al., 2009), MeOH-H_2O (1:1) (Fernandez-Megia et al., 2007) or 1% acetic acid solution (Boesel et al., 2009) with a matrix-like 2,5-dihydroxybenzoic acid (DHB) (Focher et al., 1992; Zhang et al., 1999; Kumar et al., 2005; Makino et al., 2006; Fernandez-Megia et al., 2007; Boesel et al., 2009; Popa-Nita et al., 2009) and 2-(4-hydroxyphenylazo) benzoic acid (HABA) dissolved in dioxin (Fernandez-Megia et al., 2007). The mass spectra are usually measured in positive ion mode (Zhang et al., 1999; Kumar et al., 2005), producing the most abundant monosodium/monopotassium ($[M + Na]^+$/$[M + K]^+$) carbohydrate adducts because of NaCl or KCl added as a cationating agent (Fernandez-Megia et al., 2007). Practically, MALDI-TOF PSD MS (postsource decay mass spectrometry) (Bahrke et al., 2002) has been introduced into chitin/chitosan investigations to improve the performance of this technique.

Another famous combination with a MALDI source is tandem mass spectrometry. In this respect, IT, a low-resolution analyzer characterized yet with the ability of providing MS^n spectra (Haebel et al., 2007), has been proved with several excellent features. Much more information can be achieved from MS^n analysis, making IT preferable for structure identification. Besides, another tandem technology, MALDI-TOF/TOF, has also achieved great success when applied in chitin and chitosan analysis (Cederkvist et al., 2008).

22.2.1.5.2 Electrospray Ionization

Electrospray ionization (ESI) is an ionization method based on the Coulomb fission of charged droplets, and it allows for large, nonvolatile samples to be analyzed in liquid phase. Also, it is the second most applied ionization technique for chitin/chitosan analysis (Akiyama et al., 1995; Shahgholi et al., 1997; Kerwin et al., 1999; Liew and Wei, 2002; Wu et al., 2002a,b; Li et al., 2008; Liu et al., 2008; Yao et al., 2008). The ESI ion source is usually coupled to an IT (Wu et al., 2002a), Q/QqQ (Yao et al., 2008; Trombotto et al., 2008), or TOF/QTof (Shahgholi et al., 1997; Thierry et al., 2003; Liu et al., 2006). The tandem mass spectrometry such as QqQ or QTof, as stated above, is preferred for acquiring more

structural information. QqQ tends to reach high sensitivity, while QTof is capable of providing highly accurate mass information due to its high resolution. An important application of ESI-MS in the chitosan analysis is to determine the degree of depolymerization of chitosan and to track the kinetics. In an example, the structures as well as degree of depolymerization of degraded chitosan were characterized by ESI-MS with the support of FTIR and XRD (Chen et al., 2009).

22.2.1.5.3 Fast Atom Bombardment

Other than MALDI and ESI, FAB is another soft ionization technique widely applied in chitin/chitosan derivatives investigations. Similar to MALDI, a sample matrix is needed to assist the ionization. Basically, the matrix can be the thioglycerol (Kamst et al., 1995; vanderDrift et al., 1996; Tokuyasu et al., 1997), *m*-nitrobenzyl alcohol (vanderDrift et al., 1996), glycerol (Tokuyasu et al., 1997, 2000; Kittur et al., 2005), dithiothreitol (Aronson et al., 2003), 2,4-dinitrobenzyl alcohol, or dithiothreitol-thioglycerol (1:1, v/v) (Makino et al., 2006). When operating in positive-ion mode with xenon as the bombarding gas (Aebersold and Mann, 2003; Makino et al., 2006; Fernandez-Megia et al., 2007; Mao et al., 2007), FAB often produces $[M + H]^+$ ions (Kamst et al., 1995; van der Drift et al., 1996, 1998; Tokuyasu et al., 1997) and $[M + Na]^+$ (Aronson et al., 2003; Kittur et al., 2005) or $[M + K]^+$ (Aronson et al., 2003). Generally, FAB cooperates with collision-induced dissociation tandem mass spectrometry (CID) measurements (Kamst et al., 1995; Tokuyasu et al., 1997; van der Drift et al., 1998), and MS/MS spectra could also be obtained using helium as collision gas.

22.2.1.5.4 Other MS

Some other MS methods have also been used for chitin/chitosan analysis. For instance, the CI source with ammonia (Kollar et al., 1995) or acetic acid (Dong et al., 2004) as the reactive gas, the high-temperature conversion-elemental analyzer/isotope ratio mass spectrometer (TC-EA/IRMS) (Wang et al., 2009; Verbruggen et al., 2010), the plasma desorption by the fission products of californium-252 in a time/flow biochemical mass spectrometer (Lopatin et al., 1995), and thermogravimetry-mass spectrometry (TG-MS) with a quadrupole analyzer and Channeltron detector (Lopez et al., 2008) have been applied.

22.2.2 Imaging Techniques

22.2.2.1 Scanning Electron Microscopy

Scanning electron microscopy (SEM) has the capability of producing high-resolution images of a sample surface, especially for the visual confirmation of the morphology and physical state of the surface (Varma et al., 2004). SEM images have a characteristic three-dimensional appearance for the surface structure of the sample. It provides information about the surface topography, composition, and other properties like electrical conductivity. The advantages of SEM include the high magnification, ease of sample observation, the high resolution which allows close spaced features examined, and the large depth of field which allows a large amount of the sample to be in focus at one time. However, SEM has limitations such as expensive instrument, requiring a high vacuum and sample conductivity, and being easily interfered by the presence of surfactants on the particle surfaces (Fraunhofer and Winter, 2004).

SEM has been applied for chitin/chitosan and its derivatives, for instance, to determine the surface morphology and microstructure of fungi, chitin, and chitosan in crab shells (Yen and Mau, 2007; Yen et al., 2009), deacetylated chitosan powder/films (Baskar and Kumar, 2009; Sun et al., 2009), chitosan(chitin)/cellulose biosorbents (Sun et al., 2009), the cross-section of chitosan–silica hybrid membranes (Xi et al., 2006), and vulcanized natural rubber/chitosan blends (Johns and Rao, 2009).

22.2.2.2 Transmission Electron Microscopy

Transmission electron microscopy (TEM) image is formed by transmitting a beam of electrons through the sample. TEM resolution can reach of the order of 0.2 nm, which is significantly higher than that of SEM, owing to the small de Broglie wavelength of electrons (Wang, 2000). However, SEM is able to image bulky samples and has a much greater depth of view, while the specimens for TEM must be very

thin and able to withstand the high instrument vacuum. Other drawbacks of TEM that have been mentioned with high frequency partially limit its applications. For example, it needs extensive sample preparation to make it thin enough to be electron transparent, low throughput, relatively narrow field of view which makes the characteristic of the whole sample difficult, the possibility to damage biological materials by the electron beam and expensive instrument.

In spite of these drawbacks, TEM has proved to be quite a suitable technique to image and characterize various kinds of chitinous materials (Desai and Park, 2005; Desai et al., 2006; Zhang et al., 2006a; Liang and Zhang, 2007; Wu et al., 2007; Yoksan and Chirachanchai, 2009; Tankhiwale and Bajpai, 2010; Simi and Abraham, 2010), ranging from the *N*-alkyl-*N*-dimethyl through *N*-alkyl-*N*-trimethyl chitosan derivatives (Zhang et al., 2006a) to vitamin C encapsulated tripolyphosphate-chitosan microspheres (Desai and Park, 2005; Desai et al., 2006). Another interesting work is to analyze the morphologies of bacteria treated with chitosan, in the purpose of evaluating the bactericidal activity of chitosan acetate solution against *Escherichia coli* and *Staphylococcus aureus* (Liu et al., 2004). SEM and TEM are sometimes combined together, for instance, to reveal the structural features of a chitosan/hydroxyapatite nanocomposite (Rusu et al., 2005) and to observe the changes in morphology during the *N*-deacetylation of chitin nanowhiskers to a chitosan nanoscaffold (Lertwattanaseri et al., 2009).

22.2.2.3 Atomic Force Microscopy

Atomic force microscopy (AFM) is a more recently developed high-resolution of scanning probe microscopy (Ruozi et al., 2005; Edwards and Baeumner, 2006). AFM generally has a nanometer-sized sharp probe attached to a flexible cantilever. This tip scans over a sample, the forces between the tip and the sample lead to a deflection of the cantilever according to Hooke's law. The resulting deflection is measured by a laser spot to give a high-resolution three-dimensional profile of the sample surface. With AFM, directly view of single atoms or molecules that have dimensions of a few nanometers can be obtained. Different modes of AFM are available, including contact/repulsive mode and noncontact/attractive mode.

The information provided by AFM covers the morphology, size, stability, as well as dynamic processes. It has the capability of imaging almost all types of surface, regardless of whether they are conducting, semiconducting, or nonconducting. It also allows biomolecules to be imaged not only under physiological conditions but also during biological processes, which overcome the drawbacks of x-ray crystallography and electron microscopy. With this feature, AFM opens the possibility to analyze their structural and functional properties at the submolecular level. However, when the tip is in direct contact with the actual surface, it may run into difficulties if that surface is soft, sticky, or has loose particles floating on it.

AFM has been working as a powerful technology in the fields ranging from biology and chemistry to industry. It also has extensive applications in the study of chitosan and its derivatives. Not only can AFM images provide information on the surface microstructure of chitosan-based films (Liu et al., 2009; Souza et al., 2009; Diaz-Visurraga et al., 2010; Ghosh et al., 2010), but also for other systems (Huang and Yang, 2003; Lin et al., 2009) like chitosan-based porphyrin-containing systems (Aksenova et al., 2010) and chitosan hydrogel-hydroxyapatite composite membranes (Madhumathi et al., 2009). These chitosan-based films are usually used as food package or edible films. It was also reported that FAM could be a good complementary to other established techniques such as light and electron microscopy, NMR, and x-ray crystallography.

22.2.3 Chromatographic Techniques

22.2.3.1 Gas Chromatography

The most impressive feature of chromatographic methods is to obtain separation and determination in a single run. GC is the first commercially developed instrumental chromatographic method. Commonly, it contains a compressed gas cylinder, the pneumatic system, the injection system, the column system, the detection system, the electric control system, and the data system in GC Intrumentation. Many types of

detectors can be utilized, and can be classfied into destructive (FID, NPD, MSD) and nondestructive (TCD, IRD) detectors; universal (TCD, MSD), selective (ECD, FPD), and specific (biosensor) detectors; and concentration-dependent (TCD, ECD) and mass flow-dependent (FID, NPD) detectors. A carefully chosen detector is therefore crucial to the efficiency of analysis.

With the advantages of high resolution (separating a lot of isomers), fast analysis (typically, 3 s to 30 min for a run), low analytical cost, applicable to gases, liquid, and solid samples, GC has become a routine method for many samples. However, some of its drawbacks are fatal and thus limit its application. Samples analyzed by GC must be able to evaporate but not be decompose under GC inlet conditions (typically 400–500°C). Hence, it is estimated that only 10–20% of the known compounds can be analyzed by GC. Fortunately, chitosan and its derivatives are among the list of samples suitable for GC (Holan et al., 1980; Muzzarelli et al., 1980; Stoev and Velichkov, 1991; Niola et al., 1993).

Pyrolysis-gas chromatography (PyGC) is a key method for the analysis of chitosan-based materials. It consists of a special injector used for solids and liquids with very high-boiling points. The object is to partially combust or pyrolyze the sample by heating the inlet port at a high-temperature. These materials are degraded to volatile components characteristic of the starting material. PyGC is a technique with good sensitivity and separation efficiency. It has been applied to determine the DA of chitin and chitosan (Lal and Hayes, 1984; Sato et al., 1998) and the DS in chitin derivatives (Sato et al., 2002). Besides, it has been proved an excellent tool for the quantitative and qualitative analysis of the chitin in fossil arthropods (Bierstedt et al., 1998) and the chemical composition of chitinozoans (Jacob et al., 2007). However, as an indirect method, Py-GC is unable to distinguish polysaccharide from oligosaccharide and monosaccharide (Nieto et al., 1991; Sato et al., 1998; Furuhashi et al., 2009a).

Among the various types of detectors available for GC, MS is the only one being able to provide detailed structural information. In GC/MS analysis, an EI source is often employed and the full-scan mode and the electron impact mode are commonly used (Cunha et al., 2008). Products are identified on the basis of both the GC retention parameters and the mass spectra (Nieto et al., 1991; Sato et al., 1998; Bierstedt et al., 1998; Huang and Yang, 2004). Before the GC-MS analysis of chitosan and its impurities, the target compounds have to be converted into volatile derivatives (Lee et al., 2001; Vesentini et al., 2007). Very often, the separation is accomplished by using a nonpolar or medium-polar capillary column with a gradient temperature programme (usually from ~60°C to ~280°C with different heating rates) (Sato et al., 2000).

22.2.3.2 High-Performance Liquid Chromatography

Apart from GC, liquid chromatography (LC) is another popular and robust chromatographic method for separating, analyzing, and quantifying of chitinous materials. It is a widely used separation technique to isolate target compounds from complex matrix. An HPLC instrument consists of high-pressure pump(s), an injector, analytical column(s), and a detector(s). Different components in the sample mixture pass through the column, being separated based on their hydrophobicity/polarity (reversed-phase, hydrophobic interaction chromatography), charge (cation or anion exchange chromatography), molecular mass [size-exclusion chromatography (SEC)], or other specific characteristics (affinity chromatography) (Kumirska et al., 2010). The detection system commonly employs a UV–Vis light absorbance detector, a fluorescence detector, an electrochemical detector, a diffractometer, an MS detector, or the combination of two of them. In the method development of HPLC, the detector, the type of stationary and the mobile phases, the flow rate, and the temperature need to be optimized.

Although the separation efficiency is not as high as CE, HPLC has been applied much more extensively, owing to its excellent characteristics of fast, robust, reproducible, sensitive, flexible, and modest cost. The recently developed ultraperformance LC (UPLC) can provide significant improvement in selectivity, sensitivity, and speed (64). Moreover, the two-dimensional LC (2D LC) with unprecedented separating capacity enhances the resolution of compounds in highly complex mixtures such as food matrices (Tranchida et al., 2004). Chitosan can be quantitatively determined by HPLC (Eikenes et al., 2005; Zhu et al., 2005) with DA assigned (Peng et al., 2003; Ng et al., 2006), GPC/SEC with triple detection was also used for the accurate analysis of molecular structure and weight (Heinzmann and Tartsch, 2009). These experiments also revealed a great difficulty in the analysis of chitosan-based compounds by HPLC: the large volume and high viscosity of sample molecules make the analysis not easy to accomplish.

Especially, the combination of LC to MS detector combines the advantages of both, with effective separation, sensitive detection, and structural characterization obtained in one injection. It makes LC-MS most powerful for the analysis of chitosan-based compounds. Basically, the columns used in LC-MS are a little different from those of normal LC, and the mobile phase often consists of acetonitrile or water with added formic acid (Ishimizu et al., 1999; Li et al., 2006; Watson et al., 2009). There are lots of reports on LC-MS analysis of the chitosan and its derivatives. For instance, the SEC separation followed by ESI-MS and off-line MALDI-TOF-MS (Garozzo et al., 2000; Deery et al., 2001) was employed, and acid-hydrolyzed chitin from the fungus *P. chrysogenum* growing on a minimal medium and on a medium containing added $(^{15}NH_4)_2SO_4$ was analyzed (Watson et al., 2009).

22.2.3.3 Capillary Electrophoresis

Capillary electrophoresis (CE) is a technique characterized by its high separation efficiency. Compounds with different charges, frictional forces, and hydrodynamic radius were separated in a silica capillary (usually with 10–100 μm i.d.) under high electrical field. The main components in CE instrumentation are a sample vial, buffer vials, a capillary, electrodes, a high-voltage power supply, and a detector. Two main injection methods, hydrodynamic and electrokinetic injection, can be employed. However, no matter which injection method is used, the volume of sample injected is limited to several nanoliters, due to the low capacity of the capillary. It is quite an impressive advantage as well as disadvantage of CE, depending on different purposes and conditions of analyses. The detection system can be based on UV–Vis absorbance, fluorescence, conductivity, surface-enhanced Raman spectroscopy (SERS), or MS (Vallejo-Cordoba et al., 2005). The laser-induced fluorescence (LIF) detector, one of the most sensitive detectors, is capable of enhancing the sensitivity of CE significantly.

CE comprises a family of electrokinetic separation techniques based upon different separation mechanisms. The most employed modes are capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), capillary electrochromatography (CEC), capillary isoelectric focusing (CIEF), and capillary gel electrophoresis (CGE). Of all the modes, simplicity, high speed, flexibility, and high separation efficiency are of the general advantages. However, some common drawbacks exist, including low reproducibility, throughput limitations, and limited sensitivity in terms of concentration as mentioned above.

CE has been employed to a large extent to examine chitosan and its derivatives, characterizing them in terms of size, surface charge, rigidity, permeability, and stability. One example is to separate chitosan from carboxymethyl chitosan by CZE (Fu et al., 2007). In this work, although the positively charged carboxymethyl chitosan has a considerable adsorption onto the negatively charaged capillary surface, the authors managed to eliminate the adsorption by carefully controlling pH of the separation buffer. Other applications include the CE separation of chitooligosaccharides by a positively coated capillary and indirect photometric detection (Hattori et al., 2010), the determination of high-molecular chitosan (M_r 200,000) (Ban et al., 2001), the study of the electromigration behavior of chitosan D-glucosamine and oligomers in dilute aqueous solutions using CZE (Aider et al., 2006), the quantification of *N*-acetylglucosamine and five *N*-acetyl-chitooligosaccharides by CE with capacitively coupled contactless conductivity detection (Blanes et al., 2008), the quantitation of chitin and glucan in peanut fungal pathogens and baker's yeast by CZE and CEC (Zhang et al., 2001), and so on.

22.2.4 Other Techniques

Apart from the methods stated above, there are some analytical methods that are routinely used but not introduced in details, such as viscosity measurements (Paulino et al., 2006; El-Hefian et al., 2008; Heinzmann and Tartsch, 2009; Chang et al., 2010), thermogravimetric analysis (TGA) (Cardenas and Miranda, 2004; Parize et al., 2008; Tripathi et al., 2009, 2010; Zhou and Wang, 2009; Han et al., 2010; Zawadzki and Kaczmarek, 2010), differential scanning calorimetry (DSC) (Wu, 2005; Guinesi and Cavalheiro, 2006; Xi et al., 2006; Yen and Mau, 2007; Lopez et al., 2008; Parize et al., 2008; De Souza et al., 2009; Johns and Rao, 2009; Tripathi et al., 2009; Singh et al., 2009), and isothermal titration calorimetry (ITC) (Thongngam and McClements, 2004, 2005; Klinkesorn et al., 2005; Guzey and McClements, 2006; Vieira et al., 2007; Onesippe and Lagerge, 2008; De Souza et al., 2009). Some are essential for the

characterization of new chitosan derivatives, such as viscosity measurements, while some of them, like ITC, are perfect for the study of interactions between two substances. In general, multiple methods are usually applied together to get a comprehensive understand of the chitosan-based samples.

A series of techniques, that are used to determine the DD of chitosan, include titrations (Terayama, 1952; Broussignac, 1968; Gummow and Roberts, 1985; Domszy and Roberts, 1985; Raymond et al., 1993; Jiang et al., 2003; Zhang et al., 2006b) (acid–base, colloidal, conductometric, metachromatic, and potentiometric titrations), stoichiometric dye adsorption assays (Maghami and Roberts, 1988), ninhydrin assay (Prochazkova et al., 1999), elemental analysis (GAF, 1992; Yen and Mau, 2007), and differential scanning calorimeter (Kittur et al., 2002). Kasaai (2009) classified the techniques for determining the DD into three categories: the (1) spectroscopy (IR, ^{1}H NMR, ^{13}C NMR, ^{15}N NMR, and UV); (2) conventional (various types of titration, conductometry, potentiometry, ninhydrin assay, adsorption of free amino groups of chitosan by pictric acid); (3) destructive (elemental analysis, acid or enzymatic hydrolysis of chitin/chitosan, and followed by the DA measurement by colorimetry or HPLC, pyrolysis-gas chromatography, and thermal analysis using differential scanning calorimetry) methods. The readers can resort to his review for details. It should be mentioned that the applications of these methods are not limited to the DD determination. For example, the ninhydrin assay (Prochazkova et al., 1999) can be utilized for the quantitative determination of chitosan based on reaction with ninhydrin without any pretreatment, and elemental analysis has a broad range of applications in this field (Zong et al., 2000; Cardenas et al., 2004; Holappa et al., 2004; Al Sagheer et al., 2009; Zuniga et al., 2010).

Other methods have also been applied, such as gel permeation chromatography (GPC) (Zong et al., 2000; Li et al., 2006) for the molecular weight determination, C/N analysis for the estimation of the mass of chitosan in wood (Mikalsen et al., 2001), inductively coupled plasma spectroscopy (ICP) for the determination the amount of metal ions adsorbed onto chitosan/chitin cellulose biosorbents (Gamage and Shahidi, 2007; Sun et al., 2009), circular dichroism spectroscopy (CD) for the measurement of differences in the absorption of left-handed polarized light versus right-handed polarized light that arise due to structural asymmetry (Kittur et al., 2003; Atkins and Paula, 2005), Raman spectroscopy for the structural identification of chitinous samples or membranes (Orrego et al., 2010; Roberts et al., 1995; Jacob et al., 2007; Cunha et al., 2008; Synytsya et al., 2008; Brunner et al., 2009), the contact angle measurements for the surface energy of chitin/chitosan and their respective monomeric counterparts (Cunha et al., 2008), scanning electron spectroscopy coupled with energy-dispersive spectroscopy (EDX) for examining the metal uptake mechanism on chitosan (Maruca et al., 1982), and for determining the porosity of chitosan beads and membranes as well as the diffusion of metal ions through them (Varma et al., 2004).

In summary, in the structural investigation and physicochemical characterization of chitin, chitosan, and their derivatives, more and more novel methods have been proposed. With the development of analytical methodologies of chitosan-based materials in the food industry, detailed information like the degree of *N*-acetylation, pattern of *N*-acetylation, degree of polymerization, molecular weight, crystallinity, the sequence or degree of substitution, chemical reactions processes, and intra-/intermolecular interactions can be achieved with sensitivity and accuracy.

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23

Insoluble Dietary Fiber

Leila Picolli da Silva and Melissa Walter

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The fiber content of foods has been studied for approximately two centuries. However, interest in the study of fiber content from the perspective of human nutrition is more recent and was motivated by epidemiological observations from the 1960s, which showed that diseases such as diverticulitis, hypercholesterolemia, hypertension, and colon cancer were common in industrialized countries but less prevalent in undeveloped countries. It was hypothesized that this occurred due to differences between the composition of the diets in each region: in undeveloped countries, food is nonprocessed or only slightly processed, and therefore it contains more fiber than food in industrialized countries. Over the years, several studies were conducted to validate the hypothesis that the content of fiber in the diet is related to the incidence of certain diseases, and most of them provided evidence of beneficial digestive and metabolic effects related to the higher consumption of fiber, although these effects are distinct for different sources of fiber. The impact of these studies has led to the evolution of the concept of fiber in food.

According to the Codex Alimentarius Commission (2008), dietary fiber is defined as carbohydrate polymers with 10 or more monomeric units that are not hydrolyzed by the endogenous enzymes in the small intestine of humans. However, "dietary fiber" is also widely accepted as encompassing other remnant plant compounds, such as lignin, nonhydrolyzed proteins and lipids, polyphenols, cutins and tannins, and other compounds formed during food storage or processing, for example, Maillard compounds (Lee and Prosky, 1995). The breadth of this concept stems from the fact that the fiber fraction of food does not constitute a defined chemical group but rather a combination of chemically heterogeneous substances that are indigestible in the gastrointestinal tract.

Quantitatively, most of these components are most prevalent in the cell walls of plants; to a lesser degree, they may also be present in the intercellular medium or in products secreted by vegetables to carry out certain specific functions (Cavalcanti, 1989). The distribution also varies among plant organs and tissues (Hoseley, 1990; Brunner and Freed, 1994) as well as by processing, which directly affects the composition, physicochemical properties and physiological effects of fiber.

Dietary fiber can be divided into two groups according to its water solubility, for example, insoluble and soluble fractions. Insoluble fiber (IF) is composed of the insoluble components of the plant cell wall, such as cellulose, insoluble hemicelluloses, lignin, tannins, and minority compounds (Jeraci and Van Soest, 1990), which may occur naturally or may be formed during processing (Maillard compounds, resistant starch, insoluble phenolic compounds, and phytic acid). Soluble fiber refers to water-soluble, nonstarch polysaccharides (NSPs) such as soluble hemicelluloses and lignin as well as other substances such as gums and mucilage (Olson et al., 1987; Cavalcanti, 1989; Van Soest et al., 1991). These two

groups of fiber exert different effects on humans due to their different physicochemical characteristics (Schneeman, 1987; Annison, 1993; Annison and Choct, 1994).

Currently, several methodologies are used to analyze the fiber present in food; these techniques are based on the different concepts for this fraction. For this reason, there is no homogeneity in the results, which makes the analysis and comparison of the digestive and metabolic effects of different fiber compounds difficult. These methodologies can be classified as either gravimetric or chemical. In the gravimetric methods, the residue that remains after different procedures is weighed. This group includes the earliest methods of evaluation, such as the Weende method, which determines crude fiber. More recently developed methods also fall into this category, including enzymatic–gravimetric methods in which enzymes are used to determine the total fiber content and to quantify the insoluble and soluble fractions. In the chemical methods, the fractions that correspond to the main polysaccharides in the cell-wall matrix are extracted and hydrolyzed; the concentration of these components is then determined by the quantification of their monomeric constituents.

This chapter discusses the basic composition of fiber in food, the advantages and deficiencies of the major methods used to quantify IF, and some additional measurements for the quantification of IF.

23.1 Origin and Composition of IF in Food

Most of the foods consumed by humans and animals are derived from plants. Independent of the species, all plant cells have cell wall, which constitutes the majority of ingested fiber.

The polymers that form the cell wall possess defined chemical characteristics according to the plant organ (leaves, fruits, roots, and grains), and the numerous possible interactions among them generates the large structural diversity that defines their physicochemical properties. These properties are responsible for the many different effects on the processes of digestion and absorption of nutrients, as well as effects on other metabolic processes, that have implications for human health. This heterogeneous structure of fiber is basically composed of cellulose and hemicelluloses, with small quantities of structural protein inserted into the matrix (Cosgrove, 1997). Other polymers, such as lignin, suberin, gums, tannins, and phenolic compounds, as well as water and minerals, can also be present depending on the species, organ, and stage of maturity of the plant (Hatfield, 1989; Showalter, 1993).

Usually, the cell wall can be classified as primary and secondary. The polymers that compose the primary cell wall occur in a relatively disorganized form, creating larger intracellular spaces. For this reason, primary cell-wall components are more effectively altered at the gastrointestinal level, that is, they undergo more fermentation in the ceco-colic region. In contrast, the polymers that compose the secondary cell wall are more organized and exhibit smaller intracellular spaces, with minor effects on the passage and fermentation in the gastrointestinal tract.

Independent of the type of plant tissue, cellulose is essentially insoluble, with a lower degree of crystallinity in the primary cell wall than in the secondary cell wall (McDougall et al., 1996), a property that defines its effects on digestive events. The effect of crystallinity on the digestibility of fiber has been investigated by several researchers (Jeraci and Horvath, 1989; Silva, 2002), who observed that the addition of purified cellulose (high degree of crystallinity) significantly reduces the digestibility of fiber, such that low fermentation by the microorganisms in the gastrointestinal tract is observed both *in vitro* and *in vivo*. In the same way, natural sources of fiber with high crystallinity cellulose are not well fermented in the gastrointestinal tract (Lebet et al., 1998; Silva, 2002). These compounds also have little influence on the degree of water retention and in the transit time of the digesta in the gastrointestinal tract.

Most of the hemicelluloses comprising fiber belong to the same subgroup (xylans, β-glucans, xyloglucans, and mannans) but can present different degrees of solubility according to the plant species, the maturity stage, and their localization in the plant (Table 23.1). The same observation has been made for pectic substances that present different degrees of solubility and consequently different effects on the gastrointestinal tract and on metabolism, according to their source.

Lignin is formed only after the deposition of the secondary cell wall, is associated with other polysaccharides and proteins, and is an insoluble compound that is not fermented in the ceco-colic region. In

TABLE 23.1

Mean Content of β-Glucans and Arabinoxylans in Cereal Grains and Their Water Solubility

	Cereal					
Polymer	**Barley**	**Oat**	**Rice**	**Rye**	**Triticale**	**Wheat**
Total β-glucans (%)[a]	4.36	3.37	0.10	1.89	0.65	0.65
Solubility of β-glucans (%)	66.30	63.20	—	36.00	80.00	100.00
Total arabinoxylans[a]	5.69	7.65	1.18	8.49	7.06	6.63
Solubility of arabinoxylans (%)	12.65	6.67	31.36	28.86	18.41	15.69
Ratio of total β-glucans: arabinoxylans	1:1.3	1:2.3	1:11.8	1:4.5	1:10.9	1:10.2
Ratio of soluble β-glucans: arabinoxylans	4:1	4:2.1	—	1:3.6	1:2.5	1:1.5

Source: Modified from Henry, R. J. 1985. *Journal of the Science of Food and Agriculture* 36: 1243–1253.

[a] Results are expressed as % of dry matter.

contrast, the phenolic compounds that are joined to form lignin are soluble (to different degrees) and are not necessarily associated with the cell wall. Thus, it is important to specifically identify all of these phenolic compounds and their localizations to enable a complete description of their biological effects.

The cell wall also contains proteins (extensins, proteins rich in glycine and proteins rich in proline) that can bind other components of the cell wall together, making them indigestible. Other compounds, such as silica, cutins, and tannins, are present in the cell wall, and may or may not be associated with structural polysaccharides and/or lignin. Although present in small quantities, these compounds influence the physicochemical characteristics of the cell wall and can have significant effects on the digestion and absorption of the cell content.

Food processing can also significantly influence fiber content. Resistant starch, although naturally present, can be altered through the cooking and cooling of food to increase the IF content. Different studies have demonstrated that the increase in the consumption of resistant starch has beneficial effects on health (Jenkins et al., 1998; Yue and Waring, 1998; Haralampu, 2000). Similarly, heating food to high temperatures can result in the formation of Maillard compounds, which are indigestible in the gastrointestinal tract and resist fermentation, similar to lignin. However, this fraction does not exhibit any of the beneficial characteristics attributed to fiber and may even act negatively to prevent the absorption of certain essential amino acids (Lee et al., 1977).

Overall, the wide variability in the composition of fiber requires analytical methods that are sufficiently capable of quantifying different compounds to identify precise relationships between fiber contents and specific biological effects.

23.2 Analytical Methods for IF in Food

A number of methods have been developed for the analysis of fiber (defined here as compounds that are indigestible in the gastrointestinal tract). As described above, these methods can be classified as either gravimetric or chemical.

In the gravimetric methods, the residues that remain after different procedures are weighed. In the chemical methods, monomeric constituents that are considered to be unavailable for the enzymatic action of mammals are separated and identified by different analytical methods (chromatography and colorimetry). These two groups of methodologies provide different types of information: gravimetric methods provide simplistic information, generally separating the fiber content into insoluble and soluble fractions (enzymatic–gravimetric methods), although different methodologies will yield different values for these parameters. Therefore, the choice of method is quite dependent on the information desired. Chemical methods generally provide a more detailed description of the food, especially if the aim is to study a particular component of the fiber content.

23.2.1 Gravimetric Methods

According to Van Soest and Robertson (1985), the first attempts to estimate fiber content date to 1800. In the method used at that time, the material was submitted to maceration, followed by filtration by cloth to remove the aqueous content; the final residue was considered to be the fibrous portion of the food. Around 1806, Einhof suggested that acids and alkalis could be used to estimate fiber content (Sanchez-Castillo et al., 1994). In 1820, Gorham published results obtained with a similar method, that is, successive acid/alkali digestions of the sample that left behind only the fibrous fraction of the food (Van Soest and Robertson, 1985). Henneberg and Stohmann (1864) improved upon this method, standardizing the concentrations of acid and alkali and the time of boiling; this method for the determination of crude fiber is now defined as the Weende method and was adopted by the Association of Official Analytical Chemists (AOAC) as method 962.09 (AOAC, 2007). In this method, the food is divided into six fractions, one of which is crude fiber, obtained after successive digestion with acid and alkali, and referred to as the indigestible dietary fraction. The components that remain soluble after these digestions are considered as the digestible fraction of food and are referred to as the nitrogen-free extract (Figure 23.1).

Although the Weende method was the main method used to determine the fiber content in food until the 1970s, it is severely limited. It results in the complete loss of soluble polysaccharides; furthermore, some insoluble hemicelluloses are solubilized in the acid digestion and are also extensively and variably solubilized in the alkaline digestion. Thus, these indigestible components will be considered as part of the nitrogen-free extract, causing a serious error in the calculated nutritive value of the food (Sabioni, 1989; Sanchez-Castillo et al., 1994; Van Soest, 1994). The solubilization of these components is extremely variable across the plant groups, making it impossible to specify general correction factors for this method.

These problems with the Weende method motivated the development of other methods to evaluate the IF fraction. Between 1963 and 1975, Van Soest and collaborators proposed that fiber could be quantified by detergent treatments (Goering and Van Soest, 1970). The fraction obtained by this method was considered as a good predictor of the IF present in food (Asp et al., 1983; Asp and Johansson, 1984; Sanchez-Castillo 1994; Van Soest 1994), and, together with the Weende method, it is still used extensively to evaluate the products for animal nutrition. However, the use of these methods in human nutrition studies is restricted because they do not quantify the soluble fraction of fiber.

According to Van Soest (1994), the detergent system offers a rapid way to estimate the insoluble content of the cell wall and its main components. In the neutral detergent fiber (NDF) method, the

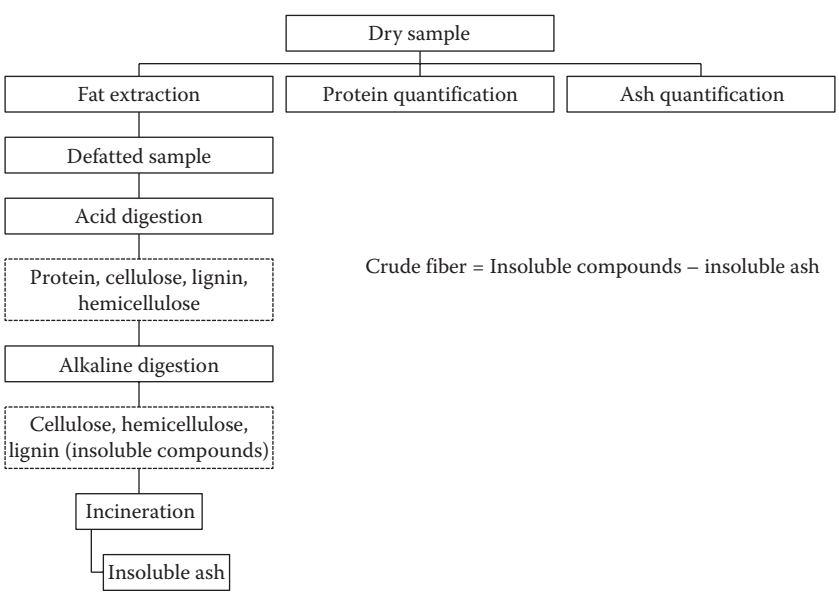

FIGURE 23.1 Schematic representation of the Weende method.

sample is treated with a sodium lauryl sulfate solution (pH 7.0) and ethylenediamine tetraacetic acid (a chelating agent that prevents metal or alkaline ions from interfering in the analysis). Because treatment in neutral detergent is not hydrolytic, the remaining residue contains the main insoluble components of the cell wall: lignin, cellulose, and hemicelluloses. Treatment with acid detergent [acid detergent fiber method (ADF)–AOAC method 973.18] containing 1 N sulfuric acid and cetyl trimethyl ammonium bromide (CTAB) can be used to evaluate the soluble (hemicelluloses and proteins of the cell wall) and insoluble (basically lignin and cellulose) fractions. The residue of the fiber obtained after acid detergent treatment is used to sequentially evaluate the contents of lignin, cutin, cellulose, indigestible nitrogen, and silica (Figure 23.2).

This methodology was adapted by introducing sodium sulfite, to eliminate the nitrogen linked to NDF, and a heat-stable amylase, to ensure the complete removal of starch (AOAC method 2002.04) (McQueen and Nicholson, 1979; Robertson and Van Soest, 1981; Van Soest et al., 1991).

Comparison of the Weende and Van Soest methods for the same food reveals that up to 50% less fiber is obtained with crude fiber method than with NDF. This variation is due to the extensive and variable solubilization of hemicelluloses and lignin in the Weende method.

Since the 1970s, as the importance of fiber in human nutrition became more clear, proposals for more sophisticated analysis methods appeared, with the aim of evaluating not only the insoluble compounds, but also the soluble compounds that are part of the fiber in food. These methods are divided into two groups, the enzymatic–gravimetric methods and the chemical or enzymatic–chemical methods.

Initially, the use of enzymes to remove available carbohydrates and proteins in the food was used by Williams and Olmsted (1935) to obtain an indigestible fraction with a better relationship to the physiological effects of the fiber content. Based on the use of enzymes, Hellendoorn et al. (1975) proposed a methodology to evaluate the fiber content of food that is considered as a milestone in the development of enzymatic–gravimetric methods (Theander and Aman, 1979). In this method, the food is submitted to sequential digestion with pepsin and pancreatin; the residue obtained represents the IF fraction. According to Asp and Johansson (1984), Furda (1977, 1981) proposed the use of chloridric acid, amylase and protease to determine the amount of IF and the use of ethanolic precipitation for soluble fiber. Asp et al. (1983) proposed the sequential use of amylase, pepsin, and pancreatin to obtain total, insoluble, and soluble fiber contents, with the soluble content obtained by ethanolic precipitation.

Based on this research, Prosky et al. (1984, 1985) suggested a method for the evaluation of total fiber, which was adopted by AOAC in 1985 as a first action (Prosky et al., 1985) and in 1986 as a final action (AOAC method 985.29). In 1988, the same researchers proposed that insoluble and soluble fiber could also be evaluated through some modifications of the first methodology (Prosky et al., 1988). Further changes to the protocol were made to correct some problems detected in studies conducted with other researchers and

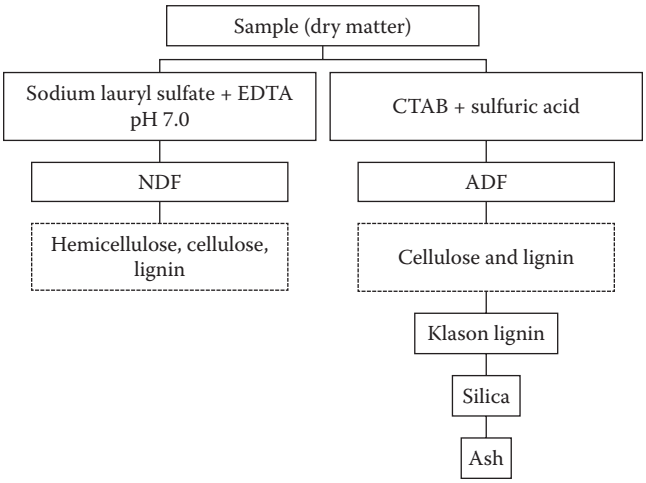

FIGURE 23.2 Schematic representation of the Van Soest method.

eventually yielded the enzymatic–gravimetric methods now indicated by AOAC (2007) for the evaluation of total, insoluble, and soluble fiber (AOAC methods 992.16, 991.42, and 993.19). In this method, the sample is submitted to enzymatic digestion with amylase, protease, and amyloglucosidase. The resulting residue can be filtered immediately to obtain the IF fraction, or the digested sample can be precipitated with ethanol to obtain the total fiber and/or soluble fiber. For each residue, protein and ash must be quantified to obtain the corrected values for each fiber fraction evaluated (Figure 23.3). For foods containing over 10% fat, an initial petroleum ether extraction is recommended to remove the fat content. In the same way, foods with high concentrations of sugar must be initially treated with an 85% methanol solution. These procedures are necessary to ensure the effective action of enzymes on the analyzed material.

Other methods were later validated and approved in collaborative studies by AOAC. Lee et al. (1992), for example, replaced the phosphate buffer used in the original method with MES-TRIS buffer (tris(hydroxymethyl)methylamine and 2-(N-morpholino)ethanesulfonic acid), resulting in AOAC method 991.43. AOAC method 992.16 was developed by Mongeau and Brassard (1993), who used successive enzymatic digestions in an autoclave followed by precipitation of the soluble fraction of the fiber with ethanol. The remaining IF is submitted to neutral detergent treatment using α-amylase from porcine pancreas.

Although the Prosky method is considered efficient for the evaluation of the total and IF fractions, Jeraci and Van Soest (1990), Mañas et al. (1994), Mañas and Saura-Calixto (1995), Silva et al. (2003), and Silva and Ciocca (2005) indicate that this method has some problems related to the gravimetric corrections used (protein, ash, and blank) as well as the ethanolic precipitation of soluble fiber. Silva et al. (2003) comment that in the first part of the analytical procedure, that is, the enzymatic digestions, a low variability is observed between the results obtained for cereal grains, with variation coefficients lower than 10% among the dry residues obtained after digestion. This finding indicates that this first step has little influence on the variability of the final results. However, the second step, which encompasses the analytical procedures for the gravimetric corrections (ash + protein + blank), has a significant effect on the variability of the final results. Research by Silva (2002) showed that the contribution of each of the corrections to the final results varies according to the nature of the sample; however, the protein correction exhibited greater variability and had the most influence on the quantification of the fiber fractions (Table 23.2). This result indicates the presence of instability in the proteolysis step across different analyses and for the analysis of different samples.

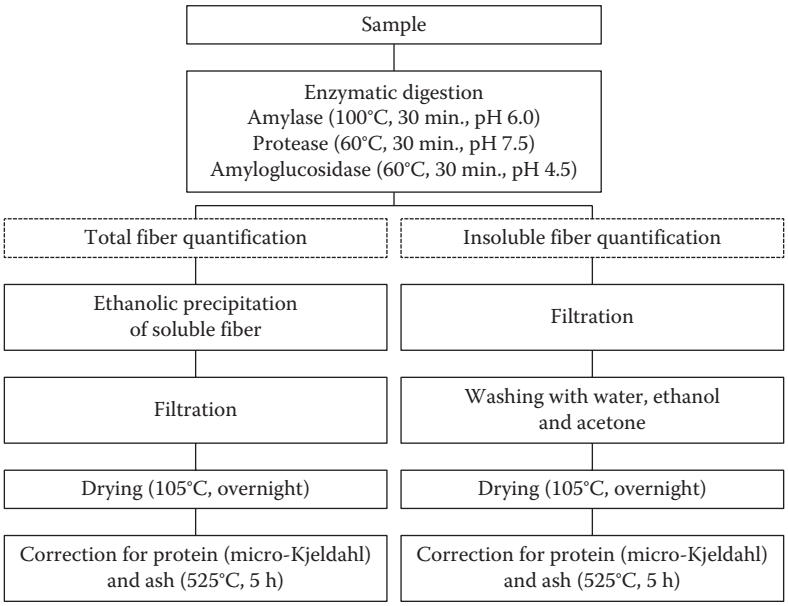

FIGURE 23.3 Schematic representation of the Prosky method.

TABLE 23.2

Mean Sample Weight, Enzymatic Digestion Residue Weight, and Gravimetric Corrections in the Quantification of TDF and IF in Maize (BR 5202 Pampa) and Oat (UFRGS 15)

	Sample	Residue		Ash		Protein		Blank		Total
Species	**mg**	**mg**	**%[a]**	**mg**	**%[b]**	**mg**	**%[b]**	**mg**	**%[b]**	**%[b]**
Maize										
TDF	1001.4 ± 0.5	168.2 ± 15.2	16.8 ± 1.5	3.3 ± 5.3	1.9 ± 3.0	23.1 ± 10.0	13.5 ± 5.0	8.7 ± 0.7	5.2 ± 0.6	20.6 ± 5.3
IF	1001.5 ± 0.7	137.1 ± 6.0	13.7 ± 0.6	–0.8 ± 1.9	–0.5 ± 1.4	16.1 ± 4.1	11.8 ± 3.0	1.7 ± 2.3	1.3 ± 1.7	12.5 ± 3.9
Oat										
TDF	978.3 ± 54.3	196.3 ± 22.9	20.0 ± 1.6	8.9 ± 4.3	4.5 ± 1.9	52.1 ± 15.0	26.4 ± 6.0	8.3 ± 2.5	4.3 ± 1.4	35.2 ± 5.8
IF	978.5 ± 54.0	129.6 ± 13.1	13.2 ± 0.8	–0.8 ± 1.5	–0.7 ± 1.2	33.8 ± 7.6	26.0 ± 4.5	2.2 ± 2.5	1.7 ± 2.0	27.0 ± 5.1

Source: Modified from Silva, L. P. 2002. Composição química de trigo e de aveia e efeito dos teores e proporções de fibra alimentar sobre a resposta biológica de frangos de corte e ratos. PhD dissertation, Universidade Federal do Rio Grande de Sul.

[a] Percentage of the residue relative to the initial sample (values expressed based on measurements of dry matter).

[b] Percentage of gravimetric correction relative to the digestion residue (values expressed based on measurements of dry matter).

The values for the gravimetric corrections for ash also exhibited great variation and were negative in 53% of the analyses of IF. This phenomenon is also mentioned in the collaborative study of this method (Prosky et al., 1992) in which the researchers ascribe these results to losses of the filtration aid and problems with the quality of the filtration aid and the fritted crucible. However, this explanation is questionable because the same behavior is observed in many other studies that have employed this methodology to quantify IF. It is more plausible that this discrepancy is caused by differences in the measurement technique performed during the second step of the method. One must consider that the value of ash in the IF residue is around 1 mg (depending on the sample analyzed) while the weight of the crucible used to obtain and incinerate the residue is between 30,000 and 50,000 mg. Thus, any small variation in weighing can result in "false" negative values, especially considering the number of decimals in the balance used for such quantifications.

The authors' research group has conducted preliminary studies to compare the methodologies used by Van Soest and by Prosky to evaluate the IF content of food. They have found that for most foods, the results obtained with NDF, using heat-stable amylase in the analytical procedure and correcting the fiber residue for the presence of protein and ash, are similar to those obtained by the enzymatic–gravimetric method proposed by Prosky et al. (1992).

The problems in the methodology proposed by Prosky and collaborators led to the proposal of other methods to quantify IF. Recently, Goñi et al. (2009) published an updated method for the analysis of fiber, including resistant protein and associated polyphenols, in food analyzed as it is consumed (raw, cooked, and fried) (Figure 23.4). This methodology deserves emphasis because it quantifies other chemical groups that are resistant to digestion but that are not typically quantified in other fiber methods, such as indigestible proteins that are part of the cell-wall structure but are usually removed from intermediate calculations by the quantification of crude protein. However, this analytical procedure is time consuming because it makes use of digestion conditions similar to the physiological ones, making it difficult to use this technique in routine analyses.

Enzymatic–chemical methods have also been extensively studied in an attempt to discover protocols that allow a better correlation between the determined IF contents and biological responses.

23.2.2 Enzymatic–Chemical Methods

The enzymatic–chemical methods in current use are based on the method proposed by Englyst et al. (1982), which was in turn based on the method proposed by Southgate (1969) and Theander and Aman (1979). Englyst et al. (1982) adopted the definition of dietary fiber as the fraction of polysaccharides that are not related to starch (or NSPs) and developed their method based on this definition. The methodology of Englyst et al. (1982) quantifies total NSPs, noncellulose polysaccharides (NCPs), and insoluble NSPs using different procedures (Figure 23.5).

The first stage is similar to all three procedures of the method and is based on the enzymatic digestion of the starch present in the sample. To quantify the total NSPs and NCPs, the digestion is followed by the addition of ethanol, solubilization using sulfuric acid (used only in the quantification of total NSPs), followed by acid hydrolysis. To quantify insoluble NSPs, the ethanol treatment is omitted, but the sample is submitted to solubilization and acid hydrolysis. After these procedures, the resulting neutral sugars are quantified by high-performance liquid chromatography or gas–liquid chromatography with acetylation and derivatization of the sugars, and the uronic acid content is quantified by colorimetry.

The omission of protease treatment in this methodology can be a source of error in the determination of the content and distribution of NSPs, depending on the sample (Mañas et al., 1994). This phenomenon is because some polysaccharides and polyphenols are associated with protein and lignin, resulting in condensed compounds, which will be part of the Klason lignin fraction.

An insufficiently long hydrolysis with sulfuric acid can prevent the complete degradation of cellulose, pectin, and protein such that some of these compounds will remain in the residue, leading to an underestimation of total fiber and its fractions (Wolters et al., 1992). In addition, this method cannot quantify resistant starch and Maillard compounds that may be present in the sample. It also has problems related to the ethanolic precipitation of soluble fiber (incomplete and with the coprecipitation of other nonfiber compounds) similar to those encountered in the method used by Prosky and collaborators.

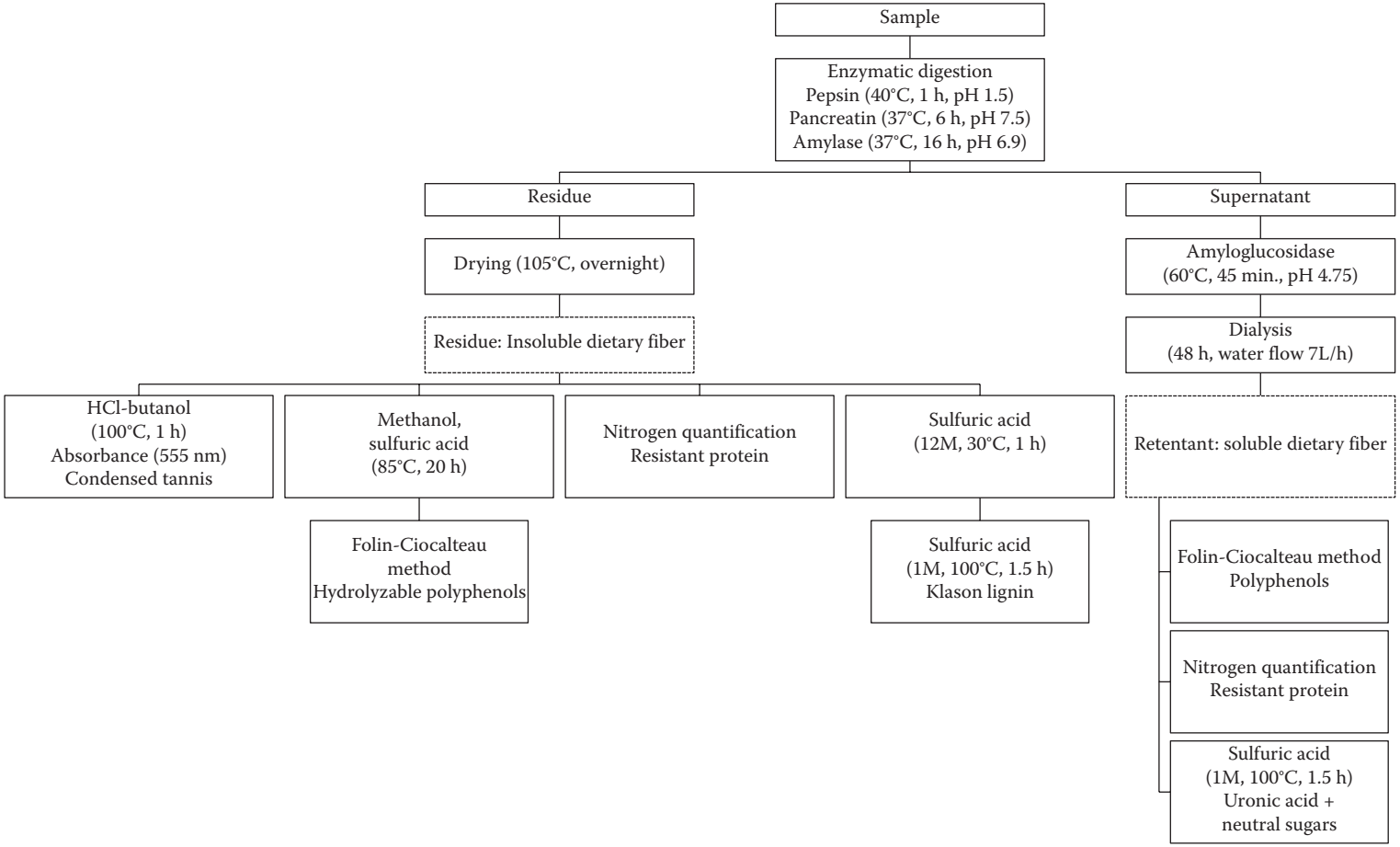

FIGURE 23.4 Schematic representation of the Goñi method.

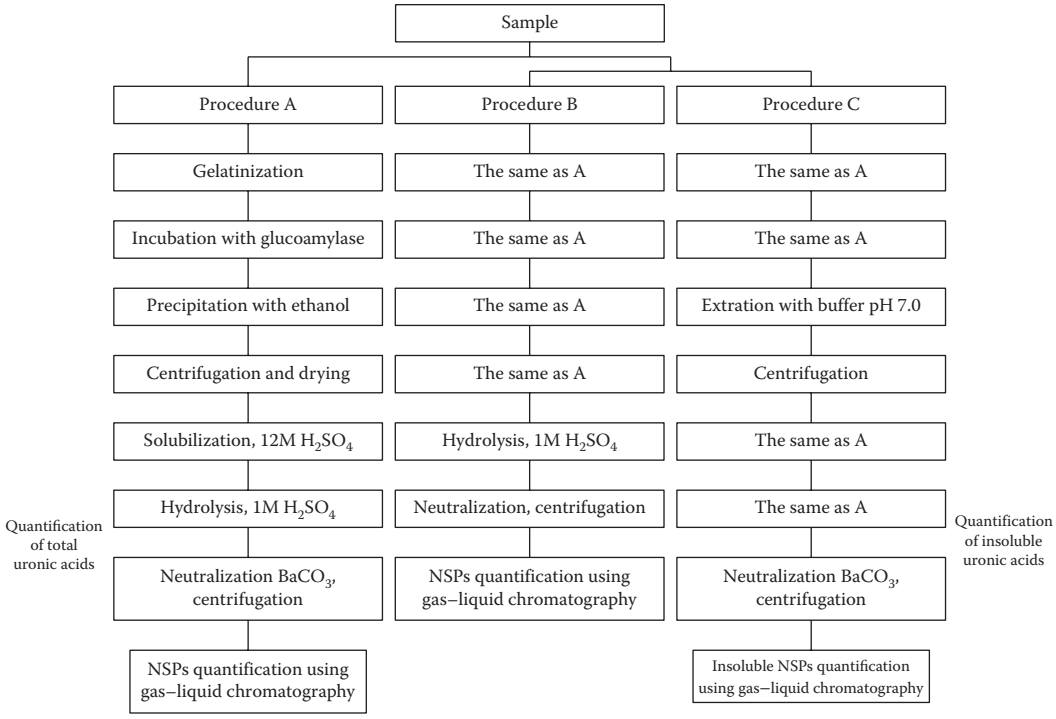

FIGURE 23.5 Schematic representation of the Englyst method to quantify nonstarch polysaccharides (NSPs), non-cellulose polysaccharides (NCPs), and isoluble NSPs.

In 1995, Theander and colleagues proposed an enzymatic–chemical method, called the Uppsala method, to quantify dietary fiber content and its composition, including neutral sugar residues, uronic acid residues, and Klason lignin (AOAC method 994.13) (Theander et al., 1995) (Figure 23.6). The method includes the digestion of the sample with heat-stable α-amylase and amyloglucosidase to remove starch followed by the precipitation of the solubilized dietary fiber components with ethanol. After acid hydrolysis of the residue, neutral polysaccharide residues are measured by gas–liquid chromatography, uronic acid residues are determined by colorimetry, and Klason lignin is measured gravimetrically. Total dietary fiber (TDF) content is calculated as the sum of NSP residues and Klason lignin.

The Uppsala method, in a similar way to that used by Englyst et al. (1982), does not require a protease digestion step and makes use of ethanol to precipitate soluble fiber compounds; this strategy may be a source of error, as discussed previously.

23.3 Additional Measurements for the Quantification of IF and Their Relationships with Biological Effects

Over the past 40 years, several studies have been conducted to test the effects of the different fractions of insoluble and soluble fiber as well as their specific constituents (e.g., β-glucans and arabinoxylans) on different aspects of human health. These have included effects on peristaltic movements, the influence on lipidic and glycemic responses, and the efficacy of fiber as a prebiotic agent to maintain the equilibrium of the intestinal microflora. Although in most cases the observed effects of fiber on digestion are positive, the relation between the type and content of the fibrous fraction in food on the biological response is not quite clear.

First, it must be considered that the study of the biological effects of a single constituent (e.g., β-glucans) of a complex food (e.g., cereal grains or oat bran), even if it is the main component of the cell wall, has

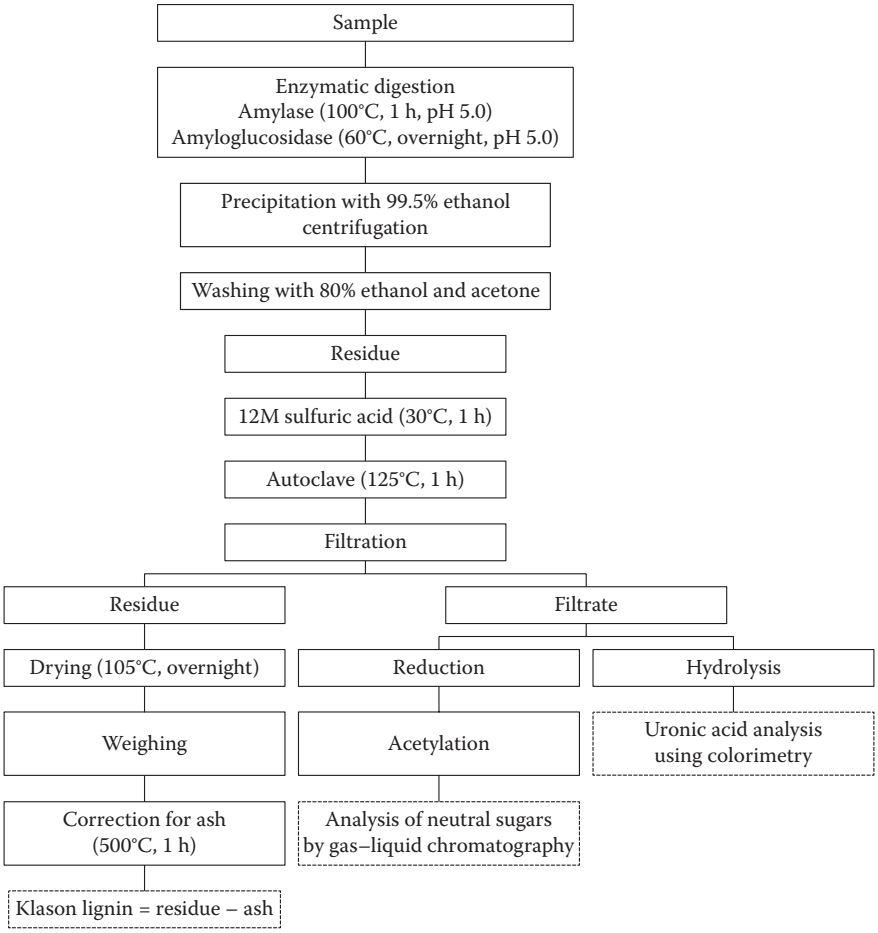

FIGURE 23.6 Schematic representation of the Uppsala method.

significant caveats. Total fiber always contains a number of constituents that act together to yield a specific structural organization and physicochemical characteristics that are unique to each plant. Therefore, it is not only one constituent but also the interaction among the components of the fiber that will provide the desirable metabolic effects on health.

It must also be pointed out that in addition to the quantification of the fiber content, it is important to know something about the physicochemical properties of this fraction of food, which are distinct among different components of the fiber. These properties can be extremely relevant to the applicability of fiber as a heath-protecting agent. For example, a study by Silva (2002) in rats (Table 23.3) showed that experimental diets with the same level of IF but derived from either purified cellulose or whole oat grains (5.49% and 5.12% of IF, respectively) stimulated completely different digestive responses in the animals; this difference was attributed mainly to the sources of fiber.

Purified cellulose has a high degree of crystallinity, with extremely reduced spaces among the individual chains of its polymers. Because of this structure, its hydration capacity and fermentation capacity are limited, which directly affects the moisture of the digesta (MD) and the feces as well as the disappearance of NDF via microbial fermentation and the energy resulting from this fermentation process. In contrast, the IF from oat grains has larger spaces within its polymers, allowing more hydration and microbial fermentation. This has a positive effect on the generation of energy through fermentation. Jeraci and Horvath (1989) also observed that purified cellulose is only weakly fermented by microorganisms in the gastrointestinal tract of humans, both *in vivo* and *in vitro*. Similarly, Lebet et al. (1998) attributed the low

TABLE 23.3

Moisture of the Digesta (MD) and of the Feces (MF), Apparent Digestibility of Neutral Detergent Fiber (ADNDF), and Apparent Digestibility of Energy (ADE), in Rats Fed with Experimental Diets with Different Sources of IF (Purified Cellulose and Whole Oat Grains)

	Purified Cellulose %	Whole Oat Grains %
MD	61.38 b	75.28 a
MF	38.04 b	59.45 a
ADNDF	1.80 b	76.87 a
ADE	91.65 b	94.13 a

Source: Modified from Silva, L. P. 2002. Composição química de trigo e de aveia e efeito dos teores e proporções de fibra alimentar sobre a resposta biológica de frangos de corte e ratos. PhD dissertation, Universidade Federal do Rio Grande de Sul.

Note: Mean values followed by different letters on the line differ by the Duncan's test at 5% significance.

yield of fermentation products from pea straw metabolized *in vitro* with the human fecal inoculum to the high degree of crystallinity of the cellulose in the cell wall. The higher fermentability of fiber in the ceco-colic region also positively affects the moisture of the feces (MF), because it is directly related to increased production of bacterial mass, and bacteria possess excellent water retention capacity (Stephen and Cummings, 1980; Jeraci and Horvath, 1989).

In these cases, the knowledge of the fiber contents, together with evaluations like porosity or hydration capacity, could be used to predict the respective physiological effects of these sources. Fiber porosity describes the space among individual chains of the polymers that form the fiber. This parameter can change based on the developmental stage of the plant or the processing to which the food is submitted (Chesson, 1997). In general, fibers with bigger pore diameters are digested more quickly because enzymes can act not only at the surface of the particle but also among the spaces (Chesson, 1993). In the case of fibrous constituents, higher porosity cannot only result in increased enzymatic accessibility to the cell content but also will allow greater ceco-colic fermentation. The hydration capacity of the fiber depends on the presence of hydrophilic groups as well as the surface area of the molecules and intracellular spaces (Annison and Choct, 1994). Insoluble structural polysaccharides such as cellulose and some xylans are linked by hydrogen bonds and act as sponges. However, their hydration capacity depends more on the particle size than on the amount of surface area that is in contact with water (Van Soest, 1994).

Another interesting example is provided by Retore (2009), who observed that in rabbits, the degree of fiber-related reduction of blood triglycerides and cholesterol is directly related to its copper-binding capacity. The main functional groups of the fiber constituents that are capable of ionic interaction with other compounds include carboxyl, amine, aliphatic, and phenolic hydroxyl groups, which are present in higher amount in pectins, lignin, and tannins (Jeraci and Van Soest, 1990). In addition to affecting the availability of some minerals (Ink and Hurt, 1987), these compounds can also retain other organic or inorganic compounds with electric charges. Thus, these results can be explained by the hypothesis advanced by Eastwood and Kay (1979) and Olson et al. (1987) that bile acids can participate in ionic interactions that alter lipid metabolism and affect cholesterol levels. This phenomenon occurs because the sequestration of a significant amount of bile salts by fiber will reduce the formation of micelles and, consequently, the absorption of fat, which will lead to reduced serum triglyceride levels. Similarly, there will be a reduction in the enterohepatic circulation of bile salts, and the organism will need to capture more circulating cholesterol to synthesize bile acid to replace what has been lost; this will cause a reduction in the levels of cholesterol in the blood. The cation-binding activity of fiber is independent of its solubility and is another characteristic that may be measured to allow better prediction of its metabolic effects in the organism.

The data presented herein describe several methodologies that have been developed over the years for the quantification of IF, separately or as TDF. Each method is best suited to analyze different fiber components, resulting in differences in the obtained results. Although the methods currently in use represent

significant improvements over earlier protocols, some caveats remain. These issues deserve continued attention from researchers, who should aim to develop methods for the better quantification of the components of fiber.

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24

Fructans Including Inulin

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24.1 Introduction

The extensive changes in nutrition concepts based on the increased understanding of various functional effects as a result of interactions between a food component and physiological functions have led to a much-appreciated and well-pursued link between colonic nutrition and human health. The radical change from a past emphasis on the absence of adverse effects to the recent prominence on the promising use of foods, thereby promoting a state of well-being, better health, and reduction of the risk of diseases, is the driving force and key factor in the present scenario. The current nutrition science has its foundation on the hypothesis that, beyond providing nutrition, diet may also modulate various functions in the body. This has led to the understanding of various functional effects that are the positive consequences of the interactions between a food component and specific genomic, biochemical, cellular, or physiological

functions with or without direct reference to any health benefit or reduction of risk of a disease. Such functional claims have already resulted in new perceptions in nutrition: prebiotics, synbiotics, colonic foods, and bifidogenic factors, to name a few (Roberfroid, 1999).

The renewed interest in the link between colonic function and human health, since the 1970s, has resulted in much attention being given to dietary foodstuffs termed nondigestible oligosaccharides (NDOs), which are prebiotic carbohydrates that are not absorbed in the vertebrate digestive system, the human digestive system in particular, and as such are available in their entirety for the abundant intestinal bacterial ecosystem (Leach, 2007). Since they are not digested in the upper intestinal tract, but rather interact with the bacterial ecosystem present in the lower intestinal tract, their consumption selectively promotes the growth of bacteria that are associated with a healthy condition (e.g., lactobacilli, bifidobacteria) and suppresses bacteria that are associated with disease (e.g., clostridia). The augmented metabolic activity of the beneficial bacteria results in the production of metabolites that are absorbed into the blood and exert beneficial effects in the rest of the body with improved resistance to infection, better skeletal bone quality, and a reduced risk of chronic diseases such as cancer, cardiovascular disease, and so on, as a direct consequence (van Loo, 2005). There has been quite a lot of research on the health benefits associated with such prebiotic oligosaccharides, mainly the fructans, both short-chained fructooligosaccharides (FOS) and the longer-chained inulin with potential in use for developing physiological functional foods.

Technically defined as a selectively fermented ingredient that allows specific changes, both in the composition and activity in the gastrointestinal (GI) microbiota that confers benefits upon host well-being and health (Gibson et al., 2004), prebiotics were ingeniously compared by Professor D. Jenkins to fertilizers of colonic bifidobacteria (Roberfroid and Milner, 1999). Common prebiotics in use include inulin, FOS, galactooligosaccharides (GOS), soya-oligosaccharides, xylo-oligosaccharides, pyrodextrins, isomalto-oligosaccharides, and lactulose. The majority of studies have thus far focused on inulin, FOS, and GOS since they have been used for a long time and are generally regarded as safe. However, there is an indiscriminate use of the term "prebiotics" for other nondigestible saccharides, beyond the original definition, in order to promote them and their effects beyond what can be scientifically proven. The appearance of a wide range of commercially available products that bear the prebiotic label but for which supportive scientific literature is sparse, and the little legislation existing that governs the use of the word "prebiotic" on functional food products, has resulted in FAO-specifying guidelines mandatory for prebiotics (Figure 24.1).

Despite a variety of new candidate prebiotics becoming available for human use, the consensus of scientists achieved as a result of the European Project ENDO (DGXII AIRII-CT94-1095) indicated β(2–1) fructans as exclusively possessing well-documented prebiotic abilities in humans (van Loo et al., 1999). The present chapter is a concise review of fructans in general, their beneficial properties, and various approaches for fructan analysis. The chapter also elaborates the published scientific data supporting the various functional effects attributed to the fructans.

24.2 Fructans

Fructans are the most abundant storage carbohydrate in plants next to starch and sucrose occurring naturally in about 15% of flowering plant species in at least one of their organs during their life cycle (Hendry, 1993). Fructan in general is a term used for any carbohydrate in which fructosyl–fructose links constitute the majority of the glycosidic bonds. They are linear or branched polymers that are either β(2–1)-linked inulins or β(2–6)-linked levans or branched group with both β(2–1) and β(2–6) fructosyl–fructose linkages in significant amounts (e.g., graminan).

The basic structure of a fructan is a trisaccharide, known as a kestose, which has a sucrose molecule linked to one additional fructose. Dicotyledonous species store inulin-type fructans, while more complex and branched-type fructans are common in monocots. They are found as oligosaccharides of 5–10 fructose molecules in the neokestose type, ~50 residues in the inulin or 1-kestose group and ~200 for the levan or 6-kestose type (Vijn and Smeekens, 1999). These naturally occurring nondigestible oligo- and polysaccharides are located in vacuoles of different organs such as onion bulbs, Jerusalem artichoke,

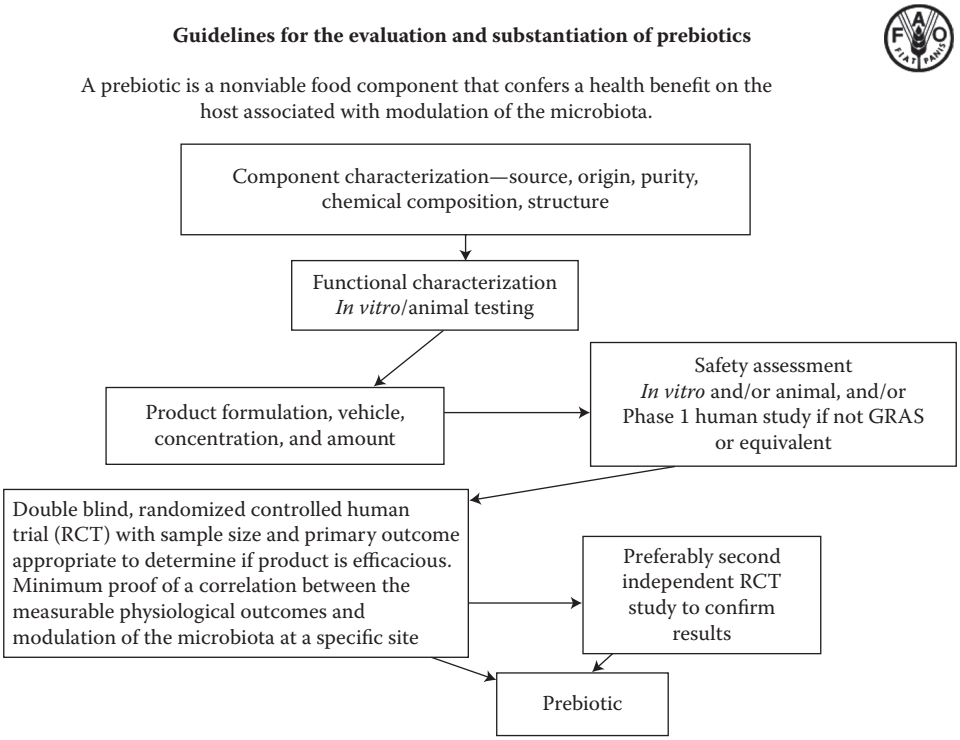

FIGURE 24.1 Guidelines for evaluation of prebiotics. (Adapted from FAO Technical Meeting report on Prebiotics, September 2007.)

dahlia tubers, chicory taproots, leaves, and in stems of wheat, barley, and so on in a wide variety of plants (Carpita et al., 1989). Asparagus, sugar beet, garlic, chicory, onion, Jerusalem artichoke, wheat, honey, banana, barley, tomato, and rye are special sources of FOS (Sangeetha et al., 2005). In addition to fruits and vegetables, prebiotics of various types can be found as natural components in milk, honey, and so on in concentrations ranging between 0.3% and 6% of fresh weight (Table 24.1).

Fructan-containing plants have always been a part of human diet for entire populations over centuries, well before the concept of prebiotic food ingredients revolutionized the nutritional research. Though fructan-containing plants were being consumed unaware as food, feed, or medicine (Leach, 2007), the field of fructan research is just about two centuries old. The first documented proof of discovery of fructans dates back to early 1800s, when Rose, a German scientist, discovered a "peculiar substance of plant origin" from a hot water extract of *Inula helenium* (Rose, 1804), which was subsequently named "inulin" by Thomson in 1818. Much of the fructan research in the early days was carried out in Germany and France. The German plant physiologist Julias Sachs (1864) contributed immensely with his studies on the microscopic identification of inulin spherocrystals in the tubers of *Dahlia*, *Helianthus*, and *Inula* species, which paved the way for further studies on the localization of fructans.

24.3 Chemical Structure

Fructans are by nature polymers of fructose and can be described as linear polydisperse carbohydrates consisting of β(2–1) fructosyl–fructose linkages with usually a glucose moiety linked to the end of the chain by an α(1–2) bond as in sucrose (Roberfroid and Delzenne, 1998). Chemically, fructans are

TABLE 24.1

Fructan Containing Plants Commonly Used by Humans

Source	Edible Part	Inulin	Oligofructose
Artichoke	Leaves-heart	3–10	<1
Banana	Fruit	0.3–0.7	0.3–0.7
Barley	Cereal	0.5–1.5[a]	0.5–1.5[a]
Burdock	Root	3.5–4.0	NA
Camas	Bulb	12–22	NA
Chicory	Root	15–20	5–10
Dandelion	Leaves	12–15	NA
Garlic	Bulb	9–16	3–6
Jerusalem artichoke	Tuber	16–20	10–15
Leek	Bulb	3–10	2–5
Murnong	Root	8–13	NA
Onion	Bulb	2–6	2–6
Rye	Cereal	0.5–1[a]	0.5–1[a]
Salsify	Root	4–11	4–11
Wheat	Cereal	1–4	1–4
Yacon	Root	3–19	3–19

Source: From van Loo J. et al. 1995. *Crit Rev Food Sci Nutr*; 35(6): 525–552. With permission.

Note: NA, data not available.

[a] Estimated value.

distinguished on the basis of the glycosidic bond that links fructose residues to each other. Originally identified as inulins, fructans were subsequently classified as inulins, levans (fleins in plants), mixed levans (gramminans in plants), and the neoseries (neoinulin and neolevan, in plants), according to the type of bond that the extended β-D-fructosyl chain forms with sucrose. As sucrose is a precursor of all types of fructans, they usually have a glucose moiety at one extreme; however, since the bond between the starting glucose and the second carbon (of fructose) can be hydrolyzed to some degree by sucrase enzymes, many fructans begin with fructose. Thus, the presence of glucose moiety is not a necessary precondition for the compound to be considered a fructan. An individual fructan having a glucose molecule preceding fructose is designated as GFm, "G" referring to the terminal glucose unit, "F" referring to fructose units, and "m" designating the number of fructose units found in the fructan chain. A fructan with no glucose can be designated as "Fm" (Figure 24.2).

Fructans can also be described by the degree of polymerization (DP). DP refers to the number of repeat units in an oligomer or polymer chain, so DP of an individual fructan would be its number of repeating fructose units and identical to "m." The DP varies from two to several hundred, with the major components of fructans being inulin (mostly DP 2–60) and FOS/oligofructose (OF) (DP 2–10).

24.4 Common Fructans

An understanding of different terms used to describe fructose-containing polymers is important as more commercial products appear in the market. Inulin, FOS, and OF are distinguished in the fructan group. Inulin (GFm) is a highly polymerized fructan of DP 10–60, extracted mainly from chicory, and consists majorly of linear chains of β(2,1)-linked D-fructofuranosyl units bound by an (α1–β2)-type linkage (as in sucrose) to a terminal glucose moiety. The basic GF_2 trimer in inulin and the shortest fructan of the inulin type is 1-kestose. All fructans found in dicotyledons, as well as some monocotyledons, are of this type. Fructans composed majorly of linear fructose units bound by a

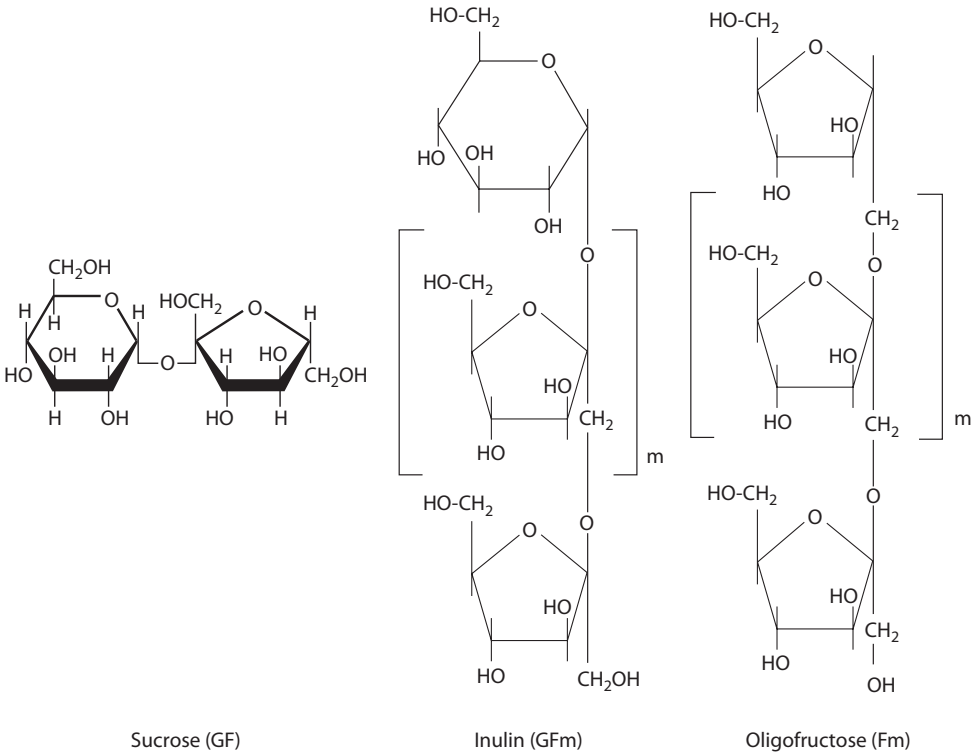

FIGURE 24.2 Chemical structure of sucrose (GF), inulin (GFm), and oligofructose (Fm).

β(2–6) glycosidic bond are typically levans that are produced by many soil and oral bacteria, yeasts, and fungi.

Small-chain oligosaccharides (DP < 9) are termed fructooligosaccharides, oligofructosides, FOS, or short-chain FOSs prepared by the transfructosylation action of a β-fructosidase of microorganisms, mainly *Aspergillus* species on sucrose. The resulting mixture has the general formula of GFm with "m" ranging from 1 to 4. The main components of this class are kestose (GF2), nystose (GF3), and fructosylnystose (GF4). OF was introduced as a synonym for fructooligosaccharides in 1989 (Coussement, 1999). OF with DP 2–9 is produced during the process of chemical degradation or controlled enzymatic hydrolysis of inulin by endoglycosidases (Gibson and Roberfroid, 1995; Roberfroid et al., 1998). OF has been defined by the IUB-IUPAC Joint Commission on Biochemical Nomenclature and the AOAC as fructose oligosaccharides containing 2–10 monosaccharide residues connected by glycosidic linkages (Niness, 1999). OF resembles FOS obtained in the transfructosylation process containing predominately molecules of the Fm-type (homopolymers of fructose bound by a β(2–1) glycosidic linkage having no terminal glucose). Thus, OF has the same structure as inulin, but the chains consist of 10 or fewer fructose units.

Neosugar is a mixture of kestose ($m = 2$), nystose ($m = 3$), and fructofuranosyl nystose ($m = 4$). Basically, these are sucrose molecules to which 1–3 additional fructose units have been added (Oku et al., 1984). Neosugar can be isolated from brans of triticale, wheat, and rye or by the action of the fungal (*Aspergillus* species) enzyme fructofuranisidase on sucrose (Fishbein et al., 1988). Short-chain fractions of FOS such as 1-kestose, the major GF, compound in chicory roots or Jerusalem artichoke, and neokestose in onion do not differ analytically (van Loo et al., 1995). Further, fructan chains linked to one of these naturally occurring trisaccharides have the β(2–1) configuration, implying that with the exception of one glycosidic linkage within the basic trisaccharide there is no difference between a fructan molecule based on 1-kestose or neokestose.

Using the most widely available and accepted nomenclature, all FOS and inulins are fructans, all FOS are inulins, but not all inulins are FOS. Those inulin molecules having a DP < 10 fructose units generally are considered to represent FOS.

24.5 Fructan Biosynthesis

In plants, fructan synthesis occurs in vacuoles with sucrose as the precursor and by the action of two or more fructosyltransferases. The biosynthesis of these polysaccharides starts with the incorporation of a fructose moiety to one of the three primary hydroxyl groups of sucrose—that is, C-1 and C-6 in the fructose moiety, and C-6 in the glucose moiety. This reaction, catalyzed by the enzyme sucrose: sucrose 1-fructosyl transferase (1-SFT) involves a transglycosylation in which one fructosyl residue is transferred from one molecule of sucrose to another, resulting in the formation of the trisaccharide, kestose. The addition of β-D-(2,1)- or β-D(2,6)-linked fructofuranosyl units to the fructose moiety of sucrose yields 1-kestose and 6-kestose, respectively, while the addition of a β-D-(2,6)-linked fructofuranosyl unit to the glucose moiety of sucrose produces neokestose (Figure 24.3).

Further elongation of the kestose-type trisaccharide proceeds by the transglycosylation action of specific enzymes named fructan, fructan fructosyl transferases (1-FFT). These enzymes transfer a fructosyl unit from a fructan molecule to position 1 or 6 in the second fructose moiety of the trisaccharide, thus forming the linear β-D-(2,1) bonds of inulins and the β-D-(2,6) linkages in levans (Figure 24.4). The action of sucrose 1-fructosyltransferase (1-SST) and 1-FFT results in the formation of a mixture of fructan molecules with different chain lengths.

Thus, inulins are linear polymers of fructose with β(2–1) bonds. 1-Kestose, the inulin precursor, is formed by the addition of fructosyl residues in a β(2–1) bond to sucrose. Levans are also linear polymers of fructose, but with β(2–6) bonds. Mixed levans (gramminans) are of plant origin and have both β(2–1)- and β(2–6)-linked fructosyl residues. Inulin neoseries characterized by the β-D-fructosyl units is linked either to C1 or to C6 of the glucose moiety of sucrose by a β(2–1) bond as in inulin and levan neoseries

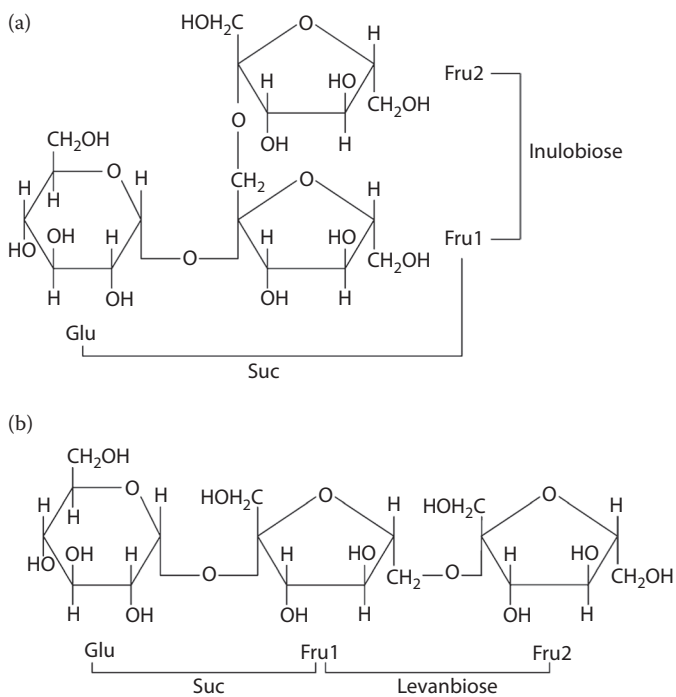

FIGURE 24.3 Structure of kestoses. (a) 1-Kestose and (b) 6-kestose.

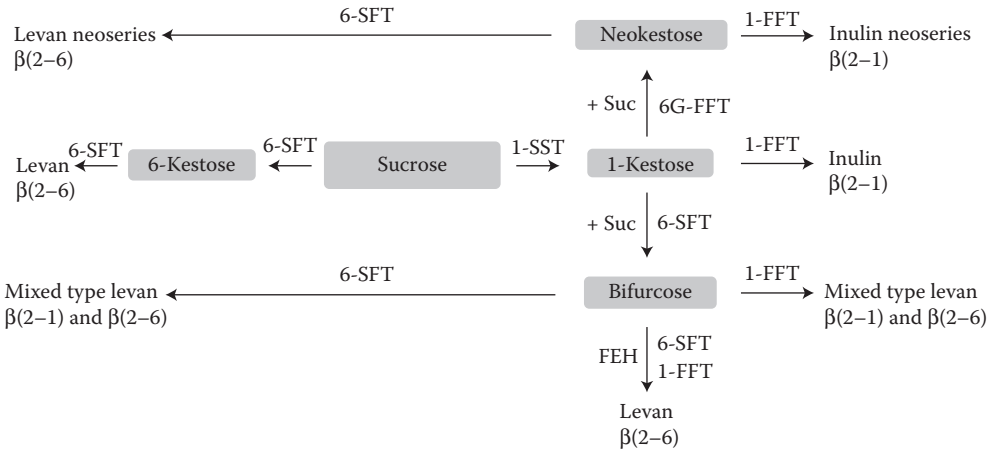

FIGURE 24.4 Fructan biosynthesis. (Adapted from Vijn I. and Smeekens S. 1999. *Plant Physiol*; 120: 351–359.) 1-FFT, fructan:fructan 1-fructosyltransferase; 1-SST, Suc:Suc 1-fructosyltransferase; Suc, sucrose 6G-FFT, fructan:fructan 6Gfructosyltransferase; 6-SFT, Suc:fructan 6-fructosyltransferase; FEH, fructan exohydrolase.

has β-D-fructosyl units linked to C1 or C6 of the glucose moiety of sucrose by a β(2–6) bond as in levan (Lewis, 1993).

24.6 Industrial Production

Inulin is extracted from chicory and Jerusalem artichoke on an industrial scale. The primary industrial source of pure inulin is the chicory root (*Cichorium intybus* L.), while FOS are commercially produced from sucrose. OF, another group of short-chain fructans, are produced through acid or enzymic hydrolysis of inulin. An entire range of fructan prebiotic products with different DP and sugar compositions in different degrees of purity are available in the market depending on the mode of production. Naturally occurring fructans are nonreducing, as are those produced from sucrose by transglycosylation. The inulin production process involves three general steps: extraction of raw inulin with hot water, purification of the raw inulin, and spray drying of the purified juice to a pure inulin powder. The hot water extraction yields ~92% inulin-type fructans of both GFm and Fm types with DP ranging from 2 to 60 and an average DP (DPav) of ~10–12. About 10% of the fructans in this minimally processed inulin might have a DP ranging from 2 to 4 and 20% might range from 5 to 9. This extract will also contain a small amount (6–10%) of free sugars (the monosaccharides fructose and glucose and the disaccharide sucrose) present in the starting root material. FOS are enzymatically synthesized from sucrose by transfructosylation using the fungal enzyme β-fructosidase, derived from *Aspergillus* spp. The enzyme sequentially adds fructose units with new β(2–1) linkages yielding short-chain fructan-type prebiotics with a DP range from 2 to 4 and DPav of ~3.6. Since the starting material is a sucrose molecule, the fructans in these mixes have higher proportions of glucose units and are designated as GFm type (Coussement, 1999; Niness, 1999; Roberfroid, 2007).

Inulin can be further processed into more purified inulin-type prebiotic products. Total enzymatic hydrolysis results in monosaccharide molecules of fructose and glucose. Partial enzymatic hydrolysis can produce mixtures with a DP ranging from a hot-water-extracted inulin to a pure mix of completely enzymatically hydrolyzed monosaccharides. An endoinulase is used for partial enzymatic hydrolysis of inulin. Using partial hydrolysis and/or physical separation techniques, products with 99% purity can be produced. Standard inulin (92% fructans and 8–10% free sugars) and low-sugar versions of inulin (99.5% fructans and ~0.5% sugars) are both referred to as inulin. FOS products also vary in their free-sugar content since it contains unreacted sucrose as well as glucose and fructose molecules formed as by-products. These free sugars can be removed or left in the finished product depending on the sweetness characteristics desired (Coussement, 1999; Niness, 1999; Roberfroid, 2007).

24.7 Role in Food, Physiological Roles

Fructans are energy-reserve carbohydrates and may act as osmoprotectants in plants. It is proposed that since they are readily soluble in water, they are osmotically active. By changing the DP of the molecules in the plant's vacuole, the plant can change the osmotic potential of its cells without altering the total amount of carbohydrates. The hydrolysis of inulin by endoinulinase to lower DP molecules allows plants to osmoregulate, surviving winter periods in cold to moderately cold and drought-stricken regions (Edelman and Jefford, 1968).

Fructans possess several properties, which make their use as food ingredients particularly attractive. They are water-soluble and mildly sweet, typically 30% as sweet as sucrose. Their relatively low sweetness is useful in food production when a bulking agent with reduced sweetness is desirable to enhance other food flavors. The viscosity and thermal stability of FOS solution is higher than that of mono- and disaccharide sucrose at the same concentration owing to the presence of the higher molecular weight of oligosaccharides, which in turn provides increased viscosity, leading to improved body and mouthfeel. Inulin/FOS are effectively used to replace fat or sugar, reduce the calories as well as to enhance taste of foods like ice cream, dairy products, confections, and baked goods, on account of their unique texturizing properties. Fructans are highly stable in the normal pH range for food (4.0–7.0) and at refrigerated temperatures over 1 year. They can be used to alter the freezing temperature of frozen foods, and to control the amount of browning due to Maillard reactions in heat-processed foods. They provide high moisture-retaining capacity, preventing excessive drying, and a low water activity, which is convenient in controlling microbial contamination (Crittenden and Playne, 1996). Fructans with their characteristic β(2–1) fructosyl–fructose glycosidic bonds resist enzymatic hydrolysis by human salivary and small intestinal digestive enzymes, which are specific for α-glycosidic bonds and as a result are indigestible but they are fermented by colonic microflora. The short-chain fatty acids (SCFA) and lactate produced by fermentation play vital roles in maintaining gut health.

24.8 Functional Properties

Fructans have a number of interesting functional properties that make them important food ingredients. For example, inulin and OF rebalance metabolic activities (e.g., lipid homeostasis), strengthen immune functions (immunostimulation), restore or stabilize colonic microflora (e.g., selective stimulation of bifidobacteria), and improve bioavailability of nutrients. The nutritional and health benefits of fructans have been the subject of a number of studies and reviews and are concisely detailed below.

24.8.1 Dietary Fiber Effect

"Dietary fibre is the edible part of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine" (AACC, 2001). The nondigestible quality of fructans makes them act similar to dietary fiber, which may benefit health through a wide range of physiological effects. They resist digestion and absorption in the stomach and small intestine of humans, as shown by their full recovery at the end of the ileum of healthy or ileostomized volunteers.

Studies in patients with a conventional ileostomy by Cherbut (2002) have shown that mean excretion of FOS at the end of ileum was about 90% of the ingested dose. Thus, they enter the large intestine where they will be available for fermentation, as demonstrated by increased breath hydrogen. Increased lactate concentration has been found in colonic and fecal contents of rats fed with FOS. Fermentation is complete and no residue has been found in human stools. They improve laxation and exhibit bulking capacity between 1.2 and 2.1 g of stool per g of ingested substrate, resulting mainly from an increase in microbial biomass in the colon. In addition, due to their fermentation properties, they also affect the intestinal epithelium that may strengthen mucosal protection and reduce the risk of GI diseases. Therefore, fructans

like FOS act as excellent dietary fiber and prevent constipation. However, excessive consumption of fructans may cause flatulence or diarrhea.

The advantages fructans have over dietary fiber are that they have a smaller daily requirement, do not cause diarrhea in recommended doses, are slightly sweet, have neither bad texture nor bad taste, are completely water soluble, do not build viscosity, do not bind minerals, are physically stable, and are easier to incorporate into processed foods and drinks (Tomomatsu, 1994).

24.8.2 Prebiotic Effect

The potential of fructans to promote the proliferation of bifidobacteria in the colon has been well understood and researched in recent years. Prebiotics are nondigestible food ingredients that selectively stimulate the growth and/or activity of potentially health-enhancing intestinal bacteria (probiotics). Since fructans are not hydrolyzed by the human digestive enzymes; they undergo fermentation in the colon and encourage the growth of beneficial bacteria in the colon. These intestinal bacteria metabolize them readily and produce large amounts of SCFA, resulting in an acidic pH in the lumen of the large intestine. Beneficial bacteria such as *Bifidobacterium* spp. and *Lactobacillus* spp. are resistant to acidic pH, whereas the harmful bacteria such as *Clostridium* spp. are sensitive to acidic conditions. Therefore the proliferation of useful bacteria is stimulated and that of harmful bacteria is suppressed, resulting in a healthy gut environment (Figure 24.5). Lactic acid bacteria (LAB) and bifidobacteria are considered to be immunomodulatory and directly or indirectly influence the GI tract and systemic defense functions.

Fructans have been demonstrated as effective prebiotics through both *in vivo* and *in vitro* assessments. Fructans are shown to increase bifidobacteria numbers in the colon at doses of <15 g/day (Crittenden and Playne, 1996). Durieux et al. (2001) have investigated the prebiotic effect of fructans by studying the metabolism of two types of chicory FOS (Fibruline Instant and Fibrulose F 97) by *Bifidobacterium longum*, *B. infantis*, and *B. angulatum*. Chromatographic analysis of the medium after 120 h revealed consumption of all the fructose oligomers present in the commercial chicory FOS by all the strains. Biomass production was highest with *B. infantis* in medium supplemented with Fibruline Instant and Fibrulose F 97. A comparative evaluation of the fermentation properties of prebiotic oligosaccharides by gut bacterial groups was undertaken by Rycroft et al. (2001). Monitoring of 24 h batch culture by fluorescent *in situ* hybridization revealed that the oligosaccharides differ in their fermentation characteristics. All prebiotics increased the numbers of bifidobacteria and most decreased clostridia, with FOS producing the highest populations of lactobacilli and least flatulence.

Shin et al. (2000) checked for the growth and activity of *Bifidobacterium* spp. (Bf-1 and Bf-6) cultures in 12% (w/w) reconstituted Nonfat Dry Milk containing 0%, 0.5%, 1.0%, 3.0%, or 5.0% (w/v) FOS. Growth, enhancement of activity, and retention of viability were found to be more with 5% FOS (w/v).

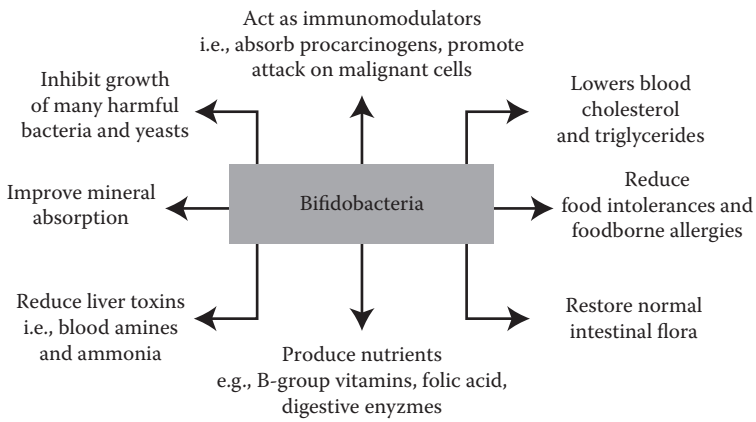

FIGURE 24.5 Beneficial bacteria and their health benefits.

Kaplan and Hutkins (2000) screened 28 strains of LAB and bifidobacteria for their ability to ferment FOS on MRS agar. Twelve of 16 *Lactobacillus* strains and 7 of 8 bifidobacterial strains tested were able to ferment the substrate. It was found that like glucose, FOS was equally a good substrate in supporting growth. The tolerance and threshold dose of FOS that significantly increased fecal bifidobacteria were assessed and the optimal dose for increased bifidobacterial counts without significant side effects such as flatulence was reported to be 10 g/day. The effect of FOS (5 g/day) on the fecal microflora, especially bifidobacteria, in healthy human subjects was investigated and compared with the ingestion of a placebo—sucrose (Rao, 2001). Samples subjected to microbial enumeration showed that ingestion of sucrose (5 g/day) had no effect on all fecal bacteria enumerated, whereas consumption of FOS (5 g/day) for 11 days resulted in close to one log cycle increase in bifidobacteria numbers.

Thus, supplementing the diet with fructans that enhance the growth and metabolic functions of probiotics boosts the defense mechanisms of the host, increases resistance to various health challenges, and accelerates recovery of the compromised GI tract in case of diseases.

24.8.3 Lipid-Lowering Effect

Fructans, besides their effect on the GI tract, are also able to exert systemic effect, by modifying the hepatic metabolism of lipids in several animal models (Delzenne et al., 2002). Fructans in diet have been found to decrease the levels of triglycerides, serum cholesterol, and lipids. Colonic fermentation of fructans results in the synthesis of SCFA, which influences the systemic lipid metabolism in human beings. Hypotriglyceridemia due to decrease in the hepatic synthesis of triglycerides and hypocholesterolemia are likely to result from the antagonistic effect of SCFA, especially propionate on cholesterol metabolism. Propionate has been found to be an inhibitor of HMG CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA reductase), an important enzyme in cholesterol synthesis (Roberfroid and Slavin, 2000). A lower hepatic lipogenesis, through a coordinate reduction of the activity and mRNA of lipogenic enzymes, is an important event in the reduction of very low-density lipoprotein triacylglycerol (TAG) secretion by FOS. Fructans can thus counteract triglyceride metabolism disorder either through dietary manipulation in animals or sometimes independently on lipogenesis modulation.

Animal studies provide strong evidence that FOS inhibit secretion of very low-density lipoprotein (VLDL) particles via inhibition of *de novo* fatty acid synthesis. Feeding rats with 10% FOS significantly lowers serum triglycerides and phospholipid concentrations. Daily feeding of OF (mean DP of 4.8) to rats at a 10% dose level resulted in significant serum triglyceride lowering after just 1 week of feeding (Fiordaliso et al., 1995). Kok et al. (1996) could confirm a significant decrease in serum triglyceride-VLDL when 10% OF (inulin) (DP = 4.8) was given in a standard diet for rats. Since liver enzyme activity was also reduced for two of the four enzymes assayed, the SCFA produced from OF fermentation was implicated in the inhibition of key enzyme activities, particularly glycerol-3-phosphate acyltransferase and fatty acid synthase, resulting in decreased *de novo* triglyceride and fatty acid synthesis. Feeding male Wistar rats on a carbohydrate-rich diet containing 10% FOS significantly lowers serum TAG and phospholipid concentration. FOS reduces postprandial triglyceridemia by 50% and avoids the increase in serum-free cholesterol level occurring in rats fed with a Western type high fat diet. FOS protects rats against steatosis (liver TAG accumulation) induced by fructose or occurring in obese Zucker rats. FOS given at the dose of 10% in the diet of male Wistar rats for 30 days reduces postprandial insulinemia by 26% (Daubiol et al., 2000). In streptozotocin-treated diabetic rats, feeding a diet containing 20% FOS for 2 months decreases postprandial glycemia despite lack of modification of the glycemic/insulinemic response to a saccharose or maltose load. Short-chain fructans have been shown to lower serum total and low-density lipoprotein cholesterol in noninsulin-dependent diabetic patients, but not in healthy subjects (Roberfroid and Delzenne, 1998).

24.8.4 Effect on Mineral Absorption

Fructans have been found to enhance the absorption of calcium and magnesium ions and maintain balance of iron and zinc ions. This has been found to be due to the osmotic effect, acidification of the colonic content due to fermentation and production of short-chain carboxylic acids, formation of calcium and

magnesium salts of these acids, and hypertrophy of the colon wall (Roberfroid and Slavin, 2000). Colonic fermentation of fructans lead to decrease in pH in the colon and this facilitates the absorption of mineral ions from the intestine, mainly calcium and magnesium.

Levrat et al. (1991) found that in rats fed a diet supplemented with a 10% inulin fraction, the cecal pool for calcium, magnesium, and phosphate was improved. A significant increase (of about 60%) in the apparent retention of calcium, magnesium, and iron was observed in rats fed a diet containing higher levels of inulin (10% inulin) by Delzenne et al. (1995). The addition of 5% FOS prevented bone loss significantly in the femur and lumbar vertebra in the presence of dietary calcium (1%). The effect may be due to enhancement of passive and active mineral transport across the intestinal epithelium, mediated by an increase in certain metabolites of the intestinal flora and a reduction in pH (Scholez Ahrens and Schrezenmeir, 2002).

The effect of FOS on protein digestibility and mineral absorption was studied by Gudieal-Urabano and Goni (2002) in rats fed with diets containing 5 g/kg FOS, 5 g/kg cellulose/FOS (1:1), or 5 g/kg cellulose as a source of dietary fiber. Cellulose/FOS enhanced apparent absorption and apparent retention of Ca, Mg, Zn, and Fe. FOS-fed rats experienced an increase in apparent absorption and apparent retention of Mg compared with cellulose-fed rats. FOS intake at the lowest dose was found to be enough to provide a desirable effect on mineral bioavailability in rats without any modification of nutritional parameters (Gudieal-Urabano and Goni, 2002).

24.8.5 FOS in Defense Functions

Fructans are known to prevent the colonization of human gut by pathogenic microorganisms because they encourage the growth of beneficial bacteria. This effect is attributed to the low-pH environment created during their fermentation in the colon and due to the secretion of antibiotic-like substances by the beneficial bacteria. Studies have shown that supplementing the diets of chicken, pigs, and rats with OF and other NDOs reduces fecal densities of *Salmonella* (Letllier et al., 2000). Supplementing the diet of mice with inulin and OF reduces the densities of *Candida* in the small intestine of mice 7 days after infection. Mice infected systemically with virulent strains of *Listeria monocytogenes* and *Salmonella typhimurium* after being fed a diet with inulin and OF (at 100 g/kg) had lower mortality than mice fed a diet with cellulose as the source of fiber (Buddington et al., 2002). Feeding mice with diets supplemented with inulin and OF increased activities of natural killer cells and phagocytes and enhanced T-lymphocyte functions compared to mice-fed diets with cellulose or lacking fiber. These results are consistent with the observations of heightened resistance to systemic infections with *Listeria* and *Salmonella*, and the lower incidence and growth of tumors after exposure to carcinogens and transplanted tumor cells, and are in agreement with enhanced innate and acquired immune functions provided by *Lactobacillus* and other LAB.

Supplementing diets with FOS increases production of SCFA, particularly butyrate, and can be predicted to strengthen mucosal defenses and enhance response to health challenges.

24.8.6 Anticancer Effect

Fructans have an indirect effect on prevention of cancer in human beings due to its prebiotic properties. This is due to immunity enhancements by the cells, cell wall components, and extracellular components of bifidobacteria (Tomomatsu, 1994). Studies with inulin and FOS have shown reduction of chemically induced aberrant crypts and prevention of colon cancer. Dietary treatment with inulin/OF (15%) incorporated in the basal diets for experimental animals resulted in the reduction of the incidence of mammary tumors induced in Sprague–Dawley rats by methylnitrosourea. The growth of transplantable malignant tumors in mice was inhibited and the incidence of lung metastases decreased in mice. It is reported that the dietary treatment with FOS/inulin significantly potentiated the effects of subtherapeutic doses of six different cytotoxic drugs commonly utilized in human cancer treatment (Taper and Roberfroid, 2002).

According to Pool-Zobel et al. (2002), in rats, a prebiotic effect resulting in the proliferation of bifidobacteria (with the major metabolites lactate or acetate) as well as of other bacteria could be responsible for the observed anticancer effects.

24.8.7 Health Benefits and Production of Nutrients

Fructans have been claimed to have no effect on blood glucose levels in case of type 2 diabetes, possibly due to effects of SCFA produced during fermentation (Alles et al., 1999; Mabel et al., 2008). It has been reported that the daily consumption of 20 g FOS decreased basal hepatic glucose production in healthy subjects without any effect on insulin-stimulated glucose metabolism (Luo et al., 2000). When the effect of chronic ingestion of FOS on plasma lipid and glucose concentrations, hepatic glucose production, and insulin resistance in type 2 diabetics was evaluated, it was found that FOS did not modify fasting plasma blood glucose and insulin concentrations or basal hepatic glucose production.

Roberfroid (1998) has reported that feeding rats with FOS (10%) for a few weeks decreased uremia in both normal and nephrectomized rats. Dietary FOS enhanced fecal nitrogen excretion and reduced renal excretion of nitrogen in rats. This occurs because these fermentable carbohydrates serve as energy source for the intestinal bacteria, which also require a source of nitrogen for protein synthesis during growth.

The presence of a good colonic environment with high bifidobacterial count leads to the production of vitamins B1, B2, B6, B12, nicotinic acid, and folic acid. Bifidobacteria ferments the prebiotics in turn resulting in improved lactose tolerance, calcium absorbability, and digestibility (Tomomatsu, 1994).

24.8.8 Low Calorific Value and Noncariogenicity

The β-configuration of the anomeric carbon, C_2, in their fructose monomers makes fructans nondigestible by human digestive enzymes, which are mostly specific for α-linkages and hence they are not utilized as an energy source in the body. However, due to colonic fermentation, they have an energy contribution to food of about 1.5 kcal/g. Inulin and OF are nondigestible by human intestinal enzymes, but they are totally fermented by colonic microflora. The SCFA and lactate produced by fermentation contribute 1.5 kcal/g of inulin or OF. This property makes them suitable for use in sweet, low-calorie diet foods and safe for consumption by individuals with diabetes. In the case of sweet foods, they may be used as bulking agents in conjunction with intense artificial sweeteners such as aspartame, phenylalanine, or sucralose, thereby masking the aftertaste produced by some of these intense sweeteners.

Unlike starch and simple sugars, fructans are not utilized by oral microflora like *Streptococcus mutans* to form acids and insoluble β-glucans, which serve as a matrix for plaque formation and are the main culprits in causing dental caries (Oku, 1994). Hence, they are presently used as noncariogenic sugar substitutes in confectionery, chewing gums, yoghurts, and drinks.

24.9 Metabolic Fate of Fructans

As mentioned earlier, fructans resist enzymatic digestion in the upper GI tract, reaching the colon virtually intact where they are fully metabolized by the colonic microflora. The end products of fermentation are gases (such as carbon dioxide and hydrogen), lactate, and SCFA (including acetate, propionate, and butyrate). Increased hydrogen concentrations can be observed by breath hydrogen testing (Rumessen et al., 1990; Alles et al., 1996). Colonic bacterial fermentation of inulin-type prebiotics, and the by-products produced, acidifies the colonic content, increases bacterial biomass (and consequently fecal mass), and modifies the composition of the microflora (Greg, 2008). The primary stimulating effect of prebiotics on gut ecology is the stimulation of bifidobacteria species growth. Although inulin-type prebiotics induce growth of bifidobacteria, they do not exert their effects in the same portion of the large bowel (Roberfroid, 2007) as DP influences where the colon fermentation occurs. The DP appears to exert considerable influence on the metabolic response to specific inulin-type prebiotic compounds since fermentation of FOS appears to occur primarily in the proximal colon (Alles et al., 1996; van de Wiele et al., 2007), whereas a higher proportion of the fructans in inulin appears to survive transit through the proximal colon. As a result, inulin might potentially have more positive effects on distal colonic fermentation and bacterial populations than shorter-chain fructan-type prebiotics like FOS or OF, which would be more metabolically active in the proximal colon. The transit time of fructans with greater DP is reportedly longer than that of short-chain fructans due to this relative resistance to enzymatic degradation in the proximal colon. The estimated average transit time for inulin (51% of fructans with DP > 12)

was 75 min compared to 30 min for OF (100% of fructans with DP < 10) in a single-blind, crossover, randomized trial with 10 healthy adults to test the difference in intestinal transit times between OF and inulin (Rumessen et al., 1990).

24.10 Dosage and Side Effects

Dietary trends indicate that high levels of fructan intake are common in the European (~12 g) and American (1–4 g) diet (Marchetti, 1993), due to increased consumption of wheat-based products, which are further enhanced by the recent discoveries of myriad health benefits of fructans. Since fructans are naturally found in artichokes (6.1 g/serving), leeks (5.9 g/serving), onion bulbs (1.01 g/serving), flour (4.0 g/serving), garlic (0.52 g/serving), watermelon (0.92 g/serving), nectarines (0.27 g/serving), and white peaches (0.50 g/serving), people typically ingest reasonable levels of fructans varying between 1 and 20 g.

Though historically, the dietary intake of fructans has been significantly higher than current-day consumption estimates, the inability of the human body to absorb beyond 5% of fructan may result in metabolic complications. Unlike glucose, fructans are not efficiently digested or absorbed by the small intestine and its malabsorption can cause GI symptoms such as heartburn, belching, abdominal pain, diarrhea, gas, and bloating, especially in cases where higher doses are ingested. The mechanism for malabsorption is related to the inability to hydrolyze the glycosidic linkages in the complex polysaccharide, resulting in the delivery of malabsorbed fructans to the large bowel. In the colon, the malabsorbed fructans are rapidly fermented, and by-products of this fermentation include H_2, CH_4, and other gases that may contribute to bowel symptoms. Furthermore, the small molecular nature of fructans results in an osmotic effect that draws more water into the small intestine and causes bloating and diarrhea. Ingestion of high daily doses of fructans (10–20 g) is especially implicated in the development of adverse effects in subjects with irritable bowel syndrome and restricting fructan intake in such cases is found to reduce the symptoms.

The maximum effective daily doses of fructans are determined to be 0.30 and 0.40 g/kg body weight for males and females, respectively. Human tolerance to fructans, as a class of compounds, is primarily dictated by chain length and dosage and abdominal symptoms increase with increasing dose and decreasing chain length. Excessive flatus follow the intake of >30 g/day and borborygmus (gaseous intestinal rumbling) and bloating occur at higher intakes (>40 g/day) with still higher doses of 50 g/day result in abdominal cramps and diarrhea. The most significant factor in determining the tolerance in humans is osmotic diarrhea, which is associated with ingestion of unavailable or unobservable oligosaccharides (Tokunaga et al., 1986; Nilsson and Bjorck, 1988). It increases as the molecular weight of the molecule decreases and therefore, human tolerance to long-chain inulin (DP > 5; average DP 23) is greater than native chicory inulin (DP of 2 to >60, modal DP 9 units), which is greater than the tolerance of FOS (DP 3–7, average 4.8), which, in turn, is greater than the tolerance of shorter-chain FOS (DP 3–5, average 3.7). The greater tolerance to inulin can also be attributed to the greater dietary exposure to the entire range of chain lengths comprising inulin since prehistoric days, than to shorter-length fructans (FOS), thus leading to the evolution of active inulinase enzymes in human and animal gut microflora.

Human tolerance to fructans has been demonstrated to be greater when they form a part of the regular diet, spread out over the course of the day, as opposed to a single dose. Absolonne et al. (1995) observed an increase in tolerance to FOS (DP 3–7) when the initial, single dose was split into two doses administered in the morning and afternoon, respectively, with maximum daily doses without adverse reactions reaching 27–31 g for men and 33–37 g for women. Shorter-chain FOS (DP 3–5), which caused adverse effects such as diarrhea when initially consumed in large amounts, were also more readily tolerated with continued consumption (Oku, 1986). The maximum dose that does not cause diarrhea was ~21–24 g/day (Takahashi et al., 1986). Kleessen et al. (1997) have shown that intakes up to 40 g/day of inulin produced no untoward effects in human subjects, especially when divided over the course of a single day. There has been no evidence of genotoxicity due to fructans as shown by *in vitro* and *in vivo* studies, the only effect noted being the occurrence of soft stools or diarrhea after ingestion of large quantities of fructans (Tomomatsu, 1994).

24.11 Market Trend

The functional food market is growing at an exponential rate, which reflects the trend toward healthier eating and lifestyles. The prebiotics market is diversifying and expanding in importance and value throughout the food and beverage industry, with several exciting developments in infant formula products, dairy and beverages, and the expansion of prebiotic ingredients into new application sectors such as snack products and meat products, according to new analysis from Frost and Sullivan (Frost and Sullivan Press Release, 2009). The prebiotics market, which earned revenues of €295.5 million in 2008, is expected to reach €766.9 million in 2015, with a compound annual growth rate of 14%. Fructans emerge as key players in the prebiotic market with a dramatic growth spurt, in part explained by the significant amount of scientific data emphasizing their functional properties and health benefits as well as the possibilities of their incorporation into diverse range of food products.

More than 400 prebiotic food products are on the market (mainly in Europe and Asia) and about 20 companies are involved in the production of oligosaccharides in Japan. Meiji Seika Kaisha Co. (Tokyo, Japan) first introduced FOS into the market as foodstuff during 1984 and they are the major producers of transfructosylation FOS. These are marketed in Japan as "Meioligo." Meiji has also established joint ventures with Beghin Say in France (Beghin–Meiji Industries, Paris) producing FOS that is marketed as "Actilight" and with Golden Technologies (Westminster Co.) in the United States, which distributes Meiji Seika's FOS as "Nutraflora." Cheil Foods and Chemicals (Seol, Korea) also manufactures FOS. FOS produced by degradation of inulin, or polyfructose, enzymatically or chemically, is mainly marketed commercially by Orafti Ltd., Tienen Belgium as "Raftilose," and by the Dutch company Cosun that markets the product as "Frutafit" or "Frutalose." The main components of this class are kestose (GF2), nystose (GF3), fructosylnystose (GF4), bifurcose (GF3), inulobiose (F2), inulotriose (F3), and inulotetraose (F4).

24.12 Analysis of Fructans

The large number of food products appearing in the market claiming to have a variety of health benefits attributed to fructans and dietary fibers necessitate precise quantification and labeling standards. Though the fructans, inulin, and OF were known to possess many of the physiological properties of dietary fiber (DF), they were not listed as DF on the labels of foods that contained them because for nutritional labeling purposes, the Food and Drug Administration (FDA) and the USDA have defined DF as the material that precipitates in 78% ethanol. Thus, the standard analytical method for DF analysis, AOAC International Method 985.29 (2000), is not suitable for measuring inulin or OF because these substances are partially precipitated in the alcohol treatment step. Subsequent to the FDA agreement in the latter part of 1995 to consider fructans as DF if an AOAC-accepted analytical method could be successfully developed, several procedures have been described for measuring fructan in plant material and food products. The need is that fructans be measured separately, accurately and precisely, and be added to total soluble DF after the fructans measured in DF residues are deducted. As a result, several specific methods have been developed to measure total fructans based on one or several enzymatic treatments of the sample and the determination of released sugars by different techniques, including high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD), reducing sugar estimation by the *p*-hydroxybenzoic acid hydrazide (PAHBAH) method, gas–liquid chromatography (GLC), and high-performance liquid chromatography with refractive index detection (HPLC-RI), respectively. Fructan content is then calculated as the difference between the sugar contents before and after hydrolysis.

Two fructan assay methods—Method 997.08 and Method 999.03—have been developed and approved by the Association of Official Analytical Chemists (AOAC) International.

24.12.1 AOAC Method 997.08

The first official method for the analysis of fructans is a modification of Hoebreg's method that employs enzymatic treatment of products with an inulinase (fructozym), followed by determination of the released

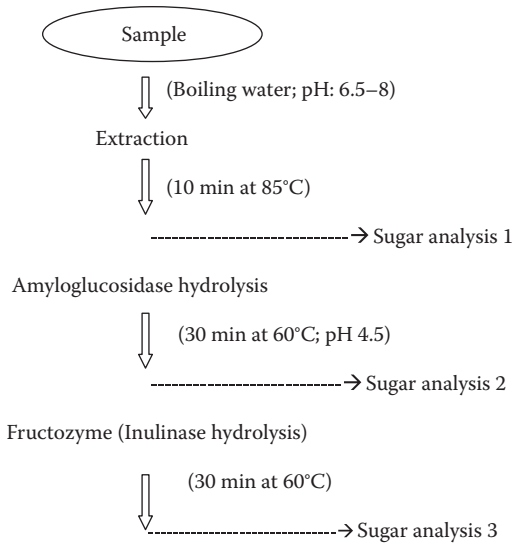

FIGURE 24.6 Schematic representation of AOAC Method 997.08. (From Hoebregs H. 1997. *J AOAC Int*; 80: 1029–1036. With permission.)

sugars using HPAEC-PAD (Hoebregs, 1997). The basic principle of the fructan analysis in depicted in Figure 24.6 (Quemener et al., 1994).

Fructans are first extracted from the product with boiling water and an aliquot of this extract is treated with amyloglucosidase or amyloglucosidase plus crude fructanase. The sugars, glucose, fructose, and sucrose, are assayed in the first and second hydrolyzates and in the initial sample by HPAEC-PAD. From the chromatographic patterns, the levels of sucrose and fructose in the untreated extract, the levels of fructose, glucose, and sucrose in the amyloglucosidase-treated extract, and the levels of fructose and glucose in the amyloglucosidase plus fructanase-treated extract are determined. The concentration of fructans is calculated by the difference in these determinations. Because OF is not recovered and a small fraction of inulin is recovered in the AOAC total dietary fiber (TDF) methods (Coussement, 1995), inulinase may be added to the enzyme complex in the TDF methods removing all fructans from the sample (Quemener et al., 1996). Alternatively, the fructan content in precipitates of the TDF methods can be calculated and this amount subtracted from the TDF amount (Hoebregs, 1997), thus ensuring that the inulin would not be counted twice.

The method is applicable to inulin and reducing and nonreducing fructooligosaccharides. The major limitation is the cost of the equipment required and the technical expertise needed. Furthermore, because fructan concentration is calculated by the difference from glucose and fructose determinations after the first hydrolysis with amyloglucosidase and the second hydrolysis with fructozyme, small inaccuracies in the determination of high glucose or sucrose values from samples containing high levels of starch, maltodextrins, or sucrose amounts can sometimes result in overestimation of glucose from fructan. There is also a possibility that the galactosyl–sucrose oligosaccharides present in the sample are also hydrolyzed to fructose and glucose (and galactose), which are measured as fructan, if the crude fructanase enzyme preparation used contains a very active α-galactosidase (and high levels of β-glucanase). This problem can be resolved by using preparations in which these enzymes have been removed or reduced to an acceptable level (Mccleary and Blakeney, 1999).

The addition of inulinase could lead to substantial losses of pectic material from the soluble fiber fraction obtained due to pectolytic activity present in the commercial preparation used. Quemener et al. (1997) proposed a modification for this method that included a heat pretreatment of commercial inulinase at 60°C for 2 h prior to its addition in the amyloglucosidase incubation step. The heat pretreatment of enzyme was found to inhibit its pectolytic activity while retaining sufficient activity to hydrolyze all the inulin from soluble fiber fraction.

A modification of AOAC Method 997.08, suggested by Andersen and Sørensen (1999), detailed a nonequipment-dependent analytical method for measuring the content of fructans as well as the contents of free glucose, free fructose, and sucrose in foods and food products. Fructans were hydrolyzed into D-glucose and D-fructose enzymatically and the released sugars quantitated using UV–visible recording spectrophotometer. Sucrose was hydrolyzed by α-glucosidase instead of β-fructosidase, and measured in the form of D-fructose instead of the typical D-glucose form. The fructanase used to hydrolyze the fructans had fewer contaminating enzyme activities. The enzymatic measurement of the released sugars was confirmed by measurements done by HPAEC-PAD, which showed reasonable agreement.

24.12.2 AOAC Method 999.03

The second AOAC method (Method 999.03) is based totally on the use of specific enzymes (see Figure 24.7).

The specific sucrase and the mixture of amylase, pullulanase, and maltase enzymes hydrolyze sucrose and starch to glucose and fructose. The reducing sugars are converted to sugar alcohols by sodium borohydride reduction. Further treatment with fructanase—pure exoinulinase and endoinulinase—liberates fructose and glucose from the fructans, which are estimated using the PAHBAH-reducing sugar method. This method is accurate, reproducible, and specific, and easy to perform, using standard laboratory equipment and highly purified and specific enzymes to hydrolyze sucrose, starch, and fructans. It is applicable to inulin and nonreducing fructooligosaccharides (e.g., native oligosaccharides and neosugars) in any matrix and may be adapted to measure fructan in plant materials and food mixtures (Mccleary et al., 2000). The specific sucrase and the highly purified *exo*- and endoinulinases used devoid of α-galactosidase and significantly reduced activity of pectinase and β-glucanase (cellulase) enable this enzyme mixture (fructanase) to be used specifically to hydrolyze the fructans in mixtures. This enzyme kit for fructan analysis is commercially available from Megazyme.

The drawback of this method is that, for highly degraded (acid or enzymic) fructan, there is an underestimation caused by sodium borohydride reduction step, which converts the reducing ends of the hydrolyzed fructooligosaccharides to sugar alcohols, resulting in their nonmeasurement in the reducing sugar assay (McCleary, 2003). Industrially, high-molecular-weight chicory fructan is subjected to controlled depolymerization by endoinulinase to produce FOS with more desirable ingredient properties (e.g., solubility). The terminal sugars at the reducing ends of hydrolyzed FOS are also reduced to sugar alcohols and they are not detected by the PAHBAH-reducing sugar test.

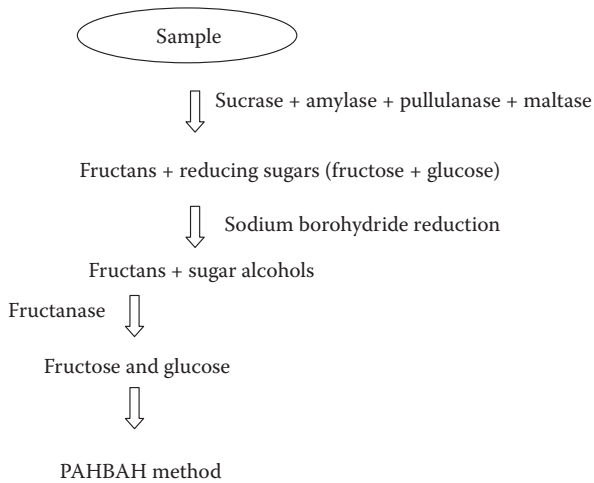

FIGURE 24.7 Schematic representation of AOAC Method 999.03. (From McCleary B.V., Murphy A., and Mugford D.C. 2000. *J AOAC Int*; 83: 356–364. With permissiom.)

An alternative approach to the measurement of fructan in the presence of sucrose is to delete the borohydride reduction step and to specifically measure glucose and fructose produced on complete enzymic hydrolysis of the fructan. McCleary and Rossiter (2004) reports a similar approach where fructans and FOS were measured with the three procedures: AOAC Method 999.03 (enzymic-PAHBAH); a second method based on the use of hexokinase, phosphoglucose isomerase, and glucose 6-phosphate dehydrogenase to measure glucose and fructose (enzymic-UV), based on the method proposed by Andersen and Sørensen (1999); and a third method in which reducing sugars are removed by borohydride reduction, followed by enzymic determination of glucose and fructose according to the second method. It was shown that for depolymerized fructan samples, the values are underestimated by the enzymic-PAHBAH method. Although the enzymic-UV method is preferred for such samples, it resulted in errors in case of samples containing high levels of glucose, fructose, and sucrose and low levels of fructan. The enzymic-PAHBAH method was preferred here with much-reduced errors but, for highly degraded fructans (e.g., Raftilose P-95), there was an ~15–20% underestimation of fructan content. For such materials, the third method wherein sucrose and reducing sugars were removed via borohydride reduction and fructose and glucose from fructan were determined enzymically (Andersen and Sørensen, 1999) proved optimal. It was suggested that with industrial materials of known degree of hydrolysis, a correction factor could be introduced into the calculations, provided that the degree of hydrolysis be known, and that this did not vary significantly between production batches. Thus, in summary, although all of the above assay formats measured fructan, each was found to be limited to some extent by the nature of the sample being analyzed (McCleary and Rossiter, 2004).

24.13 Fructans in Food and Pet Food

Stöber et al. (2004) devised a simplified method to determine total fructans in food and pet food that follows the principle of AOAC Method 997.08 wherein total fructose and total glucose released from fructans after enzymatic fructan hydrolysis was determined by HPAEC. Unlike AOAC Method 997.08, calculation of total fructans is based on the determination of fructose alone since it is difficult to accurately determine glucose since many food and pet food products contain other sources of total glucose (e.g., starch and sucrose). A correction factor *g* (1.05 by default) was suggested to take into account the theoretical contribution of glucose. The method was found suitable to quantify total fructans in various food and pet food products at concentrations ≥0.2% providing that the product does not contain other significant sources of total fructose such as free fructose or sucrose. However, at levels >5% of total fructans and in commercial fructan ingredients, both fructose and glucose can and should be accurately determined. Though limited to food products where the sucrose content does not exceed about three times the total fructan content, this procedure claims excellent compromise with regard to accuracy, applicability, and convenience.

Several analytical techniques like paper chromatography, thin layer chromatography GLC, nuclear magnetic resonance analysis, and mass spectrometry chromatography, have been discussed in the literature for fructan characterization and analysis; however, HPLC and HPAEC-PAD have been particularly applicable in rapid and accurate analysis of fructans. GLC and HPLC can be used to separate shorter fructans and HPAEC-PAD to analyze simple sugars and oligo- and polysaccharides as inulin in a variety of complex matrices, such as vegetable as well-functional foods. HPAEC-PAD is considered the most powerful tool for carbohydrate analysis as its resolution power allows the separation of each DP and their isomers.

24.14 Future Perspectives

In the current scenario, only fructan prebiotics are supported by sufficient trial results to be able to claim the wide range in functionality, whereas the manufacturers of other types of prebiotics require more documentation. The functional food ingredients market strengthened by strong legislation ensuring that only successfully registered ingredient products can claim positive effects on health and nutrition in the market will ensure the retention of only the scientifically proven products. Labeling of fructans in food

products also require uniform and clear guidelines since there is no uniform standard for naming different fructan-type prebiotics. It is difficult, if not impossible, to understand the composition, purity, and source of fructans from the generic name listed as an ingredient on food labels or the dietary supplement labels. It is currently not possible, based on name alone, to determine whether a fructan-type prebiotic product has been synthesized from sucrose or extracted from chicory root.

The area of fructan research being comparatively new, it demands concerted and intensive studies with healthy populations and populations with acute and chronic disease states to effectively understand human intestinal sensitivity and tolerance. Studies to determine differences in the physiological effects of short-, medium-, and long-chained fructans and their blends need to be conducted. Also, further research and consensus as to an effective daily prebiotic dose to exert health benefits is another aspect that needs to be addressed keeping in mind that the individual prebiotic ingredients will have different daily requirements to exert their effects.

24.15 Conclusion

The growing recognition of events taking place in the intestine and the role of microbes affecting human health has resulted in prebiotics being promoted as ideal candidates to support the growth of probiotic bacteria, to lower cholesterol and the glycemic response, to improve bone health, to lower daily energy intake, to relieve symptoms of inflammatory bowel disease, to lower colon cancer rates and in general, to maintain a healthy GI environment. The application of bifidobacteria to improve colonic health makes the bifidogenic property of fructans one of the current strongest marketing points. Prebiotics have many functions. They may be used to modify textural properties. Prebiotics with their multifunctionality finds diversified uses and novel physicochemical properties continue to be elucidated. Fructans, thus provide an edge over other functional ingredients to the food manufacturers. This coupled with the willingness of consumers to accept products shown to be beneficial for health and well-being ensure that, their production and use would continue to expand, which necessitates adequate analysis methods and cataloging so that the mass population benefit from their use.

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25

Pectin Extraction, Gelation, and Sources

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25.1 Pectin Background

Pectins are natural hydrocolloids found in higher plants, widely used as gelling agents, stabilizers, and emulsifiers in the food industry (May, 1990). This heteropolysaccharide consists of homogalacturonan ("smooth" regions), composed of an α-(1 → 4)D-GalA*p* chain, and rhamnogalacturonan ("hairy" regions) showing the repeating disaccharide GalA*p*-α-(1 → 2)-Rha*p*-α-(1 → 4)-GalA *p*α-(1 → 2)-Rha*p*. In addition, arabinose and galactose side chains can also be attached creating heteropolysaccharide complexes of rhamnogalacturonan with arabinans, galactans, and arabinogalactans (Willats et al., 2006). Pectin forms gels under certain circumstances but the gelling mechanism is highly dependent on the degree of methoxylation (DM). In this regard, the presence of methoxylated carbonyl groups gives raise to a classification of this polysaccharides into high methoxy (HM) pectin with DM > 50% and low methoxy (LM) pectin with DM < 50%. HM pectin forms gels in the presence of high sugar concentration, usually sucrose or fructose and low pH; whereas LM pectin does, in the presence of divalent ions (e.g., calcium) (Ström et al., 2007). Pectin is widely used in jams and jellies, fruit preparations, fruit drink concentrates, fruit juice, desserts, and fermented dairy products (May, 1990). Although most plant tissues contain pectin, commercial production around the world is based almost entirely on citrus and apple peel (Rascón-Chu et al., 2009).

25.2 Pectin Extraction Methods

Pectin is produced commercially from citrus peel and apple pomace. The extraction conditions vary from producers and are dependent on the pectin source and properties desired, and might produce a wide

diversity in chemical structure. Extraction most commonly occurs using a dilute mineral acid, usually hydrochloric, sulfuric, or nitric acids. Commercial pectin extraction as detailed by the International Pectin Producers Association is described as follows (IPPA, 2001). A factory receives previously washed and dried apple pomace or citrus peel from a number of sources. The material is added to hot water and a dilute mineral acid is added for extraction. Sufficient time elapses to allow extraction to occur and then the solids are separated from the pectin-containing liquid through filtration or centrifugation. The remaining solution is concentrated and mixed with an alcohol for pectin precipitation. The precipitated pectin is separated and washed with alcohol to remove the impurities. The pectin is dried, ground to a powder, and blended with other additives, if necessary. The desired gelling capability and methoxylation degree are strongly dependent on pH, temperature, and time.

In order to avoid coprecipitates of nucleic acids, proteins, and starches, wet ball milling may be used; dimethyl sulfoxide may remove starch, and surfactants such as sodium dodecyl sulfate will prevent enzymatic degradation of the pectin.

Pectin extraction by enzymatic means may improve yield; however, careful selection of the enzyme(s) is required. The use of galacturonase produces short but branched segments. Thibault et al. (1988) extracted pectin from citrus, apple, and sugar-beet pulps, with an enzyme preparation from *Bacillus subtilis*. The preparation contained endo-arabinase, endo-galactanase, and residual endo-pectate lyase. Extraction conditions were 30°C and 0.03 M sodium acetate buffer, pH 5.0, so that pectate lyase activity was minimized. Under these conditions, an appreciable amount of pectin (4.9% by the buffer and 10.8% by the enzymes) could be extracted from citrus pulp, but very little from apple and sugar-beet pulps. The citrus pectin had relatively low molecular weight (Mw) (47,000) compared to acid-extracted pectin (82,000). It was concluded that the polymer cannot efficiently be extracted with these enzymes. Accordingly, Donaghy and McKay (1994) used polygalacturonase from *Kluveromyces fragilis*. The concentrated enzyme was successfully used to release pectin from citrus peels and apple pomace but was unable to do the same for sugar-beet pulp. Instead of enzymatic process, the optimal extraction conditions for sugar-beet pulp were found through chemical hydrolysis. The hydrochloric acid was recommended to adjust pH at 1.5, and heated for 4 h at 80°C (Phatak et al., 1988). On the other hand, from watermelon, low methoxyl pectin was extracted using commercial enzyme preparations (CelluPract, Fibrilase, and Multifect XL) compared to acid extraction. CelluPract extraction resulted in significantly higher pectin yields than the other assays. Nevertheless, acid extraction resulted in the highest galacturonic acid content at an average value of 68.6% based on the colorimetric method, while CelluPract, Fibrilase, and Multifect XL yielded 47.0%, 56.2%, and 60.2%, respectively (Campbell, 2006). Evidently, enzyme-based extraction strongly depends on the raw material features.

Many scale extractions have been conducted to determine optimal pectin extraction conditions and its feasibility from a number of plant materials. In comparison, the enzymatic processes are more expensive and less efficient. For instance, the effects of temperature, time, and pH on pectin yield from orange peel using nitric acid for extraction was investigated by Aravantinos-Zafiris and Oreopoulou (1991). Optimal conditions of pH 1.6, 84°C, and 64 min resulted in yields up to nearly 26% from the dried peel weight. Galacturonic acid content, methoxyl content, and ash were reported to be independent of the extraction variables. This report on pectin yield is superior to those previously mentioned for enzymatic extraction. However, the enzyme-based process may be of importance if a special characteristic in the pectin is obtained.

Further research on enzyme technology is needed to fully understand and control enzymatic pectin extraction. There is still need for knowledge on the mechanisms involved. For instance, an endo-arabinase (ABN) (protopectinase-C) from mesophilic *Bacillus subtilis* IFO 3134 releases pectin, while a strain of *Bacillus thermodenitrificans* TS-3, with arabinan-degrading activity produces an ABN (EC 3.2.1.99) unable to release pectin (Takao et al., 2002). In sum, the extraction method, whether chemical or enzyme based, must be determined as a function of the raw material and the desired pectin type.

25.3 Pectin Analysis

Pectin functionality derives from its chemical structure. Understanding this relationship enables design of this polymer for a given application. Analyzing chemical structure helps understanding properties and

capacity of interaction of pectins with several macromolecules in diverse food and nonfood systems. The tests are generally related to structure and physical properties.

Quality and purity of this heteropolysaccharide are often determined as a function of different factors analyzed. The accepted measure of pectin purity is by determination of the anhydrogalacturonic acid (AGA) content. According to information available from the International Pectin Producers Association (IPPA, 2001), AGA percentage above 65% is considered to be the typical minimum level for pectins used for various applications. The purest citrus pectin contains 85–90% AGA (Braddock, 1999). Another factor considered for this polysaccharide is the DM. This measure will determine the usage of the pectin and whether it should be classified as high methoxyl (above 50%) or low methoxyl (below 50%). High methoxyl citrus pectin is generally at 70–80% DM (Braddock, 1999). It is also of interest to determine the ash content, Mw, and degree of amidation for a pectin sample. Mw is often determined as an indication of the gelling quality. The higher Mw is often related to firmness of the gel obtained, though methoxylation and derivatization are also strongly related. Amidation is sometimes desired in low methoxyl pectins because it can enhance gel formation. The gelling capability is standardized according to the IFT Pectin Standardization Method of 1959 (IFT Committee, 1959). This procedure utilizes a strain-induced alignment in a gel (SAG) method of standardization to measure the sugar-holding capacity of a test gel. A standard 65° Brix pectin gel is made, poured into a jelly glass, and left to dry for 20–24 h. The jelly glass is inverted and the amount of SAG is measured with a Ridgelimeter. The height of the gel deformed by its own weight is measured with a precision instrument called ridgelimeter after 2 min, and the results are reported as percent SAG. The jelly grade is determined from this measurement and the pectin is then standardized to 150 jelly grade by diluting with sugar. The term of 150 jelly grade means that 1 kg of this standardized polymer will turn 150 kg of sugar into a standard gel. Different approaches include rheological methods correlated to SAG measurements. Nielsen et al. (2001) studied the relationship between the height of gels determined by a SAG test and their elastic shear modulus (G'). Their experimental data showed a good agreement between the predictions and the results. In this manner, pectin gels can be modeled accurately as incompressible elastic materials. A standard 150° SAG pectin gel, which sags 23.5% in the SAG test, has G' moduli of 429 and 379 Pa under slip and no-slip conditions, respectively.

In general, pectins in foodstuffs are determined as part of soluble fiber. Nevertheless, the food industry, food processors, and pectin ingredient suppliers need to determine pectin content; since pectin as a food ingredient influences texture and other properties. The latter features depend on its chemical structure. In this manner, the Fourier transform infrared (FTIR) technology has potential as a quality control tool for the food industry because of easier, faster, and automatable methodology. In conjunction with other techniques, FTIR may simplify the handling of samples. Monsoor et al. (2001) reported a method called: diffuse reflectance Fourier transform infrared spectroscopy method for pectin determination. Basically, pectin calibration standards are prepared by blending polygalacturonic acid with potassium bromide to cover a range of polygalacturonic acid concentrations (10–98%). Pectin contents of various commercial pectin samples are calculated from the linear fit equation. They found results comparable to values obtained by colorimetric analysis and high-performance liquid chromatography (HPLC). This approach is quite interesting in a way that samples need no cumbersome treatment and/or preparation, for expedite analysis. In addition, Guillotin et al. (2007) reported an HPLC method using a weak anion-exchange column sensitive to the amount of substituents and their distribution within. This method is suitable for pectin companies concerned with the homogeneity of a given pectin preparation and its performance. Several examples have been published in the literature regarding traditional and new developed techniques. Some of the most common methods are mentioned below.

25.4 Pectin from Apple Fruit and Chickpea Husk

Pectin analysis is a powerful tool for innovation and derivatization of this polysaccharide, as well as raw material screening of novel pectins. Quest for new sources of this polymer is of interest for the food industry seeking for novel properties and function, as innovating food products are developed. Pectin extraction process can be of interest for developing countries looking to add value to agricultural products and by-products, by proposing new sources. Although most plant tissues contain pectin, commercial

production is based almost entirely on just a few sources that have the required properties (Thakur et al., 1997). Currently, citrus peel and apple pomace are major sources of pectic substances around the world (Willats et al., 2006). Nevertheless, novel sources of pectin are interesting for their properties and potential application in the food industry as well as in pharmaceutical and cosmetics applications.

25.4.1 Pectin from Low-Quality Apple Fruit

In Northern Mexico, apple is produced under climatically marginal conditions, especially in terms of winter chilling. This region provides more than half of the national apple production, the most important cultivar being "Golden Delicious." Considerable amounts of this production is considered as low-quality apple fruit, and destined to industrial process or not picked up in local orchards because of unprofitability in the low prices market. They remain in the orchard where they serve as animal feed, if ever. Rascón-Chu et al. (2009) reported the extraction of pectin from low-quality "Golden Delicious" apple fruit and its compositional and gelling capability, as an added value product.

25.4.1.1 Pectin Extraction and Composition

Pectin extraction by an acid hydrolysis is the most common method. Rascón et al. (2009) reported that the yield of pectin extraction from low-quality apple fruit was 16% on a dry matter basis (w pectin/w apple fruit), which is similar to that reported by Marcon et al. (2005) for pectins from apple pomace. The degree of esterification (DE) of the pectin extracted was estimated to be 57%, indicating that the polysaccharide is of the high-methoxyl type. By using a similar extraction method, Canteri-Schemin et al. (2005) reported a 69% degree of esterification in pectin from mature apple pomace. The intrinsic viscosity [η] and viscosimetric M_w of pectin gum were 307 mL/g and 112 kDa, respectively. Lower [η] and Mw were reported by Constela and Lozano (2003) in HM apple pectin recovered by a similar acid extraction. The pectin gum presented a galacturonic acid content of 65% (w/w). Residues of glucose, galactose, mannose, proteins, and ash were also detected. The levels of arabinose and galactose suggest the presence of arabinans and galactans as side chains (Marcon et al., 2005). This result indicates that this polysaccharide was mostly composed of galacturonic acid and a small quantity of neutral sugars, indicating that the molecule is pectin. The protein and ash content in this pectin are similar to that reported by Einhorn-Stoll et al. (2007) in HM pectin.

25.4.1.2 Gelation

Solubilization of this pectin in 60% (w/v) fructose and pH 2.7 produced pectin solutions at 2% and 3% (w/v) which previously heated formed soft gels after 12 h at 4°C. HM pectin, unlike LM pectin, does not contain sufficient acid groups to gel with calcium ions. It has been suggested by Oakenfull (1991) that hydrogen bonding and hydrophobic interactions are important forces in the aggregation of HM pectin molecules. Gel formation is caused by hydrogen bonding between free carboxyl groups on the pectin molecules and also between the hydroxyl groups of neighboring molecules. In a neutral or only slightly acid dispersion of pectin molecules, most of the unesterified carboxyl groups are present as partially ionized salts. Those that are ionized produce a negative charge on the molecule, which together with the hydroxyl groups causes them to attract layers of water. The repelling forces between these groups, due to their negative charge, can be sufficiently strong to prevent the formation of a pectin network. When acid is added, the carboxyl ions are converted into mostly unionized carboxylic acid groups. This decrease in the number of negative charges lowers the attraction between pectin and water molecules, and lowers the repulsion forces between pectin molecules also. Sugar further decreases hydration of the pectin by competing for water. These conditions decrease the ability of pectin to stay in a dispersed state. When cooled, the unstable dispersion of less hydrated pectin forms a gel, a continuous network of pectin holding the aqueous solution (Oakenfull, 1991). The rate at which gel formation takes place is also affected by the DE. Generally, a higher DE causes more rapid setting in most cases. Rapid set pectins (i.e., pectin with a DE of above 72%) also gel at lower soluble solids than slow set pectins (i.e., pectin with a DE of 58–65%).

Thus, HM pectin extracted from "Golden Delicious" apple can be considered as slow set pectin. The pectin gel hardness increases from 0.1 to 0.2 N by increasing the pectin concentration from 2% to 3% (w/v). The latter can be related to the polysaccharide chain aggregation phenomena, which is promoted as the polysaccharide concentration increases. The rheological stability of pectin gels at 2% and 3% (w/v) was determined after 48 h at 4°C. After storage, pectin gels hardness decreased from 0.1 to 0.08 and from 0.2 to 0.15 for the gels at 2% and 3% (w/v), respectively. This rheological evolution could be due to an increase in pectin hydration as a result of a moderate acid hydrolysis of the pectin during storage time. In fact, HM pectin gels belong to the category of physically crosslinked gels whose three-dimensional structure is stabilized mainly by multiple hydrophobic interactions and hydrogen bonds in the junction zones of the polymeric network (Schmelter et al., 2001).

Pectin with a high galacturonic acid content can be recovered from low-quality apple fruit. Under the extraction conditions used in this study, the pectin recovered presented a high intrinsic viscosity and Mw allowing the formation of a firm physical gel. Therefore, the pectin recovered could be used as food additive to texture or stabilize different food products. Further research on potential use as food additives is needed. The results demonstrate that actual low-quality apple fruit is a suitable raw material for industrial pectin extraction.

25.4.2 Pectin from Chickpea Husk

Mexico is one of the major producers of chickpea (*Cicer arietinum* L.), which is mainly exported as this grain does not represent an important constituent of the Mexican diet. In regions where chickpea is a major food legume (Southern Europe, North Africa, India, and Middle East countries), a large amount of by-products are generated. The majority of chickpea processing wastes include chickpea husk (Christodoulou et al., 2005; Maheri-Sis et al., 2008), which is used for animal nutrition. A previous research indicated that chickpea could be a source of pectin (Aisa et al., 2006). Nevertheless, the characterization and functional properties of this chickpea husk pectin have not been yet reported elsewhere. Recently, Urias-Orona et al. (2009) reported a pectin from chickpea husk.

25.4.2.1 Chickpea Husk Pectin Composition

According to Urias-Orona et al. (2009), yield of pectin extracted from chickpea husk is 8% (w/w) on a dry matter basis (w pectin/w chickpea husk), which is lower than those reported in major sources of pectic substances like apple fruit (16%) (Rascon-Chu et al., 2009). These authors reported that chickpea husk pectin contains 67% of galacturonic acid, 7.7% of arabinose, 12.3% of galactose, 1.6% of glucose, 0.4% of xylose, 0.6% of mannose, and 10.4% of rhamnose. This chickpea pectin presented an [η] of 374 mL/g, which is similar to that reported in LM pectin from yellow passion fruit (Yapo and Koffi, 2006). The viscosimetric Mw (M*v*) of chickpea husk pectin was reported to be 110 kDa. In HM pectin from low-quality apples, Rascon-Chu et al. (2009) found an M*v* value of 112 kDa. From the FTIR spectroscopy analysis, the degree of esterification of chickpea husk pectin was estimated to be 10%.

25.4.2.2 Gelation of Chickpea Husk Pectin

The formation of the pectin gel was rheologically investigated by small amplitude oscillatory shear (Figure 25.1), following the storage-modulus (G') and the loss modulus (G''). Gelation profile followed a characteristic kinetic with an initial increase of G' followed by a plateau region. The values of G' and G'' at the plateau region (60 min) were 57 and 10 Pa, respectively, which are higher than those reported for other LM pectin gels (Cardoso et al., 2003). It is possible that the attainment of a higher G' value in chickpea husk pectin gels in comparison with other LM pectins could be related to longer galacturonic acid blocks within the chain resulting in the formation of higher amounts of "egg-box" structures. The gel set time (t_g) was 3.5 min. This behavior is similar to that reported during gelation of LM pectin from olive pomace (Cardoso et al., 2003). In LM pectins, the number of sequences of nonmethoxylated galacturonic acid residues is long enough for the formation of the so-called "egg-boxes" resulting in the

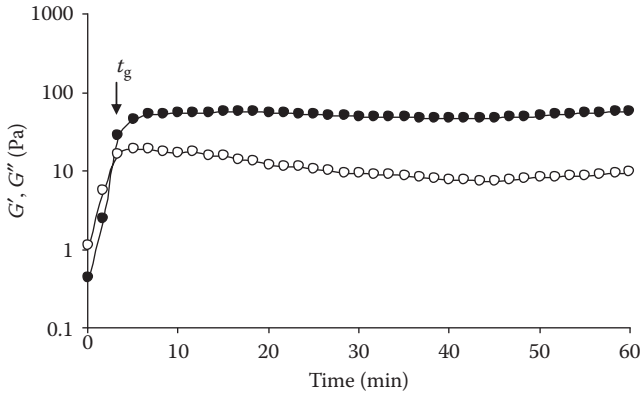

FIGURE 25.1 Gelation of chickpea husk pectin characterized by measuring the loss modulus (G'') describing the viscous character, and storage modulus (G') describing the elastic character of the system, at 2% (w/v), pH 9, and 10 mmol/L of calcium (G'•, G'' o). Measurements at 25°C, 0.25 Hz, and 2.5% strain. t_g = gel set time.

formation of the gel. Some other intermolecular interactions like hydrogen bonds could be formed, but they are much weaker as compared to the ionic cross-links formed by carboxyl groups (May, 1990). Figure 25.2 shows the mechanical spectrum of pectin gel after 60 min gelation. The mechanical spectrum was typical of solid-like material, with a linear G' independent of frequency and G'' much smaller than G' and dependent of frequency (Doublier and Cuvelier, 1996). This behavior is similar to that previously reported for a commercial LM pectin (DE 23%) (Willats et al., 2006). The tan δ (G''/G') (data not shown in Figure 25.2) value calculated at 0.25 Hz was 0.14 for pectin gels, indicating the presence of an elastic system (Ross-Murphy, 1984).

The rheological stability of pectin gels freshly made (2 h) and aged (48 h) was determined. The gels showed no significant difference on the texture profile analysis (Table 25.1), except for hardness. A decrease of 21% in the gel hardness was recorded after storage. This rheological change could be related to an increase in pectin hydration as a result of a moderate hydrolysis of the pectin during storage. Rascon-Chu et al. (2009) reported the texture profile analysis of HM apple pectin gels during storage. At the same pectin concentration, these authors found a lower hardness value (10 g) after gel set but similar

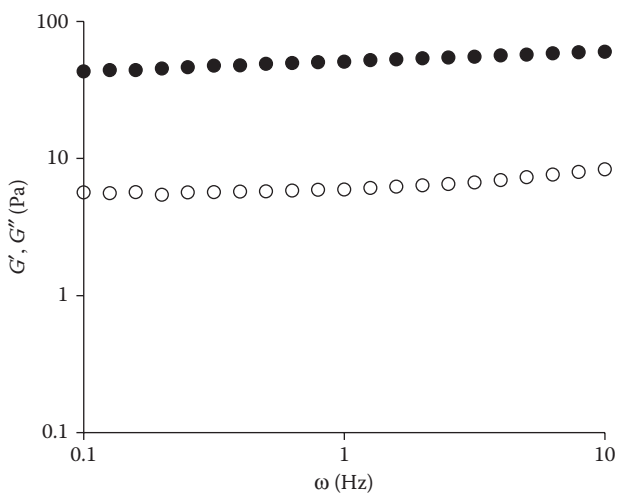

FIGURE 25.2 Mechanical spectrum (G'•, G'' o) of chickpea husk pectin at 2% (w/v), pH 9, and 10 mmol/L of calcium. Measurements at 25°C and 2.5% strain.

TABLE 25.1

Effect of Time of Storage on Texture Properties of Gels at 2% (w/v) in Pectin, pH = 9 and 10 mmol/L of Calcium[a]

	After Gel Set (2 h)	After Gel Storage (48 h)
Hardness (g)	99[a]	78[b]
Adhesiveness	−295[a]	−278[a]
Springiness	0.93[a]	0.95[a]
Cohesiveness	0.79[a]	0.84[a]
Gumminess	78[a]	76[a]
Resilience	0.04[a]	0.06[a]

Note: All results are obtained from triplicates.
[a,b] Means in a row with different letters are significantly different ($p \leq 0.05$).

hardness loss in the gel (20%) after 48 h storage at 25°C. Texture evaluation is often an important step in developing a new food product-optimizing processing variables.

25.4.2.3 Functional Properties of Chickpea Husk Pectin

Whippability, foam and emulsion stability, and reduced viscosity of pectin dispersions at different pH values are presented in Table 25.2. Whippability significantly increased from 104% to 124% as the pH increased from 5 to 9. Foam and emulsion stability significantly increased from 87% to 95% and from 75% to 96%, respectively, when the pH augmented from 5 to 9. Nevertheless, no significant difference was found in foam and emulsion stability at pH 7 and 9. This increase in whippability and foam and emulsion stability could be related to the fact that pectin solution reduced viscosity (η_{red}) values increased from 57 to 61 as the pH changed from 5 to 9. Polysaccharides contributed to the stability of foam and emulsion systems mainly by increasing the viscosity of the aqueous phase. They do not interact with the hydrophobic phase since they are not true surfactants. Coalescence of air bubbles and oil droplets in foam and emulsion systems, respectively, is hindered by a viscous aqueous phase (Temelli, 1997).

Pectin can be recovered from chickpea husk. This is an LM pectin capable of forming elastic gels by calcium addition. Chickpea husk pectin gels present a higher G' value in comparison to other LM pectins, which could be related to longer galacturonic acid blocks within the chain resulting in the formation of higher amounts of "egg-box" structures. After storage, pectin gels showed no significant difference on the texture profile analysis, except for a 20% hardness loss. Whippability, foam and emulsion stability, and viscosity increase as the pH changes from 5 to 9. The results suggest that chickpea husk could be a potential source of gelling pectin for food applications. Further research is undergoing in order to explore the structural properties of this hydrocolloid.

Chickpea husk as a source of LM pectin continue to be investigated and new information about its potential application in the food industry is being generated. Nevertheless, more research is needed to elucidate several questions, especially those concerning the structure of this polysaccharide. Additional studies will also be required in order to understand the effect of temperature, pH, pectin, and calcium concentration on the gel properties.

TABLE 25.2

Functional Properties of Chickpea Husk Pectin[a]

pH	Whippability (%)	Foam Stability (%)	Emulsion Stability (%)	Reduced Viscosity (mL/g)
5	104[c]	87[b]	75[b]	57[c]
7	112[b]	93[a]	96[a]	59[b]
9	124[a]	95[a]	96[a]	61[a]

Note: All results are obtained from triplicates.
[a,b,c] Means in a column with different letters are significantly different ($p \leq 0.05$).

25.4.3 Pectin from Cocoa Husks

Cocoa husks, a by-product of cocoa processing, were investigated as a source of pectins. Husks from different origins (Ghana and Venezuela) were used whole or minced, and pectins were extracted under various conditions. The highest yield is obtained with minced husks after 1 h of extraction at pH 2.5. In this case, samples are at the boundaries between LM and HM pectins (54.2% ± 5.2 for the titrimetric method; 46.7% ± 0.6 for the enzymatic method). In order to validate the values determined for HM and LM pectin, both methods were assayed on samples of known methoxy pectin. The enzymatic method was tested on HM commercial apple pomace pectins and gave results that agreed with the manufacturer's specifications on methoxy percentage values within experimental errors; for the titrimetric method, the errors were higher. For this reason, the enzymatic determination was chosen as the reference value. The other evaluated parameter was the acetylation degree. Also in this case, extraction conditions slightly influence results obtained for different pH and extraction times. Both samples have an acetyl content, similar to that found in sunflower pectins, lower than 4% (1.7% ± 0.4 for the extraction at pH 2.5 for 1 h; 3.2% ± 0.5 for the extraction at pH 7.0 for 3 h), which is considered a critical limit for gel formation (Mollea et al., 2008).

25.5 Modified Pectins

Structure modification of pectins are intended to enhance functional properties. Novel techniques are emerging for assesing and monitoring structural changes of a given pectin. Einhorn-Stoll et al. (2007) investigated thermal behavior of highly methoxylated citrus pectins modified chemically (demethoxylation and amidation) and mechanically (disaggregation) and examined with a combined simultaneous thermal analysis differential-scanning calorimetry (DSC), thermogravimetry (TG), and differential thermogravimetry (DTG) in a dynamic inert nitrogen atmosphere in the temperature range from 20°C to 450°C. The pectin degradation was observed in one single DSC or DTG peak, respectively, in the temperature range between 210°C and 270°C. The parameters of the degradation peak varied systematically in dependence on the degree of modification. All chemically modified pectins were more sensible to thermal degradation than their unmodified reference materials. The mechanically degraded pectins showed thermal degradation earlier and ended later with decreasing Mw. The maximum reaction enthalpy and velocity were reduced after modification. Not only experimental modifications but also the pectin origin had a considerable influence on the thermal degradation of the pectins. In combination with other methods, the thermal analysis is suitable for a relatively quick and reproducible characterization of structural changes and of state transitions, occurring during preparation and modification of pectins. Not only pectin modifications, made in laboratory scale for scientific investigations, can be followed by thermal analysis, but also in case of the composition, structure, and state transitions of pectin preparation, caused by the raw material or industrial processing.

Zouambia et al. (2009) compared the physicochemical and structural properties of hydrophobically functionalized pectin and local extracted beet and citrus pectin. FTIR spectra of citrus pectin, beet pulp pectin, and commercial pectin and their respective derivatives were carried out. In all cases, the initial pectin and their derivatives by subsequent addition of *N*-alkylamide substituents (*N*-alkylpectinamides) concordingly show spectral differences between them. The carboxyl vibration region of 1900–1500 cm^{-1} is the most important for the analysis of FTIR spectra. FTIR spectra of all *N*-alkylpectinamides showed the appearance of new band at 1560 ± 5 cm^{-1} assigned to the amide groups (CONHR). The presence of this band and the reduction in the intensity of the carboxylic group bands indicate that the substituent is bound to the pectin chain by covalent amide bond. The samples of *N*-alkylpectinamides showed more intensive C–H absorption in the region of 2970–2920 cm^{-1} than the original pectin, which can be explained by the increased C–H bond content after substitution. These structural changes in *N*-alkylpectinamides are correlated perfectly with functional features. The *N*-alkylpectinamides effectively act as surface-active hydrocolloids. In addition, pectin's hydrophobization produce and stabilize efficiently oil-in-water emulsions, at lower concentrations compared to emulsions prepared with unmodified pectins.

The hydrodynamic behavior of pectins can be achieved with different complementary techniques. Morris et al. (2002) concerned with changes during heating food processes complemented intrinsic viscosity, infinite dilution sedimentation coefficient, average Mw, and translational frictional ratio, with exclusion chromatography coupled to multiangle laser light scattering to measure average Mw and polydispersity to positively extend and confirm depolymerization of pectin at elevated temperatures. HM pectin suffers a significant depolymerization during increase in temperature as reflected by its hydrodynamic behavior. These changes are important as many food and pharmaceutical processes involve heat treatment stages.

25.6 Conclusions

Pectin analysis has been evolving as technology develops on edge molecular assessment instrumentation. Structure- and hydrodynamic-related features of pectins are becoming more complex as derivatives emerge with diversified functional properties becoming interesting in the food industry. In this regard, the analysis of pectin and derivatives has become of great importance to fully understand the implications of structural changes and functionality, as well as possible nutraceutical properties. Novel pectin from new sources, and their corresponding derivatives as amidated and acetylated, may well lead to better pectin processing and applications. A wide variety of pectins reduce fat content in many foods. Tailoring pectins to suit the food industry needs and the quest for new sources and structural features of pectin are still a developing area for research and new products in the years to come. The analysis of pectin by FTIR-based methods seems very promising. The analytical techniques developed shall follow to the enormous potential laying behind pectin and pectin derivatives development.

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26

Cyclodextrins

Katia Martina and Giancarlo Cravotto

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26.1 Introduction

Cyclodextrins (CDs) are unique molecular complexation agents. They possess a cage-like supramolecular structure, which involves intra- and intermolecular interactions where no covalent bonds are formed between interacting molecules, ions, or radicals. It is mainly a "host–guest" type phenomenon. CDs are definitively the most important supramolecular hosts found in the literature. As a result of molecular complexation, CDs are widely used in many industrial fields (cosmetics, pharmaceutics, bioremediation, etc.) and in analytical chemistry. Their high biocompatibility and negligible cytotoxicity have opened the doors to their uses such as drug excipients and agents for drug-controlled release (Stella and Rajewski 1997, Matsuda and Arima 1999), in food and flavors (Mabuchi and Ngoa 2001), cosmetics (Buschmann and Schollmeyer 2002), textiles (Buschmann et al. 2001), environment protection (Baudin et al. 2000), and fermentation and catalysis (Koukiekolo et al. 2001, Kumar et al. 2001).

CDs are cyclic oligosaccharides consisting of at least six glucopyranose units which are joined together by a (1 → 4) linkage. CDs are known as cycloamyloses, cyclomaltoses, and historically as Schardinger dextrins. They are produced as a result of an intramolecular transglycosylation reaction from the degradation of starch which is performed by the CD glucanotransferase enzyme (CGTase) (Szetjli 1998).

The first reference to the molecule, which later proved to be CD, was published by Villiers in 1891. Digesting starch with *Bacillus amylobacter*, he isolated two crystalline products, probably α- and β-CDs. In 1903, Schardinger reported the isolation of two crystalline products that he called α- and β-dextrin, in which the helix of amylose was conserved in fixed-ring structures.

From the x-ray structures, it appears that the secondary hydroxyl groups (C2 and C3) are located on the wider edge of the ring and the primary hydroxyl groups (C6) on the other edge. The apolar –CH (C3 and C5) and ether-like oxygens are on the inside of the truncated cone-shaped molecules (Figure 26.1). This results in a hydrophilic structure with an apolar cavity, which provides a hydrophobic matrix, often described as a "microheterogeneous environment." As a result of this cavity, CDs are able to form inclusion complexes with a wide variety of hydrophobic guest molecules. One or two guest molecules can be entrapped by one, two, or three CDs.

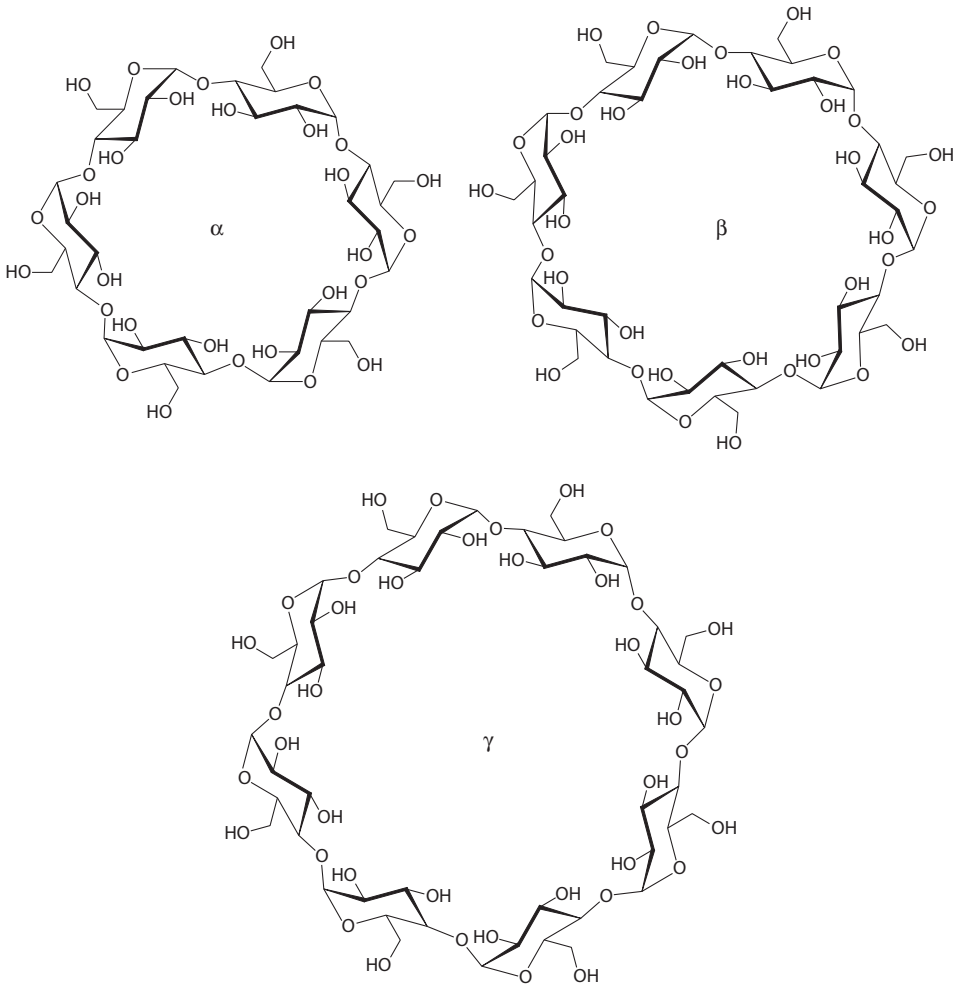

FIGURE 26.1 Chemical structure of α, β, and γ-CD.

Although CDs with up to 12 glucose units are known, only the first three homologues (α-, β-, and γ-CD) have been extensively studied and used. β-CD is the most accessible due to its low price and high versatility. The main properties of the aforementioned CDs are given in Table 26.1.

The safety profiles of the three most common natural CDs and some of their derivatives have recently been reviewed (Irie and Uekama 1997, Thompson 1997). All toxicity studies have demonstrated that orally administered CDs are practically nontoxic due to the fact that they are not absorbed by the gastrointestinal tract.

Pioneer country in the industrial applications of CDs was Japan, since 1990 it become the largest consumer in the world. Eighty percent of the annual consumption was used in the food industry and over 10% in cosmetics, <5% was used in the pharmaceutical and the agrochemical industries. The industrial usage of CDs progresses somewhat slower in Europe and America. The constant annual growth of the number of scientific papers and patents indicates the scale of research and industrial interest in this field. From a regulatory standpoint, a monograph for β-CD is available in both the *US Pharmacopoeia/National Formulary* (USP 23/NF 18, 1995) and the *European Pharmacopoeia* (3rd ed., 1997). All native CDs are listed in the generally regarded and/or recognized as safe (GRAS) list of the US-FDA for use as a food additive. β-CD was recently approved in Europe as a food additive (up to 1 g/kg food). In Japan, the native CDs were declared to be enzymatically modified starch and, therefore, their use in food products has been permitted since 1978.

TABLE 26.1

Physical Properties of α-, β-, and γ-CDs

Property	α-CD	β-CD	γ-CD
Number of glucose units	6	7	8
Mol wt. (anhydrous)	972	1135	1297
Volume of cavity ($Å^3$ in 1 mol CD)	174	262	427
Solubility in water (g 100 mL^{-1} r.t.)	14.5	1.85	23.2
Outer diameter (Å)	14.6	15.4	17.5
Cavity diameter (Å)	4.7–5.3	6.0–6.5	7.5–8.3

Apart from these naturally occurring CDs, many derivatives have been synthesized so as to improve solubility, stability to light or oxygen and control over the chemical activity of guest molecules (Eastburnand and Tao 1994, Szente and Szejtli 1999). Through partial functionalization, the applications of CDs are expanded. CDs are modified through substituting various functional compounds on the primary and/or secondary face of the molecule.

26.2 Inclusion Complex Formation

The most notable feature of CDs is their ability to form solid inclusion complexes (host–guest complexes) with a very wide range of solid, liquid, and gaseous compounds by molecular complexation (Szejtli 1982).

Since the exterior of the CDs is hydrophilic, they can include guest molecules in water solution. As depicted in Figure 26.2, the guest can be either completely or partially surrounded by the host molecule. The driving force in complex formation is the substitution of the high enthalpy water molecules by an appropriate guest (Muñoz-Botella et al. 1995). One, two, or more CDs can entrap one or more guest molecules. More frequently the host–guest ratio is 1:1; however, 2:1, 1:2, 2:2 or even more complicated associations and higher-order equilibria have been described. The packing of the CD adducts is related to the dimensions of the guest and cavity. Several factors play a role in inclusion complex formation and several interactions have been found:

a. Hydrophobic effects, which cause the apolar group of a molecule to fit into the cavity.
b. Van der Waals interactions between permanent and induced dipoles.
c. Hydrogen bonds between guest molecules and secondary hydroxyl groups at the rim of the cavity.
d. Solvent effects.

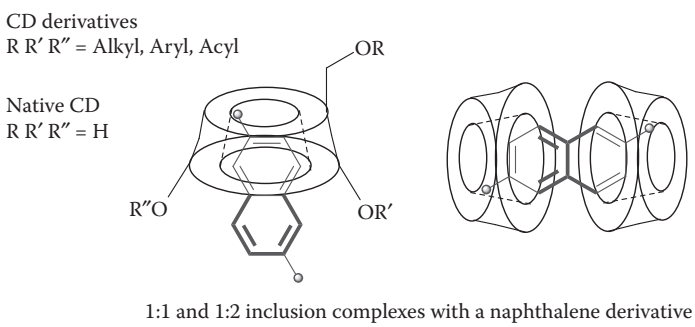

FIGURE 26.2 1:1 and 1:2 host–guest CD complexes.

Regardless of what kind of stabilizing forces are involved, the geometric characteristics and the polarity of guest molecules, the medium and temperature are the most important factors for determining the stability of the inclusion complex. Geometric rather than the chemical factors are decisive in determining the kind of guest molecules which can penetrate the cavity. If the guest is too small, it will easily pass in and out of the cavity with little or no bonding at all. Complex formation with guest molecules significantly larger than the cavity may also be possible, but the complex is formed in such a way that only certain groups or side chains penetrate the CD cavity.

Complexes can be formed either in solution or in the crystalline state and water is typically the solvent of choice. Inclusion complexation can be accomplished in cosolvent systems, also in the presence of any nonaqueous solvent. Inclusion in CDs exerts a strong effect on the physicochemical properties of guest molecules as they are temporarily locked or caged within the host cavity giving rise to beneficial modifications which are not achievable otherwise (Dodziuk 2006).

Molecular encapsulation can be responsible for the solubility enhancement of highly insoluble guests, the stabilization of labile guests against degradation and greater control over volatility and sublimation. It can also modify taste through the masking of flavors, unpleasant odors, and the controlled release of drugs and flavors. Therefore, CDs are widely used in food industry (Shaw 1990), in food packaging (Fenyvesi et al. 2007), in pharmaceuticals (Loftsson and Duchene 2007, Laze-Knoerr et al. 2010), and above all in cosmetics and toiletries (Szejtli 2006).

26.3 Applications of CD in Food

Today the nontoxicity of β-CD is well proven, the same tenet is generally accepted for the other CDs. The regulatory statuses of CDs differ in Europe, the United States, and Japan, because official processes for food approval are different. In the United States α-, β-, and γ-CD have obtained the GRAS status and can be commercialized as such. In Europe, the approval process for α-CD as Novel Food has just started and is expected to legalize the widespread application of α-CD to dietary products, including soluble fiber. In Japan, α-, β-, and γ-CDs are recognized as natural products and their commercialization in the food sector is restricted only by purity considerations. In Australia and New Zealand, α- and γ-CD have been classified as Novel Foods since 2004 and 2003, respectively.

Nowadays the application of CD-assisted molecular encapsulation in foods offers many advantages (Cravotto et al. 2006):

- Improvement in the solubility of substances.
- Protection of the active ingredients against oxidation, light-induced reactions, heat-promoted decomposition, loss by volatility, and sublimation.
- Elimination (or reduction) of undesired tastes/odors, microbiological contamination, hygroscopicity, and so on.

Typical technological advantages include, for example, stability, standardized compositions, simple dosing and handling of dry powders, reduced packing and storage costs, more economical, and manpower savings. CDs are mainly used, in food processing, as carriers for the molecular encapsulation of flavors and other sensitive ingredients. As CDs are not altered by moderate heat, they protect flavors throughout many rigorous food-processing methods such as freezing, thawing, and microwaving. β-CD preserves flavor quality and quantity to a greater extent and for a longer time compared to other encapsulants (Hirayama and Uekama 1987).

CDs can improve the chemical stability of foods by complete or partial inclusion of oxygen-sensitive components. They can be used to stabilize flavors against heat that can induce degradation and they can also be employed to prolong shelf-life by acting as stabilizers.

CDs are used for the removal or masking of undesirable components; for example, trimethylamine can be deodorized by the inclusion of a mixture of α-, β-, and γ-CDs. CDs are also used to free soybean products from their fatty smell and astringent taste. Even the debittering of citrus juices with β-CD is a long pursued goal.

CDs have an important use in the removal of cholesterol from animal products such as milk, butter, and egg yolks and have recently been studied as neutraceutics carriers to disperse and protect natural lipophylic molecules such as polyunsaturated fatty acids, Coenzyme Q10 (ubiquinone) and Vitamin K3.

26.4 Analysis of CD

26.4.1 Characterization of CD-Inclusion Complex

When molecules are inserted within the hydrophobic interior of the CDs, several weak forces between the host and guest are involved, that is, dipole–dipole interaction, electrostatic interactions, van der Waals forces, and hydrophobic and hydrogen bonding interactions. An equilibrium exists between the free and complexed guest molecules. The equilibrium constant depends on the nature of the CD and guest molecule, as well as temperature, moisture level, and so on. The inclusion complexes formed in this way can be isolated as stable crystalline substances, and precise information on their topology can be obtained from the structural x-ray analysis of single crystals (Song et al. 2009). The topology of the inclusion complex can also be determined in solution. The interactions between host and guest may lead to characteristic shifts in the ^{1}H and ^{13}C NMR spectra (Dodziuk et al. 2004, Chierotti and Gobetto 2008). Nuclear Overhauser effects (NOE) provide more precise information since their magnitudes are a measure of the distance between host and guest protons. Circular dichroism spectra give information on the topology of the adduct, when achiral guests are inserted into the chiral cavity (Silva et al. 2007). Potentiometry, calorimetry, and spectroscopic methods including fluorescence, infrared, Raman, and mass spectrometry have also been used to study inclusion complexes (Daniel et al. 2002).

The molecular encapsulation of natural essential oils, spices, and flavors such as cheese, cocoa, meat, and coffee aromas with β-CD has been known since several years. The literature has dealt with the improved physical and chemical stability of these air-, light-, and heat-sensitive flavors (Szente et al. 1988; Qi and Hedges 1995) and investigated the interaction of these compounds with CDs.

UV absorbance spectroscopy was applied to investigate hyperchromic effects induced by the addition of β-CD to a water solution of caffeine (Mejri et al. 2009). The spectroscopic and photochemical behavior of β-CD inclusion complexes with L-tyrosine were investigated by Shanmugam et al. (2008). UV–vis, fluorimetry, FT-IR, scanning electron microscope techniques, and thermodynamic parameters have been used to examine β-CD/L-tyrosine complexation.

Nishijo and Tsuchitani (2001) studied the formation of an inclusion complex between α-CD and L-tryptophan using nuclear magnetic resonance (NMR). Linde et al. (2010) investigated the complexation of amino acids by β-CD using different NMR experiments such as diffusion-ordered spectroscopy (DOSY) and rotating frame Overhauser effect spectroscopy (ROESY). This study provided molecular level information on complex structure and association-binding constants and advanced the sensorial knowledge and the development of new technologies for masking the bitter taste of peptides in functional food products.

The preparation of stable, host–guest complexes of β-CD with thymol, carvacrol, and oil of origanum has been described by LeBlanc et al. (2008). The complex was characterized by NMR and the inclusion constant was measured by fluorescence spectroscopy where 6-*p*-toluidinylnaphthalene-2-sulfonate was in competitive binding and acted as a fluorescent probe.

Caccia et al. (1998) provide the evidence of the inclusion complex between neohesperidin dihydrochalcone/β-CD by x-ray, high resolution NMR and MS spectroscopy. The association constant was determined by NMR via an iterative nonlinear fitting of the chemical shift variation of H3 in β-CD. The geometry of the binding was studied by nuclear NOEs between the proton directly involved in the host/guest interaction as well as by ROESY. The use of fast atom bombardment (FAB) gave complementary information on specific host–guest interaction, while x-ray diffractometry patterns could define the complex in solid state.

Differential scanning calorimetry (DSC), thermogravimetry analysis (TGA), or nuclear magnetic resonance (^{1}H-NMR) were employed by Marcolino et al. (2011) to study the stability of the β-CD complexes with bixin and curcumin. Owing to the huge industrial applications of natural colorants, this study aimed to compare different methods of complexes formation and evaluate their stability.

Natural and synthetic coffee flavors were included in β-CD and the complexes were analyzed by x-ray diffraction by Szente and Szejtli (1986). By thermofractometry and the loss of a volatile constituent, it was demonstrated that the volatility of these complexed flavors diminished in such a way that they could be stored for longer periods. Various spectroscopic methods have been compared, by Goubet et al. (1998, 2000), to study the competition for specific binding to β-CD. The substrates were a group of flavors which show different physicochemical properties, such as vapor pressure, water solubility, and log *P*.

Inverse gas chromatography was recently used for the direct assessment of the retention of several aroma compounds of varying chemical functionalities by high amylose corn starch, wheat starch, and β-CD (Delarue and Giampaoli 2000). The inclusion selectivity of several monoterpene alcohols with β-CD in water/alcohol mixtures was studied by Chatjigakis et al. (1999) using reverse-phase HPLC. Flavor retention in α-, β-, and γ-CDs was compared, by Reineccius et al. (2002), by the GC analysis of the released flavor compounds; quantification was accomplished using standard internal protocols.

GC-MS was used for the identification of the volatile constituents of cinnamon leaf and garlic oils before and after the microencapsulation process with β-CD (Ayala-Zavala et al. 2008). The profile of volatile substances in the β-CD microcapsules was used to evaluate the competitive equilibrium between β-CD and all volatile substances. The eugenol and allyl disulfide content of cinnamon leaf and garlic oils were used as a pattern to evaluate the efficiency in the microencapsulation process. The IR spectra of the microcapsules was employed to demonstrate the formation of intramolecular hydrogen bonds between the guest and host molecules.

Samperio et al. (2010) investigated the solubility in water and in apple juice of 23 different essential oils and 4 parabens. The study was focused on the β-CD complexes of few essential oil components (*o*-methoxycinnamaldehyde, *trans*, *trans*-2,4-decadienal, and citronellol), evaluating the increase of solubility in water and the storage stability. UV absorption spectrophotometry was performed to quantify the compound in solution. Linear regression analysis was used to calculate the concentration of test compounds in solution from day 0 to day 7.

26.4.2 Determination of CD Content

Traditionally, a variety of techniques have been developed to analyze CDs and their derivatives.

Few analytical methods for the quantification of β-CD are described in the literature. Among them are colorimetric methods, LC methods based on the use of indirect photometric detection, pulse amperometry, or refractive index experiments, affinity capillary electrophoresis, and mass spectrometry are able to provide qualitative and quantitative data when analyzing the complex CD mixtures.

26.4.2.1 The Colorimetric Method

The colorimetric method may be used as an alternative to chromatography especially at low CD concentrations, this also works in the presence of linear oligosaccharides. The colorimetric method, based on the complexation of phenolphthalein, was employed by Higuti et al. (2004) to carry out sensitive and relatively specific quantification of β-CD. A decrease in absorbance at 550 nm, due to phenolphthalein–CD complex formation, was exploited to study the optimization of the CGTase production in *Bacillus firmus*. A highly reproducible and selective α-CD determination method had already been described by Lejeune et al. (1989). This involves the formation of an inclusion complex between the α-CD and methyl orange under conditions of low pH and low temperature. The metal indicator calmagite (1-(1-hydrohy-4-methyl-phenylazo)-2-naphthol-4-sulfonic acid) interacts selectively with γ-CD and was described by Hokse (1983) to quantify a standard solution of γ-CD.

Kobayashi et al. (2008) observed that various kinds of hydrophobic food polyphenols and fatty acids could be dispersed in water containing starch by the action of GTAse (CD-producing enzyme). NMR and spectrophotometric methods were used to confirm the presence of CDs as solubilizing agents. The formation of inclusion complexes was demonstrated by using Congo Red as a model molecule in the presence of GTAse or α-, β-, and γ-CD, respectively. Major changes in the ^{1}H NMR profile of Congo Red were observed in the presence of γ- and β-CD.

On the other hand, a spectrophotometric and infrared spectroscopic study of the interaction between Orange G, a valuable clastogenic and genotoxic acid dye used as a food colorant, and β-CD has been described by Wang et al. (2007) as a method for the quantitative determination of this dye. Based on the enhancement of the absorbance of Orange G when complexed by β-CD, the authors proposed a ratiometric method, carried out spectrophotometrically, for the quantitative determination of Orange G in bulk aqueous solution. The absorbance ratio of the complex at 479 and 329 nm in a buffer solution at pH 7.0 showed a linear relationship in the range of 1.0×10^{-5} to 4.0×10^{-5} mol L^{-1}. IR spectroscopy of the complex was described to confirm the inclusion complex formation.

26.4.2.2 Chromatography

26.4.2.2.1 Thin-Layer Chromatography

One reference in the literature refers to the use of thin-layer chromatography (TLC) technique as an inexpensive, simple, and very informative method for the analysis and separation of CD inclusion complex food components. Prosek et al. (2004) isolated the inclusion complex between coenzyme Q10 (CoQ10) and β-CD and described its analysis and separation by one-dimensional, two-dimensional, and multidimensional TLC. The article described different TLC supports, mobile phases, and visualization methods in detail and the authors evaluated that 70% of the complex remained unchanged during the first semipreparative chromatography run and only a small amount of CoQ10 was lost from the complex during the TLC procedure. The results were confirmed by the use of other separation techniques such as HPLC, HPLC-MS, and NMR.

26.4.2.2.2 Liquid Chromatography, LC-MS, HPLC-MS

Liquid chromatography (LC) methods are employed for the analysis and separation of CDs and their derivatives. The separation of the complex samples containing CDs in mixture with linear oligosaccharide residual starch as well as protein salts and other substances may suffer from poor sensitivity, resolution, and long separation times. Good results can be achieved where differences in mass or polarity are found or, otherwise, will require extensive sample preparation.

Several stationary phases have been described, for example, resins modified with specific adsorbents and reverse-phase media used in combination with either refractive index detection (Berthod et al. 1998), evaporative light scattering (Caron et al. 1997, Agüeros et al. 2005), indirect photometric detection (Takeuchi et al. 1990), postcolumn complexation with phenolphthalein (Frijlink et al. 1987, Bassappa et al. 1998), polarimetric detection (Goodall 1993), or pulsed amperometric detection (Kubota et al. 1992).

López et al. (2009) described the application of LC and refractive index detection to estimate the amount of residual β-CD (>20 mg per 100 g of product) present in milk, cream, and butter after treatment with β-CD. The analyses were performed with a C18 reversed-phase silica-based LC column, α-CD was defined as an internal standard. The repeatability of the analytical method for β-CD was tested on commercial milk, cream, and butter spiked with known amounts of β-CD.

The detection limit in milk was determined to be >0.03 mg mL^{-1} of β-CD which is similar to that found by LC using amperometric detection (Kubota et al. 1992) and its reproducibility was comparable to that found in a colorimetric method for the estimation of β-CD using phenolphthalein (Basappa et al. 1998, Frijlink et al. 1987).

LC-MS coupling has led to the development of new interfaces, extending the automation of various procedures and increasing the sensitivity for high-polar and high-molecular mass compounds. New ionization techniques such as electron spray (ESI) and matrix-assisted laser desorption ionization (MALDI) (Bartsch et al. 1996, Sporn and Wang 1998) on quadrupole, magnetic sector, or time-of-flight (TOF) instruments or coupled with instruments with tandem MS (MS-MS) capabilities have also been fundamental in food applications. By coupling HPLC to isotope-ratio, MS has been proven valuable in providing precise isotopic measurements for nonvolatile species such as carbohydrates. For these reasons, the number of reported applications of LC-MS in the analysis of CD in food is rapidly increasing.

HPLC/MS analyses for the detection of minute amounts of CDs in enzyme and heat-treated, starch-containing food products were proposed by Szente et al. (2006). A suitable sensitive and selective

analytical method was studied with the aim of verifying the presence of parent β- and γ-CDs and all the three, α-, β-, and γ-branched CDs with different degrees of glycosylation in appropriately preconcentrated and purified food samples (beer samples, corn syrups, and bread). Both the HPLC-retention times and mass-spectral data were used for the identification of CDs. As the expected concentrations of CDs were very low, selected ion monitoring (SIM) was preferred to the routinely used refractive index and evaporative light scattering detection techniques as the only reliable detection method. The malto-oligomer mixture was analyzed with a detection window opened at the masses of CD sodium salts in order to enable the detection of any malto-oligomer side products.

Wang et al. (1999) proposed the efficient qualitative and quantitative analysis of food oligosaccharides by MALDI-TOF-MS. In order to optimize the method, matrices, alkali–metal adducts, response intensity, and sample preparation were all examined individually. A series of experiments were carried out by the authors to study analyte incorporation in the matrix. In a first phase of experiments, maltohexanose and γ-CD were used as reference samples to verify the suitability of 2,5-dihydroxybenzoic acid (DHB), 3-aminoquinoline (3-AQ), 4-hydroxy-a-cyanocinnamic acid (HCCA), and 2,5-dihydroxybenzoic acid (DHB), 1-hydroxy-isoquinoline (HIC), (1:1) as the matrix material. Spot-to-spot or sample-to-sample repeatability tests and the ability to achieve a good quality spectrum with a reasonable signal-to-noise ratio and the best resolution were compared. Good quality spectra and acceptable repeatability were achieved with DHB but many interfering matrix peaks were observed in the low mass region. The best results were achieved using a 2,4,6-trihydroxy-acetophenone monohydrate (THAP) matrix. The authors exploited the high solubility of THAP in acetone, its fast evaporation to fine crystals, and the homogeneous incorporation of the sample to avoid low-quality results which may be due to irregular crystallization when the substance is used directly in water.

26.4.2.3 Affinity Capillary Electrophoresis

Affinity capillary electrophoresis (ACE) techniques have been introduced more recently and are currently in rapid development. CDs have played a central role in the development of a wide variety of analytical methods based on ACE in the separation of chiral molecules. ACE also provides a powerful analytical tool for the analysis of CDs and their derivatives.

The electrophoretic separation and analysis of α-, β-, and γ-CDs have been carried out recently without modification. CDs that are charged at very high pH can be separated by the formation of inclusion complexes. Their complexes, with a large range of aromatic ions, facilitate detection by indirect UV absorbance (Larsen and Zimmermann 1998, 1999). In addition, fluorescent molecules such as 2-anilinonaphthalene-6-sulfonic have been used for the separation and detection of CDs in a ACE system (Penn et al. 1994).

Furthermore, the indirect electrophoretic determination of CD content has recently been described using periodate oxidation. The amount of produced iodate was monitored by ACE and reproducible quantitative results were obtained for α-, β-, and γ-CDs (Pumera et al. 2000). Nevertheless, ACE has not been yet exploited for the analysis of CDs in food. The major advantages of ACE compared to other analysis methods are their short analysis times and high versatility. An exhaustive review of this topic was published in 1999 (Larsen and Zimmermann 1998, 1999).

26.5 Conclusion

The use of native CDs for human consumption is growing dramatically due to their well-established safety. CDs are effective in protecting lipophilic food components from degradation during cooking and storage. In this context, several methodologies have been developed to detect, identify, and quantify CDs in food extracts and to study molecular inclusion complexes. X-ray and NMR spectroscopy afford valuable and detailed insight into the structure and the dynamics of a wide range of complexes which are not amenable to study by other analytical techniques. HPLC coupled with refractive index and evaporative light scattering detection technique is routinely used in CD food analysis and LC-MS data in this respect are particularly useful in detecting minute amounts of CDs in complex food samples.

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Section VI

Probiotics

27

Selection and Evaluation of Probiotics

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27.1 Introduction

A concise definition of probiotics describes them as "live microorganisms that, when administered in adequate amounts, confer a health benefit to the host."[1] They have been used worldwide for the last 10–15 years to prepare fermented dairy products, but they can also be found in other food products, and often they are freeze dried and sold as food supplements in sachets or capsules. This wide use of probiotics in commercial products, as well as other functional foods, sensitized consumer groups and governments against the possible indiscriminate use of their health attributes in communication media and advertisements. Therefore, national and supranational organizations are now trying to control the use of Health Claims in the labeling of functional foods or food through TOKUHO in Japan (Foods for Specified Health Use (FOSHU)), the Evidence-Based Review System for the Scientific Evaluation of Health Claims in USA[2] (FDA), the draft Standard on Nutrition, Health, and Related Claims in Australia and New Zealand (FSANZ Proposal P293, 2009), the Regulation of the European Union on Nutrition and Health Claims made on Food (Regulation 1924/2006), and the FAO report on Functional Foods.[3] Although the ruling terms may fall out of this review's scope, the criteria that they propose in order to accept and approve these claims, including safety issues (see below), have a direct incidence in the procedures for their selection.[4–7]

For a very long time, probiotics have been selected on the basis of their suitability to food's environment and technological procedures, as well as survival ability to the gastrointestinal tract passage and colonization potential. These criteria are still used. Also, safety issues constitute a general concern when microorganisms are isolated from body fluids and feces. Due to the historical track of use in food, lactic acid bacteria or species of the genus *Lactobacillus* and *Bifidobacterium* are generally regarded as safe and included in the most stringent lists of safe bacterial species,[8] although some issues must still be taken into account before their free use in food products (see below). The first issue in the assessment of safety of new potential probiotics is the correct taxonomical identification. A short list of the most commonly used probiotic species is shown in Table 27.1, although probiotics normally used in dairy products are restricted to *Streptococcus salivarius* subsp. *thermophilus* and a few species of *Lactobacillus* and *Bifidobacterium*. Methodologies for fast and efficient identification of bacterial species and for the identification and enumeration of probiotics in food have been implemented, and have been proficiently reviewed in a previous issue of this book series,[9] as well as a detailed description of probiotic commercial strains.

After intake, probiotics mix with other microbial populations in the intestinal lumen and develop an active metabolism. Lactic acid bacteria and bifidobacteria are fermentative bacteria that efficiently produce short-chain organic acids from dietary sugars (for review, see chapter by Perez-Martinez[10] of this book series) during digestion, mainly lactic acid, acetic acids, and butyric acid. They inhibit competitive bacterial species, but butyric acid acts as nutrient that stimulates epitheliocytes and many probiotic species are producing folate and vitamin B12. Furthermore, their enzymatic repertoire acts on compounds that in some individuals could raise metabolic problems, such as lactose in lactose-intolerants or bile salts in hypercholesterolemic populations.

Nowadays, most probiotic benefits have been demonstrated through clinical trials that can overcome very strict scientific "criteria of evidence."[11,12] Although they constitute a very determinant selection procedure, clinical assays impose obvious limitations in the cost and the number of strains studied. Faster and more efficient selection procedures could be designed if the mechanisms of action of probiotics were clearly established. Research studies are advancing remarkably in the discovery of metabolites or constituent elements of probiotic bacteria that interact with the host cells and which trigger biological responses.[13,14] However, different probiotic strains have different effects on health, hence different criteria and targets should be used in each case.

TABLE 27.1

Example of Microbial Species in Which Probiotic Strains Have Been Described

***Lactobacillus* spp.**	***Bifidobacterium* spp.**	**Other**
L. acidophilus	*B. bifidum*	*Escherichia coli* Nissle
L. brevis	*B. breve*	*Saccharomyces cerevisiae*[c]
L. delbrueckii[a]	*B. infantis*	*Streptococcus salivarius* subsp. *thermophilus*
L. fermentum	*B. longum*	*Streptococcus salivarius*
L. gasseri	*B. adolescentis*	*Enterococcus faecium*
L. johnsonii	*B. animalis*[b]	*Bacillus coagulans*
L. paracasei		*Bacillus clausii*
L. plantarum		
L. reuteri		
L. rhamnosus		
L. salivarius		

Source: Adapted from Council for Agricultural Science and Technology (CAST). Probiotics: Their Potential to Impact Human Health. *CAST*, *Issue Paper 36*, 2007.

[a] *L. delbrueckii* subsp. *bulgaricus* is typically used as a starter culture for yogurt.

[b] Some *Bifidobacterium animalis* strains are commonly referred to in commercial labels as *Bifidobacterium lactis.*

[c] A probiotic *Saccharomyces cerevisiae* strain is marketed as "*Saccharomyces boulardii*," which is not a valid species name.

Competition with potential pathogens is a very desirable probiotic property when searching prevention of diarrhea and even therapeutical use of probiotic strains. The criteria applied in this case range from straight forward competition in coculture, test of production of bacteriocins, or competition for attachment sites at the epithelial surface. However, during the last decade the effect of probiotics on the immune system has been widely discussed. Numerous works indicate that the intake of probiotics enhances responsiveness to antigens and most remarkably, they may play a role in restoring the normal situation in immune disorders.[15,16] The biological processes and concepts that relate the microbial populations (microbiota) in the gut with local and systemic immune processes will be further developed below. As consequence, a number of animal models (*in vivo*), epithelial cell, or lymphocyte culture systems (*in vitro*) and also cellular or tissue explants from animals and humans (*ex vivo*) model systems have been implemented to determine the possible health-promoting effects of putative bacterial isolates. They have been combined with molecular and immunological methodologies to determine protein mediators synthesized by human or animal cells in response to infectious or inflammatory stimuli and also to the probiotic challenge. Ideally, effects found through these models should be backed up by properly conducted randomized double blind clinical studies.

Therefore, the methodologies compiled in this chapter have been grouped according to four selection criteria: safety assessment, determination of strain survival and colonization potential, production of metabolites, exopolysaccharides and enzymes, and functional characterization. There are many well-established procedures for almost all of them, except for the functional characterization of their biological activity, just because their mode of action has not yet been described. In this case, methods commonly used in the research of the likely mechanisms of action and biological properties of probiotics will be shown, as they are only likely selection methods available.

Before starting with the description of methods for the selection and evaluation of probiotic bacteria, and in order to understand and apply the most appropriate selection/evaluation procedure in each case, it would be necessary to understand the role of probiotics in the gut. Also, a brief overview of the population dynamics and interaction of the microbiota with the gut's mucosa will be outlined here.

27.1.1 Gut Microbiota

The gut microbiota has begun to receive growing attention in recent years, since a remarkable relationship with gastrointestinal disorders and nonintestinal diseases has been identified. In addition to its main role (digestion of food[17]), intestinal bacteria or bacteria-derived signals maintain epithelial homeostasis,[18] modulate fat metabolism,[19] promote enteric nerve function and angiogenesis[20,21] and protect against infections,[22] and inhibit pathogens through displacement or production of antimicrobial substances, such as lactic acid or bacteriocins.[23,24]

Although epithelial surfaces in all mammalian are colonized by microorganisms, the gastrointestinal tract has the largest bacterial burden and harbors an estimated load of 10^{14} microorganisms that belong to more than 1000 species,[25] representing members of all three domains of life (eukarya, archaea, and bacteria[26]), prevailing bacterial species. Early studies on the human microbiota were based on the enumeration and characterization of culturable organisms.[27] However, a large percentage of the human microbiota are anaerobic bacteria, which lack the enzymes for the detoxification of oxygen and are difficult to culture even under ideal conditions, and therefore could not be identified. In recent years, advances in molecular-profiling methods, particularly high-throughput sequencing of 16S ribosomal RNA genes, allowed a more accurate characterization of intestinal microbial communities. These studies found that throughout the intestinal tract two phyla, *Firmicutes* and *Bacteroidetes*, sum up to 90% of the entire intestinal community.[28,29] Within the *Firmicutes* phyla, which are Gram-positive bacteria, predominantly bacteria belonging to the *Clostridia* class inhabit the intestine. Other *Firmicutes* include Enterococcaceae and Lactobacillaceae that represent a low proportion of the luminal bacteria.[29] *Bacteroidetes* are Gram-negative bacteria, which include *Bacteroides* species such as *Bacteroides thetaiotaomicron*, *Bacteroides fragilis*, and *Bacteroides ovatus*.[29] The remaining bacteria which are present in relatively low abundance include bacteria belonging to the phyla Proteobacteria, Fusobacteria, Actinobacteria, Verrucomicrobia, and Spirochaetes.[29,30]

Bacterial density increases along the GI tract.[31] Whereas colonization in stomach and duodenum is hindered by acid and bile salts and bacterial counts are in the range of 10^1–10^3 cfu/mL, bacterial density can reach up to 10^{11}–10^{12} cfu/mL in the luminal colon content.[31] Along the length of the colon, but also between luminal and mucosal-associated compartments of the colon, differences within bacterial communities have been observed.[32] Interestingly, in a recent study in mice using pyrosequencing of 16S ribosomal RNA, a much higher proportion of lactobacilli could be detected in colon mucosa associated sites than in the colon luminal contents.[33]

As mentioned before, intestinal bacteria and bacterial signals have important roles in human health. In recent years, the abnormal composition of the microbiota (dysbiosis) has been related to a variety of diseases such as inflammatory bowel disease (IBD),[34] obesity,[35] and Type-I diabetes.[36] For example, it could be demonstrated that in obese people the relative abundance in *Firmicutes* is higher than in lean people and that the proportion in *Firmicutes* decreased during the course of diet, while the relative abundance in *Bacteroidetes* increased.[35]

27.1.2 Recognition of Bacteria by the Mucosa

Some basic biological concepts can be applied when describing the relationships of bacterial communities with the gut mucosa.[37] Microbial *symbionts* are terms describing those species that establish a long, more or less stable, relationship with the host. Within this group, *mutualistic* species get and provide a mutual benefit to the interaction, *parasites* (pathogens) get benefits while the host is harmed, and *commensals* get benefits but the host is unaffected.[37] Although gut immunologists prefer to use the terms "commensal bacteria," probiotics would rather be considered as *mutualistic* bacteria, normally isolated from feces, that are commercially available.

In the gut lumen, a mixture of nutrients, food debris, and bacteria move as a bulk fluid. The elemental mucosal anatomy is constituted by an external unstirred layer of mucus where nutrients and small molecules slowly diffuse toward the primary epitelial cell layer that forms a continuous by means of the tight junctions.[38] As such, the mucosal surface is continuously exposed to potential *pathogens*, beneficial *mutualistic*, and *commensal* microorganisms. The intestinal immune system is the largest and most complex part of the immune system, comprising almost 70% of all lymphocytes of the organism, scattered in the connective tissue (lamina propria), or in organized tissues (gut-associated lymphoid tissues (GALT)) such as the Peyer's patches of the small intestine and mesenteric lymph nodes (MLNs). A clear discrimination between pathogens and harmless antigens is essential to maintain the balance between tolerance and immunity and inappropriate immune responses to harmless food antigens or bacteria will lead to inflammatory diseases. These responses have to be prevented by regulatory mechanisms, also known as "oral tolerance," to ensure the maintenance of homeostasis in the gut.[39] Oral *tolerance* refers to the *active nonresponse* to dietary and commensal enteric bacteria or food-derived antigens administered orally.[40] The mechanisms responsible for establishing and maintaining oral tolerance are still incompletely understood. The subepithelial dendritic cells (DCs), B cells and T cells, in the lamina propria efficiently acquire antigens from the intestinal lumen and express a wide range of pattern-recognition receptors (PRRs), some of them are the Toll-like receptors (TLRs). Then, DCs migrate to draining lymph nodes, where they have the unique ability to activate and influence functional differentiation of naive T cells. Secreted signals from DCs (cytokines and chemokines) determine whether tolerance or active immune responses occur to a particular antigen and furthermore influence whether a T helper (T_h) cell immune response of the type T_h1 (innate immune response), T_h2 (adaptive immune response and allergy), T_h17 or T_{reg} (lymphocyte differentiation) predominates.[41]

Furthermore, recent findings suggest that intestinal epithelial cells (IECs) are not just a simple physical barrier. They express TLRs as well as intracellular nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), and they can secrete cytokines and regulatory molecules (TSLP, TGFβ, IL-10, and so on) that regulate cytokine secretion by DCs and macrophages. Therefore, IECs actively participate in the discrimination of both pathogenic and commensal bacteria, and are crucial for maintaining intestinal immune homeostasis and mechanisms of innate defense.[38,42] Also, in epithelial cells, NF-κB signal transduction pathway has an important role in the innate immunity and inflammatory responses. NF-κB is a key multimeric regulator controlling gene expression of IL-8 and other proteins expressed

under inflammatory conditions. In the cytoplasm, it is present in an inactive form bound to a specific inhibitor, I-κB. For NF-κB activation and subsequent translocation to the nucleus, the inhibitor I-κB must be ubiquitinated and degraded by the proteasome-ubiquitin system. This degradation of I-κB and release of NF-κB is activated by TNF-α and inflammatory stimuli acting on TLR, such as bacterial pathogen ligands. Different works have demonstrated that probiotics inhibit I-κB degradation, blocking signal transduction leading to the synthesis of proinflammatory cytokines and chemokines.[43,44] Hence, model *in vitro* systems considering IECs are also valuable tools for the study of the effect of commensal/probiotic bacteria.

27.1.3 Secreted Signaling Molecules Used as Markers

The ability of probiotics to interact with the host's immune system is often measured by its potential to induce or repress cytokine and chemokine secretion. Depending on the experimental system, different typically pro- and antiinflammatory cytokine markers are used, such as TNF-α, IFN-γ, IL-6, IL-4, IL10, IL-12, and so on. The ratio of the antiinflammatory cytokine IL-10 and the proinflammatory IL-12 is frequently considered as a good estimate to predict the anti/proinflammatory potential of a certain probiotic strain in human peripheral blood mononuclear cells (PBMC), as well as monocyte-derived DCs.[45–47] In general terms, some of the above-mentioned cytokines serve as "signature" cytokines for T_h1 (e.g., IFN-γ), T_h2 (e.g., IL-4, IL-5, IL-13), T_h17 (e.g., IL-17A, IL-17F, IL-6), and T_{Reg} cells (e.g., IL-10 and TGF-β), and some human disorders are characterized by a T_h1, T_h2, or T_h17 profile. For instance, an excess of T_h1 cytokines is predominantly related to proinflammatory processes and autoimmune disease as observed in Crohn's disease or multiple sclerosis,[48,49] and an imbalance toward T_h2 cytokines is associated with the generation of IgE found in allergic diseases.[50] Hence, cytokine induction in human PBMCs or monocyte-derived DCs by probiotic bacteria is often measured in order to deduce their ability to skew these balances and favor a T_h1, T_h2, or T_h17 polarization or to induce T_{Reg} cells.[46,51,52]

Chemokines are a family of small secreted molecules that mediate leukocyte migration and therefore play an essential role in the initiation of immune responses.[53]Different chemokines can act on different subtypes of immune cells (T cells, B cells, neutrophils, monocytes, etc.) which in turn might produce larger quantities of chemokines. Additionally to immune cells, cultured epithelial intestinal cell lines, such as Caco-2 and HT-29, are frequently used for the evaluation of the immunomodulatory properties of probiotic bacteria. Intestinal epithelial cells secrete a number of chemokines, and each cell line shows some differences in the profile of secreted chemokines.[54]CXCL8, also known as IL-8, is a prototypic epithelial chemokine which is frequently used to study the impact of probiotic bacteria since it is critical in leukocyte attraction to sites of infection or inflammation.[55–57]

27.2 Methods for Selection and Evaluation of Probiotics

Classically, probiotics are selected from feces directly on selective media. Then characterization (identification) of the species constitutes the first real selective step, since the species of the selected strains directly indicate if subsequent efforts are worthwhile. At present characterization of probiotic properties in bacteria and yeasts, unlike other industrial microorganisms, has not a single qualitative or quantitative test. The definite proof comes inevitably from its use in a clinical trials or its assay in volunteers, which obviously limits the number of strains to be studied.

The now "classical procedures" recommended tests to check their survival properties on the extreme environmental conditions that suffer any ingested food component, such as stomach pH, bile acids, and various types of enzymes.[58–60] These tests do not predict the biological effect of a given strain but will certainly indicate a degree of effectiveness, assuring that they may reach the intestine in sufficient number. However, most of the recommended procedures found in the WHO/FAO report on the Guidelines for Evaluation of Probiotics in Food (2002)[1] are still valid and have been considered in this chapter (Figure 27.1).

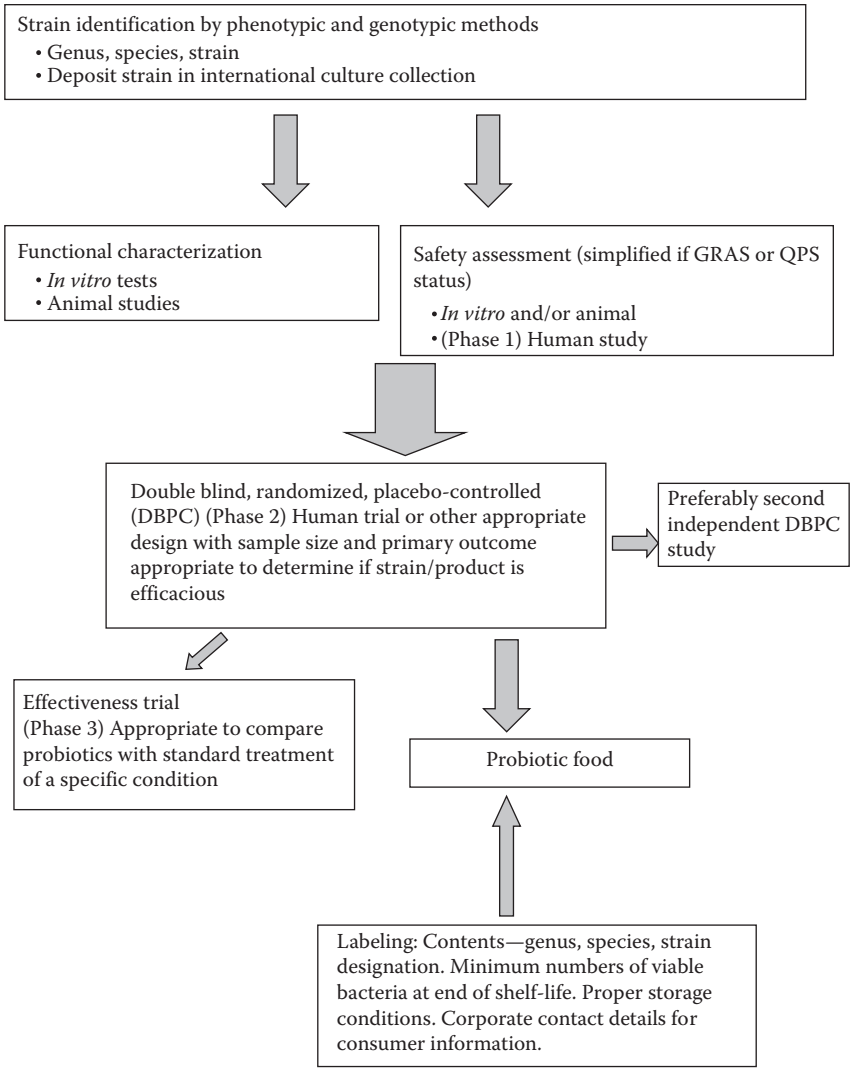

FIGURE 27.1 Flow chart summarizing the guidelines for the evaluation of probiotics for food use, as recommended by the working group report on drafting guidelines for the evaluation of probiotics in food. (Adapted from Araya, M. et al., *Guidelines for the Evaluation of Probiotics in Food*. pp. 1–11, Food and Agriculture Organization of the United Nations, World Health Organization: London, Ontario, Canada. 2002.)

Therefore, this document will describe first classical methods for selection of "effective" strains, then adhesion tests, and finally a battery of *in vitro* tests with immune cells, cultured intestinal epithelial cells and tissues that are at present the most valuable tools to assess health benefits of putative probiotics.

27.2.1 Safety Assessment

Different national or international authorities have tried to set up criteria that could help to the selection and use of microbial strains that offer some guide about their safety for food use. Regardless the simplicity of the "safety" concept, different organizations expressed it differently. FDA defined as "generally recognized as safe" (GRAS) those *food ingredients* (including microorganisms) *which were commonly used in food (prior to January 1, 1958), or by scientific procedures evaluated by qualified experts*. To obtain the GRAS status, the quantity and quality of scientific evidence required is similar

TABLE 27.2

Tests Recommended by the WHO/FAO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food for the Safety Assessment of Putative Probiotics

1. Determination of antibiotic resistance patterns.
2. Assessment of certain metabolic activities (e.g., D-lactate production, bile salt deconjugation).
3. Assessment of side effects during human studies.
4. Epidemiological surveillance of adverse incidents in consumers (postmarket).
5. If the strain under evaluation belongs to a species that is a known mammalian toxin producer, it must be tested for toxin production. One possible scheme for testing toxin production has been recommended by the EU Scientific Committee on Animal Nutrition.
6. If the strain under evaluation belongs to a species with known hemolytic potential, determination of hemolytic activity is required.

Source: Adapted from Araya, M. et al., *Guidelines for the Evaluation of Probiotics in Food,* pp. 1–11. Food and Agriculture Organization of the United Nations, World Health Organization: London, Ontario, Canada. 2002.

to that needed for the approval of a food additive or ingredient and ordinarily is to be based upon published studies.[61] However, the European Food Safety Authority (EFSA) has specifically studied the use of microorganisms in food and described a list of microorganisms with "Qualified Presumption of Safety" status (QPS) for premarket safety assessment of notified biological agents.[8,62] This essentially proposed that a *safety assessment of a defined taxonomic group (e.g., genus or group of related species) could be made based on four pillars: establishing identity, body of knowledge, possible pathogenicity, and end use*. If the taxonomic group did not raise safety concerns or, if safety concerns existed, but could be defined and excluded (the qualification), the grouping could be granted QPS status (http://www.efsa.europa.eu/en/biohaztopics/topic/qps.htm?wtrl=01). Although most microorganisms used as probiotics belong to bacterial genera and species with a long history of use in food, there are some issues that should be tested before they are used in humans, and indeed, before their commercial use. Table 27.2 extracts key issues suggested in 2002 by the FAO/WHO commission. Nowadays, antibiotic resistance is a central concern due to the Community Health implications, and procedures will be described.

27.2.1.1 Strain and Species Identification

The correct taxonomic identification of the strains used is a basic requirement for safety assessment and, therefore, for labeling purposes if the probiotic reaches the market. In fact, some studies have shown that the identity of recovered microorganisms is not always that indicated on the product label.[63,64] In addition, it can be very important to distinguish between and to identify the diversity of probiotic strains contained in commercial products available on the food markets. Furthermore, the correct species adscription will facilitate the selection of culture conditions, and functional assays to be performed due to the potential to secrete metabolites and structural composition of the cell (see below). As previously reviewed,[9] probiotics can be identified using currently available molecular methods, such as or DNA/DNA hybridization, DNA–DNA hybridization, hybridization with specific DNA probes, Randomly Amplified Polymorphic DNA (RAPD-PCR), or Pulsed Field Gel Electrophoresis (PFGE).[65–67] Most of these methods help typing strains, that is, they render information to discern strains within a given species, which is very valuable given that probiotic properties are strain dependent. Above all the molecular procedures described to precisely identify bacterial species, the easiest and most efficient 16r DNA sequencing.

27.2.1.1.1 Amplification and Sequencing of the Complete 16S and Domains I of the 16S and 23S rDNA

Amplification of the 16S rDNA from most bacteria can be achieved using the universal primers 27f (AGAGTTTGATCCTGGCTCAG) and 1492r (TACGGCACCTTGTTACGACTT).[68] PCR amplification is carried out in a thermal cycler using 30 cycles of 94°C/1 min, 55°C/45 s, 70°C/1.5 min, and a final

extension at 70°C/10 min. Taxonomically distant species will require to check the design of intermediate oligonucleotides for sequencing the complete 16S DNA fragment obtained. In the case of lactobacilli, it can be completely sequenced with the primers 27f (AGAGTTTGATCCTGGCTCAG), 1492r (TACGGCACCTTGTTACGACTT), 530f (GTGCCAGCMGCCGCGG), 1100r (GGGTTGCGCTCGTTG), and 1114f (GCAACGAGCGCAACCC). Then, PCR products are checked by agarose gel 1.2% (w/v) electrophoresis and DNA bands and purified by one of the commercial kits available (Qiagen, Amersham Pharmacia Biotech, Clontech, etc.) according to manufacture's instructions. After sequence assembly, they are subsequently aligned with specialised computer softwares (for instance, DNAMAN) with sequences retrieved, Ribosomal Database Project (RDP) libraries or GenBank, however, open free databases nowadays provide user friendly tools to upload the query sequence and get an instant solution to the species identification ("myRDP Space" at Ribosomal Database Project II (http://rdp.cme.msu.edu/)).

Frequently, the complete sequencing of the 16S ribosomal gene is not necessary to get enough information for fast species identification and sequencing just the variable regions at the 5′ end of either 16S rDNA (about 500 nucleotides) will suffice. Oligonucleotide primers generally used for amplification and sequencing of the domain I of the 16S rDNA were 27f (see above) and 558r (GTATTACCGCGGCTG) universal primers designed by Lane[68] and, optionally, those used for domain I of the 23S rDNA were 22f (CGGTGGATGCCTTGGC) and 559r (CATTMTACAAAAGGYACGC). After assembly of the sequences, alignments can be performed with sequences retrieved using BLASTN from NCIB (http://www.ncbi.nlm.nih.gov/) or the Ribosomal Database Project II as indicated above (http://rdp.cme.msu.edu/).

27.2.1.2 Biogen Amine Formation

The ability of the strain of certain lactobacilli to produce toxic biogenic amines is related to the presence of amino acid decarboxylase activity. A qualitative method can be used based on the change of the bromocresol purple indicator to purple in the presence of histidine, lysine, ornithine, and tyrosine added to the medium.[69] A more detailed determination method can be applied using on-pair high-performance liquid chromatography (HPLC) with *o*-phtalaldehyde postcolumn derivatization.[70] The media are supplemented with a "cocktail" consisting of four precursor amino acids (histidine, lysine, ornithine, and tyrosine), color changes from yellow to purple indicates the presence of the respective amino acid decarboxylase.

27.2.1.3 Antibiotic Resistance Testing

27.2.1.3.1 Agar Disk Diffusion Test

A very simple method consists in a susceptibility test based on the agar overlay disk diffusion test described by Aymerich et al.[71] In summary, strains to be tested are grown overnight (in the case of lactobacilli, MRS) and used to inoculate 7.5 mL of agar medium (e.g., MRS) that was kept molten at 50°C. Petri dishes containing 15 mL of agar medium (MRS) were overlaid with the inoculated hot medium and allowed to solidify. In order to get reproducible results, commercial antibiotic disks are recommended, however, sterile 3 MM paper disks of Ø6–10 mm can be soaked in the antibiotic solutions and used satisfactorily. Disks are then placed on the overlaid plates, which are then incubated (for lactobacilli, 24 h at 30°C, in an anaerobic chamber). Commercially available antibiotic disks with appropriate concentrations can be purchased for a very wide number of antibiotics. Most common concentrations are penicillin G (10 μg), ampicillin (10 μg), vancomycin (30 μg), tetracycline (30 μg), erythromycin (15 μg), kanamycin (30 μg) gentamycin (10 μg), and chloramphenicol (30 μg).[72]

27.2.1.3.2 Microdilution Antimicrobial Susceptibility Testing

Conceptually, this test is more accurate than the agar diffusion test, since it uses increasing concentrations of antimicrobials (antibiotics) in the appropriate media, hence minimal inhibitory concentration (MIC) can be easily determined for each antibiotic. It can be performed in microtiter ELISA plates, using aliquots of 100–200 μL of medium (LSM broth for lactobacilli and LSM broth supplemented with 0.3 g/L L-cysteine hydrochloride for bifidobacteria).[73] Those plates have an 8 × 12 wells design and

TABLE 27.3

Table of Antibiotics Frequently Tested in Bacteria for Putative Food Use

Antibiotic	Concentration Range (μg/mL)
Penicillin G	0.032–64
Ampicillin	0.032–64
Ampicillin/sulbactam (sulbactam 8 μg/mL)	0.032–64
Gentamycin	1–2048
Streptomycin	2–4096
Vancomycin	0.125–256
Teicoplanin	0.125–256
Erythromycin	0.016–32
Clindamycin	0.032–32
Quinupristin-dalfopristin (30:70 ratio)	0.032–64
Oxytetracycline	0.063–128
Chloramphenicol	0.125–256
Fusidic acid	0.063–128
Trimethoprim	0.25–512
Sulfamethoxazole/trimethoprim (19:1 ratio)	0.25–512
Ciprofloxacin	0.008–16
Moxifloxacin	0.008–16
Linezolid	0.016–32
Cefazolin	0.125–256
Kanamycin	1–2048
Neomycin	1–2048

therefore in each plate 8 strains can be tested, using increasing concentrations in 11 wells, plus one zero concentration control. Different authors use varying inoculum concentrations which are rather depending on the growth speed of each strain. In general terms, inocula giving a final concentration of 10^5–10^6 bacteria mL^{-1} or 0.5–1.0 McFarland units are recommended for lactobacilli and bifidobacteria. The list of antibiotics to be tested is also variable; however, at least one or two of each big antibiotic families should be included in the test. A list of antibiotics that can be tested is shown in Table 27.3, antibiotics in bold letter constitute an alternative reduced list. When diluting antibiotics, it should be borne in mind that some of them are not well dissolved in water, so erythromycin and fusidic acid need at least 50% methanol, trimethoprim, dimethyl formamide, oxytetracycline, 0.1 N HCl and sulfamethoxazole, 0.1 N NaOH. Strains that overgrow the highest concentration of any of the listed antibiotics (Table 27.3) are generally carrying antibiotic resistance gene/s.[73–75]

27.2.2 Determination of Strain Survival and Colonization Potential

27.2.2.1 Tolerance to Gastrointestinal Conditions

The resistance to gastric acid and to bile salts will facilitate a probiotic to survive the passage through the gastrointestinal tract[76] and the viability of probiotic strains when they reach the intestinal region will ensure their optimal functionality. Different techniques have been used to screen their tolerance to acid pH and bile.[77,78]

The simplest methods described to test sensitivity to gastric acidity are based on bacterial plate systems. Strains were grown in liquid broth (i.e., lactobacilli on MRS, at 37°C for 24 h). Then cells were harvested by centrifugation (10 min, 4°C, 12,000 rpm), washed and suspended in PBS to reach a final cell density of 10^9 cells/mL. Cell suspension is diluted in 9 mL of sterile PBS, containing 3 g/L pepsin from porcine stomach mucus and adjusted at pH 2.0 with HCl. Aliquots are taken after 0, 90, and 120 min of incubation at 37°C and the viability is determined by plate counting on culture media agar.

Plates are incubated at 37°C for 24 h and visually examined for growth. Also, sensitivity against gastric acid and bile salts can be assayed in microtiter plates similarly to the *microdilution antimicrobial susceptibility test* described before.[77] For this purpose, bacterial strains can be grown in liquid medium in microtiter plates and growth monitored measuring the absorbance in a microplate reader equipment (OD). The growth of the strains in the presence of different concentrations of bile salts (Ox-gall or bile at 0%, 1%, and 4% w/v) and other salts, as NaCl, can be determined at OD_{650} in a microplate reader at different times.

27.2.2.2 Adhesion to Mucus and Extracellular Matrix Proteins

Adhesion tests are among the classically recommended probiotic tests that are more evidently related to true biological function, since adhesion to the mucus, proteins of the intracellular matrix, or to the surface of epithelial cells would assure close contact and exposure to yet unknown receptors that would mediate the host recognition and signals thereafter.[79] Adhesion to the intestinal mucosa is regarded as a prerequisite for colonization and is an important characteristic related to the ability to modulate the immune system. For this reason, adhesion has been one of the main selection criteria for new probiotic strains.[65] However, the difficulties involved in studying bacterial adhesion *in vivo*, specifically in humans, have led to the development of different *in vitro* model systems for the preliminary selection of potentially adherent strains. *In vitro* models involving human epithelial cell lines, mostly Caco-2 and HT-29, or mucus-secreting HT-29-MTX cells as well as intestinal mucus isolated from feces, ileostomy, or resected human intestinal tissue have been used to assess the adhesion properties of potential probiotic strains.[80–85] Methods to test adhesion to human epithelial cells will be exhaustively reviewed below.

Here we will describe the methodology applied to test adhesion on mucus from mucosa and extracellular matrix proteins, such as fibrinogen (fraction I from pig plasma), fibronectin, and collagen.[81,83,86] Human mucus (0.5 mg/mL protein), fibrinogen, and fibronectin are dissolved in HEPES-Hanks (HH) buffer or 50 mM carbonate/bicarbonate buffer pH 9.6 (collagen in PBS pH 5.5) and they are immobilized into polystyrene microtiter plate wells by overnight incubation at 4°C. Adhered cells can be stained for quantification with crystal violet 1 mg/mL (100 µL/well) for 45 min. After six washes with PBS, the colorant was liberated with 50 mM citrate buffer pH 4.0 (100 µL/well) for 45 min and the absorbance at 595 nm was determined. Alternatively, radiolabeled bacteria with tritiated thymidine are centrifuged (6000 rpm, 7 min), washed twice with HH buffer and the absorbance ($A_{600\,nm}$) is adjusted in order to standardize the number of bacteria (10^7–10^8 cell/mL). Then bacteria are added into the wells and incubated for 1 h at 37°C. Subsequently, the wells are washed twice with HH to remove unattached bacteria. Adhered bacteria were released and lysed with 1% (wt/vol) SDS in 0.1 mol/L NaOH by incubation at 60°C for 1 h. The contents of the wells were transferred to microfuge tubes containing scintillation liquid and the radioactivity was measured by liquid scintillation. Adhesion was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension added to the immobilized mucus.

27.2.2.3 Antimicrobial Activity against Potentially Pathogenic Bacteria

The most extensive studies and clinical applications of probiotics have been related to the management of gastrointestinal infections caused by pathogenic microorganisms. Several metabolic compounds produced by lactic acid bacteria (including organic acids, fatty acids, hydrogen peroxide, and diacetyl) have antagonistic (antimicrobial) effects. However, bacteriocins or proteinaceous substances with specific inhibitory activity against closely related species are perhaps the most extensively studied.[87,88] Antagonistic activities reported in the literature have been evaluated with diverse sets of methodologies, degrees of sensitivity, amount of test compounds, and microbial strains, often difficult to compare. The currently available screening methods for the detection of antimicrobial activity fall into different groups, including diffusion and dilution methods. The diffusion methods are qualitative techniques since these methods will only give an idea of the presence or absence of substances with antagonistic activity. On the other hand, dilution methods are quantitative assays as they provide information on the minimal inhibitory concentrations. Bacterocins from Gram-positive bacteria are preferentially active against closely

related species and rarely against Gram-negatives, therefore in the case of probiotics, a wide spectrum of activity profile is a priority and they must show activity against enteropathogens. Therefore, the choice of the right culture media for each indicator strains is crucial for the success of the assay, so we suggest TH agar for enterococci, Trypticase soy agar for staphylococci, LB agar for coliforms, brain heart agar for *Listeria*, and brain heart agar under anaerobic conditions for clostridia. In general, to determine the antimicrobial activity of compounds secreted by bacteria, overnight cultures of each strain are used to inoculate (1% vol/vol) 10 mL of fresh culture media broth. After overnight incubation at the appropriate temperature (37°C), cells are removed by centrifugation (15 min, 4°C, 12,000 × *g*). Culture supernatants are filter sterilized (0.22 μm pore size filter) to eliminate the possible presence of viable cells and their pH are adjusted to 6.5 (neutral pH) with 1 N NaOH to exclude the effects of organic acids. It is recommended to concentrate the neutralized cell-free culture supernatants (NCSs) under vacuum or freeze drying in order to increase the sensitivity of the assay. The NCSs from bacteria are screened for antimicrobial activity by a well diffusion assay. Fifteen milliliter of appropriate culture media containing 0.7% (wt/vol) agar are inoculated with each indicator strain at a final concentration of about 10^6 cfu/mL, poured in Petri dishes, and allowed to solidify at room temperature. With the aid of a sterile cork borer or metal cylinder, wells (5 mm diameter) are made on the agar and filled with different volumes of NCS. After incubation at the conditions required by each indicator strain, the inhibition zones (millimeters) are measured.[60,89] Each assay must be performed in duplicate. Alternatively, the antimicrobial activity of NCSs can be tested using a variant of the microdilution antimicrobial susceptibility test described above using microplates. For this purpose, a volume of appropriate broth inoculated with an overnight culture of the indicator strain and 25 μL aliquots of NCS in varying dilution rates are loaded in each well and incubated at optimal temperature for up to 24 h.

27.2.3 Production of Metabolites, Exopolysaccharides, and Enzymes

27.2.3.1 Exopolysaccharides

Exopolysaccharide (EPS) secretion confers bacterial pathogens resistance to phagocytosis, since the presence of the extracellular polysaccharide capsule prevents binding of innate host opsonins, leading to an insufficient complement deposition for phagocytosis.[90] However, many microbial strains used in food as starters are producing EPS. In the case of lactic acid bacteria used as dairy starters, EPS production is essential to provide the required texture, providing a gel to ropy texture. The presence of EPS can be frequently detected directly on the colonies of producing bacteria, when grown on abundant carbohydrates. For the fast screening of numerous colonies, the ruthenium red milk plates (0.08 g/L) method is highly recommended.[91] Then more quantitative procedures can be applied to distinguish high producers, or even to purify the polysaccharide for fine compositional analysis, that use deproteinization steps and peptide precipitation with trichloroacetic acid followed by precipitation of the EPS with acetone[91] or ethanol.[92]

Many strains are producing EPS with different composition and characteristics, but in the screening of probiotics, those producing EPS may have additional functional properties. Then, some methodologies are common to other functionality assays and change according to the biological activity tested (Table 27.4).

27.2.3.2 Folate

The intestinal microbiota is known to produce vitamin K and most of the water-soluble vitamins of group B, including biotin, nicotinic acid, folates, riboflavin, thiamine, pyridoxine, panthotenic acid, and cobalamin.[93] It has been demonstrated that folate synthesized by bacteria in the human intestine is absorbed and used by the host.[94,95] In addition, probiotic bacteria, mostly belonging to the genera *Lactobacillus* and *Bifidobacterium*, confer a number of health benefits to the host, including vitamin production. Due to potentially relevant applications, the ability to produce folate has been intensively investigated in many *Lactobacillus* and *Bifidobacterium* strains. Recently, the genome sequence of an increasing number of strains of *Lactobacillus* and lactic acid bacteria has provided a major contribution to the knowledge of folate biosynthesis by these bacteria.[96,97]

TABLE 27.4

List of Biological Assays Used by Different Researchers to Determine the Biological Effect of EPS Produced by Probiotic Bacteria

EPS-Producing Probiotic	Assay	Biological Effect	Reference
L. lactis ssp. *cremoris* (viili) *L. rhamnosus* GG, *Bifidobacterium longum* NB667, *Bifidobacterium animalis* IPLA-R1	Adherence to mucus immobilized on polystyrene microtiter plate wells	Involvement of EPS in the adhesion of probiotics to mucus. Also possibly mediate binding of pathogens to mucus	130
Bifidobacterium breve A28	Binding to Caco-2 and inhibition of *E. coli* ATCC11229		131
L. delbrueckii subsp. *bulgaricus* B3 (high EPS) *L. delbrueckii* subsp. *bulgaricus* A13 (low EPS)	Induced colitis in rats with acetic acid. Determination of superoxide dismutase, catalase, total glutathione, reduced glutathione, glutathione disulfide, and lipid peroxidation	EPS-producing probiotic bacteria significantly attenuate oxidative stress and inflammation in experimental colitis	132, 133
L. delbrueckii subsp. *bulgaricus*	Cell suspensions	Higher colesterol removal by strains producing more EPS	134
L. acidophilus 606	Growth inhibition of HT-29, immunoblot, MALDI-TOF/MS	Cell bound EPS activates autophagic cell death by induction of Beclin-1, GRP78, Bcl-2, and Bak	135
L. acidophilus A4	Continuous-flow chamber models and transcriptome analysis	Released EPS inhibits *E. coli* EHEC O157:H7 biofilm formation affecting genes related to curli production (crl, csgA, and csgB) and chemotaxis (cheY)	136
Bacillus coagulans RK-02	β-carotene-linoleic acid model system, superoxide radical scavenging assays: PMS-NADH-nitroblue tetrazolium system, 1,1-diphenyl-2-picrylhydrazyl, ascorbic acid-Cu(2+)-cytochrome *c* and *in vitro* microsome peroxidation inhibition study using the thiobarbituric acid assay	EPS has a significant antioxidant and free radical scavenging activities	137
Bifidobacterium longum BCRC 14634	Proliferation of J77A.1 macrophages and secretion of IL-10, TNF-α	EPS increased macrophages proliferation and secretion of IL-10 and reduced proinflammatory and growth suppression effect of LPS	138
L. kefiranofaciens	Determination of cytokines (IL-4, IL-6, IL-10, IL-12, IFNγ, and TNFα) in the gut lamina propria, immunoglobulins in the intestinal fluid and blood serum and IgA+ and IgG+ cells in small intestine in BALB/c mice	EPS induce protective immunity, maintain intestinal homeostasis, enhance the IgA production, and influence the systemic immunity through the cytokines released to the circulating blood	139
L. lactis subsp. *cremoris* SBT1275	Feeding rats with cholesterol and EPS. Determination of HDL cholesterol/total cholesterol ratio	EPS had a beneficial effect on rat cholesterol metabolism	140
L. lactis subsp. *cremoris* KVS20	Mitogenic responses of a preparation enriched in B cells, T cells, and spleen cells of mice	EPS are a potent B-cell-dependent mitogen	141

Production of folate and other vitamins has been tested on bacterial cell extracts and culture supernatants using folate-free media.[94] For this purpose, volumes of culture were centrifuged at 13,000 × *g* for 10 min at 0°C. The supernatant was filtered through a 0.22 μm filter. The biomass was washed with 0.05 M K-phosphate buffer pH 6.5 and the wet pellet was resuspended 1:1 in the same buffer. A quantity of 0.5 g glass beads (≤106 μm, Sigma Aldrich) were added to 1 mL suspension and cells were disrupted at 1800 rpm for 10 min at 4°C in a vibration homogenizer. The cell extract was heated at 100°C for 3 min to release folate from folate-binding proteins and to precipitate proteins, then it was centrifuged at max speed at 4°C and filtered (0.22 μm). In addition, folate concentration can be analyzed using the microbiological bioassay using Bacto folic acid assay medium.[98] *Enterococcus hirae* ATCC 8043 is the test organism according to the protocol described by the medium manufacturer. The total folate concentration, including polyglutamyl folate, is analyzed after the samples are treated with human plasma, as a source of γ-glutamyl hydrolase activity, at 37°C and pH 4.8 for 4 h. Microbiological assay measurements were replicated at least 10 times.

27.2.3.3 Bile Salt Hydrolases

Bile salts are synthesized in the liver from cholesterol precursors and released to the small intestine conjugated to glycine or taurine, and their function is to solubilize fat. There are two main salts, cholic and chenodeoxycholic acid and secondary salts of lithocholic, deoxycholic, and ursodeoxycholic acids. After food digestion, they are absorbed by the intestine and return to the liver by the enterohepatic circulation. A small part of these salts is deconjugated by bile salt hydrolases (BSH) produced by different intestinal bacteria, such as *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Clostridium*, and *Enterococcus*[99] and eliminated with feces. Those bile salts will have to be synthesized again in the liver from cholesterol; hence, high BSH activity in the intestinal microbiota could help lowering endogenous cholesterol.[100]

Production of BSH by bacteria can be qualitatively determined on agar dishes, where taurodeoxycholate or glycodeoxycholate are added together with $CaCl_2$ to the required growth medium (MRS agar for lactobacillus). BSH activity will be revealed by a white halo surrounding the producing colony, as a consequence of the precipitation of the calcium salt of the deconjugated bile salt.[101]

For the quantitative determination of BSH activity, the use of HPLC is recommended.[102] The hydrolysis of tauro or deoxycholic acid in the culture medium will be proportional to the enzyme activity. For this determination, BSH activity is stopped by adding 3 μL 6 N HCl to 2 mL of culture supernatant. Then 1.2 mL isopropanol are mixed with the sample at 420 rpm for 1 h. Then, the mixture is centrifuged and the isopropanol fraction is recovered and evaporated in a vacuum centrifuge at 37°C for 2 h. Samples can then be injected to an HPLC chromatographer and separated in an 150 × 4.6 mm reverse phase C-18 column (Supelcosil 5 μm LC-18-DB or similar). The mobile phase is 70% methanol in 0.03 M Na acetate adjusted to pH 5.6 with phosphoric acid. Separation proceeds with 1 mL/min flux products can be detected with a UV detector at 210 nm.

27.2.4 Functional Characterization

A number of experimental procedures and methods are specifically used in this section, which are rather related to biomedical and immunological research tests and which are compiled in *Current Protocols in Immunology*[103] (http://onlinelibrary.wiley.com/book/10.1002/0471142735/toc). Nevertheless, they will be briefly described here but references for exhaustive description of the assays will be given.

27.2.4.1 In Vitro Assays

Probiotic mechanisms of action are suspected (see Section 27.1) to be due to more or less complex and interwoven signal interactions; therefore, the use of simple model systems would tremendously facilitate their study, and, from practical stand points, their use for the selection and evaluation of probiotics. More or less simple systems can in fact be used, although they do require special facilities and expertise, as in the case of intestinal epithelial cell or lymphocyte cultures. They are reliable procedures in the sense that, above their high subject-to-subject variability, the qualitative results—such as trends to induce or

inhibit a given cytokine—are highly reproducible for a given strain or group of them. We will describe below the most commonly used *in vitro* systems, as well as a reference to the analytical methods that are to be used. General concepts have been reviewed and experimental procedures related to immunology used can be found in a number of volumes, such as *Current Protocols in Immunology*[103] (http://onlinelibrary.wiley.com/book/10.1002/0471142735/toc).

27.2.4.1.1 Functional Properties of Probiotics Tested with IECs

Intestinal epithelial cell (IEC) cultures have become a very common tool in the investigation of functional properties of probiotics. There are very well-established methods for handling and culture[103] of a number of cell lines (e.g., HT-29, Caco-2, T83) and they are accessible through standard culture collections. These cell lines are easy to maintain because they are continuously growing, but since they are cancer cells, they require special culture rooms, cabins, and 5% CO_2 incubators. Despite of this, they have been very useful and will possibly be in the future research on a large number of functional properties of probiotics.

27.2.4.1.2 Probiotic Adhesion to Enterocytes and Inhibition of Pathogen Adhesion

EICs (HT29, Caco-2, T84) are grown to 100% confluence on six-well plates (Falcon, VWR) and washed with buffer (PBS, pH 7.4). Then, one to several milliliters of a suspension of the candidate probiotic (1×10^8 cfu/mL) was added to the wells in standard culture medium for cell culture (DMEM without FCS and without antibiotics) (100–200 bacteria per epithelial cell) and incubated for 1 h at 37°C and 5% CO_2 to allow adhesion of the strains.[104,105]

Nonadherent bacteria are removed by washing three times with the same buffer (PBS, pH 7.4). Cells with adherent bacteria are lysed with 1.5 mL of 1% (vol/ vol) Triton X-100 (Merck) and/or 200 µL trypsin/EDTA plus 200 µL PBS (10 min, 37°C). For conventional plate counts on bacteriological media, samples are serially diluted, and plated onto differential media for the probiotic species tested. Appropriate controls are essential.

Quantification of attached bacteria can also be performed by qPCR.[105] After detachment from the plastic surface, epithelial cells and the adhesive bacteria were transferred to a 1.5-mL reaction tube. The suspensions then were frozen and stored at –20°C until quantification of the bacteria by real-time PCR (qPCR). Description of methods to perform correct qPCR comparisons can be found in Schmittgen et al.[106] As a reference, 1 mL of the original bacterial cell suspensions is centrifuged and resuspended in 200 µL trypsin/EDTA plus 200 µL PBS and then frozen and stored at –20°C until quantification of the bacteria.

For radioactive adhesion assays,[105] late exponential cultures of the respective bacteria were adjusted to 1×10^8 cells/mL. A quantity of 4.5 mL of this suspension was incubated at 37°C for 30 min in the presence of 50 µCi [35S] methionine (Amersham Pharmacia Biotech, Germany). After removal of the excess radioactivity by washing four times with sterile phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) (PBS), the bacteria were resuspended in 4.5 mL DMEM and used for the adhesion assays.

After detachment of the epithelial cells from the plastic surface, the cells (epithelial cells and adhesive bacteria) are transferred to a counting vial containing 100 µL 5% (w/v) SDS in 0.5 M NaOH. After vigorous mixing, cells were lysed during incubation for 15 min at room temperature. After addition of 4 mL of scintillator counting reagent, vials are vigorously mixed and incubated for 1 h at room temperature, then the radioactivity is determined by liquid scintillation. Adhesion is calculated as the ratio between bound radioactivity to the cells and the total radioactivity of the initial amount of bacteria added at the start.

Following the incubation with probiotics, the protocol can be changed to test exclusion, competitive adhesion or displacement of pathogenic bacteria. In *exclusion assays*, the first strain incubated with the epithelial cells is the probiotic tested and second the enteropathogen. In *displacement assays*, the first strain was the pathogen and the second the probiotic, whereas in the *competitive adhesion*, both strain are incubated together. Bacterial pathogens are then inoculated to the wells (~1×10^5 cfu/well) and incubated under the same conditions. After another 1 h of incubation, nonadherent bacteria are washed as

before and cells with adherent bacteria are lysed as before, serially diluted, and plated onto differential media for the adherent pathogen tested, or quantified by qPCR with species specific primers.

Table 27.5 describes a selected number of assays and experimental systems used to test probiotic adhesion to IEC and inhibition of pathogen adhesion (exclusion, displacement, or competitive adhesion).

27.2.4.1.3 Preservation of Barrier Integrity

Monolayer barrier integrity and permeability of IEC polarized monolayers is normally determined by the transepithelial electrical resistance (TEER).[103] This method is used to assess the disruption of the epithelial layer after proinflammatory treatments and specially to monitor the invasion of enteropathogens under *in vitro* experimental conditions.[107] Hence, it has been used to show prevention of the epithelial barrier function by *Lactobacillus rhamnosus* GG when T84 were infected with enterohemorrhagic *Escherichia coli*, but confirmed by Western blot of tight junction proteins like claudin-1 and zonula

TABLE 27.5

Model *In Vitro* Systems to Test Probiotic Adhesion to Intestinal Epithelial Cells and Inhibition of Pathogen Adhesion

Probiotic Strain/s	Model System	Reference
L. rhamnosus GG and *Bifidobacterium animalis* subsp. *lactis* Bb12	Caco-2 and/or HT-29-MTX (HT-29 mucus-producing mutant)	142
L. rhamnosus DR20, *Bifidobacterium lactis* DR10, *L. acidophilus* LA-1, and *L. rhamnosus* GG	HT-29, Caco-2, and HT29-MTX adhesion and inhibition of *E. coli* adhesion	143
Natural isolates, *L. gasseri*, *L. paracasei*, *L. salivarius* CECT 5713	Caco-2 and HT-29	144–146
L. paracasei KW3110	Adhesion to Caco-2 and HT-29 was correlated to survival in human intestine	147
L. acidophilus Bar13, *L. plantarum* Bar10, *Bifidobacterium longum* Bar33, and *Bifidobacterium lactis* Bar30	Caco-2 and HT-29 and competition against enteropathogens	148
Natural isolates, *L. plantarum,* and *L. johnsonii* strains	HT-29	104,149
Natural *Lactobacillus* strains	HT-29. MUC3 mucin secretion follows adherence of Lactobacillus and reduced adherence of *E. coli*	150
L. johnsonii La1, *L. helveticus* ATCC 15009	HT29, HT29-MTX, *L. johnsonii* GroEL role in interactions with the host	118
L. helveticus R0052 and *L. rhamnosus* R0011	T84 and embryonic epithelial cells. Pretreatment with *Lactobacillus* inhibit *Campylobacter jejuni* invasion	
Several lactobacilli and bifidobacteria	Caco-2 and T84. Secreted probiotic factors ameliorate enteropathogenic infection	
Inhibition of Pathogen Adhesion		
L. acidophilus A4	HT-29. Inhibition of *E. coli* O157:H7 and reduction of inflammatory markers	151
L. acidophilus Bar13, *L. plantarum* Bar10, *Bifidobacterium longum* Bar33 and *Bifidobacterium lactis* Bar30	Caco-2 and HT-29. Competition with enteropathogens protection of enterocytes from inflammatory response	148
L. helveticus R0052	HEp-2 and T84. Surface-layer protein inhibit *E. coli* O157:H7 adhesion	117
Lactobacillus P10 (porcine strain)	Caco2. *E. coli* exclusion, displacement, and competition	152
L. rhamnosus DR20, *Bifidobacterium lactis* DR10, *L. acidophilus* LA-1, and *L. rhamnosus* GG	HT-29, Caco-2, and HT29-MTX adhesion and inhibition of adhesion of an enterotoxigenic *E. coli*	143

occludens-1 (ZO-1).[108] This effect has been confirmed to be due to two cell wall proteins (p40, p75) that, when purified, showed a similar effect on Caco-2 tight junctions.[109] A probiotic *E. coli* strain, Nissle 1927, mediates restoration of barrier integrity when coincubated with an *E. coli* EPEC, as determined by TEER), but also confirmed by Western blot and qPCR of zonula occludens-2 (ZO-2).[110]

This technique was also used to measure the barrier disruption induced by TNFα and restored by DNA from *L. rhamnosus* GG and *Bifidobacterium longum*[111] or by secreted proteins of *Bifidobacterium infantis*,[112] and those data were confirmed by the immunological analysis of tight junction proteins (claudins 1, 2, 3, and 4, zonula occludens (ZO)-1).[112]

In summary, prevention of barrier integrity by probiotics can be assessed by TEER; however, it is convenient to back up this results with other assays that determine the presence or induction of elements in the tight junctions, such as zonula occludens proteins.

27.2.4.1.4 Antiinflammatory Effect

It is known that intestinal epithelial cells have receptors that recognize luminal bacteria and they are important elements of the innate immune response process. As reviewed in the introduction of this chapter, they can synthesize cytokines and chemokines in response to pathogens and inflammatory stimuli, but intestinal epitheliocytes also take part in the detection of indigenous harmless microorganisms though an active "immune tolerance" process. Furthermore, the *in vitro* systems that are described in this section are helping to prove that some indigenous bacteria (probiotics) can actively revert inflammatory signals, through the "immune tolerance" transduction of signals. Therefore, an antiinflammatory effect of probiotics will be directly related to the decrease of signaling molecules (chemokines) linked to inflammation or to others involved in the natural inhibition of inflammatory signals. The most frequent inflammatory marker searched in the supernatant of IEC cultures is IL-8, which is normally detected by e*nzyme-linked immunosorbent assays* (ELISA),[103] the preferred method for accurate determination of secreted mediators, such as cytokines and chemokines. Other methods have been developed that, although more expensive, are highly efficient, such as flow cytometry and immunofluorescence in HT-29.[113]

As mentioned in the introduction, NF-κB signal transduction pathway participates in the innate immunity and inflammatory response in epithelial cells and its components suffer changes that can be detected by available methods. NF-κB activation/inactivation can be determined by electrophoretic mobility shift assay,[43,111,114] or through cell transfection with NF-κB-dependent luciferase reporter gene assays.[111] Degradation of I-κB, or its inhibition, can be determined by Western blotting.[43,44] The inhibition of the regulator NF-κB can be considered a solid proof of anti-inflammatory activity (Table 27.6).

27.2.1.4.5 Stimulation/Inhibition of Innate Immunity

It was described in the introduction that stimulation of TLRs by bacterial ligands induces the NF-kB signal transduction pathway that leads to an almost immediate synthesis of proinflammatory mediators in epithelial cells. Here, stimulation or repression of the synthesis of TLRs has been separated from more general antiinflammatory effects of probiotics, because the interpretation of these results is sometimes difficult. For example, *Lactobacillus plantarum* DSMZ12028 down-regulated in HT-29 TLR mRNAs, with the exception of TLR2, while *Lactobacillus paracasei* F19 up-regulated TLR2.[115] Both strains have thus far been considered probiotics. However, *L. plantarum* BFE 1685 and *L. rhamnosus* GG were shown to induce TLR2, TLR9, and TLR4 expression, both by qPCR and immunolabeling-flow cytometry.[116] Only TLR2 seems to be commonly induced by lactobacilli, which could be anticipated as it is known to mediate the recognition of peptidoglycan components of most Gram-positives. These results led to a very interesting discussion on the mechanisms of action of different probiotics; however, without further tests, TLRs cannot be considered good markers to describe probiotic effect when using intestinal epithelial cell cultures.

27.2.1.4.6 Effect of DNA and Secreted Metabolites

Due to its relative simplicity, *in vitro* experimental systems and particularly epithelial cells culture provide an excellent assay method to test the first steps in the mechanisms of action of probiotics, which includes testing the biological effect of isolated metabolites or bacterial proteins and other cell

TABLE 27.6

Model *In Vitro* Systems to Test the Anti-inflammatory Effect of Probiotics on Intestinal Epithelial Cells

Probiotic Strain/s	Model System	Reference
Bifidobacterium bifidum	Caco-2, T84, and HT29. Also two murine models of colitis	153
L. rhamnosus GG	HT29. Pathway-focused qPCR array. Modulation of IF-κBa degradation after exposure to *Vibrio cholerae*	44
L. acidophilus Bar13, *L. plantarum* Bar10, *Bifidobacterium longum* Bar33, and *Bifidobacterium lactis* Bar30	Caco-2 HT29. Adhesion properties, competition against enteropathogens and modulation of IL-8 production	148
Bifidobacterium breve Yakult and *Bifidobacterium bifidum* Yakult	HT-29, also PBMC from UC patients. Anti-inflammatory effect production of IL-10 and inhibition of IL-8	154
L. casei	HT-29. Transfected with PPRE3-tk-luciferase construct. Increased expression of PPARγ (inhibitor of NF-KB)	155
Ninety bifidobacteria isolates	HT-29. Inhibition of LPS-induced interleukin-8 (IL-8) secretion	156
E. coli, *Bifidobacterium adolescentis*, *Bifidobacterium vulgatus*, *Bifidobacterium distasonis*, and *S. salivarius*	HeLa 229, HT-29, also induced colitis in mice. *S. salivarius* inhibited the *Y. enterocolitica*-induced NF-κB activation and IL-8 production	114
Five *L. plantarum* strains and two *L. johnsonii*	HT29. Stimulation of chemokine production	104
Bifidobacterium lactis NCC362, *Bifidobacterium longum* NCC2705, *Bifidobacterium adolescentis* NCC251, *Bifidobacterium bifidum* NCC189 NCC2B, *Bifidobacterium bifidum* S16, *Bifidobacterium bifidum* S17, *Bifidobacterium infantis/longum* E18, *Bifidobacterium breve* MB226	HT-29. Reduction of expression (qPCR) of inflammatory genes IL-8, TNF-α, Cox-2, ICAM-1 expression. inhibition of LPS-induced NF-κB activation	157
Bifidobacterium infantis 35624, *Lactobacillus salivarius* UCC118	HT29, HeLa, also DC from PBMC. S. *typhimurium* inflammatory gene expression 847 gene arrays. NF-kB activation	158
L. johnsonii La1, *L. helveticus* ATCC 15009	HT29, HT29-MTX, also PBMCs. GroEL stimulates IL-8 secretion	118

components. The experimental procedures used in these tests are similar to those applied in other studies. The anti-inflammatory effect of DNA of *L. rhamnosus* GG, *Bifidobacterium longum*,[111] and VSL#3 probiotic mix[43] bacteria has been proved with HT-29 and T84 cultures, testing IL-8 secretion, attenuation of TNFα effect by reduction of IF-κBa degradation or NF-κB activation by electrophoretic mobility shift assay.[43]

Different proteins have been directly associated with the beneficial effect of some probiotics. Such is the case of the surface layer protein (Slp), a protein that is tightly packed at the surface of certain lactobacilli, shown to have lectin-like properties and antiinflammatory effect.[117] Many metabolic enzymes and regulatory proteins have been recently related to the functional properties of probiotics and have been named "moonlighting proteins" (REF). GroEL is a heat shock chaperone that has been cloned and overproduced in *E. coli*, showing that it binds to mucins and stimulates interleukin-8 secretion into test in HT-29 cells.[118] Other proteins have been characterized from *L. rhamnosus* GG (p49, p74) responsible for the inhibition of cytokine-induced apoptosis in epithelial cells[119] and prevent epithelial barrier damage in Caco-2 cells.[109]

Many Gram-positive bacteria of the family Clostridiaceae, propionibacteria, and bifidobacteria have a fermentative metabolism that yields organic acids such as lactate, acetate, butyrate, and/or propionate from simple sugars. Butyrate is known to be an excellent nutrient and repair agent for epitheliocytes, but

recently it was shown to be responsible for the reduction of the expression of TLR4 (involved in LPS recognition and native immunity) caused by *Clostridium butyricum* TO-A.[120] Also, *Propionibacterium acidipropionici* and *P. freudenreichii* were shown to induce apoptosis in human carcinoma cells (HT-29 and Caco-2), suggesting anticancer properties. Such effect was due to propionate and acetate produced by those strains.[121]

27.2.1.4.7 Anticancer and Other Effects

Other effects have been assigned to probiotics; however, evidences using *in vitro* systems are found for cytotoxic induction of apoptosis of cultured colon cancer cell lines used normally in cell culture experiments, which consequently could be considered anticancer activity. *Bifidobacterium adolescentis* SPM0212 could be demonstrated to inhibit a number of protoxigenic enzymes, such as α-glucuronidase, α-glucosidase, tryptophanase, and urease, but it can also inhibit HT-29, SW 480, and Caco-2 proliferation.[122] Short-chain fatty acids like propionate and acetate induce apoptosis in HT-29 and Caco-2 acting on adenine nucleotide translocator enzyme.[121]

Finally, through qPCR, it could be shown that *Lactobacillus acidophilus* NCFM induces opioid and cannabinoid receptors OPRM1 and CNR2, hence possibly modulating intestinal pain.[123]

27.2.1.4.8 Use of Cultured Lymphocytes

As opposed to the epithelial cell cultures, lymphocyte suspensions are heterogeneous mixtures of cell types. Whether they are extracted from blood (peripheral blood mononuclear cells, PBMCs), from mesenteric nodes, or from the lamina propria, different lymphocyte lines are always present. Frequently, they have been used as such, but fine studies describing cell differentiation processes induced by probiotics or their components require the previous separation of cell types. Monocytes, dentritic cells, T-cell subsets, and so on can be distinguished on the basis of specific surface markers—cluster of differentiation (CD*n*+) protocol, where *n* is a one or two digit number. Those markers correspond to ligands or receptors specifically recognized by monoclonal antibodies which can be used to quantify cell types by flow cytometry (see below) or even to selectively retain them using antibody-coated magnetic beads.

Flow cytometry is a technique that allows the evaluation of a population of particles or cells in suspension as function of their size and shape. Particles are forced to pass through a very small channel one at a time and to cross a laser light beam. As a consequence, light is refracted at all angles, so that the "forward scattering" of the light will determine the size and the "side scattering" will report information on the shape. Fluorescent detection techniques can be combined with flow cytometry, for instance, parts of the cell population can be labeled with fluorescent antibodies. Then, the sample can be illuminated with a certain wavelength and the emission of the fluorophor can be detected in the side scattered light through a series of filters.

There is still a method that combines epithelial cells and lymphocyte cultures as means to approximate to the intercellular communication processes occurring in the human mucosa. The Transwell coculture model[124] uses confluent monolayers of cultured epithelial cells, such as HT-29, Caco-2, or T84, in the upper (apical) side of a compartment, which is separated by a membrane filter from a lower compartment that is inoculated with 1.5 mL medium containing PBMC (2×10^6 cells/mL). Then the epithelial cells can be exposed to different stimuli, such as probiotics, added apically to the monolayer. The system allows proteins and other molecules to diffuse between both compartments but not cells. After 24 h, culture supernatants can be evaluated for cytokine secretion and immune cells are collected.

A number of representative references have been collected where either murine or human lymphocytes, most frequently PBMCs, or in occasions coculture systems, have been used to determine the effect of a very wide number of probiotic species and strains. The majority of the effects described in those works are related to the stimulation of innate and/or adaptive defense processes through the study of cell differentiation events, occurring during early stages of maturation of dendritic cells, and quantification of cell markers, IgA, and well-defined cytokines and other signaling molecules (Table 27.7). Also, some examples of experimental procedures have been found to illustrate procedures to evaluate the antiinflammatory effect of probiotics through the study of secreted signals by macrophages, DCs, and assessment of early stages of specialization of dendritic cells (Table 27.8). Furthermore, experimental procedures

TABLE 27.7

Stimulation of Innate and/or Adaptive Defence through the Study of Immune Cells and the Maturation Status of DCs

Probiotic Strain/s	Model System	Reference
L. acidophilus NCFM	Murine dendritic cells	159
L. casei DN-114001	Murine macrophages, dendritic cells, and goblet cells	160
L. casei	Mononuclear cells from Peyer patches	161
L. casei Shirota	Human PBMC	162
L. casei Shirota	Human PBMC natural killer (NK) cell activity	163,164
L. casei subsp. *casei* (symbiotic with dextran)	Human and murine PBMC	165
L. rhamnosus	DC from PBMC	166
L. paracasei subsp. *paracasei* B21060, *L. paracasei* subsp. *paracasei* F19, or *L. casei* subsp. *casei* DG	T cells purified from PBMCs and intestinal lamina propria (LP)	167
Bifidobacterium breve C50	Human DC from PBMC	168
Different gut bacteria	Human peripheral blood mononucleated cells (PBMCs)	169
VSL#3 mix (*L. acidophilus*, *Bifidobacterium longum*, *L. casei*, *Bifidobacterium breve*, *L. plantarum*, *Bifidobacterium infantis*, *L. bulgaricus*, *Streptococcus thermophilus*)	Human intestinal lamina propria mononuclear cells and PBMCs	170
Bio-Three (*Bacillus mesentericus*, *Clostridium butyricum*, *Enterococcus faecalis*)	Human DC from PBMCs	171
Bifidobacterium longum W11, NCIMB 8809, and BIF53	Human PBMC	172
L. acidophilus and *L. plantarum*	Human PBMC	173
L. rhamnosus HN001, *L. acidophilus* NCFM	Relative numbers of natural killer (NK) and NKT cells in human PBMCs	174
L. rhamnosus GG (LGG) and *Bifidobacterium breve* (Bb1, Bb2)	Transwell culture IEC (HT-29) with human PBMC	175
Bifidobacterium lactis W51, *L. acidophilus* W55, and *L. plantarum* W62	Induction of Treg cells in human PBMC	176
Mixture: (*L. acidophilus* W55, *L. casei* W56, *L. salivarius* W57, *Lactococcus* (*Lc.*) *lactis* W58, *Bifidobacterium* (*B.*) *infantis* W52, *Bifidobacterium lactis* W18, and *Bifidobacterium longum* W51	Human PBMC	177
Yogurt vs. probiotic	Determination in PBMC of volunteers of activated T cells	178
Bifidobacterium (*B*) *bifidum* W23*; Bifidobacterium breve* W6*; Bifidobacterium infantis* W52*; Bifidobacterium lactis* W18*; Bifidobacterium longum* W51*; Lactobacillus* (*Lb*) *brevis* W63*; Lb. casei* W56*; Lb. paracasei* W72*; Lb. plantarum* W59*; Lb. helveticus* W60*; Lb. rhamnosus* W71*; Lb. salivarius* W24*; Lactococcus* (*Lc*) *lactis* W58.	Human PBMCs, purified	179
VSL#3 mix (*L. acidophilus*, *Bifidobacterium longum*, *L. casei*, *Bifidobacterium breve*, *L. plantarum*, *Bifidobacterium infantis*, *L. bulgaricus*, *Streptococcus thermophilus*)	In mice, transfer of lamina propria mononuclear cell (LPMC) population from probiotic-treated mice to naive mice	180
Escherichia coli Nissle 1917	Human PBMC and lamina propria T cells. Toll-like receptor 2 (TLR-2) knockout mice	181
L. casei DN-114 001, *L. casei* no.2 DN-114 086, *L. rhamnosus* DN-116 047, *L. plantarum* DN-121 022, *Bifidobacterium animalis* DN-173 010, *Bifidobacterium adolescentis* DN-150 017, *Streptococcus thermophilus* DN-001 621, and *Bacteroides thetaiotaomicron*	Human DC from PBMC	182

TABLE 27.8

Anti-inflammatory Effect through the Study of Immune Cells

Probiotic Strain/s	Model System	Reference
Lactobacillus strains	Cultured intestinal T cells from Crohn's disease patients	183
Cocktail VSL#3	Murine bone marrow-derived dendritic cells	184
Bifidobacterium longum, *Bifidobacterium breve*, *Bifidobacterium bifidum*, and *Bifidobacterium animalis* subsp. *lactis*	Human DC from PBMC	185
Lactobacillus casei DN-114 001	Mucosal explants and mononuclear cells from Crohn's disease patients	186
VSL#3	Human PBMCs and intestinal lamina propria mononuclear cells	41
Lactobacillus plantarum 299	Intestinal lamina propria mononuclear cells	187
L. plantarum NIZO B253, *L. casei* NIZO B255, *L. reuteri* ASM20016	Human DCs from PBMCs. Transfection of HEK-293 cells	188
Saccharomyces boulardii	Human DC from PBMC	189
Lactobacillus plantarum	Murine dendritic cells (DC)	190

have also been found describing the probiotic effect on the primary prevention of allergic and modulation of allergic response (Table 27.9).

27.2.4.2 Intestine Explants: Mice or Human Intestine

The use of cultured intestinal explants in an organ culture system allows the study of probiotic effects on a whole tissue level. The tissue preserves histological and immunohistochemical features for up to 2 days, [125,126] and histiotypic relationships and interactions between different cell types remain intact. The main advantage of this technique consists of the ethical use of human intestinal material obtained after surgery or for diagnostic use.

Standard conditions of cultured intestinal explants include a humidified atmosphere containing 5% CO_2. Usually, tissues were rinsed and washed with sterile PBS, and tissues were cut into pieces of ~3–4 mm^2 in size to ensure diffusion of nutrients into the tissue. The pieces were placed onto cell strainers or filter inserts (100–500 μm mesh size), orientated with the luminal side facing upward. The authors used different standard culture media, such as DMEM or RPMI 1640,[119,127,128] and the medium was added to the wells to a point just over the epithelium. Some authors preincubate tissue fragments with antibiotics (16 μg/mL vanomycin, 2500 U/mL colistin, 50 μg/mL gentamycin) during 3 h to

TABLE 27.9

Primary Prevention and Modulation of Allergic Response

Probiotic Strain/s	Model System	Reference
	Primary Prevention of Allergic Diseases	
Bifidobacterium bifidum, *Bifidobacterium infantis*, *Lactobacillus salivarius*, *Lactococcus lactis*	Neonatal DC cord blood PBMCs	191
Lactobacillus rhamnosus GG	PBMC and cord blood mononuclear cells (CBMCs)	192
	Modulation of Allergic Response	
Lactobacillus rhamnosus GG, *Lactobacillus gasseri* (PA16/8), *Bifidobacterium bifidum* (MP20/5), and *Bifidobacterium longum* (SP07/3)	PBMCs from allergic patients and from healthy donors	193
Lactobacillus acidophilus and *Escherichia coli* Nissle	PBMC from allergic and nonallergic volunteers	194

eradicate most of the indigenous microbiota.[127] At the end of the incubation period with different experimental treatments, medium is collected and samples are processed according the protocols for the different read-outs of interest (protein or transcriptome analysis, cytokine analysis, immunohistochemical analysis, etc.).

27.2.4.3 In Vivo Tests

A simple search in the Web page of the U.S. National Library of Medicine National Institutes of Health (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed) with the words "probiotic clinical trail" retrieved more than 1300 results. Certainly, some are reviews and from the rest not all are *in vivo* assays in humans, but those numbers are very indicative of the large amount of works that have been published after testing the probiotics in humans. This chapter has not considered including a review of observational methods used in the field of probiotics. They are very similar to those used in the study of drug effects. However, the effects studied are very variable and trials using probiotics not always study the effect on disease, but try to monitor the "general health status," which is a very broad and complex concept. However, a brief description of the general methodology will be made.

The EFSA recommends that, when possible, double-blind placebo-controlled trials are performed with sufficient number of volunteers, to achieve significant results. In those experiments, psychological factors must be eliminated by hiding the clinicians and the patients/volunteers the active samples from the placebo, so that only the coordinator team will have access to all the results at the end of the experiment.

Preliminary data with acceptable significance can be designed following a crossing-over design. This type of experiment requires a shorter number of volunteers, but it is longer in time, as it has three stages. First, the group of volunteers is divided in two groups, one taking the active sample and the other one is the control (placebo). After this, a washout period is required, so that the possible biological effect of the active sample is lost and, in case of probiotics, strains that could colonize the gut are eliminated. Then, the roles are exchanges and the group that initially was administrated the active sample will now take the placebo, and vice versa.

In either case, criteria for inclusion of participants (age, health or disease condition, nutrition, etc.) must be very well established, and also the exclusion criteria (disease, drug, or antibiotic treatment).

Then, all the experimental parameters to be determined must be very clear to the clinicians (samples to be taken, determination of the symptoms profile to be followed and, if possible, quantified) and the laboratory personnel (analytical methods and number of determinations, etc.).

Other important elements of the trials are the correct information to the participant and their signature of an Informed Consent document and finally, the approval of the project by the corresponding Ethical Committee.

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Section VII

Phytoestrogens and Hormones

28

Anise Oil

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28.1 Anise—Star Anise—Anise Oil—Anethole

Anise (*Pimpinella anisum* L.) is a member of the Umbelliferae or Apiaceae. Other names for this species are anis or aniseed. It is a dainty, white-flowered annual plant with feather-like leaves. The part used are the fruits or seeds. The fruits yield after distillation 2.5–3.5% oil. *trans*-Anethole or (further in this text) anethole is the principal constituent, present to about 90% in the oil.

Star anise (*Illicium verum* (Hook, F.)) (star aniseed, badiane, Chinese star anise) from the Illiaceae or Magnoliaceae is named from the stellate form of its fruits. The essential oil resides in the pericarp. The dried fruits contain 5–8% essential oil. Overall, 85–95% of that oil is *trans*-anethole.

Oil of anise, distilled in Europe from the fruits of *P. anisum* and in Asia from the fruits of *Illicium anisatum*, is colorless to very pale yellow and has the taste and odor like the fruit. The oils of both plants are almost identical in composition. LD_{50} for anethole is 2090 mg/kg for rats, 3050 mg/kg for mice, and 2167 mg/kg for guinea pigs. Anise oil and anethole are irritants of the skin and eyes and through inhalation. Table 28.1 gives data on anise oil and anethole.

28.2 Extraction of Essential Oil

Conventionally, oils are extracted from plants by the steam distillation method. Steam is passed through the plant material. Other techniques have been evaluated for better extraction performances.

Clove bud and star anise volatile oils were isolated by supercritical CO_2 extraction coupled with a fractional separation technique (Della Porta et al., 1998). A good extraction performance was obtained operating at 90 bar and 50°C (for 630 min). The optimum fractionation was achieved in both cases by

TABLE 28.1

Nomenclature of Anethole

Compound	IUPAC-Name	Other Names	CAS-Number	Molecular Formula
Anise oil		Star anise oil	8007-70-3	
(E)-Anethole	(E)-1-methoxy-4-(1-propenyl)-benzene	*trans*-Anethole	4180-23-8	$C_{10}H_{12}O$

operating at 90 bar and −10°C in the first separator and at 15 bar and 10°C in the second. The influence of the extraction pressure and extraction time on the essential oil composition was further evaluated. A considerable difference in volatile oil composition was observed at increasing extraction times.

Star anise fruits were extracted with liquid CO_2 for essential oil (Tuan et al., 1997). Extraction with liquid CO_2 yielded 9.8% more essential oil than the conventional steam distillation method. The anethole contents obtained by the two methods were in the same range of 89–92% of the total oil.

The physical properties and chemical components of oil samples extracted from star anise by steam distillation (SD), solvent extraction (SE), and supercritical fluid extraction (SFE) were compared and analyzed by gas chromatography-mass spectrometry (Wang et al., 2007). The quality parameters of star anise essential oil from SFE were close to that of those from SD and SE. Although the extraction yield of star anise by SFE (9.2%) was close to the value from SE (9.3%), it was higher than that from SD (8.2%). For sensory evaluation, however, the three oils were significantly different. The odor and taste of the products from SFE and SE were generally more natural and vivid than the products of the distilled oil. The volatile compound revealed that significant differences of the composition existed in the distilled oil and the oleoresins prepared by SFE and SE.

A new and rapid headspace solvent microextraction (HSME), for the extraction and preconcentration of the volatile components of plant sample into a microdrop, was applied by Besharati-Seidani et al. (2005). The extraction occurred by suspending a microliter drop of the solvent from the tip of a microsyringe to the headspace of a ripe and powdered dry fruit sample (Iranian *P. anisum* seed) in a sealed vial for a preset extraction time. The microdrop was retracted back into the microsyringe and injected directly into a GC injection port. The chemical composition of the HSME extracts was confirmed according to their retention indexes and mass spectra (EI, 70 eV); and quantitative analysis was performed by gas chromatography-flame ionization detector.

Further details on extraction are discussed in Section 28.5.

28.3 Antioxidant Properties

Gülçin et al. (2003) studied the antioxidant and antimicrobial activities of water and ethanol extracts of anise seed. The antioxidant properties of both extracts were evaluated using different antioxidant tests, total antioxidant activity determination by the thiocyanate methods (Mitsuda et al., 1966), reducing power by the method of Oyaizu (1986), free radical scavenging activity by the 1,1-diphenyl-2-picryl-hydrazyl (DPPH) method (Blois, 1958), superoxide anion radical scavenging by the method of Liu et al. (1997), hydrogen peroxide scavenging according to the method of Ruch et al. (1989), and metal-chelating activity by the method of Dinis et al. (1994).

The extracts of anise seed had, for all evaluated parameters, strong activities compared with standards such as BHA (butylated hydroxyanisole), BHT (butylated hydroxytoluene), and α-tocopherol.

The antioxidant activity of the alcohol extracts (3 g/kg) of anise oils, dill seeds, and chamomile flowers in corn oil, soybean oil, beef tallow, and anhydrous butter fat was evaluated during storage at 65°C. The extent of oxidation was followed by peroxide value (PV) (Qasem and Al-Ismail, 2004).

Alcohol extracts of these plants were more effective as antioxidant than BHA (0.2 g/kg) in corn and soybean oils but less effective in beef tallow and anhydrous butter fat. Anise seed extract exhibited similar antioxidant activity as dill seed extract but had a lower-reducing activity. Chamomile flower extract was the most active as an antioxidant.

Essential oils from nine different species of Turkish plants including *P. anisum* L. were obtained by supercritical carbon dioxide extraction and steam distillation (Topal et al., 2008). Analysis was by GC-MS. The antioxidant activities of extracts were tested by means of the DPPH assay. Essential oils extracted by supercritical carbon dioxide extraction showed the highest antioxidant activities.

The acetone extract of star anise has shown excellent activity for the inhibition of primary and secondary oxidation products in rapeseed oil using peroxide, thiobarbituric acid, *p*-anisidine, and carbonyl values (Singh et al., 2006).

Other antioxidant tests (e.g., DPPH) confirmed antioxidant properties.

Two spices, star anise and black caraway, were extracted with different solvents and the relative antioxygenic activities of all fractions were evaluated, using different assays (Padmashree et al., 2006). Ethanol/water extracts exhibited higher antioxygenic activities.

The generation of free radicals is a cause of many pathological conditions such as diabetes mellitus, cancer, stroke, and so on. Free radicals cause damage to cellular DNA and initiate carcinogenesis. In the study by Yadav and Bhatnagar (2007), star anise was assessed for its anticarcinogenic potential in *N*-nitrosodiethylamine (NDEA)-initiated and phenobarbital (PB)-promoted hepatocarcinogenesis. Rats were randomly selected for eight experimental groups. The carcinogenesis was induced by injecting the rats with a single dose of NDEA (200 mg/kg body weight) intraperitoneally as initiator, followed by promotion with PB (0.05%) in drinking water for 14 consecutive weeks. The treatment with NDEA increased liver weight, while star anise treatment reduced the liver weight of rats. These results indicate that the treatment with star anise reduces the tumor burden, lowers oxidative stress, and increases the level of phase II enzymes, which may contribute to its anticarcinogenic potential.

Liu et al. (2008) studied *in vitro* antioxidant activities of 68 common Chinese herbals both for medical and food uses (including star anise), using Folin–Ciocalteu, ferric-reducing/antioxidant power (FRAP) and DPPH radical-scavenging assays. Six plant materials including Chinese white olive, clove, pricklyash peel, villous amomum fruit, Chinese star anise, and pagoda tree flower had highest total phenolic (>45 mg gallic acid equivalents (GAE)/g) and flavonoid content (>45 mg rutin equivalents (RE)/g), and had also the highest antioxidant activity (FRAP value > 2.5 mmol/g, DPPH radical-scavenging capacity > 85%), indicating they have potential for use as natural sources of antioxidant foods. The total phenolics content of these 68 plant extracts was significantly positively correlated ($r^2 = 0.9467$) with their antioxidant capacity.

28.4 Biocide Actions

28.4.1 Acaricidal Actions

Acaricidal activity of anise seed-isolated anisaldehyde, commercially available components of anise seed, and synthetic acaricides (benzyl benzoate, dibutyl phthalate, and *N*,*N*-diethyl-*m*-toluamide (DEET)) were examined against *Tyrophagus putrescentiae* adults (Lee, 2005). On the basis of LD_{50} values, anisaldehyde was the most toxic compound: $LD_{50} = 0.96$ μg/cm^2. Anethole, estragole, and myrcene had also acaracide properties.

28.4.2 Fungicidal Actions

GC-MS analysis of the volatile oil of the fruits of star anise revealed the presence of 25 components (Singh et al., 2006). Major components were *trans*-anethole (94.37%), methyl chevicol (1.82%), and *cis*-anethole (1.59%). Fifteen components were identified in its acetone extract: *trans*-anethole (51.81%), linoleic acid (11.6%), 1-(4-methoxyphenyl)-prop-2-one (6.71%), foeniculin (5.29%), and palmitic acid (1.47%). Using an inverted Petriplate method, the volatile oil completely inhibited the growth of *Fusarium moniliforme* at 6 μg/L. Inhibition of *Penicillium citrinum* and *Penicillium viridicatum* was also noticed. Other tests revealed activity against different bacteria.

Matan et al. (2008) investigated the antifungal activities of anise oil, lime oil, and tangerine oil against molds identified from rubberwood surfaces (*Aspergillus niger*, *Penicillium chrysogenum*, and *Penicillium*

sp.). The minimum inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) were determined using concentrations of essential oils between 20 and 200 μL/mL and using the broth dilution method, the dip treatment, and vacuum impregnation treatment. Anise oil was the strongest inhibitor with MIC and MFC at concentrations of 40 μL/mL against *Penicillium* sp. and *Aspergillus niger*, and 60 μL/mL against *P. chrysogenum*.

The antifungal activity of *P. anisum*, boldus, mountain thyme, clove, and griseb (poleo) essential oils against *Aspergillus* section *Flavi* was evaluated in sterile maize grain under different water activity (a_w) conditions (0.982, 0.955, and 0.90) (Bluma and Etcheverry, 2008). The effect of essential oils added to maize grains on growth rate, lag phase, and aflatoxin B_1 (AFB_1) accumulation of *Aspergillus* section *Flavi* were evaluated at different water activity conditions. The five essential oils analyzed have been shown to influence lag phase and growth rate. Their efficacy depended mainly on the essential oil concentrations and substrate water activity conditions. All essential oils showed significant impact on AFB_1 accumulation. This effect was closely dependent on the water activity, concentration, and incubation periods.

Özcan et al. (2006) determined the composition of the essential oil of *P. anisum* L. fruit by GC and GC-MS. The volatile oil content obtained by hydrodistillation was 1.91%. Ten compounds representing 98.3% of the oil were identified. The main constituents were *trans*-anethole (93.9%) and estragole (2.4%). Other constituents that were found with concentrations higher than 0.06% were (*E*)-methyeugenol, α-cuparene, α-himachalene, β-bisabolene, *p*-anisaldehyde, and *cis*-anethole.

Different concentrations of anise oil exerted varying levels of inhibitory effects on the mycelial growth of *Alternaria alternata, Aspergillus niger*, and *Aspergillus parasiticus*. The results showed that the most affected fungus by anise oil was *A. parasiticus*.

In the work of Dzamic et al. (2009), the essential oils of star anise and *Eugenia caryophyllata* were investigated as potential antifungal agents. Star anise essential oil exhibited fungicidal characteristics with MIC and MFC within a concentration range of 2.5–25 μL/mL.

28.4.3 Antimicrobial Actions

Antimicrobial activity against Gram-positive and Gram-negative bacteria was noticed when comparing with standard and strong antimicrobial compounds such as miconazole nitrate, amoxicillin-clavulanic acid, ofloxacin, and netilmicin (Gülçin et al., 2003). The method used here was disk diffusion method and the bacterial species were *Staphylococcus aureus* and *Pseudomonas aeruginosa* and the yeast *Candida albicans*.

Kubo et al. (2008) tested antimicrobial activities of anethole, anisic acid, and eugenol from aniseed against 18 organisms (bacteria and yeasts). Anethole was effective against *Saccharomyces cerevisiae* with a minimum fungicidal concentration (MFC) of 200 μg/L, but the activity was observed only when the yeast was growing on fermentable carbon sources in a hypoxic condition. On the other hand, eugenol was effective against *S. cerevisiae* with an MFC of 800 μg/L in any growing condition. Anisic acid showed fungistatic activity against the same yeast with an MIC of 400 μg/L but not fungicidal up to 1600 μg/L.

28.4.4 Insecticidal Actions

The fumigant activity of essential oil vapors distilled from anise *P. anisum* and other herbs were tested against eggs of two stored-product insects, the confused flour beetle, *Tribolium confusum*, and the Mediterranean flour moth, *Ephestia kuehniella* (Tunç et al., 2000). The exposure to vapors of essential oils from anise and cumin resulted in 100% mortality of the eggs. At a concentration of 98.5 μL anise essential oil/L air, the LT_{99} values were 60.9 and 253.0*h* for *E. kuehniella* and *T. confusum*, respectively.

Burgess et al. (2010) have found that coconut oil and anise spray gave superior efficacy for elimination of head louse infestation than 0.43% permethrin lotion.

Essential oils derived from celery, caraway, zedoary, long pepper, and Chinese star anise were investigated for adulticidal activity against mosquito vectors (Chaiyasit et al., 2006). All five essential oils exerted a promising adulticidal efficacy against both laboratory and natural field strains of *Aedes aegypti*. Although the laboratory strain was slightly more susceptible to these essential oils than the natural field strain, no statistically significant difference was observed.

The highest potential was established from caraway, followed by zedoary, celery, long pepper, and Chinese star anise, with an LC_{50} in the laboratory strain of 5.44, 5.94, 5.96, 6.21, and 8.52 μg/mg female, respectively, and 5.54, 6.02, 6.14, 6.35, and 8.83 μg/mg female, respectively, in the field strain. These essential oils may be, therefore, alternative mosquito adulticides as an effective measure used in controlling and eradicating mosquito vectors.

Vapors of essential oils extracted from cumin, anise, oregano, and eucalyptus were found to be toxic to two greenhouse pests, namely the carmine spider mite, *Tetranychus cinnabarinus* (Boisd.) and cotton aphid, *Aphis gossypii* Glov (Tunç et al., 1998). A minimum dose of 0.5 μL/L air and 2–3 days of exposure was required for 99% mortality using essential oils of the first three plants.

28.4.5 Antiviral Actions

Essential oils from star anise hyssop, thyme, ginger, chamomile, and sandalwood were screened for their inhibitory effect against herpes simplex virus type 2 (HSV-2) *in vitro* on RC-37 cells using a plaque reduction assay (Koch et al., 2008). Antiviral agents currently applied for the treatment of herpes virus infections include acyclovir and its derivatives. The inhibitory concentrations (IC_{50}) were determined at 0.016%, 0.0075%, 0.007%, 0.004%, 0.003%, and 0.0015% for anise oil, hyssop oil, thyme oil, ginger oil, chamomile oil, and sandalwood oil, respectively. A clearly dose-dependent virucidal activity against HSV-2 could be demonstrated for all essential oils tested. The results indicate that essential oils affected HSV-2 mainly before adsorption probably by interacting with the viral envelope.

28.5 Chromatographic Analysis

28.5.1 Oils of Star Anise or Anise

Volatile oils from star anise were obtained by steam distillation (Padmashree et al., 2007). Gas chromatographic analysis was performed on a Chemito GC 1000 HR (M/S Chemito, India) system (column: BP-5 (30 m × 0.25 ID—0.25 μm thickness)) connected to a flame ionization detector. Mobile phase was helium gas (30 cm/s). Injector and detector temperatures were kept at 230°C and 250°C, respectively. Sample components were identified by electron impact mass spectrometry.

Table 28.2 depicts the flavor profile of anise and star anise volatile oil from different studies.

Wang et al. (2009) used ultrasonic nebulization extraction (UNE) in combination with online gas chromatography for the determination of *trans*-anethole in fruits of star anise. In the UNE process, volatile compounds are transferred from a liquid or solid matrix to the gas phase by an inert gas flow. The extraction efficiency was compared with ultrasonic-assisted extraction (UAE) and hydrodistillation (HD). Extracts were analyzed with an HP-1 column (30.0 m × 250 μm × 0.32 μm film thickness). Nitrogen was used as the carrier gas at a flow rate of 4 mL/min.

The various fractions from clove bud and star anise obtained in different extraction and fractionation conditions were examined by GC-MS by Della Porta et al. (1998). The GC-MS was a Varian capillary GC instrument (DB-5 fused-silica column, 30 m × 0.25 mm ID, 0.25 μm). Helium was the carrier gas at 1 cm^3/min. The GC instrument was connected to an ion trap detector.

The volatile compounds of different species of *Ilicium* including Chinese star anise were analyzed by thermal desorption-GC-MS (TD-GC-MS) (Howes et al., 2009). Authors identified (E)-anethole (57.6–77.1%) and foeniculin in the pericarps.

Aniseed was extracted by SFE (CO_2—30°C—pressures of 80–180 bar) and these extracts were analyzed by GC-MS, GC-FID, and thin-layer chromatography (Rodrigues et al., 2003). The major compounds identified were

- Anethole (90%)
- γ-himachalene (2–4%)
- *p*-anisaldehyde (<1%)
- Methylchavicol (0.9–1.5%)

TABLE 28.2

Flavor Profile of Anise and Star-Anise Volatile Oil (in %)

	Star Anise (Padmashree et al., 2007)	Star Anise (Singh et al., 2006)	Star Anise (Dzamic et al., 2009)	Anise (Tabanca et al., 2006)	Anise (Rodrigues et al., 2003)
α-pinene	0.12		0.13		
β-pinene	0.03				
Myrcene	0.02				
α-phellandrene	0.04		0.11		
β-carene	0.15				
α-terpinene	0.02				
p-cymene	0.05		0.06		
Limonene	1.05		0.67		
trans-ocimene	0.09				
cis-β-ocimene	0.01				
Γ-terpenene	0.04				
Terpinolene	0.03				
Linalool	0.29		0.79		
Γ-terpineol	0.12				
4-Terpineol	0.09		0.12		
α-terpineol	0.08		0.21		
Esteragole	1.05		3.68		
cis-anethole (*Z*-anethole)	0.14	1.59	0.41	0.20	
trans-anethole	93.9	94.37	90.82	94.20	90.0
α-cubenene	0.10				
β-clemene	0.01				
β-caryophyllene	0.10		0.20		
Bergamotene	0.01				
Δ-cardimene	0.04				
A-cardinol	0.02				
Methyl chavicol		1.82		2.00	0.9–1.5
γ-chimachalene				1.40	2–4
α-zingiberene				0.20	
Valensene				0.20	
β-bisabolene				0.20	
Anisaldehyde				0.40	<1
Pseudoisoeugenyl-2-methyl butyrate				0.70	3 (*cis*) 1.3 (*trans*)
Hexadecanoic Acid				0.30	
Anisylacetone			0.24		
trans-α-bergamotene			0.17		
α-muurolol			0.07		
Foeniculin			1.00		

- *cis*-pseudoisoeugenyl-2-methylbutyrate (3%)
- *trans*-pseudoisoeugenyl-2-methylbutyrate (1.3%)

Samples of *Pimpinella* species were collected from different regions of Turkey (Tabanca et al., 2006). Air-dried fruits, stems and leaves, and roots were crushed separately using a mortar followed by water

distillation for 3 h using a modified Clevenger-type apparatus to obtain essential oils. These oils were analyzed by

1. HP GC 6890 system with an HP-Innowax FSC column (60 m × 0.25 mm ID, 0.25 μm film thickness). The carrier gas was nitrogen at 1 mL/min. Detection was by flame ionization detection.
2. HP G1800A GCD system with an HP-Innowax FSC column (60 m × 0.25 mm ID, 0.25 μm film thickness). The carrier gas was helium at 0.7 mL/min. Mass spectra were recorded at 70 eV.

Compounds identified are depicted in Table 28.2. Total identified compounds were 99.8%.

The GC-FID (Besharati-Seidani et al., 2005) analysis of the volatile components of headspace solvent microextraction was performed using a GC-14B (Shimadzu) gas chromatograph equipped with a flame ionization detector. Separations were performed on a 25 m × 0.33 mm ID fused-silica capillary column with 0.5 μm BP-5 (5% biphenyl + 95% polydimethylsiloxane) coating. The injector and detector temperatures were 270°C and 280°C, respectively.

The GC-MS analysis was performed using a Thermo Quest gas chromatograph equipped with a mass detector. The column, used for separation of the extracts, was a fused silica DB-1 (polydimethylsiloxane) capillary column, 60 m × 0.25 mm ID × 0.25 μm film thickness. The injector temperature was 250°C.

Moisture, protein, ash, acid-insoluble ash, and nonvolatile ethyl ether extracts of anise seeds were determined (Karaali and Basoglu, 1995). Essential oils were obtained by water, water-steam, and steam distillation and then they were gas chromatographed (Varian 2100) using a flame ionization detector. The column used was a stainless-steel Carbowax 20M-packed column (2 m × ¼").

The percentages of *trans*-anethole and *cis*-anethole ranged from 86.2% to 89.0% and from 1.5% to 5.8%, respectively.

Anise may contain a few mono- and sesquiterpenes (Burkhardt et al., 1986). In a 1986 study, the composition of the hydrocarbon fractions obtained from the essential oil of fruits and shoots, focusing on the sesquiterpenes was compared.

A measure of 100 g of *P. anisum* fruits was dissolved in 400 mL of CH_2C1_2 and homogenized. After filtration, the procedure was repeated twice and the combined extracts were concentrated to dryness *in vacuo*. The residue was taken up in *n*-hexane and chromatographed over silica gel using *n*-hexane as an eluent. The eluted hydrocarbon fraction was concentrated and checked by TLC (silicagel/*n*-hexane, –18°C, detection: anisaldehyde/sulfuric acid) to give seven distinct red spots.

GC was carried out on a Packard instrument model 433 using a 25 m OV 101-fused silica capillary column; nitrogen flow: 3 mL/min. Structures elucidation was by MS and ^{1}H-NMR.

Composition of sesquiterpene fractions (%) in anise fruit oil was

α-himachalene	5.32
β-himachalene	6.95
γ-himachalene	73.99
β-bisabolene	4.90
δ-elemene	1.09
ar-curcumene	1.39
Copaene	0.32

28.5.2 Determination of Anethole in Aniseed Drinks

The European Commission (EC, 2002) proposed in EC regulation No. 2091/2002 the determination of *trans*-anethole in spirit drinks by gas chromatography using 4-allylanisole as an internal standard. For further details see that regulation (EC, 2002).

If aniseed spirits contain high sugar content, there is a drawback because samples contaminate the injection liner of the GC.

Jurado et al. (2006) worked out an LC method with photodiode array detector. The column was a Teknokroma C-18 column (250 × 4.6 mm, 5 μm particle size). Isocratic elution was performed using a mobile phase (80:20, *v/v*) methanol:water at a flow rate of 1 mL/min. Column temperature was set at 30°C and detection wavelength was 257 nm.

The content of anethole determined in commercial aniseed drinks varied between 4.04 and 0.125 g/L. The average content was 1.009 g/L.

28.6 Shikimic Acid

Star anise has recently been discovered to be a major source of shikimic acid (SA), a primary raw material used to produce the anti-influenza drug Tamiflu (Liu et al., 2009).

The extraction efficiency of ultrasound-assisted and Soxhlet extraction was compared. Results showed that ultrasonic extraction was as efficient as Soxhlet extraction but was more rapid and simpler.

GC-MS analysis was performed with a GC Trans Ultra/MS-DSQ system (Thermo Electron, San Jose, USA). Compounds were separated on a 30 m length × 0.25 mm ID × 0.25 μm film DB-5 MS capillary column (J&W Scientific, St Louis, MO, USA).

The method was used for analysis of SA in Chinese star anise from different areas in Wenshan state, Yunnan province. It was found that Chinese star anise collected from Pingbian and Funing counties contained more SA than other samples, with two plants from Pingbian county containing >15% SA.

RP-LC-UV (reverse phase-liquid chromatography-ultraviolet) and LC-MS-TOF (liquid chromatography-mass spectrometry-time of flight) methods were developed for the determination of SA from the methanol extract of the fruits of star anise and various other plants (Avula et al., 2009). Separation for RP-LC-UV was achieved on a Premisphere NH_2 (250 × 4.6 mm, 5 μm particle size) column at 30°C. The mobile phase consisted of 10 mM KH_2PO_4 (pH = 4.8) (solvent A) and MeOH (solvent B) at a flow rate of 1.0 mL/min, the gradient program (linear change): 50% A/50% B hold for 12 min to 100% B in 8 min. Detection wavelength was 210 nm.

For LC-MS-TOF, the column used was a SynergiMax RP column (150 × 4.6 mm ID, 4 μm particle size). The mobile phase consisted of water with 0.1% acetic acid (A) and acetonitrile with 0.1% acetic acid (B) at a flow rate of 0.5 mL/min, with a gradient elution as follows: 0 min, 95% A/5% B held for 3 min to 100% B in the next 7 min. The mass detector was a time-of-flight with an electrospray ionization interface. The contents of SA in various species of *Illicium* were between 3.72% and 24.05% by LC-UV and between 3.56% and 24.81% by LC-MS-TOF.

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29

Occurrence and Analysis of Melatonin in Food Plants

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29.1 Introduction

In vertebrates, the essential amino acid L-tryptophan is the precursor of 5-methoxyindoleamines, or indoleamines/tryptamines, including melatonin (*N*-acetyl-5-methoxytryptamine), through the intermediate serotonin (5-HT, 5-hydroxytryptamine) and the activity of hydroxyindoleamine-*O*-methyltransferase (HIOMT) (Figure 29.1) (Axelrod and Weissbach 1961; Hardeland 2008). In mammals, melatonin is synthesized in the pineal gland, predominantly during the nighttime, though it can be also produced in other organs, such as retina, gastrointestinal tract, lymphocytes, and bone marrow cells. Conversely, light at night has an inhibitory effect on pineal melatonin biosynthesis which is initiated by the uptake of tryptophan from the circulation into pinealocytes (Reiter 1991; Hardeland 2008). Once synthesized, melatonin is not stored in the pineal cells, but it is released into the bloodstream with a circadian rhythm, from which it reaches other body fluids, including urine, saliva, cerebrospinal fluid, bile, semen, and amniotic fluid (Tan et al. 1999; Tamura et al. 2009). The circadian rhythm of melatonin secretion is generated by the biological clock, situated in the suprachiasmatic nucleus of the hypothalamus, via a neuronal pathway that begins in the retina and involves the retinohypothalamic tract (Reuss 2003). In mammals, melatonin acts in part via membrane receptors MT1 and MT2 (Dubocovich and Markowska 2005). Physiological processes regulated by melatonin presumably via a receptor-mediated mechanism include the control of the sleep/wake cycle, modulation of reproductive function, and bone metabolism (Reiter et al. 2007a). Apart from these receptor-mediated processes, melatonin and its metabolites exert a series of receptor-independent functions, mainly due to their powerful antioxidant activity (Tan et al. 1993; Peyrot and Ducrocq 2008; Hardeland et al. 2009). Melatonin can directly scavenge free radical species (both reactive oxygen and nitrogen species) and stimulate the activity of antioxidant enzymes (Reiter et al. 2009).

Although the biosynthetic pathway of melatonin has only been defined in vertebrates, its presence has been detected also in other nonvertebrate organisms (bacteria, fungi, and insects).

Outside the animal kingdom, melatonin was discovered, for the first time, in the photosynthesizing unicellular alga *Lingulodinium polyedrum* (Stein) J. D. Dodge sin. *Gonyaulax polyedra* Stein, belonging to the phylum Dinoflagellata (Balzer and Hardeland 1991, 1996; Poeggeler et al. 1991; Hardeland and Fuhrberg 1996; Hardeland and Poeggeler 2003). Since then, it was detected in higher plants, including food plants and medicinal herbs, in which the physiological and pathophysiological function of melatonin

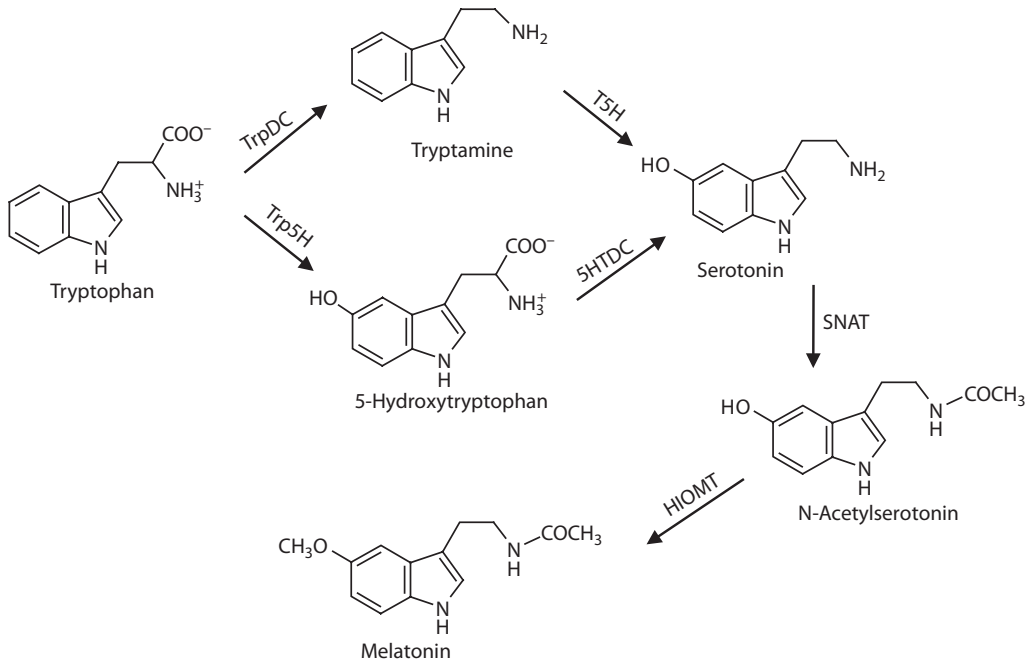

FIGURE 29.1 Biosynthetic pathway of melatonin in mammals. Enzymes involved: TrpDC, tryptophan decarboxylase; Trp5H, tryptophan 5-hydroxylase; 5HTDC, 5-hydroxytryptophan decarboxylase; T5H, tryptamine 5-hydroxylase; SNAT, serotonin *N*-acetyltransferase; and HIOMT, hidroxyindole *O*-methyltranferase.

is still unclear. Because of its structural similarity with the plant growth hormones of the auxin family, a hormone-like role has been attributed to melatonin in some plant species, as well as an action in delaying flowering, preventing chlorophyll degradation, protecting against oxidative damage, abiotic stresses, pathogens, and environmental pollutants (Arnao and Hernández-Ruiz 2006; Paredes et al. 2009; Posmyk and Janas 2009).

In this chapter, we briefly focus on the occurrence of melatonin in edible plants, with emphasis on the analytical methods suitable for assessing it in different food matrices.

29.2 Melatonin in Plant Kingdom

There are some evidences suggesting that plants are equipped with the molecular machinery for melatonin biosynthesis. The first study on the melatonin biosynthesis in plants, *Tanacetum parthenium* (L.) Sch. Bip. (feverfew) and *Hypericum perforatum* L. (St John's wort), was reported by Murch et al. (1997). The rather high quantities of the indoleamine that the authors detected in these medicinal plants led them to investigate its biosynthetic pathway in St John's wort, quantifying the incorporation of radiolabel from tryptophan into auxin and other indole metabolites under low and supplemented light conditions. The isotope tracer method showed that the carbon skeleton of ^{14}C-tryptophan was incorporated into serotonin and melatonin in higher plants. Furthermore, under low light conditions, more radiolabeled serotonin was recovered than melatonin, and, conversely, as the light intensity increased, this ratio was reversed, suggesting that the metabolism of tryptophan to melatonin is influenced by light intensity (Murch et al. 2000).

Although there were some preliminary indications (Van Tassel et al. 1993, 1995; Kolár and Macháčková 1994; Kolár et al. 1995), the first complete publications reporting melatonin in tracheophytes (vascular or higher plants) were independently provided by two research groups (Dubbels et al. 1995; Hattori et al. 1995). They found it in a number of edible plants and, since then, melatonin has been detected and quantified in roots, shoots, leaves, fruits, and seeds of a considerable variety of spermatophyte species,

as attested by numerous publications, confirming undoubtedly the presence of this molecule in the plant kingdom (Manchester et al. 2000; Reiter et al. 2001, 2007b; Reiter and Tan 2002; Caniato et al. 2003; Chen et al. 2003; Kolár and Machácková 2005).

In flowering plants (angiosperms), the occurrence of melatonin has been described in a number of families belonging to both the mono- and dicotyledons, relevant in terms of both abundance in the Plant Kingdom and nutritional value (Table 29.1) (Tettamanti et al. 2000; Reiter et al. 2001; Reiter et al. 2007b; Iriti et al., 2010). These families include the Apiaceae, Asteraceae, Brassicaceae, Fabaceae, Lamiaceae, Rosaceae, and Solanaceae among dicotyledons, Alliaceae, Poaceae, and Zingiberaceae for the monocotyledons. In spite of the large diffusion of melatonin in angiosperms, potato tuber is, to date, the only plant organ that has been reported to be devoid of detectable levels of the indoleamineamine (Dubbels et al. 1995; Badria 2002). Excluding angiosperms, the available information on the presence of melatonin in other plants is rather scarce. In fact, its occurrence has not yet been investigated in other groups of plants such as gymnosperms, ferns, and mosses.

Melatonin concentration in plants varies considerably not only from species to species, but also among varieties of the same species and in different organs of a given plant, usually ranging from pg g^{-1} to μg g^{-1} of tissue. In general, seeds and flowers present the highest levels of melatonin, whereas fruits, the lowest (Dubbels et al. 1995; Hattori et al. 1995; Kolár et al. 1997; Manchester et al. 2000; Van Tassel et al. 2001; Reiter and Tan 2002; Chen et al. 2003; Cole et al. 2008). Intriguingly, in reproductive organs, the most vulnerable to oxidative insults due to environmental pollution or UV irradiation, melatonin concentrations are several orders of magnitude higher than those normally measured in vertebrate tissues (except for the pineal gland) (Manchester et al. 2000; Afreen et al. 2006).

29.3 Analysis of Melatonin in Food Plants

The main problems concerning a reliable analysis of melatonin in edible plants are related to the sampling of plant material and to the subsequent extraction, identification, and measurement of melatonin itself in plant tissues (Reiter et al. 2001; Kolár and Machácková; Pape and Lüning 2006; Cao et al. 2006; Hardeland et al. 2007; Garcia-Parrilla et al. 2009). In general, aspects regarding melatonin detection in plants have slowed down research in this field, because scientists in plant and food science have tried to directly adopt methods from vertebrate melatonin analysis to plants. In fact, taking samples of animal blood or urine is simple, nondestructive, and results in relatively clean samples that can often be assayed directly for the presence of melatonin. On the contrary, plant samples have normally to be destructively collected, extracted by complex solvent mixtures and extensively purified before the indolic compounds can be determined (Van Tassel and O'Neill 2001). These procedures usually involve the production of a high level of oxidants, such as H_2O_2 and radicals deriving from it that can easily lead to melatonin degradation (Poeggeler and Hardeland 1994). The lack of efficient techniques to mix larger pieces of plant tissues with preserving solutions before shock-freezing, a step normally required in these determinations, represents another critical point (Hardeland and Poeggeler 2003). As an example, when comparing the melatonin content of fresh leaves from *T. parthenium* (L.) Sch. Bip. with freeze-dried and oven-dried leaves, Murch et al. (1997) observed losses during the drying process of 15% and 30%, respectively.

Besides sampling, analysis of melatonin in food plants presents some difficulties related to the extraction methods used to recover melatonin from plant material, and to the particular molecular constituents of plant tissues. Several authors have suggested that the extraction procedures from different matrices may pose a problem in the measurement of melatonin levels in plants, regardless of the subsequent quantification method used. In particular, the physicochemical properties of melatonin have been mentioned as a possible explanation for the reported variations in the amounts of melatonin in different plant extracts (see Section 29.4). Careful handling of the sample represents an essential prerequisite, because melatonin is a rather unstable molecule and a potent antioxidant able to react quickly with other food constituents. Furthermore, its amphipathic characteristics make difficult to choose a solvent yielding a nearly complete recovery. In plant material, solid–liquid extractions with different organic solvents (methanol, ethanol, ethyl acetate, ether, acetone, or perchloric acid) have been reported (Poeggeler and Hardeland 1994; Van

TABLE 29.1

Melatonin Content in Plant-Derived Foodstuffs Detected by Different Analytical Methods

Food Group Common Name	Melatonin Content	Analytical Method	Reference
Grape Products			
Grape	0.005–0.965 ng g^{-1}	ELISA, HPLC-F	Iriti et al. (2006b)
Red wine (Italian)	0.5 ng mL^{-1}	HPLC-F	Mercolini et al. (2008)
Red wine (Spanish)	50–80 pg mL^{-1}	ELISA	Guerrero (pers. comm.)
Red wine riserva (Spanish)	200 pg mL^{-1}	ELISA	Guerrero (pers. comm.)
White wine (Italian)	0.4 ng mL^{-1}	HPLC-F	Mercolini et al. (2008)
White wine (Spanish)	~50 pg mL^{-1}	ELISA	Guerrero (pers. comm.)
Sherry wine (Spanish)	<10 pg mL^{-1}	ELISA	Guerrero (pers. comm.)
Spirits			
Whisky, rum, vodka, gin	<15 pg mL^{-1}	ELISA	Guerrero (pers. comm.)
Commercial beers	52–170 pg mL^{-1}	ELISA	Maldonado et al. (2009)
Olive Oils			
Extra virgin D.O.	71–119 pg mL^{-1}	ELISA	de la Puerta et al. (2007)
Refined	53–75 pg mL^{-1}	ELISA	de la Puerta et al. (2007)
Sunflower oil	50 pg mL^{-1}	ELISA	de la Puerta et al. (2007)
Fruits			
Tart cherry	2–13.5 ng g^{-1}	HPLC-EC	Burkhardt et al. (2001)
Cherry P.D.O.	0.006–0.22 ng g^{-1}	LC/MS	Gonzales-Gomez et al. (2009)
Banana	0.46 ng g^{-1}	RIA, GC/MS	Dubbles et al. (1995)
Apple	0.05 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Pineapple	0.04 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Kiwi	0.02 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Vegetables			
Tomato (T5)	2.5–17 pg g^{-1}	GC/MS	Van Tassel et al. (2001)
Tomato (T5)	14.0 pg g^{-1}	RIA	Van Tassel et al. (2001)
Tomato	1.2 ng g^{-1}	HPLC-F	Pape and Lüning (2006)
Tomato (Microtom)	2.8–66.6 ng g^{-1}	ELISA	Okazaki and Ezura (2009)
Tomato (Rutgers)	0.16 ng g^{-1}	RIA, GC/MS	Dubbels et al. (1995)
Tomato (Sweet 100)	0.5 ng g^{-1}	RIA, GC/MS	Dubbels et al. (1995)
Tomato	0.03 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Cabbage	0.1 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Chinese cabbage	0.1 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Carrot	0.06 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Asparagus	0.01 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Onion	0.03 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Cucumber	0.02 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Turnip	0.7 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Red radish	0.6 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Grains			
Rice	1 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Oat	1.8 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Corn	1.4 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Barley	0.4 ng g^{-1}	RIA, HPLC-F	Hattori et al. (1995)
Purple wheat	4 μg kg^{-1}	LC-MS/MS	Hosseinian et al. (2008)
Seeds of edible plants	2–189 ng g^{-1}	RIA, HPLC-EC	Manchester et al. (2000)

TABLE 29.1 (continued)

Melatonin Content in Plant-Derived Foodstuffs Detected by Different Analytical Methods

Food Group Common Name	Melatonin Content	Analytical Method	Reference
Nuts			
Walnuts	3.5 ng g^{-1}	HPLC-EC	Reiter et al. (2005)
Almonds	39 ng g^{-1}	RIA, HPLC-EC	Manchester et al. (2000)
Pulse			
Lupine roots	24 ng g^{-1}	HPLC-F	Arnao and Hernandez-Ruiz (2009)
Lupine leaves	0.5 ng g^{-1}	HPLC-F	Arnao and Hernandez-Ruiz (2009)
Chinese medicinal herbs	12–3771 ng g^{-1}	HPLC-F, LC/MS	Chen et al. (2003)

Notes: Radioimmunoassay, RIA; enzyme-linked immunosorbent assay, ELISA; high-performance liquid chromatography with electrochemical detection, HPLC-EC; high-performance liquid chromatography with fluorimetric detection, HPLC-F; liquid chromatography coupled to mass spectrometry, LC/MS; gas chromatography coupled to mass spectrometry, GC/MS; and liquid chromatography-tandem mass spectrometry, LC-MS/MS.

Tassel and O'Neill 2001; Pape and Lüning 2006; Hardeland et al. 2007), as well as liquid–liquid extraction (Reiter et al. 2005; De la Puerta et al. 2007). The recovery rate of the indoleamine was evaluated by Arnao and Hernández-Ruiz (2009) comparing direct sample extraction with homogenized sample extraction. In both roots and leaves of *Lupinus albus* L. and *Hordeum vulgare* L., they found higher recovery rates with the direct sample procedure (more than 90%) than with the homogenized sample extraction, where the percentage of recovery was around 50%.

As previously introduced, because of the highly variable content of melatonin in food plants (pg g^{-1}–μg g^{-1} dry weight, DW), any analytical method should be sensitive to these variations. The most common techniques used for melatonin determination in plant foods are both immunological, the radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA), and chromatographic, high-performance liquid chromatography (HPLC) with electrochemical (HPLC-EC) or fluorimetric (HPLC-F) detection ($\lambda_{exc} = 280$; $\lambda_{em} = 345$) (Hattori et al. 1995; Harumi and Matsushima 2000; Burkhardt et al. 2001; Hernández-Ruiz and Arnao 2008a; Hernández-Ruiz et al. 2004, 2005). Additionally, HPLC or gas chromatography (GC) coupled to mass-spectrometry (MS) identification represents a powerful tool for the precise determination of melatonin in plant samples (Kolár et al. 1995). Importantly, organic solvents must be evaporated before RIA analysis to avoid antibody denaturalization.

The complex chemistry of plant tissues, which often contain great amounts of carbohydrates, lipids, and a great deal of other secondary metabolites (Iriti and Faoro 2009), makes resolution difficult, inducing, in some cases, false-positive or false-negative results. Unlike animal samples (urine, plasma, and saliva), the chemical complexity of plant extracts can interfere with the measurements, since other pigments (chlorophylls, carotenoids, and phenolic compounds) may be coextracted and partitioned with the indoleamine. This might produce false-positive results possibly due to the coelution of similar analytes in HPLC or to the cross-reactivity of coextractives with antibodies of RIA or ELISA, thus explaining several contradictory results obtained from plant materials of the same species quantified with different methods (Van Tassel and O'Neill 2001; Caniato et al. 2003; Hardeland and Poeggeler 2003; Kolár and Machácková 2005; Pape and Lüning 2006; Hardeland et al. 2007). As a consequence of these problems and of the variety of techniques and methods used, the data and concentrations reported in the literature might be, in some cases, under- or overestimated.

Considering the difficulty in melatonin extraction and quantification from plants and the high degree of interference caused by melatonin-immunodetection kits (generally developed for human samples) when using plant extracts, liquid chromatography-tandem mass spectrometry (LC-MS/MS) has been introduced as a rapid and accurate method to identify the molecule in plants, though this is a comparatively costly approach (Cao et al. 2006). LC-MS/MS with electrospray ionization has been designed as a quick method for the reproducible detection and quantification of melatonin, especially for low amounts of the compound (Cao et al. 2006). The limit of melatonin detection in plant extracts was 5 pg mL^{-1} and

the limit of quantification was 0.02 ng mL^{-1} (Cao et al. 2006). In any case, HPLC-EC and HPLC-F seem to be efficient methodological options to this sophisticated technique. This is the case of the work by Mercolini and colleagues, who have optimized an analytical method based on HPLC-F detection for the determination of melatonin (and resveratrol) in red and white Italian wines (Mercolini et al. 2008).

Finally, some authors have highlighted another difficulty related to the partitioning of melatonin in plant cells. Since it is not yet clear how much of the indoleamine resides in the large central vacuole, as compared to the cytoplasm, determinations of melatonin using fresh weight (FW) or DW as reference values may produce misleading results (Hardeland and Poeggeler 2003; Hardeland et al. 2007).

29.4 Occurrence of Melatonin in Edible Plants

As previously introduced, examination of reports on melatonin in plant materials shows that the quantity of the indoleamine varies widely according to the plant species studied, its developmental stage, and within different tissues of a given plant. For example, in *Glycyrrhiza uralensis* Fischer, a medicinal plant traditionally used for its antiviral and antitumoral properties and also as a natural sweetener, melatonin has been detected in the root tissues of 3- and 6-month-old plants, but not in the root tissues of 1-month-old seedlings nor in the stem. In addition, exposure to UV–B stimulated melatonin synthesis in root tissues (Afreen et al. 2006). Tan et al. (2007) also found differences in the tissues of *Eichhornia crassipes* (Mart.) Solms (water hyacinth), with levels of melatonin much higher in flowers than in leaves. An opposite pattern was reported in *Datura metel* L. (devil's trumpet), where the average concentration detected in leaves was roughly fivefold higher than that of unopened flowers (Cao et al. 2006). In the leaves of *Portulaca oleracea* L. (purslane), a vegetable consumed mainly in the eastern Mediterranean region, high levels of melatonin in leaves have also been reported (Simopoulos et al. 2005).

Levels of melatonin in the range of ng g^{-1}–μg g^{-1} DW have normally been reported in medicinal plants, many of them indigenous to China, Mediterranean, and alpine regions (Murch et al. 1997; Tettamanti et al. 2000; Chen et al. 2003). Of particular interest is its presence (in the order of μg g^{-1} DW) in *T. parthenium* (L.) Sch. Bip. and *H. perforatum* L., medicinal herbs traditionally used as sleep modulators in human sleeping disorders, as antidepressants or to combat jet-lag (Murch et al. 1997, 2000). Melatonin was also found in many medicinal herbs commonly used in traditional Chinese medicine, in concentrations ranging from 12 to 3771 ng g^{-1} DW (Table 29.1) (Chen et al. 2003). Again, it is very interesting that the highest melatonin concentrations were observed in the herbs used to retard aging and to treat diseases related to oxidative stress (e.g., neurological disorders) (Chen et al. 2003).

Seeds have also been reported to possess high levels of melatonin (typically in the range of ng g^{-1} tissue), although with a considerable interspecific diversity, from 189 ng g^{-1} DW found in *Brassica hirta* Moench (white mustard) to only 2 ng g^{-1} DW detected in *Silybum marianum* (L.) Gaertn. (milk thistle) (Table 29.1) (Manchester et al. 2000). The lack of central vacuoles in seed cells and, therefore, a much higher proportion of cytoplasm might be the explanation for this variation. Other reasons might be due to differences in the water content of the seeds and to the genetic variability of the plants (Manchester et al. 2000). Probably, the rationale for the rather high levels of melatonin in seeds is functional, to protect the delicate and lipid-rich tissues of the embryo from oxidative stress. Higher melatonin concentrations in the seeds would be necessary as they provide antioxidant defense in a dormant dry system, in which scavenger enzymes are poorly effective and cannot be upregulated. Thus, melatonin may be essential in protecting germ from the harmful environmental conditions (Manchester et al. 2000). Furthermore, Hosseinian and coworkers investigated colored grains of purple wheat, finding that, besides anthocyanins, these seeds also contained high amounts of melatonin (4 μg kg^{-1}) (Table 29.1) (Hosseinian et al. 2008). This suggests that purple cereal grains may be a source of different phytochemicals with additive effects on health.

Fruits seem to have a lower amount of the indoleamineamine than seeds (Dubbels et al. 1995; Hattori et al. 1995; Badria 2002), with the exception of tart cherries (*Prunus cerasus* L.), where higher concentrations of melatonin than those measured in other fruits have been reported (13.46 ng g^{-1} and 2.06 ng g^{-1} for the Montmorency and Balaton varieties, respectively) (Table 29.1) (Burkhardt et al. 2001). Nonetheless, the amount of melatonin in cherries, either Balaton or Montmorency variety, did not vary according to the time of harvesting (July and August) nor to the orchards where they were grown, although the mean

melatonin levels differed significantly regarding the tree from which the fruits were harvested (Burkhardt et al. 2001).

As mentioned above, studies performed on the same tissues of the same plant species have generated divergent data. An interesting example is represented by the species *Lycopersicon esculentum* Mill. sin. *Solanum lycopersicum* L. Hattori et al. (1995) reported 0.03 ng g^{-1} tissue, whereas Dubbels et al. (1995) measured 0.5 and 0.16 ng g^{-1} for the cultivars Sweet 100 and Rutgers California Supreme, respectively, thus showing that intraspecific differences exist among members of this species (Table 29.1). Additionally, Pape and Lüning (2006) detected a melatonin concentration of about 1.2 ng g^{-1} FW (Table 29.1). The grade of ripeness is also likely to play a role in the melatonin content of tomato. Van Tassel and colleagues harvested tomatoes at the mature green stage and allowed them to ripen under controlled conditions. Tissue samples from fruits at each stage of ripeness (mature green, breaker, turning, pink, light red, and mature red) were frozen, extracted, and HPLC fractionated. They reported that mature green tissues had the lowest amounts of melatonin, whereas the mature red ones the highest. They also obtained consistently lower values when the method of analysis was gas chromatography-mass spectrometry (GC/MS) compared with those estimated using RIA (Van Tassel et al. 2001). More recently, melatonin was detected in all tomato organs at different developmental stages. In young leaf tissues, melatonin reached a maximum of 6 ng g^{-1} FW, whereas, in berries, its content increased gradually from the mature green stage, both in pericarp and locular tissues, up to 2.8 and 3.4 ng g^{-1} FW, respectively, at mature red stage. Seeds contained the highest amount of melatonin, 66.6 ng g^{-1} FW, as previously reported for other species (Table 29.1) (Okazaki and Ezura 2009).

Interestingly, in wild tomato (currant tomato, *L. pimpinellifolium* L. (Mill.) synonym *Solanum pimpinellifolium* L.), the amount of melatonin was about fivefold lower than that in edible tomato. This variation may be associated with the different sensitivity to tropospheric ozone pollution: *S. esculentum* is more tolerant to the pollutant than the high-sensitive *S. pimpinellifolium*. Because ozone injury is mainly due to oxidative stress and melatonin is a powerful antioxidant, it was suggested that the species with higher melatonin content, that is, *S. esculentum*, experienced a lower damage after the pollutant exposure (Dubbels et al. 1995; Iriti et al. 2006a).

Similar intraspecific variations have been reported for the grape (*Vitis vinifera* L.), with the highest melatonin concentration in Nebbiolo and Croatina cultivars (0.9 ng g^{-1} and 0.8 ng g^{-1}, respectively), and the Cabernet Franc cultivar with the lowest detected values (0.005 ng g^{-1}) (Table 29.1) (Iriti et al. 2006b). Interestingly, treatment of grapevines with benzothiadiazole, an activator of the plant immune system, significantly increased the melatonin content in Merlot cultivar (Iriti et al. 2006b). Similarly, studies using *Echinacea purpurea* (L.) Moench explants revealed an accumulation of significantly higher amounts of melatonin in plants exposed to thidiazuron, a synthetic cytokinin-like plant growth regulator (Jones et al. 2007).

In the case of wine, Guerrero and colleagues have shown that the concentration of melatonin in the Spanish red wines ranged around 50–80 pg mL^{-1} (Table 29.1). Moreover, white wines exhibited slightly lower levels of melatonin than red wines. The exceptions were the Reserva type of Ribera del Duero (red wine) aged for 5 years, with an indoleamine content of around 200 pg mL^{-1}, and sherry wine, whose concentration of melatonin was below 10 pg mL^{-1} (Table 29.1) (Guerrero J. M. pers. comm.). The examined Italian wine seems to be richer in melatonin, ranging around 0.4–0.5 ng mL^{-1} (Table 29.1) (Mercolini et al. 2008). It is noteworthy that apart from genetic traits, cultivar, agrometeorological conditions, vintage and wine-making procedures can contribute to explain these differences (Iriti 2009).

Recently, melatonin has been reported in different commercial beers. In all the beers analyzed, melatonin was measured at concentrations ranging from 51.8 to 169.7 pg mL^{-1} (Table 29.1), and the more melatonin they contained, the higher their alcoholic degree, probably because of the solubility of melatonin in ethanol (Maldonado et al. 2009). A possible source of melatonin in beer may be barley, where this molecule has been identified, though *Saccharomyces cerevisiae* Meyen is able to produce melatonin in presence of tryptophan (Sprenger et al. 1999; Hernández-Ruiz and Arnao 2008b).

Extra-virgin olive oil is also a dietary source of melatonin. De la Puerta and coworkers found differences comparing refined and extra-virgin olive oils designations of origin (D.O), and also among the latter (e.g., 71 pg mL^{-1} in D.O. Bajo Aragón and 119 pg mL^{-1} in D.O. Baena) (Table 29.1). Differences in the heat treatment or chemical processing of the products may explain the diversity of concentrations measured (De la Puerta et al. 2007). Similarly, the hydrothermal treatment of *Fagopyrum esculentum*

Moench (buckwheat) seeds, followed by dehulling and milling processes, decreased the amount of melatonin by threefold on average (Zieliński et al. 2006).

Finally, another trait responsible for the above divergent findings might be the presence, also in plants, of a circadian rhythm in melatonin synthesis. In the short-flowering plant *Chenopodium rubrum* Fischer, high values of the indoleamine are reached during darkness, while low levels have been reported during the day (Kolár et al. 1997; Wolf et al. 2001). On the contrary, in *E. crassipes* (Mart.) Solms (water hyacinth), a melatonin peak occurred in the light phase of the light–dark cycle (Tan et al. 2007).

29.5 Conclusions

At the end of this brief survey, it is apparent that reliable results in melatonin determination can be obtained only if a number of factors are taken into account before carrying out any instrumental analysis. These factors include the characteristics of plant growing place, the agronomic practices, the light regime and the circadian behavior of the plant, the ripening stage, and the type of sampled tissues. Furthermore, to acquire comparable values by methodologically different procedures, researchers are urged to apply preservative conditions during extraction, controlling the yield by recovery determination. As in the case of animals, where a wide range of specific commercial products is available, it is to be hoped that methods specifically designed for plants will soon be developed, which can take into consideration the characteristics of these complex matrices.

Other main topics to be considered are the analysis of melatonin in processed foods, in order to evaluate the influence of cooking on this molecule, and its oral bioavailability. In this regard, the possibility of modulating serum melatonin levels in mammals through the ingestion of plant foods should be further highlighted and investigated, since dietary melatonin is absorbed from the gastrointestinal tract and transported in the blood stream, crossing the blood–brain barrier and the placenta (Reiter et al. 2001, 2005).

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Section VIII

Tetrapyrroles and Alkaloids

30

Chlorophylls, Chlorophyll-Related Molecules, and Open-Chain Tetrapyrroles

Benoît Schoefs

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30.1 Introduction

Epidemiological studies have established a relation between vegetable consumption and better health (Steinmetz and Potter 1996, Lampe 1999). Since World War II, the demand for food presenting additional properties to their traditional nourishment has increased. This type of food is called functional food. To satisfy these societal demands, it is necessary to find new natural sources of ingredients that offer health benefit in addition to their nutritive value. For instance, the cyanobacterium *Spirulina*, consumed since the times of pre-Columbian America (Paniagua-Michel et al. 1993; for centuries around Lake Tchad in Africa; Tremblin G, pers. comm.), is now seen as a functional food due to its antioxidant, antimicrobial, and fatty content (Eriksen 2008, Benedetti et al. 2010). Among the long list of bioactive compounds taken up from a diet rich in plant products, tetrapyrroles have been traditionally overlooked despite being present in high amounts compared to other phytochemicals. The tetrapyrrole family of compounds can be divided into two subfamilies: (i) the closed tetrapyrroles such as chlorophylls (Chl) and (ii) the open-chain tetrapyrroles such as phycobilins (Figure 30.1). Chl molecules are probably the most ubiquitous tetrapyrroles. Many tetrapyrroles are colored, making the environment attractive and, therefore, constituting an appealing force for the consumers because color modifications are often associated with inferior quality. Besides their natural presence in photosynthetic organisms, tetrapyrroles are extracted and used as natural colorants (Roman et al. 2002, Soni et al. 2006, Prasanna et al. 2007) and/or antioxidants to restore the natural level of these molecules in food products and food supplements (Benedetti et al. 2010) or to prepare fortified and functional products (Ayadi et al. 2009). According to the European regulation, Chl and their direct derivatives are designated as E140, E140i, and E140ii, the latter two designating liposoluble Chl derivatives and hydrosoluble chlorophyllins (Na^+- or K^+-chlorophyllins), respectively. These molecules could be chemically modified, for example, by replacement of Mg^{2+} with Cu^{2+}, and are designated as E141, E141i (Cu-Chl), and E141ii (Cu-chlorophyllins) (Anonymous 2 1994). According to the local legislation, the use of these compounds can be restricted or completely prohibited. For instance, the Food and Drug Administration (FDA) restricted the use of E141 compounds to citrus-based dry beverage mixes and in concentrations never exceeding 2%.

The quality/efficiency of functional food is directly or indirectly dependent on its content. To obtain such information, the tetrapyrrole composition of original products has to be established, a task that requires analytical procedures allowing the isolation, the identification, and the quantification of the tetrapyrroles present in food products. Addressing these issues is never straightforward because food can be highly complex, requiring care with process and storage conditions to ensure that the end product has unchanged level(s) of pigments, color, and taste. These methods could also be used to detect adulterations.

This chapter is dedicated to the analysis of open-chain and closed tetrapyrrole molecules. The molecular structures and the general properties of the pigments are first described. Then, pigment degradation and their roles in health are discussed. The chapter ends with a review of the invasive and noninvasive

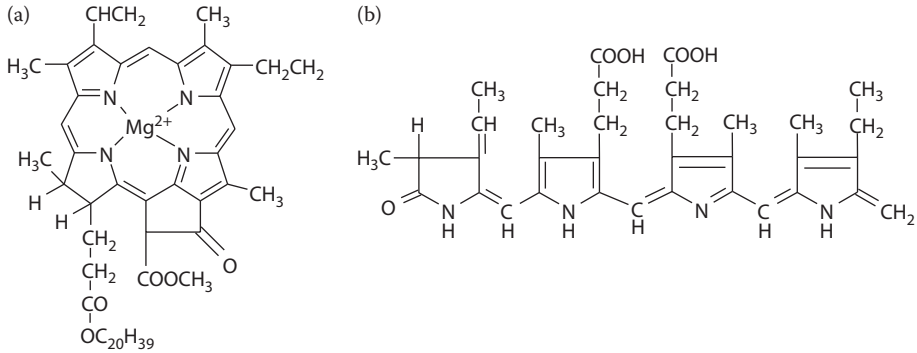


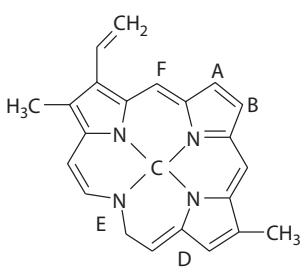
FIGURE 30.1 Structures of a (a) close and (b) open-chain tetrapyrrole of Chl *a* and phycocyanobilin, respectively.

techniques used for pigment analysis and with several examples of analytical procedures. This chapter should be considered as an update of an article published by Schoefs (2005).

30.2 Functional Characteristics: Molecular Structures and, Spectroscopies and Chemical Properties of Chlorophylls and Other Tetrapyrroles

30.2.1 Closed Tetrapyrroles

Basically, Chl molecules are conjugated tetrapyrroles, to which a cyclopentanone ring, conjoint with ring III, has been added (= a phorbin, see Figure 30.2). The macrocycle is planar. All naturally occurring Chls have a propionic acid residue at position 17. The position 17[3] is generally esterified with a long-chain alcohol, usually phytol. Chl *b* differs from Chl *a* by the presence of an aldehyde residue instead of a methyl group at position 7 (Figure 30.2). Chl *a* and Chl *b* are the most abundant pigments in terrestrial plants and in green algae. Chl *b* can be considered as typical for these organisms. The inner seed coat from Cucurbitaceae, although green, does not contain Chl molecules but protochlorophyll (Pchl) (Jones 1966, Mukaida et al. 1993). This pigment differs from Chl because pyrrole IV is not reduced (Figure 30.2), that is, Pchl belongs to the porphyrin group of compounds. Brown algae and diatoms contain pigments similar to protochlorophyllide (Pchlide), named Chl *c*. Consequently, Chl *c* does not belong to the Chl group of molecules. In addition, the nonreduced ring IV of Chl *c* is esterified at position 17 by an acrylic residue—instead of a propionic side group, the terminal carboxylic group generally being usually free (Zapata 2001). These features make Chl *c* a Pchlide, that is, Pchl without the phytol moiety. From the structures, it is clear that a "true" Chl molecule is made up of a hydrophilic part, the macrocycle, and by a hydrophobic part, the phytol chain. The most hydrophilic segment of the macrocycle is composed of the cyclopentanone ring and the propionic ester group (position 17). Therefore, nonesterified macrocycles are much more polar than the esterified ones. The nature of the other side groups as well as their configuration also modify the polarity of the molecules (reviewed by Seely 1966).



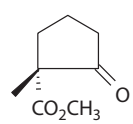
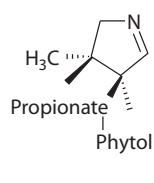
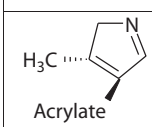
Compound name	A	B	C	D	E	F
Chl *a*	—CH_3	—C(H$_2$)—CH_3 or —C(H)=CH_2	Mg^{2+}			H
Chl *b*	—CHO					
Chl *c*	—CH_3					

FIGURE 30.2 Structures of the most abundant Chl molecules found in plants.

30.2.2 Open-Chain Tetrapyrroles

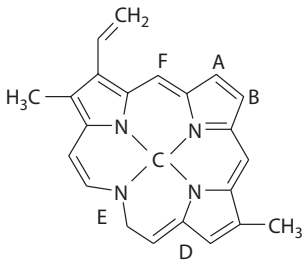
In higher plants, open-chain tetrapyrroles are not visible although they play crucial roles in plant physiology. In cyanobacteria and red algae, they are abundant enough to mask the Chl molecules and confer their color to the whole organism. These pigments are the phycobilins (Colyer et al. 2005). Two phycobilins are commonly found: phycocyanobilin (PC) and phycoerythobilin (PE). PE contains six conjugated double bonds and absorbs at lower wavelengths than PC that has eight conjugated double bonds. *In situ*, phycobilins are covalently bound to hydrophilic proteins, forming three types of phycobiliprotein: the red PE, the blue PC, and allophycocyanin (APC).

30.3 Pigment Degradation

It is recognized for a long time that Chl molecules are fragile and can be easily modified. These modifications can significantly alter their color, nutritive quality, and/or, finally, their commercial value. *In vivo*, Chl molecules are mostly destroyed through enzymatic processes (Ougham et al. 2008, Aiamla-or et al. 2010, Moser et al. 2009, Kraütler 2010). For instance, Mg-dechelatase removes the Mg^{2+} ion yielding to pheophytins (Pheo) whereas chlorophyllase cleaves the phytol ester, forming Chlide (Figure 30.3). Similar degradation may also occur during processing because the disruption of cellular structure can be accompanied by the liberation of enzymes, the activities of which destroy pigments. These enzymes can be inactivated by heating or during extraction with organic solvents. However, in some solvents, enzymes such as chlorophyllase could remain active and require several minutes at 90°C for heat inactivation (Kuroki et al. 1981). Short streaming (20–60 s), like the one used during processing of tea leaves, may not inactivate the enzymes. Inactivation is only reached during tea refining, a process that heats the leaves at 120°C for 30 min. In corn, lipoxygenases and peroxidases are inactivated after 6 and 8 min of blanching, respectively, while broccoli requires only 90 s (Barrett et al. 2000). The difference in the inactivation time may be explained by different heat-transmission capacities. Regardless of these considerations, the severity of the heating steps should be limited to maintain not only the color, texture, flavor, and nutritional quality (Lim et al. 1989, Maccarone et al. 1996), but also the pheophorbide (Pheoide) content (Pheo without phytol). In Japan, the amount of Pheoide *a* in food products is regulated by the Food and Health Administration (e.g., 1 mg/g) (Uchiyama 1991, Hwang et al. 2005). Under certain conditions, Pheo molecules can rebind divalent metals, principally copper and zinc ions (Canjura et al. 1999), regenerating the green color, which is usually brighter (LaBorde and von Elbe 1990) (Figure 30.3). This phenomenon is called regreening. Cu-Chl are denoted as Cu-Chl or chlorophyllin. The replacement capacity of Chl *b* derivatives is much less than that of Chl *a* (von Elbe et al. 1986, LaBorde and von Elbe 1990). The Chl derivatives were found to be more stable than the original Chl, especially to acids (Humphrey 1980). Formation of metallo-Chls has a potential use as a green colorant for beverages and also as a means of avoiding accumulation of Chl-degradation products during processing. Again, care should be taken not to exceed the value permitted by local FDA.

Chl and Chl-related molecules, outside their natural environment, are also very sensitive to light, acidity, temperature, enzyme actions, and oxygen. These factors are at work not only during tetrapyrroles isolation or food processing but also during storage (reviewed in Schoefs 2002, 2003, 2005, Minguez-Mosquera et al. 2008). For instance, Chl molecules can be degraded or converted to Pheos, Pheoides, or Chl oxidation products during an exposure to acid or a heat treatment; the type of products formed depends on the temperature and the length of the treatment (Schwartz et al. 1981, Wrolstad 2005, Turkmen et al. 2006). Chl *b* are less sensitive than Chl *a* (e.g., Weemaes et al. 1999). These authors have calculated the activation energy characterizing the Chl-to-Pheo and Pheo-to-further degradation compound transformations during the processing of broccoli juice. The calculations show that Pheo degradation requires more energy than the Chl-to-Pheo transformation.

The formation of Pheos or pyroPheos is often accompanied by a color change from green to olive-brown (Chen and Chen 1993, Mangos and Berger 1997). If the treatment is too strong, the molecules could be completely destroyed as reflected by the bleaching of the food product like when cottonseed oil is heated at 180°C for 60 min (Taha et al. 1988). The lipid environment of the Chl molecules seems



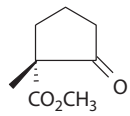
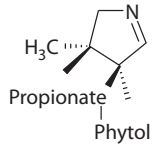
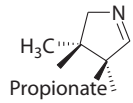
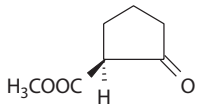
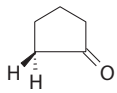
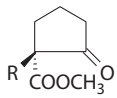
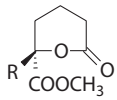
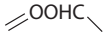
Compound name	A	B	C	D	E	F
Pheo	—CH3	—C(H2)—CH3	H			H
Chlide	or	or	Mg^{2+}			
Pheoide	—CHO	—C(H)=CH2	H			
Epimers						
Decarbo-metoxilates						
Allomers				 R = OH in 13^2 OH-Chl R = MeO in 13^2 MeOH-Chl  R = OH in 15^1 OH-lactones R = MeO in 15^1 MeOH-lactone		
Colorless degradation compounds			H			

FIGURE 30.3 Modification of the Chl structures to yield the most common Chl degradation products.

to play an important role in the process. *In vitro* experiments have shown that the highest rate of degradation is reached with methylstearate followed by methylstearate and methyllinoleate (Liu and Chen 1998). Other factors are, however, playing roles during the processing. For instance, differences in heat stability of olive oil flavored with basil or rosemary have been reported despite the fact that the latter contains more Chl molecules than the former (Ayadi et al. 2009).

Chl degradation also occurs during storage in a cold room (Schwartz and Lorenzo 1991) or at room temperature (Psomiadou and Tsimidou 1998). The degradation rate is higher in the light than in the dark

(Psomiadou and Tsimidou 1998). Regardless of the storage conditions, Chl *a* molecules are more sensitive to degradation than those of Chl *b* (Schwartz and Lorenzo 1991). Obviously, the chemical modifications undergone by Chl molecules have consequences on their physicochemical properties such as solubility and chemical reactivity. For instance, the importance of the chelated ion or the presence of a formyl group at the C7 position for antioxidant activity has been shown (Ferruzzi et al. 2002, Lanfer-Marquez et al. 2005). In addition to the color change, light sensitization of Chl molecules may trigger off-flavor of the processed compounds. For instance, even when present in sub-ppm levels, light sensitization of Chl rapidly promotes degradation of the citrus juice flavor. The promotion seems acidity dependent, being stronger at low pH (Naka and Sumitani 2009). Therefore, the presence of light during processing as well as the degree of fruit ripening has to be strictly controlled. The degree of ripening could be automatically checked using Cl fluorescence imaging (Nebdal et al. 2000).

There is only scant information about open-chain tetrapyrrole degradation in food. One typical degradation results in the bleaching of the phycobiliproteins, for instance, the typical black color of dried layer (nori, *Porphyra* sp.) turned green when toasted (Iwamoto et al. 1972) or to brown when prepared with vinegar (Amano and Nods 1993). This was due to the disruption of the protein pigment bound at low pH (Sarada et al. 1999).

30.4 Tetrapyrrole Assimilation and Health Benefits

To be considered as functional molecules, tetrapyrroles have to be assimilated. *In vitro*, open-chain tetrapyrroles exhibit antioxidant (APC: Ge et al. 2006, PC: Patel et al. 2006, PE: Soni et al. 2009), anti-inflammatory (Romay et al. 1998a,b), and hepatoprotection activity (Bhat et al. 1998, Bhat and Madyastha 2000, Soni et al. 2008). PC also reduces cancer cell growth by induction of apoptosis (Li et al. 2006, Roy et al. 2007, Wang et al. 2007). Very importantly, the capacity of oxygen radical quencher is preserved in food supplement containing PC (Pandey and Pandey 2008, Benedetti et al. 2010).

Until recently, almost nothing was known about the assimilation of close tetrapyrroles and it was simply thought that the phytol chain of the native Chl molecules is cleaved whereas the Mg ion is leached upon ingestion, the resulting Pheoide and Pheo being excreted in the feces (Baxter 1968). More recently, vegetable extracts containing Chl were shown to be antimutagenic in bacterial essay systems (Lai et al. 1980, Terwel and van der Hoeven 1985) and Ferruzzi et al. (2001) demonstrated that Pheo can be absorbed by Caco-2 human cells *in vitro*. Negishi et al. (1997) have reported the antigenotoxicity of natural Chls whereas others revealed the antioxidant activity, the modulation of xenobiotic enzyme activity, and the induction of apoptotic event in cancer cells (Dashwood 1997, Ferruzzi and Blakeslee 2007). This anticytotoxic activity is specific to natural Chl and could not be demonstrated with nonnatural Chl molecules (see below) (de Vogel et al. 2000). Despite the limited set of toxicity data on Chls, the widespread ingestion of Chl-containing products by humans, along with its limited absorption (1–3%), make Chls save compounds. Indeed, Chl derivatives such as Pheo have been found in some plants known in traditional medicine to be active against diseases such as Clinacanthus nutans-herpes simplex virus (Sakdarat et al. 2009) and Lonicera hypoglauca-hépatitis C virus (Wang et al. 2009). Chl precursors, Chl derivatives, or Chl analogs are also used in medicine for photodynamic treatments of cancers (e.g., Tang et al. 2009). Nevertheless, Chl intolerance can occur due to the liberation of phytols in patients suffering from Refsum's disease. In this pathology, phytanic acid accumulates in serum and other tissues due to a defect of the metabolic pathway for phytanic acid (Baxter 1968). This does not mean that the ingestion of tetrapyrroles is without danger. Lohrey et al. (1974) demonstrated that albino rats fed with lucerne protein concentrate, but not with ryegrass, develop photosensitization. This allergy to sunlight could be due to the accumulation of Pheoide and related pigments in tissues (Holden 1974). Similar sunlight allergic reactions were reported in humans having ingested dried laver enriched with Pheoides and pyropheophorbides (Ppheoides) (Hwang et al. 2005). Gandul-Rojas et al. (2009) have shown that these deesterified tetrapyrroles are more strongly absorbed than the phytylated ones from the food matrix by the intestinal epithelial cells, allowing a larger accumulation in the patient body. Therefore, there is a need for information on the effect of processes on tetrapyrrole bioaccessibility and bioavailability. For instance, conservation industrial processes such as freezing and canning along with cooking have a positive effect

on Chl bioavailability (Gallardo-Guerrero et al. 2008), making the pigment's quantity after processing exceeding 100% of the value measured before processing (e.g., Stahl and Sies 1992, Wrolstad 2005, Cubas et al. 2008).

Nonnatural Chls, such as Cu-Chls, Cu-Pheoide, Cu-Pheo, or chlorophyllins, are digested similar to natural Chl molecules, that is, the phytol chain is cleaved but the Cu ion is not leached from the porphyrin ring because the chelate is much more stable. The stability is only relative because an increase in Cu has been measured in the plasma following chlorophyllin ingestion (Harrison et al. 1954). Several tests suggested that oral or parenteral administration of chlorophyllins did not produce any gross adverse effect on health (Reber and Willigan 1954, Anonymous 1 2002). In addition, Hayatsu et al. (1999) indicated that chlorophyllins could prevent heterocyclic amine carcinogenesis through binding to the heterocyclic amine compounds and other mutagens (Breinholt et al. 1995, Guo et al. 1995, Park and Surth 1996). The efficiency of dephytylated compounds is less than the phytylated ones (Chernomorsky et al. 1999). However, Nelson (1992) reported that commercially available chlorophyllin can have a tumor-promoting effect and at present the chlorophyllin content in food is regulated.

30.5 Methods of Analysis: A Brief Overview

An array of methods can be used for tetrapyrrole isolation. The solvent used is crucial. There is evidence that Chl molecules are more thoroughly extracted with methanol or dimethylsulfoxide (Shoaf and Lium 1976, Wright and Sheaver 1984, Bahçeci et al. 2005). However, methanol leads to the formation of Chl epimers (Mantoura and Llewellyn 1983). Dimethylformamide (DMF) is also an efficient solvent since the pigments are stable in this solvent for up to 20 days when stored in the dark and at 4°C (Moran and Porath 1980). Alternative methods for pigment extraction and storage have been published recently (Esteban et al. 2009). The choice is usually guided by the type of information needed. Generally, the pigment must be extracted before analysis, sometimes from a complex matrix. Therefore, efficient extraction and analytical protocols are requested (for a full discussion, see Wellburn 1994). Most of the tetrapyrrole molecules have a long chain of conjugated double bonds that make them react easily with acid, base, oxygen and, light (Schoefs 2002, 2005). For instance, a solution containing Chl reacts easily with oxygen on illumination, resulting in the formation of activated oxygen species. These molecules are very reactive and able to oxidize other organic molecules, including lipids and proteins. For this reason, care should be taken during extraction and analysis. The production of singlet oxygen by Chl is not only restricted to aqueous pigment extracts, but also occurs in less polar solvents such as oil. For this reason, oil containing a high amount of Chl or related compounds should be stored in the dark and at reduced temperature.

30.5.1 Spectroscopic Methods

Tetrapyrroles present a closed circuit of conjugated double bonds, which allows them to absorb visible light (Figure 30.2). The absorbance spectrum indicates the presence of two distinct bands: one in red, corresponding to the first excited state, and another in blue, often denoted as the Soret band, and corresponding to the second excited state. Similar spectra are obtained with other Chl and open-chain tetrapyrroles. The spectra differ, however, in the blue-to-red absorbance ratio and in the position of the bands. This varies not only from pigment to pigment but also as a function of the solvent, that is, the pigment environment (Mysliwa-Kurdziel et al. 2008).

Chl and Chl-related molecules present the unique property to deexcite by emitting fluorescence and only from the first excited state because the higher singlet excited state deexcites initially by internal conversion to the first excitation state. Consequently, a fluorescence spectrum contains only one band. It should be stressed again that chemical modifications that do not affect the chromophore, such as transesterification at position 17[3], are not reflected in either absorbance or fluorescence spectra.

The absorbance spectrum reflects the organization of the conjugated double-bond system and constitutes the fingerprint of pigments (see above). Absorbance spectroscopy appears to be the simplest way of identifying major pigments present in a mixture (Schoefs 2002). Once identified, it is possible to use a set of equations to estimate their respective concentration (Beer and Eshel 1985, Wellburn 1994, Kouril

et al. 1999, Küpper et al. 2000, Sampath-Wiley and Neefus 2007). Care should be taken when choosing the equation set. For instance, the Arnon's equations underestimate the Chl *a*/*b* ratio (Wellburn 1994). As the pigment environment strongly influences the position and the shape of the spectrum, a crude absorbance spectrum is useless for direct measurements of the pigment concentration. Alternatively, a new equation set can be established using specific (or molar) absorbance coefficients. With these data, it is always possible to establish a new equation set, adapted to the particular situation. The precision of the method depends on the type of device used, the ability to determine with precision the absorbance maxima, and, of course, the accuracy of the absorption coefficient used for the calculation (Wellburn 1994, Sampath-Wiley and Neefus 2007). It is therefore advisable to regularly check the literature for new values and equation sets. Identification of pigments on the sole basis of an absorbance spectrum, however, presents strong limitations. The overlapping of the absorbance bands of pigments in a mixture complicates the estimation of individual pigment concentration and is much less efficient especially if the number of pigments is higher than three. Another limitation of the spectrophotometric methods relies on their inability to distinguish esterified pigments from ones, that is, Chl and Chlide or Pheo and Pheoide because they display the same spectrum. Therefore, the quantification of these compounds requires the use of separation methods. Equations based on fluorescence measurements have also been published (Moberg et al. 2001, Schoefs 2005).

The absorbance spectrum of a full extract is of little use when there is a dominance of one pigment or if several compounds have similar spectra. Fluorescence spectroscopy can be more diagnostically helpful due to the selective excitation of a number of Chls and Chl-related pigments. Fluorescence can also be used for the determination of tetrapyrrole concentrations in a mixture. One of the advantages of the method is the possibility to excite the pigments of the mixture separately, allowing the determination of their quantity using a calibrated curve (Welschmeyer 1994). Care should, however, be taken because other pigments could also absorb the excitation light and contribute to the fluorescence, creating potential errors in the concentration determination (Chl *b*: Lorenzen 1981, Trees et al. 1985, Kouril et al. 1999, Pheo: Welschmeyer 1994). The limitations of the spectroscopic methods are easy to determine using a green beverage. As it is green, it is expected that Chl *a* molecules are conferring the green color to the product. The absorption spectrum of the beverage presents several maxima: at 413 and 634 nm. They could belong to chlorophyllin, Pheo, and Pchl(ide), whereas the one at 437 nm could reflect the presence of little amount of Chl *a*. Therefore, additional analyses are required to precisely determine the nature of the green pigment(s) (see below). In conclusion, spectroscopic methods usually permit a crude identification of the pigments present in an extract, but specific composition remains obscure. To resolve a pigment mixture, chromatographic methods should be used.

30.5.2 Chromatographic Separation

30.5.2.1 Open Column

Various phases such as powdered sucrose, diethylaminoethyl-sepharose, cellulose, and MgO/Hyflosupercel have been used to achieve Chl and carotenoid separation (Wasley et al. 1970, Omata and Murata 1983, Soni et al. 2008).

30.5.2.2 Thin-Layer Chromatography

Thin-layer chromatography (TLC) and high-performance TLC are often used for the separation and isolation of individual classes of molecules as TLC is fast, effective, and relatively cheap. Special care should be taken when the separation is done on silica gel: it is necessary to neutralize the acidity of silica in advance to avoid Chl-pheophytinization. Unfortunately, separation of compounds with a similar structure is usually rather difficult. This is well illustrated by the comparison of the chromatograms obtained for pumpkin seed oil pigments separated by TLC and high-performance liquid chromatography (HPLC) (Schoefs 2005). The TLC method allows the separation of four bands, whereas 14 peaks were obtained with HPLC. One can see that the TLC methodology can be supplemented by more efficient separation techniques, such as HPLC coupled with UV–Vis and/or fluorometric detection.

30.5.2.3 High-Performance Liquid Chromatography

Chls and carotenoids have a clear hydrophobic character and analysis by C30 reverse-phase (RP) columns is preferred. However, when a mixture is complex, coelutions become rapidly limiting (Khachik et al. 1996). A less selective stationary phase, such as the C18-RP, is preferably used for less detailed analysis. To improve pigment separation, the heating of the column is sometimes proposed. This procedure is, however, not recommended because it can trigger epimerization and/or allomerization of Chl molecules. These modifications may not be detected using usual HPLC methods (Hyvärinen and Hynninen 1999). During allomerization, Chl molecules are oxygenated (Woolley et al. 1997). Care should be taken to employ proper chromatography conditions to ensure that pigments do not escape the analysis. This is illustrated when the analysis of pumpkin seed oil using two different RP HPLC methods, both of which are recommended for the analysis of plant pigments, were compared (Schoefs 2005). For the phycobiliproteins, methods using C4 columns have been proposed (reviewed by Colyer et al. 2005). Supplementing HPLC with a diode-array detector together with the availability of powerful computers has especially increased the analytical power of HPLC. Using such detectors, it is now possible to simultaneously follow the elution on the full UV–Vis range (190–800 nm). This guarantees that each pigment or pigment–protein complex can be followed at its absorbance maximum, that is, with maximum sensitivity.

None of the methods described above is entirely suitable for an exhaustive separation of a complex mixture of pigments. The tetrapyrrole fingerprint is often sufficient to identify its chromophore; it does not contain enough information to determine the complete structure of the pigment (e.g., Schoefs 2002, 2005). This observation can be partly deduced from chromatographic behavior and from the comparison of the obtained retention data with the literature. Other methods such as mass spectrometry (MS) will have to be applied to fully characterize the structure of the molecule (Schoefs 2005). Recent developments in MS that allow the analysis of subnanogram quantities have made this technique attractive for foodstuff research.

30.5.3 Analysis of Tetrapyrroles by MS

Chls have long presented special analytical challenges to MS because of their high mass, low volatility, and thermal instability. Thus, Chl *a* and Chl *b* have given only pyrrolytic fragments in electron impact (EI) MS. This could be explained by the exceptional stability of the π-electron system of porphyrin rings and the strong magnesium–oxygen coordination. Similarly, Pheo has only one weak molecular ion signal in EI-MS. Introduction of desorption–ionization MS, such as chemical ionization, secondary ion MS, fast-atom bombardment, field, plasma, and recently matrix-assisted laser desorption (MALDI), opened ways for molecular ion detection and, thus, for direct molecular weight determination. In all these methods, the necessity of sample vaporization prior to ionization is avoided. Rather, the desorption and ionization processes occur directly from the condensed phase. For details on desorption ionization techniques, the interested reader is referred to the comprehensive review by Hunt and Michalski (1991). When the matrix contains salts, addition of alkali metal(s) can also be observed. Occurrence of additional signals in the lower mass region reflects the presence of Chl degradation products, such as 10-OH Chl *a* (*m*/*z* 908) or Pheo *a* (*m*/*z* 870) and signals at *m*/*z* 482, *m*/*z* 556, and *m*/*z* 615. However, it is difficult to decide whether they were present in the sample before the analysis or if they are a result of sample preparation and/or analysis techniques. Regardless of the origin of these degradation products, the determination of their molecular mass can be used to build up a fragmentation scheme of the target compound. It is obvious that such schemes can be very useful for the determination of the structure of Chl, especially degradation products, such as changes in the esterification by fatty acids at the $C17^2$ position and Chl halogenation at the C20 position (Hunt and Michalski 1991). Using MALDI, Suzuki et al. (2009) obtained the molecular ions corresponding to Chls and most of its derivative products, whether present in a matrix or not. The most recent progress in MS analysis of tetrapyrroles has been obtained with the development of atmospheric ionization methods, that is, atmospheric pressure chemical ionization (APCI) (Harris et al. 1995, Huang et al. 2008) and electrospray ionization (ESI) (Vandell and Limbach 1998). Under APCI, the mechanism of ionization involves the formation of a charged adduct, for example, by protonation. With reference to the molecule orbital description of the porphyrin

π-system (Adar 1978), the protonation of the nitrogen heteroatoms of Chl is not favored because the one-pair electrons of the N-metal ligand contribute to the π-system. In Pheo, this is not the case and the nitrogen heteroatom is more readily available for protonation. Therefore, a way of enhancing the sensitivity of MS analysis toward Chl type of pigment would involve converting Chls to Pheo (Spooner et al. 1994, Villanueva et al. 1994a,b). Alternatively, Chl molecules can be converted online to Pheo by acidification of the elution mixture (Airs and Kelly 2000). The APCI technique, in combination with RP-HPLC, has proved to be efficient in detecting Chl *a* and many degradation products (Eckardt et al. 1991, Huang et al. 2008). The procedure is ~1000 times more sensitive than thermospray ionization (Eckardt et al. 1991). To demonstrate the potential of electrospray MS in Chl research, an ESI interface was employed with an ion trap mass spectrometer as a mass analyzer (Schoefs 2001). ESI is a mild ionization technology feature, allowing the detection of Chl *a*-protonated molecular ion $(M + H)^+$, *m/z* 893.5, which dominates and is partly accompanied only by a molecular radical ion ($M^{+\cdot}$ *m/z* = 892.5). It is remarkable that the spectrum was obtained with ~2 pmol of the compound. Further structural information has been obtained with the unique MS^N capability of the ion trap analyzer, allowing consecutive dissociation of side-chain functional groups from the selected precursor ion (up to eight steps; Schoefs et al. 2000, Schoefs 2001). The MS^N procedure is highly selective and enables consecutive cleavage of at least seven functional groups around the porphyrin, providing valuable structural information about the tetrapyrrole. Using MS methods, Chl allomers and their derivatives, produced during fruit and vegetable processing (Minguez-Mosquera et al. 1995), have been isolated and their structures have been elucidated (Hyvärinen and Hynninen 1999).

The metalloporphyrins can also be detected and quantified *in vitro* using antibodies (Cochran and Schultz 1990, Mantrova et al. 1994, Savitsky et al. 1994).

All the methods described above are invasive techniques that consume sample, time, and money. Alternatively, noninvasive procedures of analysis have also been developed to follow pigment content in food products to estimate its impact on product perception. They can also be used to evaluate the freshness of unprocessed samples.

30.5.4 Nuclear Magnetic Resonance

Nuclear magnetic resonance (NMR) spectroscopy was used to identify and determine the structural properties of Chl and Chl derivatives (e.g., Smith et al. 1984, Sakdarat et al. 2009, Charlton 2010). Later, ^{1}H NMR was used to establish the metabolic profiles of crude extracts from plants. This type of analysis generates a huge quantity of complementary information that can be selectively analyzed (Fan 1996, Sobolev et al. 2005). Valverde and This (2008) reported the analysis of photosynthetic pigments extracted from green beans by ^{1}H NMR. Chls, Chl epimers, and Pheos were identified and quantified. The precision was lower than using spectrophotometry but the amount of Chl epimers could be determined, which was not possible using spectrophotometry.

30.5.5 Noninvasive Methods

30.5.5.1 Evaluation of Color and Its Perception

The visual impression is mainly a function of the pigments present but is also affected by morphological factors like epidermal hair or cuticular waxes and the shape and the orientation of the cells in the epidermis and the subepidermis. In fact, pigments and surface topography selectively absorb, reflect, and refract the incident visible light sensed by the eye. The signals, generated at the retina level, are transduced through the optic nerve to the brain and interpreted as color. When light strikes the eye, it is detected by one of the three color sensors of the retinas: a red, a green, or a blue. It is known that the information is not sent as individual color but as a red/green signal, a yellow/blue signal, or a black/white signal. When all the wavelengths are reflected by an object, the eyes see it as white, whereas when all are absorbed, the object appears black. It is out of the scope of this review to explain in detail the basis of color measurement (reviewed by Wyszecki and Stiles 1987). In brief, each color can be described by a set of three parameters, that is, hue, the dominant shade, saturation (or chroma), how much color is present,

and lightness, the degree of darkness of a particular color. Therefore, it is necessary to describe a different hue, saturation, and lightness for each unique set of illuminant or observed conditions. By using a standard illuminant and a standard observer, the amount of light reflected by an object can be converted into the hue, saturation, and lightness values. Additionally, a sample can be compared to any standard with these three attributes. In 1976, the Commission Internationale de l'Electricité adopted a standard method of calculating color attributes, known as the Commission Internationale de l'Electricité Lab Colour Space. The lightness coefficient L^* ranges from black (= 0) to white (= 100) while the coordinates a^* and b^* designate the color on a rectangular-coordinate grid perpendicular to the L^* axis. The color at the grid origin is achromatic (i.e., gray; $a^* = 0$, $b^* = 0$). On the horizontal axis, positive and negative a values indicate the hue of redness (positive values) and greenness (negative values), whereas on the vertical axis, b^* indicates yellowness (positive values) and blueness (negative values). There are conflicting reports in the literature on the correlation between color measurement and pigment composition. This is especially true when more than one pigment has to be monitored. Several mathematical combinations of the parameters are used to predict pigment modifications occurring during food processing (e.g., Steet and Tong 1996, Ma and Shimokawa 1998). Some authors also use the hue angle (Wyszecki and Stiles 1987) to characterize the color modifications (De Ell and Toivonien 1999). Other methods, based on reflected and scattered light, were used to derive estimated pigment content and to predict color (McClements et al. 1998).

30.5.5.2 Tetrapyrrole Content

Recently, nondestructive optical methods, based on the absorption and/or reflectance of light by intact leaf, have been developed for the determination of the Chl content (Markwell et al. 1995, Hawkins et al. 2007, Cassol et al. 2008). In addition to allow the use of the samples for other measurements, these methods are quick and could be used in the field. These optical methods yield a "Chl index" value that expresses the relative Chl content and not an absolute value. To obtain absolute values, the device should be calibrated. One of the limitations of the nondestructive methods of analysis resides in the fact that the internal and/or external structure of the sample, such as the nonuniformity in the distribution of the Chl molecules or anatomical characters, may modify the absorbance properties and the uniformity of the radiations (Uddling et al. 2007). Such methods could, however, be useful to assess the freshness of food products as well as their quality. For instance, Haripriya Arrand and Byju (2008) used such a method to determine the Chl content, leaf color, and yield of cassava (*Manihot esculenta*), the most important root crop and also an important source of food calories (Cock 1982).

30.5.5.3 Determination of the Freshness/Health of Plant Products

The freshness of vegetables can be assessed using Chl fluorescence kinetic measurements, which reflect photosynthetic activity (for a review, see Rohacek et al. 2008). It is out of the scope of this review to explain in detail the background of this method and the interested reader is referred to recent reviews (Sayed 2003, Rohacek et al. 2008). In brief, photon energy absorbed by the photosynthetic pigments drives the photochemical reactions of photosynthesis. The energy conversion is usually very effective (>90%) and only a small part of the absorbed energy is emitted in the environment as heat of fluorescence. The Chl fluorescence yield depends on the redox state of the photosystem II primary acceptor—quinone A (Q_A). When all the Q_A are oxidized, for instance, after a period of complete darkness, the level of fluorescence, denoted F_0, is minimum. In contrast, when all the Q_A are reduced, for example, during a saturating light pulse, the level of fluorescence, denoted F_m, is maximum. The state of the photosynthetic apparatus can be estimated using the F_v/F_m ratio with $F_v = F_m - F_0$. Healthy plants exhibit an F_v/F_m ratio of ~0.8. When the photosynthetic process is inactivated, such as in senescent plants or in cold-stored plants, the ratio decreases (De Ell and Toivonen 1999, De Ell et al. 1999). Tian et al. (1996) showed that the ratio could be a sensitive indicator of responses of broccoli to hot-water treatment, even before visual changes were noted. Other parameters, such as the fluorescence decrease ratio, can be obtained from Chl fluorescence kinetic to characterize the physiological state of the plants (Lichtenthaler and Rinderle 1988, Sayed 2003, Rohacek et al. 2008). Imaging devices are also available (for a review, see Rohacek et al. 2008).

30.6 Pigment Identification and Quantification

Definitive pigment identification and quantification usually require the development of standards (Table 30.1), which are critical to food pigment analysis. Those standards that are not commercially available need to be prepared from scratch. Schiedt and Liaanen-Jensen (1995) have defined the minimum criteria for the identification of carotenoids: the absorbance spectra in the UV–Vis region, obtained in at least two different solvents, should be in agreement with the chromophore suggested; the chromatographic properties must be identical in TLC (Rf) and HPLC (t'_R) and must be coeluted with authentic samples; a mass spectrum should be obtained that allows at least confirmation of the molecular mass. Although such "rules" were not specified for the identification of tetrapyrrole molecules, similar criteria are suggested.

30.6.1 Pigments from Juices and Drinks

30.6.1.1 Extraction

The juice is first mixed with tetrahydrofuran. The nonpolar pigments are transferred to petroleum ether. The water phase is discarded and the organic phase is washed with water. This is repeated until the water phase becomes colorless. The ether extracts are pooled and dried on anhydrous sodium sulfate. When the juice contains pulp, it is advisable to remove it by centrifugation. The isolated pulp is dispersed in distilled water and extracted as explained above (Arena et al. 2000). Mendolia et al. (2008) injected the juice directly on the HPLC column, except when it contained gas. In that case, the sample was degassed prior to the injection.

30.6.1.2 Analysis

When the extract is rich in Chl derivatives, the HPLC method described by Canjura et al. (1999) may be used. It involves an isocratic elution (9 min) of a hexane/isopropanol (98.3:1.7 v/v) mixture, followed by

TABLE 30.1

Comparison with Pigment Standards Constitutes a Very Crucial Step in Tetrapyrrole Analysis. They Can Be either Commercially Available or Prepared in the Laboratory

Pigment	Commercially Available	Preparative Method
Chl *a*	Yes (e.g., Sigma, DHI Water & Environment)	
Chl *b*	Yes (e.g., Sigma, DHI Water & Environment)	
Chl *a′*	No	Katz et al. (1968)
Chl *b′*	No	Katz et al. (1968)
Chlide *a*	No	Almela et al. (2000), Roca et al. (2003)
Chlide *b*	No	Almela et al. (2000), Roca et al. (2003)
Pheo *a*	Yes (e.g., Wako Chemicals)	Lynn et al. (1967), Sievers and Hynninen (1977), Airs and Keely (2000)
Pheo *b*	No	Lynn et al. (1967)
Pheoide *a*	Yes (e.g., Frontier Scientific)	Almela et al. (2000)
Pheoide *b*	No	Almela et al. (2000)
PPheo	Yes (e.g., Wako Chemicals, Tama Biochemicals)	
PPheoide	Yes (e.g., Frontier Scientific)	
C13-epimer Chl *a*	No	Watanabe et al. (1984)
C13-epimer Chl *b*		
132-OH-Chl *a*	No	Laitalainen et al. (1990)
132-OH-Chl *b*		
151-OH-lactone-Chl *a*	No	Minguez-Mosquera and Gandul-Rojas (1995)
151-OH-lactone Chl *b*		

a linear gradient (2 min), to yield a mobile phase with equal parts of hexane/isopropanol (98.3:1.7 v/v) and hexane/isopropanol (98.3:3.0 v/v). This mixture is held for 7 min to increase hexane/isopropanol (98.3/3.0) fraction to 100%. When the Chl molecules are part of a complex mixture with molecules other than carotenoids, the method described by Mendolia et al. (2008) could give interesting results.

30.6.2 Pigments from Oil

30.6.2.1 Extraction

The lipid matrix can be removed from the extract using a silica gel column or TLC (Ellsworth 1971). For a separate analysis of the fatty acid moiety, the pigments are saponified with KOH–methanol (10% w/v) and left for 10 min with periodic shaking at room temperature. Then, the long-chain alcohols are transferred by addition of water to diethyl ether. The aqueous phase is reextracted twice. The diethyl ether is dried and stored. To avoid these extra sample treatments, a normal solid phase can be used to analyze the Chl content (olive oil: Psomiadou and Tsimidou 1998). Another possibility would consist in first dissolving the oil in DMF and then transferring the lipids and carotenes in a hexane phase. When DMF is treated with a solution of NaCl 10% (w/v), the Chls and xanthophylls can be transferred in a hexane/diethyl ether mixture (1:1 v/v). The aqueous layer is washed with diethyl ether and discarded (Roca et al. 2010).

30.6.2.2 Analysis

The oil can be diluted in the HPLC mobile phase (Goulson and Warthesen 1999) and can also be directly injected into an HPLC column (Schoefs 2003, 2005). As during the olive oil processing, many Chl degradation products appear, a dedicated HPLC elution program has been developed (reviewed in Minguez-Mosquera et al. 2008). When adulterants are searched, particular methods must be used (e.g., Cu-Pheo: Inoue et al. 1988, E141i: Scotter et al. 2005, Chl-derivatives + adulterants: Roca et al. 2010).

30.6.3 Pigments from Leaf and Vegetable Puree

30.6.3.1 Extraction

The pigments are extracted with acetone. The solution is filtered through a filter paper. The pigments are then dried over a bed of anhydrous Na_2SO_4. The sample can be frozen and pulverized. The powder can be mixed with DMF containing $NaCO_3$ to prevent Chl-to-Pheo transformation (Sibley et al. 1996). The mixture is homogenized and the pigment extracted (Cubas et al. 2008).

30.6.3.2 Analysis

For tetrapyrroles, a method separating Chl *a*, Chl *b,* and their degradation products is recommended (e.g., Schoefs et al. 1995, Darko et al. 2000).

30.6.4 Pigments from Fruits

Usually, the Chl molecules are degraded during fruit ripening. Andersson et al. (2009) have used Pheo as a maturity marker of sea buckthorn fruit, this fruit entering in the composition of functional foods. The fruits were lyophilized and seeds or stones, when present, were removed. The lyophilized products were ground for 5 s at 20,000 rpm in a lab mill and the pigments were extracted in a solvent mixture of ethanol and *n*-hexane (4:3 v/v) containing 0.01% butylated hydroxy toluene (BHT). The sample was placed in an orbital shaker for 20 h at 4°C in the dark. At the end of the incubation period, the samples are centrifuged for 10 min at 10,000*g* and the supernatant was collected for analysis.

30.6.5 Pigments from Digesta and from Micellar Fractions

Frozen aliquots were thawed and the Chl pigments were extracted with 2 mL acetone (0.2% BHT), 2 mL diethyl ether, and 2 mL of a 10% NaCl solution. The mixture was vortexed for 1 min and then centrifuged at 4500*g* for 5 min. The diethyl ether phase is collected. The diethyl ether extraction is repeated three times. The fractions are pooled, dried under N_2, and kept until used (Gallardo-Guerrero et al. 2008).

30.6.6 Open-Chain Tetrapyrroles from the Red Algae Nori

30.6.6.1 Extraction

The algae are ground in a mortar and the phycobiliproteins are extracted with phosphate buffer (0.1 M, pH 6.3). The pigment–protein complexes are precipitated using ammonium sulfate and recovered by centrifugation (25,000*g*, 15 min). The pellet is dialyzed against distilled water.

30.6.6.2 Extraction

The extraction is performed either by HPLC using a C4 column or by using sodium dodecyl sulfate polyacrylamide gel electrophoresis or isoelectric focusing (Amano and Noda 1993, Colyer et al. 2005).

30.7 Future Trends

In food analysis, continued study of various techniques offers the best possibility of finding the most powerful method for monitoring the pigment separation for high quality and safety of food products. For instance, the versatility of HPLC and MS as analytical tools offers an almost endless number of applications for food analysis. The versatility of HPLC techniques also makes it an ideal procedure for use by analytical quality control and research and development laboratories in the food and beverage industry. Objective, physicochemical methods to determine the quality of food products are often destructive and time-consuming. Simultaneous development of rapid and nondestructive indicators of quality must be continued. In establishing these tools, it is necessary to correlate physical parameters, for example, firmness, soluble solids, dry matter, and color with physiological data (e.g., respiration and photosynthesis). These methods could involve techniques such as delayed luminescence (Triglia et al. 1998) or delayed fluorescence (Bodemer 2004), near infrared (Slaughter et al. 1996), supercritical fluid or ultrasound-assisted extraction (Macias-Sanchez et al. 2009), and so on, which were, in the past, more restricted to basic research.

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Section IX

Minerals and Trace Elements

31

Minerals

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31.1 Introduction

In the past few years, nutrition science, traditionally concentrated on identifying a balanced diet, has emphasized "optimized" nutrition, that is, maximizing life expectancy and quality by identifying food ingredients that, when added to a balanced diet, improve the host capacity to resist disease and enhance health. These ingredients include mineral elements—bioactive agents that form part of the composition of foods, or which can be added to foods.

The addition of specific nutrients to food or water has been shown to be an effective strategy to combat nutrition deficiencies, contributing to the eradication of most vitamin and mineral deficiencies in industrialized countries. Food fortification has been defined as the addition of one or more essential nutrients to a food, whether or not such nutrients are normally contained in the food, for the purpose of preventing or correcting a demonstrated deficiency of one or more nutrients in the population or in specific population groups. Enrichment has been used interchangeably with the term fortification (FAO/WHO 1994). Mineral fortification of commonly used foods is common in both developing and industrialized countries, and is an important strategy for dealing with micronutrient malnutrition. The Micronutrient Initiative is dedicated to ensuring that the world's most vulnerable population groups—especially women and children in developing countries—obtain the vitamins and minerals they need to survive and thrive (http://www.micronutrient.org).

One of the critical points of mineral fortification is the selection of the food to be fortified. Population food habits, as well as specific populations at risk, have to be taken into account. The ideal food carrier or vehicle is consumed throughout the year in amounts sufficient to provide adequate mineral amounts for the populations at greatest risk, without compromising the safety of those consuming larger amounts of the food. If only a specific group of the population is at risk for a particular nutrient deficiency, it must be possible to select a food consumed only by this specific group. However, if the cost of fortification is low (as is usually the case), extension of the fortification program to the entire population may be administratively more practical and still economically feasible. It is not always possible to define an ideal food vehicle for fortification in every situation. Staple foods (milk and milk products, wheat flour, bread, fats, and oils) and also condiments such as salt are the most adequate examples for fortification, because they are consumed by large population groups. Salt is an ideal carrier of micronutrients due to its almost universal and uniform regional consumption (Diosady et al. 2002; Salgueiro et al. 2002).

The biofortification of crops, through targeted genetic manipulation and/or the application of mineral fertilizers, combined with breeding varieties with an increased ability to acquire mineral elements, is an alternative approach to fortifying foods with minerals. In addition, since mineral elements in edible portions of biofortified crops must be bioavailable to humans, parallel attempts are proposed to increase the contents of "promoter" compounds such as vitamin C, β-carotene, cysteine-rich polypeptides, and certain organic and amino acids, which favor the absorption of essential mineral elements in the gut, and to reduce the concentrations of antinutrients, such as oxalate, polyphenolics, or phytates (White and Broadley 2009).

The most common elements added to foods are calcium, iodine, iron, selenium, and zinc. In choosing the compounds to be added, solubility, stability in the food to which they are added, possible changes in the food sensorial properties as a result of element addition, mineral bioavailability, and safety—especially in terms of potentially adverse nutrient interactions such as those that can occur between minerals (e.g., calcium and iron)—have to be taken into account (Abrams and Atkinson 2003).

Several mineral elements, including Ca, P, Fe, Zn, Se, I, and F, are authorized for addition to foods and for use in food supplements (Annex I and II of Regulation (EC) No. 1170/2009). At present, in the European Union, elements such as calcium (Regulation 983/2009) and phosphorus (Regulation 1024/2009) have been authorized in relation to health claims regarding growth and bone development in children.

In the United States, minerals used for the fortification of foods are classified by the Food and Drug Administration as being Generally Recognized as Safe (http://www.fda.gov/Food/FoodIngredientsPackaging/GenerallyRecognizedasSafeGRAS/default.htm).

31.2 Sample Treatment

In the case of simple food matrixes (salt, tea, water, etc.), only sample dilution is required for mineral determination.

Routine mineral food analysis is performed using traditional dry ash or acid digestion procedures. These procedures are reliable but have drawbacks. Dry ashing (oxygen atmosphere at a temperature of 450–600°C) is safe, requires little attention from the analyst, and permits a large number of samples to be processed. However, the technique is time consuming (2–3 days to prepare an analytical solution). Conventional acid digestions are typically faster (normally 3–4 h) than dry ashing, but require constant operator attention. Ternary acid digestions (nitric/perchloric/sulfuric acids) offer more complete digestion, but safety concerns and hazardous waste regulations make the use of perchloric acid unattractive. Microwave digestion offers many advantages over conventional digestion procedures. It is usually performed with nitric acid in a closed high-pressure polytetrafluoroethylene-lined vessel. These features reduce acid consumption, contamination, and preparation time. Microwave digestion is usually complete within 1 h. The microwave system can be controlled and operated unattended, and is easily transferred to other laboratories. However, the mass of the analytical portion must be carefully selected to prevent excessive pressure during the digestion. Thus, each food matrix may require a different microwave program, or the analytical portion mass is restricted (Dolan and Capar 2002; Alegría et al. 2004). A single microwave

digestion program for multielemental analysis in foods by inductively coupled plasma absorption emission spectroscopy (ICP-AES) was developed to digest a variety of food matrixes at the same time. This method was enabled by an analytical portion mass based on the food's energy content. Method validation has shown that it is applicable to different elements, including Ca, Fe, P, and Zn (Dolan and Capar 2002).

Microwave-assisted extraction (MAE) is an extraction technique that combines microwave and traditional solvent extraction. MAE has many advantages, such as shorter time, less solvent, higher extraction rate, lower cost, and the advantage of not requiring tedious clean-up.

An MAE procedure for Zn and Fe determination in celery using HNO_3 as the extraction solvent based on a multivariate technique, response surface methodology (RSM), has recently been described. RSM represents a more economical approach, as the number of experiments can be significantly reduced, and the interaction between variables can be evaluated (Khajeh and Sanchooli 2010).

Tables 31.1 through 31.7 show the sample pretreatment conditions commonly used in different analytical methods.

31.3 Calcium

Most of the governments and health organizations (National Institute of Health 1994; Guéguen and Pointillart 2000) emphasize the importance of calcium enrichment of food products. Adequate calcium intakes are required to achieve adequate growth and bone mass density (McCarron and Heaney 2004, WHO/FAO 2006).

Calcium fortification has been shown to be an economical way to obtain additional calcium, and it eliminates compliance problems with regard to remembering to take a supplement (Keller et al. 2002). To give consumers more opportunity to increase their calcium intake without resorting to supplementation, manufacturers in the United States have been encouraged to fortify foods and beverages with calcium if certain criteria can be met (Henry and Heppell 2002): the food needs to be commonly consumed, the added ingredient should be compatible with the product, and the technology to fortify the food should be simple, safe, and cost effective.

Several commercial calcium salts (carbonate, chloride, phosphate, tribasic calcium phosphate, citrate malate, lactate, gluconate, lactate gluconate, and natural milk calcium) have been used to fortify milk/beverages, which are the foods of choice for calcium addition. Calcium gluconate, lactate, sulfate, acetate, citrate, and carbonate are approved for fortification, and their calcium absorbability is similar to that of calcium from milk (Fairweather-Tait and Teucher 2002). Milk satisfies all the prerequisites for an ideal food carrier: it has a high nutritional value, and most of the calcium is present as caseinate–phosphate complex from which calcium is readily released during digestion, and hence has high potential bioavailability (Guéguen and Pointillart 2000).

The application areas for adding calcium to dairy products include yoghurt, yoghurt drinks, ice cream, cottage cheese, sour cream, cream cheese preparations, and desserts, among others (Pirkul et al. 1997; Gerstner 2002; Van der Hee et al. 2009). Calcium enhancement makes these products even more attractive nutritionally.

However, the addition of calcium to milk and milk products poses problems. Attempts to produce calcium-fortified protein-containing beverages have resulted in products with low soluble calcium contents, suspension settlement problems, poor textures as a consequence of added stabilizers or chelating agents, or a combination of these drawbacks. Studies have been made to solve these problems. Accordingly, calcium chloride, calcium lactate, and calcium gluconate have been the calcium salts assayed in the case of cow and buffalo milks (Ranjan et al. 2005; Singh et al. 2007), and calcium carbonate and tricalcium phosphate have been the salts employed in soybean milk (Chaiwanon et al. 2000). Stabilizers and emulsifiers have been used in these products to maintain calcium in suspension so as to improve its sensory acceptance, calcium bioavailability, and heat stability. It is possible to increase the calcium content up to 50 mg/100 mL in fruit yoghurt by adding calcium lactate, without affecting the sensorial properties (Singh and Muthukumarappan 2008). Heat stability, organoleptic properties of milk, and calcium bioavailability are affected by the type of calcium salt used; as such, they are the critical factors in choosing calcium salts to be used as fortifying agents.

TABLE 31.1

Main Techniques for the Determination of Calcium in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Titrimetry			
Ca-chloride-, Ca-lactate-, and Ca-gluconate-fortified buffalo milk and whey[a]	20% (w/v) Trichloroacetic acid (TCA) and filtering. Precipitation as calcium oxalate. Dissolution with HCl	Back titration of EDTA (0.05 M) excess with MgAc (0.015 M) Indicator: Calmagite solution	Ranjan et al. (2005)
Ca-chloride-, Ca-lactate-, and Ca-gluconate-fortified cow milk[a]			Singh et al. (2007)
Ca-lactate-fortified mango yoghurt			Singh and Muthukumarappan (2008)
Fortified flour	Dry ashing 550°C. Precipitation with oxalic acid. Dissolution with H_2SO_4 (AOAC: 944.03)	Titration with $KMnO_4$	AOAC (2002)
Poultry and beef	Wet digestion: HCl. Addition of EDTA (pH = 12.5) (AOAC: 983.19)	Back titration with Ca_2CO_3 Indicator: naphthol blue	AOAC (2002)
Canned vegetables	Dry ashing 525°C (AOAC: 968.31)	Titration of EDTA (pH = 12.5–13) Indicator: hydroxynaphthol blue	AOAC (2002)
Spectrometry			
Enriched whole and skimmed milk	Dilution with water (1:100). Addition of pH 8.5 0.2 M Tris buffer solution. Immersion of the ionophore-treated test strip	Visible ($\lambda = 655$ nm)	Capitán-Vallvey et al. (2004)
X-Ray Fluorescence			
Ca-lactate-fortified vegetables (eggplant, carrot, oyster mushroom) by vacuum impregnation	—	ED-XRF	Gras et al. (2003)
Ion Selective			
Ca-chloride- and Ca from milk-enriched skim milk	—	Ca-ISE	Canabady-Rochelle et al. (2007)
Chromatography			
Ca-lactate-enriched sliced carrots	—	IC: Metrosep C2 150 column (250 mm × 4 mm); amperometric detector; mobile phase: aqueous tartaric acid (4 mM/L) and dipicolinic acid (0.75 mM/L) at 1 mL/min	Rico et al. (2007b)
Ca-lactate-enriched sliced apples	Dry ashing 550°C. Dissolution with HNO_3	IC	Barrera et al. (2009)

TABLE 31.1 (continued)

Main Techniques for the Determination of Calcium in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Atomic Spectrometry			
Ca-carbonate- and tri-Ca-phosphate-enriched soybean milk[a]	Dry ashing 450°C	FAAS	Chaiwanon et al. (2000)
Cheese	Dry ashing 525°C (AOAC: 991.25)	FAAS	AOAC (2002)
Ca-lactate- and Ca-chloride-fortified rice	Dry ashing 550°C. Dissolution with HNO_3	FAAS (5% lanthanum)	Porasuphatana et al. (2008)
Fortified fruit juices containing milk and cereals[a]	Dry ashing 450°C. Dissolution with HCl	FAAS (0.2% lanthanum)	Perales et al. (2005)
Fortified cow milk[a]			Perales et al. (2006)
Ca-lactate-enriched mango slices	Dry ashing 500°C. Dissolution with HNO_3 (65%) (AOAC: 975.03)	FAAS	Torres et al. (2008)
Ca-lactate- and Ca-gluconate-enriched apple slices	Drying 80°C. Microwave digestion. Dry ashing 550–600°C. Dissolution with HNO_3	FAAS	Anino et al. (2006)
	Drying 60°C. Microwave digestion HNO_3	FAAS ($LaCl_3$, 6500 μg/g)	Salvatori et al. (2007) González-Fésler et al. (2008)
Ca-gluconate-fortified yoghurt	24% TCA. Filtering	FAAS (5% La_2O_3)	Fligner et al. (1988)
Ca-lactate- and/or Ca-gluconate-enriched yoghurt	Wet digestion: $HNO_3 + H_2O_2$	FAAS	Pirkul et al. (1997)
Fortified cereal-based complementary infant foods	—	FAAS	Uvere et al. (2010)
Fortified orange juice concentrate, vegetable juice, butter substitute, dry mix beverage (also similar items without added Ca). Standard reference material (SRM)	Wet digestion: $HNO_3 + H_2O_2$. Residue dissolved in 0.5% lanthanum 0.1 M HCl	FAAS	Cerklewski (2005)
Ca-chloride- and Ca from milk-enriched skim milk	—	FAAS	Canabady-Rochelle et al. (2007)
Ca-carbonate-enriched cow milk	Dry ashing	FAAS	López-Huertas et al. (2006)
Fortified ice cream formulations			Van der Hee et al. (2009)
Fruit juices	a. Photo-Fenton reaction + microwave + UV radiation: $Fe^{2+} + H_2O_2$	ICP-OES	Gromboni et al. (2010)
	b. Microwave digestion: $HNO_3 + H_2O_2$		
Osmodehydrated melons by Ca-lactate	Drying 100°C, 1 h. Dry ashing 525°C. Dissolution with HNO_3 (AOAC: 991.25)	ICP-MS	Ferrari et al. (2010)

[a] Bioavailability study.

TABLE 31.2

Main Techniques for the Determination of Phosphorus in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Gravimetry			
Baking powders	Sample + $MgNO_3$ ignition, dissolution with HNO_3	Precipitation with MoO_3 in NH_4OH, weight as $Mg_2P_2O_7$ (AOAC 965.18)	AOAC (2002)
Titrimetry			
Flour	a. Dry ashing 550°C with $MgNO_3$ b. Dry ashing 550°C with Na_2CO_3	Precipitation with ammonium molybdate, dissolution in alkali standard, titration with acid standard (AOAC 948.09)	AOAC (2002)
Spectrometry			
Cheese	Dry ashing 525°C	Ammonium molybdate/ ammonium metavanadate ($\lambda = 400$ nm) (AOAC 991.25)	AOAC (2002)
Cheese and cheese products	Wet digestion: $H_2SO_4 + H_2O_2$	Molybdate/ascorbic acid ($\lambda = 820$ nm) (AOAC 990.24)	AOAC (2002)
Meat and meat products	Wet digestion: $H_2SO_4 + H_2O_2$	Molybdate/ascorbic acid ($\lambda = 890$ nm) (AOAC 991.27)	AOAC (2002)
Milk, skim milk powder	—	2,4–Diaminophenol dihydrochloride ($\lambda = 750$ nm)	De la Fuente and Juarez (1995)
Ca-fortification compounds SRM 1549 nonfat milk powder	Wet digestion: $HNO_3 + H_2O_2$	Ammonium molybdate/ aminonaphtolsulfonic acid	Cerklewski (2005)
Ca-fortified milk	—	—	Singh et al. (2007)
Soy and dairy products	Dry ashing 450°C	Ammonium molybdate/ hydrazine sulfate ($\lambda = 730$ nm)	Jastrzebska et al. (2003)
X-Ray Fluorescence			
Soy and dairy products	Drying 200°C	WD XRF—analytical line P-K_α 0.58 nm, counting time 60 s	Jastrzebska et al. (2003)

Although a variety of calcium compounds have been approved for calcium fortification, a significant fraction of calcium-fortified foods uses tricalcium phosphate. Dibasic calcium phosphate is also used as an anticaking agent for dry-mix drink powders. The relevance of the new recommendation for higher calcium intake compared to adequate phosphorus is that the potential negative effect of a high phosphorus diet on bone health can be negated by a high calcium intake. In addition, a higher calcium intake will reduce phosphorus absorption to some degree. Only the use of a phosphorus-free calcium compound for calcium fortification of foods will make it possible to achieve a higher calcium intake relative to adequate phosphorus (Cerklewski 2005).

Besides dairy products, other products such as some ingredients for preparing complementary foods for infants and young children [quick-cooking rice (Porasuphatana et al. 2008), maize, and bambara groundnut food (Uvere et al. 2010)] have recently been found to be fortified in calcium in order to prevent low calcium intakes/malnutrition in this population. In contrast, in industrialized countries, calcium

TABLE 31.3

Main Techniques for the Determination of Iron in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Spectrometry			
Milk, soft drinks	Milk samples diluted 1:10 or 1:100	Ascorbic acid (1% w/v) + NaAc (pH = 4.5) + *o*-phenanthroline	Oliveira et al. (2000)
	Ground dried solid samples + H_2O + Triton X-100		
	Sequential injection system with online sample decomposition (HNO_3 and microwave)		
Flour	Dry ashing 550°C + $Mg(NO_3)_2$	$NH_2OH.HCl$ 5 min. *o*-phenanthroline or α,α′-dipyridyl. λ = 510 nm (AOAC 944.02)	AOAC (2002)
	Wet digestion: $H_2SO_4 + HNO_3$	$NH_2OH.HCl$ 5 min. *o*-phenanthroline. λ = 510 nm (AOAC 944.02)	
Fortified wheat flour, powdered drink mixes, rice	Extracting solution: 1.2 M HCl, 0.6 M TCA, 0.7 M $NH_2OH.HCl$. Boiling bath, 15 min	Chromogen reagent: 0.03% bathophenantroline disulfonic acid/3 M NaAc. λ = 535 nm	Kosse et al. (2001)
Legumes	Ground sample + H_2O. Agitation 5 min + TCA (boiling bath, 10 min). Centrifugation (1800*g*, 10 min) → soluble fraction	Fe (II) and Fe (III) of total soluble iron Addition's method to interferences elimination $NH_2OH.HCl$/ bathophenanthroline. λ = 535 nm	Quinteros et al. (2001)
Fortified and unfortified wheat flour	a. Total iron: 5–10 g. Dry ashing at 600°C/12 h aided with HNO_3 and HCl	a. H_2SO_4 30%/$K_2S_2O_8$ 7%/ KSCN 40%. λ = 530 nm b. Iron bioavailability: α,α′-dipyridyl method	Nayak and Nair (2003)
Bioaccessible fraction of food dishes	*In vitro* digestion → bioaccesible fraction	Fe (II) and Fe (III) from bioaccessible fraction TCA + $NH_2OH.HCl$/ bathophenanthroline. λ = 535 nm	Cámara et al. (2005)
Fish	Freeze-dried samples + citrate buffer (pH 5.5). Ascorbic acid + TCA. Centrifugation	Addition of ammonium acetate, ferrozine color reagent Filtration. λ = 562 nm	Roos et al. (2007)
Milk, tea, foodstuffs	a. Milk or tea: drying. Dry ashing 450–500°C. Dissolution with HNO_3 b. Foodstuffs: Drying. Wet ashing with $HNO_3 + HClO_4$ Dissolution with HCl	Fe (II): digested solution + PBN/ EtOH + thiosemicarbazide + phosphate buffer (pH = 6.0) λ = 550 nm	Sharma and Singh (2009)
Dual-fortified salt (1000 mg/kg several iron form and 100 mg/kg potassium iodide (KI) encapsulated)	Water dilution Wet digestion: concentrated H_2SO_4	3% 1,10-Phenanthroline Buffer solution 0.2 M potassium biphthalate Total iron: λ = 396 nm Ferrous iron: λ = 512 nm Ferric iron: $\Delta_{396-512\ nm}$	Diosady et al. (2006)

continued

TABLE 31.3 (continued)

Main Techniques for the Determination of Iron in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Atomic Spectrometry			
Fortified and unfortified rice (brown and white)	Drying 70°C/72 h and dry ashing 500°C	FAAS	Prom-u-thai et al. (2009)
Rice	Dry ashing 450°C	FAAS	Jorhem et al. (2008)
Fish	a. Dry ashing 550°C b. Microwave solvent extraction system with 65% HNO_3	FAAS	Roos et al. (2007)
Pumpkin	Bioavailability assay	FAAS (AOAC 953.01)	Escalada et al. (2009)
Water, lettuce, onion, rice, spices, and SRM 1568a rice flour	a. Vegetables: dried and homogenized sample + HNO_3. Evaporation 130°C/3 h. Addition of H_2O_2 to dryness. Resolution with diluted HNO_3. Preconcentration as their *N*-benzoyl-*N*-phenylhydroxylamine complexes on Amberlite XAD-1180 resin. Elution 1 M HCl in acetone	FAAS	Tokalıoğlu and Livkebabcı (2009)
Dual-fortified salt (ferrous fumarate and potassium iodide or potassium iodate encapsulated)	Total iron: digestion: HNO_3:HCl (1:1 v/v). Dilution to 40 mL with H_2O *In vitro* solubility iron: contact with 1 N HCl 30 min	FAAS ($\lambda = 248.3$ nm, hollow cathode lamp)	Diosady et al. (2002)
Triple-fortified salt microencapsulated (ferric pyrophosphate/KIO_3/vitamin A)	Dissolution and filtration	FAAS	Wegmüller et al. (2006)
Dual-fortified wheat-based biscuit (ferrous sulfate and KIO_3 or NutraFine™ [a])	—	FAAS	Biebinger et al. (2009)
Legumes and nuts; SRM	Wet digestion: dried sample + 65% HNO_3 + V_2O_5 60°C/30 min + 120°C/60 min	ETAAS	Cabrera et al. (2003)
Fruits, SRMs	Sample + Triton X-100 → slurry	ETAAS Standard's addition method	Cabrera et al. (1995)
Olives fruit	a. Dried samples + HNO_3 + HCl + H_2O_2/90°C/17 h b. Dried samples + HF + HNO_3/90°C/8 h + HNO_3 + HCl + H_2O_2/90°C/17 h	ETAAS	Soares et al. (2006)
Chocolate milk powder, infant cereals, corn bran, dietetic milk powder. SRMs	Microwave digestion (closed- or open-vessel system): HNO_3	ICP-AES Internal standard: Sr ($\lambda = 338.071$ nm), or Y ($\lambda = 371.028$ nm) or Cr ($\lambda = 283.563$ nm)	Poitevin et al. (2009)

TABLE 31.3 (continued)

Main Techniques for the Determination of Iron in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Wheat-flour and flour-based ready-oven foods	a. Microwave digestion: sample + HNO_3 (PTFA digestion tube). 80% power 10 min; 100% (700 W), 10 min b. Slurry: sample + Triton X-100 + HNO_3/ultrasonic bath/15 min	ICP-OES a. Aqueous standards b. Standard addition with slurries	Cernohorsky et al. (2009)
Fish	Microwave digestion in closed Teflon vessels ($HNO_3 + H_2O_2$) pressure 200 psi, ramp time 25 min, $T^a = 210°C$, maximum power 300 W, hold time 10 min	ICP-AES	Türkmen et al. (2009)

[a] NutraFine™ Comercial H-reduced Fe.

addition has been observed not only in dairy products, but also in foods of diverse origin [orange juice concentrate, vegetable juice, butter substitute, dry mix beverages (Cerklewski 2005)].

Moreover, calcium salts have been employed before or after harvest in order to delay ripening and senescence and to prevent physiological disorders, extending the shelf-life of several fruits and vegetables. Furthermore, the use of calcium salts recently has been aimed at tissue structural preservation and the development of functional foods (Martin-Diana et al. 2007).

Two main techniques for calcium application to fresh-like minimally processed fruits and vegetables have been reported: calcium salt dips in combination with mild heat treatments and impregnation methods with the addition of calcium salts to the osmotic solution under vacuum or atmospheric conditions. With the latter approaches, porous food matrixes can be impregnated with solutions or suspensions of

TABLE 31.4

Main Techniques for the Determination of Zinc in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Spectrometry			
Foods	Wet digestion: $HNO_3 + HClO_4$	Diphenylthiocarbazone/pH 8-8-2/Extraction CCl_4. $\lambda = 540$ nm (AOAC 944.09)	AOAC (2002)
Atomic Spectrometry			
Foods	a. Dry ashing 525°C	FAAS (AOAC 969.32)	AOAC (2002)
	b. Wet ashing: $HNO_3 + H_2SO_4$		
	c. Dry ashing 450°C	FAAS (AOAC 999.11)	
Foods	Microwave digestion: $HNO_3 + H_2O_2$	FAAS (AOAC 999.10)	AOAC (2002)
Foods	Dry ashing 650°C	FAAS	Ghaedi et al. (2009)
	SPE: pH 7, SDS-coated with alumina- bis (2-hydroxyacetophenone)-1,3-propanediimine (BHAPN)		
Celery	Microwave-assisted extraction: HNO_3	FAAS	Kjajeh and Sanchooli (2010)
Wheat grains	Wet digestion: $HNO_3 + HClO_4$	FAAS	Chhuneja et al. (2006)
Multiple-fortified quick-cooking	Fe and Zn: wet digestion: $HNO_3 + HClO_4$	FAAS	Porasuphatana et al. (2008)
	Ca: dry ashing 550°C		

TABLE 31.5

Main Techniques for the Determination of Selenium in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Spectrofluorometry			
Human food	Wet digestion: $HNO_3 + H_2SO_4 + HClO_4$	EDTA/NH_4OH/DAN 100°C 2 min/room temperature 1–2 h/cyclohexane extraction. λ_{exc} = 366 nm, λ_{em} = 525 nm (AOAC 974.15)	AOAC (2002)
Neutron Activation Analysis			
Food matrixes. SRM	—	RSINAA thermal neutral flux 1.4 to 4.5 × 10^{16} nm^{-2} s^{-1}; irradiation time 10–20 s, detector Ge	Ventura et al. (2009)
Food matrixes. SRM	—	PC-INAA-AC thermal neutral flux 5 × 10^{11} cm^{-2} s^{-1}; irradiation time 30 s, detector HPGe	Zhang and Chatt (2009)
Atomic Spectrometry			
Enriched sprouts	Total Se: Microwave digestion: $HNO_3 + H_2O_2$	ICP-MS	Lintschinger et al. (2000)
	Se speciation: Four extraction procedures: H_2O, HCl (0.1 M), NaOH (0.1 M), or nonspecific protease. Centrifugation	AE-HPLC-ICP-MS	
Oysters. SRM	Total Se: Microwave digestion: $HNO_3 + H_2O_2$	HGAAS	Moreno et al. (2001)
	Se speciation: aqueous extraction (pH 7.5 room temperature). Enzymatic hydrolysis (pronase E in Tris media)	IE-HPLC-ICP-MS	
Enriched yeast. SRMs	Total Se: enzymatic hydrolysis: protease XIV. Sonication (5 s 20 W) and centrifugation	ICP-MS	Capelo et al. (2004)
	Se speciation: enzymatic probe sonication as total Se (sonication 30 s 20 W)	HPLC-ICP-MS	
Chicken (muscle, liver, and kidney)	Total Se: a. Microwave digestion: $HNO_3 + H_2O_2$ b. Enzymatic probe sonication (protease *S. griseus*, sonication 120 s 200 W and centrifugation 40,000 rpm)	ICP-MS	Cabañero et al. (2005)
	Se speciation: a. Enzymatic hydrolysis (*S. griseus*, pronase E) b. Enzymatic probe sonication	HPLC-ICP-MS	
Enriched plants	Total Se: a. Microwave digestion: $HNO_3 + H_2O_2$ b. Three extraction procedures: HCl (0.1 M), buffer extraction (pH 5.6), or enzymatic hydrolysis (protease) with/without probe sonication	ICP-MS	Montes-Bayon et al. (2006)
	Se speciation: Extraction procedures as in b	RP-IP-HPLC/ SEC/IE-HPLC	
Enriched potatoes	Total Se: Wet digestion: $HNO_3 + H_2SO_4 + H_2O_2$	HGAFS	Cuderman et al. (2008)
	Se speciation: Extraction a. Water b. Pronase XIV c. Amylase d. Pronase XIV + amylase with stirring incubation or ultrasound probe	HPLC-ICP-MS	

TABLE 31.5 (continued)

Main Techniques for the Determination of Selenium in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Enriched food supplements and yeast. SRM	Total Se: a. Ultrasonic-assisted enzymatic digestion (protease XIV, ultrasonication 120 s, centrifugation 4000 rpm 3 min), b. Microwave digestion: $HNO_3 + H_2O_2$	ETAAS	Vale et al. (2010)
	Se speciation: ultrasonic-assisted enzymatic digestion	AE-HPLC-ETAAS	

bioactive compounds without destroying the initial food matrix, and minimizing the processing of foods. So, impregnated products can be commercialized as minimally processed fresh functional foods or can be dried osmotically or by air in order to obtain more stability (Fito et al. 2001; Alzamora et al. 2005; Martin-Diana et al. 2007; Rico et al. 2007a).

31.3.1 Determination

The application of several techniques for calcium determination in some products is shown in Table 31.1.

Among the official methods, titrimetry and atomic spectrometry are the most commonly used options, usually with ashing as sample preparation.

A selective optical strip test based on an ion exchange (IE) mechanism to determine calcium in several types of milk has been developed. This test trio contains a polymeric film of plasticized polyvinyl chloride that contains all the reagents necessary to produce a response to calcium, among them the new ionophore, 4,13-bis[(*N*-adamantylcarbamoyl)propionyl]-1,7,10,16-tetraoxa-4,13-diazacyclooctadecane, measuring the absorbance at 655 nm. The procedure was applied to the determination of calcium ion in different types of milks (whole, skimmed, skimmed with calcium added, special types), validating the results against atomic absorption spectrometry (AAS) as a reference method (Capitán-Vallvey et al. 2004).

Calcium distribution between the soluble and the colloidal phases of reconstituted skimmed milk supplemented with different salts (such as Ca chloride and Ca from milk) has been studied. Total Ca and soluble Ca were quantified by AAS and ionized Ca by ion-selective electrode (ISE) (Canabady-Rochelle et al. 2007).

In a recent study (Gromboni et al. 2010), different sample preparation techniques (photo-Fenton reaction coupled with microwave radiation and common acid digestion using microwave radiation) were compared with direct determination by inductively coupled plasma optical emission spectrometry (ICP-OES) using chemometrics for elementary analysis, including calcium, in fruit juices. The use of the reaction between Fe^{2+} and hydrogen peroxide, coupled with ultraviolet (UV) radiation, known as the photo-Fenton reaction, is widely used in the treatment of residues with high carbon content due to its high efficiency, low cost, and the use of reagents with low toxic effects. The volume of Fe^{2+} and H_2O_2 and the reaction time were the variables for photo-Fenton system evaluation.

The difference presented, when chemometrics were applied, for direct analysis in comparison with the other two methods is due to the quantity of carbon present in the samples. This elevated carbon quantity causes interference in the spectral analysis. The values of mineral element evaluated in this work showed that sample preparation of juice is necessary for ICP-OES determination. All methods presented satisfactory accuracy for all levels tested when addition and recovery tests were performed; therefore, it could be applied to mineral-enriched fruit juices as well. Although direct analysis was the method that presented worst recovery values, it can be inferred from these results that the higher carbon content originally present in the samples may be interfered in the analysis. The photo-Fenton reaction proved to be an interesting alternative procedure for sample preparation because its reagents are cheaper and have lower toxicity than acid digestion. Microwave and photo-Fenton reaction presented satisfactory and concordant results.

Calcium distribution in plant tissues, using calcium lactate for fortification by the vacuum impregnation technique, was evaluated directly without sample preparation by energy dispersive x-ray fluorescence (ED-XRF) (Gras et al. 2003).

TABLE 31.6

Main Techniques for the Determination of Fluoride in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Titrimetry			
Foods, baking powders	Ashing with $Ca(OH)_2$, isolation by Willard–Winter distillation	Back titration with $Th(NO_3)_4$ (AOAC 944.08)	AOAC (2002)
Ion Selective			
Foods	Total F: a. Open ashing 500°C, isolation by overnight diffusion with $HClO_4$ at 60°C. b. Oxygen-bomb-reverse extraction: incineration and extraction in diphenylsilanediol and reverse extraction in NaOH Acid-diffusible F (ionic + acid-labile): a. Heat-facilitated diffusion (overnight diffusion with $HClO_4$ at 60°C, the diffused fluoride was trapped in NaOH 0.5N), b. Silicone-facilitated diffusion: concentrated $HClO_4$ and collect the diffused fluoride with 0.5 N NaOH	Fluoride ISE (pH 5.0)	Singer and Ophaug (1986)
SRM shrimps, tea, krill, fish feed	Dry ashing 525°C with 8 M NaOH	ISE (pH 5.2–5.4)	Kjellevold Malde et al. (2001)
Diet samples. SRM	Ashing with NaOH and alkali carbonate fusion with $KNaCO_3$	ISE (pH 6.0) using the semiautomatic multiple known addition technique	Ponikvar et al. (2007)
Tea infusions. SRM	Direct determination	ISE (pH 5.2)	Malinowska et al. (2008)
Drinking waters, beverages	Dilution 1:1 with TISABII	ISE	Viswanathan et al. (2009)
Chromatography			
Foods. SRM	a. Alkali fusion (550°C), b. Alkali fusion + CaO (550°C), c. Direct diffusion, d. Closed-vessel concentrated HNO_3 digestion. Fluoride extraction with chlorodimethylphenilsilane in cyclohexane	GC-FID: Capillary column (30 m × 0.5 mm × 1 μm). Carrier gas He (0.5 bar), temperature programmed oven. Injection volume 2.5 μL. FID = 200°C GC-MS: Capillary column (30 m × 0.25 mm × 0.25 μm). Carrier gas He (0.8 bar), EI +, *m/z* 50–300	Haldimann and Zimmerli (1993)
Tea infusions	Direct determination	AE-LC: column (15 × 4.6 mm × 5 μm), guard column (5 × 4 mm × 5 μm). Isocratic elution: 3.2 mM Na_2CO_3 + 1.0 mM $NaHCO_3$. Flow rate 0.7 mL/min. Injection volume 20 μL. Suppressor and conductivity detector	Michalski (2006)

Calcium lactate enrichment of melon pieces by osmodehydration was determined by inductively coupled plasma mass spectrometry (ICP-MS), and structural changes, mechanical properties, and sensory preferences were also evaluated (Ferrari et al. 2010). The effect of calcium, measured by ion chromatography (IC), on the textural properties of calcium-fortified apple slices and sliced carrots during storage has been studied by Rico et al. (2007b) and Barrera et al. (2009). Ashing treatments, as sample preparation,

TABLE 31.7

Main Techniques for the Determination of Iodine in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Titrimetry			
Fortified salt (KIO_3)	Water dilution	pH adjusted 2.8 with 0.6% HCl. KI (to convert KIO_3 to I_2). 0.004 N NaS_2O_3/starch indicator solution. Relative standard deviation (RSD) < 1%	Diosady et al. (1997, 1998)
Saturated systems solutions		KI/0.3 M HCl. 0.1 N NaS_2O_3/ starch indicator solution	Winger and Ren (2009)
Dual-fortified salt (several iron form and KIO_3 encapsulated)		Iodide crystals + HCl or H_2SO_4 drops NaS_2O_3 known concentration/ starch solution	Diosady et al. (2006.)
Double-fortified salt and iodized salt		2 N H_2SO_4/10% KI. Dark 10 min. 0.005 M NaS_2O_3/starch indicator solution	Sullivan et al. (1995)
Double-fortified salt (ferrous sulfate and KIO_3) and iodized refined and common salts (KIO_3)		1% KI/4N H_3PO_4. Dark 10 min. 0.005 M NaS_2O_3/starch indicator solution	Ranganathan et al. (2007)
Iodized salt (AOAC 92.556)		Br-H_2O/ bleach boiling 100°C. Add cristal salicylic acid. 85% H_3PO_4/KI. 0.0025 M NaS_2O_3/ starch indicator solution	AOAC (2002)
Mixed Feeds (AOAC 93.514)	Na_2CO_3 solid/NaOH (1 + 1)/ alcohol/100°C 30 min. Dry ashing 500°C/15 min. H_2O/100°C 10 min. Filter		
Spectrometry			
Dual-fortified salt (several iron form and KI or KIO_3)	Ultrasound water solution	Ammonium persulfate 95°C 65 min/0.1 M arsenious acid/15 min/0.08 M ceric ammonium. $\lambda = 405$ nm at 33 min	Haldiman et al. (2003)
Dual-fortified salt (several iron form and KI or KIO_3)	—		Wegmüller et al. (2003)
Iodized salt (25 μg iodine/g salt)	—		Wegmüller et al. (2006)
Foods (uncooked and cooked)	Dry ashing: dry 110°C. Na_2CO_3/600°C 2 h		Goindi et al. (1995)
Food	Dry ashing: 30% (w/v) $K_2CO_3 + Zn_2SO_4$. Dry 95°C, 100–500°C in 90 min and	Potassium thiocyanate solution/ ammonium iron(III) sulfate/ sodium nitrite. $\lambda = 450$ nm.	Moxon and Dixon (1980[a])
Dairy products. SRM	maintain 1 h. Added Zn_2SO_4 and		Cressey (2003)
Fortified pork (impregnated wheat fiber or soy isolate) and iodized salt. Iodization: KI or KIO_3	repeat drying and ashing procedure. Centrifugation 50 Hz 5 min to remove carbonaceous matter		Waszkowiak and Szymandera-Buszka (2008[b])
Fortified pork (impregnated collage) and iodised salt. Iodisation: KI			Waszkowiak and Szymandera-Buszka (2007)
Fortified milled rice (KI or KIO_3 or mixture of KI and KIO_3) and nonfortified milled rice			Tulyathan et al. (2007)

continued

TABLE 31.7 (continued)

Main Techniques for the Determination of Iodine in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Fortified freshwater fish emulsion sausage (refined tuna oil with KI)	Dry ashing: 30% (w/v) potassium carbonate solution/550°C 3 h		Panpipat and Yongsawatdigul (2008)
Neutron Activation Analysis			
Fortified salt (KIO_3)	Polyethylene vial shielded with Cd (to decrease interference of high concentration of chlorine)	NAA: Irradiation 1 kW. Neutron flux 5.0×10^{11} cm^{-2} s^{-1} 3 min. Gamma ray spectrometer 44.3 keV. Calibration 0–250 mg iodine/kg salt RSD < 5%	Diosady et al. (1997, 1998)
Dual-fortified salt (several iron form and KI or KIO_3 encapsulated)			Diosady et al. (2002)
Dual-fortified salt (several iron form and KI encapsulated)		EINAA: Irradiation 1 kW. Neutron flux 5.0×10^{11} cm^{-2} s^{-1} 3 min. Gamma ray spectrometer 44.3 keV. Calibration 0–1000 mg iodine/kg salt RSD < 5%	Diosady et al. (2006)
Diet	—	NAA	Haldimann et al. (2000)
Food. SRMs	—	EINAA: Epi-cadmium flux 2×10^{11} n/cm^2 s. Irradiation time 18 min, decay time 3 min, counting time 10 min	Serfor-Armah et al. (2003)
Electroanalysis			
Table salt, seaweed, eggs	Oxygen flask combustion 2% acidified sodium formate as absorbent	CSV: Ag/AgCl, 0.95 to –0.2 V at 250 mV/s	Yang et al. (1991)
Chromatography			
Pasteurized liquid milk, skim milk powder	Water dilution Ultrafiltration (10,000–30,000 MW, 900–1000 rcf 15–20 min)	Reverse phase for quantitation 20.3 µg/L iodide. Mobile phase: 10 mM dibasic sodium phosphate, 1 mN cetyl trimethylammonium chloride, 32% acetonitrile pH 6.8. Electrochemical detector with silver working electrode or pulsed amperometric detector Flow 2 mL/min. Injection volume: 50–200 µL	AOAC (2002)
Atomic Spectrometry			
Vegetables. SRM	Wet digestion: HNO_3/N_2 (high-pressure asher-autoclave: 80–110°C 30 min and 230°C 90 min (130 bar))	ICP-MS: Rf power/W 1050; Argon flow L/min (outer 15, intermediate 0.8, nebulizer 1). Cinnabar minocyclonic spray chamber and a Micromist low-uptake nebulizer	Haldimann et al. (2000)
Foods			Haldimann et al. (2005)
Dual-fortified wheat-based biscuit (ferrous sulfate and KIO_3 encapsulated or 20 mg NutraFine[c TM])			Biebinger et al. (2009)

TABLE 31.7 (continued)

Main Techniques for the Determination of Iodine in Fortified and Nonfortified Foods

Food	Sample Preparation	Determination Conditions	Reference
Dual-fortified salt (several iron form and KI or KIO_3) added and nonadded of national institute of standards and technology (NIST) SRM 4949C ^{129}I radioactivity standard	Ultrasound water solution		Haldimann et al. (2003)
Triple-fortified salt microencapsulated (ferric pyrophosphate/KIO_3/ vitamin A)			Wegmüller et al. (2006)
Food. SRMs	Wet digestion: 65% HNO_3 + 30% H_2O_2.Microwave oven (20 min, 650 W, 30 bar). Internal standard (tellurium) Adjusted pH 9–10 3% NH_3	ICP-MS (Detection limit 16.2 µg I/kg or 0.081 µg I/L sample solution)	Eckhoff and Maage (1997)
Food	a. Extraction iodine: tetramethylammonium hydroxide/bottle closed gas-tight dry oven 90 ± 3°C 3 h. Undissolved particles removed by centrifugation or filtration (5 µm). Internal standard (tellurium) b. Wet digestion: HNO_3-$HCLO_4$ in high-pressure asher	ICP-MS: Rf power 1350 W; Ar flow L/min (cooling 13, auxiliary 1.2, nebulizer 0.9). Sample skimmer Mi; cyclone spray chamber; concentric nebulizer	Fecher et al. (1998)
Salt	—	ICP-OES	Wegmüller et al. (2003)
Skimmed milk powder and human milk	Wet digestion: HNO_3 + H_2O_2. Microwave	ICP-ORC-MS	De la Flor et al. (2004)
Whey fractions of human milk, raw cow milk, UHT cow milk and infant formulas. SRM	Centrifugation (30,000*g* 60 min 5°C). Milk whey SEC	ICP-QMS: Meinhard (concentric nebulizer). Double pass/ Peltier spray chamber, cooled 2°C. Ni sampler and skimmer cones	Rivero Martino et al. (2002)
Nonfat milk powder	Solid sample	ETV-ICP-MS. ETV: Prereduced Pd (0.5 µg) (120/1000/20°C)/sample 0.5–2 g (120/700/2500/2700°C). Interfaced to the Ar plasma: 80 cm length (6 mm id) Teflon tubing. Ni sampler and skimmer cones: 1.0 and 0.75 mm	Resano et al. (2005)
Nonfat milk powder, cow milk, milk powder	a. Slurry sonication: *aqua regia* b. Microwave wet digestion: HNO_3 + H_2O_2 c. Direct analysis previous dilution with water. Comparison of digestion methods	ICP-MS: Cross flow nebulizer. Ni sampler and skimmer cones: 1.14 and 0.89 mm	Cava Montesinos et al. (2005)

[a] Manual or automated determination.

[b] Products containing KIO_3, 1% ascorbic acid was used for reduction to KI.

[c] NutraFine™ Commercial H-reduced Fe.

and flame atomic absorption spectrometry (FAAS) for calcium measurement, were employed by Anino et al. (2006), Salvatori et al. (2007), González-Fésler et al. (2008), and Torres et al. (2008) for their studies on several fruits enriched with calcium.

31.4 Phosphorus

Phosphorus oxoacids and their salts (as inorganic phosphates and polyphosphates) are the common phosphorus species in foods (Jastrzebska et al. 2003).

The European Food Safety Agency (EFSA) Panel on Dietetic Products, Nutrition, and Allergies concludes that a cause–effect relationship has been established between the dietary intake of phosphorus and normal function of cell membranes, normal energy-yielding metabolism, and the maintenance of normal bone and teeth conditions (EFSA 2009a).

31.4.1 Determination

Different analytical methods have been developed for phosphorus determination in food samples. The methods of the Association of Official Analytical Chemists (AOAC) include a gravimetric and a titrimetric method for P determination in baking powders and flour, respectively (AOAC 2002). Visible spectrophotometry is often simple and less expensive than other methods. Some applications of phosphorus determination by the most commonly used techniques are summarized in Table 31.2.

Total phosphorus content has been measured in milk, dairy products, and infant formulas through the formation of a complex with molybdate or the reduction of amidol after digestion of the sample. Spectrophotometric determination of P in dairy products following microwave digestion ($HClO_4 + H_2O_2$) is a simple, rapid, and low-cost method when compared to ICP-AES, when only P is to be measured—making it a suitable procedure for routine determination (Alegría et al. 2010).

A sequential injection system for the in-line digestion (thermal/UV-induced digestion) and colorimetric determination of P in milk has been developed. The detection limit was 2 mg P/L. The advantages of the method are its speed, with 3.5 min/sample (digestion and determination) versus 4 h for the classical procedures, and the possibility of total automatization—thereby eliminating human errors and saving time (Reis-Lima et al. 2002).

A microdetermination technique applied to P in foods, based on the formation of a phosphomolybdate, has been proposed and validated. The detection limit of 0.04 μg in assay (1.1 mg P/100 g sample) is lower than that recorded in milk or infant formulas by techniques such as electrothermal atomic absorption spectrometry (ETAAS), ICP-AES, or neutron activation analysis (NAA) (Miquel et al. 2004).

Atomic spectrometric methods (FAAS, ICP-MS, ICP-AES) have been applied to P determination in different foods (Alegría et al. 2004).

Others methods for P determination such as flow injection analysis, capillary electrophoresis, high-resolution ^{31}P nuclear resonance magnetic, and x-ray fluorescence (XRF) have been described. The attractive features of XRF, compared to other analytical methods, are the rapidity of the determination, and the fact that it is a nondestructive and easily automated procedure. Wavelength dispersive x-ray fluorescence (WD XRF) has been applied to different foods for P determination, and has been compared to spectrophotometric methods with ammonium molybdate. The XRF method appeared to be more accurate and precise than the standard method, as well as less time consuming, and sample preparation proved simple (Jastrzebska et al. 2003).

31.5 Iron

Iron deficiency is a major global public health problem, and is particularly common in developing countries—affecting more than two billion people worldwide (WHO/FAO 2006).

Food fortification has been identified as one of the most cost-effective and sustainable approaches to control iron deficiency anemia. Cereals (flours, bread, bakery products, breakfast cereals, cereal-based foods), dairy products (milk, beverages, fermented milks, cheeses), and to a lesser extent sugar, salt, and condiments are the products most commonly used for iron enrichment (Martínez-Navarrete et al. 2002; Salgueiro et al. 2002).

Cereal flours are currently the most frequently used option, and are even obligatory in some countries, but the efficacy of this measure is not clear, mainly owing to differences in the form and level of iron used. Breakfast cereals and cereal-based foods for infants are often enriched with iron. Beverage powders and chocolate drinks have been enriched with compounds such as ferrous fumarate, ferrous succinate, and ferric pyrophosphate. The addition of disodium ethylene diamine tetraacetic acid (EDTA) to iron-fortified foods appears to counteract the inhibitory effects of phytate and polyphenols, but only when the iron fortificant is soluble (the molar ratio of EDTA to iron is important—ratios of 1:2 and 1:1 improving iron absorption) (Fairweather-Tait and Teucher 2002).

Several soluble (ferrous sulfate, ferrous gluconate, ferrous lactate, ferrous ammonic citrate), poorly soluble (ferrous fumarate, ferrous succinate, ferrous saccharate), and insoluble iron compounds (ferric orthophosphate, ferric ammonic orthophosphate, ferric pyrophosphate, elemental iron powder, electrolytically reduced carbonyl iron) have been used to fortify foods (Salgueiro et al. 2002). Although soluble iron compounds have high bioavailability, they often result in unacceptable color and flavor. Ferrous sulfate is a water-soluble compound that has the highest relative bioavailability among conventional iron compounds. It is commonly added to foods, but has been reported to cause a metallic taste in fruit drinks, and it produces strong color changes in many foods (Hurrell 2002; Hilty et al. 2009).

Ferric pyrophosphate is a water-insoluble iron compound often used to fortify infant cereals and chocolate drink powders, as it causes no adverse color or flavor changes in food vehicles. However, its absorption in humans is low. Recently, novel ferric pyrophosphate compounds have been developed, based on small particle size ferric pyrophosphate and encapsulation with a mixture of emulsifiers, so that they remain in suspension in liquid products. These novel compounds offer iron absorption similar to that of ferrous sulfate from fortified infant cereals, as well as from yoghurt drinks (Fidler et al. 2004).

Ferrous bis-glycinate has the advantage of being soluble in water and does not change the organoleptic properties of the food vehicle. This compound is being increasingly considered in programmers for iron fortification of foods and beverages because it prevents iron from binding to inhibitors in food (Olivares and Pizarro 2001, Miglioranza et al. 2003). Encapsulation of iron compounds for food fortification may protect against adverse sensory changes and reduce interactions of Fe with other food components, but at the same time may reduce bioavailability (Wegmüller et al. 2004).

The need for high bioavailability forms of iron that do not react with food matrixes favors the proposed use of iron-protected compounds (hemoglobin, EDTA-Fe (III), amino acid chelates, stabilized ferrous sulfate) that nevertheless are not free of drawbacks. The intense color in the case of hemoglobin and the safety concerns that its use raises constitute an example. The risk of an increased absorption of toxic metals with the use of EDTA-Fe (III) is another potential concern (Salgueiro et al. 2002).

Encapsulation of iron offers the potential to help overcome several major challenges in the iron fortification of foods. It may decrease unwanted sensory changes in fortified products and reduce interactions of iron with food inhibitors of iron absorption capable of affecting its bioavailability. However, the effect of encapsulation per se upon iron bioavailability is a concern. The addition of encapsulated iron to foods or condiments also poses problems. Accordingly, in the case of addition to salt, the capsule design should be improved to increase resistance to moisture and abrasion, while maintaining bioavailability. In staple cereals, a potential barrier to their use is the relatively low-melting point of the capsules, which may cause unwanted changes during food preparation. The cost of the encapsulation process likewise may be a limit to the application of this method (Zimmermann 2004).

Some studies have demonstrated that reducing the particle size of low-solubility Fe compounds to the μm scale may improve their bioavailability from foods (Wegmüller et al. 2004; Arredondo et al. 2006). Nanostructured Fe compounds produce significantly less color change than ferrous sulfate, ferrous fumarate, or reduced Fe when added to the foods. Thus, nanostructured powders may prove valuable for fortification of color-sensitive foods, such as extruded artificial rice grains, salt, chocolate-based

drinks, and fruit yoghurts. Because iron and zinc deficiencies often coexist in populations, combined Fe/Zn-containing nanostructured compounds may be useful for nutritional applications. The new nanostructured powders produce minimal color changes when added to dairy products containing chocolate or fruit compared to the changes produced when ferrous sulfate or ferrous fumarate is added to these foods. These powders are promising for food fortification and other nutritional applications (Hilty et al. 2009).

31.5.1 Determination

Several reference methods for quantifying iron are available using analytical techniques such as spectrometry, AAS, and ICP-AES. Some applications of iron determination by the most commonly used techniques are summarized in Table 31.3.

Among the many colorimetric methods for iron determination, the phenantroline method is the most commonly used technique because of its simplicity and the stability of the colored iron complex formed. This method quantitatively determines ferrous iron content by the reaction of ferrous iron with 1,10-phenantroline (Yuan et al. 2008).

Kosse et al. (2001) developed and evaluated a rapid method for iron determination in fortified and unfortified foods. The method was adapted from a spectrophotometric method used in the determination of serum iron concentration in human blood. The proposed method produced accurate results that were not significantly different from the standard laboratory method (a modification of an AOAC method (AOAC: 14.013)), where iron contents were analyzed by ICP-AES after sample digestion with HNO_3/$HClO_4$ for the majority of the food samples tested. It was necessary to determine the optimum-heating time for the method, since heating affects the accuracy of the rapid method. Heating for an insufficient time resulted in low iron extraction in some samples. However, heating for a prolonged time caused browning of the solution. Fifteen minutes of heating was sufficient to extract all of the iron in most samples. The rapid method underestimated the iron content of enriched flour and cornmeal but not the unenriched counterparts. This might be due to the slow dissolution of the elemental iron fortificants in dilute acids. The proposed method takes a little over an hour to complete, while most standard laboratory methods for iron determination can take days to complete.

A spectrophotometric method for iron determination in foods with an ethanolic solution of 2,6 bis(1-hydroxy-2-naphthylazo)pyridine (PBN) has been proposed. The method is very simple, highly selective, reproducible, and relatively inexpensive. The comparison with AAS determination reveals that PBN can be successfully used for iron determination in diverse samples of foods (Sharma and Singh 2009).

Iron exists mainly in the iron(III) form, and it is well known that iron(II) is more bioavailable than iron(III). Therefore, it would be interesting to estimate the different oxidation states of the iron added to fortified products to complete the study and knowledge of its bioavailability. A spectrophotometric method using batho-phenantroline as reagent has been optimized for iron speciation [ionic Fe (II) and Fe (III)] in the soluble mineral from cooked legumes (Quinteros et al. 2001) and from the fraction obtained from the *in vitro* digestion of food dishes (bioaccessible fraction) (Cámara et al. 2005).

A semiquantitative test kit for the determination of microencapsulated iron in double-fortified salt has been developed using a mixture of 5% heptane and 95% tetrachloroethylene to free the iron, which in turn was determined by the 1,10-phenanthroline method. This method has a detection range of 0–2 ppm of iron. The results obtained with the kit correlated well with those obtained by methods recommended by the AOAC (AOAC 14.011, 14.012, 14.013) (Yuan et al. 2008).

The optothermal window technique is a suitable choice for iron(II) determination once it overcomes the fact that sample opacity prevents the use of conventional techniques such as the traditional optical spectroscopic methods. This methodology has been applied to the determination iron(II) content in fortified commercial milk (Cardoso et al. 2003).

In iron-fortified foods, AAS is useful after wet or dry digestion. The direct determination of extremely low concentrations by atomic spectroscopic methods, such as FAAS and ICP-AES, is often difficult because of insufficient sensitivity and selectivity of the methods used. For this reason, iron preconcentration as *N*-benzoyl-*N*-phenyl hydroxylamine complexes on Amberlite XAD-1180 resin and determination

by FAAS has been proposed as a simple, sensitive, precise, reliable, and accurate technique for iron determination in foods (Tokanoglu and Livkebaber 2009).

ICP-AES is one of the most commonly used techniques especially within the food industry for accurate and cost-efficient routine analyses of nutritional minerals in food products. Among the different papers published on iron determination in food by ICP-AES, mention must be made of the study of Poitevin et al. (2009), made to improve and update AOAC official method 984.27, and including a single-laboratory validation and a ring trial to determine nine nutritional elements in food products (calcium, copper, iron, potassium, magnesium, manganese, sodium, phosphorus, and zinc). The improvements involved optimized microwave digestion, selected analytical lines, internal standardization, and ion buffering. Selectivity, sensitivity, linearity, accuracy, precision, recovery, ruggedness, and uncertainty were estimated. The results indicate that the method proposed can determine, with sufficient precision, from 169.9 to 16.4 mg/kg. In addition to the microwave digestion proposed, the main improvements compared to the AOAC Method 984.27 are the use of appropriate analytical wavelengths for each element of interest, and the automatic addition of a solution of appropriate internal standard and ionization buffer to correct for physical and chemical interferences (i.e., to compensate for matrix effects induced by the complexity of the food samples). Neither internal standardization nor ion buffer is used for AOAC Method 984.27. The application for all food matrixes covered all nine AOAC food (fat–protein–carbohydrate) triangle sectors, including infant formulas, which were the only types of matrixes validated for AOAC Method 984.27.

ED-XRF could be a good alternative for the analysis of iron versus AAS or ICP-AES because sample preparation and analysis are nondestructive, rapid, and simple; the technique can be installed near the production line; and the installation of ED-XRF does not require any water connections for cooling. The setting up of the calibration standards is a really important step in the installation of ED-XRF. A delay of several weeks is generally necessary to collect these calibration samples and to complete the reference method measurements. A key point for a successful application is that a suitable range of products containing the adequate range of iron must be available to obtain robust calibrations. This technique has been applied to determine iron in infant cereals and comparing with ICP-AES, with no significant bias between the two methods. The total analytical time for iron is <20 min (Perring and Blanc 2007).

31.6 Zinc

Zinc deficiency is widespread through the world. The major problem in zinc fortification is finding a suitable zinc compound. The choice of the zinc compound to be added, considering zinc bioavailability, side effects, and technological aspects, is a question that remains to be solved.

The zinc compounds most commonly used in food fortification are zinc sulfate and zinc oxide, but both have drawbacks. Zinc sulfate affects food sensorial properties (mainly food flavor), and the solubility of zinc oxide is low, precipitating in liquid foods, while in solid foods, differences in particle size and density between zinc oxide and the food to which it is added can lead to the depositing of zinc oxide at the bottom of the package. Zinc oxide is a commonly used source of supplementary zinc in diets of animals and humans (Salgueiro et al. 2002; Rosado 2003).

Nowadays, efforts are made to develop protected fortifying agents similar to those developed for iron, and in some cases, given that both deficiencies frequently coexist, iron and zinc fortification is proposed. Fe-containing and atomically mixed Fe/Zn-containing nanostructured compounds show promise for nutritional applications. These compounds demonstrated superior sensory qualities in reactive food matrixes at solubility equivalent to commercial ferrous sulfate and Zn sulfate—the reference compounds for food fortification in humans (Hilty et al. 2009).

The EFSA Panel on Dietetic Products, Nutrition, and Allergies concludes that a cause–effect relationship has been established between the dietary intake of zinc and normal function of the immune system, normal DNA synthesis and cell division, the protection of DNA, proteins and lipids from oxidative damage, maintenance of normal bone, normal cognitive function, normal fertility and reproduction, normal

metabolism of fatty acids, normal acid–base metabolism, normal vitamin A metabolism, and the maintenance of normal vision (EFSA 2009b).

31.6.1 Determination

A novel and simple spectrophotometric method (based on the complexation reaction with bromopyrogallol) via ratio spectra-continuous wavelength transformation for the simultaneous determination of Ca, Mg, and Zn without prior separation steps has been recently applied to ultra-high temperature (UHT) cow's milk and milk powder (Afkhami et al. 2008).

AAS, ETAAS, ICP-AES, and ICP-MS have been proposed for Zn determination in foods. FAAS is one of the most widespread traditional analytical techniques, but it is often limited by its low sensitivity. Zn determination at very low concentrations requires the use of preconcentration methods coupled to spectroscopic methods such as ICP-AES and FAAS.

The traditional separation and preconcentration methods include liquid–liquid extraction, coprecipitation, IE, and so on. These methods often require large amounts of high-purity organic solvents, some of which are harmful to health and cause environmental problems. Magnetically assisted chemical separation, which uses micro- and nanoparticles, is suitable for this purpose. It combines the selectivity and efficient separation offered by chemical extraction with magnetic recovery of extractant for the selective separation of metal ions. A procedure for Zn determination in foods, after extraction by magnetic nanoparticles by ICP-OES, has been developed. This method has a detection limit of 0.8 μg/L (Khajeh 2009).

Solid-phase extraction (SPE) allows wide research activity in the development of alternative methods capable of high selectivity in the removal of trace amounts of metal ions from solutions containing complicated matrixes, with minimal usage of organic solvents. The technique can be easily adapted for FAAS to improve the detection limit and selectivity of the determinations (Ghaedi et al. 2009).

A critical study of different procedures such as dry ashing (with or without the addition of H_3BO_3, $Mg(NO_3)_2$, and H_2SO_4) and wet dissolution procedures (hot-plate, high-pressure asher, and open and closed microwave-heated systems) for the decomposition of milk samples was made, taking into account zinc recovery and residual carbon content. Digestions yielding lower residual carbon content values can be obtained on a hot plate with a mixture of HNO_3, H_2SO_4, and H_2O_2. The same mixture also provided maximum sample digestion in the open-vessel-focused microwave system. In closed-vessel microwave systems, replacement of low-pressure vessels by medium-pressure vessels, or the addition of H_2O_2 or H_2SO_4, reduces the residual carbon content. High-pressure digestion together with a high-temperature program completely destroys the organic matter only with HNO_3. Similarly, the dry ashing procedure is simple and exhaustive (Alegría et al. 2010).

For Fe and Zn determinations in yoghurts by AAS dry ashing (500°C, 3 h), wet ashing and microwave digestions were compared. For the two elements, the lowest detection limit was obtained by microwave digestion, although iron in the yoghurt samples was not completely released when microwave destruction was used (Yaman and Durak 2005).

Choi et al. (2007) have adapted spectrophotometric methodologies to generate a simple, cheap, and semiquantitative analytical method to help screen germplasm for wheat, maize, rice, cassava, sweet potato, and potato biofortification breeding programs. Using a common extraction procedure with 0.5 M HCl, Fe^{2+} was analyzed using 2,2 α-dipyridyl. Zn was analyzed after reaction with potassium cyanide, zincon (a colorimetric reagent for the detection of Zn and Cu), and cyclohexanone. These two methods showed good correlation with ICP-OES analyses in wheat.

Some applications of zinc determination by the most commonly used techniques are summarized in Table 31.4.

31.7 Selenium

Selenium is ubiquitous in soils, but exists mainly in insoluble forms in high-Fe, low-pH, and certain leached soils, and hence is often of limited availability to plants. Therefore, it is often supplied by plants to animals

and human consumers at levels too low for optimum health. Selenium deficiency probably affects at least a billion individuals and is associated with health disorders, including oxidative stress-related conditions, reduced fertility and immune functions, and an increased risk of cancer (FAO/WHO 2006).

Food sources of selenium can be characterized as follows: good sources include selenium-enriched yeast and wheat, moderate sources include most plant materials, and poor sources are most meat and fish products and soybean. Selenium is frequently included in food supplements. Some examples of such supplements are the following Se-enriched foods: broccoli, yeast, and potatoes (Vale et al. 2010).

The major forms of selenium in foods are probably the amino acids selenomethionine and selenocysteine bound to proteins. Selenomethionine is presumably the prevalent form in diets from plant sources, whereas proteins from animal tissue contain both amino acids in various proportions. Inorganic selenium species are used in supplements, but it is not likely that they are present in food (Vale et al. 2010).

To prevent selenium deficiency, biofortification of crops can be an effective way for producing Se-rich foodstuffs. This procedure has been adopted in Finland and its potential has been evaluated in the United Kingdom (Lyons et al. 2003; Broadley et al. 2006).

31.7.1 Determination

Fluorimetric determination has been applied to selenium assay in foods. The main problem encountered is organic matter destruction. With foods that have high protein contents, complete digestion is more difficult than in other biological samples. Organic matter destruction must be complete, without losses due to volatilization, and all Se must be reduced to the Se(IV) state. Wet digestion with HNO_3–$HClO_4$ is efficient and did not cause any interferences with the spectrofluorimetric technique. The HNO_3–H_2SO_4 mixture is effective in decomposing resistant organic material, but H_2SO_4 caused the formation of crystallized 2,3-diaminonaphthalene (DAN). The detection limit in Se spectrofluorometry determination (0.001 μg/g) is lower than in hydride generation atomic absorption spectrometry (HGAAS) (0.033 μg/g) (Alegría et al. 2010).

Hydride generation coupled to AAS, atomic fluorescence spectrometry (AFS), ICP-OES, and ICP–MS is commonly used in the determination of Se. This combination usually requires sample pretreatment for food samples that includes harsh digestion with acids, including perchloric acid, as some Se species are very resistant to digestion. Furthermore, a reduction step is required, since Se(VI), resulting from the oxidation of the organoselenium species, does not form hydride. HCl or HCl plus HBr or Br^- solutions are used for the reduction of Se. Treatment with *aqua regia*, assisted by microwave irradiation and sonication, is able to reduce all Se species present in the samples to Se(IV), being a faster and simpler alternative to other recommended procedures. Digestion and reduction occur in the same *aqua regia* medium, avoiding the use of a different reducer. Determination of Se in biological samples, including foods, by ICP-OES after digestion with *aqua regia* and online chemical vapor generation has been optimized. The quantitation limit of Se is 0.1 μg/g (dos Santos et al. 2009).

Ultrasonic liquid extraction (USLE) has been applied for total selenium studies in combination with either ultrasonic bath or ultrasonic probe under different acid conditions. The state of art concerning sample treatment using USLE for total selenium determination in several foods has been described by Capelo et al. (2004).

Enzymatic probe sonication as a tool for solid–liquid extraction for total Se determination in foods by ETAAS has been described by Vale et al. (2007). $Ni(NO_3)_2$ and $Pd(NO_3)_2$ were studied as matrix modifiers in conjunction with H_2O_2, the best results being obtained with $Pd(NO_3)_2$ plus H_2O_2. The presence of H_2O_2 as matrix modifier increases the lifetime of the graphite tubes up to 66%, by avoiding the building up of carbonaceous residues. The enzymes used must be carefully chosen. Protease XIV is the recommended protease-type enzyme. Activity of the enzyme must be regularly checked, since low activities lead to low extractability. Fresh protease must be used as soon as possible, and USLE must be performed at room temperature, since cooling diminishes enzyme activity.

NAA, while less used for Se determination than atomic methods, constitutes an alternative when the levels of Se in foods are too low for other methods. The main advantages are the significant reduction in experimental time, and generally better sensitivity ratings. Replicate sample neutron activation analysis (RSINAA) is based on the fact that the signal from a short-lived radionuclide of interest can be improved

by summing the spectra from a number of replicates, in the same way that the total spectrum in cyclic activation can give better sensitivity and detection limits. This method has been applied to different foods with lower or higher Se contents, and is considered to be a reliable method for total Se determination in foods (Ventura et al. 2009). The anticoincidence counting technique in combination with pseudo-cyclic neutron activation (PC-INAA-AC) provides a more reliable measurement in terms of improved precision and detection limits for short-lived nuclides compared to both one-shot and PC-INAA coupled to conventional counting for Se determination in foods, without the need of dissolving the samples or tedious chemical treatments. A lower detection limit is always obtained in anticoincidence counting for the same number of cycles compared with conventional counting (Zhang and Chatt 2009).

In addition to the total selenium concentration in foods, the identification and quantification of the elemental Se species is crucial in the understanding of the metabolism of this element and its importance in biology, toxicology, clinical chemistry, and nutrition (Lintschinger et al. 2000).

For selenium speciation, different approaches such as enzymatic and basic hydrolysis have been proposed, the former being the most widely used option. However, the process sometimes requires two consecutive steps, each lasting 24 h at 37°C. This methodology is extremely time consuming, the efficiency of the process is far from being quantitative, and the risk of selenium interconversion is very high (Capelo et al. 2004).

Different solvents (H_2O, 0.1 M, 0.1 M NaOH) or a solution containing nonspecific protease was used to obtain the information about the chemical form of selenium present in selenium-enriched sprouts. Under acidic conditions and with the protease solution, no increase in extraction ratio was obtained, whereas with NaOH the extraction efficiency increased by ~10–15% compared to the water extract (extraction efficiency 64–72%) (Lintschinger et al. 2000).

The application of pronase, proteinase-K, subtilisin, pepsin, and trypsin for Se speciation has been reported. These methods offer certain advantages in terms of the moderate conditions of temperature and pH involved, preventing elemental losses by volatilization, and their selectivity, and it is possible to distinguish among fractions of elements bonded to different components of the sample matrix. This methodology is extremely time consuming, and therefore the risk of selenium species interconversion could be high (Cabañero et al. 2005).

Selenium compounds from oysters and their determination by high-performance liquid chromatography inductively coupled plasma mass spectrometry (HPLC-ICP-MS) were evaluated by Moreno et al. (2001). Extraction of Se species in aqueous media (with studies of aqueous solvents, pH, and extraction temperatures) was optimized. Water as extraction medium, pH 7.5, and room temperature are the best extraction conditions. Also, enzymatic hydrolysis (subtilisin in phosphate medium or pronase E in Tris media) was evaluated. When pronase E was used, a precipitate of $Ca(PO_4)_2$ was obtained. Enzymatic hydrolysis with subtilisin is the method of choice due to the higher enzymatic activity for this enzyme. The use of enzymatic hydrolysis to catalyze the breakdown of selenoproteins into selenoamino acids prior to fractionation of the samples by aqueous extraction is an efficient method for selenium speciation in foods.

An enzymatic process assisted by ultrasonic probe energy applied to selenium speciation was first presented by Capelo et al. (2004). The catalytic activity of the proteolytic enzyme was dramatically improved in kinetic terms of the extraction, and no interconversion of selenium species was detected. The extraction is performed in water, minimizing contamination risk and without further pH adjustment, and no control temperature is needed. Moreover, the time of enzymatic incubation using the ultrasonic probe is substantially shortened.

Enzymatic probe sonication extraction of Se in animal-based food samples has been described by Cabañero et al. (2005). Parameters influencing extraction (sonication time, extracting media, temperature, sample mass ultrasound amplitude, and sample/enzyme mass ratio) were investigated. The advantages of the proposed method over traditional hydrolysis are speed, simplicity, and safety of the procedure.

An excellent review of ultrasonic-assisted enzymatic digestion for total elemental determination and elemental speciation has recently been published (Vale et al. 2008).

Three extraction procedures [acid extraction (0.1 M HCl), buffer extraction (25 mM ammonium acetate buffer, pH 5.6), and enzymatic hydrolysis (protease) with and without ultrasonic probe] for Se extraction from selenium-enriched plants have been studied by Montes-Bayón et al. (2006). No statistically

significant differences among extraction procedures were observed when no ultrasonic probe was applied. However, with ultrasound, slightly higher yields were observed with protease, and the extraction time was dramatically shortened. For Se speciation, reverse-phase ion-pairing high-performance liquid chromatography inductively coupled plasma mass spectrometry (RP-IP-HPLC-ICP-MS) or size exclusion/ion exchange high-performance liquid chromatography (SEC/IE-HPLC) was suitable for isolating the main extractable Se species.

Selenium water-soluble compounds extracted from Se-enriched potatoes by water or enzymatic hydrolysis (protease XIV, amylase, or both) at different times of extraction (2–24 h) for stirring incubation or ultrasound-assisted extraction were determined by Cuderman et al. (2008). No differences were observed using an ultrasound probe or incubation for the extraction of Se compounds. The extraction efficiency was found to be higher when using protease XIV and amylase together.

Regarding Se speciation, lower detection limits can be achieved with recently developed powerful analytical tools such as ICP-MS or liquid chromatography tandem mass spectrometry. A critical review for selenium speciation from food using mass spectrometry has been published by Dumont et al. (2006). Although these techniques using mass detection can offer better understanding of selenium species identification, they are costly and expensive to maintain. The hyphenation of common techniques such as chromatography and AAS or AFS is an alternative to mass spectrometry techniques for control in Se-enriched supplements and is the option of choice in Se-enriched supplements where the concentration of Se species is high enough to be determined by less expensive techniques (Capelo et al. 2006).

In general, the literature describing Se speciation in unknown samples recommends the use of two different chromatographic columns, for instance, an anion exchange column and a column combining IE and SEC, to discriminate SeCys from SeMetO—since both species elute at the same time when only an anion exchange column is used. It has also been recommended not to use only retention times of standards in a single chromatographic method as a single method to identify the presence of certain Se species. However, in routine controls, when certain Se species can be anticipated or the number of expected species is low, the use of a single chromatographic column is still a valuable option (Vale et al. 2010).

Table 31.5 summarizes the main techniques for total selenium and speciation in foods.

31.8 Fluoride

The effect of fluoride on humans is dual: it is an essential trace element at low levels, and is a potentially toxic element at higher levels. Natural fluorides from drinking water and food, especially tea infusions and high fluoride salts, are the main sources of total fluoride intake in the population. The inverse relationship between fluoride exposure and the prevalence of dental caries is well established. There are a number of ways in which fluoride intakes can be increased: fluoride can be added to water supplies at the point of supply, or added to toothpaste. Hexa-fluoro-silicate acid is the most commonly used fluoride compound for large-scale water fortification. It is added as a concentrated aqueous solution. The fluoridation of salt and the enrichment of milk with fluoride are alternative options that have been used in some parts of the world (WHO/FAO 2006). A recently published study has shown that fluoridated milk has no negative effect upon the availability of trace elements (Zn, Cr, Fe, Mo, and Se), except Cu (Zohoori et al. 2009).

31.8.1 Determination

Fluoride-selective electrodes have proven to be a convenient and efficient tool for analyzing fluoride in water samples, where fluoride is present in the ionic form as free fluoride. Fluoride in foods is present in both ionic and bound forms, so total decomposition of the sample seems to be the critical step for total fluoride determination (Ponikvar et al. 2007). The most common methods for the decomposition of biological materials are open ashing, fusion, oxygen combustion, and acid digestion. Fluoride is most often determined by ISE or by gas chromatography (GC) (Kjellevold Malde et al. 2001). Methods based on the separation of fluoride from an unashed sample by acidic diffusion and determination of the isolated fluoride with colorimetric methods may yield erroneous results (Singer and Ophaug 1986).

Different sample treatments such as ashing procedures (open-ashing-heat-facilitated diffusion, oxygen-bomb-reverse extraction) and nonashing procedures (heat-facilitated diffusion, silicone-facilitated diffusion) for fluoride analysis [acidic diffusible (ionic plus acid-labile) and total fluoride (acidic diffusible plus nonionic forms)] in foods by ISE have been studied by Singer and Ophaugh (1986). For acid-diffusible fluoride content in foods, the simplicity of the silicone-facilitated diffusion method (unashed sample) and its low cost and easy application to large numbers of samples make it the recommended technique. For total fluorine content, ashing is required, with recommendation of the oxygen-bomb-reverse extraction technique, despite the fact that it is time consuming, complex, and limited in the number of samples that can be analyzed.

The influence of the concentration and volume of sodium hydroxide as ashing aid, sample weight, lifetime of the electrode, and storage time of the sample solutions for fluoride determination in foods by alkali fusion and fluoride ISE has been evaluated by Kjellevold Malde et al. (2001). Analytical parameters (accuracy: percentage recovery 81–105%), relative standard deviation (RSD) (%) 2.2–7.0, and detection limit (0.02 μg/mL) showed the alkali fusion method to be well suited for analyzing fluoride in food. The use of automated standard addition technique versus direct calibration for the determination of fluoride at low levels by ISE allows minimization of matrix effects, greater precision of the analysis, a spike recovery test on each sample, and a shorter time of analysis (Ponikvar et al. 2007).

Fluoride GC determination is based on the principle that appropriate chloroalkyl- or chloroarylsilanes are converted by water into the corresponding silanol. This reacts selectively to form the fluorosilane, which can be extracted from the acidified solution by an organic solvent and determined by GC. In contrast to the ISE, which directly measures fluoride activity, dissolved metal ions with a tendency to form fluorocomplexes do not interfere in this procedure. The potential of GC combined with different ashing procedures, including high-pressure closed-vessel acid digestion, was evaluated to determine trace amounts of fluoride in biological materials, including foods. Alkaline fusion (KOH) for plant materials and alkali fusion plus a retaining agent (CaO) for nonplant materials showed the best results. However, the limit of quantitation (0.3 μg/g) is relatively high owing to the concentration of fluoride in CaO. Glassy carbon crucibles are advantageous in comparison to platinum ones because of their inertness. GC measurement of fluoride via organosilane is accurate and specific, and the results obtained were similar when compared with a standard method using an ISE (Haldimann and Zimmerli 1993).

IC allows the simultaneous determination of a few ions, including fluoride, in a short time, with good reproducibility, high sensitivity, and a small sample volume—with the possibility of using conductimetric or mass spectrometry detectors (Michalski 2006).

A suppressed IC method for the simultaneous determination of common inorganic anions (including fluoride) in tea infusions has been developed and validated. Separation was performed on an anion exchange column and determination by suppressed conductivity. Accuracy (recovery assays ~100%, detection limit 0.14 mg/L, and precision RSD 2.3%) showed that the method is well suited for the rapid, accurate, and simultaneous analysis of organic anions (including fluoride) in tea infusions (Michalski 2006).

Some applications of fluoride determination by the most commonly used techniques are summarized in Table 31.6.

31.9 Iodine

Although iodine deficiency is easily treated, it is still considered to be a public health problem. Nearly two billion people (35.2% of the world population) have inadequate iodine nutrition (Anon 2004). More specific data on iodine deficiency can be obtained from Web pages of the WHO (WHO 2004).

The International Council for the Control of Iodine Deficiency Disorders is a nonprofit, nongovernment organization for the sustainable elimination of iodine deficiency and the promotion of optimal iodine nutrition worldwide (http://www.iccidd.org/).

The major cause of low iodine status is insufficient iodine intake. Iodination of salt has been considered to be the most efficient way to improve iodine intake. Salt iodination programs have led to significant reductions in iodine deficiency disorders. Because salt is cheap and consumed daily at a fairly steady

level, even by population groups in poor remote areas, it could be a promising food vehicle for fortification with iodine (Wegmüller et al. 2006).

Dual fortification of salt with iodine and iron can be an effective approach to combating iodine and iron deficiency. The National Institute of Nutrition has developed a double-fortified salt. Its characteristics of formulation, stability, bioavailability, acceptability, efficacy, safety, cost, and so on have recently been published in a review (Ranganathan and Sesikeran 2008).

The critical problem with iodine stability is the potential for reducing (iodate form) or oxidizing (iodide form) the iodine in the salt to elemental iodine. This can happen rapidly depending on the environmental conditions, manufacturing parameters, distribution, material packaging, and the presence of moisture and impurities (Diosady et al. 1997, 1998, 2002; Haldimann et al. 2003). It is theoretically possible that reactions in foods involving iodine and its salts may cause color reactions, increase oxidative reactions and hence reduce the shelf-life, and decrease the bioavailability of iodine and other nutritionally important substances. A review of the literature related to the addition or use of iodine and its salts in processed foods has recently been published (Winger et al. 2008).

Microencapsulation of iodine in the form of either potassium iodide or potassium iodate can protect double-fortified salt from iodine loss even under severe storage conditions (Diosady et al. 2002, 2006; Wegmüller et al. 2003).

In Asia and in less developed countries, rice cereal is another food that has been fortified with iodine because rice remains the main dietary staple (Tulyathan et al. 2007).

On the other hand, there are the nutritional recommendations promoting a reduced intake of salt. Thus, alternative carriers (collagen, wheat fiber, soy isolate) that could fortify foodstuffs with iodine are being explored (Szymandera-Buszka and Waszkowiak 2007; Waszkowiak and Szymandera-Buszka. 2007, 2008; Panpipat and Yongsawardigui 2008).

31.9.1 Determination

For iodine determination, destruction of the organic matrix by dry ashing under alkaline conditions (calcium carbonate) is necessary to avoid or reduce losses of iodine. Wet digestion with HNO_3 under strong oxidizing conditions and in closed flasks is commonly used to avoid losses.

The most commonly used methods for iodine determination have been colorimetric methods based on the catalytic effect of iodide (Sandell and Kolthoff 1937; Moxon and Dixon 1980), and titrimetric methods with freshly prepared sodium thiosulfate solution. Voltammetry and liquid chromatography (LC) also offer sufficient detection capacity, but they require time-consuming sample preparation steps. Various other methods have also been reported, such as NAA and ICP-MS. In terms of productivity, sensitivity, precision, accuracy, and operating ease, the colorimetric methods appear to be the most suitable option for several requirements. At present, ICP-MS is the analytical technique usually applied. Generally, thermal instrumental NAA for low levels of iodine suffers from the high background activities induced by activation products of Na, K, Mn, Br, and Cl, which could be present in foods (Moxon and Dixon 1980; Serfor-Armah et al. 2003). All these methods are reflected in Table 31.7.

The most practical application of a kinetic procedure for iodine determination is based on the catalytic effect of iodide on the reduction of ceric (Ce^{5+}) ammonium sulfate by arsenious acid (As^{3+}), which yields colorless cerrous (Ce^{3+}) ammonium sulfate and arsenic acid (As^{5+})—the decrease in absorption at 405 nm being proportional to the iodine concentrations (Sandell and Kolthoff 1937; Haldimann et al. 2003). However, the synergistic catalytic effect of iron on iodide kinetic reactions has not been studied in detail. The applicability of these reactions to the determination of iodine in dual-fortified salt samples enriched with different chemical forms of iron and iodine (as iodide or iodate) has been studied using ICP-MS as reference method. No influence of iron on the catalytic reaction was detected. This method is useful for iodine monitoring in salt fortified with iron and iodine (Haldimann et al. 2003).

Another colorimetric method, in which iodide catalyzes the destruction of iron thiocyanate by nitrite, with an accompanying decrease in the color of the iron(III) thiocyanate produced by the addition of iron(III) ions, has sufficient sensitivity and precision for the simple determination of total iodine in food. This technique has been evaluated and automated to be applied to several foods. In order to automate this

technique and make it applicable to a wide variety of foodstuffs, optimum conditions for the destruction of organic matter (different ashing aids: sodium carbonate, potassium hydroxide, and potassium carbonate) and the release of iodine (the effect of alkali on the colorimetric reaction) have been investigated, and a set of conditions (baselines, interferences by bubble, sampler wash solution) applicable to the automated end technique have been developed. The method offers a precision of about 10%, a detection limit for iodine of 1 μg/100 g food, a mean recovery of 90%, and an output of about 70 samples per week (Moxon and Dixon 1980).

Both colorimetric methods have been tested with a reference sample of nonfat milk powder. The thiocyanate-nitrite assay consistently showed greater sensitivity and precision, and less variability (Garwin et al. 1994).

One rapid and low-cost official analytical method for iodate determination in iodized salt is a titrimetric redox technique with sodium thiosulfate (AOAC 2002). Generally, the iodine content of iodized salt is estimated by conventional iodometric titration using sulfuric acid reagent (Sullivan et al. 1995). However, the titrimetric method has a potential for large positive or negative errors if the salt contains significant amounts of oxidizing or reducing impurities—giving rise to wide variations in the iodine content, and under/overestimation of iodine values has been observed in freshly prepared fortified salt (Ranganathan and Karmarkar 2006). This research group tested the new method to more accurately estimate the iodine content in double-fortified salt (iodine and iron) by using orthophosphoric acid instead of sulfuric acid in the titration procedure, reducing the quantity of KI and changing the order of addition of reagent. In this sense, iodine should be freed only after sufficient iodide is present in the solution to minimize the loss of iodine by volatilization. Excellent agreement was recorded with the conventional titration method for the estimation of iodine both in the KIO_3 standard at different iodine levels and in iodized salt from the factory to market. Therefore, conventional iodometric titration cannot be used for the estimation of iodine in dual-fortified salt (Ranganathan et al. 2007).

An iodine test kit (starch-based test used on a semiquantitative basis) has been evaluated versus the iodometric titration method in order to simplify the methodology of the titrimetric method. Due to the low specificity and resulting high numbers of false-positive results when used by multiple observers, the kit is seen to overestimate the iodized salt. Until a valid alternative becomes available, the titration method should be used for monitoring the iodine content of salt at all levels, from producer to consumer (Pandav et al. 2000).

At present, two methods are used for the determination of different iodine species in foods: NAA or a combined IC/ICP-MS system. However, no published studies have yet established the efficiency of the recovery of iodine and its salts from complex solid food matrixes. Determination of iodine species (iodate, iodide, and elemental iodine) is not possible by these routine measurements (Winger et al. 2008). The NAA has a high RSD (~5%), but is not subject to interference from reducing and oxidizing agents. Therefore, it was used to confirm the results of titrimetric measurements of iodine in salt (Diosady et al. 1998) and iodine determinations by ICP-MS in foods (Haldimann et al. 2000). NAA has been applied to evaluate iodine stability in salt (Diosady et al. 1997, 1998) and salt fortified with different iron compounds (ferric lactate, ferrous ammonium citrate, fumarate, or sulfate), and potassium iodide or potassium iodate encapsulated in modified starches, gelatin, sodium hexametaphosphate, and purified sodium chloride by spray drying and fluidized bed drying (Diosady et al. 2002, 2006).

Haldimann et al. (2000) proposed a new ICP-MS method for the determination of iodine in foods (milk, egg, vegetables, meat, wheat flour) digested with nitric acid in the high-pressure asher-autoclave that prevented losses of iodine. A spray chamber of reduced volume and a concentric glass nebulizer designed for low sample uptakes has been used, and is operated in a self-aspirating mode. The wash-out was accelerated by this aspiration mode over conventional systems. The isotope dilution technique (^{129}I) was applied to obtain freedom from matrix effects. The method was validated with several reference material standards and by comparison with NAA.

The aforementioned ICP-MS technique has been used to evaluate the accuracy of the colorimetric methods of Sandell and Kolthoff (1937) applied to salt samples (Haldimann et al. 2003).

The development and application of a simple alkaline extraction method employing tetramethylammonium hydroxide at high temperatures (90°C) and ICP-MS for the determination of iodine in food

(detection limit 30 mg/g) was accepted as a German reference method for the determination of this element in dietetic foodstuffs. The parameters of the extraction procedure (time, temperature) were studied versus wet digestion with HNO_3–$HClO_4$. The method was validated by a collaborative study involving 15 laboratories in two dietetic child nutrition food samples enriched with iodine, and the analysis of different certified reference materials (Fecher et al. 1998).

The LC method is an official method (992.22) applied to measure iodine in milk only in the form of iodide. Protein and insoluble material are removed by passing through a membrane. Iodide in clear filtrate is separated by reverse-phase ion-pair LC, and is selectively detected electrochemically (AOAC 2002).

A review of the application of IC to the determination of inorganic ion in food has been published by Buldini et al. (1997). The review includes application to the determination of inorganic anions, including iodine and iodate determination usually by UV–Vis and/or amperometric detection (Buldini et al. 1997).

The speciation of iodine species in milk and infant formulas by SEC with online selective detection by ICP-MS, with a detection limit of 1 μg/L, has been published (Sanchez and Spuznar 1999).

Some studies as examples of the speciation of iodine of nutritional interest in dairy products by using hyphenated techniques comprise the following:

1. The speciation of iodine species in milk and infant formulas by SEC with online selective detection by ICP-MS, with a detection limit of 1 μg/L (Sanchez and Spuznar 1999)
2. Combination of IC and ICP-MS to determine iodine species in milk samples with a limit of determination for iodide and iodates of <2 μg/L (Leiterer et al. 2001)
3. Iodine in three fractions (whey, fat, and caseins) of human milk and infant formulas using ICP-MS (Fernández-Sánchez 2007)

The extensive annual literature review in the section titled "Atomic Spectrometry Update on Clinical and Biological Material, Food and Beverages" of the *Journal of Analytical Atomic Spectrometry* (www.rsc.org/jaas) reflects the major role that atomic spectroscopy has played in the development of current databases for minerals in foods. Comprehensive reviews of methods focus on the progress for individual elements, sampling and sample preparation, reference materials, and developments in analytical methodology and instrumentation.

Recently, different methodologies for the determination of the aforementioned elements in different foods have been reviewed (Alegría et al. 2004, Alegría et al. 2009, 2010).

31.10 Conclusions

Mineral elements of nutritional interest are considered bioactive compounds, and therefore searching for ideal food products as good dietetic sources or as minerals carriers is of interest. These aspects have been the subject of public health programs and policies, and have led to the development of new technologies such as impregnation, encapsulation, or nanoparticulation for mineral fortification.

For Ca, P, Fe, Zn, Se, F, and I determination in fortified or nonfortified foods, sample treatment is still the key step in most of the techniques. The most widely used methods for all the aforementioned elements are atomic techniques. ICP-MS, NAA, and X-RF are reliable techniques for multielemental analysis. The conventional methods (spectrometry, titrimetry) remain valid for Ca, P, Fe, and I determination in enriched foods.

The hyphenated techniques, such as HPLC-ICP-MS, are able to define the chemical forms (speciation) of the element in the food—this being necessary in order to evaluate mineral bioavailability.

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Section X

Lipid Compounds

32

Omega 3 and Omega 6 Fatty Acids

Ken D. Stark

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32.1 Introduction

Omega 3 and omega 6 fatty acids are chemically classified as polyunsaturated fatty acids. As is common to fatty acids, they consist of a hydrocarbon chain with a hydrophilic carboxylic acid group at the "alpha" terminus and a hydrophobic methyl group at the "omega" terminus. As a polyunsaturated fatty acid, omega 3 and omega 6 fatty acids have more than one carbon–carbon methylene-interrupted *cis* double bonds in the hydrocarbon chain. The "omega 3" designates that the carbon–carbon double bond located closest to the omega terminus is in the third position, whereas the double bond is in the sixth position for the "omega 6" fatty acids (Figure 32.1). This terminus labeling reflects a bias of mammalian fatty acid metabolism and biochemistry as typical mammalian enzymology is not capable of introducing carbon–carbon double bonds in the omega 3 and omega 6 positions. Chemical-based nomenclature would locate the carbon–carbon double bonds using the "alpha" carboxy terminus as the reference point, and given that the "alpha" end of the molecule is the site of carbon addition (elongation) and removal (β-oxidation), an omega-3 bond in an 18-carbon fatty acid would be in the Δ15 position, in a 20-carbon fatty acid it

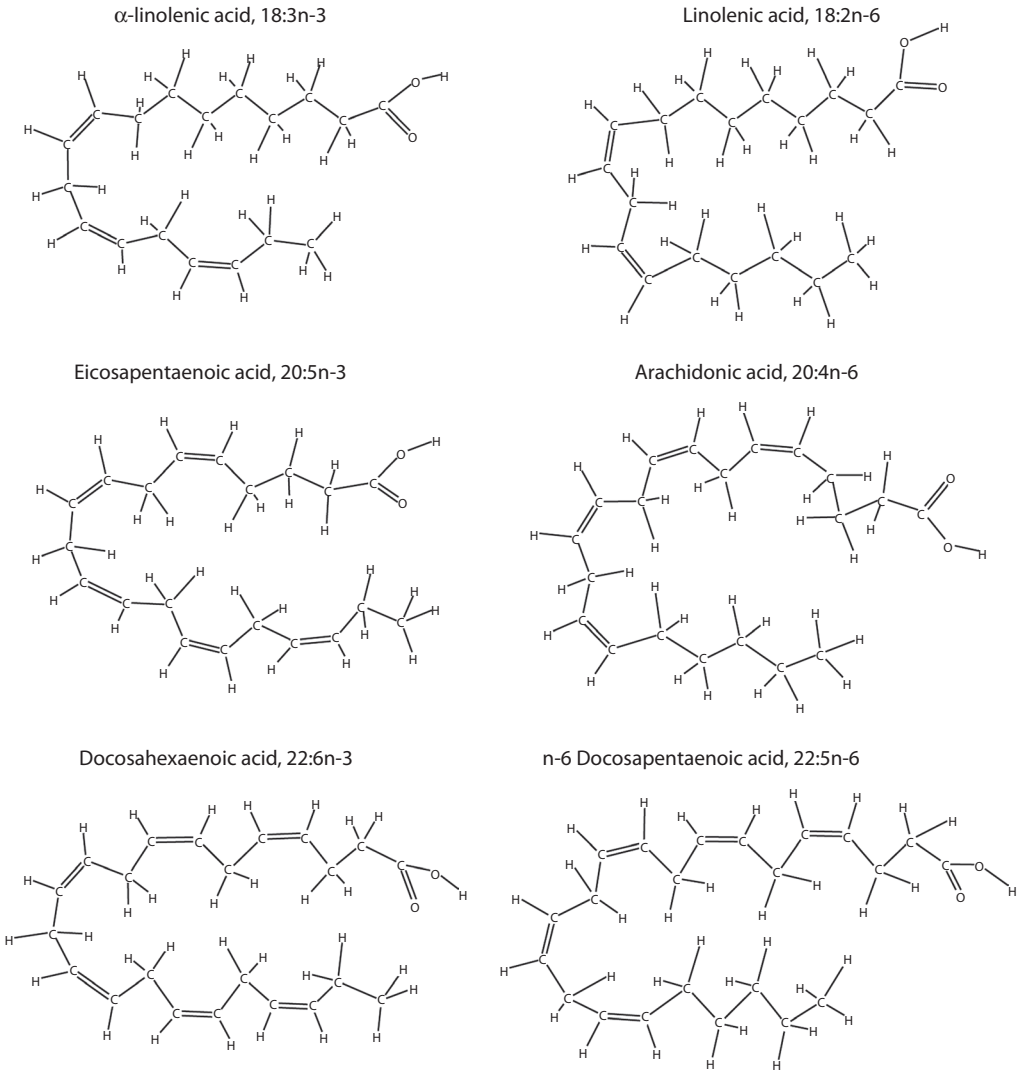

FIGURE 32.1 Structures of omega 3 and omega 6 fatty acids.

would be in the Δ17, and in a 22-carbon fatty acid it would be in the Δ19 position. Using the "omega" methyl terminology, distinct and important biological fatty acid classes are established and, therefore, in humans, an omega 3 fatty acid remains an omega 3 fatty acid and an omega 6 fatty acid remains an omega 6 fatty acid despite various metabolic changes. In biological applications and the literature, fatty acid nomenclature is typically a mix of modified systematic and trivial naming systems. A common approach is a numerical abbreviation where digits preceding a colon represent the number of carbons in the fatty acid and the number following indicates the number of carbon–carbon double bonds. For unsaturated fatty acids, the number of double bonds is also followed by a locant number from the methyl end. For example, all-*cis*-9,12 octadecadienoic acid or linoleic acid is 18:2n-6. Generally, for biological fatty acids, one assumes that carbon–carbon double bonds are *cis* and methylene interrupted unless otherwise identified.

Polyunsaturated fatty acids are associated with health benefits, particularly a reduction in cardiovascular disease risk mediated through a reduction in circulating cholesterol levels when polyunsaturated fatty acids are substituted for saturated fatty acids (Mensink et al. 2003). However, there is a controversy

suggesting that the high intakes of omega 6 polyunsaturated fatty acids relative to omega 3 in most industrialized societies may potentiate inflammation and contribute to several disease paradigms (Lands 2008). In contrast, an increased intake of omega 3 fatty acids is associated with several beneficial health effects. The most dramatic health benefit associated with increased omega 3 fatty acid intake is a significant reduction in the risk of sudden cardiac death (GISSI-Prevenzione Investigators 1999, Mozaffarian and Rimm 2006). This is largely attributed to antiarrhythmic effects of eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3) in the heart (Kang and Leaf 1996, 2000, Leaf et al. 2005). There is also considerable evidence that 22:6n-3 supports fetal neural development (Connor et al. 1993, Innis et al. 2001, Lim et al. 2005a,b, Makrides and Gibson 2000) and there is accumulating evidence that omega 3 fatty acid intake may support various aspects of neural function such as preventing depression, cognitive decline, and dementia in adults (Beydoun et al. 2008, Dullemeijer et al. 2007, Nurk et al. 2007, Schaefer et al. 2006, van Gelder et al. 2007, Whalley et al. 2008).

An examination of the dietary intakes of individual fatty acids within various populations (Bang et al. 1976, Bang et al. 1980, Denomme et al. 2005, Fratesi et al. 2009, Kuriki et al. 2003, Stark et al. 2002) indicates that ~90% of fatty acids in the human food supply consist of just four fatty acids, namely, palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1n-9), and linoleic acid (18:2n-6). The intake of 18:2n-6 has been estimated to make up ~20% of all fatty acids consumed, whereas total omega 3 fatty acids are usually under 3% with most of the omega 3 fatty acid intake as α-linolenic acid (18:3n-3). As such, functional food strategies to increase polyunsaturated fatty acids are typically focused on increasing omega 3 fatty acid content, although there are some exceptions.

32.2 Omega 3 and Omega 6 Polyunsaturated Fatty Acids

Humans are not able to biosynthesize omega 3 and omega 6 fatty acids *de novo* and must consume these essential fatty acids from food. Fatty acid synthesis, elongation, and desaturation reactions are universal in animals and plants and most bacteria (Gunstone et al. 1994, Shanklin and Cahoon 1998). *De novo* synthesis of omega 3 and omega 6 fatty acids tends to be restricted to plants and to some insects and bacteria. Fatty acid synthesis in animals and plants involve various complexes, but generally result in the production of unesterified palmitic acid (16:0) in animals and stearoyl–acyl carrier protein complex in plants. In animals, elongation to stearic acid (18:0) and Δ9 desaturation (steroyl-CoA desaturase) leads to the production of oleic acid (18:1n-9). Additional carbon–carbon double bonds can be introduced to fatty acids by animals, but only through "front-end" desaturases such as Δ5 and Δ6 desaturases. Plants produce 18:1n-9 through Δ9 desaturation (steroyl–acyl carrier protein desaturase), but also express "methyl-end" desaturases such as Δ12 and Δ15 that desaturate fatty acids beyond the Δ9 position to generate the "omega 6" and "omega 3" fatty acids. The Δ12 desaturase enzymes have substrate selectivity for Δ9 double bond fatty acid substrates, while Δ15 desaturase enzymes are specific for Δ9, Δ12 double bond fatty acid substrates. The Δ12 and Δ15 desaturase enzymes appear to have developed in plants from cyanobacteria and are localized in plasmids and endoplasmic reticulum (Sperling et al. 2003). These desaturation reactions utilizes complex lipids as substrates rather than acyl complexes with 18:1n-9 in phosphatidylcholine being converted to linoleic acid (18:2n-6) and possibly α-linolenic acid (18:3n-3) in the endoplasmic reticulum of seeds, whereas in chloroplasts, Δ12 desaturase and Δ15 desaturase activity is in plasmids and utilizes 18:1n-9 in monogalactosyldiacylglycerol for 18:2n-6 and 18:3n-3 production. Chloroplast membranes tend to have a high content of 18:3n-3, possibly to enable proper membrane function with low temperatures (Hugly and Somerville 1992). As such, 18:2n-6 tends to be high in cultivated terrestrial seed oils, while leafy greens, although low in total fatty acids, tend to have a high proportion of 18:3n-3.

Humans have the ability to convert preformed 18:3n-3 and 18:2n-6 to longer-chain polyunsaturated fatty acids through elongation and desaturation reactions (Figure 32.2). However, this metabolism is competitive between omega 6 and omega 3 fatty acids (omega 9 fatty acids can compete but are poor substrates), and these 18-carbon fatty acids are still utilized as energy substrates via mitochondrial oxidation at a relative high rate (McCloy et al. 2004). Therefore, 18:3n-3 is in competition with 18:2n-6 for Δ6 desaturation to stearidonic acid (18:4n-3) and γ-linolenic acid (18:3n-6), respectively, and for the following rapid elongation to eicosatetraenoic acid (20:4n-3) and di-homo-γ-linolenic acid (20:3n-6),

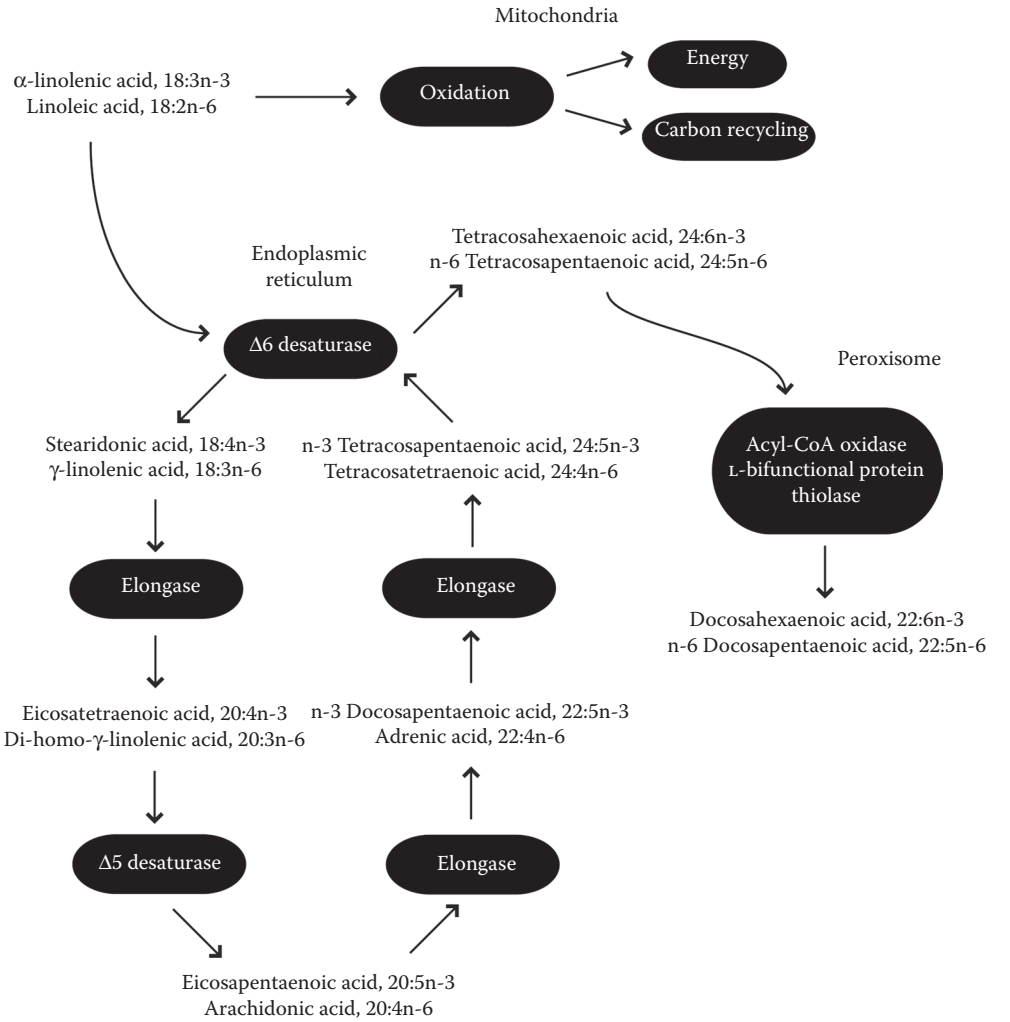

FIGURE 32.2 Pathways for conversion of omega 3 and omega 6 fatty acids.

respectively (Kitson et al. 2010). Eicosapentaenoic acid (20:5n-3) and arachidonic acid (20:4n-6) can then be synthesized via Δ5 desaturation, and another round of elongation can then produce n-3 docosapentaenoic acid (22:5n-3) and adrenic acid (22:4n-6). The next chemical step required to produce docosahexaenoic acid (22:6n-3) and n-6 docosapentaenoic acid (22:5n-6) is Δ4 desaturation, but Δ4 desaturase activity is absent in human hepatocytes (Kitson et al. 2010, Sprecher 1999). As such, 22:5n-3 and 22:4n-6 are elongated to n-3 tetracosapentaenoic acid (24:5n-3) and tetracosatetraenoic acid (24:4n-6), respectively, desaturated at the Δ6 position to tetracosahexaenoic acid (24:6n-3) and n-6 tetracosapentaenoic acid (24:5n-6), respectively, and then undergo one round of β-oxidization to form 22:6n-3 and 22:5n-6. There are two important characteristics regarding this round about metabolic pathway or "shunt" (Leonard et al. 2004, Sprecher et al. 1995). First, this second round of desaturation at the Δ6 position appears to be mediated by the same desaturase enzyme in the endoplasmic reticulum (Stroud et al. 2009); therefore, 18:3n-3, 18:2n-6, 24:5n-3, and 24:4n-6 are in direct competition with each other. Second, the chain-shortening step requires the fatty acid to move from the endoplasmic reticulum to the peroxisome where it undergoes a single-round nonenergetic β-oxidization (Sprecher et al. 1995).

Polyunsaturated fatty acid biosynthesis can influence, but does not entirely dictate, the fatty acid composition of biological tissues and food supply. Fatty acids must be incorporated into lipid constructs

such as triacylglycerols and glycerophospholipids that can occur during *de novo* synthesis of the lipid or through acyl remodeling by transferases (Holub et al. 1987). For the omega 3 and omega 6 fatty acids, 18:3n-3 and 18:2n-6 will accumulate in triacylglycerols and in the *sn*-1 positions of glycerophospholipids in competition with 16:0, 18:0, and 18:1n-9 (Stark 2008). For the elongation and desaturation products, 20:5n-3, 22:6n-3, and 20:4n-6 tend to accumulate in the *sn*-2 position of glycerophospholipids (Metherel et al. 2009a). Under certain dietary conditions, other polyunsaturated fatty acids such as 20:3n-6, 22:5n-3, and 22:5n-6 can accumulate but will vary depending on the lipid constructs and the parent tissue (Stark et al. 2007a).

In the diets of Western societies, ~80% of the polyunsaturated fatty acids consumed consist of 18:2n-6, whereas 18:3n-3 makes up ~12% (Denomme et al. 2005, Fratesi et al. 2009, Stark et al. 2005a). Therefore, the bioconversion of 18:3n-3 to longer-chain omega 3 fatty acids is largely suppressed and possibly inadequate to provide the human tissues levels of 20:5n-3 and especially 22:6n-3 that are associated with significant health benefits. Increasing the dietary intake of 18:3n-3 can increase blood levels of 20:5n-3 and even 22:5n-3, but not 22:6n-3 (Barcelo-Coblijn et al. 2008, Barcelo-Coblijn and Murphy 2009, Burdge and Calder 2005). This may be a result of overwhelming competition for Δ6 desaturation. Decreasing the dietary intake of 18:2n-6 could reduce the competition for Δ6 desaturation and potentially result in greater 22:6n-3 synthesis from 18:3n-3, (Lands 2005); however, this is a difficult task. A large amount of 18:2n-6 intake is through "hidden" fats that are contained in manufactured and processed foods, so reducing 18:2n-6 intake level at the population level requires industrial intervention. Alternatively, tissue levels of longer-chain omega 3 fatty acids can be increased by consuming preformed longer-chain omega 3 fatty acids. This has been demonstrated to be effective through various fish oil supplementation studies (Fekete et al. 2009, Holub and Holub 2004, Metherel et al. 2009a, Stark et al. 2000). The blood levels of omega 3 fatty acids of the population of Japan are often cited as a standard for Western societies. The Japanese have similar dietary intake levels of 18:2n-6, slightly higher intakes of 18:3n-6, and dramatically higher levels 20:5n-3 and 22:6n-3 as compared to Western nations with the high intakes of 20:5n-3 and 22:6n-3 in Japan attributed to seafood consumption (Kuriki et al. 2003).

In Western countries, the intake of fish and seafood is limited due to several challenges, including limited availability, lack of experience with fish preparation, and negative associations to smell and taste (Patterson and Stark 2008). In addition, recent concerns about containments in fish such as methyl mercury, polychlorinated biphenyls, and dioxins, and environmental sustainability of fish stocks work against efforts to increase fish intakes (Jenkins et al. 2009). There is increasing interest and consumer demand for alternative sources of longer-chain omega 3 fatty acids. Therefore, novel functional foods containing eicosapentaenoic acid (20:5n-3) or "EPA" and docosahexaenoic acid (22:6n-3) or "DHA" are rapidly entering the food supply.

32.3 Food Supply

Traditionally, research attention has been focused on three omega 3 fatty acids: α-linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3), and docosahexaenoic acid (22:6n-3) (Figure 32.1). In Western society, dietary intakes of 20:5n-3 and 22:6n-3 tend to be lower than 120 mg/day combined while 18:3n-3 intakes are ~1.3 g/day (Denomme et al. 2005, Fratesi et al. 2009, Stark et al. 2005a). Although 18:3n-3 is associated with the chloroplast (Hugly and Somerville 1992), the low overall oil content of leafy green plants results in low contributions to the human diet from this source, and a large amount of dietary 18:3n-3 comes from 18:3n-3 in specialized seed oils such as canola, flaxseed, and perilla oil. However, the bulk 18:3n-3 intake is through the high consumption of soybean oil, but this is overshadowed by a much higher content of 18:2n-6 (Whelan and Rust 2006). The bulk of cultivated seed oils (such as corn and safflower) in Western diets have high 18:2n-6 content and as a result 18:2n-6 intakes in Western intakes are in the 6–7 g/day range (Denomme et al. 2005, Fratesi et al. 2009, Stark et al. 2005a). The intake of all other omega 6 and omega 3 fatty acids are dramatically lower. In particular, the intakes of other omega 6 fatty acids are very low. Some selected vegetable oils such as evening primrose, borage, black currant, and hemp seed contain γ-linolenic acid (18:3n-6), but their intakes are relatively minor in most Western diets. The intakes of longer-chain omega 6 fatty acids, namely, arachidonic acid (20:4n-6)

have limited variation across various human populations (Bang et al. 1976, Denomme et al. 2005, Fratesi et al. 2009, Kuriki et al. 2003, Stark et al. 2005a). The intakes of longer-chain omega 6 fatty acids are relatively low and are generally associated with the consumption of animal tissues. In contrast, the intakes of 20:5n-3 and 22:6n-3 can vary considerably in human populations with a strong association with marine fish and seafood intake. In populations that refrain from seafood consumption, chicken and eggs may actually provide a significant proportion of the dietary intake of 20:5n-3 and 22:6n-3 (Stark et al. 2005b). Other omega 3 fatty acids detectable in the human diet include stearidonic acid (18:4n-3), eicosatetraenoic acid (20:4n-3), and n-3 docosapentaenoic acid (22:5n-3). Docosapentaenoic acid (22:5n-3) intake can actually exceed 20:5n-3 intake when fish intake is low (Denomme et al. 2005, Fratesi et al. 2009, Stark et al. 2005a). In addition, 22:5n-3 content has been demonstrated to be particularly high in marine mammal oils, particularly seal (Conquer et al. 1999, Murphy et al. 1999).

The potential health benefits of omega 3 fatty acids and challenges in getting 20:5n-3 and 22:6n-3 from fish consumption have led to the development of alternative dietary strategies and nontraditional food sources (Patterson and Stark 2008). Encapsulated fish oil or "nutraceutical" delivery is one strategy to allow for increased intake of omega-3-rich oils without actually consuming fish. Alternatively, the content of omega 3 fatty acids can be increased or enriched in various food products to generate novel "functional foods." Strategies to increase omega 3 fatty acid levels vary from direct addition of the oils to the product to manipulation of environmental conditions that result in more accumulation of omega 3 fatty acids in complex lipids to genetic modulation to increase the production of omega 3 fatty acids. Direct addition of these fatty acids is difficult because of the potential for off flavors that can be generated with trace levels of oxidation; however, microencapsulation can protect omega 3 fatty acids and enable enrichment.

The demand for omega 3 fatty acids has also resulted in the cultivation of new dietary sources. Oils rich in stearidonic acid (18:4n-3) are being produced as this fatty acid may have higher bioconversion rates to 20:5n-3 and possibly 22:6n-3 relative to 18:3n-3, given that it bypasses the Δ6 desaturase. Stearidonic acid can be obtained by the cultivation of specific seed oils such as echium and black currant seeds, and by the genetic modification of existing crop seeds such as canola and soybeans (Eckert et al. 2006, Harris et al. 2008). Microalgae species *Crypthecodinium cohnii* and *Schizochytrium* are cultivated as sources of 22:6n-3 (Abril et al. 2003, Hammond et al. 2001, Hauvermale et al. 2006, Spolaore et al. 2006, Stark et al. 2007a, Sukenik et al. 1994) and the unicellular fungus *Mortierella alpine* is a source of 20:4n-6 (Hempenius et al. 2000, Wynn and Ratledge 2000). Efforts to identify other long-chain polyunsaturated organisms and attempts to transfer genes to allow for increased production for food supply are ongoing (Chi et al. 2008, Leonard et al. 2004, Spolaore et al. 2006).

32.4 Chemical Occurrence of Omega 3 and Omega 6 Fatty Acids within Food

In general, 18-carbon omega 3 and omega 6 fatty acids (primarily 18:2n-6 and 18:3n-3, but also 18:3n-6 and 18:4n-3) are consumed as a component of triacylglycerols from plant seeds, while animal triacylglycerol depots can also contain significant amounts of 18:2n-6 and 18:3n-3. The ≥20-carbon omega 3 and omega 6 fatty acids are generally associated with animal products and can accumulate in both phospholipid and triacylglycerols fractions. When the overall content of omega 3 fatty acids is low within total fatty acids, as in terrestrial mammals, the longer-chain omega 3 fatty acids will preferentially accumulate in the phospholipid fraction as compared with the triacylglycerol fraction. The fatty acid composition of triacylglycerol-rich tissues such as adipose has been demonstrated to comprise of only 0.5% long-chain polyunsaturates (Stark et al. 2007a), whereas skeletal muscle content can be over 20% long-chain polyunsaturates (Stark et al. 2007b). This is of significant analytical importance as triacylglycerols are neutral or nonpolar lipids and phospholipids are polar lipids. Therefore, appropriate nonpolar, polar, or nonpolar/polar mixtures must be used when appropriate to ensure accurate quantitation. The technique in which the omega 3 and/or omega 6 fatty acid content of food products are manipulated can influence the parent lipid class. Biological manipulations such as feeding, altered temperature (decreased temperature can increase long-chain omega 3 content of algae), or genetic modification result in incorporation of these fatty acids into the triacylglycerol and phospholipid pools of the food matrix. Direct addition of

fatty acids to an existing food product such as deodorized or microencapsulated fish oil is typically added as triacylglycerols.

32.5 Analytical Challenge

Dietary relevant omega 3 and omega 6 fatty acids are 18–22-carbon polyunsaturated fatty acids that can be distributed in a variety of different lipid classes and can be in various food matrices with varying concentrations of total fat. Typically for routine food analysis, determining the concentration of omega 3 and omega 6 fatty acids in a serving of a food product is desirable and involves the determination of the fatty acid composition of the total lipid fraction. The identification and quantification of the individual fatty acids are usually determined by the analysis of fatty acid methyl esters by gas chromatography (GC) with flame ionization detection (FID) and will be the focus of the present discussion. Other analytical instruments and derivatization techniques can be used to determine the fatty acids such as high-performance liquid chromatography, GC with mass spectrometry, nuclear magnetic resonance, Fourier transform infrared spectroscopy, and others as reviewed elsewhere (Christie 2003, Firestone and Mossoba 1997, Myher and Kuksis 1995, Zamora and Hidalgo 2004). In addition, it may be desirable to determine the fatty acid composition of individual lipid classes and/or the positional distribution of individual fatty acids within a complex lipid. This can usually be accomplished by additional manipulation and processing of an isolated total lipid extract and is also covered elsewhere (Christie 2003, Firestone and Mossoba 1997, Zamora and Hidalgo 2004).

Omega 3 and omega 6 fatty acid determinations of food samples require the isolation of the fatty acids from the lipid component followed by quantitative analysis. Various techniques exist to isolate these fatty acids and prepare fatty acid methyl esters for gas chromatographic analysis. Classic techniques involve lipid extraction, hydrolysis of fatty acids from various lipids, and esterification to form fatty acid methyl esters. There are also several transesterification techniques that generate fatty acid methyl esters directly from the parent lipid without prior hydrolysis as well as "one-step" or "direct" extraction/derivatization techniques. The food matrix and parent lipid class of the fatty acid to be analyzed can dictate the most appropriate preparation technique. For functional foods, there may be additional technological manipulations of the food matrix that can influence the most appropriate analytical strategy or require additional treatments to accurately determine the concentration of omega 3 and omega 6 fatty acids.

32.5.1 Standards and Quantitation

Quantitation is obviously dependent on optimization of analytical instruments. Typically, standard curves should be generated for each analyte on the analytical instrument. This can be quite cumbersome for fatty acid analyses of biological samples as over 20 individual fatty acids are regularly detected. GC coupled to FID analysis has a distinctive advantage in that the flame ionization detector tends to have a linear response for fatty acids. While individual standard curves for each individual fatty acid are not necessary, it is important to regularly verify the linearity and quantitation of the instruments and column. This can be done by the routine analysis of fatty acid standard mixtures or "external standards" to verify retentions times and calculate individual response factors for the various fatty acids of interest (Ackman and Sipos 1964). The external standard should include a range of saturated, monounsaturated, and polyunsaturated fatty acids with carbon chain lengths resembling the fatty acids in the food product to be analyzed. For foods containing omega 3 and omega 6 fatty acids, typically 10–24-carbon fatty acids are sufficient. If the goal is to identify all fatty acids in a food product, an external standard with shorter-chain fatty acids may be necessary when analyzing certain food products such as milk and dairy products (Zamora and Hidalgo 2004). For determining the linear response of the instrument, an external standard with equimolar or equal weight distribution of each fatty acid is preferable, while for retention time verification, a standard with a molar or weight distribution similar to the fatty acid composition of the food sample will provide tighter estimates of the observed retention times.

Quantitation of total fat in food products is often determined by gravimetric means after isolating and drying the lipid extract to remove all solvents. The "fat" determined in this manner is a mixture of

lipophilic compounds that may or may not contain fatty acids. In order to determine the fatty acid content, typically, a fatty acid needs to be added to the sample to serve as a standard to correlate instrument response with mass or moles. This standard is often described simply as an "internal standard" in the literature. Presently, we prefer to use the term "surrogate internal standard" to clearly indicate that this fatty acid standard is preferably added to the sample before any chemical or mechanical processing. Alternatively, the term "internal standard" could refer to a known amount of a derivatized fatty acid added to the prepared analyte just before analysis, which we will define herein as a "preinjection internal standard." Both standards provide a known concentration associated with the analytical response to the standard fatty acid, thereby allowing the analytical responses to other fatty acids to be converted to concentrations. In principle, the surrogate internal standard should mimic the existing fatty acids of the initial food sample. In theory, losses of fatty acids during sample homogenization, chemical preparation, and handling will be accounted for by proportional losses of the surrogate internal standard. For this to be true in practice, the surrogate internal standard should be in a chemical structure similar to the bulk of the fatty acids in the sample to be analyzed. For food products, the majority of fatty acids are esterified into triacylglycerols; however, some animal products and potentially some functional foods may have the bulk of the fatty acids as components of phospholipids.

A preinjection internal standard for fatty acid analysis is typically a fatty acid methyl ester for gas chromatographic analysis. This type of standard allows for the quantitation of the fatty acids in the sample after mechanical and chemical processing without any adjustment for losses. Fatty acid methyl esters are often used as surrogate internal standards when examining mixed fatty acid containing lipids; however, the use of fatty acid methyl esters as surrogates will typically account only for volume losses and not account for different extraction and derivatization efficiencies typically associated with the native lipid in which the fatty acid was esterified. A potential alternative to methyl esters is the use of ethyl ester fatty acid surrogate internal standards to verify derivatization as a fatty acid ethyl ester will have a shifted retention time relative to the similar fatty acid methyl ester by GC. However, an ethyl ester internal standard will not work for all esterification/transesterification reagents. The addition of a surrogate and a preinjection internal standard within a single sample can allow for the quantitation of sample-processing losses, but this practice is rarely reported in fatty acid determinations.

The specific fatty acid to be used as an internal standard can be debated. Ideally, the fatty acid should not naturally occur in the sample to be analyzed and resolve independently of other fatty acids during analysis. Typically, odd-chain fatty acids synthesized by bacteria are used as surrogate internal standards, given that the animal and plant fatty acids are largely even-chain because synthesis and oxidation are based on two-carbon addition and removal. Therefore, methyl esters of and various lipids containing pentadecanoic acid (15:0), heptadecanoic acid (17:0), or nonadecanoic acid (19:0) are often used as internal surrogate standards because they tend to be rare in biological samples and they are relatively affordable to purchase. The use of 15:0 and 17:0 as internal standards can be problematic when determining the fatty acid composition of ruminant food products, as dairy and animal fat from beef and lamb can contain small but significant amounts of these fats (Wolk et al. 2001). When the focus of the fatty acid analyses is for determining very long-chain polyunsaturates, heneicosanoic acid (21:0) and tricosanoic acid (23:0) are often used as their longer carbon chain length may provide a more appropriate analytical response for quantitation. Docosatrienoic acid (22:3n-3) has also been used when targeting the quantitation of very long omega 3 fatty acids such as 20:5n-3 and 22:6n-3 (Stark et al. 2005a). These longer-chain standards can be financially costly and commercial availability can be limited to methyl esters and triacylglycerols.

32.5.2 Lipid Extraction

Lipid extraction can be completed through a variety of techniques. In academic laboratory research, chloroform- and methanol-based extractions such as the method of Folch, Lees, and Sloane-Stanley (Folch et al. 1957) and the method of Bligh and Dyer (1959) are typically used, while within industrial settings, official methods of the Association of Official Analytical Chemists (AOAC) (AOAC Official Method 996.06 2005) and the International Organization for Standardization (ISO) (Luque-Garcia and Luque de Castro 2004) are used. The Folch, Lees, and Sloane-Stanley method was originally developed for examining biological tissues such as mammalian brain and liver, which all have relatively high fat

contents, whereas the Bligh and Dyer method was developed for the rapid and large-scale analysis of lean fish muscle. There are several variations and modifications of these methods, but generally, the Folch, Lees, and Sloane-Stanley method is preferred for samples with significant percentages of total fat, while the Bligh and Dyer method is reserved for samples with low total fat. Both methods are routinely used for specific circumstances in food analysis. In contrast, both the AOAC and ISO methods were developed originally for gravimetric determinations of total fats in foods. As the triacylglycerol fraction is the dominant fatty acid-containing lipid in foods, these official methods focused on extraction of this nonpolar lipid component and use primarily nonpolar solvent such as petroleum ether (AOAC) and hexane (ISO). Therefore, for the analysis of omega 3 and omega 6 fatty acids that do partition into nonpolar lipids, it is highly recommended that the extraction system contains a mixture of nonpolar and polar solvents such as chloroform and methanol. The AOAC method for the determination of saturated and unsaturated fatty acid in foods uses diethyl ether in addition to petroleum ether; however, the Soxhlet-based ISO method uses only hexane. The ISO procedure is well designed to extract fats from triacylglycerol-rich seeds, but the use of only hexane may not efficiently extract polar lipids such as phospholipids typically found in animal products. The addition of polar solvents such as isopropanol to hexane has been demonstrated to significantly improve fatty acid yields (Metherel et al. 2009b). Regardless of the method used for extraction, the excess solvent should be evaporated from the lipid sample with an inert gas such as nitrogen at a temperature below 40°C. The concentrated lipids should be stored in a small volume of chloroform with 0.005% antioxidant such as butylated hydroxytoluene (2,6-di-*tert*-butyl-*p*-cresol) at –20°C or lower.

32.5.2.1 Method of Folch, Lees, and Sloane-Stanley

There are several variations and modifications of the original method developed by Folch, Lees, and Sloane-Stanley for extracting lipids. For discriminating saturated, monounsaturated, and polyunsaturated fatty acids in several matrices, the sample can be homogenized with chloroform/methanol (2:1 ratio by volume) although there are variations where chloroform and methanol are introduced independently with repeated rounds of homogenization (Christie 2003, Zamora and Hidalgo 2004). For food analysis, samples of 1 g are typically extracted with 20 mL of solvent; however, if the food matrix is homogeneous, sample masses can be dramatically reduced and the amount of solvent reduced. This is particularly relevant given the environmental impact of the use of chloroform/methanol. After the sample has been homogenized, it is necessary to achieve a chloroform/methanol/water ratio of 8:4:3 to separate the organic and aqueous phases. This is usually achieved by the addition of an aqueous buffer equal to 0.2 of the total solvent volume. After the aqueous (3:48:47, chloroform/methanol/water) and organic phases (86:14:1, chloroform/methanol/water) have formed, there are several variations in collecting the lipid extract that can include filtration, washing, reextraction, and increasing the amount of solvent/homogenate incubation time. Extensive washing of the aqueous phase and reextraction of the homogenate has been shown to increase gravimetric yields of total fat (Ways and Hanahan 1964), but this can be cumbersome for routine analyses. Allowing the chloroform/methanol and sample homogenate mix to incubate for 24–48 h prior to the collection of the organic phase allows for increased lipid partitioning and can increase lipid gravimetric yields (Taha, Metherel and Stark, unpublished observations). Often a single collection of the lower organic phase is completed immediately after homogenization for the goal of relative fatty acid composition and under the assumption that quantitative losses will be accounted for by the internal surrogate standard; however, this is not recommended as it will typically underestimate total lipid concentrations.

32.5.2.2 Method of Bligh and Dyer

The main advantage of the Bligh and Dyer method is a reduced solvent volume to sample mass ratio. For the Folch, Lees, and Sloane-Stanley method, 20 mL solvent/g sample is used as compared with 4 mL solvent/g sample for the Bligh and Dyer method. The Bligh and Dyer method was developed for samples low in fat (1%) with a relatively high water content (80% of the sample mass). When the Bligh and Dyer method is applied to samples with increasing lipid content (>2%), there can be significant underestimations of total lipid content (Iverson et al. 2001). Typically, three volumes of chloroform/methanol (1:2 ratio by volume) are added to the sample to achieve a monophasic mixture with a chloroform/methanol/water

ratio of 1:2:0.8 for homogenization. Following homogenization, the sample is diluted with an additional one volume of chloroform and one volume of water to generate a biphasic mixture with the aqueous phase on top and the organic phase on the bottom. The options for isolating the final lipid extract from the biphasic mixture are similar to those described for the Folch, Lees, and Sloane-Stanley above.

32.5.2.3 Method of the Association of Official Analytical Chemists

The AOAC method for the determination of total, saturated, and unsaturated fats in foods uses a hydrolysis technique to degrade proteins that may interact with fatty acids prior to lipid extraction. Usually, acid hydrolysis (hydrochloric acid) is applied; however, for most dairy products, alkaline hydrolysis (ammonium hydroxide) is applied, and for cheese, a combination of alkaline, followed by acid hydrolysis, is applied. Pyrogallic acid is added prior to hydrolysis to prevent fatty acid oxidation, and protein hydrolysis is completed by mixing and heating the sample in a Mojonnier flask. After hydrolysis, ethanol is added to dilute the sample and terminate the reaction. The lipids are then extracted by additions of diethyl ether and petroleum ether with mixing after each addition. The mixture can be centrifuged or allowed to stand for 1 h to facilitate the separation of the aqueous and organic phases. The top organic phase (1:1 diethyl ether/petroleum ether mixture containing total lipids) can be decanted and dried under nitrogen. The lipid residue should then be dissolved in a small volume of chloroform and stored as described in previous sections.

32.5.2.4 Method of the International Organization for Standardization

The ISO method utilizes a Soxhlet apparatus. This consists of a still pot with hexane seated in a hot plate with an extraction and siphon chamber and a condensing unit. The sample is loaded into a paper thimble and then inserted into the extraction chamber. The hexane solvent is heated in the still pot, after which it evaporates, condenses in the condenser, and then drips into the extraction chamber. When the solvent reaches the top of the siphon tube, the solvent and extract empty from the extraction chamber into the still pot. The hexane cycles through this process of evaporation, condensation, extraction, and siphoning, while the extracted lipids accumulate in the lower still pot. After ~2 h, the hexane solvent is collected and the 2 h cycle is typically repeated 2–4 times with fresh hexane. The hexane extractions are then combined and evaporated under nitrogen, and the resulting lipid residue can be stored with chloroform and antioxidant as described previously.

32.5.2.5 Instrument-Assisted Extraction

In addition to these manual techniques, there is an increasing application of various instruments in lipid extraction (Christie 2003, Firestone and Mossoba 1997). Typically, the application of these instruments is to increase analytical throughput and include automation of previously described procedures such as the Soxhlet-based extraction. The application of microwave energy has been examined to accelerate heating for reactions such as digestion (Ganzler et al. 1986, Leray et al. 1995), and ultrasound has been used to assist lipid extraction as ultrasonic energy can produce heat, pressure, and mechanical shear that can promote rapid lipid partitioning (Metherel et al. 2009b). Instruments are also available that allow solvents to be pressurized, allowing for rapid extractions with lower volumes (Schafer 1998) and recently the supercritical fluid extraction using CO_2 has been demonstrated in determining omega 3 fatty acid content in fish by-products (Rubio-Rodriguez et al. 2008). A common challenge with the application of these instruments to the analysis of polyunsaturated fatty acids is the potential for oxidation, particularly of the omega 3 fatty acids.

32.5.3 Preparation of Derivatives

The conversion of fatty acids to nonpolar derivatives greatly enhances the ability to separate and detect fatty acids by chromatographic procedures. For fatty acids, methyl ester derivatives are almost used exclusively with routine GC-FID analyses for separation, and identification and quantification of individual fatty acids. Reviews of common methylation techniques are available (Carrapiso and Garcia 2000,

Liu 1994). Although, nonesterified fatty acids are present in samples, typically, their levels are very low in food samples and they will have minimal impact on the quantitative estimate of the amount of omega 3 and omega 6 fatty acids in a food product. As such, the majority of the fatty acids will be esterified to complex lipids such as triacylglycerols and various phospholipids. Derivatization, therefore, requires hydrolysis to release the fatty acid and then esterification to form the ester derivative. Depending on the planned esterification procedure, prior hydrolysis may be required. The previously isolated lipid extract is saponified with an aqueous ethanolic alkali such as potassium hydroxide in 95% water (Christie 2003) and the nonesterified fatty acids are isolated after extraction with a mixture of polar and nonpolar solvents, and washing with water. In contrast, there are several "transesterification" protocols whereby hydrolysis occurs during the esterification process. Base-catalyzed reactions are by definition transesterification reactions as they are typically not capable of esterifying nonesterified fatty acids. In the past, acid-catalyzed reactions were often coupled to saponification, but for the determination of the total content of omega 3 and omega 6 fatty acids in a biological product, acid-catalyzed reactions are capable of transesterification.

32.5.3.1 Acid-Catalyzed Esterification and Transesterification

Acid catalyzation can methylate nonesterified fatty acids and transesterify fatty acids in triacylglycerols and phospholipids, although the transesterification of the amide linkage of sphingolipids can require longer-heating times. The lipid extract is typically added to an excess of methanol with an acid catalyst and heated. For most lipid extracts, it is also necessary to add a nonpolar solvent such as hexane or toluene to solubilize the nonpolar lipids such as triacylglycerols.

Methanolic hydrogen chloride (5%) and sulfuric acid in methanol (1%) are classically used as catalysts (Christie 2003, Zamora and Hidalgo 2004). For the determination of omega 3 and omega 6 fatty acids in total lipids, the lipid sample (0.3 mg) is dissolved in toluene (1 mL) and then 2 mL of methanolic hydrogen chloride or sulfuric acid in methanol is added in a test tube with a Teflon-coated cap. The sample is heated overnight at 50°C. Alternatively, the sample can be loaded into a condenser flask and refluxed for 2 h. The solution is then allowed to cool and 2.5 mL of an aqueous buffer such as 5% sodium chloride is added and additional hexane is added. The hexane is collected to isolate the fatty acid methyl esters. The hexane can be washed, dried, and further concentrated if necessary.

Boron trifluoride (12–14% w/v) is also commonly used as an acid catalyst (Morrison and Smith 1964). Boron trifluoride protocols can esterify nonesterified fatty acids and transesterify most ester-linked fatty acids in ~15 min when heated at 90°C. For the transesterification of the amide-linked fatty acids of sphingolipids, 1 h of heating at 90°C is typically required. There are some disadvantages of the use of boron trifluoride as pointed out by Christie (Christie 2003). Boron trifluoride is a harsh reaction as compared with methanolic hydrogen chloride and sulfuric acid in methanol, as the acid concentrations and the temperature used to drive the reaction are much higher. As a result, the risk of producing fatty acid and butylated hydroxytoluene artifacts and decreasing polyunsaturated fatty acid yields are increased. These risks can be minimized by storing boron trifluoride in the refrigerator, and avoiding old or concentrated boron trifluoride. There is direct evidence that the boron trifluoride-catalyzed transesterification reactions for the determination of omega 3 fatty acids in fish oil samples are accurate and precise and suitable for routine analyses (Ackman 1998). Briefly, lipid extracts (0.3 mg) dissolved in 0.3 mL of hexane is added to 1 mL of 14% boron trifluoride in methanol in a test tube and with a Teflon-lined cap. Samples are heated at 90°C for 1 h and then allowed to cool to room temperature. Water (1 mL) and additional hexane (1 mL) are added to isolate the organic phase containing the fatty acid methyl esters. The sample can be washed, dried, and concentrated if necessary.

32.5.3.2 Base-Catalyzed Transesterification

Base-catalyzed reactions should be considered strictly transesterification reactions of glycerol esters and are not suitable for esterification of nonesterified fatty acids and transesterification of amide linkages. The lipid sample (0.3 mg) is dissolved in 1 mL toluene, and 2 mL of methanolic 0.5 M sodium methoxide is added and heated at 50°C for 10 min. The reaction is quenched by cooling and neutralizing with

0.1 mL of acetic acid. The organic phase with the fatty acid methyl esters is isolated by adding water or aqueous buffer (mL) and extracting twice with hexane (5 mL). The extract is dried and concentrated if necessary. Alternatively, methyl esters can also be generated by heating an oil sample with tetramethylguanidine in methanol (1:4 v/v) at 100°C for 2 min. The methyl esters can then be extracted with petroleum ether after the addition of saturated sodium chloride. This guanidinium ion-driven base reaction has been demonstrated to be suitable for methylating nonesterified fatty acids and fatty acids in triacylglycerols in oils with high acid values (Schuchardt and Lopes 1988), but it has significant limitations with methylating glycerophospholipids and sphingolipids. Base-catalyzed reactions have been demonstrated to result in no isomerization of conjugated dienes and no production of methoxy artifacts and as such are well suited for the determination of conjugated linoleic acid (Christie 2003, Zamora and Hidalgo 2004).

32.5.3.3 Diazomethane Esterification

Diazomethane can be used to rapidly methylate nonesterified fatty acids. However, diazomethane must be prepared fresh by the reaction of a base with a nitrosamide in diethyl ether and the reagent is toxic, carcinogenic, and potentially explosive. Diazomethane use may limit the isomerization of conjugated dienes and the production of methoxy artifacts (Kramer et al. 1997). For the determination of omega 3 and omega 6 fatty acids in total lipids of foods, the use of diazomethane is generally avoided and other derivatization techniques are utilized.

32.5.4 One-Step Extraction and Derivatization Techniques

One-step extraction and derivatization or "*in situ* transesterification" techniques allow for significant gains in analytical throughput as compared with conventional techniques. These one-step techniques allow for the preparation of fatty acid methyl esters directly from the sample without prior lipid extraction. The direct transmethylation of *in situ* lipids to determine fatty acid composition was first reported in 1963 for the classification of microorganisms (Abel et al. 1963). The use of these one-step techniques was limited; however, the development of gas chromatographs with automated sample injectors has enabled increased analytical throughput and rapid sample-processing procedures have become highly desirable. In general, one-step transesterification techniques generally utilize the same esterification and transesterification reagents as described in the previous section with some additional considerations (Carrapiso and Garcia 2000, Liu 1994). With *in situ* transesterification, the food sample matrix must be considered as the lipids must be available to react with the reagents, and other compounds should not interfere with the desired reaction. Appropriate solvents must be used to solubilize the lipids in the food sample to allow the transesterification reaction to occur and the water content of the food sample must be considered.

32.5.4.1 Water Content and One-Step Transesterification

With *in situ* transesterification, the moisture content of the sample and the amount of sample analyzed determines the amount of water present. If a sample has low lipid content, a greater amount of sample is required and, therefore, the risk of high water content increases, unless the sample has very low moisture content or the sample has been dried prior to analysis. Water can decrease the solubility of lipids, particularly polar lipids such as triacylglycerols that are relatively high in food products, and water is a stronger electron donor as compared with methanol, the most common esterification/transesterification reagent. Base-catalyzed reactions appear to be particularly sensitive to moisture as the water content of soybean oil of >8% results in dramatic decreases in fatty acid methyl ester recovery after direct transesterification of the oil with 0.8 N sodium hydroxide in methanol in the presence of hexane (3:2, v/v) (Long et al. 1988). In acid-catalyzed reactions using methanol, water content <20% can be tolerated without an effect on fatty acid methyl ester recovery (Lepage and Roy 1986, Sukhija and Palmquist 1988).

Food samples with high moisture contents can be treated to remove water. For the analysis of polyunsaturated fatty acids, it is often recommended to avoid the use of heat to prevent oxidation and employ freeze drying of the sample to remove water. However, direct evidence supporting these recommendations is difficult to find and treatment of tissues with heat may actually denature enzymes involved in lipolysis

and fatty acid degradation and prevent losses of 20:5n-3 and 22:6n-3 (Williams et al. 1995). It is possible that when fatty acids are in intact biological matrices, in parent lipids and/or in solvents, omega 3 and omega 6 fatty acids have some degree of protection from oxidation unless they are exposed to oxygen and heat for prolonged periods of time, but further investigation is required. Alternatively, prior treatment with 2,2-dimethoxypropane has been demonstrated to scavenge water, although it is recommended that acetone and unreacted 2,2-dimethoxypropane be removed prior to the addition of the transesterification catalyst (Shimasaki et al. 1977). For food samples with high moisture content, the steps required to eliminate or reduce water content to tolerable levels may actually be more tedious than conventional sample preparation procedures involving prior lipid extraction.

32.5.4.2 Acid-Catalyzed Direct Transesterification

Acid catalysis requires heating and typically longer reaction times than base catalysis, however, it is well suited for one-step transesterification reactions because it has the ability to derivatize all the fatty acids required for a quantitative total fatty acid profile. Methanolic hydrogen chloride tends to be the acid catalyst used most often for *in situ* transesterification likely due to a significant tolerance to water content, although it was not the first acid catalyst used *in situ*. Initial hydrogen chloride *in situ* experiments used benzene and 5% methanolic hydrogen chloride and heat for 2 h at 70°C to prepare fatty acid methyl esters from dried feed, digesta, and fecal samples (Outen et al. 1976). Modifications, that have been applied to this method, include the replacement of benzene that is highly toxic with toluene (Sukhija and Palmquist 1988), and reduction of the reaction time to 1 h at 100°C (Lepage and Roy 1984, 1986). Recently, we demonstrated that a stock solution of methanol (1.7 mL/sample), acetyl chloride (0.1 mL/sample), and a fatty acid internal standard could be prepared in advance and added to the sample in single step, eliminating the need for drop wise addition of acetyl chloride to methanol (Masood et al. 2005). In addition, replacing a portion of the methanol with toluene (0.3 mL/sample), reducing the temperature to 80°C and increasing time of reaction to 2 h, allows for the reaction to occur in an open tube, thereby increasing the amenability to commercially available robotics platforms (Masood et al. 2005). After transesterification is complete, the fatty acid methyl esters can be recovered by extracting with an appropriate solvent such as hexane.

Methanolic sulfuric acid is often preferred as an acid catalyst as it can be stored longer and prepared easily as compared with methanolic hydrogen chloride. However, reports of the use of methanolic sulfuric acid *in situ* are much more limited. Generally, methanolic sulfuric acid catalysis requires heating times equal to or in excess of 3 h (Harrington and Darcyevans 1985, Welch 1975), although the original use of methanolic sulfuric acid *in situ* involved the addition of concentrated sulfuric acid to the sample in diethyl ether at low temperature followed by additions of methanol, and methanolic potassium hydroxide (Dugan et al. 1966). In these analyses, the length of time and/or the series of steps required defeat the typical primary objective of increasing analytical throughput by *in situ* transesterification.

The rapid transesterification capacity of methanolic boron trifluoride is an attractive catalyst to employ for *in situ* transesterification to increase the analytical throughput. Boron trifluoride, however, has several disadvantages as described in the previous section. In addition, boron trifluoride is particularly sensitive to moisture, as water content >0.5% can result in decreased fatty acid methyl ester recovery in triacylglycerols as nonesterified fatty acids are produced through hydrolysis (Morrison and Smith 1964). However, boron trifluoride was the initial catalyst used *in situ* (Abel et al. 1963) and it has been demonstrated to be suitable for *in situ* analysis of anhydrous samples such as thin layer chromatography scrapings (Ohta et al. 1990, Sattler et al. 1996) and freeze-dried samples (Sattler et al. 1991). Recently, we have demonstrated that direct transesterification of whole-blood total lipids could be completed with boron trifluoride using similar conditions used for total lipid extracts (Armstrong et al. 2008). However, the use of boron trifluoride *in situ* for food analysis is problematic due to typical moisture contents of foods, although there has been a successful application to adipose and freeze-dried animal tissues (Rule 1997).

32.5.4.3 Base-Catalyzed Direct Transesterification

Base-catalyzed reactions are typically rapid and require mild heating, if any; however, they are not capable of esterifying nonesterified fatty acids or transesterifying sphingolipids, and in the presence of

water, may result in fatty acid hydrolysis rather than transesterification (Carrapiso and Garcia 2000). For food analysis, moisture is definitely a consideration as described previously, while the limitations in regard to nonesterified fatty acids and fatty acids amide linked to sphingolipids may or may not be an issue dependent on the lipid class composition of the food to be analyzed. While limiting the application of heat during the preparation of samples containing polyunsaturated fatty acids is generally considered beneficial, for *in situ* direct transesterification, the absence of heat could possibly decrease the lipid solubilization in certain food matrices. The application of heat through convection (Dubrow et al. 1973), microwave (Leray et al. 1995, Virot et al. 2007), or as a component of sonication (Metherel et al. 2009b) has been demonstrated to either increase lipid extraction yields or greatly reduce the time required for extraction. With base-catalyzed reactions, increasing the time of reaction and/or the addition of heat to increase lipid solubilization is not an option as it would shift the reaction toward saponification and may increase the risk of degradation of polyunsaturated fatty acids (Carrapiso and Garcia 2000).

Sodium methoxide-based *in situ* transesterification has been demonstrated to generate similar results to conventional techniques for several matrices, including food (Cantellops et al. 1999, Hougen and Bodo 1973, Long et al. 1988, Suter et al. 1997a,b). The use of boron trifluoride to esterify nonesterified fatty acids after initial transesterification with methanolic sodium methoxide has also been demonstrated for several food matrices, including infant formula, eggs, and animal tissues (Park and Goins 1994).

For base catalysis, tetramethylguanidine may be better suited for *in situ* transesterification as it has the ability to esterify nonesterified fatty acids unlike sodium methoxide. Reports of successful application of tetramethylguanidine for *in situ* transesterification are relatively limited; however, it has been used to produce fatty acid methyl esters from degummed and refined palm, soybean, and peanut oils (Schuchardt and Lopes 1988). Recently, tetramethylguanidine attached onto a silica gel surface has been used as a reusable solid catalyst for the generation of fatty acid methyl esters from soybean oil for biodiesel production (Faria et al. 2008).

32.5.5 Extraction and Derivatization of Microencapsulated Oils

Microencapsulation of oil powder is a novel mechanism to incorporate fatty acids into foods while protecting the fats from oxidization and potentially preventing the formation of off flavors and odors. Novel functional foods with enriched levels of 20:5n-3 and 22:6n-3 will often contain microencapsulated fish oil.

The process of encapsulating oils typically involves emulsification of the oil with an encapsulation material that can include various proteins, starches, gums, cellulose derivatives, chitosans, cyclodextrins, waxes, and other fats (Curtis et al. 2008). The emulsified oil can then be spray dried to form a powder and additional coatings can be applied. Typically, this type of microencapsulation results in oil percentages of 20–50% of the powder weight. There are various factors that can affect extraction efficiencies of microencapsulated oils. Increased microcapsule particle size results in decreased surface area-to-volume ratios and can, therefore, reduce solvent penetration and lipid solubilization during analysis. Increasing the oil load of microcapsules can increase the proportion of surface oil and potentially increase the size of vacuoles and pores, resulting in potentially greater fatty acid recovery yields. Drying temperatures may also affect the microcapsule architecture with higher drying temperatures possibly reducing particle size (Drusch and Berg 2008). Microencapsulation strategies that better protect the fatty acid from oxidation will generally increase the difficulty to extract and quantify the fatty acid content. Drusch and Berg (2008) examined total oil content and nonencapsulated oil content of fish oil microencapsulated using a modified starch and glucose syrup emulsification and spray dry coating using the AOAC dairy method (Mojonnier flask with ammonium hydroxide) and the ISO method (Soxhlet apparatus with various passes of solvent), respectively.

A complex coacervation microencapsulation method is also being utilized for commercially available fish oil powder (MEG-3®, Ocean Nutrition Canada Ltd., Dartmouth, Nova Scotia) and has been described previously (Curtis et al. 2008). With this process, single oil droplets or clusters of small oil droplets can be formed with relatively thick gelatin shell coatings that are further stabilized by enzymatic treatment to cross-link proteins. These particles can be spray dried to form a powder and then incorporated in various food products. The particles are stable in boiling water and breaking down the coating presents

a significant analytical challenge. Presently, there is only one report in the literature examining the determination of the omega 3 content of coacervation-encapsulated fish oil. Briefly, Curtis et al. (2008) demonstrated that a methanolic hydrogen chloride catalyzed direct *in situ* transesterification followed by fatty acid methyl ester extraction with toluene provided similar values for gravimetric total fat determinations and concentrations of 20:5n-3 and 22:6n-3 of the microencapsulated fish oil powder as compared with a laborious enzymatic digestion/extraction of the microencapsulated fish oil. This one-step, acid-catalyzed transesterification method was also shown to have application for the analysis of freeze-dried microencapsulated fish oil-enriched infant formula. Furthermore, it is indicated (but data is not presented) that various available extraction methods, including Soxhlet-based, chloroform methanol-based, pressurized fluid-based, and supercritical fluid-based techniques, result in poor fatty acid recoveries without prior treatment of the shell coating with acid or enzyme digestion, or by mechanical planetary ball mill disruption.

We have applied the one-step, methanolic hydrogen chloride transesterification method to various commercially available functional foods containing microencapsulated fish oil with intermittent success in obtaining accurate analyses (Kishi and Stark, unpublished observations). In general, direct methanolic hydrogen chloride transesterification results in accurate fatty acid estimates for foods with high fat content and, therefore, low, if any, moisture, such as margarine enriched with microencapsulated fish oil. In contrast, this direct acid-catalyzed transesterification method severely underestimated omega 3 fatty acids from food products enriched with microencapsulated fish oil with relatively low fat and high water content such as fruit juices. The failure of the one-step methanolic hydrogen chloride transesterification is most likely due to the interference of water with the catalysis reaction as described in Section 5.4. Curtis et al. (2008) only reported successful application of this direct transesterification method in anhydrous samples: the microencapsulated powder and freeze-dried infant formula. Therefore, freeze drying of low-fat samples to remove moisture should allow the acid-catalyzed *in situ* transesterification reaction to proceed fully. A pretreatment of low-fat/high-moisture samples with acid to digest microencapsulation coatings followed by a lipid extraction protocol that accounts for existing water content and finally transesterification treatment of the recovered extract to generate fatty acid methyl esters may theoretically result in accurate fatty acid determinations as well.

32.5.6 Gas Chromatographic Analyses

A detailed review of the various GC techniques available to determine fatty acid methyl esters would be cumbersome and a significant distraction from the present focus on the analysis of functional foods. In addition, it would be largely redundant given the availability of detailed descriptions of the principles and application of GC to fatty acid and lipid analysis (Christie 1989, Christie 2003, Christie and Han 2010, Zamora and Hidalgo 2004).

For omega 3 and omega 6 fatty acids, capillary columns are used almost exclusively due to their ability to resolve complex fatty acid mixture. Various polar stationary-phase columns can be used, although columns with an intermediate polarity phase such as polyethylene glycol tend to be a suitable compromise between resolution capacity that tends to increase with the degree of polarity and column life span that tends to decrease with increasing polarity.

It is recommended that helium or hydrogen be used as the carrier gas based on Van Deemter plot of theoretical plate height with carrier gas velocity. In the past, helium was the preferred choice due to the risk of explosion associated with hydrogen. Modern gas chromatographs have all but eliminated the risk of explosion with electronic flow controls and automatic shutdowns. Hydrogen has some advantages. The use of helium is a significant financial consumable as compared with hydrogen, especially given that dry, high-purity hydrogen can be produced onsite by a hydrogen generator with a reasonable initial infrastructure investment. In addition, the hydrogen Van Deemter plot remains flat at higher carrier gas velocity, thus enabling faster flow rates. In the past, these flow rates were beyond the capacity of the gas chromatograph, but again modern gas chromatographs can support these flow rates that have enabled the development of fast and ultrafast GC.

Fast GC can dramatically decrease instrument analysis time and it is accomplished mainly through column miniaturization (Stark and Salem 2005c). Reducing the internal diameter of the column increases

the theoretical plate numbers and reducing the film thickness of the column reduces the theoretical plate height, both of which increase greater resolution. The smaller internal column diameter also requires a proportionate increase in the pressure drop between inlet and outlet and, therefore, increased carrier gas velocity and reduced analyte transit time through the column. The increased resolution also allows for the column to be shortened, thus, further reducing analysis time. At high pressure and fast carrier gas velocity, aggressive temperature ramping is also required. One drawback of a miniaturized column is an increased risk of overloading; however, an increase in the injection split ratio can compensate for this.

32.6 Concluding Remarks

The analysis of omega 3 and omega 6 fatty acids in functional foods is similar to routine fatty acid analytical techniques with a few exceptions. With the large variation in the types of food matrices, it is recommended that the analyst start with conventional techniques that have been proven to be accurate and precise for a specific food matrix previously. When dealing with a functional food with a fat content that has been altered or enriched with omega 3 and/or omega 6 fatty acids, it is important to identify the technique used to incorporate the fats into the food. If the omega 3 and omega 6 fatty acids were increased through biological incorporation into existing complex lipids such as triacylglycerols and phospholipids, conventional techniques will often suffice, although the degree and length of time of heating protocols should be scrutinized, particularly if the traditional form of the food was initially low in polyunsaturates. If the omega 3 and/or omega 6 fatty acids have been enriched in the food through food-processing manipulation, the analyst must be prepared to make adaptations to conventional and standard methods to achieve accurate fatty acid determinations. Conventional methods should suffice when the omega 3 and/or omega 6 fatty acid contents are increased with simple emulsifications that are sometimes used for the 18-carbon omega 3 and omega 6 fatty acids. The practice of microencapsulating polyunsaturated fatty acids to decrease the risk of oxidation and subsequent development of off flavors and odors presents an analytical challenge that may require additional or prior treatment to allow for lipid solubilization and accurate fatty acid determinations.

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33

Conjugated Linoleic Acid

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33.1 Introduction

Conjugated linoleic acid (CLA) is a family of positional (6–8 to 13–15) and geometric (*cis–cis*, *trans–trans*, *cis–trans*, and *trans–cis*) isomers of linoleic acid containing conjugated double bonds. Multiple interactions between biologically active CLA isomers and a range of metabolic signaling pathways could be responsible for the various physiological effects that have been reported for CLA. For instance, two CLA isomers, *cis*-9, *trans*-11 (rumenic acid, RA) and *trans*-10, *cis*-12, may in particular be sources of biological effects (Lock et al. 2009; Tissot-Favre and Waldron 2009). The beneficial effects of RA suggested by animal models include anticarcinogenic action, immunomodulation, and antiatherosclerosis. At the same time, the *trans*-10, *cis*-12 isomer has been shown to be a potent inhibitor of milk fat synthesis and has been implicated in diet-induced milk fat depression in dairy cows. Additionally, a growing body of evidence suggests that this isomer may adversely influence various aspects of human health, chief among them being insulin sensitivity and blood lipid levels, while also eliciting procarcinogenic effects in animal models of colon and prostate cancer (Wahle et al. 2004; Tricon et al. 2005). Interestingly, no such detrimental health effects have been reported for the more commonly employed 50:50 blends of *trans*-10, *cis*-12 and RA (Wahle et al. 2004). Studies of other isomers, for example, *cis*-9, *cis*-11 (Tanmahasamut et al. 2004) and *trans*-9, *trans*-11 (Lai et al. 2005), have held out the potential for antitumor properties, but evidence is still scanty. In contrast, the activity of key enzymes in cell energy pathways may be adversely affected when mitochondrial cardiolipin contains *cis*-11, *trans*-13 (Hoch 1992). In these circumstances, further study to determine the beneficial and detrimental effects of each individual CLA isomer is needed, and studies of this kind would be highly topical.

CLA isomers are produced by bacteria in the rumen as intermediates in the biohydrogenation of polyunsaturated fatty acids (FAs), specifically linoleic acid (*cis*-9, *cis*-12 C18:2), and they thus occur naturally in foods obtained from ruminants (Lock et al. 2009). RA makes up about 70–90% of all the CLA present, but a large number of other, minor geometric and positional isomers ranging from 7–9 to 13–15 are also formed as products of incomplete rumen biohydrogenation. Additionally, two other such products, vaccenic acid (*trans*-11, C18:1) and *trans*-7 C18:1, can be converted to RA and *trans*-7, *cis*-9 C18:2,

respectively, via delta-9 desaturase in mammalian tissues. Milk and other biological products contain mostly RA; by contrast, synthetic CLA mixtures prepared by alkali isomerization of linoleic acid or oils rich in linoleic acid, for example, sunflower, soybean, and safflower oil, tend to comprise blends that contain similar proportions of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 C18:2 along with small quantities of *cis–cis* and *trans–trans* isomers (Sæbø 2003; Kellersmann et al. 2006).

Conversion to fatty acid methyl esters (FAMEs) is the conventional derivatization method employed in gas chromatographic (GC) analysis of FAs with flame ionization detection (FID). This procedure has enabled the total CLA content to be determined in different substrates. Still, the separation, identification, and quantification of CLA isomers represent a daunting analytical challenge, especially for dairy foods and biological samples. Starting in the second half of the 1990s, considerable research has been undertaken in this field, and a series of reviews have dealt with a range of aspects of this question in detail (Fritsche and Steinhart 1998; Roach et al. 2002; Adlof 2003, 2006; Dobson 2003; Cruz-Hernández et al. 2004; Delmonte et al. 2004a, 2006, 2009; Kramer et al. 2004). At the present time, GC with mass spectrometry (MS) and silver-ion HPLC (Ag^+-HPLC) with columns connected in series and UV detection of FAMEs are the two most widely used analytical methods for elucidating the CLA isomer profiles (De la Fuente et al. 2006; Christie et al. 2007). The geometry (*cis* or *trans*) of the double bonds in CLA isomers can be disclosed by GC in combination with Fourier transform infrared spectroscopy (FTIR) (Mossoba 2001; Mossoba et al. 2004; Christy 2006). Nuclear magnetic resonance (NMR) (Davis et al. 1999; Jie 2001), attenuated total reflectance-FTIR (Kadamne et al. 2009), and Raman spectroscopy (Meurens et al. 2005; Bernuy et al. 2009) can also yield substantial information that can be extremely useful, but these techniques are not widely available. Accordingly, this chapter is intended to review the principal chromatographic methods most commonly used for CLA determination over the past 15 years, discussing the latest trends and developments and reporting on recent advances in the field while considering the advantages and limitations associated with the different approaches (summarized in Table 33.1).

33.2 Production and Generation of Standards

Analysis of CLA isomers has been hampered by a dearth of well-characterized reference materials. In recent years the unavailability of commercial standards for most CLA isomers has often resulted in misidentification. Most commercial CLA blends contain only four major isomers (*trans*-8, *cis*-10; *cis*-9, *trans*-11; *trans*-10, *cis*-12; and *cis*-11, *trans*-13), with smaller amounts of the corresponding *cis–cis* and *trans–trans* isomers of those same positional isomers. Only a few pure isomers, that is, *cis*-9, *trans*-11; *trans*-10, *cis*-12; *cis*-9, *cis*-11; *cis*-11, *trans*-13; and *trans*-9, *trans*-11 can be purchased commercially.

Other CLA isomers from 7–9 to 12–14 C18:2 have been synthesized using a combination of sigmatropic rearrangement followed by selenium-catalyzed geometric isomerization of known CLA isomers (Destaillats and Angers 2003). Mixtures of the *cis*-6, *trans*-8; *trans*-7, *cis*-9; *cis*-9, *trans*-11; and *trans*-10, *cis*-12 C18:2 have been generated by partial hydrogenation of γ-linolenic acid (*cis*-6, *cis*-9, *cis*-12 C18:3) with hydrazine followed by conjugation with KOH in ethylene glycol (Figure 33.1) and then isolating the different isomers by preparative Ag^+-HPLC (Delmonte et al. 2003, 2004b). Applying this same procedure to α-linolenic acid (*cis*-9, *cis*-12, *cis*-15 C18:3) yielded a mixture of *cis*-9, *trans*-11; *trans*-10, *cis*-12; *cis*-12, *trans*-14; and *trans*-13, *cis*-15 C18:2 (Delmonte et al. 2004b).

The geometric CLAs from positions 6–8 to 13–15 derivatized to FAMEs have thus far been generated as mixtures by treating either reference mixtures of commercially available pure CLA compounds or synthetic mixtures prepared from α- and γ-linolenic acid with I_2 (Eulitz et al. 1999). For instance, the four geometric isomers (*trans–trans*, *cis–cis*, *cis–trans*, and *trans–cis*) of 9–11 C18:2 are readily prepared by treating commercial RA with light and I_2. However, even though all these synthetic preparations have been developed as analytical standards (Delmonte et al. 2009), these isomers are still not commercially available, and further modification will be called for if production processes are to be scaled up for commercial purposes.

TABLE 33.1

Main Chromatographic Procedures Used to Determine Conjugated Linoleic Acid Isomers in Different Foodstuffs and Biological Substrates Reported in the Past 15 Years

Food/Biological Substrate	Fatty Acid Derivatives	Separation + Detection	Reference
Milk and dairy products	FAME	GC + FID	Sehat et al. (1998a); Kramer et al. (2002); Roach et al. (2000); Luna et al. (2005a); Buccioni et al. (2010)
Human milk and plasma	FAME	GC + FID	Moltó-Puigmartí et al. (2007); Bondía-Pons et al. (2007)
Beef	FAME	GC + FID	Fritsche et al. (2001); Nuernberg et al. (2002, 2007); Dannenberger et al. (2004); Aldai et al. (2006); Dugan et al. (2007); Kraft et al. (2008); Alves and Bessa (2009); Alfaia et al. (2009)
Liver	FAME	GC + FID	Zabala et al. (2007)
Ruminal fluid	FAME	GC + FID/ Ag^+-HPLC+ UV (233 nm)	Luna et al. (2008)
Milk	FAME	GC + MS + chemometric deconvolution	Blaško et al. (2008, 2009)
Milk and dairy products/ Human milk	FAME and DMOX	GC + MS	Sehat et al. (1998a); Yurawecz et al. (1998); Roach et al. (2000); Luna et al. (2005a)
Adipose tissue/ Pig tissues	FAME and DMOX	GC + MS	Kramer et al. (1998); Yurawecz et al. (1998)
Soybean oil	DMOX	GC + MS	Jung and Jung (2002)
Milk	FAME	GC + acetonitrile CACI-MS/MS	Brenna (2006); Gómez-Cortés et al. (2009)
Dairy products/ Human milk/ Biological tissues	FAME and DMOX	GC-FTIR	Fritsche et al. (1997); Kramer et al. (1998); Sehat et al. (1998a); Yurawecz et al. (1998); Rickert et al. (1999)
Dairy products	FAME	Ag^+-HPLC+ UV (233 nm)	Sehat et al. (1998a, 1999); Rickert et al. (1999); Delmonte et al. (2005); Luna et al. (2005a); Buccioni et al. (2010)
Beef/Pig tissues	FAME	Ag^+-HPLC+ UV (233 nm)	Kramer et al. (1998); Sehat et al. (1999); Nuernberg et al. (2002, 2007); Dannenberger et al. (2004) Kraft et al. (2008)
Dairy products	9-Anthrylmethylesters	RP-HPLC + fluorescence (412 nm)	Nishimura et al. (2005)
Beef and pig fat blend (EU standard material)	*p*-Methoxy-phenacyl esters	Ag^+-HPLC + UV (270 nm)	Nikolova-Damyanova et al. (2000)
Pig adipose tissue and muscle	FFA (No derivatization)	Ag^+-HPLC + UV (233 nm)	Ostrowska et al. (2000)

Note: Ag^+-HPLC: Silver-ion HPLC; CACI-MS/MS: covalent adduct chemical ionization tandem mass spectrometry; DMOX: dimethyloxazolyne derivatives; EU: European Union; FAME: fatty acid methyl esters; FFA: free fatty acids; FID: flame ionization detector; FTIR: Fourier transform infrared spectroscopy; GC: gas chromatography; MS: mass spectrometry; RP-HPLC: reverse-phase HPLC; TAG: triacylglycerol; TLC: thin-layer chromatography.

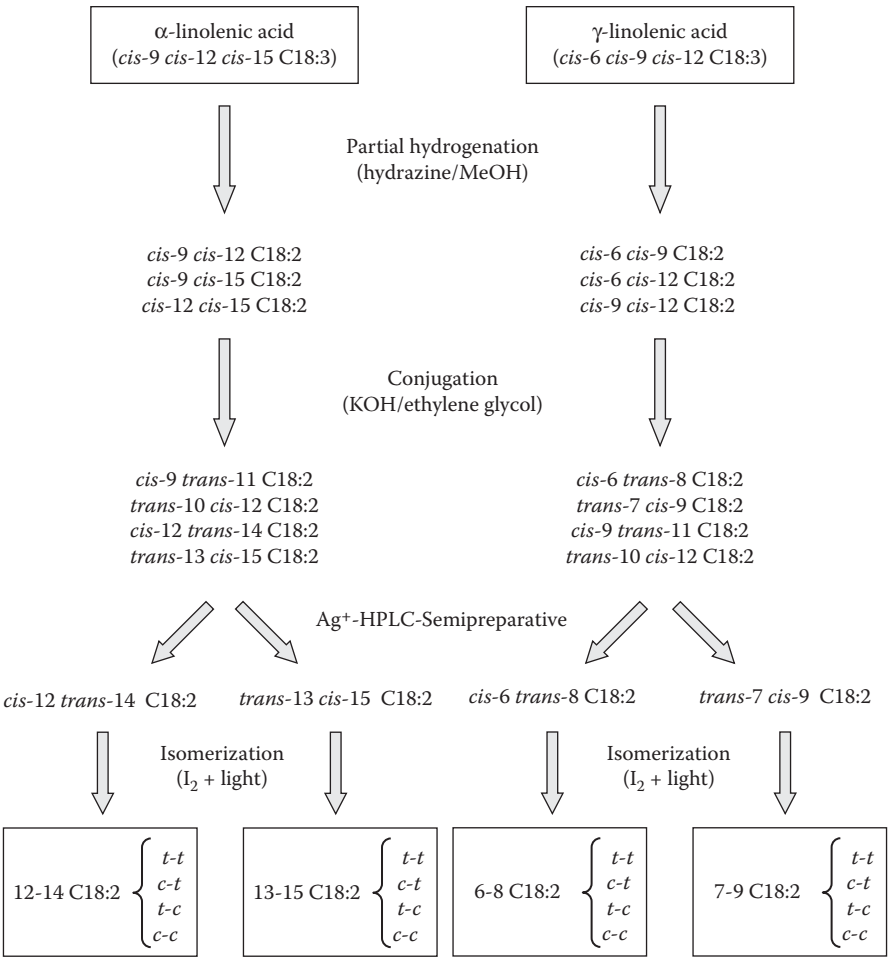

FIGURE 33.1 Synthesis of CLA isomers according to the procedures proposed by Delmonte et al. (2003, 2004b) *c*: *cis*; *t*: *trans*. (From De la Fuente, M.A., Luna, P. and Juárez, M. 2006. *Tr. Anal. Chem.* 25: 917–926. With permission.)

33.3 Gas Chromatography

33.3.1 Fatty Acid Derivatization

Most CLA molecules in foodstuffs and biological samples are in free form and, mainly, esterified to form triacylglycerols (TAGs). Conventional analysis of FAs from lipids has employed derivatization to form less polar, more volatile compounds. Preparation of volatile derivatives is often a preliminary step in the GC analysis of FAs. Transforming the FAs from TAGs into methyl esters is the most commonly used method of derivatization. Base-catalyzed methylation methods using $NaOCH_3$ or KOH in methanol at room temperature are widely regarded as being most reliable for determining CLA isomer distributions, because they do not result in double-bond isomerization and do not produce methoxy artifacts (Kramer et al. 1997).

Acid-catalyzed methylation methods employing BF_3, HCl, or H_2SO_4 yield extensive isomerization of conjugated dienes and contribute to the formation of allylic methoxy artifacts (Yamasaki et al. 1999) that can interfere with the chromatographic analysis. Increasing the temperature and/or incubation time for both base- and acid-catalyzed methylation decreased the content of RA and *trans*-10, *cis*-12 but increased the *trans*-9, *trans*-11 and *trans*-10, *trans*-12 concentrations along with artifacts (Park et al. 2001, 2002).

Using Ag^+-HPLC, Ostrowska et al. (2000) observed that all acid catalysts—and particularly HCl—were associated with significantly higher levels of *trans–trans* isomers of CLA and lower levels of *cis/trans (cis–trans* plus *trans–cis)* isomers. Acid-catalyzed methylation with BF_3 was extensively used to analyze the CLA content of dairy foods in the early 1990s and high levels of CLA isomers other than RA, principally *trans–trans* isomers, were observed in some studies (Ha et al. 1989; Werner et al. 1992; García-López et al. 1994).

For milk fat, with its appreciable short-chain FA content, procedures able to minimize the loss of such volatile FAs as butyric acid (C4:0) and caproic acid (C6:0) are advisable. The standard procedure uses KOH in methanol at room temperature as catalyst (ISO-IDF 2002) to derivatize milk fat to study the FA profile. This method reduces losses of short-chain FAs and does not result in CLA isomerization. However, this catalyst does not react with free fatty acids (FFAs) and does not completely methylate phospholipids, making the method unsuitable for substrates such as ruminal liquid or tissues with high levels of these compounds (Kramer et al. 1997; Kraft et al. 2008). As a rule, this shortcoming does not adversely affect the analyses of dairy fat, except in the case of milks with poor bacteriological quality and cheeses with high FFA levels. For samples of this kind, acid-catalyzed methods employing mild conditions are recommendable. Christie et al. (2001) reported that catalysis with H_2SO_4–methanol at room temperature achieved complete methylation of CLA isomers in FFA form without forming either artifacts or unusual components that might interfere with chromatographic analysis. This technique has also been shown to be analytically viable to analyze conjugated diene-containing FFAs in ruminant digestive fluids (Luna et al. 2008).

Reliably determining the CLA profile in meat also requires base-catalyzed methylation (Fritsche et al. 2001; Dugan et al. 2007; Kraft et al. 2008). However, thorough FA analysis requires acid methylation followed by additional GC analysis (Kraft et al. 2008), because base-catalyzed methylation does not derivatize sphingolipids, FFAs, or plasmalogenic lipids, which make up about 10–15% of the total lipids in muscle tissue. One can perform these reactions (base- and acid-catalyzed methylations) separately (Kramer and Zhou, 2001; Alfaia et al. 2009), and then merge the two results using either native C16:0 or C18:0 in the sample as internal standard. This approach has the advantage that all lipids will be methylated. The combination of a base-catalyzed method followed by an acid-catalyzed procedure could also be a suitable alternative (Nuernberg et al. 2002; Dannenberger et al. 2004; Alves and Bessa 2009). Compared with other methods, for the most abundant CLA isomers in beef lipids, this approach yielded a satisfactory level of repeatability, higher recovery rates with low variability, and higher concentrations of all isomers (Nuernberg et al. 2007).

33.3.2 CLA Analysis on Capillary Columns

GC-FID is by far the most widely used method for FA analysis and indeed today is still the only tool used by many researchers to determine total CLA contents or levels of the main isomers, RA and *trans*-10, *cis*-12 C18:2, present in blends (Aldai et al. 2006, 2007; Bondía-Pons et al. 2007; Moltó-Puigmartí et al. 2007; Zabala et al. 2007). However, identification of minor CLA isomers using this method is often based solely on comparing retention times and may thus be tentative at best, given the limited availability of standards. The various commercially available 100 m highly polar cyanosilicone capillary columns (BPX-70, CP Sil-88, and SP-2560) are best for attempting to resolve the most closely related CLA isomers. On the CP-Sil 88 column, CLA isomers elute in the region just after α-linolenic methyl ester (Kramer et al. 2002; Alves and Bessa 2009). The elution order of the positional isomers of CLA on this column is first the *cis/trans* isomers, followed by the *cis–cis* isomers, and lastly the *trans–trans* isomers. For a given positional isomer, *cis–trans* elutes before the *trans–cis* geometric isomer.

Theoretically, it should be possible to resolve the four geometric isomers of all positional species. Figure 33.2 depicts gas chromatograms showing standards of all the CLA isomers from 6–8 to 13–15 previously synthesized and isolated as described by Delmonte et al. (2003, 2004b), expressed in terms of the relative retention time/γ-linolenic acid ratio. Figure 33.2 shows that most geometric isomers with the same positional configuration were in fact discriminated. Actually, GC should be able to resolve mixtures of commercial standards of only two major isomers like RA plus *trans-10, cis*-12. However, for a mixture of roughly similar proportions of up to eight isomers, for instance, like the mixture obtained by

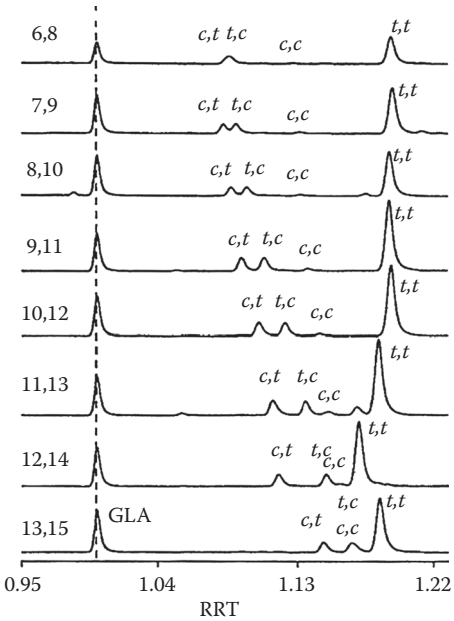

FIGURE 33.2 Gas chromatograms of the 6–8 to 13–15 CLA isomers in terms of the relative retention time (RRT)/γ-linolenic acid (GLA) ratio. *c*: *cis*; *t*: *trans*. (From Delmonte, P. et al. 2004b. *Lipids* 39: 185–191. With permission.)

I_2 isomerization of a commercial mixture of four CLA isomers (*trans*-8, *cis*-10; *cis*-9, *trans*-11; *trans*-10, *cis*-12; and *cis*-11, *trans*-13), GC is just barely able to separate the isomers but fails to resolve them distinctly. Furthermore, separations of this type are not feasible when a single isomer predominates, as is usually the case for RA in natural products (Figure 33.3). Whenever the relative concentrations are unequal, the predominant isomers will mask the minor CLA components.

The second most abundant CLA isomer in dairy products, *trans*-7, *cis*-9, is a particular concern. The 100 m capillary GC columns just described do not resolve this isomer from RA. GC separation of *trans*-7, *cis*-9, RA, and *trans*-8, *cis*-10 isomers using an isothermal oven program at 130°C has also been studied on a 200 m CP-Sil 88 capillary column (Blaško et al. 2008). The level of resolution of these CLA isomers achieved under these experimental conditions was not high enough for direct quantitative analysis but did suffice to determine their peak areas using commercial deconvolution software. The relative retention times and resolution factors for CLA isomers with overlapping peaks in separations of a commercial mixture of CLA standards and for CLA isomer fractions obtained by semipreparative HPLC of ewe's milk were used as input data for the deconvolution procedure (Blaško et al. 2008). This approach allowed successful determination of the CLA isomer contents in ovine and bovine milk samples. More recently, a new application of this method on a 100 m CP-Sil 88 capillary column at 160°C (Figure 33.3) proved useful in evaluating the *trans*-7, *cis*-9, RA, and *trans*-8, *cis*-10 C18:2 contents of dairy samples (Blaško et al. 2009) and confirmed the presence in milk fat of *cis*-7, *trans*-9 C18:2, a minor CLA previously reported in ewe's (Luna et al. 2005a) and cow's (Brenna 2006) milk.

33.3.3 Infrared Spectroscopy in CLA Determination

Infrared spectroscopic methods can be particularly useful in verifying the presence of *trans* double bonds in FAs, but they are of no help in locating the specific position of the bonds in the FA chain. To do this, a pure single component has to be isolated by means of a chromatographic procedure. Potentially, GC combined with FTIR spectroscopy is a powerful technique for determining the geometric configuration of the double bonds in FAs. The infrared spectra of CLA molecules exhibit many common features along with some bands that are highly characteristic of the double-bond geometry of a particular CLA

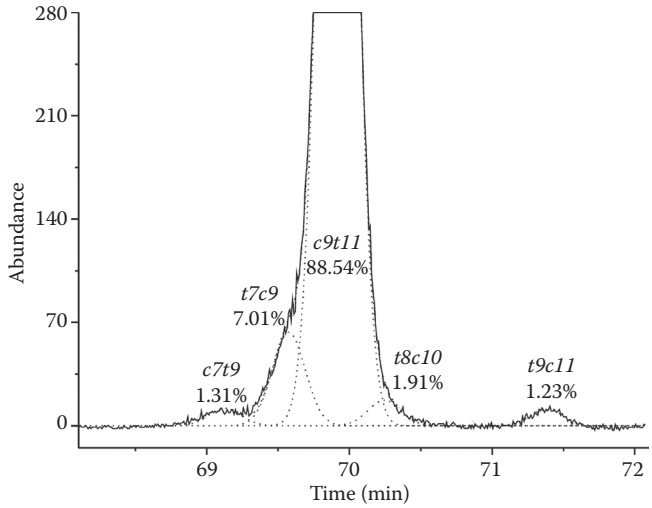

FIGURE 33.3 Separation of methyl esters of *cis*-7, *trans*-9; *trans*-7, *cis*-9; *cis*-9, *trans*-11; *trans*-8, *cis*-10; and *trans*-9, *cis*-11 C18:2 from cow's milk fat on a 100 m capillary column at 160°C. The solid line chromatogram is from the original record; dotted lines were obtained by an inbuilt peak-fitting module using Microcal Origin 7.5 software for the deconvolution of unresolved peaks. *c*: *cis*; *t*: *trans*. (From Blaško, J. et al. 2009. *J. Chromatogr. A* 1216: 2757–2761. With permission.)

isomer that can be utilized successfully to discriminate among closely related configurations. Thus, GC-FTIR was used to identify *cis/trans*, *cis–cis*, and *trans–trans* isomers. Although this method is unable to distinguish between *cis–trans* and *trans–cis* isomers, assignments made on the basis of GC retention time data offer a reasonable degree of certainty. GC-FTIR spectroscopy has been employed as a complementary tool and has been successfully applied to identify blends of CLA geometric isomers present in foods (Yurawecz et al. 1998; Jung and Jung 2002) and animal tissues (Fritsche et al. 1997; Kramer et al. 1998). Nevertheless, while GC-FTIR can yield valuable structural information, the method requires complex and expensive instrumentation and well-trained, experienced personnel capable of interpreting the spectra.

33.3.4 GC-MS

GC-MS combines high-resolution gas chromatographic separation with a sensitive and selective detection procedure and can be a valuable tool for identifying CLA isomers where suitable derivatives are used. FAME can furnish useful information on the molecular weight of FA molecules and on the number of double bonds present. High-resolution selected-ion recording MS of the molecular ion ($m/z = 294$) can be used to discriminate CLA from unknown or interfering FAs. Qualitatively, the mass spectra of CLA FAME isomers are indistinguishable from one another and from methylene-interrupted octadecadienoic acid methyl esters such as linoleate. Nevertheless, because the 294 ion is selective for C18:2 methyl esters, it could be suitable for discriminating CLA species from other FAs that elute in the same region of the chromatogram. The CLA region obtained using GC on a 100 m CP-Sil 88 column has been shown to be relatively free from interfering FAMEs, except for one peak corresponding to C21:0 (Figure 33.4). This FA has been reported as coeluting in GC analysis with *trans*-10 *cis*-12 (Roach et al. 2000) or in the *cis–cis* area (Kramer et al. 1998; Luna et al. 2005a) liver lipid and milk fats, respectively. Some signals for the molecular ions for C20:2 isomers, lower than those for minor CLA isomers, have also been reported in the *trans–trans* CLA region (Roach et al. 2000).

Other specific derivatives of the conjugated diene system have been assayed to distinguish between different CLA positional isomers. Spitzer (1999) compiled derivatization techniques that obtain structurally

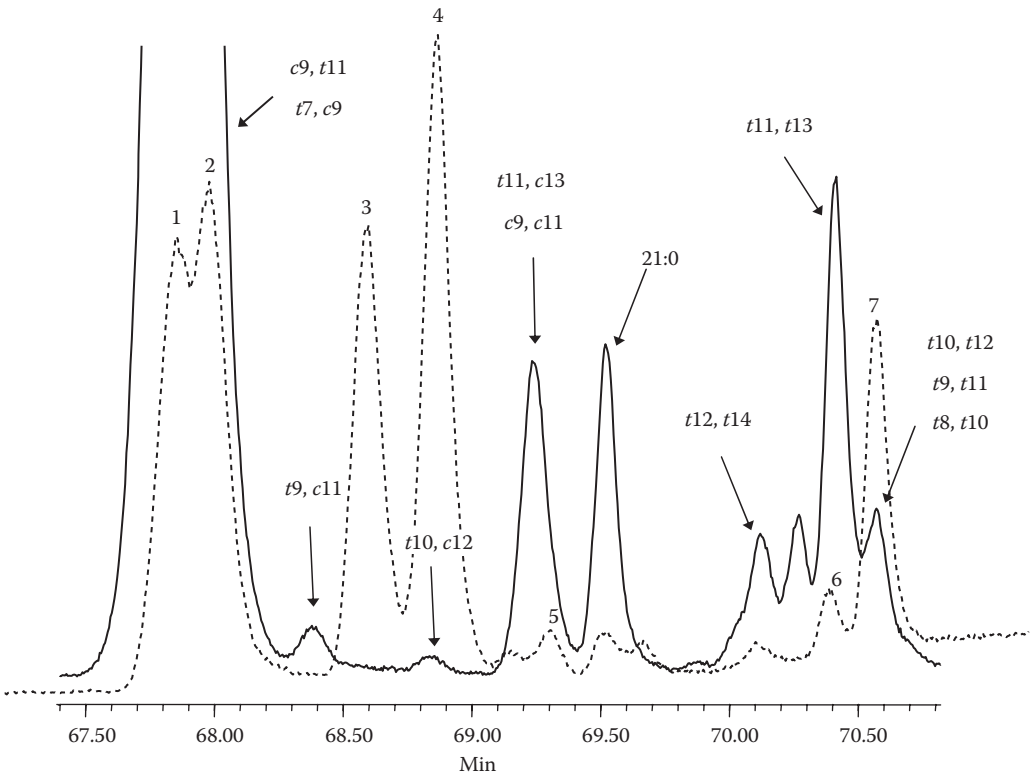

FIGURE 33.4 Partial GC-MS (total ion) chromatograms depicting the conjugated fatty acid methyl ester profiles from processed cheese fat (solid line) and a standard mixture (dotted line). Standard CLA isomer mixture: 1: *cis*-9, *trans*-11; 2: *trans*-8, *cis*-10; 3: *cis*-11, *trans*-13; 4: *trans*-10, *cis*-12; 5: *cis*-9, *cis*-11; 6: *trans*-11, *trans*-13; 7: *trans*-8, *trans*-10 + *trans*-9, *trans*-11 + *trans*-10, *trans*-12. *c*: *cis*; *t*: *trans*. (From Luna, P., De la Fuente, M.A. and Juárez, M. 2005b. *J. Agric. Food Chem.* 53: 2690–2695. With permission.)

useful data for CLA and recommended transforming unsaturated FAs into dimethyloxazolyne (DMOX) derivatives by adding 2-amino-2-methyl-1-propanol or forming 4-methyl-1,2,4-triazolyn-3,5-diones (MTADs) by means of the Diels–Alder reaction (Reaney et al. 2001). Pyrrolidides and picolinyl esters are other derivatives that have also been documented (Dobson 2003).

DMOX derivatives are strongly preferred, even though their preparation requires higher temperatures than MTADs. CLA DMOX separations are influenced by both the geometry and the position of the double bonds of the CLA isomers, hence DMOX derivatives afford superior resolution of CLA isomers compared to MTADs (Roach et al. 2002). Furthermore, the separations obtained using the corresponding DMOX derivatives are as good as or better than the chromatographic separations obtained using FAMEs (Mossoba et al. 1997; Sébédio et al. 1997).

DMOX derivatives of FAs have proven to be extremely suitable for determining the double-bond location, especially in polyunsaturated and conjugated systems, in particular for establishing the positions of the double bonds in CLA isomers in a range of materials (Kramer et al. 1998; Sehat et al. 1998a; Yurawecz et al. 1998; Jung and Jung 2002; Luna et al. 2005a; Gómez-Cortés et al. 2009). Roach (1999) published the distinctive electron impact mass spectra of the most common CLA positional isomers (from 7–9 to 12–14), and Fritsche et al. (2001) characterized the more unusual 13–15 and 6–8 C18:2 species. All the mass spectra of CLA DMOX derivatives have intense ions at *m/z* 113 and 126 and a molecular ion at *m/z* 333. They are also characterized by a loss of 15 atomic mass units (amu) from the molecular ion and successive losses of 14 amu except for the conjugated diene system, which may be located in the chain by its characteristic loss sequence of 12, 14, and 12 amu. Carbons in the allyl chain of the conjugated diene system are favored radical sites and yield a higher degree of fragmentation than other positions on

the chain (Roach 1999). The abundant ion fragments flanking a loss sequence of 12, 14, and 12 amu are helpful in assigning the positions of the double bonds in the carbon chain. Roach et al. (2002) reported that there were seven significant diagnostic ions per isomer for a total of 28 ions potentially useful in identifying positional isomers ranging from 6–8 to 13–15 C18:2.

Certain diagnostic ions are highly characteristic, and their abundance can be used to detect the presence of some positional isomers. This technique has been employed to find the *trans*-7, *cis*-9 CLA isomer in different foods and biological materials (Yurawecz et al. 1998). The elution sequence of CLA isomers on 100-m CP Sil 88 columns, together with the CLA DMOX spectra, has also detected trace amounts of the *cis*-7, *trans*-9 C18:2 geometric isomer in ovine milk fat (Figure 33.5) (Luna et al. 2005a). Nevertheless, most of the diagnostic ions used to distinguish CLA isomers are also present in the DMOX derivative mass spectra of many other FAs. Thus, obtaining a representative mass spectrum identifying a given CLA isomer in the form of its DMOX derivative requires sufficient chromatographic separation from other congeners and coextractives. Furthermore, although the *cis*- or *trans*-configurations can be inferred from their chromatographic retention times, geometric isomers cannot be differentiated on the basis of their GC-MS spectra.

Acetonitrile covalent adduct chemical ionization tandem mass spectrometry (CACI-MS/MS) with GC has been observed to be an alternative method for identifying the double bond position and geometric configuration of methyl esters of CLA (Michaud et al. 2003, 2005; Brenna 2006; Michaud and Brenna 2006; Gómez-Cortés et al. 2009). CACI-MS/MS analysis of FAs is a multistage procedure. In the first step, the (1-methyleneimino)-1-ethenylium ion ($m/z = 54$) generated by self-reaction of acetonitrile under mass spectrometer chemical ionization conditions reacts with unsaturated FAs to yield an $[M + 54]^+$ ion. Collisionally activated dissociation of the $[M + 54]^+$ ion then yields two diagnostic ions corresponding to bond cleavage at specific locations on the corresponding unsaturated FAs. Michaud et al. (2003) used standards to determine the acetonitrile CACI-MS/MS diagnostic ions expected for CLA with different double-bond positions (3–5 to 14–16). These ions were generated by C–C cleavage at a vinylic position on either side of the conjugated diene unit, yielding an α diagnostic ion containing the ester group and an ω diagnostic ion containing the terminal methyl group. In CLA with mixed double bond geometry, for steric reasons the $m/z = 54$ ion adds preferentially across the *cis* double bond, resulting in a higher abundance of the diagnostic ion in which C–C cleavage occurs vinylic to the original *trans* double bond (Michaud et al. 2005). Accordingly, the α/ω ion abundance ratio is characteristic of the double bond geometry of CLAs. Table 33.2 shows the α/ω ratio to be an order of magnitude lower in *trans–cis* than in *cis–trans* isomers.

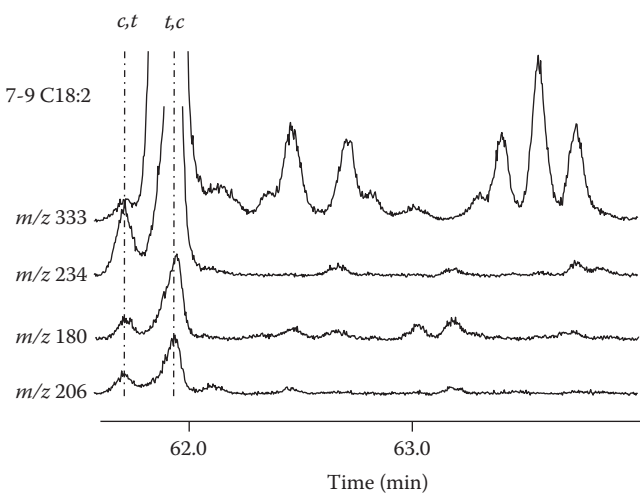

FIGURE 33.5 Reconstructed ion chromatograms include ion *m/z* 333 and characteristic ions of the 7–9 C18:2 positional isomer (*m/z* 234, 180, and 206) using dimethyloxazoline derivatives from ewe's milk fatty acids. *c*: *cis*; *t*: *trans*. (From Luna, P. et al. 2005a. *J. Dairy Res.* 72: 415–424. With permission.)

TABLE 33.2

α/ω Diagnostic Ion Ratios for Methyl Esters of *cis–trans*, *trans–cis, cis–cis, and trans–trans* Positional Isomer Standards of Conjugated Linoleic Acid Ranging from 7–9 to 11–13 C18:2

	α/ω Diagnostic Ion Ratios			
Positional Isomer	***cis–trans***	***trans–cis***	***cis–cis***	***trans–trans***
7–9	10	0.3	1.1	3.1
8–10	5.3	0.4	1.0	2.3
9–11	4.9	0.1	2.4	2.1
10–12	11	0.1	1.4	2.1
11–13	6.8	0.2	3.2	1.9

Source: Adapted from Michaud, A.M. et al. 2003. *Anal. Chem.* 75: 4925–4930.

Consequently, acetonitrile CACI-MS/MS can, by itself, discriminate three CLA double-bond geometries: *cis–trans, trans–cis*, and *cis–cis* plus *trans–trans* (Figure 33.6). The *cis–cis* and *trans–trans* double-bond geometry can be inferred from the GC relative retention times. In combination with GC retention time data, acetonitrile CACI-MS/MS has provided positive identification of 13 CLA FAMEs from a sample of milk fat (Brenna 2006). Subsequent acquisition of CLA standards and of the corresponding mass spectra showed that every peak in the milk fat sample had been properly assigned without the standards. More recently, CACI-MS/MS has been applied to elucidate the positional and geometric configuration of other minor conjugated and nonconjugated polyunsaturated FAs in milk fat (Gómez-Cortés et al. 2009).

33.4 Ag^+-HPLC

Argentation chromatography is able to separate FAs according to both their configuration and the number and position of their double bonds. Adlof (2003) published a fairly comprehensive listing of examples of CLA analysis for a variety of substrates using silver-ion chromatography (HPLC and TLC). His overview also extended to such aspects as CLA-enriched TAGs and using semipreparative Ag^+-HPLC to isolate CLA isomers. In this review, the focus will be on analyzing FAMEs by Ag^+-HPLC. Combining this technique with GC is currently the most effective way of separating and quantifying individual CLA isomers. Stable Ag^+ columns in which the silver ions bond ionically to phenylsulfonic acid moieties, which are in turn bound to a silica matrix, have been developed for HPLC and have been used extensively to separate CLA isomers (Table 33.1).

The use of Ag^+-HPLC to complement GC in the assay of CLA was first reported by Sehat et al. (1998b) based on the previous work of Adlof (1997). General Ag^+-HPLC CLA analysis conditions were standardized by the late 1990s (Sehat et al. 1998a,b, 1999). Systems used for isocratic separations of CLA FAMEs were equipped with commercial *ChromSpher 5 Lipids* silver-impregnated columns (250 mm × 4.6 mm i.d. stainless steel; 5 μm particle size) with 0.1% acetonitrile in hexane as the mobile phase. CLA FAMEs are selectively detected by their characteristic UV absorbance at 233 nm, and direct fat extraction followed by measuring absorbance at this wavelength is by far the simplest and most viable means of rapidly determining the CLA isomer profile.

Isomer identification on HPLC chromatograms is achieved by coinjecting known reference materials obtained from commercial or synthesized sources. Ag^+-HPLC profiles exhibit a first chromatographic zone where the different *trans–trans* compounds separate, followed by a zone where the *cis/trans* compounds elute. Although the geometric isomers of these compounds are not resolved, species that differ in positional double bonds elute separately. Finally, after the *cis/trans* area *cis–cis* CLA isomers located. In theory, these isomers could be individually quantified. However, in some substrates such as milk fat, the *cis–cis* zone is sometimes masked by a wide peak (Sehat et al. 1999; Luna

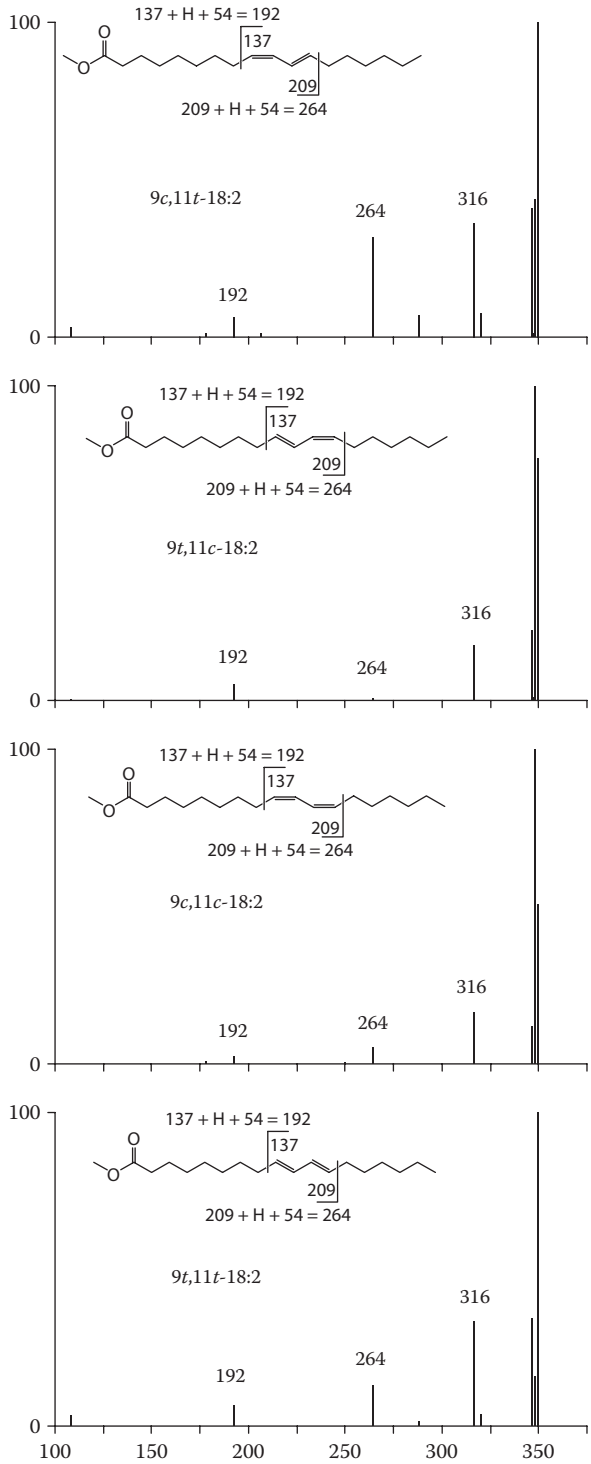

FIGURE 33.6 Acetonitrile covalent adduct chemical ionization tandem mass spectrometry spectra for the 9–11 C18:2 geometrical isomers. *c*: *cis*; *t*: *trans*. (From Michaud, A.M. et al. 2003. *Anal. Chem.* 75: 4925–4930. With permission.)

et al. 2005a) (Figure 33.7). This peak has been attributed to absorption by methyl oleate when high amounts of this FA are present. Employing a dual UV wavelength detector has enabled CLA to be selectively detected at 233 nm and other fatty acids to be detected simultaneously at 205 nm. Other minor sources of interference in the CLA zone have also been reported (Delmonte et al. 2004a).

Sehat et al. (1999) and Rickert et al. (1999) were able to improve the resolution of methyl esters of CLA isomeric mixtures from natural and commercial products progressively by operating up to six Ag^+-HPLC columns in series. However, using three columns appears to be a reasonable trade-off for achieving timely resolution of most CLA isomers from biological substrates. The lengthy run times needed for all components to elute and high mobile phase consumption militate against the use of more than three columns, and this approach should only be used to resolve specific critical pairs of isomers. Furthermore three columns can resolve the 11–13 pair of *cis/trans* geometric CLA isomers.

The main problem with Ag^+-HPLC is the retention volume (RV) drift that occurs over time. This nonreproducibility of RV means that peak identification is difficult. Factors influencing the RV and peak shape of individual CLA isomers include column preconditioning and sample loading, column temperature, double-bond positions relative to each other and to the FA carbonyl group, as well as double-bond geometry and, especially, solvent composition.

To obtain reproducible results for Ag^+-HPLC runs, potential sources of error, such as solvent composition, need to be addressed. Because of the difference in polarity, the solubility of acetonitrile in hexane is <5%, so the solution resulting from mixing these two solvents is unstable. Increasing the amount of acetonitrile in the hexane decreases FAME retention times, and small variations in the percentage acetonitrile in the elution solvent cause large variations in CLA isomer RV. This drift does not affect the relative resolution of CLA isomers, but retention times for the isomer FAMEs can increase with each

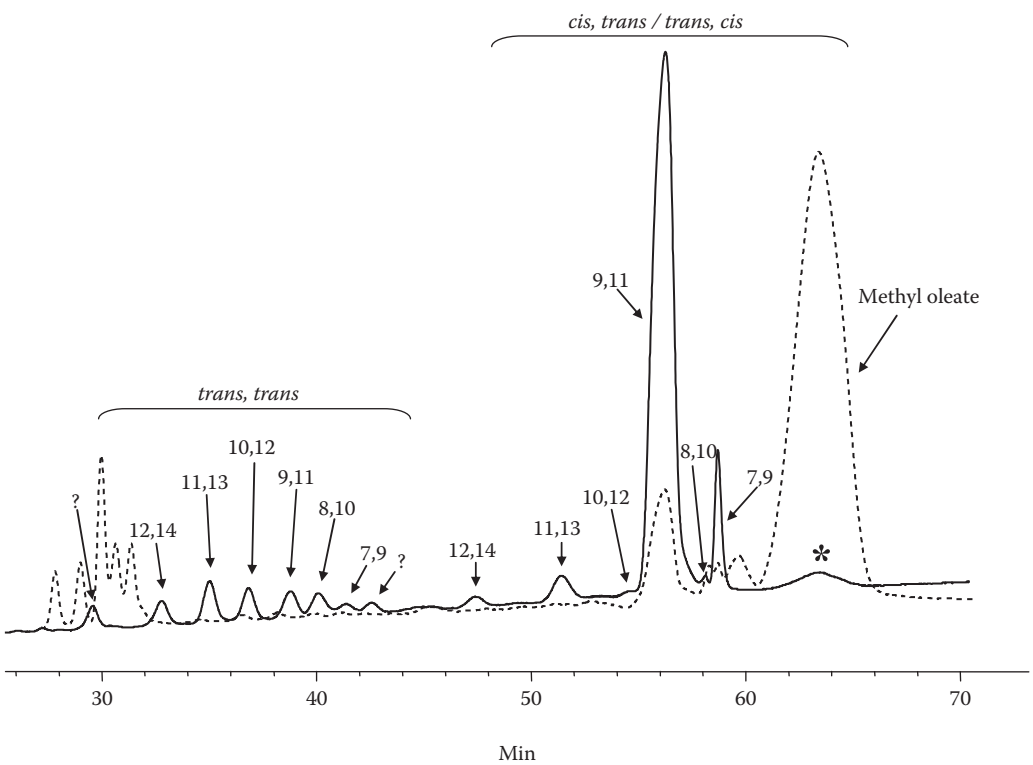

FIGURE 33.7 Silver ion HPLC profile for ewe's milk fatty acid methyl esters obtained using three *ChromSpher 5 Lipids* columns in series and a UV detector at 233 nm (solid line) and 205 nm (broken line). Asterisk represents methyl oleate. (From Luna, P. et al. 2005a. *J. Dairy Res.* 72: 415–424. With permission.)

successive run. As an alternative, Delmonte et al. (2003) suggested adding diethyl ether to the mobile phase to partly stabilize the solvent mixture. Still, this elution system (0.1% acetonitrile/0.5% diethyl ether in hexane) did not totally eliminate the retention time shift.

Using a commercial CLA mixture and two *ChromSpher 5 Lipids* columns in series, Müller et al. (2006) evaluated 13 solvent systems from a perspective of retention time stability and resolution. The eluent system composed of 0.2% propionitrile in hexane yielded the highest degree of stability compared with the reference acetonitrile system. This stability was attributed to the better solubility of propionitrile in hexane. Unfortunately, propionitrile is a toxic reagent, and it also failed to achieve the same optimum resolution of CLA as acetonitrile did. In order to improve resolution with this eluent system, use of a third column has been suggested (Müller et al. 2006).

To circumvent this problem, Delmonte et al. (2004a, 2005) proposed another approach based on relative retention volumes (RRVs). These researchers observed that by using toluene as a reference to approximate the dead volume in the Ag^+-HPLC system and RA as a retention time reference value, the chromatographic data could be recalculated into a reproducible format using the formula:

$$RRV_i = (RV_i - RV_{toluene})/(RV_{RA} - RV_{toluene})$$

where RRV_i is the RRV of an individual CLA isomer.

This approach is based on the assumption that changes in the composition of the mobile phase affect elution of all CLA isomers in the same way. Expressing CLA isomer elution in terms of their RRVs goes a long way toward standardizing each CLA isomer, resulting in coefficients of variation of <2% for a wide variety of substrates (Delmonte et al. 2005). Figure 33.8 depicts Ag^+-HPLC chromatograms transformed using the above equation. The *cis*-10, *trans*-12 and *trans*-10, *cis*-12 isomers have the same RRV. For a given position from 6–8 to 9–11, the RRVs of the *trans–cis* isomers are higher than those of the corresponding positional *cis–trans* isomers, whereas for the positions from 11–13 to 13–15, the RRVs of the *cis–trans* isomers are higher than those of the *trans–cis* isomers.

Delmonte et al. (2005) tested 2% acetic acid/hexane as an alternative mobile phase and obtained a different but complementary elution pattern for CLA FAMEs. Advantages include discrimination of the two *cis/trans* 10–12 isomers and partial resolution of the *cis*-6, *trans*-8 isomer from the *trans*-7, *cis*-9 isomer. However, chromatographic runs take longer. More recently, the time of analysis was reduced from 90 min to 45 min to 35 min by the addition of 0.05% or 0.1% (v/v) 2-propanol, respectively to acetonitrile (0.1% v/v) and diethylether (0.5% v/v) in hexane with no effect on resolution of the 17 individual isomers of CLA mixtures (Kuhnt et al. 2010). This approach would be useful to shorten the time of analysis, save solvents, and reduce costs.

Ag^+-HPLC has also been adapted for the purpose of analyzing CLA isomers in the form of FFAs, thus removing the need for methylating before assays (Cross et al. 2000; Ostrowska et al. 2000). Using mobile phases that had previously been optimized for methylated CLA analysis, retention times were overly long and a competing acid was required, and ultimately a mobile phase of 2.5% acetic acid and 0.025% acetonitrile in hexane was selected as the optimum mobile phase with a view to shortening run times somewhat (Cross et al. 2000). This procedure yielded smaller RVs and better resolution than CLA FAME separation on a single *ChromSpher 5 Lipids* column. To our knowledge, this method has not been used for samples naturally enriched with FFAs, but potentially it could be very useful for substrates of this type.

Yet another procedure for analyzing CLA isomers by Ag^+-HPLC is based on transforming FAs into aromatic esters (Nikolova-Damyanova et al. 2000). The results obtained demonstrated the beneficial effects of using *p*-methoxyphenacyl esters for isomeric CLA separation. Using only a single column rather than the multiple columns employed by other methods and stepwise gradient elution with hexane/dichloromethane/acetonitrile and UV detection at 270 nm, saturated, CLA, *cis*-monoenes, and methylene-interrupted C18:2 were satisfactorily separated in a single chromatographic run. However, a drawback of this procedure is that it is nonspecific. Non-CLA esters present in a sample are detected with the same degree of sensitivity as CLA esters, and therefore both cleanup after ester formation and isolation of CLA esters from non-CLA esters by preparative liquid chromatography are required before Ag^+-HPLC.

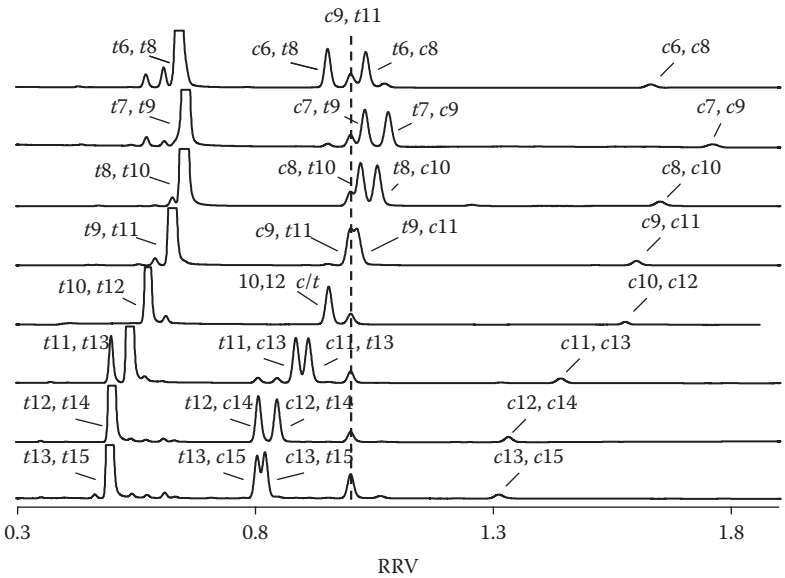

FIGURE 33.8 Silver ion HPLC chromatograms of all the geometric CLA isomers FAMEs from 6–8 to 13–15 C18:2 using 0.1% acetonitrile/0.5% diethyl ether/hexane elution and UV detection at 233 nm. *cis*-9, *trans*-11 was added to each positional mixture for reference. RRV: relative retention volume; *c*: *cis*; *t*: *trans*. (From Delmonte, P. et al. 2005. *Lipids* 40: 509–514. With permission.)

In the studies discussed above, the Ag^+-HPLC columns were held at temperatures between 20°C and 30°C. Lowering the elution temperature increases retention times for CLA isomers but at the same time increases isomer resolution, and for this reason other researchers (Adlof 2006; Delmonte et al. 2006) have addressed the option of working at lower column temperatures. Additional combinations of columns in series, elution solvents, and temperatures remain to be investigated with a view to improving chromatographic resolution, but thus far a system comprising three *ChromSpher 5 Lipids* columns in series at –15°C and a 2% acetic acid/hexane mobile phase at a flow rate of 1 mL/min has been found to be optimal for separating CLA FAMEs (Delmonte et al. 2006). This elution temperature increases the separation between the *trans*-10, *cis*-12 and *cis*-10, *trans*-12 isomers as well as between *trans*-9, *cis*-11 and RA, and at the same time all the other *cis/trans* CLA isomers are well resolved. Only *cis*-8, *trans*-10 appears as a shoulder with *cis-9, trans-11* C18:2 (Figure 33.9). A further decrease in the elution temperature was found to increase retention times for the CLA FAMEs without achieving any further improvement in resolution (Delmonte et al. 2006).

33.5 Reverse-Phase HPLC

Reverse-phase (RP) HPLC is based on a different separation mechanism and is thus complementary to Ag^+-HPLC as a method of analyzing CLA. It holds out great potential for semipreparative isolations of FAs. Ag^+-HPLC separates FAMEs based on the number of double bonds, geometric configuration, and position, but compared to RP-HPLC it is less selective with regard to FA aliphatic chain length. Based on this selectivity for FA chain length, RP-HPLC has been used successfully to separate and quantify CLA metabolites formed by elongation and desaturation in animal tissues (Banni et al. 1996; Murru et al. 2003). Separations were carried out on an octadecylsilyl column with a particle size of 5 μm using a diode array detector. Unsaturated nonconjugated FAMEs were detected at 200 nm and conjugated diene FAMEs at 234 nm.

RP-HPLC yields CLA isomer separations unlike the separations obtained using Ag^+-HPLC, with *trans–trans* moieties eluting at the end of the chromatogram (Figure 33.10). Delmonte et al. (2006)

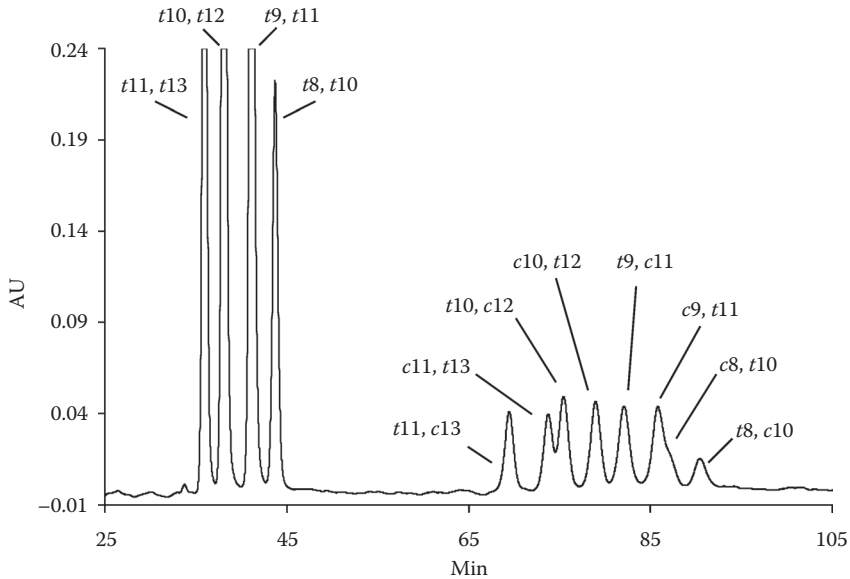

FIGURE 33.9 Partial chromatogram of the fatty acid methyl esters of an I_2 isomerized CLA mixture (Nu-Chek Prep., Inc.). Chromatographic conditions were three *ChromSpher 5 Lipids* columns in series at –15°C, a 2% acetic acid in hexane mobile phase at a flow rate of 1.0 mL/min, and UV detection at 233 nm. (From Delmonte, P. et al. 2006. *Advances in Conjugated Linoleic Acid Research*, Vol. 3, M.P. Yurawecz, et al. (eds), pp. 95–118. Champaign: American Oil Chemists Society Press. With permission.)

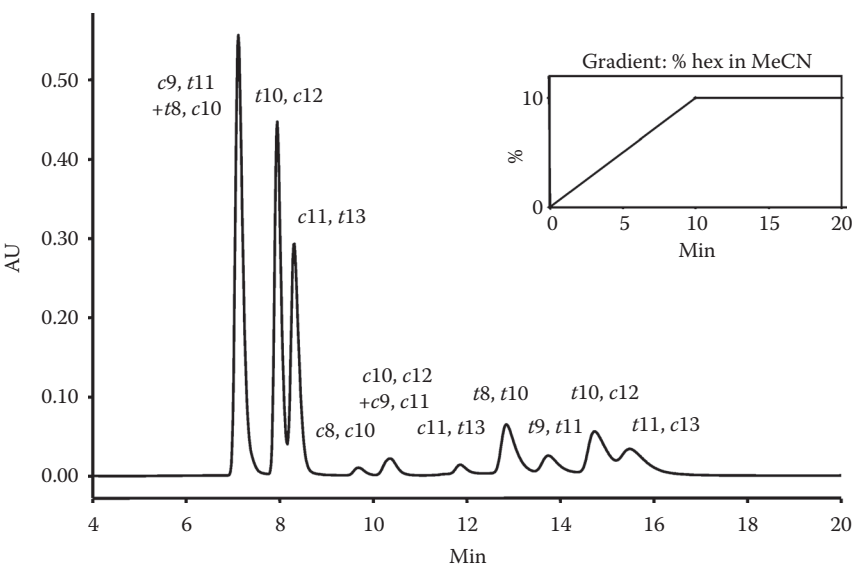

FIGURE 33.10 Partial reverse-phase HPLC chromatogram of a conjugated linoleic acid methyl esters reference mixture (Nu-Chek Prep., Inc.). Chromatographic conditions were a single Vydac 201TP54 column at 5°C, a hexane (hex) in acetonitrile (MeCN) elution gradient at a flow rate of 1.0 mL/min, and UV detection at 233 nm. (From Delmonte, P., et al. 2006. *Advances in Conjugated Linoleic Acid Research*, Vol. 3, M.P. Yurawecz, et al. (eds), pp. 95–118. Champaign: American Oil Chemists Society Press. With permission.)

observed RP-HPLC separations of CLA isomers to be systematically affected by column temperature and to be column specific. RVs were inversely correlated with column temperature, resulting in large differences in resolution between CLA isomers. Systems based on RP-HPLC octadecylsilylated silica columns (250 mm × 4.6 mm i.d., 5 μm particle size) and elution gradients including acetonitrile, hexane, and tetrahydrofuran have been found useful for separation of the *trans*-10 *cis*-12, *trans*-9 *cis*-11, *cis*-6 *trans*-8 and *trans*-6 *cis*-8 18:2 isomers (Delmonte et al. 2006).

Another RP-HPLC method based on separation of the 9-anthrylmethyl ester derivatives of conjugated and nonconjugated FAs with fluorescence detection has been employed to determine total CLA content and other FAs in dairy products (Nishimura et al. 2005). CLA reacted readily with 9-anthryldiazomethane at room temperature to produce 9-anthrylmethyl esters without isomerization and/or decomposition of the conjugated double bonds. Resolution of the individual FAs in the form of their 9-anthrylmethyl esters was achieved on an octadecylsilylated silica column (150 mm × 3 mm i.d., 3 μm particle size) using stepwise gradient elution with methanol-water.

33.6 Conclusions

CLA analysis can be either a simple or a complex task, depending on analytical requirements. Conventional base-catalyzed FAME derivatization for GC-FID FA analysis is able to determine the total CLA content of different substrates. However, at present, there is a real need to be able to determine CLA isomer distribution, in as much as the different isomers may perform different biological functions. GC-MS using 100 m long highly polar capillary columns has been employed to characterize different CLA isomers. GC-MS of DMOX derivatives can identify positional isomers of CLA with double bonds located at carbons 6–8 to 13–15, whereas GC with acetonitrile CACI-MS/MS or FTIR can elucidate double-bond position and geometric configuration. However, GC alone is not capable of separating all the CLA isomers known to occur in foodstuffs and biological substrates. Over the past 15 years, analysis of FAMEs by Ag^+-HPLC with an acetonitrile:hexane elution system has become one of the most widely used methods in CLA analysis complementary to GC. This technique has played, and will continue to play, a prominent role in the isolation and separation of individual CLA isomers. The availability of new CLA standards and the use of RRV and/or alternative eluent systems in Ag^+-HPLC separations could in future greatly improve CLA isomer identification.

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34

Lecithin

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34.1 Introduction

Researchers working in the field of oils and fats, particularly the technicians of the fat-processing industry, call all phospholipids "lecithins," which are considered by many authors as a group of compounds with the common property of having a phosphoric acid in their molecule, for which two different categories are distinguished: glycerophospholipids, the derivatives of glycerol, and sphingophospholipids, those that do not contain it (Figure 34.1). The concept of lecithins being lipids that contain a molecule of phosphoric acid (mainly glycerophospholipids) is widespread among the industry staff who work with these compounds (Graciani Constante 2006).

The first researcher to find the phosphoric group among the components of brain tissue fats was Vauquelin M. in the early-nineteenth century (Vauquelin 1812). Between 1846 and 1847, Gobley detected the presence of a lipid from which he could obtain glycerophosphoric acid and fatty acids in egg yolk for the first time, and he called it "lecithin," a term derived from the Greek "lekithos" (egg yolk) (Gobley 1846, Gobley 1847a,b). Diakanow (1867a,b) and Strecker (1868) showed that this lipid contained a choline base that had been originally isolated from pig liver by Strecker (1867), and they deduced a temporary structure for the lecithin. Subsequently, lecithins were studied by other authors (Ansell and Spanner 1982), thus contributing to extent the documentation on them. In 1884, Thudichum differentiated the term lecithin from similar compounds, the "cephalins," for their different behavior to crystallize in alcohol (the cephalins are more insoluble); and it was he who, in 1874, assigned the name "phosphatides" to all these compounds containing phosphoric acid, fatty acids, glycerin, and a nitrogen base in the molecule (Graciani Constante 2006). In addition, this researcher identified a new phospholipid that does not have glycerin, nowadays called sphingomielin (Graciani Constante 2006). In 1910, the term "phospholipines" was introduced by J.B. Leathes to designate the compounds discussed above and this terminology was followed by other authors, while others called them phosphatides (Graciani Constante 2006). Then, the name "phospholipid" (or "phospholipide") was adopted in the United States as the most appropriate (Graciani Constante 2006).

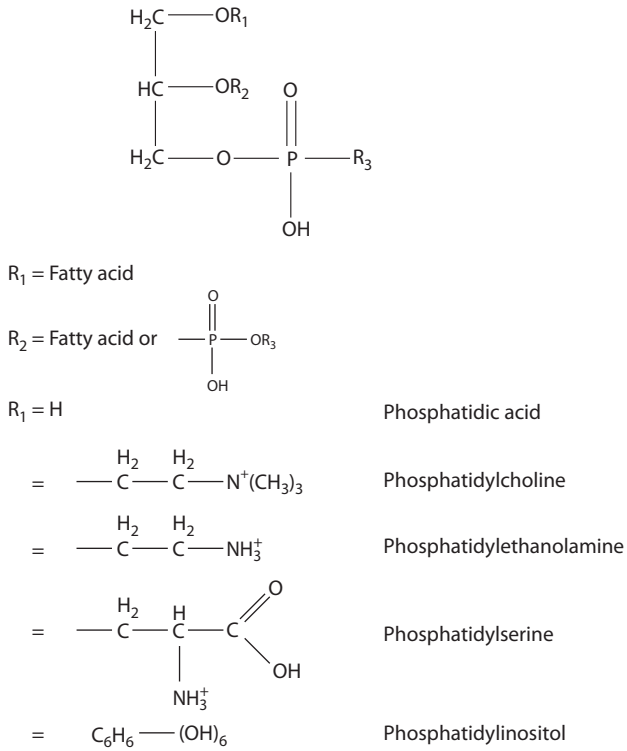

FIGURE 34.1 Chemical structures of the same phospholipids.

The nomenclature of phospholipids underwent numerous modifications, until 1967 when the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry (IUB) Commission on Biochemical Nomenclature published a document for discussion on the Nomenclature of Lipids (IUPAC 1967). It was followed in 1976 by revised Recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature (IUPAC 1977). In these documents, the common glycerophospholipids are called derivatives of phosphatidic acid, for example, 3-*sn*-phosphatidylcholine (this term is preferred to the trivial name, lecithin; the systematic name is 1,2-diacyl-*sn*-glycero-3-phosphocholine); 3-*sn*-phosphatidylserine; 1-phosphatidylinositol 1,3-bis(3-*sn*-phosphatidyl)glycerol.

34.2 Role of Lecithin in the Fat Industry and Its Effects on Health

Practically, all vegetal oils extracted with solvents contain lecithin or phosphatides in their composition. These compounds have been considered as undesirable by the industries of refining or fat transformation, having to be eliminated before the refining process of fats (Bernardini 1981). This previous depuration has a fundamental importance, since the good operation of the refining process and the final quality of refined oils largely depend on the complete elimination of these substances (Cherry and Kramer 1989).

The elimination process of phosphatides or lecithins consists of hydration in an acid medium (sulfuric, phosphoric, chloridric, oxalic, citric acids, etc.) to 60–80°C and the flocculation which is aided by decanters (Bernardini 1981). This step is previous to either the neutralization process in chemical refining or bleaching in physical refining, as shown in Figure 34.2.

Owing to surfactant, emulsifier, and emollient properties, lecithins have multiple food applications like bakery goods, chocolate, instant products, and margarine. Besides, there are nonfood applications such as drugs, calf milk replacer, paints, leather treatment, and mosquito control (Van Nieuwenhuyzen 1981).

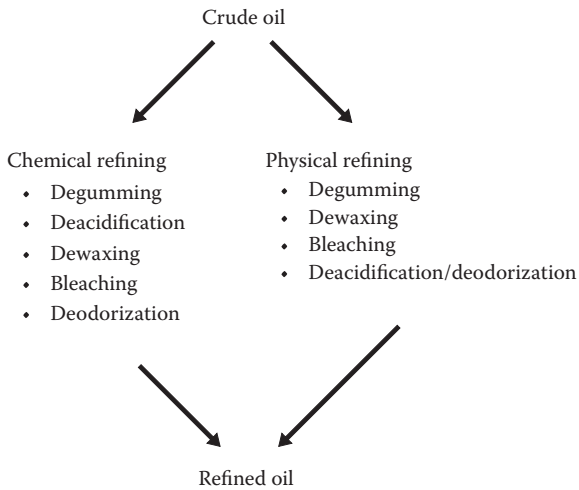

FIGURE 34.2 Scheme of chemical and physical refining process of oil seeds.

As lecithins are important constituents of all human cells, they can play important roles in human diseases. A great number of researches have been using lecithin as a dietary supplement for the purpose of treating or preventing several diseases. Some of these therapeutic uses of lecithin are reviewed below.

In a study of a disease of the nervous system called Tardive Dyskinesia, Gelenberg et al. (1979) found that lecithin improved abnormal movements in all patients with this disease, since it compensated for the increased dopaminergic activity of the central nervous system. Leo and Rao (1988) carried out a pilot study in which they studied the effects of intravenous lecithin on memory loss in multiple sclerosis, concluding that lecithin may be useful in treating memory disturbance due to this disease, since a significant improvement in verbal memory was observed in several patients. Later, other researchers (Holford and Peace 1994) demonstrated that lecithin has beneficial effects on the cognitive status in patients with Alzheimer's disease.

Several authors (Aabdallah and Eid 2004) have attributed an antioxidant action comparable to that of α-tocopherol to lecithin, concluding that it has a possible neuroprotective effect partly through this property. However, Olcott and Mattill (1936) showed that purified lecithin is not an oxidation inhibitor, but purified cephalin is, and that the particular portion of this molecule responsible for its antioxygenic action is probably the mono-basic phosphoric acid radical.

The action of lecithin in mechanisms of lipid transport has been widely studied. Rampone and Machida (1981) concluded that lecithin suppresses cholesterol absorption by some additional mechanism, such as by a direct effect on cell membranes or by holding the cholesterol in micellar form. Some years later, Knuiman et al. (1989) showed that the consumption of lecithin has a more beneficial effect on serum cholesterol than the consumption of equivalent amounts of polyunsaturated oils. A study made in humans suggests that soybean lecithin could be considered an effective nutrient useful in the dietary treatment of mild hypercholesterolemia (Polichetti et al. 1998). Spilburg et al. (2003) found that fat-free food supplemented with soy stanol-lecithin powder reduced cholesterol absorption and low-density lipoprotein cholesterol.

Other researchers give a possible explanation to the reduction in plasma cholesterol induced by lecithin. One of them is that polyunsaturated lecithin causes a reduction in plasma cholesterol in rats possibly through an increased formation of high-density lipoprotein particles (Jimenez et al. 1990). In addition to the studies on the effect of lecithin on cholesterol, others show the effects of this on the secretion of biliary lipids. Dietary lecithin can stimulate bile formation and biliary lipid secretion, particularly cholesterol output in bile and might modify hepatic cholesterol homeostasis and lipoprotein metabolism (LeBlanc et al. 2003). Choline contributes to the beneficial effect of a lecithin diet on bile secretion, and this effect may be attributed to the modulation of HDL and an enhancement of the cholesterol and

phospholipid pools destined for biliary secretion (LeBlanc et al. 1998). In addition, lecithin can remove fatty deposits from arteries and is able to prevent blood clotting in the arteries (Morrison 1958). All the factors mentioned above suggest potential beneficial effects of dietary lecithin supplementation for treating vascular disease.

On the other hand, lecithin reduces plasma membrane disruption through hydrophobic bile salts, and so it may play a key role in preventing the bile salt injury of biliary and gastrointestinal epithelia (Narain et al. 1997). In some cases of psoriasis, the feeding of soybean lecithin mixture produces improvements in patients with this disease (Goldman 1942).

In spite of the beneficial effects of the lecithin ingest, there are some authors who have reported some detrimental effects (Bell and Lundberg 1985, Bell et al. 1986a,b). In one of these studies, the results indicate that dietary soy lecithin preparation (SLP) enrichment during development leads to behavioral and neurochemical abnormalities in the exposed offspring and the animals exposed to lifelong 5% or 2% SLPs were hypoactive, had poor postural reflexes, and showed attenuated morphine analgesia (Bell and Lundberg 1985). In 1986, a study showed that exposure of developing rats to SLPs influenced macromolecular constituents of immature brain cells and caused abnormal behavioral patterns (Bell et al. 1986a). In this same year, the data obtained in another study indicated that dietary supplementation with SLP throughout perinatal development alters synaptic characteristics in a manner consistent with disturbances in neural functions (Bell et al. 1986b).

34.3 Methods for Analysis of Lecithin

Given the industrial importance and health effects that are attributed to phospholipids, efficient analytical methods for evaluating the lecithin or phosphatide content are necessary such as in the industrial processing of fats and in the foods that contain them. In order to make a description of the analytical methods used in the determination of phosphatides, they will be grouped according to the technique used for their quantification.

34.3.1 Gravimetric Methods

Traditionally, phospholipids have been determined by gravimetric methods either as a previous technique of separation and subsequent mineralization and gravimetric or volumetric determination of the inorganic phosphorus derivatives or by employing the gravimetry as an analytical technique to evaluate them directly without previous mineralization.

In the first group, fat or oil samples are dissolved in acetone and the phosphatides precipitate. In another type of samples, the lipid fraction is extracted previously with solvents such as petroleum ether (Wittcoff 1951), ethyl ether, or alcohol (Bloor 1929) and then after removing these solvents, the phospholipids are precipitated through the addition of acetone. In both cases, the precipitated phosphatides are collected by centrifuge and subsequently the precipitate is mineralized by humid (oxidation) (Bloor 1929) or by a dry method (calcination) (A.O.C.S. 1997). Finally, the inorganic phosphorus is determined forming phosphomolybdic compounds gravimetrically (A.O.A.C. 1980) or by titration (A.O.C.S. 1989). The methods that use the mineralization of phosphatides precipitated with acetone are long and tedious; however, they are more reliable than those that directly determine the phosphatides by gravimetry. This is due to the fact that when acetone is used alone to precipitate the phosphatides, waxes can precipitate together with them, producing errors in the quantification by excess. To solve this problem, the phospholipids and the waxes precipitated by the excess of acetone are dissolved in benzene and separated through chromatography on a single column of aluminum oxide, where the phospholipids are retained (UNE 55-115-81).

Sinram (1986) developed a procedure to measure the phosphorus content of soybean and corn oil samples using nephelometry (turbidity). This method measures turbidity in oil–acetone mixtures due to phospholipids, which is correlated to the phosphorus level. This is 30 times faster than following colorimetric methods.

34.3.2 Spectroscopic Methods

34.3.2.1 Molecular Absorption Spectroscopy

Most colorimetric methods for the determination of phosphorus depend on the conversion of phosphoric acid to phosphomolybdic acid and the reduction of this by a suitable reagent to blue molybdic oxide, the intensity of which is proportional to the concentration of the phosphate. Osmond (1887) published the first colorimetric method for the estimation of phosphorus in which molybdic oxide is used and the washed precipitated ammonium phosphomolybdate is reduced with stannous chloride giving a blue color that could be measured colorimetrically. Although this method was quite extensively used, subsequent modifications of it were made, one of them by Taylor and Miller (1914) in which phenylhydrazine was used as the reducing agent. Bell and Doisy (1920) found that hydroxilamine and many phenols had this selective reducing action in weak acid solutions at room temperature and they selected hydroquinone as the most suitable-reducing agent. In addition, they reported that the molybdenum present as phosphomolybdic acid could be colorimetrically determined in the presence of an excess of molybdic acid, and so it was unnecessary to isolate the phosphate as ammonium phosphomolybdate. Briggs (1922) found that the Bell and Doisy method had one objection and this was that the alkaline blue color which was used for comparison in the colorimeter faded rather quickly. He modified the method by adding a little sodium sulfite to the reagent and carried out the reaction in an acid medium, thus obtaining greater stability in the color obtained. At the same time, Randles and Knudson (1922) described a procedure for applying the Bell and Doisy method to the estimation of lipoid phosphoric acid in blood and found that varying the acidity during the color development had a marked effect. Whitehorn (1924) found that the chief difficulty in carrying out this procedure was in the control of the digestion process, and so he proposed that the driving off of sulfuric acid by vigorous digestion could reduce acidity enough to permit a greater color yield.

Based on the method of Bell and Doisy (1920), Fiske and Subbarow (1925) described a procedure for the colorimetric determination of phosphorus, which had applications in the field of lipids and employed 1-amino-2-hydroxynaphthalene-4-sulfonic acid as the reducing agent because it was more active than hydroquinone and quickly yielded an intense stable color.

A very convenient modification of the Briggs procedure was made by Martland and Robinson (1926), in which the color was allowed to develop at acidity higher than that normally used, and the acid and molybdate were kept separated. In the same year that Martland and Robinson published this modification, Roe et al. (1926) found the following conditions to greatly influence the production of the blue color; the concentration of the molybdic acid; the concentration of the reducing agent; the time allowed for the completion of the reaction, the hydrogen ion concentration; the presence of salts; and the amount of phosphate in the sample to be analyzed.

Kuttner and Cohen (1927) described a method based on the selective reduction of phosphomolybdic acid by stannous chloride when definite concentrations of reagents were maintained, since it was more stable than others used. This method was more sensitive.

Horecker et al. (1940) suggested the use of a larger quantity of sulfuric acid to assure complete digestion and to avoid any loss of phosphorus. Furthermore, they used a photoelectric spectrophotometer, whereby as little as one gamma of phosphorus could be determined.

Leiboff (1928) worked out a different colorimetric procedure in which uranium acetate was used to precipitate the phosphate. Then, the uranium phosphate was dissolved in trichloroacetic acid and reacted with potassium ferrocyanide to develop a color that could be compared with a known standard.

Combinations of several methods were used too; the one developed by King (1932), which is a combination of the Martland and Robison (1926) and the Fiske and Subbarow (1925) procedures. He retained the high acidity recommended by Martland and Robison (1926) and kept the acid and molybdate separated. At the same time, he used the aminonaphtosuphonic acid as a reducing agent as Fiske and Subbarow (1925) suggested, but this was made up in a slightly different way. In addition, he replaced sulfuric acid by perchloric acid, which is a much better oxidizing agent for the destruction of the organic material in total phosphorus determinations.

Other authors employed enzymatic reactions and determined the lecithin as choline instead of phosphorus. Thaxton and Bowie (1968) described a method using lecithinase D in an *in vitro* system for the

hydrolysis of plasma lecithin to choline. After this enzymatic reaction, the ether-soluble phosphatidic acid was removed and the choline was precipitated from the aqueous phase as the reineckate. This salt was soluble in acetone and was then measured colorimetrically. Artiss et al. (1979) published a procedure for the direct determination of the micromolar quantities in lecithin employing enzymes as reagents. They used phospholipase D to generate phosphatidic acid and choline, which was subsequently oxidized by choline oxidase, with the formation of hydrogen peroxide. Then, a red dye was produced by the peroxidase-catalyzed coupling of sodium 2-hydroxy-3,5-dichlorobenzenesulfonate to 4-aminoantipyrine with hydrogen peroxide. This method is relatively quick, simple, and inexpensive, and involves no extractions.

34.3.2.2 Atomic Absorption Spectroscopy

Atomic absorption spectrometry (AAS) is an alternative to wet chemical methods because it is a rapid, accurate, and standardized method for the determination of phosphorus, which is very important for quality control in the industry. Flame mode has low sensitivity, and so a direct measurement of traces of phosphorus is unfeasible in this mode (Marmer 1985). In spite of this, Hoft et al. (1979) applied this mode in fertilizers that contained high amounts of phosphorus, obtaining excellent precision and accuracy.

In 1977, Prevot and Gente-Jaumiaux (1977) published a rapid method for the determination of phosphorus in oils and fats by flameless atomic absorption, which consisted of using a programmed drying temperature (20–300°C; 30 s), mineralization temperature (300–1300°C; 2 min), and graphite furnace atomization at 2800°C. He combined this with an electrodeless discharge lamp and obtained sensitivity in the 0.5–1 ppm range. The sensitivity of this method was increased in a study made by the same authors 2 years later (Gente-Jauniaux and Prevot 1979). In this study, the determination of phosphorus in vegetable oils by flameless AAS after dilution to 50% in methylisobutyl ketone was improved by the addition of lanthanum as lanthanum cyclohexanebutyrate.

Vigler et al. (1978) investigated the analysis of phosphorus in organic compounds, finding it necessary to change the organic phosphorus to inorganic phosphorus before measuring it by AAS. The organic phosphate was converted to $Mg_2P_2O_7$ and subsequently dissolved in nitric acid, diluted to desire concentration, and then injected into the heated graphite furnace in the presence of a lanthanum nitrate solution. In 1981, this method was applied to the determination of phosphorus in orchard and tomato leaves, and in bovine and oyster tissue (Langmyhr and Dahl 1981).

The results of a collaborative study of a standardized method for the determination of phosphorus in crude and refined oils and fats by direct graphite furnace AAS were published by Hendrikse and Dieffenbacher (1991). The method studied in this collaborative study was based on an earlier work (Slikkerveer et al. 1980). They found comparable results using an electrodeless discharge lamp and a hollow cathode lamp, although in the latter case the precision was not so good and the mode of atomization had no influence on the results. Due to the repeatability and reproducibility of the results obtained in this study, the Commission on Oils, Fats, and Derivatives decided to adopt the method (IUPAC 1991).

34.3.3 Chromatographic Methods

In the previous sections, we have described the analytical methods to determine the group of lecithin without differentiating between families of compounds or molecular species. These methods are based on the similar physicochemical properties of phospholipids such as precipitation in determinate solvents, colorimetric reactions, or atomic absorption of the common element, phosphorus, to all these compounds.

To be able to characterize and evaluate independently each of the different compounds that belong to lecithin, it is necessary to take into account the elevated number of compounds that constitute this family and the complexity of the matrix where these phosphatides use to be, formed also by other substances with a different nature. To approach this matter with success, the analytical chemist has had valuable tools for some time, namely chromatographic techniques, which are essentially separation techniques, adequate to achieve the separation of compounds in a complex matrix such as foods and lipids that

contain them. Subsequently, these techniques have developed turning into analytical techniques for quantification, using certain physicochemical properties of the substances that would be quantitatively proportional to their concentration.

Since the use of the first chromatographic techniques in column or thin layer until the development of the high-performance gas or liquid chromatography, the chromatographic methods of analysis of phospholipids have undergone an important evolution, being able to dissolve and quantify their different molecular species with great accuracy and precision. These techniques are numerous and varied as explained later in this chapter. However, the problems that analytical chemists need to solve when they want to select or carry out a certain method to approach the studies of their samples are always the same: the different solubility of this kind of compound, which depends on the type of lecithin, in the different mobile and stationary phases that can be used, and the trend of these compounds to form micelles and emulsions.

In this sense, the old principle *Corpora non agunt nisi soluta* (the substances that are not dissolved do not react) must always be applied to select the technique. Hence, "a dissolved problem is a resolved problem."

Consequently, we describe the chromatographic techniques more frequently used, grouped also by the type of mobile phase, stationary phase, or used detection method.

34.3.3.1 Thin-Layer Chromatographic Methods

In 1951, the thin-layer chromatography (TLC) technique using adsorbent coated on glass strips was used for the first time (Kirchner et al. 1951). From this moment, the technique has been widely used for the separation of phospholipids as a separation technique previous to the quantification of these by another procedure or even for their direct quantification by densitometry or thincography (Szuhaj 2003). In any case, a better or worse separation of the different groups is obtained depending on the type of mobile phase used and whether the TLC is in one or two dimensions.

Several procedures for the analysis of phospholipids using one-dimensional TLC have been reported from a number of authors. In them, separations are achieved on plates of silica gel. However, different mobile phases and methods of detection or quantification are used.

All procedures are developed as follows: First, the plates are prepared with silica gel. A relatively thick layer of silica gel is recommended. The plates are immediately activated at high temperatures and, after this, stored in a desiccator or used immediately. The samples are applied in single spots with micropipettes and treated with a mixture of solvents that is allowed to ascend until it reaches a distance of 1–2 cm from the top of the plate. After chromatography, the plate is dried and sprayed to detect any spots. Figure 34.3 illustrates an example of one-dimensional TLC of phospholipids, in which the spots correspond to the different groups of phospholipids.

Finally, a method of quantification can be used for phosphorus determination (Robinson and Phillips 1963, Seminario de Bohner et al. 1965) or densitometry (Vaysse et al. 1985, Masella and Cantafora 1988). Table 34.1 shows a summary of the different mobile phases and sprays used in the separation of phospholipids by TLC.

In an attempt to improve this separation, the stationary phase has been modified by some authors adding silver nitrate (Arvidson 1965, Kennerly 1986), sodium acetate (Skipski et al. 1962), sodium carbonate (Skipski et al. 1962, Gentner et al. 1981), calcium sulfate (Kahovcová and Odavic, 1969), or phosphoric acid (Lendrath et al. 1990).

In many cases and when the samples are very complex with a high number of compounds, which can have similar values of R_F, it is necessary to improve the separation between the different classes of phospholipids. In this sense and with the objective of improving the resolution in the analysis using TLC, some authors have used two-dimensional TLC, which consists of developing the plate a second time with another type of solvent and perpendicular to the first development carried out (Lepage 1963). Figure 34.4 shows a general scheme of two-dimensional TLC.

Generally, the use of both mobile phases results in a change in polarity and pH which produces a better separation, especially of those nonpolar lipids extracted jointly with phospholipids (Broekhuyse 1968). In Table 34.2, a summary of the different mobile phases used in the separation of phospholipids by two-dimensional TLC is shown.

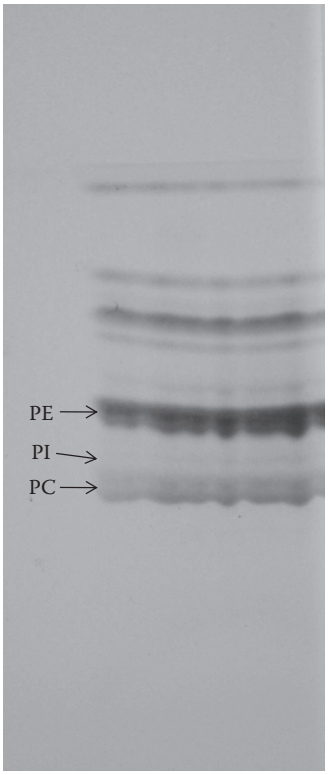

FIGURE 34.3 Separation of a commercial lecithin by thin-layer silica gel plate PE: phosphatidylethanolamine; PI: phosphatidylinositol; PC: phosphatidylcholine. mobile phase: chloroform/methanol/water (65:25:4 v/v/v), spray: 50% sulfuric acid in water.

TABLE 34.1

Different Mobile Phases and Sprays Used in Thin-Layer Chromatographic Separation of Phospholipids

Reference	Mobile Phase	Spray
Vogel et al. (1962)	$Cl_3CH/CH_3OH/H_2O$ 80:25:3	2′,7′-Dichlorofluorescein
Skipski et al. (1962)	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 65:25:8:4	Ninhydrin, rhodamine 6G or B, and ammonium molybdate
Robinson and Phillips (1963), Phillips and Robinson (1963)	$Cl_3CH/CH_3OH/H_2O$ 65:25:4	Ammonium molybdate
Arvidson (1965)	$Cl_3CH/C_2H_5OC_2H_5/CH_3COOH$ 97:2.3:0.5	Dibromo-*R*-fluorescein
Kahovcová and Odavic (1969)	$Cl_3CH/CH_3OH/H_2O$ 65:25:4	Sulfuric acid
Gentner et al. (1981)	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 25:15:4:2	Iodine and molybdophosphoric acid
Touchstone et al. (1984)	$CH_3CH_2OH/Cl_3CH/C_6H_{15}N/H_2O$ 35:30:30:8	Phosphoric acid
Juaneda and Rocquelin (1985)	$C_6H_{14}/C_2H_5OC_2H_5/CH_3OH/CH_3COOH$ 90:20:5:2	Iodine
Vaysse et al. (1985)	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 100:55:16:6	Iodine
Marsella and Cantafora (1988)	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 25:15:4:2	Molybdenum blue
Lendrath et al. (1990)	Cl_3CH/CH_3OH/acetate buffer 0.2 M 65:25:4.3(pH 4)	Ninhydrin, rhodamine 6G, and molybdenum blue
Nzai and Proctor (1998)	$Cl_3CH/CH_3OH/ H_2O$ 75:25:3	Ammonium molybdate and iodine

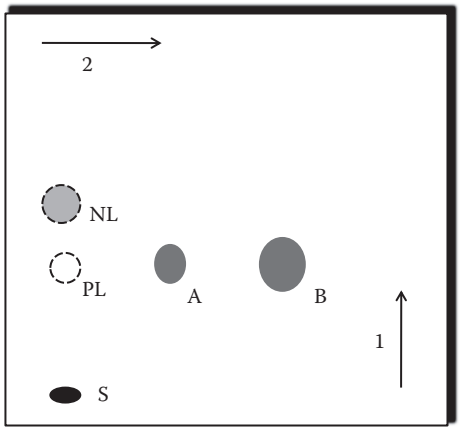

FIGURE 34.4 Scheme of a two-dimensional thin-layer chromatogram of lipids; S: initial sample; PL: polar lipids; NL: neutral lipids: A and B: different phospholipids; 1: first dimension; 2: second dimension.

34.3.3.2 High-Performance Liquid Chromatographic Methods

In the previous section, we have seen that chromatographic techniques are the most appropriate for approaching the study of lecithin composition and different classes of phospholipids, obviously obtaining more information than with the use of colorimetric or gravimetric methods. However, TLC, both one and two dimensional, has some drawbacks, such as a limited capacity to resolve the phosphatides in their different molecular species, techniques of detection more or less complex with significant errors in the quantification and a relatively long-time period required for the analysis. Therefore, the methods that use

TABLE 34.2

Different Mobile Phases Used in Two-Dimensional Thin-Layer Chromatographic Separation of Phospholipids

Reference	Phase 1	Phase 2
Lepage (1963)	$Cl_3CH/CH_3OH/H_2O$ 65:25:4	DIBK/CH_3COOH/H_2O 80:50:10
Broekhuyse (1968)	$Cl_3CH/CH_3OH/NH_3$ 7M 90:54:11	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 90:40:12:2
Getz et al. (1970)	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 52:20:7:3	$Cl_3CH/CH_3OH/CH_3NH_2/H_2O$ 13:7:1:1
Erdahl et al. (1973)	$Cl_3CH/CH_3OH/NH_3$ 7N 65/30:4	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 170:25:25:6
Goswami and Frey (1973)	IO/IP/EA/CH_3COOH 40:20:10:10	$Cl_3CH/CH_3OH/H_2O$ 65:35:4
Rodríguez de Turco and Bazán (1977)	$Cl_3CH/CH_3OH/NH_3$ 65:25:15	Cl_3CH/ACETONE/$CH_3OH/CH_3COOH/H_2O$ 6:8:2:2:1
Portoukalian et al. (1978)	THF/ACETONE/$CH_3OH/H_2O/NH_3$ 55:25:43:7:1	Cl_3CH/ACETONE/$CH_3OH/CH_3COOH/H_2O$ 50:20:10:10:4.5
Blass et al. (1980)	$Cl_2CH_2/CH_3CH_2OH/H_2O$ 100:25:3	$Cl_3CH/CH_3OH/NH_3$ 170:20:3
Chapman (1980)	$Cl_3CH/CH_3OH/NH_3$ 7N 65:30:4	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 170:25:25:4
AOCS (Ja 7–86)	$Cl_3CH/CH_3OH/NH_3$ 7N 130:60:8	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 170:25:25:6
Fine and Sprecher (1982)	$Cl_3CH/CH_3OH/NH_3$ 65:25:5	Cl_3CH/ACETONE/$CH_3OH/CH_3COOH/H_2O$ 3:4:1:1:0.5
Kraus et al. (1987)	$Cl_3CH/CH_3OH/CH_3COOH/H_2O$ 70:30:4:3	$Cl_3CH/CH_3OH/CH_3COOH/CH_3CH_2OH/H_2O$ 60:30:4:10:3

Note: EA: ethyl acetate; IO: isooctane; IP: isopropyl alcohol; THF: tetrahydrofurane.

TLC as a way of identifying phospholipids, despite their rapid detection, were soon relegated to simple techniques of previous separation, being replaced by the new emergent technique of high-performance liquid chromatography (HPLC), which evolved as this instrumentation has been developed and improved. The separation obtained with HPLC is similar to that obtained with TLC when working in the absorption mode (straight phase), and even superior when partition chromatography is used (reverse phase). In this case, the molecular species of each class of phospholipids can be perfectly resolved, employing a shorter time and a lower quantity of mobile phase reaching values of detection limits lower than in the case of TLC, since the systems of detection used are much more sensitive that will be discussed later.

In the literature study carried out on this issue, it can be observed that a large amount of mobile phases have been used to separate the different phospholipid classes, as in TLC; all of them quite successful in terms of resolution. Nevertheless, unfortunately not all phases have the same effectiveness for the different types of samples. On the other hand, it is deduced that the composition of the mobile phase can become critical for each type of matrix.

A detailed study of the different mobile and stationary phases more frequently used is described below.

As in the case of TLC, the separation of phospholipid classes using HPLC has been carried out employing the absorption chromatography in silica columns. However, depending on the technique of detection used, many TLC solvent systems for phospholipids are incompatible with some of the detectors used. This is the case of the use of the detection system of ultraviolet (UV) absorbance between 202 and 210 nm, in which chloroform and acetic acid cannot be used (Yandrasitz et al. 1981). The studies developed in this technique have been aimed at searching mobile phases to obtain a good separation that is compatible with the detection technique used.

One of the big problems of HPLC is that there is not a simple, universal detector with a wide range of linearity like in the case of the flame ionization detector in gas chromatography. This detector has been used in some cases in HPLC, though without satisfactory results (Moreau et al. 1990).

The most widely used detector is UV, which presents more sensibility, making possible the use of gradients of elution to improve the resolution. Different wavelengths have been proven, but the most frequently used are 205 and 208 nm (absorption of the bond ester).

In these detectors, one of the problems, as indicated above, is the absorption of some mobile phases at these wavelengths, and so there are solvents that cannot be used.

On the other hand, the absorption of phospholipids depends on the number of unsaturations in the fatty acids along with the possible isomerization and conjugation of theses double bonds. In Table 34.3, the different mobile phases most commonly used with this type of detector are shown.

An alternative to the UV detector is the evaporative light-scattering detector, which presents the advantage of simplicity and universality. In this detector, the eluent of the column is carried with a stream of N_2 through a nebulizer. Later, the spray is heated and the solvent is evaporated. Then, the diffusion of light by solute drops is measured, which is proportional to the concentration of the solute.

Although the response must not be influenced by the chemical nature of the analyte, it has been verified that such influences do actually exist.

TABLE 34.3

Different Mobile Phases Used in the Separation of Phospholipid Classes by HPLC with Si Column and UV-Detection

Reference	Programme	Mobile Phase
Yandrasitz et al. (1981)	Isocratic	Hexane–isopropanol–water–sulfuric acid
Patton et al. (1982)	Isocratic	Hexane–isopropanol–phosphate buffer–ethanol–acetic acid
Chen and Kou (1982)	Isocratic	Acetonitrile–methanol–phosphoric acid
Kaduce et al. (1983)	Isocratic	Acetonitrile–methanol–sulfuric acid
Demandre et al. (1985)	Linear gradient	A: isopropanol–hexane; B: isopropanol–hexane–water
Kang and Row (2002)	Isocratic	Hexane–methanol–isopropanol
Kivini et al. (2004)	Linear gradient	A: *n*–hexane; B: isopropanol–chloroform; C: isopropanol–water
Wang et al. (2009)	Linear gradient	A: *n*–hexane–isopropanol; B: *n*–hexane–isopropanol–ammonium acetate; C: *n*–hexane–isopropanol–water

At the same time, the linearity range is approximately between 50 and 250 μg. Below 50 μg, the sensibility decreases rapidly (due to the fact that the particle size is of the order of magnitude of the wavelength); for this reason, in the analysis of a real sample, where minority components are presented, the response factors will change.

Therefore, to use this detector in quantitative analysis, the response factors of the phospholipids must be calculated.

It is important to keep in mind the gas flow, the boiling point, the extenuation, and the nature of the solvent used for the response factor. Otherwise, this detector presents very good resolution, does not show solvent peaks, and is not affected by solvent changes.

In recent years, the use of this detection technique has been promoted in the analysis of phospholipid classes, obtaining very good separation with programmed linear gradients using different mobile phases (Descalzo et al. 2003, Kivini et al. 2004, Avalli and Contarini 2005, Boselli et al. 2008). Figure 34.5 shows a classic separation of phospholipid classes using silica column and evaporative light scattering detector (ELSD).

Other stationary phases have been used for the separation of phospholipid classes, such as amino columns, using a mixture of acetonitrile–methanol–water-acetic acid–ammonia (630:350:15:0.3:1.3, v/v/v/v/v) in an isocratic mode and a UV detection at 205 nm (Zamora and Hidalgo 2003). In Figure 34.6, a chromatogram of a mixture of phospholipid standards obtained with this technique is shown, where an acceptable separation is observed. However, this phase has been scarcely used due to its need to be periodically regenerated with ammonia solutions. Although good separations are obtained for phosphatides of vegetal origin, this is not so for those of animal origin, in which some classes are not well resolved, such as sphyngomyelins and cardiolipins. In spite of this, an attempt to use this stationary phase has been carried out using the fluorescence of phospholipids achieved by the postcolumn formation of mixed micelles with diphenyl–hexatriene as the detection system (Bernhard et al. 1994).

In previous sections, the separation of different classes of phospholipids using a straight phase has been described. The use of HPLC in reverse phase allows for the separation of the different molecular species of each phospholipid class. Normally, the different phospholipid classes that are separated by some of the methods described above are collected.

Depending on the resolution desired and the mobile phase used, one or two columns in a series (Brouwers et al. 1998) can be used. The most commonly used mobile phase in reverse-phase HPLC using an RP-18 column is acetonitrile–methanol–water (Patton et al. 1982, Demandre et al. 1985, Abidi and Mounts 1997), which has been modified to obtain a better resolution with additives such as tetraalkylammonium

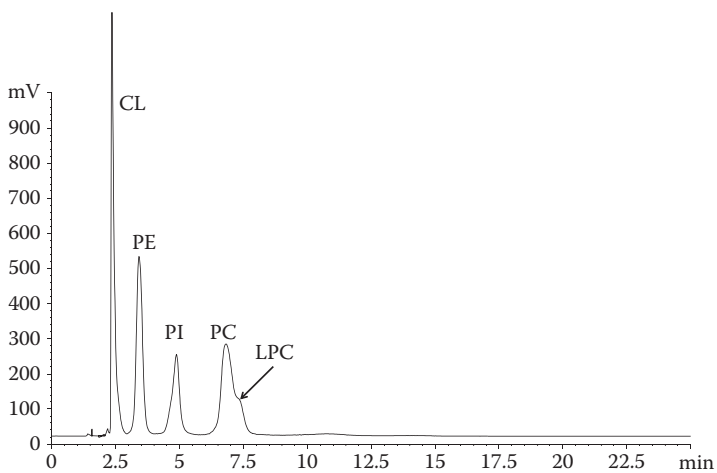

FIGURE 34.5 High-performance liquid chromatogram of a mixture of phospholipid standards. CL: cardiolipin; PE: phosphatidylethanolamine; PI: phosphatidylinositol; PC: phosphatidylcholine; LPC: lysophosphatidylcholine. Column, LiChrospher Si 60 5 μm 4.6 × 250 mm; mobile phase: chloroform/methanol/ammonia/water (300:150:9:10, v/v/v/v); Flow rate, 1 mL/min; detection with evaporative light-scattering detector, gas flow: 1.5 mL/min; temperature: 70°C.

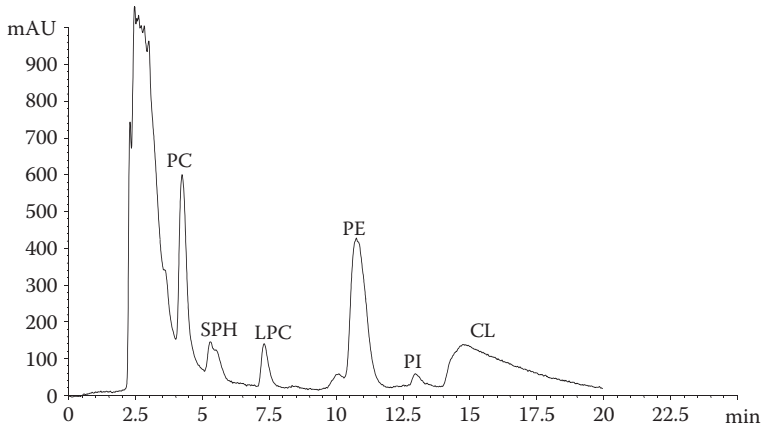

FIGURE 34.6 High-performance liquid chromatogram of a mixture of phospholipid standards. CL: cardiolipin; PE: phosphatidylethanolamine; SPH: sphingomyelin; PI: phosphatidylinositol; PC: phosphatidylcholine; LPC: lysophosphatidylcholine. Column, LiChrospher 100-NH_2 5 μm 4.6 × 250 mm; mobile phase: acetonitrile/methanol/water/acetic acid/ammonia (630:350:15:0.3:1.3 v/v/v/v/v); flow rate, 1 mL/min; detection, UV (205 nm).

phosphates (Abidi and Mounts 1992, 1996, Abidi et al. 1993) or triethylamine (Brouwers et al. 1998). Using high concentrations of tetraalkylammonium phosphates in this mobile phase facilitates elution of components, improves peak symmetry, and increases the efficiency of the column (Abidi and Mounts 1992). Similarly, increasing the percentage of triethylamine decreases the retention of the species on the stationary phase and improves the resolution (Brouwers et al. 1998).

The methods developed to analyze molecular species of phospholipids using RP-18 column have been proven effective and useful, especially for samples with low concentrations of these substances. The most widely used detectors with reverse-phase HPLC technique are, as in the separation of phospholipid classes, UV detector and ELSD, which are currently more frequently used due to an improvement in technology. Only in the case of the use of electrolytes in the mobile phase is this detector unsuitable and incompatible.

The molecular species elution in RP-18 reverse-phase columns using mobile phases of acetronitrile–methanol–water with nitrogenous bases (tetraalkylammonium phosphates or triethylamine) takes place so that the retention time tends to be inversely proportional to unsaturation in the total number of phospholipid (Abidi and Mounts 1992, 1996, Abidi et al. 1993). An example of lipid separation of molecular species from soybean phosphatidylcholine is shown in Figure 34.7.

Other attempts at separating the different species of phospholipids have been carried out by some authors (Abidi and Mounts 1992, Lin et al. 1998; 2000) using reverse phase with a shorter-alkyl-chain-bonded silica column (C-8) instead of larger-alkyl-bonded silica (C-18). Nevertheless, although the separation of molecular species is generally similar in both stationary phases, the degree of resolution of the different components is better when a C-18 column is used (Abidi and Mounts 1992).

34.3.3.3 Gas–Liquid Chromatographic Methods

Gas–liquid chromatography (GLC) is the principal technique in lipid analysis. The analysis of the majority of lipids can be made through the use of this technique. However, GLC has no relevance in phospholipid analysis because phospholipids are not volatile compounds. Despite this fact, GLC has been used, albeit indirectly, in the phospholipid analysis.

The main application of GLC in phospholipid analysis was the determination of their fatty acid composition. The methods that employ this technique for the analysis of different molecular species are based on the use of HPLC silica column (straight phase) as a previous separation technique of the different phospholipids classes, recovered by a fraction collector, preparation of fatty acid methyl esters, and GLC analysis by a high-polarity column (Kivini et al. 2004). Before HPLC can be carried out, prior separation of the phospholipid fraction is made by liquid–liquid extraction (Patton et al. 1982, Jungalwala

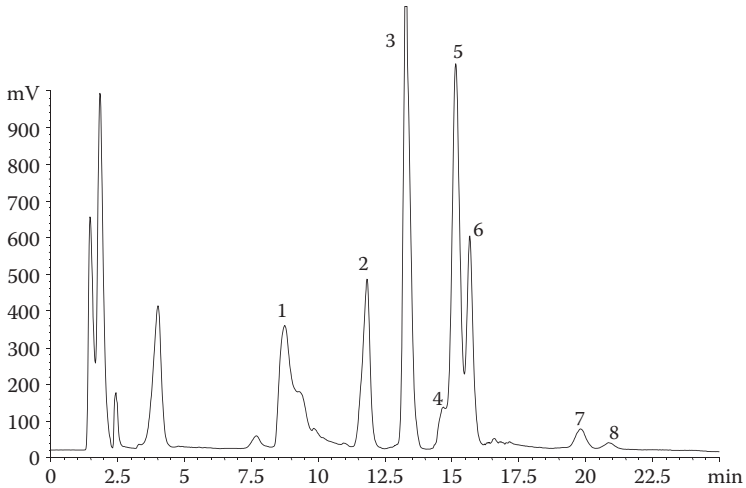

FIGURE 34.7 HPLC separation of molecular species of soybean PC with the stationary phase of two Lichrosphere 100 RP-18 columns in series. Mobile phase consisting of acetonitrile–methanol–water–triethylamine (69:28:2:1 v/v/v/v). Elution was performed at a flow rate of 1 mL/min. Detection with evaporative light scattering. 1: 18:3–18:3; 2: 18:2–18:3; 3: 18:2–18:2; 4: 16:0–18:3; 5: 18:1–18:2; 6: 16:0–18:2; 7: 18:0–18:2; 8: 18:0–18:1.

et al. 1984, Kivini et al. 2004), by column chromatography (Demandre et al. 1985, Eder et al. 1992), or by solid-phase extraction (Kaluzny et al. 1985).

However, the GLC analysis of 1,2-diacylglycerols is a better technique and provides more information from the different phospholipid classes once they have been separated by HPLC in the silica column. Once phospholipid classes have been separated, the 1,2-diacylglycerols are determined as trimethylsilyl ethers using a low-polarity column type SE-30 (Horning et al. 1969) or more recently in polarizable capillary columns (65% phenyl–35% methyl polysiloxane) (Olsson and Kaufmann 1992). Also, the analysis can be performed in a nonpolar methylsilicone column (Tserng and Griffin 2003), although its length has to be lower than in the case of polarizable columns.

Obtaining 1,2-diacylglycerols can be carried out by means of different procedures. One procedure is heating the phospholipids dissolved in diphenyl ether at 250°C for about 4–5 min (Horning et al. 1969). This method has not been widely used, because the reaction time should fit well to prevent the decomposition of the phospholipids. Enzymatic hydrolysis with phospholipase C (Olsson and Kaufmann 1992) is still the most common procedure. This method has been well studied and all variables have been optimized. Other enzymes have been used such as sphingomyelinase to analyze the sphingomyelins (Tserng and Griffin 2003).

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35

Sterols

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35.1 Introduction

Sterols are a group of C-27–C-29 secondary alcohols of plant or animal origin which differed from common alcohols in being crystalline solids at room temperature [1]. Cholesterol is the unique sterol of vertebrates and ergosterol the principal sterol of most fungi; however, a mixture of various sterols is present in higher plants, with sitosterol usually predominating [2,3].

Plant sterols are synthesized from the mevalonate pathway similar to cholesterol biosynthesis [4]. The major plant sterol end products are sitosterol (stigmasta-5-en-3β-ol), stigmasterol [(24E)-stigmasta-5,22-dien-3β-ol], and campesterol (campest-5-en-3β-ol) [5]. It has been reported that their isoprene units are supplied exclusively from the mevalonate pathway which is located in the cytoplasm [6].

Sterols can be grouped in two ways: first according to their substituted moiety at the C-3 position, and second according to the number of methyl groups at the C-4 position [7]. Following the first classification, there are four groups: free sterols which has a 3β-hydroxyl group; steryl ester in which the 3β-hydroxyl group is esterified to a fatty acid or a hydroxycinnamic acid; steryl glycoside which contains a carbohydrate moiety (usually glucose) at C-3; and the acylated steryl glycosides, which are linked to a fatty acid via ester linkage at the C-6 position of the sugar moiety [7,8]. In the steryl phenolic acid esters, the sterol hydroxyl group is esterified with a phenolic acid, most often ferulic acid [9]. However, according to the number of methyl groups at the C-4 position (Figure 35.1), sterols are divided into three groups: the 4,4-dimethylsterols (triterpene alcohols), the 4-monomethylsterols, and the 4-demethylsterols [7].

Plant sterols are also classified according to double bond positions in the ring skeleton into two groups: Δ5-sterols and Δ-7-sterols. Most plants contain predominantly Δ5-sterols with only trace amounts of Δ7-sterols. The predominance of Δ7-sterols appears to be restricted to only a few plant families, for example, Cucurbitaceae and Theaceae [10].

The structure of sterols and their biosynthetic pathway differ significantly among fungi, animals, and plants [2,3,5]. In higher plants, the conversion of cycloartenol into functional phytosterols involves the removal of the two methyl groups at C-4. Although the first C-4-methyl group is removed early from a 4,4-dimethyl-9β,19-cyclopropylsterol precursor, the second C-4-methyl group is eliminated by numerous steps later. In contrast, in animals and fungi, both methyl groups are removed successively early in

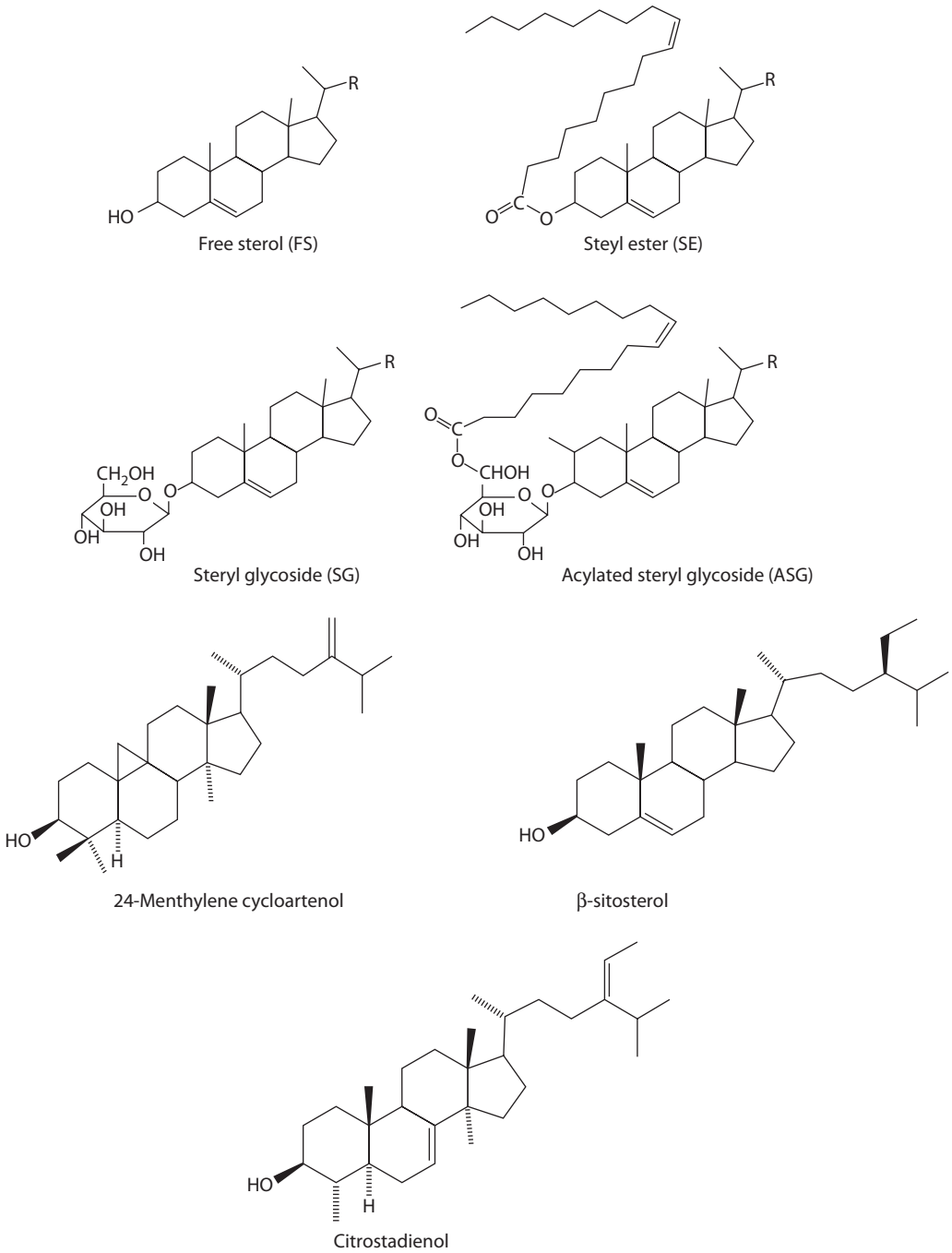

FIGURE 35.1 Examples of plant sterol structures: free sterol, steryl ester, steryl glycoside, acylated steryl glycoside, 24-menthylene cycloartenol, β-sistosterol, and citrostadienol.

the biosynthetic pathway [11]. The sterol molecule becomes functional as a structural membrane component only after the removal of the two methyl groups at C-4.

Both cholesterol and phytosterols contain an unsaturated ring structure and are thus susceptible to undergo oxidation in the presence of oxygen. The main oxidation products are hydroxyl, ketone, epoxyl, and triol derivatives, all of which are polar products [12]. Oxidation is accelerated by heating, exposure to

ionizing radiation, light, chemical catalysts, or enzymatic processes [13–17]. The oxidative stability of different phytosterol compounds during pan-frying was evaluated [18]. Rapeseed oil, rapeseed oil-based liquid margarine, and butter oil were used as lipid matrices. Gas chromatographic–mass spectrometric analyses indicated that pan-frying may induce phytosterol oxidation but has no marked effect on phytostanol oxidation; up to 5.1% of original sitosterol and 0.1% of original sitostanol were found as oxides.

Plant sterols were found to be minor constituents of vegetable oils. They are also characteristic of the genuineness of vegetable oils [19]. Recently, interest in those compounds has greatly increased and much progress has been made in the different steps of phytosterol determination. The qualitative and quantitative characterization of 4,4-dimethylsterols and 4-monomethylsterols seems to be useful for many aspects of vegetable oil production, including detection of adulteration, quality control, and product development. Phytosterol composition has been used to characterize vegetable oils and to detect admixture of vegetable oils [20–22]. The 4,4-dimethylsterol fractions have been used as markers to detect virgin olive oil adulteration with hazelnut oil [21].

Plant sterols are bioactive compounds that have positive effects on human health. They are used in the pharmaceutical industry as reagent for the synthesis of steroids, pesticides, and emulsifiers and are expected to add value in functional foods [23]. Clinical studies have shown that the dietary intake of phytosterols may decrease the blood cholesterol levels inhibiting its absorption from the small intestine [25,26]. Moreover, it has been suggested [26] that sterols show anti-inflammatory, antibacterial, antifugal, antiulcerative, and antioxidant activities. They have been recognized as cancer-preventive biologically active substances together with other secondary plant product [27]. Steryl ferulates are effective antioxidants by virtue of the phenolic hydroxyl group of ferulic acid [28,29], and steryl glycosides have been suggested to have effects on benign prostate hyperplasia and various other biological functions [30]. Plant sterols are less efficiently absorbed than cholesterol (2–5% and 60%, respectively) [31,32], while phytostanols are more poorly absorbed than phytosterols.

Diminishing of plasmatic cholesterol levels is vital for the prevention of cardiovascular diseases, which are the main cause of death in Europe [33]. Therefore, the development of food technology has created some foods enriched with phytosterols. At present, several functional food product types such as yoghurts and milk with added plant sterols and stanols are available on the market [8]. The currently available food products enriched with plant sterols and stanols use sterols derived from the deodorized distillate by-product of vegetable oil refining [34]. The major sources of plant sterols used for incorporation into commercial products are tall oil, which contains up to 80% β-sitosterol, and the by-products of soybean oil production [35]. Phytosterols exert their effects either as enriched food ingredients in functional foods [36,37] or as natural components of regular diet [38].

35.2 Contents of Sterol in Foods

In foods, phytosterols occur as free sterols, fatty acyl esters, glycosides, and fatty acyl glycosides, with substitutions occurring at C3. Therefore, the total sterol content is defined by the sum of all of them, whereas cholesterol occurs either as free alcoholic sterol or as cholesteryl esters [39,40].

Reported sterol data for some foods of animal origin have shown that cholesterol is the major sterol [41–45]. Cholesterol contents in meat, fish, milk, eggs, and their products consumed in Finland have been reported [46]. The mean cholesterol amount of eggs was 366 mg/100 g. In meat and sausages, the cholesterol amounts ranged from 45 to 84 mg/100 g and from 36 to 75 mg/100 g, respectively. In most fish species, the cholesterol contents were slightly higher ranging from 49 to 92 mg/100 g and they did not correlate with the fat contents. The contents in liquid milk products correlated with their fat contents ranging from 6.2 (milk with 1.5% fat) to 77 mg/100 g (cream with 38% fat). The corresponding range for cheese was 33–82 mg/100 g. The dietary cholesterol intake should be <300 mg/day [47].

Phytosterol compounds exist in all food items of plant origin. 4-Demethylsterols such as sitosterol, campesterol, and stigmasterol are the most abundant sterols in plants; however, 4-monomethyl and 4,4-dimethyl sterols are usually present in much lower amounts [48–50]. Sterol content and composition of vegetable oils can vary due to tissue type [51–55], or the ripening stage of the seed [50,56]. The genetic variability, parts, and harvest seasons could significantly affect the sterol composition in the bamboo shoots [57].

Phytosterols were found to be major component of the unsaponifiable fraction. There are various studies reporting the phytosterol concentration in plant foods and vegetable oils [9,58–61] (Table 35.2). Phytosterols occur mainly as free or esterified sterols in oils and fats, the forms accumulated in the membranes and in oil droplets, respectively [62]. Compared to free sterols and steryl fatty acid esters, steryl glycosides and steryl ferulates are far less studied, since in oil seeds and most vegetables they are either absent or present in very low amounts [9]. However, each form may contribute about 10–15% of all sterols in cereals.

The analysis of the free and esterified sterol contents including individual values for sitosterol, campesterol, stigmasterol, brassicasterol, and Δ5-avenasterols was performed for several vegetable oils [63]. A large variation in the content and distribution of the phytosterol fraction between different vegetable oils was observed. 4-Desmethylsterol was the predominant class, with sitosterol usually contributing more than 50% of the total phytosterol content. The highest phytosterol content was detected in corn and rapeseed oils, with a total phytosterol content ranging between 770 and 920 mg/100 g. Corn and rapeseed oils were very rich in steryl esters (56–60% of total sterol content); however, the majority of other vegetable oils (soybean, sunflower, palm oil, etc.) contained a much lower esterified sterol content (25–40%).

The plant sterol contents of 14 vegetable and nine industrial fats and oils available currently on the Finnish market were determined [64]. This study reported that phytosterol contents ranged from 69 mg/100 g in a frying fat to 4240 mg/100 g in wheat germ oil. Organic rapeseed oil, the second best source of plant sterols, contained 887 mg/100 g.

Cereals are among the most important natural sources of plant sterols in human diets, their contents, expressed on a fresh weight basis, being higher than in vegetables [8]. The total sterol contents of rye (95.5 mg/100 g), wheat (69.0), barley (76.1), and oat (44.7) have been reported in Finland [48]. The median total amount of plant sterols in some cereal foods commonly consumed in Sweden and in the Netherlands was 49 mg/100 g (range 4.1–344) edible portion. β-Sitosterol is generally the dominant form (62%), followed by campesterol (21%) and, in smaller concentrations, stigmasterol (4%) [65]. Jiang and Wang [52] studied various milling by-products as sources of plant sterols and showed that rice and wheat bran, wheat germ, and corn fiber are rich in plant sterols, and could be used as sources for extraction of these valuable beneficial compounds, if they were not included in the final product. Large differences in the sterol content and composition of different rye and wheat milling fractions were demonstrated [9]. Phytosterol composition of different fractions of adlay (*Coxi lachrymal-jobi*) collected from Laos, Thailand, Vietnam, and Taiwan was determined [53]. Adlay bran had a higher content of phytosterols (4733 mg/kg) than had hull (1114 mg/kg) or polished adlay (1154 mg/kg). In all adlay samples investigated, β-sitosterol was the main plant sterol, ranging from 43.2% to 55.3% of the total sterol contents, followed by ergostanol accounted for 16.7–30.3% and campesterol, for 7.38–12.4% of total sterols. Total phytosterol levels of 500 and 900 mg/kg were determined, respectively, in sorghum and corn grains [66]. Free and esterified sterol contents of 52.7 and 52.8 mg/100 g, respectively, have been reported in spelt (*Triticum spelta*) and winter wheat (*T. aestivum*), which also contain 123.8 and 112.6 mg/100 g, respectively, of glycosylated sterols [67].

Phytosterols have been quantified in vegetables and fruits commonly consumed in China [59]. The median total amount of plant sterols in the vegetables was 13.4 mg/100 g edible portion (e.p.) ranging 1.1–53.7 mg/100 g (e.p). The higher concentration of above 30 mg/100 g (e.p) was determined in pea (53.7 mg/100 g e.p.), cauliflower (42.8 mg/100 g e.p.), broccoli (40.9 mg/100 g e.p.), and romaine lettuce (30.9 mg/100 g e.p.). Some vegetables such as potato, sweet pepper, eggplant, and wax gourd had very low plant sterols, which was less than 5 mg/100 g (e.p). The total phytosterol contents in fruits ranged between 1.6 and 32.6 mg/100 g e.p., and the highest level was found in navel orange. Tangerine, mango, orange, and hawthorn also had total plant sterols higher than 20 mg/100 g e.p. The lowest plant sterol contents were found in melon, such as Fengtian melon (3.2 mg/100 g e.p.), small watermelon (2.1 mg/100 g e.p.), and Jingxin watermelon (1.6 mg/100 g e.p.). β-sitosterol was found to be the predominant sterols, especially in cauliflower, which constituted more than 80% of total plant sterols.

Phytosterols have been quantified in 12 nuts and seeds commonly consumed in Sweden and the Netherlands; the median value was 144 mg/100 g, with minimum 68 mg/100 g in coconut rasps and maximum 404 mg/100 g in sesame seeds [61]. β-sitosterol (19 mg/100 g), campesterol (19 mg/100 g), and Δ5-avenasterol (17 mg/100 g) were found to be the predominant forms. In walnuts, a campesterol content of 6 mg/100 g and trace levels of stigmasterol have been reported [70]. In general, β-sitosterol

TABLE 35.1

Phytosterol Concentrations in Some Foods

Food	Phytosterols (mg/100 g Edible Portion)
Corn oil	952
Sunflower oil	725
Safflower oil	444
Soybean oil	221
Olive oil	176
Almonds	143
Beans	76
Corn	70
Wheat	69
Palm oil	49
Lettuce	38
Banana	16
Tomato	7

Source: Adapted from Athyros, V.G., et al. 2011. *Nutr. Metab. Cardiovasc. Dis.,* 21(3), 213–221.

was the most abundant sterol in nuts and the total sterol contents ranging from 99.12 to 207.17 mg/100 g oil. There were only small differences between the campesterol and stigmasterol contents [69]. Phytosterols have also been quantified in nuts and seeds commonly consumed in the United States; the highest and lowest phytosterol contents corresponding to sesame seed and wheat germ (400–413 mg/100 g), and the lowest to Brazil nuts (95 mg/100 g). In walnuts, phytosterol represents 0.1–0.2% of the total lipid fraction; about 87% of the total phytosterol is represented by sitosterol. Macadamia nuts contain about 1.3 mg of phytosterol per gram of lipids [70]. Individual sterol components of *Pinus pinea* L. oil extracted from seven Mediterranean populations were identified and quantified [60]. *P. pinea* oil unsaponifiable matter contained very high levels of phytosterols (>4298 mg kg^{-1} of total extracted lipids), of which β-sitosterol was the most abundant (74%).

Commercial margarines formulated with certain levels of phytosterols are currently available in several countries [71]. Thus, margarines constitute an important natural source of plant sterols in human diets [59,72–75]. The median total content of phytosterols for the 42 fat spreads and margarines, commonly consumed in Sweden and the Netherlands, was 204 mg/100 g with minimum 73 mg/100 g in a low-fat margarine and maximum 775 mg/100 g in a polyunsaturated fat spread [61].

Several studies on phytosterol oxides' content and their formation in different types of foods have been performed. The highest phytosterol oxide content was measured in saturated butter oil enriched with free phytosterols. Oxidation products of stigmasterol were characterized during thermooxidation at 180°C for different time periods by using solid-phase extraction (SPE) and high-performance size-exclusion chromatography methods [76]. For phytosterol concentrations in some foods see Table 35.1 [159].

35.3 Average Intakes of Sterols

The most important natural sources of plant sterols in human diets are oils and margarines, although they are also found in a range of seeds, legumes, vegetables, and unrefined vegetable oils [72]. Numerous data show that vegetable oils and cereals are the best natural sources of dietary phytosterols. In fact, due to the higher amount of consumed cereals, they may contribute about 40% of the daily intake of plant sterols [77]. The consumption of fruits and vegetables containing a relatively lower concentration of phytosterols also contributes substantially to the total sterol intake [59,78]. Fats and oils and derived

products are a major source of plant sterols in the Western diet. They contribute to 26%, 39%, and 50% of the total plant sterol intake in the Netherlands, Finland, and Spain, respectively [77–79].

Naturally occurring phytosterols even in the habitual dietary intake range of 150–450 mg/day are negatively correlated with cholesterol absorption [80]. The average dietary consumption of phytosterols and phytostanols is approximately 250 and 25 mg/day, respectively [81]. The estimated daily dietary intakes of plant sterols among different populations range from 160 to 400 mg [82]. The average intakes of phytosterols have been estimated between 140 and 360 mg/day in Finland [83] and 163 mg/day in the United Kingdom [84]. In the Netherlands, the mean intakes (mg/day) of cholesterol (202), campesterol (27), stigmasterol (15), and β-sitosterol (102) have been estimated [85]. On the other hand, the Netherlands Cohort Study on Diet and Cancer reported a mean intake of 359 mg phytosterols/day, of which 64% corresponded to β-sitosterol, 18% to campesterol, and 9% to stigmasterol [78].

35.4 Analysis of Sterols

In general, the analysis of individual sterols includes the extraction of lipids, saponification or acid hydrolysis and saponification to liberate sterols, extraction of unsaponifiable matter and separation/partial purification of sterols, the formation of sterol derivatives, and their analysis by chromatographic techniques [8,40,62,71,86].

Sample preparation depends on whether free or total sterols have to be analyzed. Protocols for preparation for both cases are described [21,55,63]. There are several approaches to deconjugate plant sterols in different plant food matrices including alkaline, acid, and enzymatic hydrolyses [12,48,64,75]. Since fats and oils contain free and esterified sterols only alkaline hydrolysis (i.e., saponification) is required and acid hydrolysis that is needed to hydrolyze glycosylated forms could be omitted [40].

35.4.1 Saponification

Saponification is an essential step in the sterol analysis since it allows the transformation of liposoluble triacylglycerols into water-soluble compounds, facilitating the posterior extraction of phytosterols and phytostanols by an apolar solvent that can then be determined as free compounds [40]. To free the esterified sterols and to remove the glycerol lipids, the total lipid extract is saponified. Methods for saponification either at room temperature (cold saponification) or with heating (hot saponification) have been largely described [8,21,33,50,52,63,87].

For saponification of the lipid extract or vegetable oils, an aliquot of the oil sample (5 g) is saponified with 50 mL of 12% ethanolic potassium hydroxide (w/v) and heating at 60°C for 1.30 h. After cooling, 50 mL of water was added and the unsaponifiable fraction was extracted four times with 50 mL of petroleum ether. The combined ether extract was washed with 50 mL of ethanol-distilled water EtOH-H_2O (1:1). The extracted ether was dried over anhydrous sodium sulfate Na_2SO_4 and evaporated to dryness using N_2. The dry residues were dissolved in chloroform for chromatographic analysis. In saponification at room temperature, an aliquot of lipid extract or oil sample is stirred overnight at room temperature with 1 M ethanolic potassium hydroxide [71,88]. The mixture is diluted with water and extracted with three portions of diethyl ether. The unsaponifiable matter is isolated preferably using the diethyl ether procedure that allows the total extraction of sterols [89]. The unsaponifiable lipids of lupin oil were determined gravimetrically after saponification with 20% (w/v) of methalonic KOH, kept overnight at room temperature [90].

In view of the fact that the acetal bound between the sterol and the carbohydrate moiety cannot be hydrolyzed in alkaline conditions, direct saponification methods fail to quantify steryl glycosides [91]. To obtain total free, esterified, and glycosidic sterols, acid hydrolysis has to be applied to the lipid extract. Acid hydrolysis is carried out with 0.5 M methanolic HCl in a Teflon screwcap test tube with a leak-proof cap at 75 ± 2°C for 22 h, with constant shaking at 145 rpm [92]. Acid hydrolysis (6 M HCl) and alkaline saponification (96% ethanolic KOH) have been used for the determination of phytosterols and phytostanols in cereal products [73].

A combination of acid hydrolysis and alkaline hydrolysis is superior to alkaline hydrolysis alone [40,75]. However, other data reported that chemical protocols using acidic hydrolysis of the glycosides

prior to gas chromatography (GC) analysis are not applicable due to the acid lability of Δ7-phytosterols [93]. Some study have revealed that Δ7-phytosterol already decompose or isomerize after a short time of acidic hydrolysis [10].

For milk and yoghurt samples, saponification temperatures and times that gave the best results were 80°C/45 min and 60°C/90 min, respectively [33]. Ethanolic solutions of KOH with concentrations of 2.0 and 2.5 M were selected as the best saponification reagents for milk and yoghurt, respectively. It was also verified that volumes of 1500 and 2500 μL were sufficient to saponify 250 μL of milk and 100 μL of yogurt. Hot saponification with ethanolic potassium hydroxide is adequate for most matrices having added phytosterols/phytostanols, such as spread, milk, and yoghurt [86].

35.4.2 Thin-Layer Chromatography, Column Chromatography, and SPE

Several methods have been used to isolate sterols: Thin-layer chromatography (TLC), column chromatography (CC), and SPE. Currently, Solid-phase microextraction is the widely used separation/preparation method for biological samples [49].

TLC is the conventional method to separate the sterol fractions. TLC on silica gel with suitable mobile phases has been used to fractionate unsaponifiable lipid extracts [21,50,56,90]. The unsaponifiable matter (4% in $CHCl_3$) containing 1% (w/w) each of 5-α-cholestanol and lanosterol as the internal standard for 4-desmethylsterols and dimethylsterols, respectively, was applied on the silica gel plates in 3 cm bands. To correctly identify the sterols bands, a reference sample of purified sterol (5-α-cholestanol and lanosterol) was applied on the left and the right sides of the TLC plates. The plate was developed twice in hexane/diethyl ether/ acetic acid (70:30:1) [21]. Various developing liquids can be used to isolate sterol fractions (4,4-dimethylsterols, 4-monomethylsterols, and 4-desmethylsterols) by TLC on silica gel [50,56,90,94–97].

After development, the plate was sprayed with 2′,7′-dichlorofluorescein and viewed under ultraviolet (UV) light or with 50% sulfuric acid in ethanol (or 80% potassium dichromate in sulfuric acid) followed by destructive carbonization [98]. On the basis of the reference spots, the sterols bands were identified. The bands corresponding to sterol fractions were scraped off separately and each fraction was extracted three times with $CHCl_3$–Et2O (1:1), filtered to remove the residual silica, dried in a rotary evaporator, and stored at –10°C for further analysis.

The CC method, such us TLC, is adequate for sample cleanup, purification, qualitative assays, and preliminary sterol estimation studies [98]. To isolate sterol fractions, the CC method has been widely described [94,99–103]. CC and TLC are accessible and affordable separation techniques [10,104,10], the choice depending on the amount of sample to be processed. The polarity difference between free and esterified sterols was exploited for their separation by the CC method. Oil sample (1.5 g) is loaded onto a silica gel column and elution started with 75 mL hexane/ethylacetate (90:10 v/v) to collect the steryl ester fraction, followed by elution with 75 mL hexane/diethyl ether/ ethanol (25:25:50 by vol) for collection of the free sterol fraction [63].

TLC and CC are the classic methods for sterol separation, but these techniques are very time consuming and, therefore, not suitable for routine analysis [8]. The TLC method for separation of sterol fractions has some drawbacks such as low recovery and time consuming [22,105,106].

SPE has been shown to separate total sterols in some vegetable oils more effectively and conveniently than traditional TLC separation [107]. In fact, it is a simple and inexpensive chromatographic method; it can be done in a short time and uses only small volume of solvent [98]. SPE is a rapid method for the separation of sterols, providing faster fractionation and using smaller volumes of solvent [8]. Therefore, SPE has been widely used to extract and purify total sterols from other unsaponifiable compounds [107,109]. Neutral alumina SPE cartridges have been used for the determination of both free and esterified sterols [109]. The SPE method was developed with stepwise elution by increasing the polarity of solvents mixture: *n*-hexane and diethyl ether [22] to separate sterol fractions of hazelnut and virgin olive oils. The dissolved unsaponifiables in hexane were loaded onto an SPE silica cartridge preconditioned with *n*-hexane. Elution with *n*-hexane–diethyl ether (99:1), (98:2), and (60:40) affords 4,4-dimethylsterols, 4-monomethylsterols, and 4-desmethylsterols, respectively. The recovery of spiked authentic sample of 4-desmethylsterols in oil was higher with the SPE method (94%) compared with the TLC method (62%).

The amount of 4,4-dimethylsterols and 4-desmethylsterols separated with SPE in both hazelnut and virgin olive oil samples were at least 75% and 35%, respectively, higher than that of TLC. Generally, both methods obtained similar results for 4-monomethylsterols of the two oils.

SPE has been used in reverse-phase (RP) mode (octadecylsilica) to isolate the sterol fraction from the unsaponifiable extract, and in normal-phase mode to separate trimethylsilyl (TMS) derivatives from a nonsaponified vegetable oil sample [108]. Silica SPE cartridges have been used to separate free sterols from steryl glycosides [10,110].

35.4.3 Preparation of TMS Ether Derivatives of Sterol Fractions

Most of the sterols and their metabolites are polar compounds containing one or more functional groups (–OH, >C = O, and –COOH) [111]. Prior to GC analysis, the free hydroxyl group of sterols was silylated to reduce their polarity and to increase their volatility and their thermal stability [23]. Derivatizing agents mostly used: *N*-methyl-*N*-trimethylsilyltrifluoroacetamide and bis(trimethylsilyl)-trifluoroacetamide [8,63,90,112–115]. Trimethylsilylation involves the introduction of a TMS group into a molecule; TMS derivatives of sterols are stable, giving informative fragmentation ions under positive electron ionization (EI). This allows identification and quantification of sterols in complex matrices at a low concentration, if sufficient separation from chemical noise is achieved [111].

35.4.4 Gas Chromatography

GC is most often employed for the separation and quantification of sterols. The analysis of sterols in edible oils and fats by chromatographic methods has been reviewed [8,98]. The conventional method for quantifying sterols and triterpenic alcohols involves capillary GC analysis with FID, of the fraction isolated as TMS derivatives [116–118]. Numerous publications described the sterol determination by GC with different detectors [119–122] and with mass spectrometry (MS) [54,55,123–125].

In several studies, sterols have been identified by coupling GC, and more recently high-performance liquid chromatography (HPLC), with MS [27,126–128]. Mass detection was selected due to its properties, namely specificity, in order to identify the different compounds based on their derivatized mass spectra. Identification of phytosterols was performed by comparing the retention time and mass spectra with those of the purchased phytosterol standards. Table 35.2 listed the values of *m–z* derivatives of the most common phytosterols found in food.

TABLE 35.2

Mass Spectrometric Data for Trimethylsilyl Derivatives of the Most Common Phytosterols Found in Food, Identified by GC-MS

Compounds	Formulas	Molecular and Prominent Fragments Ions (*m/z* values)
Obtusifoliol	$C_{33}H_{58}OSi$	498; 483; 393;109
β-amyrine	$C_{33}H_{58}OSi$	498; 483; 393; 218
Gramisterol	$C_{32}H_{56}OSi$	484; 469; 357
Cycloartenol	$C_{33}H_{58}OSi$	498; 408; 393; 365
24-Methylenecycloartenol	$C_{34}H_{61}OSi$	512; 497; 422; 383
Citrostadienol	$C_{33}H_{58}OSi$	498; 400; 357
Cholesterol	$C_{30}H_{40}OSi$	458; 329; 255; 129
Campesterol	$C_{31}H_{56}OSi$	472; 382; 343; 129
Campestanol	$C_{31}H_{58}OSi$	474; 305; 215
Stigmasterol	$C_{32}H_{56}OSi$	484; 355; 255; 129
β-sitosterol	$C_{32}H_{58}OSi$	486; 357; 255; 129
Stigmastanol	$C_{32}H_{60}OSi$	488; 305; 215

Separation and subsequent identification of phytosterols in cereals and cereal-based food products have always been performed by gas chromatography/mass spectrometry (GC/MS) as reported, for example, in the works of Piironen et al. and Dutta [48,72].

Analysis of phytosterols in vegetable oils can be carried out by capillary electrochromatographic (CEC) preferably with a C18 column in an aqueous mobile phase, whereas oryzanols (sterol ferulates) in rice bran oils can best be analyzed by CEC with a C30 column in a nonaqueous mobile phase [129]. Few data, dealing with the separation of these compounds by capillary zone electrophoresis or CEC, are also available [130,131]. Supercritical fluid chromatography (SFC) employs inert supercritical carbon dioxide as the mobile phase eluent, which is suitable for the analysis of phytosterols in vegetable oils [132].

The major disadvantages of GC, which are recommended by official methods, are the requirement of both thermally stable columns and chemical derivatization prior analyses [130]. Some methods show good separation and quantification of underivatized phytosterols [56,133]. The identification of triterpenes and sterols of pterogyne nitens was performed by high-resoluation GC using a method which does not require prederivatizations [133]. The GC-FID method used in this study employed two different columns: SPB-5 and SPB-50, which were used due to their low polarity, inertness toward organic compounds, and high-temperature limit. Such features make them ideal for the analysis of underivatized semivolatile plant extracts [133]. Although quantitative GC analysis of underivatized phytosterols appears to be accurate and reliable, some experts prefer derivatizing phytosterols to prevent dehydration and decomposition, which may result in peak tailing and poor resolution.

GC-MS using positive-ion EI and negative-ion chemical ionization has been shown applicable in characterization of intact sterol esters [134]. Ammonium adducts of sterol esters [M+ NH4]+ have been used successfully for structural elucidation of sterol esters by chromatography-chemical ionization MS [135].

Analysis of phytosterols by GC-MS in enriched milk and yoghurts that are used as functional foods was described [33]. A Hewlett–Packard (HP) chromatographic system (Soquimica, Lisbon, Portugal) controlled by an HP Vectra VL2 4/50 computer, an automatic HP6890 injector, and a GC HP5890 equipped with an HP-5MS column (30 m × 0.25 mm i.d. × 0.25 μm film thickness), connected to an HP5972 mass detector, were used.

Although HPLC provides good resolution for cholesterol oxides, this technique is not suitable for phytosterol oxides analysis because of excessive interference, modest sensitivity, and limited chromatographic resolution [136,137]. For this reason, separation and identification of phytosterol oxides in food matrix are always carried out by GC-FID or GC-MS [138–143]. For an example of GC-MS see Figure 35.2. The analysis of sterol oxides in foods and biological samples has been reviewed [144,145].

35.4.5 HPLC and Nuclear Magnetic Resonance Spectroscopy

HPLC with different detection systems has been widely used to determine and quantify major plant sterols in foods [10,146–148]. NP HPLC with an evaporative light-scattering detector has been used successfully to separate phytosterol fatty acyl esters, free phytosterols, and ferulate phytosterol esters from other lipid classes in corn fractions [51,149].

MS and tandem mass spectrometry (MS/MS) are powerful detection methods, which are suitable for GC and HPLC systems. These detectors are not only superior in terms of sensitivity but are also highly specific compared with flame ionization, UV, and RI detectors [150]. GC-MS has been widely accepted as a reliable analytical method for the determination of sterols in foods. However, during the past decade, HPLC-MS or HPLC-MS/MS has also come to be used conveniently because these methods do not always require deconjugation and derivatization steps before analysis [151,152].

The identification by LC-MS of some sterols in soybean oil [153] and in edible seaweed [154] has been reported. LC-MS with atmospheric pressure chemical ionization was used to identify and characterize sterol fraction in the olive oil sample [27] and in spelt and winter wheat fine bran [155].

Recently, Rocco and Fanali [156] reported the applicability of nano-LC for the quantification of phytosterols in extra-virgin olive oil. The UV detector was used for quantitation, while an ion-trap mass spectrometer was coupled with the nano-LC apparatus for mass determination in order to identify separated compounds. This method can be useful for both qualitative and quantitative determination of sterols after sample treatment with good sensitivity, precision, and in short analysis time. The separation of all

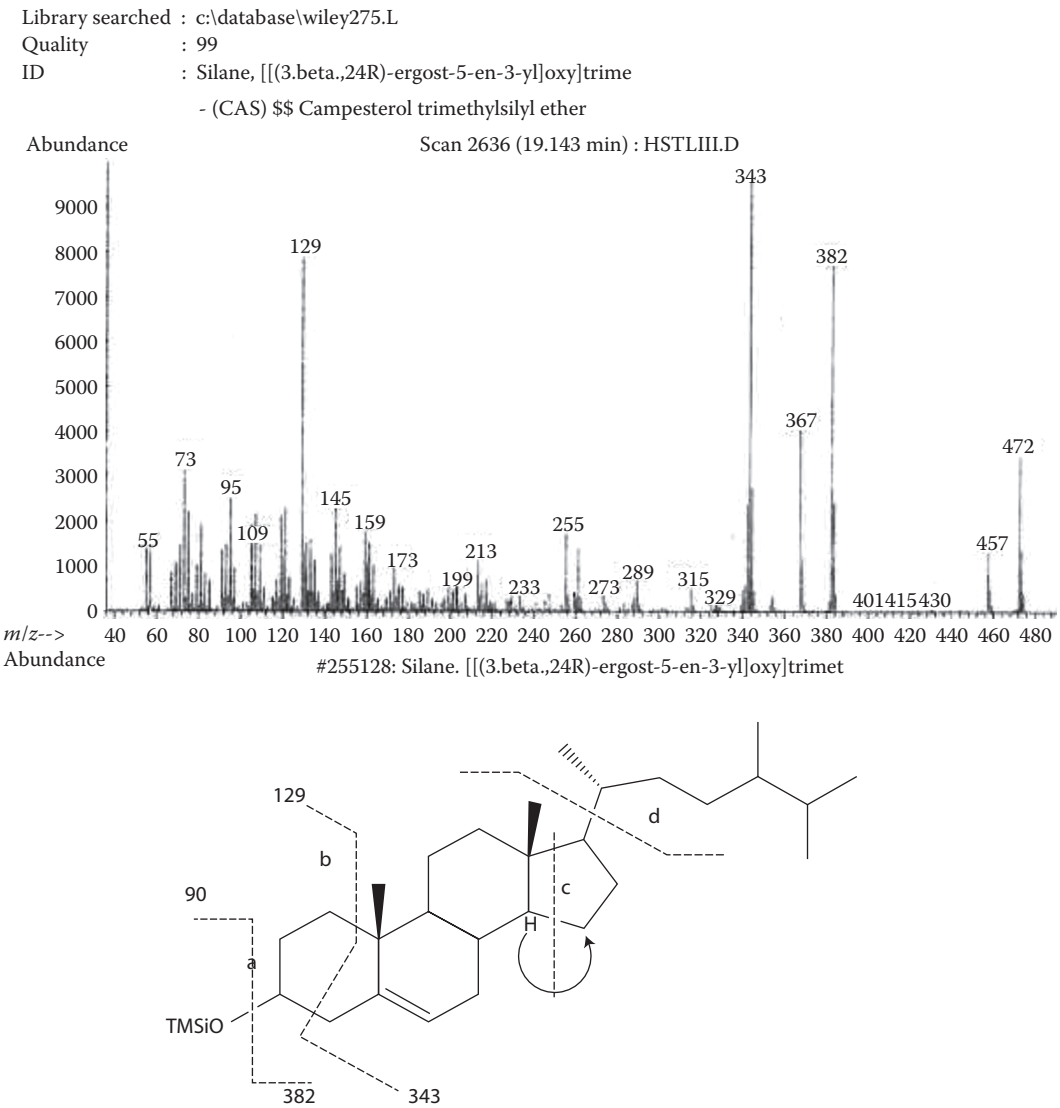

FIGURE 35.2 GC-MS mass spectra and possible fission processes of campesterol trimethylsilylether.

compounds was obtained in about 20 min, employing a capillary column packed with a C18-RP (sub-2 μm particles) stationary phase for 15 cm. Methanol only was used as the mobile phase.

The separation of free sterols at a high temperature reduces considerably their analysis times and also improves the separation factors of certain pairs of these sterols more difficult to separate. Increasing the column temperature accelerates markedly elution in HPLC. Thus, many types of stationary phases (graphitic carbon, polymeric C18 silica, and zirconia-based adsorbent) that can withstand high temperatures have been tested and compared for the separation of a group of sterols extracted from fruit juices [159]. Measurements of retention data were made at up to 150°C with mobile phases of different compositions. The graphitic carbon column studied provides the best separation factors. The polymeric, reversed-phase column studied gives particularly advantageous results when operated at high temperatures because the water concentration of the mobile phase can be increased, which slightly improves its selectivity, a result which has not yet been observed in reversed-phase chromatography. The zirconia-based column coated with a thin layer of carbon yielded good results, except for the separation of cholesterol and sitosterol. It

allowed the positive identification of the sterols extracted from fruit juices and their quantitation in <1 min. Although at the expense of a longer analysis time, the graphitic carbon column produced the best separation of the sterols in this study.

Nuclear magnetic resonance (NMR) spectroscopy applied for the determination of free and esterified sterols, without any previous separation, in 24 extra-virgin olive oil samples from various regions of Greece was reported [158]. Free sterols were determined by ^{31}P NMR upon derivatization of free sterols with a phosphorous reagent, whereas total (free + esterified) sterols were obtained from ^{1}H NMR. This method combines ^{1}H NMR for the determination of the total fraction of both free and esterified sterols and ^{31}P NMR for the quantification of free sterols. The NMR method shows three major advantages when compared with GC. First, no calibration with standards is needed prior to the analysis rendering the NMR method faster than GC; second, it gives well-resolved signals in 1H and 31P NMR spectra facilitating integration of the sterols signals and thereby furnishing results with higher precision and accuracy, and third the duration of the analysis is much shorter than GC; the NMR analysis for both esterified and free sterols lasts about 45 min, whereas that by using GC requires at least one day.

According to their sensitivity and precision, the currently available techniques for sterol analysis can be classified as follows: GC > HPLC > SFC [8]. The sensitivity order may vary depending on the sterol structures and detectors coupled to the chromatographic instruments. GC-FID (or MS, when peak identity confirmation is needed) can be considered the method of choice for the determination of phytosterols in foods and diets.

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36

Stanols

Harrabi Saoussem

CONTENTS

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36.1 Introduction

Phytostanols are a completely saturated subgroup of phytosterols and lack the carbon–carbon double bonds found in cholesterol and phytosterols [1–5] (Figure 36.1). Saturated plant sterol derivatives (campestanol and sitostanol) are produced by the hydrogenation of sterols and are not abundant in nature. Chemical hydrogenation reduces all double bonds, that is, from sitosterol and stigmasterol to sitostanol, and campesterol and brassicasterol to campestanol [6]. The conversion of campesterol into campestanol involves a three-step process that includes 24-methylcholest-4-en-3β-ol, 24-methylcholest-4-en-3-one, and 24-methyl-5α-cholestan-3-one as intermediates [7]. Moreover, when seeds from transgenic cotton plants were analyzed for levels of 3-hydroxysteroid oxidase and sterol composition, a direct correlation between enzyme levels and phytostanol levels was observed, suggesting that the enzyme is responsible for formation of the latter [6].

In plant cells, stanols are converted into brassinosteroids which are steroidal hormones that regulate the growth of immature tissue. Campestanol is the precursor of C28 brassinosteroids; however, stigmastanol is the precursor of C29 brassinosteroids [8]. It was verified that the seeds of pumpkin contain campestanol as a biosynthetic precursor of brassinosteroids [9].

Most studies comparing the activity of phytostanols and phytosterols conclude that their ability to reduce serum cholesterol levels is equal, although some consider that plant stanols are more efficient [10–12]. This better effectiveness is related to the fact that phytostanols, which have practically no absorption, remain for longer period in the intestinal lumen where they interfere continually and in a more efficient way with the absorption of cholesterol [1,3]. Sitosterol, the most abundant phytosterol, is much less well absorbed than cholesterol (<5% vs. 20–60%). In comparison with sitostanol, the 5α-saturated derivative of sitosterol is virtually unabsorbable at <2% [10,11,13]. Other researches have estimated the absorption rate of sitostanol to be in the range of 0–3% [14]. While the intestinal absorption of phytostanols is lower than that of phytosterols, campesterol is more efficiently absorbed than sitosterol. Thus, from the perspective of maximizing cholesterol-reducing activity of phytosterols, sitostanol offers the best prospect as a component of a cholesterol-lowering dietary supplement and/or functional food [6]. Plant sterol/stanol absorption is described in Table 36.1 [15].

The putative mechanisms by which plant sterols and stanols reduce serum cholesterol include (a) the inhibition of cholesterol absorption in the gastrointestinal tract by displacing cholesterol from micelles, (b) limiting the intestinal solubility of cholesterol, and (c) decreasing the hydrolysis of cholesterol esters in the small intestine [16]. This reduced absorption of cholesterol lowers serum cholesterol despite the

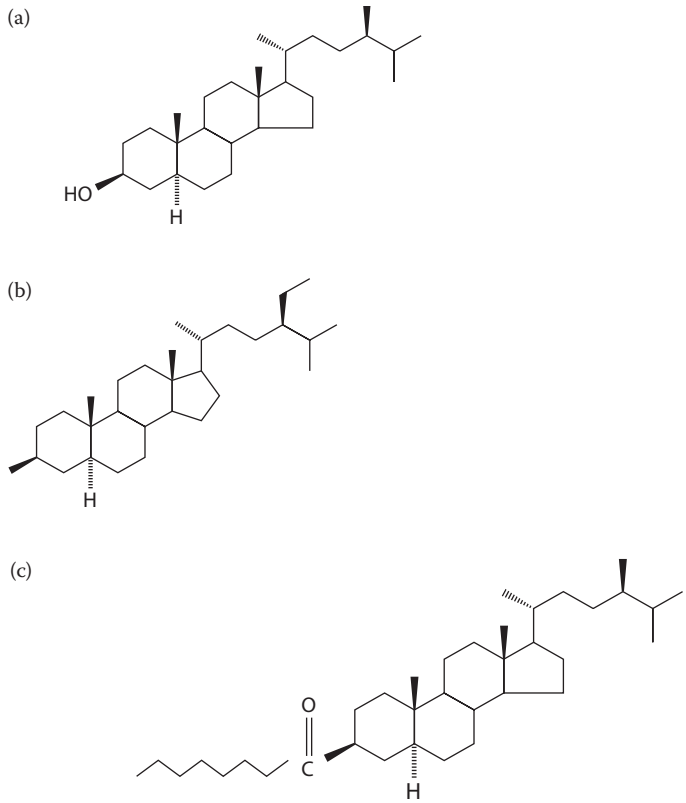

FIGURE 36.1 Structures of campestanol (a), sitostanol (b), and stanyl ester (c).

compensatory increase in cholesterol synthesis which occurs in the liver and in other tissues [16–18]. Increased plant stanol concentrations within the enterocyte also activate cholesterol efflux through the ATP-binding cassette A1 system back into the intestinal lumen [19].

Diminishing of plasmatic cholesterol levels is vital for the prevention of cardiovascular diseases, which are the main cause of death in Europe [12]. Therefore, the development of food technology has created some foods enriched with phytostanols and phytosterols. According to the decisions 2004/4289 [20], 2004/333 [21], 2004/334 [22], 2004/336 [23], and 2000/500 [24] of the European community, the products that can be enriched with phytostanols, phytosterols, and phytosterol esters are as follows: yellow fat

TABLE 36.1

Absorption of Sterols-Stanols

	Absorption (%)
Sitostanol	0.04
Sitosterol	0.51
Campestanol	0.16
Campesterol	1.9
Cholesterol	56.0

Source: Adapted from Athyros, V. G. et al. 2011. *Nutrition, Metabolism and Cardiovascular Diseases*, 2(3), 213–221.

spreads, salad dressings, milk-type products, yogurt-type products, spicy sauces, milk-based fruit drinks, soybean drinks, and cheese-type products.

The European Commission adopted specific rules for labeling "foods and food ingredients with added phytosterols, phytosterol esters, phytostanols, and/or phytostanol esters" in Commission Regulation (EC) No. 608/2004 and declared that "this food should be part of a balanced diet, including regular consumption of fruits and vegetables to help maintain plasma carotenoids levels" [25, p. 45].

At present, several functional food product types such as yoghurts and milk with added plant sterols and stanols are available in the market [26]. Both sterols and stanols are frequently used in esterified forms, as fatty acid esters: this increases their solubility and allows their incorporation into lipid-based foods [27]. In particular, it has been demonstrated that plant stanol esters with low fat milk are almost three times more effective than with bread and breakfast cereals [4]. Vegetable oil processing is the major source of phytostanols in several food products currently sold [6].

Phytostanols are either absent or are found at trace levels. Saturation of phytosterols by commercial hydrogenation processes, including the saturation of sitosterol and campesterol at the 5α-ring position, results in phytostanol compounds such as sitostanol and campestanol [14]. The first advantage is that the hydrogenation of sterols to their corresponding stanols makes them almost unabsorbable in the gut.

Phytostanols are currently produced by processing deodorized distillate and hydrogenating the phytosterols; however, such chemical modifications add to the cost of manufacturing the final product. Therefore, modification of phytosterols to phytostanols in planta would be a more economical means of producing vegetable oil distillates with significant nutritional value [6]. Thus, 3-hydroxysteroid oxidase from *Streptomyces hygroscopicus* was utilized to engineer oilseeds from rapeseed (*Brassica napus*) and soybean (*Glycine max*), respectively, to modify the relative amounts of specific sterols to stanols. Consequently, each of the major phytosterols had its C-5 double bond selectively reduced to the corresponding phytostanol without affecting other functionalities, such as the C-22 double bond of stigmasterol in soybean seed and of brassicasterol in rapeseed. This has, consequently, led to the formation of the phytostanols, stigmastanol and brassicastanol, normally not observed in nature. Those novel phytostanols obtained are not produced by chemical hydrogenation of phytosterols normally present in plants [6].

The esterified forms of phytosterols have also been used as cholesterol-lowering agents [28]. Numerous researches have focused on the esterified forms of plant stanols, that is, plant stanol esters [15,29–38]. In contrast to free sterols and stanols, which are crystalline and largely insoluble, esters of the same sterols and stanols are easily dissolved in different fat-containing foodstuffs. The commercial esterification of plant sterols and stanols with fatty acids from vegetable oil has made it possible to produce spreads and other foods containing the desired esters. Recently, several functional food product types such as spreadable fats, yoghurts and milk, with free phytosterols or phytosteryl fatty acid esters or phytostanyl fatty acid esters added at high levels, are available in the market—especially in several European countries [39]. Oil-based products enriched with plant stanol esters can lower low-density lipoprotein (LDL) cholesterol concentrations by 10–14% [36,40,41].

In a recent study, the plant stanol ester spread reduced (by 1 month) total cholesterol (–14%), LDL cholesterol ((LDL-C))(–16%), high-sensitivity C-reactive protein (–17%), and estimated cardiovascular disease risk (26–30%) [15].

36.2 Stanol Contents in Foods

Phytostanols, fully saturated forms of phytosterols, occur in trace levels in many plant species and they occur in high levels in tissues of only a few cereal species [50]. Plant stanols are intrinsic constituents of cereals (corn, wheat, rye, and rice), fruits, and vegetables, but their concentrations are generally lower than those of unsaturated plant sterols [51,39]. Phytostanols are less abundant in nature than plant sterols [44]. In cotton plants engineered for insect resistance by expressing a 3-hydroxysteroid oxidase from *S. hygroscopicus* A19249 in plastids, there was a significant increase in campestanol and sitostanol levels [52].

Cereals seem to be an important dietary source of phytostanols, as demonstrated by the high concentrations found in certain cereal foods [46]. Phytostanols are suggested to exist only in substantial amounts

in the endosperm of the grain [53]. Conversely, high phytostanol concentrations were detected in dry and coarse wheat bran (82 and 58 mg/100 g e.p., respectively). In corn kernels, stanols were localized mainly in the aleurone cells [54]. Further, wheat germs had a medium concentration (17 mg/100 g e.p.), while no phytostanols were detected in wheat flour [46]. In adlay samples, ergostanol accounted for 16.7–30.3% of total sterol content [55].

The composition of total sterols (free + bound) in rye, oats, barley, wheat, corn, and other grains has been determined [56]. They found that all grains contained significant levels of phytostanols (sitostanol and campestanol) in the total phytosterol fractions. Most of the phytostanols in corn are esterified in either steryl fatty acid ester or hydroxycinnamate steryl ester (HSE) [57]. All of this later form is localized in the aleurone cells which form a single layer in corn, and fractionates into the corn fiber fraction during wet milling. Since commercial corn oil is obtained by extracting corn germ, the levels of HSE and phytostanols in corn germ oil are very low [57,58].

The content of stanols was investigated in a small number of samples of hydrogenated fats and oils, and in the "free" and "bound" lipids of various samples of cereals [51]. Among the hydrogenated fats and oils, coconut oil contained the largest amounts of sitostanol followed by soybean oil (80 and 20 g kg^{-1} of total unsaponifiables, respectively). No sitostanol was detected in hydrogenated palm oil under the used analytical conditions. Both "free" and "bound" lipids in various samples of wheat, except for wheat germ, contained c 70–120 g kg^{-1} campestanol and 100–150 g kg^{-1} sitostanol in total unsaponifiables. In lipids of oats and barley, no campestanol or sitostanol could be detected. Rye total lipids contained 60–90 g kg^{-1} of campestanol and 100–150 g kg^{-1} of sitostanol of total unsaponifiables in "free" and "bound" lipids, respectively.

The phytostanol amounts of various vegetable oils, including individual concentrations of campestanol and sitostanol, have been reported [46,59–62] (Table 36.2). In *Pinus pinea* L. oil isolated from seven Mediterranean populations, a campestanol content of 5.17 mg/100g and a stigmastanol amount of 21.06 mg/100 g of oil have been reported [59]. Stitostanol contents (mg/kg of oil) were determined in a virgin olive oil (4.4), a refined olive oil (7.7), an olive-pomace oil (38.9), and a crude olive-pomace oil (44.9) [61]. Phytostanol composition of olive oil can be used to assess the degree of purity of the oil and the absence of other plant oils. This determination also permits characterization

TABLE 36.2

Some Reported Stanol Concentrations in Selected Foods and Vegetable Oils

Food Product	Sitostanols	Campestanol	Reference
Pea (mg 100 g edible portion)	2.7		[67]
Carrot	0.2	0.2	[67]
Potato	0.7		[67]
Tomato	0.7		[67]
Cucumber	0.7	0.1	[67]
Orange	0.5		[67]
Banana	0.2		[67]
Virgin olive oil (mg/kg)	4.4		[61]
Refined olive oil	7.7		[61]
Olive-pomace oil	38.9		[61]
Crude olive-pomace oil	44.9		[61]
Almonds (mg/100 g)	3.9		[60]
Walnuts			[60]
Hazelnuts	6.5		[60]
Peanut oil	3.3		[60]
Coconut oil	3.6		[60]
Baking fat	4.3	2.7	[60]
Margarine AH	3.1	2.2	[60]
Peanut butter	2.2		[60]

of the type of olive oil in question: extra virgin, virgin, refined, and so on. In extra virgin oil, the median proportions of campestanol and sitostanol were 0.08% and 1.34% of total 4-desmethylsterols have been reported [63]. Oils and fats may contain naturally small amounts of 5α-cholestane [64]. Corn germ oil had lower levels of phytostanols (27–46 mg/100 g) [62] than wheat germ oil which had 150 mg/100 g of oil [65]. Among the three corn kernel fractions, endosperm oil had the most amounts of phytostanols (6793.3–7720.2 mg/kg of oil) [62]. In the oil obtained from three corn kernel fractions, sitostanol was the predominant stanols (77–87%), followed by campestanol (13–23%). This contrasts with the composition of wheat germ oil where sitostanol and campestanol amounted 55.3% and 44.7% of total phytostanols, respectively [65]. The change in phytostanol content during corn kernel maturation has been studied [66]. The highest level of phytostanol was detected at 20 days after pollination.

The amounts of campestanol and sitostanol were determined in 87 fatty foods (vegetable oils, spreads, and nuts) consumed in Sweden and the Netherlands [60]. Campestanol was detected in only a few fat spreads, with the highest concentration (10% of total sterols) in a polyunsaturated margarine from Sweden.

Phytostanol concentrations in 34 kinds of vegetables and 33 kinds of fruits commonly consumed in China have been also reported [67]. Sitostanol was not detectable in seven vegetables and in six kinds of fruits. Campestanol was only detectable in nine kinds of vegetables and it was not detectable in all the fruits examined. The sitostanol content ranging from 0.1 to 3.1 mg/100 g edible portion, and the highest level was found in romaine lettuce. However, the highest level of campestanol ranging from 0.1 to 0.7 mg/100 g edible portion was detected in spinach.

Direct GC/MS analysis of the hexane extracts of fruit juices demonstrated that very different sterol patterns exist in the juices of pineapple, passion fruit, and two citrus fruits, orange and grapefruit [68]. Ergostanol and stigmastanol were found to be the sterol markers for pineapple juice. Sitostanol, the hydrogenated form of sitosterol, constitutes about 20% of the total plant sterols in pine oil [14].

Sitostanol and campestanol are saturated plant sterols, which are found in nature in much smaller amounts than plant sterols. Because of their cholesterol-lowering effects, these components are incorporated nowadays into a wide variety of food products, referred to as functional foods [69]. Phytostanol concentrations in enriched milk and yoghurts that are used as functional foods were determined [12].

36.3 Average Intake of Phytostanols

Since phytostanols are not synthesized by the human body, dietary consumption is the only source of plasma phytostanols. Several studies have shown that the intake of 2–3 g/day of plant stanols as plant stanol esters reduce serum total and LDL cholesterol concentrations by 10–15% [42]. Recently, clinical studies suggested that the high intake of plant stanols (9 g/day) reduced LDL cholesterol values without any other side effects than reduction of serum β-carotene concentration [43]. Consequently, the plant stanol ester intake can be increased to induce a greater cholesterol-lowering effect. The average dietary consumption of phytostanols is approximately 25 mg/day [44].

In the Netherlands Cohort Study, the correlation coefficients between campestanol and sitostanol intake and dietary fiber from cereals were 0.97 and 0.98, respectively [45]. This adds support to the finding that phytostanols exist in higher concentrations in a fiber-rich fraction of the cereal, rather than in the endosperm [46].

Plant stanols are potent hypocholesterolemic agents and a daily consumption of 2–3 g lowers LDL cholesterol concentrations in hyper- and nonhypercholesteromic adults and children by 10–14% without changing high-density lipoprotein cholesterol or triacylglycerol concentrations. Low-fat yoghurt enriched with plant stanol esters lowers within 1 week LDL cholesterol to the same extent as oil-based products [47]. The National Cholesterol Education Program (NCEP) Adult Treatment Panel (ATP) III report [48] recommends that plant stanols/sterols (2 g/day) should be incorporated into a diet aiming at LDL-C lowering by 10–15%. In the large sample of the EPIC study (European Prospective Investigation into Cancer), the dietary intake of plant stanols is about 50 mg/day [49].

36.4 Analysis of Stanols

Phytostanols are saturated phytosterols, which are detected in 4-desmethylsterol fraction [62,70]. Thus the analysis of these saturated compounds needed the same steps employed for unsaturated sterol analysis. In general, the analysis of individual stanols includes the extraction of lipids, saponification or acid hydrolysis and saponification to liberate stanols, extraction of unsaponifiable matter and separation/partial purification of stanols, the formation of stanols derivatives, and their analysis by chromatographic techniques.

Two methods were applied to determine phytostanol/phytosterol contents of several sterol-enriched functional food products and phytostanyl/phytosteryl fatty acid ester ingredients [39]. A method based on hot saponification of a sample with ethanolic potassium hydroxide in the presence of an internal standard (5β-cholestan-3α-ol) is adequate for most matrices, such as spread, milk, and yoghurt. Some matrices, such as pasta, need acid hydrolysis in order to release matrix-incorporated bound sterols or sterols from steryl glycosides before the saponification step. After saponification, the unsaponifiable fraction containing phytostanols and phytosterols is extracted into an organic solvent (e.g., heptane), followed by evaporation of the solvent to dryness. Sterols are separated as their trimethylsilyl (TMS) ether derivatives with a gas–liquid chromatograph (GLC), on a column coated with 5% phenyl/95% dimethylpolysiloxane, and detected with a flame ionization detector. The GC conditions used offer efficient separation of the most abundant phytostanols/phytosterols in 15 min, a wide linear range of stanols/sterols without the need of defining sterol response factors.

Analysis of these complex mixtures of stanols and 4-desmethylsterols is generally accomplished by capillary column GLC and a combination of GC with mass spectrometry (MS).

Medium-polarity columns such as Suppelcowax 10 have been shown to separate the common Δ5-unsaturated sterols from their saturated sterols in the lipid fraction [71]. A fused-silica capillary column DB-1701 (14% cyanopropyl-phenyl-methylpolysiloxane; 30 m × 0.25 mm, 0.25 μm) of low-medium polarity was used for separation of campesterol and campestanol, and sitosterol, sitostanol [45,72,73]. To accomplish this separation, other columns were also tested DB-1, 30, and 60 m long, and both having 0.25 mm ID and 0.25 μm film thickness, and a column of DB-5MS, 12 m × 0.2 mm ID and 0.33 μm film thickness. However, none of these columns could provide a good separation of the sitostanol, sitosterol, and Δ-5 avenasterol [72].

The separation of campestanol from campesterol and sitostanol from sitosterol is not satisfactory with very nonpolar and nonpolar GLC columns [60]. As it is not only the polarity of the capillary column materials that is important for separation of closely eluting peaks, other factors such as length, internal diameter, and film thickness should also be considered. OV-1-type liquid phase (100% methylpolysiloxane) and OV-5 (5% phenyl–95% methylpolysiloxane) capillary columns of various dimensions are used for the quantitative analysis of sterols. These types of columns may not be able to separate a complex mixture of sterols [51,74,75].

The use of capillary HP-5 column (5% phenyl methyl siloxane; 30 × 0.25 mm, 0.25 μm film thickness) provides a good separation of campesterol and campestanol, and of sitosterol and sitostanol in corn oil [62,66]. This separation was achieved in cereal by-products by an SAC capillary column (0.25 mm ID, 30 m long, and 0.25 μm film thickness) with the following temperature program: held at 250°C for 5 min, the temperature increased to 265°C at a rate of 1°C/min, and held at 265°C for 25 min [65].

The capillary column fused-silica SE52 (30 m × 0.25 mm ID × 0.10 μm film thickness) (Mega, Milan, Italy), coated with 5% diphenyl–95% dimethylpolysiloxane, was used for the separation of campeaterol and campestanol, and sitosterol and sitostanol in the olive oil samples. The oven temperature was programmed from 240°C to 325°C at a rate of 1.5°C/min and kept at 325°C for 10 min. The injector and detector temperatures were both set at 325°C [63].

Although the separation of sterols and stanols as such without derivatization is possible [76], the resolution of a sterol and its corresponding stanol is not as good as that of their TMS derivatives. Thus, usually prior to capillary GC, sterols and stanols are conventionally transformed into derivatives that yield improved peak shape, resolution, and sensitivity, and a higher stability for the thermally labile

unsaturated sterols [39]. Sterols are commonly analyzed as their TMS or acetate derivatives. The former are more suitable for the GC-MS characterization and quantitation of sterols.

Structural confirmation of phytostanols was manly done by GC-MS. Mass detection was elected due to its properties, namely specificity, in order to identify the different compounds based on their derivatized mass spectra [12]. This approach also provides better sensitivity when compared to other GC detectors, such as flame ionization detection (FID). The mass spectra of some stanols of interest (Figure 36.2) show the abundances of different characteristic ion fragments concurring with published results [9,72,78].

Analysis of phytostanols by GC-MS in enriched milk and yoghurts that are used as functional foods was described by Santos et al. [12]. A Hewlett–Packard (HP) chromatographic system (Soquimica, Lisbon, Portugal) controlled by an HP Vectra VL2 4/50 computer, an automatic HP6890 injector, and a GC HP5890 Série II equipped with an HP-5MS column (30 m × 0.25 mm ID × 0.25 lm film thickness), connected to an HP5972 mass detector, were used.

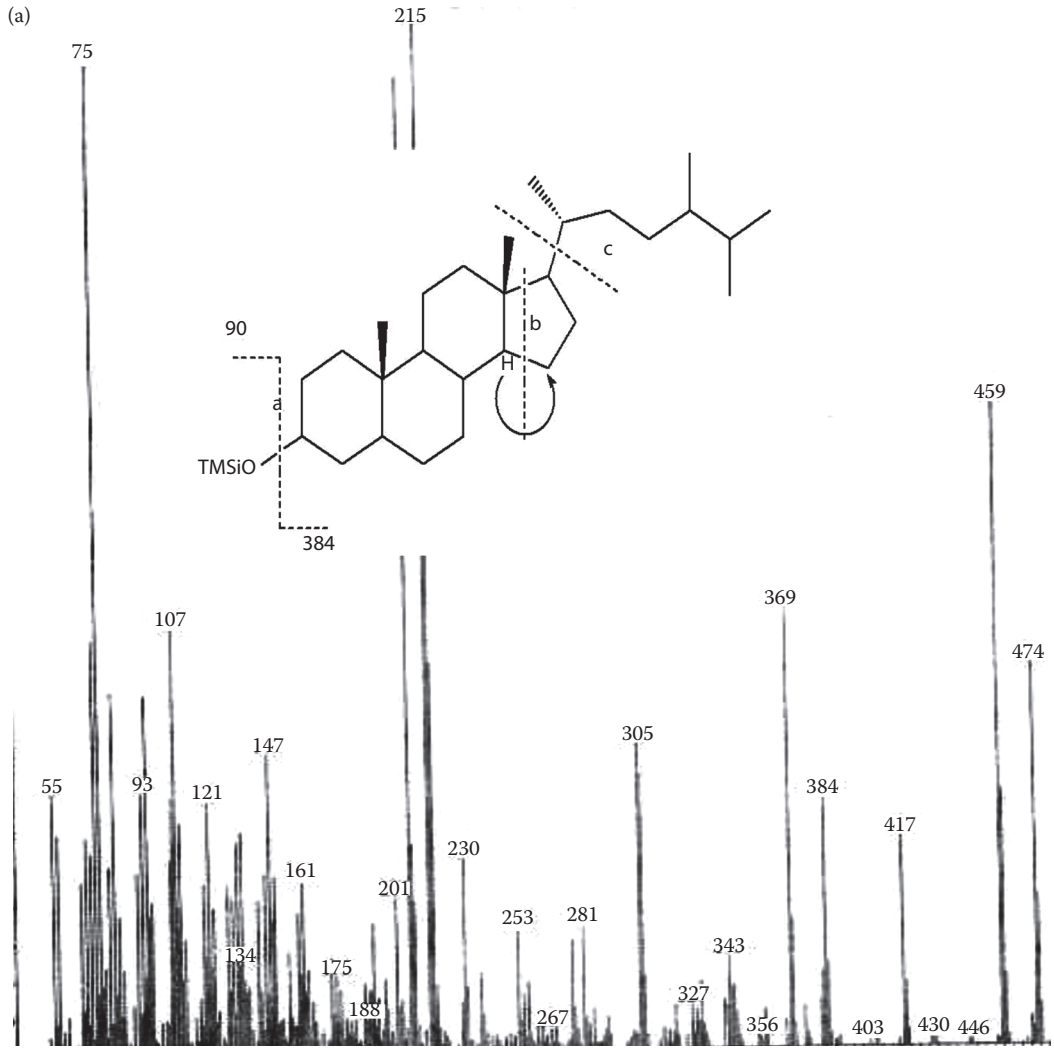

FIGURE 36.2 Full scan mass spectra of some common stanols from the unsaponifiable fraction of corn lipids. (a) a full scan mass spectrum of the TMS ether derivative of campestanol showing the molecular ion ($M^+ = 474$) and the most typical ion fragment at *m/z* 215 [M^+-side chain – 42 –(CH)SiOH]; (b) a full scan mass spectrum of the TMS ether derivative of sitostanol showing the molecular ion ($M^+ = 488$) and the most typical ion fragment at *m/z* 215 [M^+ -side chain – 42 –(CH) SiOH].

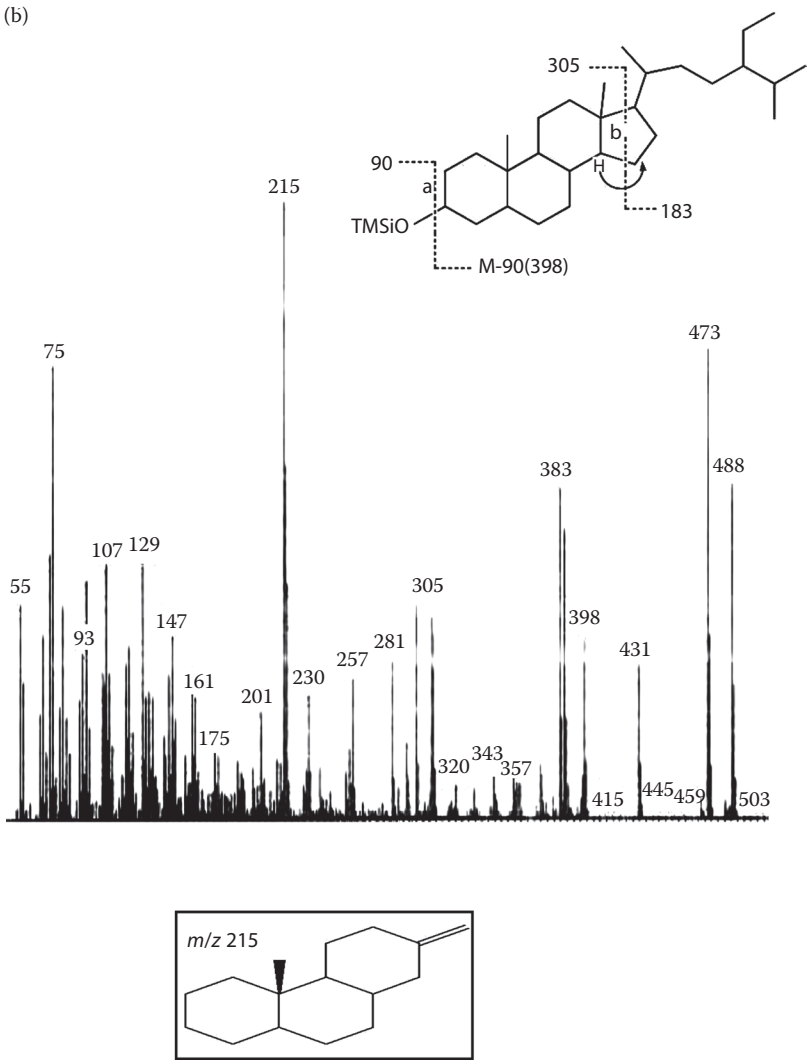

FIGURE 36.2 Continued.

The content of stanols was investigated in a small number of samples of hydrogenated fats and oils, and in the "free" and "bound" lipids of various samples of cereals [51]. The sterols, after saponification of the total lipids, were analyzed as TMS derivatives by GC and identified by GC-MS.

Phytosterols and phytostanols, including brassicastanol, were detected in *B. napus* seeds using reversed-phase HPLC high-performance liquid chromatography [6] and structural confirmation was done by GC-MS as described by Goad and Akihisa (1997). Phytosterols and phytostanols were then separated from one another based on their structural properties, such as the number of double bonds in the rings and side chain, and also based on the number of methyl groups on the side chain, that is, 24-methyl from 24-ethyl. The column used was a glass capillary DB-5 column (50 m) with a film thickness of 0.25 μm.

A validated and repeatable HPLC method with online evaporative light scattering (ELSD) was developed for the quantitative analysis of the combination, stigmastanol, stigmasterol, and β-sitosterol [44]. ELSD is needed for detection of stigmastanol, which is transparent in the ultraviolet range. This method is based on the separation of the three marker compounds on a C8 column (Phenomenex Luna, 5 μm, 150 mm × 4.6 mm ID) using methanol:water (95:5 v/v) as the mobile phase, and a flow rate of 1 mL/min

to separate all the marker compounds within 12 min. Cholesterol (50 μg/mL) was used as internal standard and methanol as the extraction solvent. The ELSD response parameters were optimized and the limits of detection (2 μg/mL) and quantification (5 μg/mL) were determined, which is more sensitive than obtained by photodiode array detection (5 and 7 μg/mL). This method was used to assay commercially available products formulated as oral dosage forms purported to contain African Potato and associated sterols and stanols and proved to be suitable for the routine analysis and quality control of such products. ELSD is relatively inexpensive and easily operable compared to GC and MS detection. This simple, rapid, precise, and accurate method was successfully applied in the analysis of commercially available solid dosage forms.

Another study employed HPLC-MS, specifically atmospheric pressure chemical ionization mass spectroscopy (APCI-MS), for the separation of sitosterol and stostanol in the olive oil sample [61]. Characteristic fragment-ions observed in the ESI and APCI mass spectra of sterols in positive mode have been reported. Sterols in the nonsaponificable fractions of the olive oil were isolated with a gradient of acetonitrile/water (0.01% acetic acid) at a flow of 0.5 mL/min. The gradient LC conditions were: the mobile phase was water (acetic acid 0.01%) (Phase A) and acetonitrile (Phase B) and the solvent gradient changed according to the following conditions: from 0 to 2 min, 30% (A):70% (B) to 0% (A):100% (B); this value was maintained for 28 min and then, from 30 to 31 min, 0% (A):100% (B) to 30% (A):70% (B) using this percentage during 4 min, and the run was ended.

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37

Analytical Methods for Determination of α-Lipoic Acid, Dihydrolipoic Acid, and Lipoyllysine in Dietary Supplements and Foodstuffs

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37.1 Introduction

α-lipoic acid (α-LA), 1,2-dithiolane-3-pentanoic acid (Figure 37.1), is a naturally occurring compound that can be synthesized by plants and animals (Reed 1957, Packer et al. 2001) in low amounts, and it is absorbed by humans from different dietary sources. The dominant form in food is the (*R*)-enantiomer, but most commercial preparations of α-LA consist of a racemic mixture of the (*R*)- and (*S*)-enantiomers.

α-LA may occur naturally in a free form, bound to proteins by hydrogen bonds, or in a protein-bound form via an amide linkage between the ε-amino group of a lysine residue and the carboxylic group of α-LA (Reed et al. 1958, Bradford et al. 1987). It may also form mixed disulfides (Reed et al. 1953) and disulfide polymers (Thomas and Reed 1956) or occur in the reduced form, dihydrolipoic acid (DHLA) (Sen et al. 1999). In bovine kidney and heart, α-LA is present in the enzyme dihydrolipoyltransacetylase

FIGURE 37.1 Structure of (*R*)-α-lipoic acid.

containing one lipoyl moiety/polypeptide chain (White et al. 1980). α-LA is also a coenzyme for other enzymes, for example, pyruvate dehydrogenase and α-ketoglutarate dehydrogenase.

α-LA has been reported to be a potent biological antioxidant, a detoxification agent, and a diabetes medicine, and it has also been applied to improve age-associated cardiovascular, cognitive, and neuromuscular deficits (Biewenga et al. 1997, Bustamante et al. 1998, Packer et al. 2001, Navarri-Izzo et al. 2002, Wollin and Jones 2003, Shay et al. 2009). The antioxidative activity of free α-LA has been studied in detail, but the antioxidative potential of the protein-bound lipoic acid has hardly been elucidated (Matsugo et al. 2003).

Despite several analytical methods for the determination of α-LA, there is a lack of data to estimate its daily intake. In general, it can be assumed that the amounts consumed in Western diet are too low to cause an increase of α-LA in plasma and cells. However, consumption of dietary supplements containing 50–600 mg of free α-LA (Shay et al. 2009) leads to a significant increase in plasma and cells even though the bioavailability of the (*R*)- and (*S*)-form has been estimated to be only 38% and 20%, respectively, after a 200 mg dose (Hermann et al. 1996, Teichert et al. 1998). Simultaneous food intake reduces the bioavailability of α-LA (Gleiter et al. 1996). Regarding supplementation, there is a need to determine the border between beneficial dose and overdose with regard to human health (Wollin and Jones 2003). For these reasons, analytical methods for the determination of α-LA are important. Still, it is also important to determine DHLA, the reduced form of α-LA, and lipoyllysine (LLys) because they may act as sources of free lipoic acid.

37.2 Analytical Methods

In this section, an overview of the analytical methods used for the determination of α-LA, DHLA, and LLys in dietary supplements and food from animal and vegetable sources is given (Table 37.1).

37.2.1 Dietary Supplements

37.2.1.1 Sample Preparation

Dietary supplements contain free α-LA in a relatively high amount (50–600 mg). The samples can be dissolved in an appropriate solvent (e.g., water, methanol, acetone, ethanol/water) at nearly quantitative recovery (96–98%) and the contained α-LA can be determined by several analytical methods (Table 37.1). In principle, one has to distinguish between methods with and without separation.

37.2.1.2 Differential Pulse Voltammetry

α-LA is directly oxidized at a glassy carbon electrode and the resulting current could be measured by differential pulse voltammetry (DPV). The limit of detection (LOD) and the limit of quantification (LOQ) ranged from 1.8 to 6.1 μM, respectively (Corduneanu et al. 2007). In principle, DPV is a very simple method. However, α-LA adsorbs on the surface of the electrode, thereby influencing the response of the electrode. Hence, it has to be removed by washing the glassy carbon electrode with ethanol followed by few scans of electrochemical cleaning in the supporting electrolyte. In addition, other compounds in samples that are oxidized at lower potentials, for example, ascorbic, glutamic, and uric acid, other disulfides, as well as proteins, can interfere. Even if such compounds are absent, it is advisable to apply the standard addition method to exclude the influence of other matrix compounds on the response. The use of

TABLE 37.1

Overview of Analytical Methods and Parameters as well as the Content of α-Lipoic Acid, Dihydrolipoic Acid, and Lipoyllysine

Method	Analytical Parameters	Sample type Sample Preparation Analyte	Content Recovery	Reference
DPV	LR: <75 μM LOD_{ST}: 1.8 μM LOQ_{ST}: 6.1 μM	Dietary supplements EX by ST in water/ethanol α-LA	119 mg/L 59.0 mg/capsule R: 98%	Corduneanu et al. (2007)
LSV	AR: 26–180 μM LOQ_{ST}: 19 μM	Tablets Dissolved in water α-LA	11.9 and 24.8 mg/tablet	Ziyatdinova et al. (2009)
Potentiometry/FIA	CR: 0.1–10,000 μM LOD_{ST}: 0.09 μM	Tablets/capsules Dissolved in 0.1 M NaOH, precipitated in 0.1 M sulfuric acid, dissolved in 0.1 M NaOH α-LA	600 and 300 mg/tablet or capsule R: 99%	Abbas and Radwan (2008)
CZE/UV	LR: 500–4500 μM	Tablets EX by ST in phosphate buffer α-LA	51 mg/tablet	Trentin et al. (2002)
CZE/UV	LR: 48.5–4360 μM LOD_{ST}: 3.9 μM LOQ_{ST}: 12 μM	Tablets EX in methanol α-LA	49.1 mg/tablet R: 98%	Sitton et al. (2004)
HPLC/UV	CR: 48.5–2423 μM LOD_{ST}: 21 μM LOQ_{ST}: 81 μM	Dietary supplements EX by SON in methanol or acetonitrile α-LA	150–250 mg/tablet R: 99%	Aboul-Enein and Hoenen (2004)
HPLC/UV	LR: 0.12–4.85 μM LOD_{ST}: 0.12 μM	Dietary supplements EX by SON in methanol α-LA	0.4 and 12.1 mg/g tablet R: 96%	Durrani et al. (2007)
HPLC/FLD	LR: 0.1–10 μM LOD_{ST}: 0.03 μM LOQ_{ST}: 0.1 μM	Dietary supplements EX by SON in acetone Derivatization: TCEP/SBD-F α-LA	20 mg/tablet R: 97%	Satoh et al. (2007)
HPLC/FLD	CR: 0.75–120 μM LOD^*_{ST}: 0.003 μM	Dietary supplement Dissolved in methanol Derivatization: TBP/PIAA α-LA	33 mg/tablet R: 98.0%	Inoue et al. (2009)
HPLC/CEAD	LR: 0.048–4.85 μM LOD_{ST}: 0.024 μM	Dietary supplements EX by SON in methanol α-LA	0.7–33.6 mg/g tablet R: 96%	Durrani et al. (2007)
HPLC/ESI-MS	LR: 0.024–4.85 μM LOD_{ST}: 0.015 μM	Dietary supplements Ex by SON in methanol α-LA	0.7–33.6 mg/g tablet R: 96%	Durrani et al. (2007)
Microbiological assay	WR_T: 1.0–9.7 pmol/culture WR_V: 24.2–242 pmol/culture	Bacteria AH sulfuric acid (12 M) α-LA	0.1–25 μg/g DW R: 90–109%	Herbert and Guest (1975)

continued

TABLE 37.1 (continued)

Overview of Analytical Methods and Parameters as well as the Content of α-Lipoic Acid, Dihydrolipoic Acid, and Lipoyllysine

Method	Analytical Parameters	Sample type Sample Preparation Analyte	Content Recovery	Reference
Titration	WR: 0.24–9.7 μM	Protein hydrolysates α-LA		Wronski (1987)
Colorimetric assay	WR: 0–111 μM	Animal tissues AH: hydrochloric acid (6 M) EX: ethyl acetate Reaction: DBQC α-LA		Garganta and Wolf (1996)
Enzymatic assay	LR: 1–5 (10) μM LOD_{ST}: 0.1 μM	Kidney, liver (bovine, rabbit) EH: proteases LLys	2.7 and 2.4 μg/g WT 0.8 and 0.9 μg/g WT	Konishi et al. (1996); Akiba et al. (1998)
Enzyme immunoassay	LOD_{ST}: 5 μM	Model assay α-LA		MacLean and Bachas (1991)
TLC	LR: 6.8–34.5 μg/spot	Wheat flour, wheat germ EX: chloroform/methanol/water α-LA	285–588 μg/g flour 7550–8469 μg/g germ R: 91%	Swatditat and Tsen (1973)
TLC	LOD_{TLC}: 5 and 10 μg/spot	Brewers and bakers yeast	2.6–8.3 μg/g DW	Kozma-Kovacs et al. (1991)
GC/FID		AH: sulfuric acid (12 M) EX: dichloromethane Derivatization: methyl ester α-LA	R: 30%	
GC/FID		Chick liver, fresh eggs	5–10 μg/g	Shih and Steinsberger (1981)
		AH: sulfuric acid (12 M) EX: benzene Derivatization: methyl ester	1–2 μg/g R: 34%	
GC/FID	WR: 0–97 μM	Cow's milk, bacteria AH: hydrochloric acid (8 M) EX: dichloromethane Derivatization: *S*,*S*-dibenzyl-methyl ester α-LA	0.01 μg/g WW 2.3–11.8 μg/g WW R>87%	White (1981)
GC/FDP	LLR: 1–24 μM LOD_{ST}: 0.25 μM LOD_S: 10 ng/g	Egg yolk, chicken, pork, beef, yellow tail, cuttlefish BH: potassium hydroxide 2–3 M EX: dichloromethane Derivatization: *S*,*S*-diethoxy-carbonyl methyl ester (DEOC) α-LA	1.24; 0.91; 1.07; 2.36, 0.75; 0.55 μg/g WW R: 50–60%	Kataoka et al. (1993) Kataoka et al. (1997) Kataoka (1998)
GC/MS	LR: 0.05–97 μM LOD_{ST}: 0.05 μM	Liver, kidney, heart, muscle AH: sulfuric acid (2 M)	0.07–1.6 μg/g WW R: 60–70%	Mattulat and Baltes (1992)

TABLE 37.1 (continued)

Overview of Analytical Methods and Parameters as well as the Content of α-Lipoic Acid, Dihydrolipoic Acid, and Lipoyllysine

Method	Analytical Parameters	Sample type Sample Preparation Analyte	Content Recovery	Reference
		EX: diethylether/sodium bicarbonate/diethylether Derivatization: silyl derivative (MBDSTFA) α-LA		
HPLC/UV		Beef heart α-ketoglutarate dehydrogenase(α-KGDH) and pyruvate dehydrogenase (PDH) EH: pronase LLys	0.55 mg/g α-KGDH 0.83 mg/g PDH R: 116.8–119.5%	Hayakawa and Oizumi (1989)
HPLC/FLD	LR: 0.02–3.0 μM LOQ_{ST}: 0.02 μM	Animal tissue SON in phosphate buffer (10 mM) EX: diethylether Derivatization: monobromobimane (mBBr) derivative	R: 61%	Witt and Rüstow (1998)
HPLC/FLD	LCL: 0–0.3 μM LOD_{ST}: 0.03 μM	Tomato juice, green tea, broccoli, sauerkraut, dried yeast, liver, rice bran, nato, spinach, egg yolk AH: sulfuric acid (9 M) Purification: charcoal Derivatization: 9-anthryldiazo-methane (ADAM) ester α-LA Derivatization: ammonium-4-fluoro-2,1,3-benzoxazole-7-sulfonate (SBD-F) LLys	0.007–6.65 mg/g DW or WW or mg/mL R: 70 %	Hayakawa et al. (2007)
HPLC/ECD	0.039–97 μM LOD_S: 0.005 μM	Plasma EH: alcalase, subtilisin EX: phenyl cartridge (SPE) α-LA, DHLA	1.4–11.6 ng/mL R: 82%	Teichert and Preiss (1997)
HPLC/ECD	WR: 1–24 μM	Wheat, tomatoes AH: hydrochloric acid (6 M) EX: dichloromethane, chloroform α-LA, DHLA	Wheat: ~1.5–5 μg LA/g DW ~10–30 μg DHLA/g DW Tomatoes: 2.5–4 μg LA/g DW ~8.51–17.1 μg DHLA/g DW	D'Amico et al. (2004); Incerti et al. (2006); Sgherri et al. (2008)
HPLC/ECD	CR: 0–30 μM LOD_S: 0.1 μg/g	Kidney, heart, liver, spinach, broccoli, tomato, garden pea, Brussels sprout, rice bran, yeast, egg yolk SON in Tris-HCl (pH 7.6)	2.64, 1.51, 0.86, 3.15, 0.94, 0.56, 0.39, 0.39, 0.16; 0.27, 0.05 μg/g DW	Lodge et al. (1997)

continued

TABLE 37.1 (continued)

Overview of Analytical Methods and Parameters as well as the Content of α-Lipoic Acid, Dihydrolipoic Acid, and Lipoyllysine

Method	Analytical Parameters	Sample type Sample Preparation Analyte	Content Recovery	Reference
		EH: pronase, subtilisin EX: ethanol LLys	R: 100%	
HPLC/CDD	CR: 1–60 μM α-LA CR: 1–30 μM DHLA LOD_{ST}: 0.01–0.05 μM	Cells Deproteinization with mono-chloroacetic acid α-LA, DHLA		Sen et al. (1999)
HPLC/CEAD	CR: 0.048–4.85 μM LOD_{ST}: 0.014 μM LOQ_S: 0.1–0.3 μg/g WW	Egg yolk, dried egg powder, mayonnaise, fresh potatoes, canned peas EX by SON in acetic acid (0.5%) /methanol α-LA	0.5–0.9; 1.3; 0.5–0.6, 1.5–4.2: 0.5–1 μg/g WW R: 67–94%	Durrani et al. (2010)

Note: AR: analytical range; CR: calibration range; LCL: linear calibration line; LR: linear range; LLR: logarithmic linear range; WR: working range; WR_T: working range (turbidimetry); WR_V: working range (voltammetry); LOD_{ST}: detection limit of the standard solution; LOD_S: limit of detection of the sample; LOQ_{ST}: limit of quantitation of the standard solution; LOQ_S: limit of quantitation of the sample; R: recovery; ST: stirring; SON: sonication; AH: acid hydrolysis; BH: base hydrolysis; EH: enzymatic hydrolysis; WW: wet weight; DW: dry weight; WT: wet tissue; DPV: differential pulse voltammetry; LSV: linear sweep voltammetry; FIA: flow injection analysis; CZE: capillary zone electrophoresis; TLC: thin layer chromatography; GC: gas chromatography, FID: flame ionization detector; FPD: flame photometric detector; MS: mass spectrometry; HPLC: high-performance liquid chromatography, UV: ultraviolet detection; FLD: fluorescence detection; ECD: electrochemical detection; CDD: coulometric dual electrode detection; CEAD: coulometric electrode array detection; ESI-MS: electrospray ionization mass spectrometry; TCEP: tris(2-carboxy-ethyl) phosphine hydrochloride; TBP: tributylphosphine; SBD-F: ammonium 4-fluoro-2,1,3-benzoxadiazole-7-sulfate; PIAA: *N*-(1-pyrene) iodoacetamide; ADAM: 9-anthryldiazo-methane: monobromobimane (mBBr); MBDSTFA: *N*-(*tert*-butyldimethylsilyl)*N*-methyl-trifluoroacetamide; DBQ: 2,6--dibromoquinone-4-chlorimide.

a glassy carbon electrode modified with multiwalled carbon nanotubes allows a reduction of the oxidation potential, increases the current of α-LA, and extends the linear range of the current on the concentration. α-LA was not adsorbed onto the modified glassy carbon electrode (Ziyatdinova et al. 2009) when pharmaceuticals were analyzed. The relatively high detection limit of 19 μM was due to the application of linear sweep instead of the more sensitive differential pulse voltammetry.

37.2.1.3 Potentiometry

A potentiometric lipoate-selective sensor (Abbas and Radwan 2008) also fulfills the demand for the determination of α-LA in dietary supplements. This lipoate membrane sensor can be used for direct potentiometric determination where it shows a response over a concentration range of 0.1–10,000 μM and a detection limit of 0.09 μM. Sampling rates greater than 30 injections per hour could be achieved by positioning the sensor in a flow-through cell and coupling it with flow injection analysis (FIA). Such a sensor has a life span of at least 6 weeks. The method is simple, and slightly expensive, but to exclude the influence of other ions present in dietary supplements on the response, the selectivity of the sensor has to be investigated.

The drawback of lacking selectivity of the direct methods for the determination of α-LA can be overcome by applying separation methods with appropriate detectors (Kataoka 1998).

37.2.1.4 Capillary Zone Electrophoresis

Methods have been published for the quantitative determination of α-LA in dietary supplements based on capillary electrophoresis (CZE) using an uncoated capillary and UV detection at 208 and 214 nm, respectively (Trentin et al. 2002, Sitton et al. 2004). α-LA was well separated from other compounds, such as ascorbic acid or cysteine, the analysis time was short, and it could be detected with sufficient sensitivity. The limit of detection and the limit of quantitation at 208 nm were 3.9 and 12 μM, respectively. A drawback was the adsorption of α-LA to the capillary wall after a few injections so that the capillary needed to be washed for further use (Sitton et al. 2004). The addition of β-cyclodextrin (β-CD) to the running buffer (20 mM phosphate buffer, pH 9.0, 1 mM β-CD) prevented the adsorption of α-LA on the surface of the capillary because β-cyclodextrin included α-LA as a guest in its cavity. Thus, α-LA could be reliably quantified in dietary supplements by the standard addition method (Trentin et al. 2002).

37.2.1.5 HPLC/UV

A reversed-phase chromatographic method was developed and validated for the determination of α-LA and other compounds in pharmaceutical dosage forms and dietary supplement tablets using UV detection at 332 nm (Aboul-Enein and Hoenen 2004). The calibration function was linear over a concentration range of 48.5–2423 μM. Capsules (150–250 mg) were analyzed and interferences by excipients in the tablets were not observed. In comparison to CZE-UV, the LOD and LOQ were high, 21 and 81 μM, respectively. Recently, Durrani et al. (2007) reported a reversed-phase chromatographic method with UV detection at 215 nm. In spite of the lack of a strong chromophore, the LOD was 0.12 μM and even dietary supplements containing low amounts of α-LA (0.4 mg/g) could be analyzed. The chromatograms were more complex than those of other high-performance liquid chromatography (HPLC) methods (Figure 37.2) but α-LA was well separated from matrix compounds.

37.2.1.6 HPLC/FLD

Fluorimetric detection after derivatization is more sensitive and selective than UV detection. Inoue et al. (2009) reduced α-LA to the corresponding dithiol compound with tributyl-phosphine (TBP) and derivatized the product with *N*-(1-pyrene) iodoacetamide (PIAA) to a dipyrene-labeled derivative. This intramolecular excimer-forming fluorescence precolumn derivatization method proved to be fast, simple, and suitable for HPLC analysis of α-LA in dietary supplements. Due to the high sensitivity and selectivity of the method, biological samples (urine) could also be analyzed. The detection limit of 0.003 μM was significantly lower than that of the HPLC/UV method by Durrani et al. (2007). A disadvantage may be that the direct determination of α-LA and DHLA cannot be performed, which would be of interest in biological samples.

A simultaneous determination of α-LA and DHLA is possible by labeling DHLA with 4-(aminosulfonyl)-7-fluoro-2,1,3-benzoxadiazole (ABD-F), then reducing α-LA to DHLA using tris(2-carboxyethyl)-phosphine hydrochloride (TCEP) and finally derivatizing the formed DHLA with ammonium 4-fluoro-2,1,3-benzoxadiazole-7-sulfate (SBD-F). Both derivatization reactions are relatively mild (Satoh et al. 2007). The derivatives were separated on a reversed-phase column by gradient elution and detected by fluorimetry (λ_{ex} = 380 nm, λ_{em} = 510 nm). The LODs were 0.03 μM and the LOQs were <0.1 μM. The recovery of α-LA determined by the standard addition method was 96%. Still, as DHLA is usually not included in nutritional supplements, the simultaneous determination of both forms is of greater importance for biological samples.

37.2.1.7 HPLC/CEAD

In 2007, a method based on reversed-phase chromatography coupled with a coulometric electrode array detector for the determination of α-LA was published (Durrani et al. 2007). It was shown that

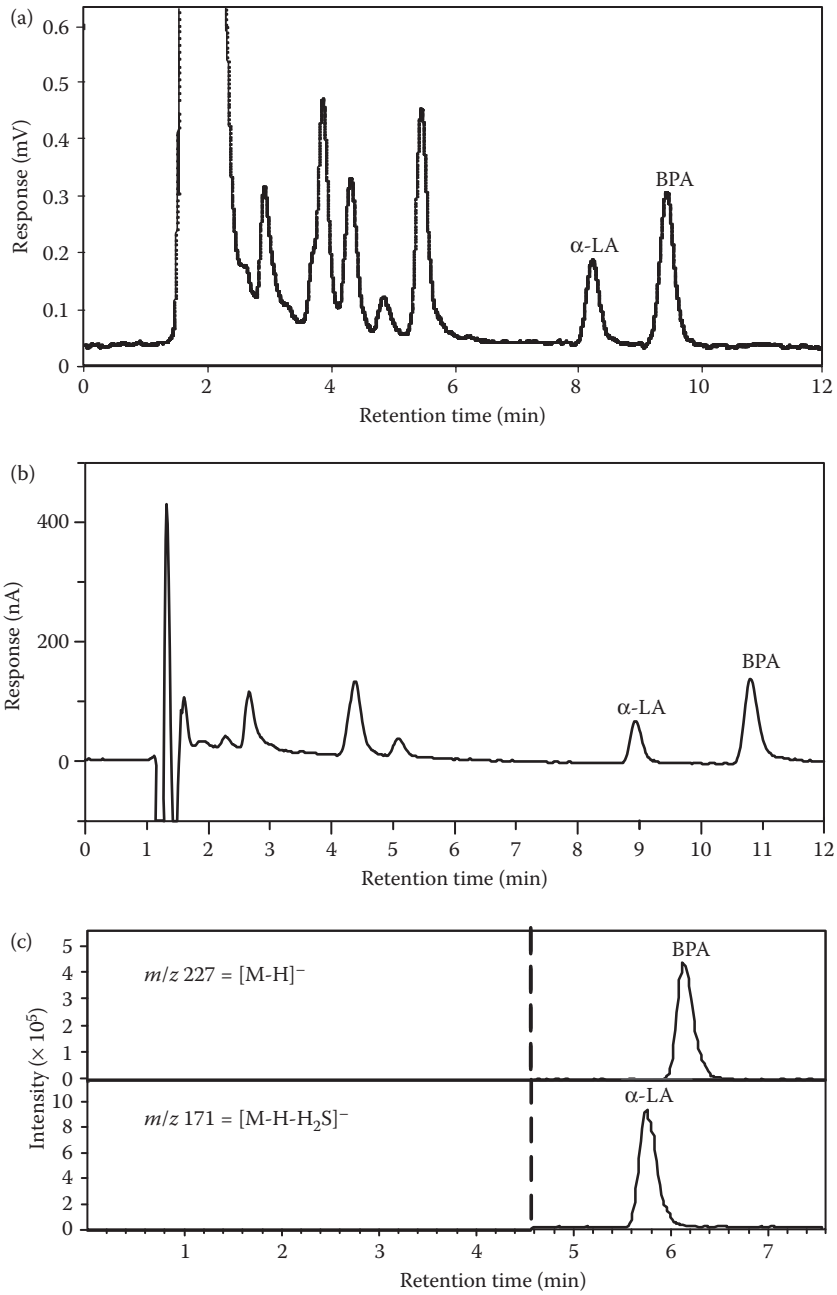

FIGURE 37.2 Chromatograms of a supplement extract measured with (a) UV, (b) CEAD, and (c) ESI-MS detectors. BPA: bisphenol A (internal standard).

α-LA can be detected at lower potentials (+500 and +550 mV) than with conventional electrochemical detection because the optimal detection potential corresponds approximately to the half-wave potential of the compound. The lower working electrode potentials are responsible for a decreased noise, a low detection limit (0.024 μM), and a decreased susceptibility to electrode fouling due to the large surface of the working electrodes. Due to this benefit, standard addition method and external calibration deliver similar data. The developed method is rapid and simple and the detection mode is more

selective and sensitive than UV detection (Figure 37.2) but not as sensitive as fluorescence detection (Inoue et al. 2009).

37.2.1.8 HPLC/ESI-MS

Durrani et al. (2007) also published an HPLC/ESI-MS method for the determination of α-LA in different dietary supplements. The authors showed that applying the negative multiple reaction monitoring (MRM) mode offered the possibility of complete fragmentation of the deprotonated molecular ion (*m/z* 205) into the fragment ion (*m/z* 171) that forms in part already in the ion source. This ion can be isolated in the ion trap and the corresponding peak area can be used for quantitation. This approach offers both higher selectivity due to the measurement of the fragment ion and higher sensitivity because the original ion and the fragment ion created from the deprotonated molecular ion in the trap contribute to the total response. The chromatograms were clean (Figure 37.2) due to the high selectivity of the detection mode. The detection limit was 0.015 μM. Comparison of the contents of α-LA in six dietary supplements obtained with HPLC/CEAD and HPLC/UV confirmed the reliability of the method.

37.2.2 Food from Animal and Vegetable Sources

37.2.2.1 Sample Preparation

The analysis of α-LA in food of vegetable or animal origin may be a complicated issue because α-LA can occur not only in the free form but may also be bound to proteins. In addition, DHLA, the reduced form of lipoic acid, and LLys may be present both in biological and in vegetable samples. Therefore, different sample preparation methods are necessary to determine α-LA, DHLA, LLys, and the total lipoic acid content in those samples. One can distinguish between nonhydrolytic methods for the determination of free α-LA and hydrolytic methods (Figure 37.3) to analyze total or protein-bound α-LA.

37.2.2.1.1 Nonhydrolytic Methods

Swatditat and Tsen (1973) extracted α-LA from wheat flour and wheat germ with a mixture of chloroform/methanol/water. The extractability of α-LA indicates that at least one part of the total α-LA content is rather weakly associated with proteins than covalently linked. The extraction method was rapid and recovered more than 91% of α-LA from wheat flour.

α-LA can also be extracted with 0.5% acetic acid in water/methanol or water/ethanol by accelerated solvent extraction (ASE) or ultrasonication (Durrani 2008). Accelerated solvent extraction gave cloudy extracts in some cases so that a solid-phase extraction clean-up step was necessary. Extracts obtained by sonication did not require any purification and could be injected into the HPLC system after evaporation and uptake into the mobile phase. Under these experimental conditions, the protein-bound lipoic acid is not cleaved so that the free α-LA can be determined. The recovery of free α-LA applying ultrasonication at room temperature was 96%.

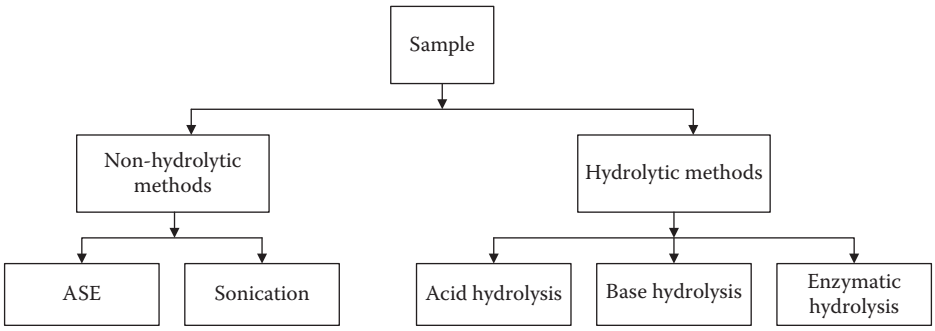

FIGURE 37.3 Schematic representation of hydrolysis methods. ASE: accelerated solvent extraction.

37.2.2.1.2 Hydrolytic Methods

These methods offer the possibility to analyze the total α-LA content or the protein-bound LLys.

Acid hydrolysis: In order to cleave covalently protein-bound α-LA in food samples, more drastic conditions have to be chosen. Shih and Steinsberger (1981) hydrolyzed eggs and livers of chicken with 12 M sulfuric acid for 6 h at 125°C, extracted the hydrolysate with benzene, and determined α-LA by GC with a flame ionization detector after derivatization. The overall recovery using ^{14}C lipoic acid was found to be 34%.

Swatditat and Tsen (1973) observed that losses of α-LA extracted from wheat flour depended on the concentration of hydrochloric or sulfuric acid (1–6 M). Maximum losses were up to 74%. On the other hand, White (1981), after hydrolyzing bacteria, tissues, and cow's milk with 6 M hydrochloric acid at 120°C for 4 h under nitrogen in the presence of a mixture of lipoic acid homologs (e.g., DL-1,2-dithiolane-3 butyric acid), obtained recoveries >87%.

Mattulat and Baltes (1992) reduced the concentration of sulfuric acid to 2 M and hydrolyzed liver, kidney, and meat samples at 120°C for 7 h, extracted α-LA with diethyl ether, cleaned the extract by solid-phase extraction (SPE), and determined α-LA by GC/MS after derivatization. Due to the less drastic conditions, the recovery was between 60% and 70%. Similar recoveries (70%) were obtained for different foods after hydrolysis with 9 M sulfuric acid at 120°C for 1 h (Hayakawa et al. 2007).

Base hydrolysis: Base hydrolysis can also be used to release α-LA from complex samples. Kataoka et al. (1997) hydrolyzed food samples in 2 M potassium hydroxide solution at 110°C for 3 h in the presence of 4% bovine serum albumin. They then added 2-mercapto-ethanol to prevent oxidation and hydrochloric acid for acidification and subsequently extracted α-LA with dichloromethane. The pooled dichloromethane extracts were evaporated to dryness and the residue was redissolved in sodium hydroxide solution prior to derivatization and analysis by GC with flame photometric detection (FDP). Although α-LA is more resistant under basic conditions than under acidic conditions and the addition of albumin prior to extraction prevents the oxidation of α-LA to thiosulfinate or thiosulfonate, it was estimated that 20–30% of α-LA present in the sample was lost during base hydrolysis (Kataoka et al. 1993, 1997).

Due to the instability of α-LA and DHLA under strong acidic or basic conditions, several authors recommended to perform acid and base hydrolysis in the presence of an inert gas (Teichert and Preiss 1995, 1997, D'Amico et al. 2004, Sgherri et al. 2008).

Enzymatic hydrolysis: To prevent α-LA and LLys from damage, and also to discriminate the free from the protein-bound α-LA, enzymatic hydrolysis can be recommended. The enzyme subtilisin can be used to hydrolyze lipoic acid bound to lysine in human plasma (Teichert and Preiss 1997). The α-LA-containing mixture has to be then purified on a polyamide column before analysis. Protein-bound lipoyl groups of bovine or rabbit tissue can be liberated in the form of LLys by protease digestion and assayed for LLys (Akiba et al. 1998).

Satoh et al. (2008) hydrolyzed animal tissues with several enzymes such as pronase E and subtilisin A to liberate LLys from proteins. In the case of spinach, cellulase was additionally added to break down the cellulose cell walls. This was found to double the amount of LLys released from those samples. After deproteinization, centrifugation, and fluorescence derivatization, the filtered sample solution could be analyzed without further purification. The recovery of LLys was between 99% and 107% and the relative standard deviation of the method ranged from 4.5% to 17.2%. Hayakawa and Oizumi (1989) used only pronase to liberate LLys from beef heart α-ketoglutarate dehydrogenase and detected α-LA by HPLC/UV. However, as reported by Lodge et al. (1997), the content of LLys determined by mild enzymatic hydrolysis may be underestimated because some LLys may remain inaccessible to protease digestion.

37.2.2.2 Microbiological Assay

A microbiological assay (Herbert and Guest 1975) is performed by incubating LA-deficient organisms, which contain apo-enzymes of the pyruvate and α-ketoglutarate dehydrogenase complexes with test extracts or standard samples of DL-α-lipoic acid. When α-LA is incorporated into the dehydrogenase complexes, pyruvate added in excess is oxidized. The oxidation of pyruvate is detected by voltammetric measurement of the current at an oyxgen electrode. The response of the electrode correlates with the α-LA concentration. This assay is accurate over the range of 24.2–242 pmol of α-LA/culture. The turbidimetric assay is based on the ability of the inoculum to give an increase of turbidity with test extracts and standard DL-α-LA, which can be measured over a range of 1–9.7 pmol of α-LA/culture.

37.2.2.3 Titration

If lipoic acid is present in concentrations between 0.24 and 9.7 μM in protein hydrolysates, it can be reduced with Sn(II) and titrated with *o*-hydroxymercurybenzoic acid in the presence of ethylenediaminetetraacetic acid (EDTA) and the indicator dithiofluorescein. The relative standard deviation of this method was about 20% (Wronski 1987).

37.2.2.4 Colorimetric Assay

This method involves acidification of the sample (animal tissue homogenate) with hydrochloric acid and separation of α-LA from LLys by extraction into ethyl acetate (Garganta and Wolf 1996). Lipoic acid reacts with 2,6-dibromoquinone-4-chlorimide (DBQC) to a colored product. The absorbance of the product was measured at 440 nm and a linear relationship between absorbance and α-LA concentration up to 111 μM was observed. A drawback of this method is that the rapid color formation is followed by slow degradation of the colored product. In addition, the formation of the colored product is influenced by the concentration of hydrochloric acid and the content of water in ethyl acetate.

The above-mentioned methods are limited by a lack of sensitivity and specificity. The specificity can be enhanced by using enzymes and antilipoic acid antibodies.

37.2.2.5 Enzymatic Assay

The enzyme lipoamide dehydrogenase catalyzes the reduction of LLys to dihydrolipoyllysine in the presence of NAD^+ and glutathione disulfide. The absorbance of NADH at 340 nm can be used for the quantification of lipoyl groups in the concentration range 1–5 μM (Konishi et al. 1996).

37.2.2.6 Enzyme Immunoassay

To avoid the decomposition of α-LA in strong acidic or basic conditions, MacLean and Bachas (1991) developed a homogenous immunoassay using pyruvate dehydrogenase complex. This complex is composed of three enzymes, pyruvate dehydrogenase, dihydrolipoyl transacetylase, and dihydrolipoyl dehydrogenase. The enzyme dihydrolipoyl transacetylase contains one lipoic acid as covalently attached prosthetic group, so that the complex can be considered as an enzyme–lipoic acid conjugate. Depending on the concentration of free α-LA incubated with antilipoic acid antibodies, an inhibition of the activity of the added pyruvate dehydrogenase complex is observed. This enzyme catalyzes the reaction of pyruvate with coenzyme A and NAD^+ to hydrogen carbonate, acetyl coenzyme A, and NADH. The production of NADH allows the reaction to be monitored at 340 nm. The detection limit was found to be 5 μM lipoic acid. This assay is highly specific and structurally similar compounds like biotin and biocytin do not disturb the concentration range of the assay. However, this method is not very sensitive and the preparation of the antilipoic acid antibodies is time consuming.

37.2.2.7 Thin Layer Chromatography

In complex hydrolysis mixtures, chromatographic methods offer the possibility to separate potentially disturbing compounds and to gain selectivity in this way.

Kozma-Kovacs et al. (1991) used thin layer chromatography (TLC) for qualitative analysis of α-LA from an acid yeast extract. The chromatogram was developed on a Kieselgel 60 F_{254} plate using butanol/ethanol/25% ammonia solution (80:10:10, v:v:v) and α-LA was detected with chromium trioxide or with molybdophosphoric acid. The detection limits of α-LA were 10 and 5 μg/spot, respectively. Yet, in spite of the development of a two-dimensional TLC, this method cannot be recommended for the determination of α-LA in the presence of fatty compounds.

Another example of a TLC method is the separation of the nonhydrolytic extract of wheat flour and germ on a silicagel G plate using ethylether/petrolether/acetic acid (60:40:20, v:v:v) (Swatditat and Tsen 1973). α-LA is then visualized by spraying the air-dried plate with potassium dichromate solution. A linear plot of peak area versus concentration was obtained from densitometry in a range of 6.8–34.5 μg of α-LA/spot.

37.2.2.8 Gas Chromatographic Methods

When the resolving power of TLC is not sufficient, instrumental chromatographic techniques such as HPLC or capillary gas chromatography are required. Capillary GC distinguishes itself by a high number of theoretical plates and, therefore, greater peak capacities and high resolution. If the selectivity of the GC method has to be enhanced, more selective detectors or more selective derivatization reagents have to be applied.

Derivatization: White (1981) extracted an acid hydrolysis mixture in the presence of internal standards with dichloromethane, reduced α-LA, and its homologs with sodium borohydride and converted these compounds into the *S*,*S*-dibenzylmethyl ester derivatives with benzylchloride and diazomethane. Alternatively, dihydrolipoic acid can be derivatized with ethyl chloroformate and methanol/hydrochloric acid to its *S*,*S*-diethoxycarbonylmethyl ester (DEOC) within 30 min (Kataoka et al. 1993, 1997). α-LA can also be derivatized with *N*-(*tert*-butyldimethylsilyl) *N*-methyl-trifluoroacetamide (MBDSTFA) after extraction of the acid hydrolysis mixture with diethylether, followed by purification with a saturated sodium bicarbonate solution and reextraction of α-LA in diethylether (Mattulat and Baltes 1992).

GC/FID: After derivatization to its methyl ester, the α-LA contained in brewer yeast extracts can be separated from matrix compounds on a capillary column (Supelcowax 10). The universal flame ionization detector gave rise to complex chromatograms (Kozma-Kovacs et al. 1991), so that the α-LA content could only be estimated. White (1981), on the other hand, separated α-LA in the presence of a mixture of homologs (C_7, C_8, and C_9 compounds) on a glass column packed with 3% OV-1 on Gas Chrom Q and corrected the influence of decomposition, extraction, and derivatization on the analysis result by adding 1,2-dithiolane-3-butyric acid and/or 1,2-dithiolane-3-caproic acid as internal standards. Samples containing more than 50 ng of lipoic acid/g could be easily assayed.

GC/FPD: After derivatization to *S*,*S*-diethoxycarbonylmethyl ester (DEOC), α-LA contained in the alkaline extracts of food samples could be selectively separated on a fused silica capillary of cross-linked DB-210 and sensitively detected with a flame photometric detector without any interference (Kataoka et al. 1993, 1997, Kataoka 1998). The calibration graph was linear between 1 and 24 μM and the detection limit was about 0.25 μM. The overall recoveries of the method were between 50% and 60%. This method is selective and sensitive, but the total method is time consuming (about 5 h).

GC/MS: Mattulat and Baltes (1992) reported the separation of the *N*-(*tert*-butyldimethylsilyl) *N*-methyl-trifluoroacetamide (MBDSTFA) derivative from other compounds on a DB-5 fused silica capillary column and detected them by a mass selective detector in the chemical ionization mode with methane as reactant gas. α-LA was identified by comparing the retention time and mass spectrum of the lipoic acid derivative with those of the standard. The quantitative analysis of α-LA was performed using the base peak (*m*/*z* 189). This method combines the high resolving power of the GC separation on a capillary column with the selective MS detection mode and offers a low detection limit (0.05 μM) and moderate recoveries (60–70%) after acid hydrolysis.

37.2.2.9 Liquid Chromatographic Methods

Gas chromatographic methods are sensitive and—depending on the derivatization reagent and on the detector—more or less selective but they lack the ability to distinguish between the different forms of α-LA. Liquid chromatography with several detectors offers the possibility to detect α-LA, DHLA, and LLys. These substances lack a strong chromophore needed for ultraviolet or fluorescence detection but in the case of α-LA and DHLA, this problem can be overcome by derivatization. Powerful alternative detection techniques include mass spectrometry and coulometric electrode array detection.

HPLC/UV: Even though UV detection mode at 340 nm is not sensitive, Hayakawa and Oizumi (1989) could determine LLys in beef heart α-ketoglutarate dehydrogenase and pyruvate dehydrogenase. This method has the advantage of increasing recoveries of endogenous lipoic acid, but the detection mode was neither selective nor sensitive enough to detect small amounts of LLys in the presence of matrix compounds in a tissue hydrolysate.

HPLC/FLD: Determination of α-LA, DHLA, and LLys by HPLC coupled with fluorescence detection requires derivatization with a fluorophore.

Witt and Rüstow (1998) converted α-LA into its monobromobimane (mBBr) [3-(bromomethyl)-2,5,6-trimethyl-1*H*,7*H*-pyrazolo[1,2*a*]-pyrazole-1,7-dione] derivative by first opening the dithiolane ring of LA by reduction with sodium borohydride and then derivatizing it with mBBr. The authors then separated the stable fluorescent adduct isocratically on a reversed-phase column and detected it by measuring the fluorescence (λ_{ex}: 385 nm, λ_{em}: 470 nm). The concentration–response curve was linear over two orders of magnitude, resulting in a useful range of quantitation from 0.02 to 3 μM. The recovery of ^{3}[H]LA found for animal tissue was 61%. The higher loss of lipoic acid was due to the additional steps necessary for derivatization. This method allowed a differentiation between α-LA and DHLA. In addition, the presence of other natural sulfohydrils in animal tissues such as glutathione and cysteine did not interfere with the detection of lipoic acid.

In order to determine α-LA in different foods, Hayakawa et al. (2007) performed acid extraction, derivatized α-LA with 9-anthryl-diazomethane (ADAM), and separated the lipoic acid–ADAM ester by affinity chromatography on a trypsin-treated avidin gel column. The eluent was a linearly increasing gradient of propanol in an acidic phosphate buffer system containing sodium chloride (pH 2.4). The fluorescence signal was measured at 412 nm after excitation at 365 nm. The detection limit was 0.03 μM. The recovery of the whole method was 70% (Hayakaswa et al. 2007).

A method for the direct determination of LLys in animal tissues and plant samples using fluorescence labeling method was published by Satoh et al. (2008). The authors reduced the disulfide bond in LLys with tris(2-carboxyethyl)phosphine to the dithiol form and subsequently labeled with SBD-F. The fluorescent derivative (SBD-LLys) was completely separated by a linear gradient on a reversed-phase column without any interference by endogenous substances in the sample and sensitively detected at 510 nm. The limits of detection and quantitation of lipollysine were approximately 0.007 and 0.022 μM, respectively. The whole method is characterized by a high recovery (99–107%).

HPLC/ECD and HPLC/CEAD: Electrochemical detectors coupled with HPLC offer the possibility to determine free α-LA, DHLA, and LLys (Lodge et al. 1997, Kataoka 1998) with sufficient sensitivity without any derivatization steps.

Teichert and Preiss (1995, 1997) used HPLC with amperometric detection at a glassy carbon electrode at +1.1 V to determine α-LA in human plasma after enzymatic hydrolysis. They also suggested that DHLA can be detected after chromatographic separation. The initial sensitivity was high but due to electrode fouling at the high detector potential a severe loss of electrode sensitivity was observed. Later, the same authors overcame this drawback by applying pulsed amperometric detection (Teichert and Preiss 2002).

The above-mentioned method was modified and applied to the determination of α-LA and DHLA in wheat roots and shoots. After acid hydrolysis, both compounds could be separated isocratically on a

reversed-phase column and the effect of irrigation with 20% sea water on the concentration of DHLA and α-LA, ascorbate, and glutathione was studied (D'Amico et al. 2004). HPLC with amperometric detection (ECD) was also used to investigate the influence of salt and stress conditions on the content of DHLA and α-LA in tomato berries with ripening (Incerti et al. 2006).

A further application of HPLC/ECD is the determination of α-LA and DHLA at nanomolar levels in biological samples using a dual Hg–Au electrode system (Handelman et al. 1994, Han et al. 1995). At the first electrode, α-LA was reduced to DHLA at –900 mV and then detected as DHLA on the downstream electrode at +50 mV. One major limitation was that the electrodes lost sensitivity after 30–50 injections and had to be reconditioned. This method was modified for the determination of LLys in foods from animal and plant sources by Lodge et al. (1997). At the flow-through system with dual gold electrodes coated with mercury, LLys was detected at +50 mV. Its presence was additionally confirmed by fast atomic bombardment (FAB) mass spectrometry. The total method had a high recovery (100 ± 5%), which was, however, attributed to the uncertainty present in the integrating software and the noise of the detector. A further problem was the spread of peak areas for different standard solutions on different days resulting in mediocre interday repeatability. The detection limit of this method is 0.1 μg/g sample.

HPLC with dual electrode coulometric detection on porous carbon electrodes was used to determine oxidized and reduced forms of α-LA in biological samples but a high working electrode potential (+850 mV) was necessary (Sen et al. 1999). Calibration functions for α-LA and DHLA were linear from 1 to 60 and 1 to 30 μM, respectively. An advantage was the low detection limit of 0.01 to 0.05 μM, which was at least 10 times lower than that of Au/Hg electrode-based detection (Han et al. 1995, Handelman et al. 1994).

One way to minimize the problem of electrode fouling and to reduce the oxidation potential for α-LA is to apply porous graphite working electrodes with great surfaces to enable full conversion of the substance at an array of flow-through cells, where the porous graphite working electrodes are set at increasing potentials (e.g., +300 to +700 mV). This detection mode enables the most sensitive detection of a compound at its half-wave potential. Durrani et al. (2010) published a simple but nevertheless selective HPLC/CEAD method coupled with a mild ultrasonic-assisted extraction for the determination of free α-LA in several foodstuffs. Free α-LA could be detected at +500 and +550 mV (Figure 37.4) and showed a linear relation between response and concentration in the investigated concentration range (0.048–4.85 μM). The limit of detection in standard solutions was 0.014 μM. The limit of quantitation (0.1–0.3 μg/g) and the recovery (67–94%) depended on the food matrix.

HPLC-ESI-MS: A simple LC-MS/MS method for the determination of α-LA in plasma of rats was developed by Trivedi et al. (2004). α-LA could be detected in the negative ionization

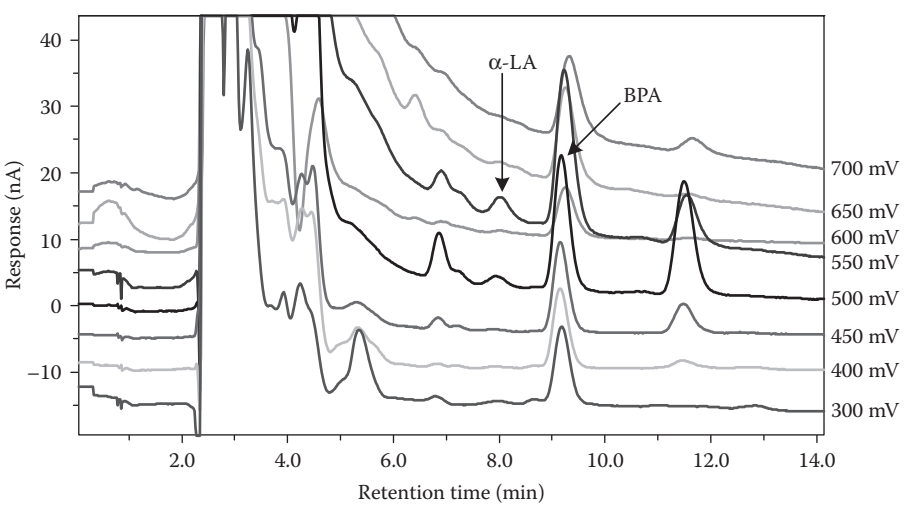

FIGURE 37.4 Coulometric electrode array chromatogram of fresh potatoes extracted by sonication.

multiple reaction monitoring (MRM) mode using electrospray ionization. The limit of quantitation was 0.024 μM. Recently, Durrani et al. (2010) published a method where the acetic acid/methanol extracts of foodstuffs were separated on a reversed-phase column and α-LA was determined by electrospray ionization mass spectrometry in the negative ionization mode. Analyses were carried out in the scan mode (70–240 *m/z*) and in the MRM mode (detection of fragment ion 171 $[M–H–H_2S]^-$ using bisphenol A as internal standard). In this way, the presence of α-LA in eggs, dried egg powder, mayonnaise, fresh potatoes, and canned peas could be confirmed.

37.3 Content of α-LA, DHLA, and LLys in Foods

α-LA was reported to be present in wheat flour in concentrations between 1 and 1000 ppm (Dahle and Sullivan 1960, Sullivan et al. 1961, Morrison and Coussin 1962, Swatditat and Tsen 1973). These highly different values can rather be attributed to the methods applied than to variety differences. In 1994, Vianey-Liaud et al. (1994) determined α-LA content in wheat germ of about 0.1 μg/g, but they could not detect α-LA in wheat flour. Shih and Steinsberger (1981) analyzed α-LA in chick livers and fresh chicken eggs. The levels of α-LA were 5–10 μg/g and 1–2 μg/g, respectively. Recently Durrani et al. (2010) determined free α-LA in fresh eggs, dried egg powder, mayonnaise, fine peas, and potatoes in concentrations ranging from 0.5 to 4.2 μg/g. In hearts, livers, kidneys, and muscles of bovines, calves, lambs, and pigs, values between 1.6 and 0.07 μg/g were found with the highest contents in hearts and the lowest values in muscles (Mattulat and Baltes 1992, Lodge et al. 1997). Red ripe tomatoes (D'Amico et al. 2004) contained more DHLA (8.51–17.1 μg/g DW) than α-LA (2.5–4 μg/g DW). Kataoka et al. (1997) did not detect α-LA in milk or egg white but found the highest concentration in beef tissue (2.36 μg/g) and lower concentrations in egg yolk (1.24 μg/g), pork (1.07 μg/g), or chicken (0.91 μg/g). Lipoyllysine levels (Akiba et al. 1998) were determined in rabbit and bovine tissues. Kidney and liver of cattle and rabbit were found to have the highest content of lipoyllysine in the range 2.4–2.7 and 0.8–0.9 μg/g, respectively. The content of LLys in heart was higher in cattle (1.3 μg/g) than in rabbit (0.4 μg/g).

To sum up, lipoic acid occurs in different forms in the human diet. It is found in abundance in animal tissues with high metabolic activity such as heart, liver, and kidney and to a lesser extent in fruits, vegetables, wheat flour, and rice bran. An overview of the content of α-LA, DHLA, and LLys is given in Table 37.1. The concentration ranges and limits of detection and quantitation were converted into uniform units to assure comparability. The definitions of the terms "linear, analytical, calibration, and working range" as well as of "limit of detection" and "limit of quantitation" were adopted from the original papers without any changes.

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38

Alliin and Allicin

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38.1 Garlic

Garlic (*Allium sativum* L.) has been used as a spice and medicine since ancient times.

Allicin (thio-2-propene-1-sulfinic acid *S*-allyl ester) is the main biologically active component of garlic (Rabinkov et al., 1998). Its biological activity is attributed to either antioxidant activity or thiol disulfide exchange. Antioxidant properties of both allicin and its precursor alliin (+*S*-allyl-L-cysteine sulfoxide) may be demonstrated by the Fenton oxygen-radical generating system.

Biological functions and health benefits of garlic include reduction of cancer risk in humans, improving immune system, antimicrobial, antioxidant, and antihypertensive activities.

One can find a great number of studies on all properties of garlic even though they are outside the scope of this chapter. A few examples are given in the next sections.

Chung (2006) investigated antioxidant properties of garlic compounds, alliin, allyl cysteine, allyl disulfide, and allicin. Alliin scavenged superoxide, while allyl cysteine and allyl disulfide did not react with superoxide. Allicin suppressed the formation of superoxide by the xanthine/xanthine oxidase system. Alliin, allyl cysteine, and allyl disulfide all scavenged hydroxyl radicals. Contrary to the previous reports, allicin did not exhibit hydroxyl radical scavenging activity. Alliin, allicin, and allyl cysteine did not prevent induced microsomal lipid peroxidation, but both alliin and allyl cysteine were hydroxyl scavengers, and allyl disulfide was a lipid peroxidation terminator.

The polyphenol contents and antioxidant activity of garlic and its ready-to-eat products were evaluated by Queiroz et al. (2009). Total phenolics were determined using Folin-Ciocalteu reagent. Antioxidant activity was measured by the DPPH assay (DPPH = 2,2-diphenyl-1-picrylhydrazyl radical), β-carotene/linoleic assay, and the Rancimat® method. The Rancimat method measures the preservation against induced oxidative rancidity. The result is a protection factor that may be used as a criterion for the effectiveness of antioxidants. Fresh garlic and ready-to-eat garlic products have antioxidant properties which are affected by processing factors and storage time. Fried garlic extract exhibited the best performance in all tests.

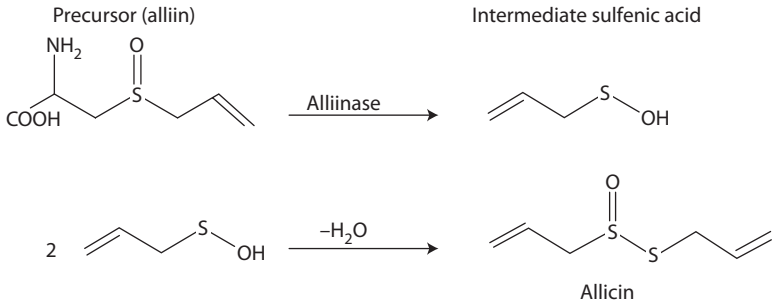

FIGURE 38.1 From alliin to allicin. (From Bocchini, P. et al. *Anal. Chim. Acta*, 441, 37–43, 2001. With permission.)

Onion and garlic (Lanzotti, 2006) are common food plants, rich in several phytonutrients, recognized as important elements of the Mediterranean diet. They are also used in the treatment and prevention of a number of diseases such as cancer, coronary heart disease, hypercholesterolemia, and many others. Thiosulfinates, volatile sulfur compounds, are involved in these processes. Other more polar compounds of phenolic and steroidal origin also show interesting pharmacological properties. These nonvolatile compounds include sapogenins and saponins.

38.2 Alliin and Allicin

By cutting or crushing a garlic clove, alliinase (alliin lyase—EC 4.4.1.4) transforms alliin into allicin, which is characteristic for the garlic flavor (Figure 38.1).

This is very unstable and degrades to organosulfur compounds. In Table 38.1, alliin, allicin, and related compounds are enumerated.

38.3 Detection Methods

The most frequently used method to quantify alliin and allicin is high-performance liquid chromatography (HPLC) in combination with UV-detection. Table 38.2 lists a number of methods published until now. Only a few methods deal with gas chromatography (GC) or other analysis techniques.

TABLE 38.1

Alliin, Allicin, and Related Compounds

Alliin	: *S*-Allyl-L-cysteine sulfoxide
SAC	: *S*-Allyl-L-cysteine-deoxyalliin
GLUAICs	: γ-Glutamyl-*S*-allyl-L-cysteine
GLUPeCs	: γ-Glutamyl-(*trans*-1-propenyl)-L-cysteine
GLUPPheAla	: γ-Glutamyl phenylalanine
Allicin	: Diallyl thiosulfonate
Methiin	: *S*-Methylcysteine-*S*-oxide
Isoalliin	: *S*-(1-Propenyl)cysteine-*S*-oxide
Propiin	: *S*-Propylcysteine-*S*-oxide
Ethiin	: *S*-Ethylcysteine-*S*-oxide
Butiin	: *S*-Butylcysteine-*S*-oxide

TABLE 38.2

Recent Analysis Methods of Alliin, Allicin, and Related Products

Chromatographic Technique/ Detection	Column Type/ Wavelengths or ΔE/ Elution Mode	Remark	Compounds Tested	Reference
HPLC-UV	RP-C_{18}/220 nm/isocratic	—	2, 3, 4	Lawson et al. (1991)
HPLC-UV	RPTMS/210 nm/isocratic	—	1	Mochizuki et al. (1989)
HPIPC-Fl	RP-C_{18}/405–480 nm/ isocratic	Ion-pairing reagent: tetra-n-butylammonium bromine	1	
GC-MS	Porous-layer open tubular/-/-	—	4	Saito et al. (1989)
HPLC-UV	RP-C_{18}/337 nm/SMGE and isocratic	Precolumn derivatization: OPA-tert.-BuSH	1	Ziegler and Sticher (1989)
HPLC-ED	RP-C_{18}/750 mV/SMGE and isocratic	—		
HPLC-Fl	RP-C_{18}/230–420 nm/ SMGE and isocratic	—		
HPLC-DAD	RP/260 and 337 nm/ gradient and isocratic	Precolumn derivatization: OPA-tert.-BuSH	1, 2, 3	Mütsch-Eckner et al. (1992)
HPLC-FL	RP/230–420 nm/gradient and isocratic	—		
HPLC-DAD	RP-C_{18}/240 nm/isocratic	—	4	Ferary et al. (1996)
HPLC-MS	RP-C_{18}/-/isocratic	—		
GC-FID	Wall-coated open tubular/-/-	Precolumn derivatization: ECF-NaI + acetyl chloride	1	Kubec et al. (1999)
HPLC-UV	RP-C_{18}/335 nm/gradient	Precolumn derivatization: OPA-2-methylpropanethiol	1	Krest et al. (2000)
HPLC-DAD	RP-C_{18}/254 nm/isocratic	Postcolumn photochemical	4	Bocchini et al. (2001)
HPLC-ECD	RP-C_{18}/1,7 V/isocratic			
HPLC-UV	RP-C_{18}/337 nm/isocratic	Precolumn derivatization: OPA-tert.-BuSH	1	Iberl et al. (1990)
HPLC-UV	RP-C_{18}/254 nm/isocratic	—	4	
HPLC-DAD	RP-C_{18}/254 nm/gradient	Precolumn derivatization: PITC	1	Auger et al. (1993)

Source: From Arnault, I. et al. *J. Chromatogr. A*, 991(1), 69–75, 2003. With permission.

Note: 1: Alliin; 2: GLUAICs; 3: GLUPeCs; 4: Allicin; OPA: *o*-phthaldialdehyde; PITC: phenylisothiocyante; DAD: diode-array detection; FL: fluorimetric detection; RP: reversed-phase; ED: electrochemical detection; ECF: ethyl chloroformate; IP: ion-pair; SMGE: selective multisolvent gradient elution; FID: flame ionization detection; -: no data.

38.3.1 Spectrophotometric Methods

A simple spectrophotometric assay for the determination of allicin and alliinase activity was described. This assay was based on the reaction between 2-nitro-5-thiobenzoate (NTB) and allicin but this reagent is not commercially available and must be synthesized (Miron et al., 1998).

NTB reacts with the activated disulfide bond –S(O)–S– of allicin, forming the mixed-disulfide allylmercapto-NTB, and may be characterized by NMR. The method can be used for determination of allicin and total thiosulfinates in garlic preparations and garlic-derived products. The method was applied for determination of pure alliinase activity and for the assay of the enzyme activity in crude garlic extracts.

Miron et al. (2002) described the quantitative analysis of alliin and allicin, as well as of alliinase activity with 4-mercaptopyridine (4-MP), a commercially available chromogenic thiol. The assay is based on the reaction of 4-MP ($\lambda_{max} = 324$ nm) with the activated disulfide bond of thiosulfinates –S(O)–S–, forming the mixed disulfide, 4-allylmercaptothiopyridine, which has no absorbance at this region. The structure of 4-allylmercaptothiopyridine was confirmed by mass spectrometry. The method

was used for the determination of alliin and allicin concentrations in their pure form as well as of alliin and total thiosulfinates concentrations in crude garlic preparations and garlic-derived products, at micromolar concentrations. The 4-MP assay is an easy, sensitive, fast, cost-effective, and highly efficient throughput assay of allicin, alliin, and alliinase in garlic preparations.

38.3.2 High-Performance Thin-Layer Chromatography

A precise, specific, sensitive, and accurate high-performance thin-layer chromatography method was developed by Kanaki and Rajani (2005). Alliin was resolved on silica plates with *n*-butanol–acetic acid–water (6 + 2 + 2, v/v) as the mobile phase. After derivatizing with ninhydrin, peak areas were measured at 540 nm. A measure of 0.5 g of fresh garlic clove was crushed in 17.5 mL methanol for the inactivation of alliinase. After addition of 7.5 mL water, the mixture was kept at room temperature for 1 h. After filtration, the retained solid was further macerated with methanol–water (7 + 3, v/v) and filtered again.

Garlic preparations (10 capsules or 10 tablets) were mixed together. Samples were macerated with methanol–water (7 + 3, v/v) at room temperature for 1 h.

The content of alliin in raw garlic was 1.58 ± 0.05% (w/w) and in formulations between 2.35 and 4.81 mg/unit.

38.3.3 Gas Chromatography

Steam distillation (SD), simultaneous distillation and solvent extraction, solid-phase trapping solvent extraction, and headspace solid-phase microextraction (HS-SPME) were compared as extraction techniques for the determination of Korean garlic flavor components by Lee et al. (2003). Determination was performed by GC-MS. The predominant flavor components were diallyl disulfide, allyl sulfide, and diallyl trisulfide. The most efficient fiber of five types assayed in HS-SPME was DVB/CAR/PDMS.

38.3.4 Liquid Chromatography

Ichikawa et al. (2006) developed an HPLC method to determine four sulfoxides and three γ-glutamyl peptides in garlic. Extraction was with 90% methanol solution containing 0.01 M HCl. Alliin, isoalliin, methiin, and cycloalliin were separated by NP-HPLC using an aminopropyl-bonded column.

A high-performance ion-exchange chromatography method was worked out to assay alliin (Yuan et al., 2009). Analysis was carried out on an SCX column (250 mm × 4.6 mm, 5 μm). The mobile phase was a phosphate buffer solution (pH 2.0). UV detection was at 214 nm.

Samples of fresh garlic of different areas in Xinjiang were determined by HPLC (Tang et al., 2007). Garlic samples were disposed in microwave for alliinase deactivation. The content of alliin ranged between 0.657% and 2.474%.

Apawu (2009) developed an HPLC method for alliin detection. The mobile phase consisted of 30:70% methanol:water and 0.05% sodium dodecylsulfate. The column was C18 (3.9 × 150 mm, 5 μm). UV detection was at 210 nm.

Min et al. (2009) worked out an HPLC-UV method for the detection of alliin and allicin in garlic. Allicin was analyzed with a YMC-Pack ODS C18 (25 cm × 4.6 mm) column and a methanol–water mobile phase (20–80 v/v). Alliin was analyzed on a similar column and a mobile phase methanol–water (85–15 v/v). Both compounds were detected at 214 nm. The contents of alliin and allicin in garlic were 1.67% and 0.93%, respectively.

The conditions of the RP-HPLC method (Xuesong et al., 2005) were Hypersil 0DS C18 (250 × 4.6 mm) column; UV detection at 200 nm and mobile phase of 5% methanol and 95% phosphate buffer (pH 5). Alliin content found in fresh garlic was 1.28 ± 0.07%.

After heating in boiling water, homogenization, and centrifugation of garlic and garlic product samples, the alliin content was determined by liquid chromatography (Zorbax TMS column—water as the mobile phase) and UV detection at 210 nm (Mochizuki et al., 1988). An alternative was fluorometric detection by ion-pairing chromatography (Nucleosil 5 C_{18} column) with tetra-*n*-butylammonium bromide.

Five *S*-alk(en)ylcysteine *S*-oxides are typically present in commonly consumed alliaceous plants, namely *S*-methyl-, *S*-allyl-, *(E)-S*-(1-propenyl)-, *S*-propyl-, and *S*-ethylcysteine *S*-oxides (methiin, alliin, isoalliin, propiin, and ethiin) (Kubec and Dadáková, 2009). HPLC, capillary electrophoresis, and GC analyses were used for their analysis. The developed HPLC/DnsCl (dansyl chloride) method gave good results for the analysis of various *S*-substituted cysteine derivatives. A C18 reverse-phase column (Rainin Microsorb-MV 100 Å, 250 mm × 4.6 mm, 5 μm) was used.

Twenty-four garlic ecotypes collected in Iran were analyzed by HPLC to evaluate the allicin content (Baghalian et al., 2005). HPLC consisted of Knauer C18 column (150 mm × 4.6 mm) and the mobile phase was methanol–water (50:50) at a flow rate of 0.7 mL/min. Detection was at 254 nm. Garlic was dried, homogenized, and extracted in 1% (v/v) solution of anhydrous formic acid in methanol (40/60). Allicin content varied from 1.61% to 13.03%.

HPLC analysis was carried out on the extracts of crushed leaves of Chinese chive (*A. tuberosum* Rottler) to detect methiin and alliin (Yabuki et al., 2010). HPLC separation was performed on a Cap-cell Pak SCX UG80 cation-exchangeable column (4.6 mm ID × 150 mm) with a mobile phase of 25 mM potassium dihydrogen phosphate (pH 2.5) at a flow rate of 0.4 mL min. Detection was at 220 nm. Detection limits for methiin and alliin were 80 ng and 100 ng, respectively. Sample preparation had different steps: crushing of leaves, extraction in ca. 73% ethanol, sonication, and centrifugation.

A quantitative method is described for the determination of allicin in garlic, using standard additions of alliin in conjunction with supercritical fluid extraction and HPLC with UV–vis detection (Rybak et al., 2004). The use of an internal standard, allyl phenyl sulfone, resulted in a slight improvement of recovery and precision. Standard additions of alliin were converted to allicin *in situ* by endogeneous alliinase. This method was a convenient alternative for assessing the amount of allicin in fresh and powdered garlic.

38.3.5 Capillary Electrophoresis

Ma et al. (2009) developed a high-performance capillary electrophoresis method for determining alliin content in *A. sativum* L.

A fused-silica capillary tube (50 μm ID—length 100 cm) (GL Science) was used to measure methiin and alliin in vegetables (Horie and Yamashita, 2006). The experiments were performed with an Agilent CE system (SD-CE) with a diode-array detector. The detected alliin and methiin contents in *Allium* were 12.67 and 1.18 g/kg, respectively.

Kubec and Dadáková (2008) developed an electrokinetic capillary chromatographic method for the determination of the whole range of *S*-alk(en)ylcysteine-*S*-oxides (methiin, alliin, isoalliin, propiin, ethiin, and butiin). Analyses were carried out on a Spectraphoresis 2000 connected to a UV–vis scanning detector (Thermo Separation Products, Fremont, CA, USA). The authors used a Supelco fused-silica capillary (70 cm × 75 μm ID). Detection wavelength was 265 nm. The separation buffer, pH 9.2, consisted of 20 mM sodium tetraborate, 20 mM sodium dodecyl sulfate, and 10% (v/v) MeOH. Analytes were extracted from vegetables with MeOH and derivatized with fluorenylmethyl chloroformate. The method was evaluated in *Allium* and Brassicaceae species.

38.3.6 Biosensors

Alliinase catalyzes the reaction of cysteine sulfoxides (e.g., alliin) into thiosulfinates (e.g., allicin), pyruvic acid, and ammonia. Ammonia may be used for biosensoric detection of cysteine sulfoxides (Keugen et al., 2003a). The amount of enzymatically formed ammonia is proportional to the content of alliin or other cysteine sulfoxides. The ammonia is determined by an ammonia gas electrode (Figure 38.2). The working conditions of an Orion™ 95-12 ammonia electrode have been optimized. Further on, a pH-sensitive electrolyte/insulator/semiconductor (EIS) was also combined with immobilized alliinase. A measure of 0.2–0.8 g of fresh garlic were heated for 10 min in 20 mL MeOH under reflux, crushed in a mortar, and further extracted in MeOH with the addition of 20 mL H_2O. The extract was filtered and washed with 3 × 3 mL MeOH. The evaporated filtrates were redissolved in phosphate buffer (pH 7.0, 0.06 M with the addition of 0.17 M NaCl).

In real samples, the enzyme is highly specific for alliin and isoalliin.

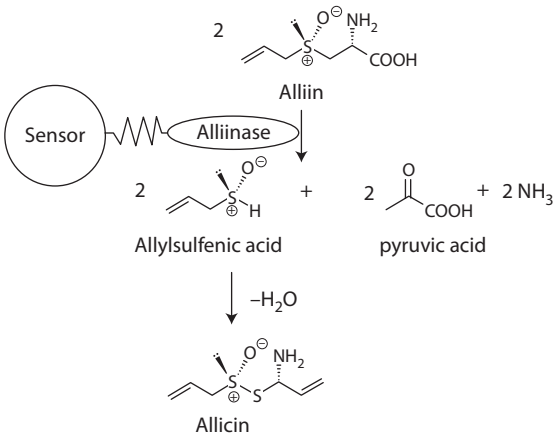

FIGURE 38.2 Alliinase catalyzed reaction of cystein sulfoxides (e.g., alliin) into thiosulfates (e.g., allicin), pyruvic acid, and ammonia. (From Keugen, M., et al. *Biosens. Bioelectron.*, 18, 805–812, 2003a. With permission.)

The same authors (Keugen et al., 2003b) developed an alliin-specific biosensor exploiting immobilized alliinase. The formed ammonia was detected by a potentiometric sensor based on an ammonia electrode or a pH-sensitive EIS layer structure made of Al/*p*-Si/SiO_2/Si_3N_4. The obtained sensitivities were comparable to those obtained by HPLC. Alliin concentrations below 6×10^{-6}M could be quantified.

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Section XI

Sweeteners

39

Methods of Analysis of Acesulfame-K and Aspartame

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39.1 Introduction

Acesulfame-K (ACS-K) and aspartame (ASP) are characterized as artificial high-intensity sweeteners. They are also called nonnutritive sweeteners. These two are commonly used in foods, beverages, and confectionery products. They are exclusively used for low-calorie intake that helps obese consumers to maintain their weight. It is also medically suggested to diabetics to use foods containing these two artificial sweeteners instead of sugar.

Aspartame (*N*-L-α-aspartyl-L-phenylalanine-1-methylester) is a dipeptide (Figure 39.1). It is 200 times sweeter than sugar and its sweet taste is almost identical to that of sucrose but it lacks in aftertaste. This sweetener was discovered accidentally in 1965 by James M. Schlatter at G.D. Searle Co. This nutritive sweetener provides 4 cal/g, but the amount required to give the same sweetness as sugar is only 0.5% of the calories (Sardesai and Waldshan 1991). It is a white crystalline powder with a molar mass of 294.31 and a calorie value 17 kJ/g. Because of its high sweetness, the amounts that can be used are small. Although it is relatively stable in a dry form, the compound can undergo pH- and temperature-dependent degradation; for this reason, ASP is undesirable as a baking sweetening agent. Below pH 3, ASP is unstable and hydrolyzes to produce aspartylphenylalanine and above pH 6, it changes to form 5-benzyl-3,6-dioxo-2-piperazineacetic acid (Zygler et al. 2009). ASP was approved in 1981 and produced for the market as "Nutra-Sweet." For the first time, dairy products (ice cream, yogurt, etc.) were calorie reduced and could be sold with the prefixes "diet" or "light" (Weihrauch and Diehl 2004).

The metabolism of ASP was extensively studied. The major point that was concluded from metabolism studies is that ASP is broken down in the gastrointestinal tract to its constituents (i.e., aspartic acid, phenylalanine, and methanol) (Sardesai and Waldshan 1991).

ASP is probably the most controversial artificial high-intensity sweetener on the market. It has been reported that ASP can cause medical effects to the consumers; these medical effects are multiple sclerosis, systemic lupus, brain tumors, and methanol toxicity (Zygler et al. 2009). Recent reports suggest that ASP can be related with cancer, lymphomas, and leukemia after investigations using rats. In 2006 and 2007, these findings were published by the European Ramazzini Foundation of Oncology and Environmental Sciences (Soffritti et al. 2007). These allegations regarding ASP and its effects on humans have been carefully evaluated by scientists at regulatory agencies around the world including the European Union and the United States. The conclusion was that ASP does not cause anything of the effects mentioned. In March 2009, European food safety association (EFSA) investigated the results of these studies and found no indications of any genotoxic or carcinogenic potential of ASP (EFSA 2009).

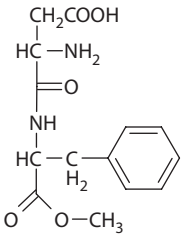

FIGURE 39.1 Chemical structure of aspartame.

The only disadvantage of ASP is that it cannot be used by individuals suffering from phenylketonuria. For this reason, the foodstuff in which ASP is a constituent must be labeled so that it cannot be used for phenylketonurics as it contains phenylalanine. The intake of ASP must be limited in these individuals (MacKinnon 2003).

Acesulfame-K (6-methyl-1,2,3-oxathiazin-4(3H)-one-2,2-dioxide, MW 201.24) was an accidental discovery in 1967 from studies at Hoechst Corporation in West Germany on novel ring compounds (Sardesai and Waldshan 1991). Its full name is potassium acesulfame and consists of a 1,2,3-oxathiazine ring, a six-heterocyclic system in which oxygen, sulfur, and nitrogen atoms are adjacent to each another (Figure 39.2). ACS-K is about 200 times sweeter than sucrose, but has a slight bitter aftertaste (especially at high concentrations); it is soluble in water and has an extremely long storage life. Unlike ASP, it is stable at high temperatures, which makes it ideal for use in baking (Zygler et al. 2009). ACS-K is not metabolized by the body and is excreted by the kidneys unchanged. A large number of pharmacological and toxicological studies have been conducted and the sweetener has been found to be safe (Sardesai and Waldshan 1991). ACS-K can be used as a sweetening agent in a wide range of products (low-calorie products, diabetic foods, sugarless products). ACS-K is suitable for low-calorie beverages because it has a pronounced stability in aqueous solutions and is even suitable for diet soft drinks that maintain a low pH.

A substantial number of low-calorie beverages, however, are sweetened with mixtures of ACS-K and ASP. The combination of ACS-K and ASP has led to an improvement of the quality of sweetened products. In soft drinks, a combination of these two has found wide application. The production and use of combination of these two sweeteners to create a mixture in which each molecule contains both sweeteners is constantly rising. The compounds that contain combined sweeteners are called "twinsweets." The reasons for marketing these compounds are obvious. These two sweeteners together offer two very important advantages for the food industries. "Twinsweets'' offer a greater sweetness stability and longer stability as compared with the individual use of ASP or ACS-K. ASP and ACS-K exhibit quantitative synergy. When these two artificial sweeteners are used together, they provide a more potent sweetener than that when used independently. These beverages benefit from a synergism and an improved taste that is provided by such blends. For example, a sweetness level equivalent to approximately 10% of sucrose in beverage is replaced by concentrations in the range of 500–600 mg/L of ACS-K or ASP. If a blend of the two sweeteners is used, the same sweetness level can be achieved by using only 160 mg/L of each of these sweeteners. The blend to achieve this is ca. 60:40 (%) of ASP–ACS-K, which in reality is a unimolar ratio. ACS-K has good solubility in water and, therefore, highly concentrated solutions suitable for household use can be manufactured. No problems of stability have to be anticipated for solutions in normal storage conditions. Similarly, no problems have been reported for the dissolution of tablets or powders. When blending ACS-K with other intense sweeteners for beverage applications, the blend ratio

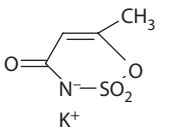

FIGURE 39.2 Chemical structure of ACS-K.

may depend on different factors, including the flavor or flavor type. For this reason, in orange-flavored beverages, considering the time intensity curves of sweetness and fruitness similar to sucrose-sweetened beverages, blends of ACS-K, and ASP (40:60) have been used. In raspberry-flavored beverages containing natural flavors, 40/60-to-25/75 (ACS-K/ASP) blend ratios were considered optimum, whereas beverages with artificial raspberry flavors are considered optimum with blend ratios of 50/50 to 20/80. Nowadays, emerging trends in beverages include replacement of sugar in fully sugared beverages with intense sweeteners like ACS-K.

These two sweeteners are authorized for use in the EU and the United States.

ASP is referred to as a "first-generation sweetener." This generation was followed by a second generation of sweeteners. ACS-K belongs to this second generation. However, the new sweeteners have similar limitations to the older ones. The taste is often accompanied by a bitter and metallic aftertaste and does not provide the same taste of regular sugar. A key attribute that distinguishes sweeteners from other ingredients is their characteristic and pleasurable sweet taste and intensity. The standard for comparing sweetness is sucrose. The sweetness value assigned to sucrose is either 100 or 1.0, depending on the scale or scoring system used (Helstad 2006).

The approvals of new-generation sweeteners like ACS-K are too recent to establish any epidemiological evidence about possible carcinogenic risks (Weihrauch and Diehl 2004). Following the adoption of the European Commission (EC) Sweeteners Directive in 1994 (Commission of the European Communities 1994) and its implementation into the national laws, member states are required to establish a system of consumer surveys to monitor additive intake (European Commission 1994). For this reason, intake data are required through developed and validated methods for the determination of these two artificial sweeteners.

The Acceptable Daily Intake (ADI) has been defined by the World Health Organization (WHO) as "an estimate by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) of the amount of a food additive, expressed on a bodyweight basis, that can be ingested daily over a lifetime without appreciable health risk," and is based on an evaluation of available toxicological data. For example, in Europe, the ADI is set at 9 mg/kg of body weight/day for ACS-K (Wilson et al. 1999). For ASP, there is a safety margin, even in high-consuming diabetics (Ilbäck et al. 2003). The Food and Drug Administration (FDA) has set the ADI for ASP at 50 mg/kg of body weight/day. An ADI of 40 mg/kg body weight per day set by the committee of experts of the Food and Agriculture Organization (FAO) and the WHO is not likely to be exceeded, even by children and diabetics. An EC report gives a theoretical maximum estimate for adults' consumption of 21.3 mg/kg body weight per day of ASP. However, the actual consumption is likely to be lower, even for high consumers of ASP. The report also gives refined estimates for children, which show that they consume 1–40% of the ADI. People with diabetes are high consumers of foods containing ASP; their highest reported intake varies between 7.8 and 10.1 mg/kg body weight per day. At the international level (JECFA), as well as for the United States (FDA), the ADI for ACS-K has been set at 15 mg/kg body weight. As per the European level, on March 13, 2000, the ADI has been set at 9 mg/kg body weight [scientific committee on food (SCF)].

Generally speaking, in the EU, sweeteners are thoroughly assessed for safety by the EFSA before they are authorized for use. EU Directives 94/35/EC (European Commission 1994), 96/83/EC (European Commission 1996), 2003/115/EC (European Commission 2003), 2006/52/EC (European Commission 2006) define which sweetener has approved to be added to food products and beverages.

Today, ACS-K and ASP are used in foods, including baked goods (dry bases for mixes), beverages (dairy beverages, instant tea, instant coffee, and fruit-based beverages), soft drinks (colas, citrus-flavored drinks, and fruit-based soft drinks), sugar preserves and confectionery (calorie-free dustings, frostings, icings, toppings, fillings, and syrups), alcoholic drinks (beer), vinegar, pickles, and sauces (sandwich spreads and salad dressings), dairy products (yoghurt and yoghurt-type products, puddings, desserts and dairy analogues, and sugar-free ice-cream), fruits, vegetables, nut products, sugar-free jams and marmalades, low-calorie preserves, and other food products (i.e., chewing gums, liquid concentrates, and frozen and refrigerated desserts). Hard-boiled candies can be manufactured using ACS-K as the intense sweetener. ACS-K brings the taste close to standard, sugar-containing products. In chocolate and related products, ACS-K can be added at the beginning of the production process (e.g., before rolling). It withstands all treatments including conching without detectable decomposition (Baron and Hanger 1998). In reduced-calorie baked goods, bulking agents like polydextrose substitute for sugar and flour may help

reduce the level of fats. ACS-K combines well with suitable bulking ingredients and bulk sweeteners and, therefore, allows production of sweet-tasting baked goods having fewer calories. In diabetic products, combinations of ACS-K and sugar alcohols such as isomalt, lactitol, maltitol, or sorbitol can provide volume and sweetness. Texture and sweetness intensity can be similar to sucrose-containing products.

A number of analytical methods based on different principles are available for the determination of ACS-K and ASP in a broad range of food matrices. The aim here is to present the available methodology for sample pretreatment and the available protocols of analysis.

39.2 Sample Pretreatment

Generally speaking, sample pretreatment cannot be avoided in most of the analytical methods for the determination of food additives. This step is very important because without it the food sample cannot be directly analyzed. With the term "sample pretreatment," we refer to sample preparation or/and sample cleanup prior to analysis.

The determination of sweeteners directly in foods can often not be achieved due to interferences. Often sample pretreatment is the most time-consuming step of the method of analysis. For most assays, the weight of the sample taken for quantitative analysis has to be known. Then, some preparative operations are likely necessary before an extraction can be performed. For the extraction of sweeteners, these operations that make the complete extraction easier are usually the change of volume and the change of pH prior to extraction.

Food samples are characterized as difficult matrices. The food matrix presents a great variability in its composition. Carbohydrates, proteins, lipids, minerals, preservatives, colors, thickeners, and vitamins may stand alone or coexist in a food matrix. All the components can interfere in the determination of sweeteners. Sample pretreatment procedures must be suitable with the method that the analyst will use, considering the instrumentation that he has available in the laboratory. The success of the method is often totally owed to the effectiveness of sample pretreatment and it depends on the accuracy (quantitative or/and qualitative) that the analyst wants to obtain ("fit for purpose").

The chemical and the physical properties of food vary. The variability in composition of a given food sample can be minimized with proper sample preparation. Generally speaking, preparation of food samples is achieved in four steps: (1) homogenization, (2) extraction, (3) cleanup, and (4) preconcentration.

Analysis of liquid samples does not require the homogenization step, because of their liquid state. After the sample preparation, some matrix components may still be present interfering in the analysis. They may coextract with analytes due to similar solubility in the solvents used for extraction. The presence of matrix interferences in a sample extract can contribute to problems on the accuracy of the method. The only way to resolve this problem is to further clean up the sample for analysis. Cleanup is achieved commonly by (1) solid-phase extraction (SPE), (2) dialysis, (3) liquid–liquid extraction (LLE), (4) precipitation, and (5) filtration.

Appropriate extraction and cleanup procedures maximize the recovery of the analytes. Optimal sample preparation can reduce analysis time, enhance sensitivity, and enable confirmation and quantification of analytes. Extraction, cleanup, and/or purification might be necessary, depending on the complexity of the sample and the sensitivity and selectivity of the method used (Self 2005).

The approach of determination of ACS-K and ASP in simple matrix is much easier and less time consuming than the determination of these two sweeteners in more complicated food sample matrix. Bulk samples of sweeteners have a much more simple pretreatment stage than complex food matrices. The interference of other food additives in the determination of sweeteners is more common in complicated food sample matrix. These additives may be in the same level of magnitude as the sweeteners or in a much higher level of magnitude causing interference in the determination. In bulk samples (e.g., tabletop solid tablets), additive compounds exist in a much lower quantity. Usually, three types of additives are added in bulk powders to deal with possible problems during the preparation of tablets. Glidants are added for dealing with a poorly flowing material. Pure lubricants give more effective mixing and antiadherents prevent the tablet adhering to the die. When we want to determine sweeteners in tabletop solid tablets, these are turned into powder. A portion of the powder is being weighed, directly dissolved in

ultrapure water, and transferred into volumetric flasks. Then according to the methods protocol, the next steps of determining ACS-K or/and ASP follow. Samples characterized by relatively simple matrix like liquid sweeteners and beverages can simply be diluted or dissolved in deionized water or in an appropriate buffer. In the case of carbonated drinks, the samples have to be degassed. Degassing is being done by sonication, by sparging with nitrogen or under vacuum.

All samples are filtered prior to the analysis and the extracts may need centrifugation. This simple sample preparation procedure is found in published procedures. It is very quick and cheap. In order to preconcentrate the analytes or/and remove the chemical interferences, we must take advantage of the SPE technique. It fractions, the compounds of our choice based on the affinity of the compound or a group of compounds to the stationary phase. The most frequently used SPE cartridges are the nonpolar C_{18} SPE cartridges. Their stationary packing material is constituted of nonpolar C_{18} chains. The protocol of an SPE procedure consists of four steps: (1) cartridge conditioning, (2) sample load, (3) cartridge wash, and (4) elution of analytes.

In the most common mode of SPE, an aliquot of the sample extract is loaded onto a previously conditioned SPE cartridge. The type of SPE-packing material, solvents, pH, and the flow rates need to be properly selected to retain analytes effectively within the cartridge. The interfering substances should be retained very strongly or not retained at all. As a result, weakly retained substances are readily removed from the cartridge during sample load and/or cartridge wash. Analytes are eluted during the elution step and interfering substances having a strong affinity to the sorbent stay adsorbed within the cartridge. The sensitivity of a final determination can easily be enhanced by evaporating the final SPE extract to dryness and reconstituting it with a smaller amount of a solvent of choice (preconcentration). SPE-based sample-preparation protocols seem to be the best available choice. They are simple, reproducible, reasonably quick, and inexpensive. They are universal and compatible with the most popular techniques used in food analysis (Tunick 2005).

Sweetened beverages include two types of beverages with carbon dioxide and two types of beverages without carbon dioxide. Beverages with carbon dioxide include soft drinks "light" and soft drinks sugar sweetened. Beverages without carbon dioxide also include the same types of beverages, soft drinks "light" and soft drinks sugar sweetened. In soft "light" drinks with carbon dioxide, a mixture of ASP and ACS-K is more often used to sweeten the products (Leth et al. 2007). Micellar electrokinetic capillary chromatography (MEKC) is a rapid method for the determination of artificial sweeteners in low-calorie soft drinks and is often used. The sample solutions of "light" soft drinks are prepared by diluting the products with an appropriate amount of deionized water. The solutions are then simply filtered through a 0.45 μm cellulose acetate filter before analysis (Thompson et al. 1995).

Today, the most common technique for the determination of artificial sweeteners in soft drinks is HPLC analysis. The sample preparation for HPLC analysis includes filtering through a 0.45 mm membrane filter and then the sample is ultrasonicated before the analysis. First, nectars are centrifuged, filtered through membrane filters, and then ultrasonicated (Lino et al. 2008). For the determination in Cola Drink, first a volume is accurately weighed into a volumetric flask, and then degassed in an ultrasonic bath. If the determination is obtained through HPLC analysis, the volume of cola drink is directly diluted with the mobile phase (Demiralay et al. 2006).

Gum samples are prepared by placing the sample in a flask and extracting with a mixture of glacial acetic acid, water, and chloroform. Hard- or soft-candy samples are shaken with water until dissolved (Biemer 1989). Milk and dairy products are homogenized prior to analysis and an aliquot of a homogeneous sample is transferred to a flask followed by the addition of distilled water. The mixture is thoroughly stirred and allowed to stand, and then filtered (Ni et al. 2009).

Sweeteners are determined in diet jams by mixing the jam with water, and sonicating the mixture. The mixture is made up to volume and then filtered through a 0.45 mm filter (Boyce 1999). Preserved fruit is grounded and homogenized. Then it is weighed into a volumetric flask, and water is added. This mixture is extracted ultrasonically and diluted to volume with water after cooling to room temperature. A volume of supernatant is applied to a conditioned Sep-Pak C_{18} cartridge (Chen and Wang 2001) and the subsequent SPE follows according to the previous protocol.

The determination of sweeteners with flow-injection analysis (FIA) methods is very common. It is reported that beverages, juices, strawberry sweets, and tomato sauce containing ACS-K and/or ASP can

be analyzed with the use of an FIA setup coupled with an ultraviolet (UV) detector. An adequate amount of beverage is taken, degassed, and diluted, adjusting the same conditions as the carrier. Finally, it is filtered through a 0.2 mm Millipore filter. For the analysis in juices, prior to the step of filtering, a centrifuge step may be necessary. In the case of strawberry sweets, an adequate amount is weighed and thoroughly crushed in a glass mortar, then dissolved in water with the aid of an ultrasonic bath, adjusting to the same conditions as the carrier. After that, it is centrifuged and filtered as above. Finally, in the case of tomato sauce, the amount is suspended in water, and then a portion is diluted, centrifuged, and then the same procedure is followed as for the beverages (Jiménez et al. 2009).

Fourier transform infrared (FTIR) spectroscopy is reported as a quick method for the determination of ACS-K in commercial diet food samples without the use of even an extraction procedure prior to analysis. Samples that are characterized as difficult food matrices, such as chocolate syrup, coffee drink, coffee creamer, cranberry juice, ice cream, and instant chocolate milk, can be easily analyzed. The ice cream sample is converted to mixture at 60 C in a water bath with continuous agitation. All other samples are used without any pretreatments. A portion of each food sample is mixed thoroughly with ultrapure water. The biggest advantages of direct measurement through FTIR spectroscopy are speed and lack of time-consuming sample pretreatment. The samples are then maintained at 40°C in an incubator followed by ultrasonication. Carrez I (3.6 gr potassium hexacyanoferrate trihydrate dissolved and made up to 100 mL with distilled water) and Carrez II reagent (7.2 g zinc sulfate heptahydrate dissolved to 100 mL with distilled water) solutions are added to samples, followed by centrifugation at 4°C. The supernatant is carefully separated using a syringe to avoid the fat layer for each sample and filtered. The water-soluble extract obtained is directly poured onto the surface of attenuated total reflectance–FTIR (Shim et al. 2008).

The simultaneous determination of other additives including ACS-K and ASP in some food samples is today very common. For example, a simultaneous determination in a food sample of soy sauce has been reported. For the pretreatment of this sample, it is placed in a volumetric flask and then diluted with water. After mixing thoroughly, a portion of the solution is added to a Sep-Pak C_{18} cartridge. Some additives that are not adsorbed by the packing material come through the cartridge and are collected as eluate A. Then water is passed through the cartridge to remove the interfering substances. The other additives that are absorbed onto the packing material were then eluted with acetonitrile–water (2:3 v/v) and collected as eluate B. Both eluates A and B are combined and poured into a volumetric flask, and diluted to volume with acetonitrile–H_2O (2:3 v/v). The same procedure can be followed for food sample of dried roast beef and sugared fruit. The only difference is that they are previously ground into fines with a grinder (Chen and Fu 1995).

39.3 Analytical Methodology

The number of methods for the determination of ASP and ACS-K in food samples is large and is classified according to the detection method. The method is selected as a compromise between the following factors: (1) accuracy, (2) precision, (3) cost, (4) detection limits, (5) selectivity, (6) safety, (7) sample throughput, (8) consumption of sample and reagents, (9) simple operation (automated or not), and (10) contamination risks.

However, three main parameters are important to select the method of analysis and these are as follows: (1) the reagents and apparatus of the method must, respectively, have a low cost; (2) we do not need a very sensitive method with a low-detection limit since the sweeteners fluctuate in the micromolar range and sensitive methods often are costly; (3) the sample throughput and the consumption of sample and reagents must be relatively low. The following analytical and detection techniques meet these criteria:

1. Biosensors
2. Spectrophotometry
3. Electroanalysis
4. Chromatography

These detection techniques mentioned above have been applied to the simultaneous determination of several kinds of sweeteners in foods (Cantarelli et al. 2009). The magnitude of sensitivity and selectivity of the method (including sample preparation prior to analysis) used is different when only one of these two sweeteners in foodstuffs is determined and different when the two sweeteners simultaneously with other food additives are determined. When an analyst wants to simultaneously determine a number of compounds, he must compromise the best analytical parameters of each compound. In this case, it is common to lose magnitude of sensitivity and selectivity of the majority of the compounds analyzed simultaneously. On the other hand, this does not occur when a method for the determination of one and only compound is developed.

For routine analysis of complex food matrix samples, micellar electrokinetic chromatography (MEKC), capillary zone electrophoresis, high-performance liquid chromatography (HPLC), and ion chromatography (IC) are preferred. Even thin-layer chromatography (TLC) (Baranowska et al. 2004) has been reported for the determination of the two sweeteners. These instrumental methods are important reference methods for food sample analysis and are based on expensive analytical instruments and reagents. Liquid chromatographic determination in foods is simple, because beverages or aqueous extracts from foods can often be injected into the columns immediately after filtration. The most usually used methods are basically reverse-phase HPLC separations coupled with a UV light detector.

In the past few years, there has been an interest in the development of analytical devices for the detection and monitoring of various biological and chemical analytes. For several decades, analytical chemists were inspired from the biological sciences and nowadays the detection of analytes using biosensors is very common. Biosensors offer the capability to develop a method for the rapid screening of these sweeteners with a respective low cost (Nikolelis and Pantoulias 2000). Lipid films can be used for the rapid detection or continuous monitoring of a wide range of compounds in foods and in the environment. Such electrochemical detectors are simple to fabricate and can provide a fast response and high sensitivity. Investigations have taken place for the interactions of the artificial sweeteners ACS-K with freely suspended bilayer lipid membranes (BLMs) that can be used for the direct sensing of these sweeteners. The interactions of the sweeteners with these BLMs produce transient electrochemical current signals, the magnitude of which could be used to quantify the concentration of the sweetener. Determination of the mechanism of signal generation involves analysis of the structural effects caused by interactions of the sweeteners with model lipid membranes. Differential scanning calorimetry studies have shown that the interactions of the sweeteners with lipid vesicles stabilize the gel phase of the lipid films. Monolayer compression techniques at an air–water interface have revealed an increase of the molecular area of lipids when sweeteners are added to the aqueous subphase. Such structural changes are known to cause electrostatic and permeability changes with lipid membranes.

A conductometric method based on the use of surface-stabilized bilayer lipid membranes (s-BLMs) prepared from egg phosphatidylcholine (PC) is developed for monitoring ACS-K and other sweeteners. The interactions of the sweeteners with s-BLMs produce a reproducible electrochemical ion current signal increase that appears within a few seconds after exposure of the membranes to the sweetener. The current signal increases relatively to the concentration of the sweetener in bulk solution in the micromolar range. The alterations in the current signal are observed even from low concentrations between 0.4 and 7 μM in electrolyte solutions. The s-BLM-based biosensor is stable for long periods of time (over 48 h) and can be easily constructed at low cost (and, therefore, can be used as a disposable sensor) with fast response times of the order of a few seconds. The lipid layer is deposited into a nascent metallic surface while immersing the metal wire into the lipid solution. The wire with the lipid layer is immersed into KCl aqueous solution, and ionic current is stabilized over a period of 10–15 min depending on the diameter of the silver wire. Calibrations are subsequently done by stepwise additions of 0.1 or 1.0 mM sweetener standard solution added to the KCl electrolyte while continuously stirring. Once the calibration plot or its equation is set up, the unknown sweetener concentration of a solution can be independently determined using a fresh BLM on a nascent metallic surface and the procedure of immersing the wire with BLM into a KCl solution and so on is repeated. The detection limit of ACS-K is 1 μm and the reproducibility is of the order of ±4–8% in a 95% confidence level. The recovery ranged between 96% and 106% and shows no interferences from the matrix (Nikolelis et al. 2001).

Enzyme electrodes coupled with FIA have also been reported. An ammonia-sensitive electrode L-aspartase or carboxypeptidase A with aspartate ammonia lyase immobilized and a three-enzyme system including aspartate, aminotransferase, glutamate oxidase, and ASP hydrolysing enzyme coupled with an H_2O_2 probe have been reported. The major drawback of these biosensors is the interferences from L-aspartate, which is usually present in samples (Compagnone et al. 1997).

Potentiometry is the procedure in which a single measurement of electrode potential is employed to determine the concentration of an ionic species in a solution. A potentiometric method is developed for the titration of ASP. It makes use of a selective PVC membrane electrode. This electrode is based on the electroactive species of cetylpyridinium (CP^+) and 2,4,6-trinitrobenzenesulfonate ($TNBS^-$). The electrode exhibits a rapid and Nernstian response to $TNBS^-$ from 5.0×10^{-5} M to 1.0×10^{-2} M at 25 ± 0.1°C. The response is unaffected by the change of pH over the range 2–12. The electrode is successfully applied to the determination of ASP in pure solutions with a precision and accuracy of 0.9–1.3% without interference from other constituents. The selectivity of the ion-pair-based membrane electrodes depends on the selectivity of the ion-exchange process at the membrane-test solution interface and the mobilities of the respective ions in the membrane. The CP-TNBS electrode is highly selective for the TNBS anion. The organic and inorganic anions do not interfere due to the differences in their mobilities and permeabilities as compared to the TNBS anions. It is found that the periodate ion and picric acid interfere with the CP-TNBS electrode. In the case of sugars, the high selectivity is mainly attributed to the difference in polarity and the lipophilic nature of their molecules relative to the TNBS anion or CP cation. This method has a high degree of accuracy, a mean recovery of 99.8–101%, and excellent precision by the small values of the relative standard deviation (RSD). It is found that TNBS reacts with the investigated molecules in the ratio of 1:1. The analysis indicates the high accuracy and precision depending on less complicated instrumentation and time-consuming pretreatment steps. The combination of sensitivity, selectivity, and simplicity of ion-selective electrode potentiometry makes it an excellent and versatile analysis technique (Buduwy et al. 1996). An established potentiometric assay for the indication of ACS-K is performed by nonaqueous titration with 0.1 N perchloric acid in glacial acetic acid (Joint FAO/WHO 2001).

Spectrophotometry is a detection technique widely used for the determination of sweeteners. This technique is not usually used in food samples because it is sensitive to interference due to food additives. Spectrophotometric procedures for determining ASP involve different chemical reagents, such as ninhydrin, chloroanilic acid, diethyldithiocarbamate, and *p*-dimethylaminobenzaldehyde (Fatibello-Filho et al. 1999).

A screening flow-injection spectrophotometric method in tabletop sweeteners and in food samples (pudding, gelatin, and refreshment) using ninhydrin as a colorimetric reagent is reported. The reaction is conducted in a 1:1 v/v methanol–isopropanol medium also containing potassium hydroxide. The absorbance measurements are made at 603 nm. The results obtained for the determination of ASP have a good correlation coefficient, $r = 0.998$. Thirty-six samples can be analyzed per hour, and the RSD is <3.5% ($n = 6$) for all samples. The detection limit is 0.0381 mM of ASP (Nobrega et al. 1994).

Another flow-injection spectrophotometric method is developed for determining ASP in tabletop sweeteners without interference of saccharin, ACS-K, and cyclamate. Samples are dissolved in water and a portion of the solution is injected into a carrier stream of 5.0×10^{-3} M sodium borate solution (pH 9.0). The sample flows through a column packed with $Cu_3(PO_4)_2$ immobilized in a matrix of polyester resin. Then Cu(II) ions are released from the solid-phase reactor by the formation of Cu(II) $(ASP)_2$ complex. The mixture is merged with a stream of borate buffer solution (pH 9.0) containing 0.02% (w/w) alizarin red S and the Cu(II)–alizarin red complex formed is measured spectrophotometrically at 550 nm. The calibration graph for ASP is linear in the 20–80 μg/mL concentration range, with a detection limit of 2 μg/mL of ASP. The RSD is 0.2% for a solution containing 40 μg/mL ASP ($n = 10$) and 70 measurements are obtained per hour. The column is stable for at least 8 h of continuous use (500 injections) at 25°C (Fatibello-Filho et al. 1999).

However, these methods are time consuming or do not have the selectivity required for ASP determination in some commercial samples. Spectrophotometry is usually coupled with an FIA system. The analysis of analyte mixtures by means of FIA systems has been accomplished in different ways:

1. Use of a microcolumn after flow cell to retain one analyte preferentially, the other being transiently retained in the solid support placed in the flow-through cell (Ruiz-Medina et al. 2001).

2. Use of differences in transient retention for both analytes at the flow cell. The transient retention of one analyte in the upper part of the flow cell, away from the measuring area, makes it possible to measure what is less retained (Capitan-Vallvey et al. 2004).
3. Use of retention of only one analyte in the solid phase that fills the flow cell, while the second analyte is measured when it flows along the interstitial solution through the particles.
4. Use of a chemometric approach without separation of the analytes prior to the detection step (Jiménez et al. 2009).

A multianalyte flow-through sensor spectrophotometric method achieved the simultaneous determination of ASP and ACS-K in tabletop sweeteners. The procedure is based on the transient retention of ACS-K in the ion exchanger Sephadex DEAE A-25 placed in the flow-through cell of a monochannel FIA setup using a pH 2.7 prepared from orthophosphoric acid/sodium dihydrogen phosphate buffer (0.06 M) as carrier. In these conditions, ASP is very weakly retained, which makes it possible to measure the intrinsic UV absorbance of first ASP at 226 nm and then ACS-K at 205 nm after desorption by the carrier itself. The linear concentration range for ASP is from 10 to 100 μg/mL, the detection limit is 5.65 μg/mL, and the RSD is 3.4% (at 50 μg/mL). The linear concentration range for ACS-K is from 40 to 100 μg/mL, the detection limit is 11.9 μg/mL, and the RSD is 1.61% (at 50 μg/mL). No interference is caused by glucose, sucrose, lactose, maltose, fructose, glycine, and leucine even when present in concentrations higher than those commonly found in the tabletop sweeteners analyzed. The level of the solid phase in the flow cell should be that needed to fill it up to a sufficient height, allowing the radiation beam to pass completely through the solid layer. The height of the solid support considerably influences the separation of both sweeteners (Jiménez et al. 2006).

A multianalyte flow-through method is proposed for the simultaneous determination of ASP, ACS-K, and saccharin in several food and soft drink samples. The procedure is based on the transient retention of the three sweeteners in a commercial quaternary amine ion exchanger monolithic column, placed in its specific holder, and allocated in a monochannel FIA setup using pH 9.0 prepared from Tris buffer 0.03 M, NaCl 0.4 M, and $NaClO_4$ 0.005 M as carriers. In these conditions, ASP is very weakly retained, while ACS-K is more strongly retained, making it possible to measure the intrinsic UV absorbance of ASP and then ACS-K after desorption by the carrier itself. The linear concentration range for ASP is from 9.5 to 130.0 μg/mL, the detection limit is 2.87 μg/mL, and the RSD is 1.46% (at 65 μg/mL). The linear concentration range for acesulfame-K is from 2.2 to 600.0 μg/mL, the detection limit is 1.0 μg/mL, and the RSD is 0.08% (at 300 μg/mL). The method is applied and validated satisfactorily for the determination of ASP and acesulfam-K in foods and soft drink samples, comparing the results with an HPLC reference method (Jiménez et al. 2009).

A new method of determining mixtures of two sweeteners, ASP and ACS-K, in commercial sweeteners is proposed making use of chemometrics. A classical 5^2 full factorial design for standards is used for calibration in the concentration matrix. Salicylic acid is used as an internal standard to evaluate the adjustment of the real samples in the PLS-2 model. This model is obtained from UV spectral data, validated by internal cross-validation, and is used to find the concentration of analytes in the sweetener samples. The mean value of recovery degree is 99.2% with a standard deviation of 3.2%. The proposed procedure is applied successfully to the determination of mixtures of ASP and ACS-K in bulk samples (Cantarelli et al. 2009).

HPLC and gas chromatography (GC) are commonly used for food sample analysis. A common HPLC method that is used nowadays as a basic procedure for sweetener determination and used as a reference method for validation is the following: the method uses a UV light detector by using absorbance measurements at 205 nm. Seven standard solutions and five replicates are prepared for both ASP and ACS-K. The HPLC carrier flow is 0.75 mL/min. Every sample is accurately weighted in a volumetric flask and diluted to the volume with 0.02 M KH_2PO_4: acetonitrile (90:10 v/v). Then every sample is sonicated for 5 min in an ultrasonic water bath to extract sweeteners from the matrix. One milliliter of the extract is diluted and filtered through a 0.22 μm nylon membrane. The RSD% values are better than 0.3% for ASP and 0.1% for ACS-K (Armenta et al. 2004). For the estimation intake of intense sweeteners from nonalcoholic beverages in Denmark, sweeteners including ACS-K and ASP are separated by HPLC on a C18 column (5 mm, 250 × 4.6 mm) equipped with a guard column, eluted isocratically with a mixture of methanol and water

buffered with potassium hydrogen phosphate and measured spectrophotometrically at 220 nm (Leth et al. 2007). For the estimation of the intake of ACS-K and ASP, from soft drinks for a group of Portuguese teenage students, reversed-phase liquid chromatography was used with an Hichrom C_{18} column (5 μm, 250 × 4.6 mm) and a buffered mobile phase (KH_2PO_4 0.02 M/ACN (90:10, v/v)/phosphoric acid) at 1 mL/min. The pH was rigorously controlled at 4.2–4.3 for an adequate resolution between ASP and benzoic acid, which is present in most analyzed samples. Detection was performed with a UV detector at 220 nm. The external standard method is used for quantification (Lino et al. 2008). An isocratic separation of some food additives with HPLC analysis was carried out on a Shimadzu class liquid chromatography-vapor phase (LC-VP) HPLC system with an autosampler (SIL-10ADVP) and a diode-array detector (DAD) (SPD-M 10A VP). A 5 μm YMC ODS-Pack AM column (250 × 4.6 mm I.D.) was used for the analysis. In this study, three different values of the mobile-phase acetonitrile content (15%, 20%, 25%, v/v) were prepared. The pH values were adjusted to 4.0 with glacial acetic acid. The flow rate was 0.8 mL/min and the volume injected was 20 μL. For ACS-K detection followed at 230 nm and for ASP detection followed at 203 nm. This HPLC-UV procedure was able to separate ACS-K and ASP from other additives in Cola drinks and in instant powder drinks. The recovery achieved is 99–101%, the limit of detection (LOD) is 0.2–3.1 μg/g, and the RSD is 1.0–2.2% for all the analytes determined (Demiralay et al. 2006).

The establishment of analytical conditions for the determination of ASP by means of high-performance liquid chromatography electrospray ionization mass spectrometry (HPLC-ESI-MS) has been studied. Mass spectrometry is a powerful qualitative and quantitative analytical technique that has been introduced in many analytical and research laboratories in the last 10 years. The combination of HPLC with tandem MS yields a particularly powerful tool and it is now the method of choice for analysis. However, HPLC-ESI-MS methods are not completely without problems that can compromise the quality of the results. ASP is analyzed in the positive-ion mode at 3.05 kV probe voltage with a methanol/water mobile phase (30:70, pH 3.00 by the addition of acetic acid). Mass spectra at different spraying capillary voltages are reported and discussed. A detection limit of 5 pg and a linear response for ASP in aqueous solution in the range of 1–200 pg are obtained. The method is applied to the detection of ASP in three soft drinks commercially available in Italy. HPLC-ESI-MS provides an additional tool for the sensitive and selective detection of ASP and for the improvement of possibly critical separations such as that of ASP from caffeine (Galletti et al. 1996).

ASP can be determined by HPLC with electrochemical detection. ASP, which is electrochemically inactive, is made oxidizable in the range 0.1–1.1 V after postcolumn irradiation at 254 nm. A detection limit of 0.5 mg/L (signal-to-noise ratio 3:1) is attained using a coulometric detector with the working cell set at 0.8 V and a C_6 column (150 × 4.6 mm I.D.) operated under isocratic conditions with 0.1% perchloric acid–methanol (85:15, v/v) as the eluent at a flow rate of 1 mL/min. A linear response for aqueous solutions of ASP in the range 1–20 mg/L and a 5% standard deviation for five replicate injections were obtained. The method was applied to the determination of ASP in two diet colas (Galletti and Bocchini 1996).

An HPLC-UV procedure was able to separate ASP and ACS-K from saccharin, benzoic acid, sorbic acid, Ponceau 4R, Sunset Yellow, and Tartrazine in soft drinks using a LiChrosorb C_{18} column (10 μm, 250 × 4.6 mm) and a mobile phase consisted of MeOH-phosphate buffer (pH 4). The recovery achieved is 98.6–102.3% with a limit of detection of 0.1–3 mg/L for all the analytes determined (Dossi et al. 2006).

An HPLC evaporative light scattering detector (ELSD) procedure was able to separate ASP and ACS-K from saccharin, cyclamate, sucralose, dulcin, alitame, neotame and neohesperidine dihydrochalcone in noncarbonated soft drinks, canned or bottled fruits and yoghurts using a C_{18} stationary phase, and a mobile phase consisted of TEA formate buffer-MeOH–ACN. The limit of detection is 15 μg/g, and the RSD is 0.9–4.5% for all the analytes determined (Wasik et al. 2007). An ion-paired HPLC-UV procedure is able to separate ACS-K from saccharin, dulcin, preservatives, antioxidants in sugared fruits, soy sauces, and dried roast beef. A Shoko stainless-steel $5C_{18}$ (5 μm, 250 × 4.6 mm) was used as a stationary phase and a mobile phase consisted of ACN-aqueous α-hydroxyisobutyric acid solution containing hexadecyltrimethylammonium bromide. The recovery is 81.9–103.27%, the limit of detection achieved is 0.15–3 μg/g, and the RSD is 0.3–5.69% for all the analytes determined (Chen and Fu 1995). A high-performance ion chromatography–UV-electrolytic conductivity detector (HPIC-UV-ELCD) procedure is able to separate ACS-K and ASP from saccharin, cyclamate, and citric acid in drinks and powdered tabletop sweeteners. A Dionex Ion Pac AS4A-SC (254 × 4 mm) was used as a stationary phase

and a mobile phase consisted of Na_2CO_3. The recovery is 93–107%, the limit of detection is 0.019–0.044 mg/L, and the RSD is 0.84–1.38% for all the analytes determined (Chen et al. 1997). An HPIC-UV procedure is able to separate ACS-K and ASP from saccharin, benzoic acid, sorbic acid, caffeine, theobromine, and theophylline in drinks, juices, fermented milk drinks, preserved fruits, tablets drinks, and powdered tabletop sweeteners. A Shim-pack IC-A3 (5 μm, 150 × 4.6 mm) was used as a stationary phase and a mobile phase consisted of NaH_2PO_4 (pH 8.20)-ACN. The recovery is 85–104%, the limit of detection is 4–30 mg/L and the RSD is 1–5% (Chen and Wang 2001). Finally, an HPIC-suppressed conductivity detector procedure is able to determine ACS-K and ASP in the presence of saccharin and cyclamate in carbonated cola drinks, fruit-juice drinks, and preserved fruits. A Dionex Ionpac AS11 (250 × 2 mm) was used as a stationary phase and a mobile phase consisted of KOH. The recovery is 97.96–105.42% and the limit of detection is 0.019–0.89 mg/L for all the analytes determined (Zhu et al. 2005).

An MEKC-UV procedure is able to determine ASP and ACS-K with saccharin, dulcin, alitame, caffeine, benzoic acid, and sorbic acid in low-energy soft drinks, cordials, tomato sauce, marmalades, jams, and tabletop sweeteners. An uncoated fused-silica capillary (75 cm × 75 μm) was used as a stationary phase and a mobile phase consisted of buffer consisting of sodium deoxycholate, potassium-dihydrogenorthophosphate, and sodium borate (pH 8.6). The recovery is 104–112% and the RSD is 0.63–2.6% for all the analytes determined (Thompson et al. 1995). Another MEKC-UV procedure is able to determine ASP and ACS-K with saccharin, preservatives, and antioxidants in cola beverages and low-energy jams. A fused-silica capillary (52 cm × 75 μm) was used as a stationary phase and a mobile phase consisted of borate buffer with Na cholate, dodecyl sulfate, and MeOH (pH 9.3). The recovery is 98.9–100.86% and the RSD is 0.9–1.5% for all the analytes determined (Boyce 1999). Finally, an MEKC-UV procedure is able to determine ASP and ACS-K with saccharin, preservatives, and colors in soft drinks. An uncoated fused-silica capillary (48.5 cm × 50 μm) is used as a stationary phase and a mobile phase consisted of carbonate buffer (pH 9.5) with sodium dodecyl sulfate. The LOD is 0.005 mg/mL for all the analytes determined (Frazier et al. 2000).

The separation and determination of the sweetener ASP by IC coupled with electrochemical amperometric detection is reported. Typically in IC, electrochemical detection is employed, such as amperometric or conductivity detector. On the other hand, UV-absorbing excipients such as flavors and dyes may not give any electrochemical response and can, therefore, be eliminated as an interferent in quantitation of the analyte. Thus, IC offers the opportunity to streamline methods' development and increase sample throughput. Sodium saccharin, ACS-K, and ASP were separated using 27.5 mM NaOH isocratic elution on a Dionex IonPac AS4A-SC separation column. ASP can be determined by integrated amperometric detection without interference from the other two sweeteners. The method can be applied to the determination of ASP in tabletop, fruit juice, and carbonated beverage samples, and the results obtained by integrated amperometry are in agreement with those obtained using a UV detection method. The recoveries for samples ranged from 77.4% to 94.5%. The peak area response for ASP is linear in the range 0.1–10 μg/mL. For seven consecutive injections of a standard solution with a concentration of 5 μg/mL, the RSD is 1.29% and the detection limit (signal-to-noise ratio of 3:1) is 0.031 μg/mL for ASP (Qu et al. 1999).

A simple method for the simultaneous determination of five artificial sweeteners, alitame, ACS-K, saccharin, ASP, and dulcin in various foods by HPLC and detecting the species at 210 nm was reported. The recoveries of the five sweeteners from various kinds of foods spiked at 200 mg/g ranged from 77% to 102%. The detection limits of the five sweeteners were 10 mg/g (Kobayashi et al. 1999).

The most popular reference method used in bibliography is the HPLC-DAD method proposed by Lawrence and Charbonneau. A 5 mm C_8 silica in a 150 × 4.6 mm column was used as a stationary phase in this method, with a mobile phase gradient ranging from 3% acetonitrile in 0.02 M KH_2PO_4 (pH 5) to 20% acetonitrile in 0.02 M KH_2PO_4 (pH 3.5) at a constant flow rate of 1.0 mL/min. The chromatograms were obtained at a wavelength of 210 nm. In order to obtain the calibration function, six different concentration levels and three replicates of each one of the standard solutions are analyzed using peak area as the analytical parameter (Lawrence and Charboneau 1988).

Quantitative determination by GC is impossible due to the low volatility of ACS-K and due to the fact that methylation produces differing ratios of methyl derivatives. A method of determining ASP and its degradation products has been reported in 1975 by GC (Furda et al. 1975).

Capillary electrophoresis is an interesting alternative to HPLC. The resolving power of this technique is in many cases comparable with that of HPLC and, frequently, their running costs are lower. High-pressure capillary electrophoresis appears to be a viable method for the determination of ASP in real commercial products. The analysis time is significantly faster than that reported for HPLC methods and no interferences were detected in the soft drink samples tested. Caffeine has a different migration time from ASP and its low sensitivity at the detection wavelength precludes the appearance of a peak in the electropherogram. The linear calibration curve developed is compatible with the range of ASP concentrations or amounts in the products tested. It appears that a small amount of ASP adsorption occurs on the capillary walls, but this effects quantitative determinations only well below the useful range for typical commercial samples. A linear calibration curve between 25 and 150 μg/mL for the analyte solution is established, which can be used for quantitative determinations of ASP in typical food and beverage products. Six commercial samples are analyzed, and one diet cola with a known ASP concentration gives an RSD of 2.6% from the manufacturer's value (Qu et al. 1999).

A method for isotachophoretic determination of sweeteners of different character in candies and chewing gums is developed. A capillary made of fluorinated ethylene–propylene copolymer with internal diameter of 0.8 mm and effective length of 90 mm is filled with an electrolyte system consisting of a leading electrolyte (10 mM HCl with 14 mM Tris, pH 7.7) and a terminating electrolyte (5 mM L-histidine with 5 mM Tris, pH 8.3). The analysis is performed at a driving current of 200 μA, and for detection current, the magnitude is decreased to 100 μA. Boric acid is added to the aqueous sample solution to form borate complexes with substances of polyhydroxyl nature and make them to migrate isotachophoretically. Using conductivity detection, the calibration curves in the tested concentration range up to 2.5 mM and are linear for all components of interest: ACS-K, saccharine, ASP, cyclamate, sorbitol, mannitol, lactitol, and xylitol. The concentration detection limits for the determined compounds range between 0.024 and 0.081 mM. Good precision of the method is evidenced by favorable RSD values for all compounds ranging from 0.8% to 2.8% obtained at the analyte concentration of 1.0 mM ($n = 6$). The analysis time is about 20 min. Simplicity, accuracy, and low cost of analyses make this an alternative procedure to methods used thus far for the determination of ionizable sweeteners (Herrmannová et al. 2006).

TLC methods have been developed for analysis of sweeteners. ASP, ACS-K, sodium cyclamine, and benzoic acid are separated on thin layers of silica gel G with 10% (v/v) ethanol—40% (v/v) isopropanol—1% (v/v) (12.5%) aqueous ammonia. This chromatographic system was applied to the analysis of sweeteners in 23 sparkling and nonsparkling drinks (Baranowska et al. 2004).

A new chromatographic modality that does not require high pressures and also allows renewal of the stationary phase as desired is reported. The technique is based on a thin-layer paramagnetic stationary phase (Fe_3O_4–SiO_2) retained on the inner wall of a minicolumn through the action of an external magnetic field, which also plays an important role in separating the analytes. Accordingly, the name "renewable stationary phase liquid magnetochromatography," or renewable stationary phase-liquid magnetochromatography (RSP-LMC), has been proposed for it. The technique is used to separate and quantify the sugar substitute ASP and its constituent amino acids (hydrolysis products), L-aspartic acid and L-phenylalanine, in diet fizzy soft drinks. When the results obtained for ASP were compared with those obtained using the HPLC reference method of Lawrence and Charbonneau, no significant differences were observed. The system proposed is fully automated, making it an economic, competitive alternative to conventional methods of determining ASP and its amino acid components (Barrado et al. 2006).

The determination of ASP and ACS-K in tabletop samples, as was previously described, has been achieved by Fourier transform middle infrared (FTIR) spectrometry. With the use of a fully mechanized online extraction, the contact of the operator with toxic solvents was avoided and differentiates between samples that contain ASP and ACS-K and those that include only ASP, reducing the time needed for the analysis of the last kind of samples to 5 min. The method involves the extraction of both active principles by sonication of samples with 25:75 v/v $CHCl_3/CH_3OH$ and direct measurement of the peak height values at 1751 cm^{-1}, corrected using a baseline defined at 1850 cm^{-1} for ASP, and measurement of the peak height at 1170 cm^{-1} in the first-order derivative spectra, corrected by using a horizontal baseline established at 1850 cm^{-1}, for ACS-K. Limit of detection values of 0.10% and 0.9% w/w and RSDs of 0.17% and 0.5% are found for ASP and ACS-K, respectively. On the other hand, an HPLC method needs approximately 35 min for completion (Armenta et al. 2004).

The determination of ACS-K by FTIR spectra has been achieved using an advanced spectrometer equipped with a multibounce horizontal attenuated total reflectance accessory. The instrument can produce 12 reflections with a penetration depth (infrared beam) of 2.0 μm. The accessory comprised a ZnSe crystal mounted in a shallow trough for sample containment with an aperture angle of 45° and a refractive index of 2.4 at 1000 cm^{-1}. Single-beam spectra (4000–400 cm^{-1}) of the ACS-K extract from real samples are obtained and corrected against the background spectrum of pure Milli-Q water. All spectra are collected in triplicate and averaged before subjecting to multivariate analysis (Shim et al. 2008).

Electrochemistry at the liquid–liquid interface enables the detection of nonredoxactive species with electroanalytical techniques and the electrochemical behavior of two food additives, ASP and ACS-K, can be investigated. Both ions were found to undergo ion-transfer voltammetry at the liquid–liquid interface. Liquid–liquid electrochemistry as an analytical approach in food analysis is very suitable. Differential pulse voltammetry was used for the preparation of calibration curves over the concentration range of 30–350 μM with a detection limit of 30 μM. The standard addition method was applied to the determination of their concentrations in food and beverage samples such as sweeteners and sugar-free beverages. Selective electrochemically modulated LLE of these species in both laboratory solutions and in beverage samples was achieved (Herzog et al. 2008).

39.4 Conclusion

The determination of artificial sweeteners individually or simultaneously in mixtures is very important for legal aspects. Researchers focus their efforts on developing analytical methods for simple, rapid, and low-cost-sensitive determination. Sensitive and robust analytical methods are essential to meet the needs of growing markets in quality control and consumer safety. Scientists have applied a wide variety of instrumental techniques. Today, the method of choice for the determination of artificial sweeteners in different food matrices is HPLC because of its multianalyte capability, compatibility with the physicochemical properties of sweeteners, high sensitivity, and robustness. Due to a constant rising demand for alternative methods for determination of sweeteners, and due to the rising development of mass spectrometry or tandem mass spectrometry methods, we will witness a number of procedures based on mass spectrometry coupled with liquid chromatography for the determination of ACS-K and ASP.

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40

Methods of Analysis of Saccharin

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40.1 Introduction

Saccharin (SAC) is characterized as an artificial high-intensity sweetener. It is also called nonnutritive sweetener and today is commonly used in the food industry. However, in the past, SAC was used in a variety of applications apart from its major known use as a sweetener today. It was first used as an antiseptic and preservative to retard fermentation in food. Later on, SAC was used in the plastic industry as an antistatic agent and as a modifier. SAC has even been used as a brightener in nickel-plated automobile bumpers (Arnold et al. 1983). Nowadays, it is exclusively used for low-calorie food intake that helps consumers to maintain their weight. It is also medically suggested to diabetics to consume foods containing SAC instead of sugar.

SAC (1,2-benzisothiazol-3(2*H*)-on-1,1-dioxide) is the oldest sweetener on the market. It was discovered accidentally in 1879 by Constantin Fahlberg, a graduate student at Johns Hopkins University. He was working on the synthesis of toluene derivatives. One day, while having his lunch, he had a sweet taste in his bread (Sardesai and Waldshan 1991). This sweet taste was attributed to a compound today known as SAC (Figure 40.1).

It is a white crystalline powder with intensely sweet taste and is available in three forms (acid SAC, sodium SAC, calcium SAC). Aqueous solutions of SAC are slightly acidic. Calcium and sodium salts of SAC are neutral and slightly basic. The melting point of acid SAC is 228.8–229.7°C, whereas the salts decompose above 300°C. SAC is characterized by its high solubility, stability, and bitter metallic aftertaste. SAC is ca. 300–500 times sweeter than sugar. The excellent stability of SAC in food processing makes it ideally suited in many different products. It is used in a wide range of cases where heat processing is required (e.g., jams, canned products). However, at low pH (pH 2.5), it can slowly hydrolyze to 2-sulfobenzoic acid and 2-sulfoamylobenzoic acid (Pearson 1991). SAC is used in a wide variety of food products including baked goods (dry bases for mixes), beverages (dairy beverages, instant tea, instant coffee, fruit-based beverages), soft drinks (colas, citrus-flavored drinks, fruit-based soft drinks), sugar preserves and confectionery (calorie-free dustings, frostings, icings, toppings, fillings, syrups), alcoholic drinks (beer), vinegar, pickles and sauces (sandwich spreads, salad dressings), dairy products (yoghurt and yoghurt-type products, puddings, desserts and dairy analogs, sugar-free ice-cream), fruit, vegetables, nut products, sugar-free jams and marmalades, low-calorie preserves, and other food products (i.e., chewing gums, liquid concentrates, and frozen and refrigerated desserts).

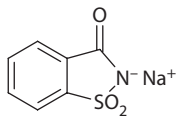

FIGURE.40.1 Chemical structure of saccharin (sodium salt).

Even today there is a controversy over its safety even if SAC is widely used. Numerous toxicological studies have taken place with a variety of animal species. SAC is excreted unchanged except for a small percentage that is accumulated in the bladder (Renwick 1985). It is shown that the compound is not genotoxic, and it does not bind to DNA. The greatest concern relating to metabolic effects of SAC has been the uncertainty about its carcinogenicity to humans (Whysner and Williams 1996). Several early carcinogenicity studies in rats that included *in utero* exposure had indicated that feeding of SAC may lead to bladder tumors (Cohen-Addad et al. 1986). On the contrary, studies in monkeys have shown that SAC does not pose carcinogenic effects on the primate urinary tract (Takayama et al. 1998). A number of extensive epidemiological studies in humans have shown no association between SAC consumption and urinary bladder cancer (Weihrauch and Diehl 2004). SAC may reasonably be anticipated to be a carcinogen, whereas a lethal dose in mice and rats is set for this compound (The Merck Index 1996).

The U.S. Food and Drug Administration delisted SAC in 1972, because of the uncertainty of the safety of SAC and its use in foods and beverages was proposed to be banned in 1977. However, public protest led to impose a moratorium on the ban that has been extended up to the present (Pearson 1991; Kroger et al. 2006). In Canada, SAC was banned in 1977 for use in foods (Arnold 1984). However, it is permitted to be sold in pharmacies as a table-top sweetener. In other countries, SAC is permitted although its use is restricted to varying degrees. EU Directives 94/35/EC (European Commission 1994), 96/83/EC (European Commission 1996), 2003/115/EC (European Commission 2003), 2006/52/EC (European Commission 2006) define in which food products and in what quantity SAC can be used.

Consumer safety demands the monitoring of SAC intake. A number of analytical methods based on different principles are available for the determination of SAC in a broad range of food matrices. The aim here is to present the available methodologies for sample pretreatment and the available protocols of analysis.

40.2 Sample Preparation Procedures

Determination of food additives demands in most cases sample preparation or/and sample cleanup prior to analysis. This step cannot be avoided because the determination of sweeteners directly in food can often not be achieved due to interferences from the food matrix. Generally speaking, sample pretreatment is the most time-consuming step of the analytical method. Food samples are characterized as difficult matrices. The food matrix presents a great variability in its composition. Carbohydrates, proteins, lipids, minerals, preservatives, colors, thickeners, and vitamins may stand alone or coexist in a food matrix. All the components can interfere in the determination of SAC. The success of determining SAC is often totally dependent on the effectiveness of the sample pretreatment and it is tailored to the accuracy (quantitative or/and qualitative) that the analyst wants to obtain "fit for purpose." The chemical and the physical properties of food vary. The variability in the effects of a given food sample composition can be minimized with proper sample preparation, which mainly consists of three steps. First, the food sample needs homogenization. Second, SAC should be extracted from the food matrix. As a third step, further cleanup of the extract prior to analysis could be performed (if needed). A fourth and last step could be the preconcentration of the final extract. Analysis of liquid samples usually does not require the homogenization step.

After the sample preparation, some matrix components may still be present interfering in the analysis. They may be coextracted with the analyte due to similar solubility in the solvents used for extraction. The presence of matrix interferences in a sample extract can contribute to problems on the accuracy of the method. The only way to resolve this problem is to further clean up the sample for analysis. Cleanup

is achieved commonly by solid-phase extraction (SPE), dialysis, liquid–liquid extraction, precipitation, and filtration. Samples may need centrifugation prior to analysis for even better cleanup.

Appropriate extraction and cleanup procedures maximize the recovery of SAC. For its efficient extraction, the analyst should optimize the solvent volume and its pH prior to extraction. Optimal sample preparation can reduce analysis time, enhance sensitivity, and optimize confirmation and quantification of SAC. The most common sample preparation procedures that are found nowadays in published procedures use the SPE technique. It is simple, reproducible, quick, and inexpensive. SPE technique fractions the compounds of our choice based on the affinity of the compound or a group of compounds to the stationary phase. The protocol of an SPE procedure consists mainly of four steps. First, the conditioning of the cartridge precedes, followed by the loading of the food extract, washing of the cartridge follows and finally, elution of the analytes is performed by adequate solvent(s). The type of SPE-packing material, solvents, pH, and the flow rates need to be properly selected to retain the analytes effectively within the cartridge. The interfering substances should be retained very strongly or not retained at all. As a result, weakly retained substances are removed from the cartridge during sample load and/or cartridge wash. Analytes are eluted during the elution step and interfering substances having a strong affinity to the sorbent stay adsorbed within the cartridge. SPE procedures are universal and compatible with the most popular techniques used in food analysis (Tunick 2005). In published procedures for the determination of SAC, one can witness a variety of food matrices being suitably treated prior to analysis (e.g., Kobayashi et al. 1999). Cola drinks are usually degassed in an ultrasonic bath (Demiralay et al. 2006). The same applies to every carbonated drink. Degassing is performed by sonication, by sparging with nitrogen or under vacuum. Chewing gum samples are prepared by placing them in a flask and extracting with a mixture of glacial acetic acid, water, and chloroform. Hard- or soft-candy samples are shaken with water until dissolved (Biemer 1989). Milk and dairy products are homogenized prior to analysis and an aliquot of a homogeneous sample is transferred to a flask followed by the addition of distilled water. The mixture is thoroughly stirred and then filtered (Ni et al. 2009). SAC is determined in diet jams by mixing the jam with water, and sonicating the mixture. The mixture is then made up to a certain volume and filtered (Boyce 1999). Preserved fruits are grounded and homogenized. Then water is added and the formed mixture is extracted ultrasonically and diluted to a certain volume with water (Chen and Wang 2001). For the analysis of juice before filtration, a centrifuge step may be necessary. In the case of strawberry sweets, an adequate amount is weighed and thoroughly crushed in a glass mortar, then dissolved in water with the aid of an ultrasonic bath. Then, it is centrifuged and filtered. In the case of tomato sauce, the amount is suspended in water, and then a portion is diluted and centrifuged (Jiménez et al. 2009). When the determination of SAC in table-top solid tablets is needed, tablets are turned into powder. A portion of the powder is being weighed, and directly dissolved in ultrapure water. Samples characterized by relatively simple matrix like liquid sweeteners can simply be diluted or dissolved in deionized water or in an appropriate buffer. The approaches for the determination of SAC in simple matrices are much easier and less time consuming than the determination of SAC in more complicated food sample matrices.

40.3 Analytical Techniques

The number of techniques used for the determination of SAC in food samples is large. The choice of the best-suited technique is done by the analyst after thoroughly investigating 10 principal parameters. These parameters are the following: apparatus availability, accuracy, and precision that must be achieved, costs, detection limits, selectivity, safety, sample throughput, consumption of sample and reagents, simple operation (automated or not), and contamination risks. However, three main parameters are the most critical for choosing the appropriate method of analysis:

1. The reagents and apparatus intended for use must have a low cost.
2. A sensitive method with a low-detection limit is needed, since the sweeteners generally fluctuate in food in the micromolar range and sensitive methods often are costly.
3. The sample throughput and the consumption of sample and reagents must be relatively low.

For the determination of SAC in food, the following analytical techniques that meet the criteria as previously described are

1. Biosensors
2. Spectrophotometry
3. Electroanalysis
4. Chromatography

These detection techniques have been applied to the simultaneous determination of several kinds of sweeteners in foods (Cantarelli et al. 2009). In Table 40.1, the analytical parameters of biosensors, spectrophotometry, and electroanalysis are presented from published methods for the determination of SAC in foods. In Tables 40.1 and 40.2, the abbreviations LOD and RSD are used for limit of detection and relative standard deviation, respectively. The magnitude of sensitivity and selectivity of the method (including sample preparation prior to analysis) used is usually different when only SAC in the foodstuff is determined and different when it is determined along with other food additives (may be simultaneously).

In the last few years, there has been an interest in the development of analytical devices for the detection and monitoring of various biological and chemical analytes. For several decades, analytical chemists were inspired from the biological sciences and nowadays the detection of analytes using biosensors is very common. Biosensors offer the capability to develop methods for the rapid screening of SAC with a relatively lower cost (Nikolelis and Pantoulias 2000). Lipid films can be used for the rapid detection or continuous monitoring of a wide range of compounds in foods. Such electrochemical detectors are simple to fabricate and can provide a fast response and high sensitivity. The major interference from proteins can be eliminated by modulation of the carrier solution that does not allow adsorption of these compounds in bilayer lipid membranes (BLMs) (Nikolelis and Pantoulias 2001b). A conductimetric method based on the use of surface-stabilized bilayer lipid membranes (s-BLMs) is developed for monitoring SAC and other sweeteners. The interactions of sweeteners with s-BLMs produce a reproducible electrochemical ion current signal increase that appears within a few seconds after exposure of the membranes to the sweetener. The current signal increases relatively to the concentration of the sweetener in bulk solution in the micromolar range (Nikolelis et al. 2001a; Nikolelis and Pantoulias 2001b). Recently, a nanohybrid membrane sensor was proposed (artificial BLMs) and was compared with the s-BLMs offering higher stability (Chalkias and Giannelis 2007).

Potentiometry is the technique in which a single measurement of electrode potential is employed to determine the concentration of an ionic species in a solution. A potentiometric technique is characterized by the selectivity, the stability, and the response time of the electrode used. The selectivity of the electrode is generally evaluated considering some important ionic species normally found as components in food (e.g., Fo and Guarita Dos Santos 1993). Stabilization and response times are evaluated by recording the potential response of the membrane versus time, as the concentration of SAC increases in solution. Drift is determined by measuring changes in the electrode potential with time, in a fixed SAC concentration solution. In the literature, a number of electrodes are proposed for the potentiometric determination of SAC in foodstuff with specific selectivity, stability, and response time properties in each case. A polymer (silsesquioxane 3-*n*-propylpyridinium chloride)-coated graphite rod ion-selective electrode with fast response times has been proposed. The electrode response was based on the ion pair formed between saccharinate acid and the 3-*n*-propylpyridinium cation from the silsequioxane polymer (Alfaya et al. 2000). A silver wire electrode (Filho et al. 2003) and a saccharinate ion-sensitive electrode immobilized in a graphite matrix (Santini et al. 2008) have also been proposed, demonstrating great stability and lifetime. An electrode based on a polypyrrole-doped membrane is developed, exhibiting high selectivity toward SAC in the presence of other compounds commonly found in food (Álvarez-Romero et al. 2010).

Spectrophotometry is a detection technique widely used for the determination of sweeteners. This technique is not usually used in food samples because it is sensitive to interference due to food additives. In the literature, a number of sensitive methods have been proposed for overcoming the drawbacks of interferences. A rapid, sensitive, and selective spectrophotometric method is developed using a formation of SAC with Nile Blue (Cordoba et al. 1985). The formation of SAC with dyes is a smart way of enhancing

TABLE 40.1

Analytical Techniques (Biosensors, Spectrometry, Electroanalysis) for the Determination of Saccharin in Functional Foods

Determined Analytes	Matrix	Technique	LOD	RSD	Recovery and Linear Range of SAC	References
SAC, CYC, ACS-K	Solid sweeteners	Biosensors	0.3 μM	N/A	96–106% and 0.4–7 μM	Nikolelis and Pantoulias (2000)
SAC, CYC, ACS-K	Solid sweeteners	Biosensors	0.1 μM	N/A	N/A and 0.35–3.5 μM	Nikolelis et al. (2001a)
SAC	Artificial table top sweeteners	Potentiometry	N/A	N/A	N/A and 6.9×10^{-6} -5.3×10^{-3} mol/L	Alfaya et al. (2000)
ACS-K, SAC	Beverages	Differential pulse polarography	<1 ppm	N/A	N/A and 1–200 ppm	Hannisdal and Schrøder (1993)
SAC, CYC, ACS-K	Cola drinks, fruit drinks, milk, dairy products	Differential kinetic spectrophotometry	0.0312 μg/mL	N/A	N/A and N/A	Ni et al. (2009)
SAC, CYC	Table-top sweeteners	Partial least square regression/ Fourier transform (PLS/ FT)-Raman	0.2% w/w	0.8%	N/A and N/A	Armenta et al. (2004)
SAC	Commercial artificial sweeteners	Potentiometry	2.5 mg/mL	3%	97–102.6% and N/A	Filho et al. (2003)
SAC	Solid artificial sweeteners	FIA with AAS	3 μg/mL	2.7%	N/A and 5–75 μg/mL	Yebra et al. (1995)
SAC	Artificial sweeteners	Potentiometry	3.6×10^{-4} mol/L	0.6%	101.5–102.5% and N/A	Álvarez-Romero et al. (2010)
SAC	Instant tea powders, diet soft drinks, strawberry dietetic jam	Potentiometry	3.9×10^{-7} mol/L	1.8–2.3%	96.7–102% and 5×10^{-7}–1×10^{-2} mol/L	Santini et al. (2008)
SAC	Tablets, liquid sweeteners, soft drinks	Spectrophotometry	N/A	1.3%	99–105% and 0.1–3.5 μg/mL	Cordoba et al. (1985)
SAC	Liquid sweeteners, powder sweeteners, low-calorie soft drinks	Potentiometry	0.5 mg/mL	N/A	95.2–103.2% and N/A	Orlando et al. (1993)
SAC	Soft drinks, juice, bakery products, table-top sweeteners	FIA with spectrophotometric detection	0.2 μg/mL	0.78%	98–104% and 1–200 μg/mL	Capitán-Vallvey et al. (2004)
SAC	Dietary products	Potentiometry	N/A	N/A	98.2–103.1% and 1×10^{-1}–5×10^{-5} mol/L	Elmosallamy et al. (2005)
SAC	Solid and liquid sweeteners	Spectrophotometry	1.55×10^{-5} M	0.5–1.6%	99.2–104.3% and N/A	Weinert et al. (2004)

Note: AAS, atomic absorption spectrometry; ACS-K, acesulfame-k; CYC, cyclamate; FIA, flow injection analysis; LOD, limit of detection; RSD, relative standard deviation; SAC, saccharin.

TABLE 40.2

Chromatographic Techniques for the Determination of Saccharin in Functional Foods

Determined Analytes	Matrix	Technique	Mobile Phase/ Electrolyte	Column/Capillary	LOD	RSD	Recovery and Linear Range	References
ACS-K, ASP, SAC	Soft drinks, juice, tomato sauce, strawberry sweets	FIA with on-line monolithic element	Water (0.4 M in NaCl, 5×10^{-3} M $NaClO_4$) (pH = 9)	Quaternary amine ion exchanger monolithic column	0.9 μg/mL	0.09%	97.6–103.4% and 3–600 μg/ml	Jiménez et al. (2009)
ACS-K, ASP, SAC, vanillin, sorbic acid, benzoic acid	Cola drinks, instant-powder drinks	HPLC-UV	Acetonitrile (ACN)-Ammonium acetate buffer (pH = 4)	Yamamura chemicals lab corporation-octadecylsilane (YMC-ODS) Pack AM (5 μm × 250 mm × 4 mm I.D)	0.2–3.1 μg/g	1.0–2.2%	99–101% and N/A	Demiralay et al. (2006)
ACS-K, SAC, CYC	Gum	IC-UV	300 mg/L Sodium carbonate	Dionex AS4A Separator column	N/A	1.1%	99.8–102.5%, 0.0262 –0.0022 mg/mL	Biemer et al. (1989)
DUL, ACS-K, SAC, preservatives, antioxidants	Soy sauce, sugared fruits, dried roast beef	Ion-paired LC-UV	ACN-aqueous a-hydroxy-iso butyric acid solution (pH = 4.5) containing hexade-cyltrime-thylammonium bromide	Stainless-steel Shoko $5C_{18}$column (5 μm × 25 mm × 4.6 mm I.D)	0.5 μg/g	N/A	81.9–89.64% and N/A	Chen and Fu (1995)
ACS-K, SAC, ASP, antioxidants, preservatives	Beverages, low-calorie jam	MEKC-UV	Sodium tetraborate solution (pH = 9.5) with Na cholate, dodecyl sulfate-10% ACN or isopropanol or MeOH	Fused silica capillary (60 cm × 75 μm I.D)	N/A	N/A	98.9–100.86% and N/A	Boyce (1999)
SAC, ASP, ACS-K, sorbic acid, benzoic acid, caffeine, theobromin, theophylline	Cola drinks, preserved fruits, tablets, fermented milk, fruit juice	IC-UV	Aqueous NaH_2PO_4 (pH = 8.20)–4%(v/v) ACN	IC-A3 Shim-Pac (5 μm × 150 mm × 4.6 mm I.D)	20 ng/mL	1.5%	85–104% and N/A	Chen and Wang (2001)

SAC, ASP, ACS-K, CYC, citric acid	Drinks, powdered tabletop sweeteners	IC-UV-evaporative light scattering detector (ELCD)	Na_2CO_3 Solution	Dionex IonPac AS4A-SC (250 mm × 4 mm I.D)	0.26 µg/mL	0.84%	93–107% and 2–100 mg/mL	Chen et al. (1997)
ACS-K, SAC, Benzoic acid, sorbic acid	Beverages, jams	HPLC-UV	8% MeOH in phosphate buffer (pH = 6.7)	Spherisorb C_{18} ODS-1 (5 µm × 250 mm × 4.6 mm I.D)	< 0.1 mg/100 mL	N/A	100.4–103.2% and 0–100 mg/L	Hannisdal (1992)
SAC, ASP, CYC, ACS-K	Carbonated cola drinks, fruit juice, preserved fruit	IC-suppressed conductivity detector	KOH	Dionex Ion Pack AS 11 (250 mm × 2 mm I.D)	0.045 mg/L	N/A	98.5–102.4% and N/A	Zhu et al. (2005)
SAC, ASP	Carbonated beverages, soft drinks, strawberry jam	FIA with on-line SPE	Dihydrogen phosphate buffer 3.75×10^{-3} mol/L	N/A	1.4 µg/mL	1.6%	99–101% and 10–200 µg/mL	Capitán-Vallvey et al. (2006)
ACS-K, SAC, ASP, CYC, sorbitol, mannitol, lactitol, xylitol	Chewing gum, candy	CITP	Two electrolytes used: HCl-Tris (pH = 7.7)(E1) L-histidine-Tris (pH = 8.3)(E2)	Capillary ethylene propylene copolymer (90 mm length)	0.052 mM	2.6%	98.2–102.5% and N/A	Herrmannová et al. (2006)
SAC, preservatives	Coffee drink	Ion-pair chromato-graphy	ACN-Water-0.2 M phosphate buffer (pH = 3.6) (7:12:1)	Nucleosil5C_{18}	10 µg/g	1.18%	102.4% and N/A	Terada and Sakabe (1985)
SAC, ASP, CYC, ALI, ACS-K, DUL, NEO, SCL, NHDC	Soft drinks, canned and bottled fruits, yogurt	HPLC-ELSD	Triethylamine (TEA) formate buffer-methanol-acetone	Nucleodur C_{18} Pyramid (5 µm × 250 mm × 3 mm I.D)	15 µg/g	0.9–4.5%	93–109% and N/A	Wasik et al. (2007)
ACS-K, SAC, ASP, benzoic acid, sorbic acid, ponceau 4R, sunset yellow, tartrazine	Soft drinks	HPLC-UV	MeOH-phosphate buffer (pH = 4)	Lichrosorb RP_{18} (10 µ, 250 × 4.6 mm I.D)	0.1–3 mg/L	N/A	98.6–102.3% and N/A	Dossi et al. (2006)
SAC, ASP, benzoic acid, sorbic acid	Soft drinks	HPLC	Phosphate buffer (pH = 4.5)-ACN	LichrosorbC_{18} (5 µm × 25 cm × 4 mm I.D)	N/A	<3.2%	>95% and N/A	Moors et al. (1991)

continued

TABLE 40.2 (continued)

Chromatographic Techniques for the Determination of Saccharin in Functional Foods

Determined Analytes	Matrix	Technique	Mobile Phase/ Electrolyte	Column/Capillary	LOD	RSD	Recovery and Linear Range	References
AK, SCL, SAC, CYC, ASP, DUL, GA, STV, REB	Solid and liquid food matrices	Liquid chromatography-mass spectrometry (LC-MS)	Acetonitrile-water (8:2)	Zorbax EclipseXDB-C_{18} (150 mm × 2.1 mm I.D)	N/A	N/A	75.7–109.2% and N/A	Koyama et al. 2005
SAC, sorbic acid, benzoic acid, *p*-HBA ethyl, *p*-HBA isopropyl, *p*-HBA propyl, *p*-HBA isobutyl, *p*-HBA butyl	Solid and liquid food matrices	liquid chromatography-tandem mass spectrometry (LC-MS-MS)	0.01% formic acid solution-acetonitrile	TSKgel ODS8OTs (150 mm × 4.6 mm I.D)	10 µg/g	N/A	78–120% and N/A	Ujiie et al. (2007)
ASP, SAC	Dietary foods	HPLC-UV	Solution TEA-phosphate buffer (pH = 3)-MeOH-tetrahydrofuran (THF)	Hypersil C_{18} (10 µm, 250 mm × 4 mm I.D)	N/A	N/A	95–97% and N/A	Di Pietra et al. (1990)
AK, SCL, SAC, CYC, ASP, DUL, GA, STV, REB	Beverages, canned fruit, cakes	HPLC-ESI-MS	TEA formate buffer-MeOH-ACN	Spherogel C_{18} (5 µm × 250 mm × 4.5 mm I.D)	<0.10 µg/mL	N/A	95.4–104.3% and 0.05–5.00 µg/mL	Yang and Chen (2009)

Note: ACS-K, acesulfame-k; ALI, alitame; ASP, aspartame; CITP, capillary isotachophoresis; CYC, cyclamate; DUL, dulcin; FIA, flow injection analysis; GA, glycyrrhizic acid; HPLC-UV, high-performance liquid chromatography–ultraviolet; IC, ion chromatography; IC-UV, ion chromatography–ultraviolet; MEKC-UV, micellar electrokinetic chromatography–ultraviolet; NEO, neotame; NHDC, neoesperidine dihydrochalcone; *p*-HBA, *p*-hydroxybenzoic acid; REB, rebaudioside; SAC, saccharin; SCL, sucralose; SPE, solid-phase extraction;STV, stevioside.

the sensitivity. Spectrophotometry coupled with a flow injection analysis (FIA) system offers reproducible and accurate results (Capitán-Vallvey et al. 2004). Recently, a sensitive spectrophotometric method was proposed taking advantage of the different kinetic rates of SAC and other sweeteners in their oxidative reaction with $KMnO_4$. The data obtained from the kinetic rates of each analyte were processed using chemometrics (Ni et al. 2009).

For the routine analysis of complex food matrix samples, micellar electrokinetic chromatography (MEKC), high-performance liquid chromatography (HPLC), and ion chromatography (IC) are preferred (Table 40.2). These instrumental methods are important reference methods for food sample analysis. HPLC is commonly used for food sample analysis. Liquid chromatographic determination of SAC in food is simple because beverages or aqueous extracts from foods can often be injected into a column immediately after filtration. The most applicable method for the determination of SAC is reverse-phase HPLC coupled with ultraviolet (UV) light detector. The most popular reference method mentioned in the bibliography is the HPLC-diode array detector (DAD) method proposed by Lawrence and Charbonneau in 1988. A 5 μm C_8-bonded silica in a 150 mm × 4.6 mm column was used as a stationary phase in this method, with a mobile phase gradient ranging from 3% acetonitrile in 0.02 M KH_2PO_4 (pH 5) to 20% acetonitrile in 0.02 M KH_2PO_4 (pH 3.5) at a constant flow rate of 1.0 mL/min. The chromatograms were obtained at a wavelength of 210 nm (Lawrence and Charbonneau 1988).

The analytical conditions for the determination of SAC by means of HPLC coupled to electrospray mass spectrometry (HPLC-ESI-MS) have been studied recently. Mass spectrometry (MS) is a powerful qualitative and quantitative analytical technique that has been introduced in many analytical and research laboratories in the last 10 years. The combination of HPLC with tandem MS yields a particularly powerful tool and it is the future method of choice for the determination of sweeteners. A simple and rapid method for the simultaneous determination of nine sweeteners, including SAC, in various foods by high-performance liquid chromatography–electrospray mass spectrometry (HPLC-ESI-MS) is developed. Mass spectral acquisition is done in the negative ionization mode by applying selected ion monitoring. The sweeteners are extracted from foods with 0.08 mol/L phosphate buffer (pH 7.0)—ethanol (1:1), and the extract is cleaned up on a Sep-pak Vac C_{18} cartridge after the addition of tetrabutylammonium bromide and phosphate buffer (pH 3.0). The quantification limit of SAC is 1 mg/kg (Koyama et al. 2005). Another simultaneous determination method of SAC and other additives in foods by Liquid chromatography-electrospray ionization (in negative mode)-tandem mass spectrometry (LC-ESI(–)-MS/MS) is proposed. A mixture of acetonitrile–water (1:1) was used to extract these additives from solid food matrices and acetonitrile was used to extract them from liquid food matrices. SAC was identified and determined in the negative ionization mode, using single-reaction monitoring of product ion ($m/z = 106$) from its precursor ion ($m/z = 182$) (Ujiie et al. 2007).

Capillary electrophoresis (CE) is an interesting alternative to HPLC. The resolving power of this technique is comparable with that of HPLC. Different types of CE have been used. MEKC (Boyce 1999) and capillary isotachophoresis (CITP) (Herrmannová et al. 2006) are the types of CE most used for the determination of SAC.

In the past, the preferred technique used for screening SAC and other sweeteners was thin-layer chromatography (TLC) and for quantification, was gas chromatography (GC). TLC methods were developed for separating sweeteners from other impurities using generally layers of polyamide. A TLC method that was developed in 1970 detected SAC in a quantity of 2 μg in various foods (Takeshita 1972). In the literature, there are a number of methods that determine SAC and other sweeteners by TLC-UV and are characterized by a sensitivity that fluctuates in the microgram range (Nagasawa et al. 1970). Artificial sweeteners including SAC have been determined by GC methods, which are usually sensitive and selective. The main drawback of GC methods is the time-consuming derivatization step. This step is necessary for the conversion of sweeteners in volatile compounds so that we can determine them with GC. The derivatization of SAC is commonly achieved with trimethylsilylation (Dickes 1979).

40.4 Conclusions

The determination of SAC individually or simultaneously with other sweeteners in mixtures is very important for consumer safety. Researchers focus their efforts on developing analytical methods for

simple, rapid, and low-cost-sensitive determination. Sensitive and robust analytical methods are essential to meet the needs of growing markets in quality control and consumer safety. Scientists have applied a wide variety of instrumental techniques. Today, the method of choice for the determination of artificial sweeteners in different complex food matrices is HPLC because of its multianalyte capability, compatibility with the physicochemical properties of sweeteners, high sensitivity, and robustness. However, biosensor technology has offered some methods of direct detection in simpler and cheaper matrices, without sample preparation. Due to a constant rising demand for alternative methods for the determination of SAC, and due to the increasing development of MS or tandem mass spectrometric methods, a number of procedures based on MS coupled with liquid chromatography for the determination of SAC will be expected to appear.

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Section XII

Salt Replacers and Taste Modifying Compounds

41

Sodium Replacers

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41.1 Introduction

There is a clear evidence that current high salt intake in Western societies constitutes a major factor in the increase of blood pressure and, thus, the increased incidence of several diseases such as diabetes, heart disease, stroke, and kidney disease (He et al., 2010; Doyle and Glass, 2010). The governments of Western countries are imposing regulations and programmes to control salt by establishing limits of salt in processed foods and thus moderate its daily intake by consumers. Such measures are trying to encourage food industry to reduce salt in processed foods for both the improvement of the health of the population and the reduction in the long-term health-related costs. A reduction in salt intake toward less than 5–6 g/day would be very beneficial to consumers, and would save a large amount of money to healthcare services (Cobiac et al., 2010). Some countries like Finland and the United Kingdom have already reduced the amount of salt consumed, being the action based on a combined action for salt reduction by the food industry, a clear labeling on food products, and an effective communication to consumers about potential harmful effects of salt on health (He et al., 2010).

Food industry is trying to reduce salt and a sodium reduction between 20% and 30% has been in general achieved in a large variety of foods. The reduction in sodium intake should be considered as a dietary advice for all consumers with moderate risk of cardiovascular disease but should be strongly recommended for those at high risk. However, consumers are not fully aware for this. A recent report based on health-style surveys in the United States during the period 2005–2008 revealed that about half of hypertensive patients reported that they were reducing their intake of sodium and also were reading food labels for salt (Ayala et al., 2010). However, recent data from the Third National Health and Nutrition Examination Survey showed that over 95% of men and over 75% of women exceeded the recommended daily tolerable upper intake of sodium (Doyle and Glass, 2010). On the other hand, a significant amount of potassium, which is present in fruits, vegetables, and low-fat dairy products, has been shown to reduce blood pressure (Adrogué and Madias, 2008) even though potassium intake should not exceed a certain level.

Some of the difficulties found by consumers when trying to reduce salt consumption is that most of the salt consumed comes from salt added during cooking, salt present in processed foods, or from salt-containing sauces. It was estimated that <10% is attributed to salt added during home cooking while more than 75% proceeds from processed foods and foods served at restaurants (Mattes and Donnelly, 1991). For instance, recent data on the sodium content in more than 7000 Australian processed foods indicated

that breads, processed meats, and sauces were especially above the recommended levels (Webster et al., 2010). Breads and cereals may contribute up to 50% of the total sodium intake depending on the countries (Brown et al., 2009).

The major food sources of sodium in diverse East Asian and Western population samples were recently assessed based on data from the INTERMAP Study (Anderson et al., 2010). The reported results indicated that dietary sodium was mostly from salt added in home cooking in China while, on the other hand, it mostly came from processed foods in Japan, the United Kingdom, and the United States. The authors concluded that different strategies are needed depending on the countries. So, it would be necessary to reduce the presence of salt in processed foods for an effective sodium intake reduction in Western countries and Japan, while less salt used in home cooking would be necessary in China.

The data on sodium intake may proceed from either the estimates based on urinary sodium excretion or from the estimates based on dietary survey (Brown et al., 2009). Recent studies of sodium in the urine of certain Belgian (45–65 years old (Vandevijvere et al., 2010)), Slovenian (25–65 years old (Ribic et al., 2010)), and young Swedish (18–20 years old (Hulthen et al., 2010)) consumers revealed that salt intake in men was alarmingly high and equivalent to more or less twice the recommended salt intake.

41.2 The Relevance of Salt in Foods

Salt is extensively used in the food industry for many reasons. It constitutes an excellent preservative, extending shelf-life and preventing the growth of spoilage microorganisms, contributes to water activity reduction, helps to control the enzyme activity, contributes to retain water and to the partial solubilization of certain proteins, and gives a typical salty taste to foods while also enhancing the flavor of food products. In fact, taste is one of the most important challenges for reducing salt content in foods containing it to achieve the same perceived saltiness with less sodium content (Dötsch et al., 2009).

It must be kept in mind that salt not only plays an important role in processed foods against pathogenic and spoilage microorganisms providing preservative action (Hutton, 2002; Durack et al., 2008), but also plays secondary roles, like a better enzyme stability in meat products (Toldrá, 2006), so that caution must be taken when replacing salt by other substances. For instance, sodium chloride is able to inhibit the growth and toxin production by *Clostridium botulinum* in processed meats and cheeses (Taomina, 2010).

The increase in the water-holding capacity (WHC) of meat, poultry, and fish products, and thus its process yields, is another benefit associated with the use of salt (Offer and Trinick, 1983). Furthermore, proteins are solubilized and the viscosity of meat batter increases (Desmond, 2006).

Sodium chloride is an effective agent for the control of enzyme activity in muscle foods, with inhibitory action against muscle proteases like cathepsins, dipeptidylpeptidases, and aminopeptidases (Toldrá and Flores, 1998). A salt replacer like potassium chloride was reported to exert a similar effect as sodium chloride but calcium and magnesium chlorides exerted a more inhibitory effect (Armenteros et al., 2009a).

Salts replacing sodium chloride may affect the release of volatile aroma compounds. So, the binding ability of sarcoplasmic protein extracts to branched aldehydes, hexanal, and methional was reported to be reduced by NaCl and KCl but no effect was detected on octanal and 2-pentanone (Pérez-Juan et al., 2006). Much lower effects were observed with $MgCl_2$ and $CaCl_2$. The headspace concentration of volatile compounds in water solutions was also studied. It was reported that KCl produced a similar salting-out effect as NaCl but $MgCl_2$ and $CaCl_2$ produced a much lower salting-out effect (Pérez-Juan et al., 2007).

41.3 Technology for Salt Reduction

Different strategies for sodium reduction can be adopted by the food industry (Toldrá, 2007; Dötsch et al., 2009): (1) the replacement of sodium chloride by other salts like potassium chloride. However, this solution has its limitations due to the bitter and metallic taste imparted by potassium, especially at replacement levels above 40%; other chloride salts like calcium and/or magnesium chloride can also partially replace sodium chloride but only at minor levels because they give a metallic aftertaste to the

food; (2) the gradual salt reduction in foods, if it does not affect its shelf-life, in order to reduce the sodium intake and make consumers adapted to low-sodium levels and thus, less sensitive to salty taste and less demanding for salt. However, this approach takes time and has limitations when low levels might affect sensory quality; and (3) the education of consumers toward the use of lower amounts of salt in home cooking and their demand and/or request for low salt use in restaurants and take-away businesses. The understanding of the human taste receptors would explain more about the salty taste perception and the influence of genetic variability (Dötsch et al., 2009).

Numerous patents have been filed for the replacement of sodium by other cations. The most extended practice consists of the use of potassium chloride up to a certain level, where its bitter taste detection is not reached. Other salts used are calcium and/or magnesium based even though at lower levels because of their metallic taste. These alternative salts are usually based on chloride, but lactate, citrate, ascorbate, carbonate, and sulfate have also been used. Flavoring additives such as pepper, onion, garlic, tomato, sweet pepper, and so on may be added to mask such unpleasant tastes associated to bitterness and/or metallic aftertastes (Toldrá and Barat, 2009). Also, a taste enhancer like a dehydrated proteolyzed protein from milk or cereals may be used; the enhancing effect has been attributed to the basic amino acids. Other recent enhancing substances such as alapyridain, alkyldienamides, and high ribonucleotide yeast extracts have been reported (Dötsch et al., 2009).

41.4 Applications of Sodium Reduction in Foods

The reduction of sodium represents some difficulties, especially for foods where salt plays important technological functions such as preservation, WHC, or salty taste. Anyway, different low-sodium foods have been developed in recent years even though the final degree of reduction is not always as high as desirable. The reduction of sodium in cheese is not easy but reductions of up to 0.5% salt in Cheddar cheese and up to 35% in cottage cheese have been reported (Johnson et al., 2009). The dough with reduced salt content could be managed without affecting its rheological properties, but the complete absence of salt was reported to affect the structural quality of dough and bread (Lynch et al., 2009). A brown bread with 32% less sodium and acceptable quality could be produced by mixing potassium chloride, calcium carbonate, magnesium chloride, and magnesium sulfate (Charlton et al., 2007). Sauerkraut with good quality could be produced with less than half of the typical salt concentrations of 2–2.5% by adding a starter culture of *Leuconostocmesenteroides*to cabbage (Johanningsmeier et al., 2007). The sodium content could be reduced in dry-cured pork loin down to 50% by using a mixture of KCl, $MgCl_2$, and $CaCl_2$ without significantly affecting either the sensory and/or safety quality of the final product (Aliño et al., 2009; Armenteros et al., 2009b,c). Sodium content could also be reduced in dry-cured ham by about 40% by similar mixtures of chloride salts keeping similar physicochemical properties and low microbiological development (Blesa et al., 2008; Aliño et al., 2010; Ripollés et al., 2011).

41.5 Analysis of Cations

As previously mentioned, one of the most relevant strategies used by industry consists of the use of potassium salts with addition of calcium and magnesium salts as partial replacement of sodium chloride. These alternative salts are usually based on chloride but lactate, citrate, ascorbate, carbonate, sulfate, and so on have also been used. The details for the analysis of cations such as sodium replacers by ion chromatography (IC) are described below. The use of atomic absorption spectrometry or inductively coupled plasma-optical emission spectrometry can constitute an alternative but these methodologies are described elsewhere (see Chapter 31).

41.5.1 Sample Preparation

The sample pretreatment previous to the determination of cations by IC techniques for simple food matrices like drinking water or beverages involves only simple operations, such as filtration, sample dilution, or

pH adjustment. In the case of foods, typical sample preparation consists of the extraction of ground sample with warm water, or low-concentration HCl solution (i.e., –0.5 N HCl), by homogenization for a period of time around 10–15 min under refrigeration with either a stomacher or stirring (Armenteros et al., 2009b). Once the suspension is cooled, it may be deproteinized (i.e., with Carrez solutions I and II) and centrifuged at 5000–10,000 × *g* for 20 min under refrigeration. The supernatant is filtered through glass wool to remove any fat on the surface and the pH is adjusted if required. The final soluble extract is filtered through a 0.2 μm nylon membrane filter before it is injected into the chromatograph.

Other extraction techniques, especially for solid foods where divalent cations may be bound to matrix substances, include accelerated solvent extraction with high-pressure and high-temperature or microwave-assisted extraction, where samples are digested with mineral acids (Klampf, 2004). Even though in most of the cases the amount of cations exceed the concentration required for the analysis and must be diluted, in some cases the cations' concentration in the initial food may be low and need preconcentration before the analysis. To this aim, solid-phase extraction is a technique that permits the concentration of ions and eliminates possible interferences in four steps. The first step consists of the conditioning of the packing material, the second step is the injection of the sample in the cartridge, the third stage is the washing of the cartridge with an adequate solvent, and the fourth step is the elution of the analytes with an adequate solvent. Different materials like chelating or ion exchange may be used as sorbent packagings (Camel, 2003). Reverse-phase cartridges have been also used to remove organic components (Spiro and Lam, 1995).

41.5.2 Chromatography

Since the introduction of IC in 1975, it has gained new areas of application with continuous improvements in the efficiency and selectivity (Lucy, 1996). The improved convenience of IC makes it a standard analytical methodology for the analysis of cations in foods.

IC is typically used in water analysis for the determination of anions and cations in drinking water and natural mineral waters of different composition and different total mineralization (Gros and Gorenc, 1997; Walter and Gordon, 1997; López-Ruiz, 2000). In fact, IC was recommended in the United States by the Environmental Protection Agency as the official method for analysis of drinking water samples (Romano and Krol, 1992). It has shown good performance even in the analysis of trace or ultratrace concentrations of certain ions in complex water samples with large quantities of matrix ions (Frankenberger et al., 1990).

The separation of heavy and transition metals with ion exchange requires complexation of the metal ions in the mobile phase to reduce their effective charge density and to reinforce the differences in selectivity among them. The simultaneous determination of certain minerals of nutritional relevance (copper, nickel, zinc, cobalt, magnesium, and iron) was performed in foods by IC coupled with UV–vis detection (Fredrikson et al., 2002). The methodology was based on the formation of mineral complexes by pyridine-2,6-dicarboxylic acid in the mobile phase, which were then postcolumn derivatized with 4-(2-pyridylazo)resorcinol (PAR), resulting in mineral–PAR complexes detectable at 500 nm. The column used was a CSSA (Dionex, Sunnyvale, CA). Kelkar et al. (2010) also reported the determination of alkali, alkali earth, and transition metal ions in powders and pellets.

The availability of column packing like zirconia with good thermal stability (up to 90%) has allowed a reduction of the separation time of most typical cations by means of increasing the temperature and flow rate during the separation. So, up to 60% in the separation time of Li^+, Na^+, NH_4^+, K^+, Rb^+, Cs^+, Mg^{2+}, Ca^{2+}, and Sr^{2+} was achieved with a CS12A column (Dionex, 150 × 3 mm) at 60°C and 1.3 mL/min. The chromatograph had to be modified to be compatible with the flow rate and temperature conditions. Detection was achieved by suppressed conductivity (Chong et al., 2003). Typical running times up to 20 min could be reduced to <5 min. The increases in column temperature reduces the solvent viscosity, and thus results in a decrease in the backpressure. So, higher flow rates may be used, giving an increase in separation speed. Separation efficiency was also reported to improve with higher temperature due to enhanced mass transfer (Chong et al., 2003).

So, IC constitutes a useful analytical tool for the simultaneous determination of a wide range of cations in food samples that include those in which formulas for salt reduction have been applied. A few examples

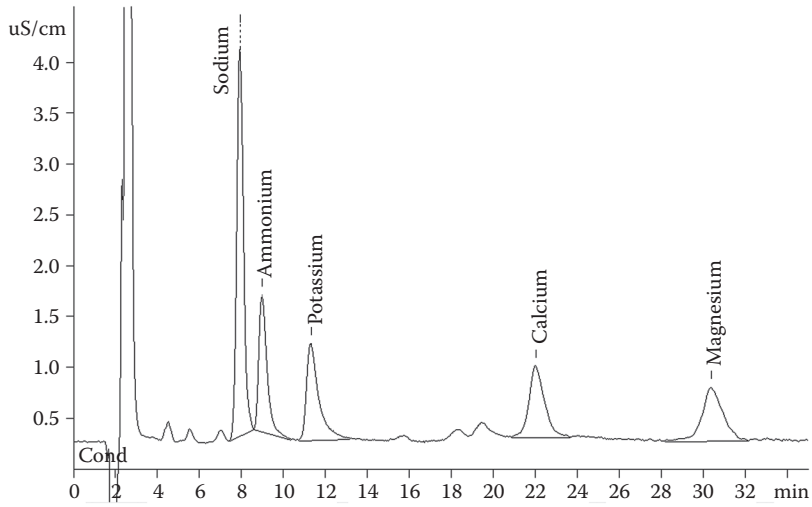

FIGURE 41.1 Chromatogram of dry-cured ham with partial replacement of sodium chloride by a mixture of potassium, calcium, and magnesium chloride. The separation was performed by IC (Advanced Compact IC 861, Metrohm® Lt., Herisau, Switzerland) with a conductivity detector using an analytical column Metrosep C3-250A (250 mm × 4.0 mm) of polyvinyl alcohol with carboxyl groups with particle size 5 μm, at 40°C. The mobile phase that consisted of 3 mM nitric acid with 50 mL/L acetone was eluted at 1 mL/min.

of its use and applications, especially for the analysis of cations replacing sodium in low-salt foods, are given below.

Typical formulas for sodium replacement may contain salts of potassium, calcium, and/or magnesium. These cations have been successfully analyzed by IC in low-salt dry-cured loins (Armenteros et al., 2009b) and low-salt dry-cured hams (Armenteros et al., 2009c). The separations were performed by IC with a conductivity detector and using an analytical column of polyvinyl alcohol with carboxyl groups (Metrosep C3-250A from Metrohm, Herisan, Switzerland), 250 mm × 4.0 mm, with particle size 5 μm, maintained at 40°C. The mobile phase that consisted of 3 mM nitric acid with 50 mL/L acetone was eluted at 1 mL/min. An example of chromatogram for a low-salt dry-cured ham is shown in Figure 41.1. Another recent commercially available column is a sulfonate polystyrene–divinylbenzene copolymer (Metrosep C 5 column from Metrohm, 150 mm × 4.6 mm, particle size 12 μm) to be eluted with a solution of 6.0 M oxalic acid, 3.0 M citric acid, pH 4.2.

Alkali and alkali earth metal ions plus ammonium were also analyzed in milk whey by IC using a carboxylate-functionalized capillary column IonPacCS12A (Dionex, 250 × 4 mm, particle size 8 μm) at 25°C and eluted at 1 mL/min with 20 mM methanesulfonic acid, and coupled with an electrochemical suppressed conductivity detector using a cation self-regenerating suppressor CSRS-I (4 mm) (Dionex). The whey-soluble sample, after whey proteins precipitation with the Carrez solutions I and II, was separated by centrifugation at 5000 rpm for 1 h and the supernatant was collected and diluted with water, and then filtered and injected into the system (Cataldi et al., 2003). Similar conditions were successfully used in the analysis of such cations in fruit juices and purées (Trifirò et al., 1996). K^+, Mg^{2+}, and Ca^{2+} were analyzed in tea with an IonPac CS3 (Dionex) column using 27.5 mM HCl + 4.5 mM 2,3 diaminopropionic acid monohydrochloride as the eluent (Spiro and Lam, 1995).

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42

Triterpene Glycosides

Markus Ganzera

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42.1 Introduction

Like all terpenoid compounds, triterpenes are composed of isoprene units. Via farnesyl-diphosphate, six of them form squalene, an acyclic intermediate that is synthesized in all living organisms. From this compound, two biosynthesis pathways originate. Through epoxidation, cyclization, and conformational changes, one leads to the formation of cycloartenol and cholesterol (which is the precursor of steroids, steroid alkaloids, and cardenolides), and the other one to triterpenes. The latter are a large group of natural compounds, with ~1700 derivatives known thus far. They share the same number of carbons in their scaffold (30), and depending on the number of rings they can be separated in tetra- and pentacyclic derivatives [1–3]. Examples for the first group are dammaranes and lanostanes; they both derive from gonane, the steroid nucleus. Oleananes, ursanes, and lupanes are pentacyclic triterpenes (Figure 42.1).

The large number of naturally occurring triterpenes is not only explainable by their different scaffolds, but also by an enormous structural variability. They can occur as lipophilic aglycones (e.g., anti-inflammatory boswellic acids in *Boswellia serrata* resin [4], or the widely distributed betulinic acid [5]), oxidized derivatives with medium polarity (such as bitter tasting cucurbitacines in *Bryonia cretica* or *Ecballium elaterium* [6]), or hydrophilic triterpene glycosides. Depending on the number of sugar chains linked with the aglycone monodesmosides (one chain), bisdesmosides (two chains), or rarely occurring tridesmosides (three chains) are differentiated. The individual sugar chains are branched or unbranched, and they can be composed of a varying number of different monosaccharides (most common are glucose, galactose, xylose, and arabinose), acylated sugar moieties, or uronic acids. Because of biosynthesis, sugar residues are most commonly attached to positions C-3 and C-28 of the aglycone [2].

Triterpene glycosides are assigned to the group of saponins, a class of natural compounds that also includes steroid glycosides and steroid alkaloid glycosides. The term saponin is deduced from the Latin

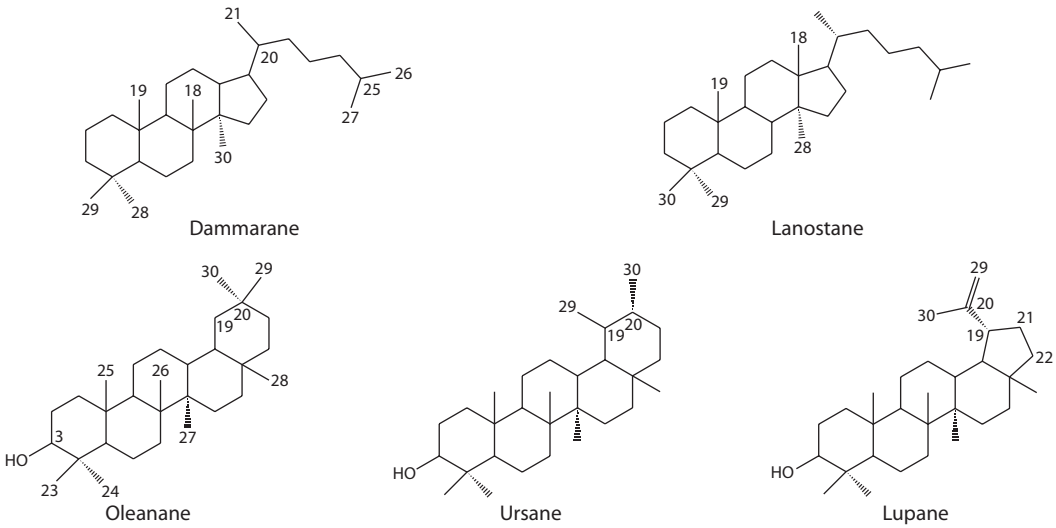

FIGURE 42.1 Most common tetra- and pentacyclic triterpene aglyca.

word "sapo" (soap), and describes some of the commonly observed characteristics of saponins. Due to their hydrophobic (aglycone or sapogenin) and hydrophilic (glycosidic side chain(s)) nature, they are able to reduce surface tension, resulting in detergent and emulsifying properties, fish toxicity, and hemolysis. Especially, the "soap-like" properties have been utilized for hundreds if not thousands of years, as indicated by plant names like soapwort (*Saponaria officinalis*) or soapbark (*Quillaja saponaria*) [7]. But these physical properties are not equally distributed among saponins, so that they will not be applicable to all representatives. Thus, saponins are nowadays classified based on structural features rather than these typical properties.

Saponins including triterpene glycosides are widely distributed in nature. They mostly occur in plants (three out of four plants are assumed to contain saponins, usual concentration ranges in dried plants are 0.1–30%; [3]), but they have been reported from marine organisms like sea cucumbers as well [8,9]. In plants, triterpene saponins are only found in dicotyledons, particularly in species belonging to the Caryophyllaceae, Primulaceae, Polygalaceae, Hippocastanaceae, Fabaceae, and Sapotaceae families. Their occurrence in edible plants is usually undesirable because of a sometimes bitter, displeasing taste and toxic effects, so that the saponin content in many fruits and vegetables (e.g., cucumber) was reduced over the centuries by breeding [10,11]. Nevertheless, saponins show a multitude of highly interesting biological activities, and many of them contribute to/explain the health benefits of herbal remedies and dietary supplements. Therefore, this introduction is followed by a brief survey of the biological activities of saponins and a section reporting on general challenges and options associated with their analysis. In the chapters thereafter, a selection of most relevant medicinal plants containing triterpene glycosides and analytical methods for their analysis are presented.

42.2 Pharmacological Properties of Saponins

Saponins show a broad spectrum of biological activities, but their main indications are expectorant, anti-inflammatory, adaptogenic, and hypocholesterolemic. Typical examples for saponin drugs with expectorant properties are common ivy (*Hedera helix*), cowslip (*Primula veris*), or licorice (*Glycyrrhiza glabra*). It has been postulated that active ingredients in the plants (triterpene saponins) increase mucus production in bronchia via stimulation of the parasympathetic nervous system in the stomach. This is unlikely to a certain extent, because the amount of respective compounds in herbal drugs and preparations is not high enough to trigger nerval excitation. Thus, the actual mode of action remains unclear till date [3].

Anti-inflammatory activities of saponin drugs like horse chestnut (*Aesculus hippocastanum* [12]) and Asian ginseng (*Panax ginseng* [13]) can be explained by antioxidant properties or a reduced liberation of inflammation mediators like cytokines. Additionally, some saponins such as ginsenosides or glycyrrhizic acid also increase ACTH levels, and thereby show an indirect glucocorticoid activity [14]. The term adaptogen is used for herbal preparations that are intended to increase the body's resistance to stress, anxiety, and fatigue. These health problems comprehend a number of different elicitors, so that remedies with diverse biological activities (e.g., antioxidative, immuno-modulating, or neuroprotective) are utilized [15]. Several adaptogenic herbs contain triterpene glycosides as active ingredients, prominent examples are ginseng, gotu kola (*Centella asiatica*), or *Astragalus membranaceus.*

Cholesterol-lowering effects of saponins have especially been studied and described for soy (*Glycine max*). The beans contain ~6% of triterpene glycosides, compounds that form nonabsorbable complexes with cholesterol. Thus, a diet rich in soybean products results in lower blood and liver cholesterol concentrations [16]. Indeed, there is a negative correlation between soy consumption and the occurrence of cardiovascular diseases [17], and recent pharmacological studies indicated that soy isoflavones might significantly contribute to these effects [18].

42.3 Analytical Challenges in Saponin Analysis

The analysis of triterpene saponins has been described by biological and nonbiological methods. The first ones include the determination of hemolytic effects or immunoassays (ELISA, antibodies); the latter are mainly based on chromatographic or electrophoretic techniques (TLC, thin-layer chromatography; GC, gas chromatography; HPLC, high-performance liquid chromatography; CE, capillary electrophoresis) [19]. A common feature of many saponins is the absence of a (strong) chromophore in the molecule. Thus, when using an UV/vis detector, most of these compounds can be detected only between 200 and 210 nm. This unspecific range not only puts special requirements on the mobile phase used (e.g., UV-cut-off of solvents), but also hampers separation due to possibly interfering compounds. This is especially relevant when complex matrices such as plant extracts are assayed. Only a few saponins such as glycyrrhizic acid or cucurbitacines can be monitored directly at 254 nm.

Approaches to overcome the weak UV absorbance of saponins are either derivatization of the analytes or the use of a different detection technique. Many derivatization procedures create some practical problems, however. Commonly their points of contact on the target are OH-groups, and saponins usually possess several of them. If the applied procedure does not readily derivatize all of them in one step, a single saponin might yield a number of reaction products, so that separation and identification are even more difficult. Nevertheless, saponins have successfully been derivatized with reagents such as 4-bromophenacyl bromide or benzoyl chloride, and detected by UV at 260 nm thereafter [20,21]. Concerning change of the detection technique, several options are possible. Refractive index detection is one of them, but as it can be employed only under isocratic conditions, its use is drastically limited. Much more versatile in this respect are evaporative light-scattering detection (ELSD) or mass spectrometry (MS). Because of many desirable features, MS is increasingly becoming popular, but due to high acquisition and maintenance costs, this methodology is not affordable for many institutions. ELSD is a much simpler but still powerful technique that is especially popular for saponin analysis (see applications). Its principle can be described as follows. In a first step, the HPLC effluent is vaporized with the aid of a carrier gas (nebulization), then the solvent is evaporated in a heated tube, and finally the remaining sample particles (which are flushed through the instrument by a carrier gas) are detected by light scattering. Compounds can be detected with this "universal" detector, regardless of their chemical or physical properties; they only need to be nonvolatile to a certain extent (this is depending on the evaporation temperature). A significant disadvantage of ELSD compared to MS is a reduction in sensitivity, which usually is in the range of 50–100 ng on-column [22].

A prerequisite for any compound to be analyzed by GC is volatility. Saponins, being polar and rather large molecules do not fulfill this requirement by nature, and therefore they require derivatization prior to analysis. Suitable procedures in this respect are the formation of acetyl, methyl, or trimethylsilyl ethers [23]. In most of the related publications, only sapogenins were assayed by this approach after hydrolysis of the glycoside, one of the few reports on the analysis of intact saponins is the determination of ginsenosides

after trimethylsilylation [24]. CE has been used for saponin analysis as well, but one of the major drawbacks for this method is the already mentioned detection issue. This becomes especially relevant considering the minute amounts (several nL) of the sample that are injected. On the other hand, CE offers many advantages such as extremely high efficiency, rapid separations, and low sample and solvent consumption, so that the benefits of CE actually prevail. Separations by CE are always based on the differential movement of charged analytes in the electric field. Triterpene saponins are, unless they show a carboxylic residue, neutral; thus they migrate unresolved with the electroosmotic flow. Micellar electrokinetic chromatography (MEKC) is a technique to separate charged as well as uncharged molecules, and therefore commonly applied for saponin analysis [25]. Ionic detergents such as sodium dodecyl sulfate are added to the running buffer at a concentration above the critical micellar concentration. This results in the formation of charged micelles that move in the applied electric field. They interact with neutral as well as charged analytes (hydrophobic/hydrophilic interaction) and thereby enable their separation.

Last but not least, TLC can be applied for saponin analysis. It usually served as a simple approach to confirm purity and identity of the compounds, so that only a limited number of quantitative TLC applications can be found in literature [26]. Most of the time, densitometric techniques were used for detection, implying the necessity of derivatization with suitable spray reagents (e.g., vanillin/sulfuric acid) prior to analysis.

42.4 Medicinal Plants Containing Triterpene Glycosides and Their Analysis

42.4.1 *Aesculus hippocastanum* (Horse Chestnut)

The horse chestnut tree, which can reach a height up to 35 m and has characteristic, palmately divided leaves, is widely distributed in the temperate northern hemisphere (Greece to Scandinavia, North America). Of medicinal relevance are mostly the saponin rich, shiny brown, globular, and 1–2 cm in diameter large seeds of the plant [27]. They contain 3–5% of a complex mixture (more than 30) of triterpene glycosides called β-aescin or escin. Based on two aglyca, protoaescigenin and barringtogenol C, the different derivatives possess varying sugar residues on position C-3, and the OH-groups at positions C-21 and C-22 are esterified (e.g., with angelic acid, tiglic acid, or isobutyric acid). β-Aescin preparations are widely used as anti-inflammatory and vasoprotective, as well as in cosmetic and dermatological preparations [28].

The complex composition of β-aescin renders the analysis of individual saponins difficult. Thus, the method described in the *German Pharmacopoeia* uses a mixture of $FeCl_3$, acetic acid, and sulfuric acid to convert all saponins in red-colored derivatives, which can be determined photometrically at 540 nm. Prior to analysis, sample preparation by solvent partitioning is required, in order to remove the interfering sugars and flavonoids [27]. Another possible approach for analysis was described by Wagner in 1985. This author suggested hydrolysis of the saponin mixture by refluxing the sample with 0.1 N KOH for 15 min, followed by SPE cleanup on C-18 cartridges. The resulting deacylsaponins were then assayed by reverse-phase HPLC (RP-HPLC), using an acidic mobile phase comprising water and acetonitrile (ACN); detection was performed at 200 nm [29]. This method enabled the differentiation between protoaescigenin- and barringtogenol-glycosides, and the quantitative characterization of ß-aescin and plant extracts. The individual peaks obtained after hydrolysis were however not structurally identified.

The analysis of native aescin by HPLC was described by Kockar et al. [30]. They used isocratic conditions for analysis (35% acetonitrile in water with 40 mL 1 N H_3PO_4/l) on Zorbax ODS material, and simply summarized all signals that were visible in an aescin standard to construct a calibration curve. Individual peaks were not identified, yet the quantitative results of different extracts were in good agreement to those obtained by TLC. For the latter, a mobile phase composed of chloroform, methanol, acetic acid, and water (ratio 60:32:12:18) was well suited, spots were visualized by sulfuric acid spray reagent, and then the plate scanned at 537 nm. Another suitable TLC procedure (including method validation) was reported more recently, utilizing a mixture of acetic acid, water, and butanol (10:40:50) as the mobile phase and anisaldehyde reagent for detection [31].

Three LC-MS assays for the analysis of aescin can be found in literature. Griffini and coworkers identified 32 different saponins using thermospray MS for detection [32]. Separation was performed under

reversed-phase conditions (mobile phase: water and acetonitrile, both containing 0.01% acetic acid; gradient: in 50 min from 30% to 45% of ACN), and the compounds of interest were readily assignable as $[M + Na]^+$ adducts. This and a more recent study [33] focused on the identification of individual saponins, so that quantitative results and method validation are missing. Even though the investigated species was *A. chinensis*, the method published by Chen et al. [34] should be mentioned as well. These authors separated and quantified four major saponins (escin 1a and 1b, isoescin 1a and 1b) by RP-HPLC in diverse plant specimens, and confirmed their structures by ESI-TOF-MS.

42.4.2 *Astragalus membranaceus* (Astragalus)

The genus *Astragalus* comprises more than 1500 species, of which *A. membranaceus* (*huan qi* in Chinese) is medicinally most valued and used. The roots of this species contain bioactive polysaccharides, isoflavones, and triterpene saponins (astragalosides); the latter showed neuroprotective [35] and anticancer effects [36]. The plant is an important ingredient in many traditional Chinese medicine (TCM) preparations used as tonic and to treat nephritis and diabetes. Astragalosides are cycloartane glycosides, compounds in which carbons C-9 and C-10 of the aglycone are connected by a characteristic cyclopropyl group. Like most saponins, they show basically no UV absorbance, so that MS or ELSD were used for their detection (Table 42.1).

TABLE 42.1

Analytical Procedures for Assaying Triterpene Glycosides in *A. membranaceus*

Analytes	Method	Separation Conditions	Detection	Val.	Appl.	Reference
Astragalosides I, II, IV (+10 other saponins)	HPLC	Luna C-18, 5 µm, water, and acetonitrile	ELSD	1–4	S, SA +	[37]
Astragalosides I, II, IV, acetyl-astragaloside I (+6 isoflavones)	HPLC	Zorbax ODS, 5 µm, 0.3% FA in water and ACN	ELSD, UV	1–4	S, SA +	[38]
Astragalosides I, II, IV, acetyl-astragaloside I, alexandroside I (+12flavonoids)	HPLC	Shimpak ODS, 5 µm, 0.3% acetic acid in water, and acetonitrile	ELSD, UV	1–4	S, SA +	[39]
Astragalosides I, II, IV (+11 isoflavones)	HPLC	Xterra MS C8, 0.2% TFA in water and ACN	ELSD, UV	1–4	S, SA +	[40]
Astragaloside IV in bilogical samples	HPLC	Cosmosil C18, 5 µm, 5 µM Na-acetate in water and ACN	ESI-MS	1–4	S, SA +	[41]
7 Astraglosides	HPLC	Zorbax Extend C18, 5 µm, 0.1% FA in water and ACN	ESI-TOF-MS	1–4	S, SA +	[42]
6 Astragalosides (+7isoflavones)	HPLC	Zorbax Extend C18, 5 µm, 0.2% FA in water and ACN	ESI-TOF-MS	1–4	S, SA +	[43]
10 Astragalosides (+20isoflavones)	HPLC	Hypersil C18, 5 µm, water and acetonitrile	APCI-MS	—	SA	[44]
6 Astragalosides (+7isoflavones, ferulic acid)	UPLC	Zorbax SB-C18, 1.8 µm, 0.2% FA in water and ACN	ESI-TOF-MS	1–4	S, SA +	[45]
8 Astragalosides (+isoflavones, chalcones, organic acids)	UPLC	Zorbax SB-C18, 1.8 µm, 0.3% FA in water and ACN	ESI-TOF-MS	—	SA	[46]

Note: Appl., application; S, standard; SA, sample; +, quantification; Val., validated (1, sensitivity; 2, specificity; 3, accuracy; 4, precision).

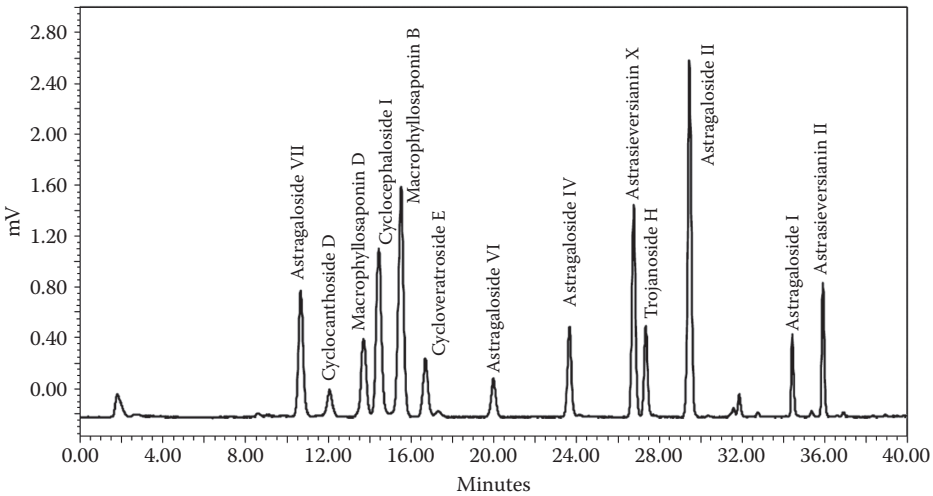

FIGURE 42.2 Separation of 13 *Astragalus* saponins by HPLC-ELSD, analytical conditions according to Ganzera et al. [37].

The first analytical procedure to assess all major astragalosides in *A. membranaceus* by HPLC was published by Ganzera et al. [37]. The described method not only enabled the quantitative determination of astragalosides I, II, and IV, but also of 10 other triterpene saponins (e.g., astragaloside VII, cyclocephaloside I, or trojanoside H; Figure 42.2). The latter were used to differentiate several Turkish *Astragalus* species. Separations were performed using a Luna C-18 stationary phase and an ACN/water gradient; a Sedex 55 ELS detector was utilized to monitor the saponins. Detection limits were in the range between 15 and 20 μg/mL, and the required analysis time was 40 min. Comparable methods were reported thereafter, most of them focusing on the simultaneous analysis of saponins and flavonoids [38–40]. For example, the procedure reported by Qi et al. [38] enabled the determination of four saponins and six isoflavonoids (e.g., calycosin, formononetin, and ononin), the method developed by Yu et al. [39] increased the number of identified compounds to 12 flavonoids and five saponins. All of these assays used reversed-phase stationary phases, acidic eluants, and the detection of the flavonoids was performed with a diode array detector at 210 or 280 nm.

The analysis of *Astragalus* saponins by MS has been described using time of flight (TOF) or triple–quadrupole instruments. In one case, ionization was performed by atmospheric pressure chemical ionization (APCI), otherwise electrospray ionization (ESI) in positive and/or negative mode was employed. The reported applications range from the determination of single saponins in biological samples [41], analysis of astragalosides in plant material and herbal preparations [42], as well as the simultaneous identification of isoflavones and saponins [43–46]. The latter were usually assigned at *m/z* values corresponding to $[M + H]^+$, $[M + Na]^+$, or $[M + HCOOH–H]^-$. Besides the already mentioned benefit of sensitivity (e.g., detection limits from 10 to 40 ng/mL have been observed when using TOF-MS; [43]), an additional advantage of MS is improved specificity. In contrast to MS, ELS detectors provide no spectroscopic information, and therefore peak purity cannot be verified, unless coeluting compounds are visible as shoulder. Most of the described LC-MS methods were validated and they showed to be well suited for quantitative investigations. Respective experiments were performed using external or internal standards (e.g., scutellarin or notoginsenoside R1 [38,43]). Separation conditions were comparable to those already mentioned, with the exemption of two recently published ultra performance liquid chromatography (UPLC) methods. The procedure described by Qi et al. [45] facilitated the baseline separation of six astragalosides and other plant constituents (all together 15 compounds) in <17 min. This is approximately four times faster than conventional HPLC. The stationary phase employed was a Zorbax SB-C18 (50 × 4.6 mm, 1.8 μm particle size) from Agilent, with flow rate, column temperature, and injected sample volume set to 0.6 mL/min, 25°C, and 2 μL, respectively.

42.4.3 *Centella asiatica* (Gotu Kola)

The leaves of this species, which is synonymous to *Hydrocotyle asiatica* (Apiaceae), are not only a common vegetable in Sri Lanka and Malaysia, but also important ingredients in many ayurvedic and TCM herbal preparations. Even in Western countries, dietary supplements containing gotu kola extracts are easily available. Their medical properties are described as tonic, diuretic, and the treatment of skin diseases and infections [47]. In the *Chinese Pharmacopoeia*, one triterpene saponin (asiaticoside) is listed as identity and quality marker of the drug, but besides this compound, several other similar triterpenes (e.g., madecassoside, asiaticoside B, asiatic acid, and madecassic acid) have been isolated and assayed. The employed techniques for their determination range from TLC, over high-speed countercurrent chromatography (HSCCC) to HPLC.

Gupta et al. [48] described an high performance thin layer chromatography (HPTLC) assay for the quantitation of asiaticoside, using a mobile phase comprising ethyl acetate, methanol, and water in the ratio of 60:12:8, the procedure reported by the group of Bonfill enabled the separation of four triterpenes (two glycosides, two aglyca) in plant material [49]. They used silica–gel plates and a mixture of ethyl acetate and methanol (6:4) for separation; the compounds were assigned by comparison with references and MS data. For the latter, respective TLC spots were scraped off, the adhered compounds dissolved in methanol, the solvent evaporated, and the residue analyzed by matrix-assisted laser desorption ionization-TOF-MS (MALDI-TOF-MS). The absorbing matrix for these experiments was α-cyano-4-hydroxycinnamic acid. The isolation of the same four triterpenes by HSCCC was published by Du et al. [50], utilizing a biphasic liquid system composed of *n*-hexane, *n*-butanol, and 0.05 M NaOH. The lower phase of this mixture served as stationary phase, the upper layer as initial mobile phase.

Most reports on the analysis of *Centella* triterpenes described the use of HPLC in combination with different detectors, in a similar fashion as already mentioned in previous chapters. All of them utilized C-18 phases for separation (e.g., Zorbax SB-C18, Cosmosil $5C_{18}$-MS II, or Aqua C18), applying isocratic (e.g., water, acetonitrile, and methyl tertiary-butyl ether in the ratio of 80:18:2 [51]) or gradient conditions (e.g., 0.1% acetic acid/acetonitrile [52]; water/acetonitrile [53]). The compounds of interest were either directly detected at low UV-wavelengths (206 nm [53]), after derivatization with benzoyl chloride in pyridine at 235 nm [54], by ELSD [55], or using MS [52]. For the latter, an ion trap as well as a TOF MS-instrument were successfully utilized in positive ESI mode, showing the most abundant signals for the saponins at $[M + Na]^+$ and $[M + NH_4]^+$, respectively.

42.4.4 *Cimicifuga racemosa* (Black Cohosh)

Possibly due to the widespread use of black cohosh products for the treatment of dysmenorrheal and climacteric symptoms, *C. racemosa* is well studied in analytical as well as pharmacological aspects. The nature of bioactive constituents (triterpene glycosides, isoflavones such as formononetin, or cimicifugic acids) and possible modes of action (e.g., estrogenic/serotonergic activity, bone nodule formation) are still under discussion [56,57]. Nevertheless, because of their unique structure and restricted occurrence, triterpene glycosides such as actein and 23-epi-26-deoxyactein (syn. 27-deoxyactein) are most commonly analyzed in order to assure the identity and quality of herbal material and commercial products (Table 42.2).

Triterpene glycosides of the cycloartane type in black cohosh challenge the analyst with the same problem as structurally similar constituents, which is the absence of any measurable UV absorbance. Several manuscripts described the successful use of ELS detectors for their analysis, beginning with Ganzera et al. [58] in 2000, followed by Li et al. [59] and Avula et al. [60]. For example, in Li's manuscript, the separation and quantitative analysis of 18 compounds is described, including 13 saponins (e.g., 26R and 26S actein, cimifugosides H-1 and H-2, and cimigenol derivatives), organic acids, and flavonoids (e.g., formononetin). A Sedex 75 ELS detector (temperature: 43°C, nebulizer: 3.4 bar nitrogen) provided enough sensitivity to assess all the above-mentioned compounds in plant material and herbal products [limit of detection (LOD): 25–50 ng on-column]. In the latter, the authors found large deviations concerning the content of triterpene glycosides (0.13–5.97 mg per serving) but no formononetin; some of the products did not meet the label claims. Compared to the previously mentioned assay, the UPLC procedure described by Avula et al. [60] required only 1/10 of analysis time (6 min versus 60 min).

TABLE 42.2

Analytical Procedures for Assaying Triterpene Glycosides in *C. racemosa*

Analytes	Method	Separation Conditions	Detection	Val.	Appl.	Reference
Actein, 26-deoxyactein, cimiracemoside A	HPLC	Discovery C-18, water, ACN, and reagent alcohol	ELSD, MS	1–4	S, SA +	[58]
10 Saponins (incl. cimifugosides), organic acids, flavonoids	HPLC	YMC ODS-AQ RP18, 0.05% TFA in water, ACN, and water	ELSD	1–4	S, SA +	[59]
Actein, 23-epi-26-deoxyactein, cimiracemoside A, formononetin	UPLC	Acquity UPLC BEH C18, water, and ACN/methanol (7/3)	ELSD, MS	1–4	S, SA +	[60]
Actein, 26-deoxyactein, cimifugoside, cimifugoside M	HPLC	Hypersil ODS, water, and acetonitrile	APCI-MS, UV	—	S, SA	[61]
5 Triterpenes (e.g., cimifugoside H, acetylshengmanol), + fingerprint	HPLC	Synergi-hydro, 10 mM ammonium acetate, ACN	TIS-MS	—	S, SA +	[62]
Triterpene glycosides, organic acids, flavonoids	HPLC	Aqua C18, ACN, and water	APCI-MS, UV	1–4	S, SA +	[63]
9 Saponins (e.g., cimigenol and shengmanol derivatives), 8 phenols, + fingerprint	HPLC	Zorbax DBS, 0.1% acetic acid, and acetonitrile	APCI-MS	—	SA	[64]

Note: Appl., application; S, standard; SA, sample; +, quantification; Val., validated (1, sensitivity; 2, specificity; 3, accuracy; 4, precision).

Nevertheless, the number of determined analytes was only four (actein, 23-epi-26-deoxyactein, cimiracemoside A, and formononetin), so that the whole triterpene-spectrum occurring in this species will not be reflected actually. All three assays [58–60] were fully validated and therefore can be used for quality control purposes.

In other studies, HPLC-MS was utilized for investigating the quality of black cohosh products or to distinguish among different *Cimicifuga* species [61–64]. *C. racemosa*, which is wildly occurring in the eastern parts of North America, shares the same habitat with *C. americana* and *C. rubifolia*. These three and other related Asian species (*C. dahurica*, *C. foetida*, or *C. heracleifolia*) are difficult to differentiate by morphological means. As an example, Jiang et al. [63] found cimifugin instead of cimiracemoside C in several products which claimed to contain black cohosh only. Cimifugin is a typical marker for Asian *Cimifuga* species; therefore, the authors concluded a possible adulteration or wrong identification of the plant material used. Analyses were performed on a C-18 stationary phase (Aqua C18), employing a water/acetonitrile gradient over a runtime of 55 min. Like in most of the related studies mentioned above, triterpene glycosides were directly assignable in positive APCI ionization mode at *m/z* values of $[M + H]^+$, and their quantification was feasible at 203 nm (LOD: 0.11–0.38 μg; not defined if μg/injection or μg/mL). Another interesting observation is reported in the manuscript by He et al. [64]. They noticed significant changes in the triterpene fingerprints of a freshly prepared ethanolic *Cimicifuga* extract compared to the same extract after concentration under the influence of heat; at room temperature, the same changes were noticed after 2 months. Based on MS data, these authors postulated the conversion of 23-*O*-acetylshengmanol derivatives to cimigenol-type constituents, emphasizing the importance of the manufacturing process for product quality and consistency.

42.4.5 *Glycine max* (Soy)

Depending on variety, geographic conditions and maturity soybean seeds contain 0.6–6.0% saponins, a complex mixture of triterpene glycosides mainly found in the germ and cotyledons. On the basis of their

aglycone (soyasapogenol A and B), these triterpene glycosides are divided into two groups. Soyasaponins A are bisdesmosides and supposed to be responsible for the bitter and adstringent taste of soy. Representatives of the B-type are monodesmosidic and found in intact plant tissue mainly as conjugates with 2,3-dihydro-2,5-dihydroxy-6-methyl-4H-pyran-4-one (DDMP). Besides isoflavones, especially type B saponins are of great relevance for the health benefits of soy (e.g., hypocholesterolemic, antimutagenic, and immunostimulant). Other saponins such as group E saponins are possibly artifacts arising from extraction or processing of the raw material [65].

A simple and fast procedure for the estimation of total saponins in legumes including soy was published by Gurfinkel and Rao [66]. The compounds of interest were extracted from plant material (e.g., soy flour) with methanol, nonsaponins were removed by precipitation with 0.4 M ammonium sulfate solution, and the saponins purified on an Oasis HLB SPE cartridge. The so-obtained material was then assayed by TLC (mobile phase: chloroform/methanol/water = 65/35/10 (lower phase), stationary phase: silica gel), the spots visualized by spraying with sulfuric acid, and the results evaluated densitometrically (reflectance mode) in comparison with a standard. The results obtained (0.58% saponins in soy) were accurate, precise, and in good agreement to literature data. Hubert et al. [67] assayed soyasapogenol A and B by HPLC. For that purpose, the samples (43 soybean varieties and soy dietary supplements) were treated with 1 N HCl (6.5 h at 85°C), and the resulting aglyca analyzed on C-18 material (mobile phase: acetonitrile, 1-propanol, water, and acetic acid; detection wavelength: 205 nm). According to previous studies, no correlation in the ratio of the two aglyca was observed within different samples.

More detailed investigations regarding the exact composition of group B saponins in soybeans enabled three other HPLC methods [68–70]. All of them focused on the quantitative determination of soyasaponins I, II, III, and IV, with [69] allowing the simultaneous analysis of isoflavones, and [70] enabling the determination of DDMP-derivatives and isoflavones as well. For quantifying DDMP conjugates, the authors reported an interesting approach. Based on the fact that the total soyasaponin content remains constant when the conjugated saponins are degraded to non-DDMP forms by alkaline hydrolysis, a response factor (peak area before hydrolysis versus peak area after hydrolysis) was determined. Using this factor, the amount of DDMP derivatives was then calculated using calibration curves of the respective non-DDMP saponins.

The most comprehensive study on soy saponins was published by Decroos et al. in 2005 [71]. They reported the simultaneous quantification of differently glycosylated and acetylated triterpenes (group A and B) including respective DDMP-forms. Separations were performed on an Aquasil RP C-18 from Thermo-Hypersil, the mobile phase comprised water and acetonitrile, both containing 0.001% acetic acid (required analysis time: 80 min). At the HPLC outlet, the eluate was split in the ratio of 5:95, so that five parts were submitted to MS analysis (Finnigan Mat 95 ion-trap, ESI in positive mode) for compound identification; the rest was analyzed and quantified by ELSD (Sedex 55, 40°C, gas pressure 2.3 bar). The method was validated, and then used to assess the content and composition of triterpene glycosides in diverse soygerm samples. Quantifications were performed using an internal standard (equilenin). They showed that 80% of group B saponins are present as DDMP derivatives, and considerable amounts (30%) of A-type representatives are acetylated; in most of the samples, the content of group A saponins was higher (1.5–2.8 mg/100 g sample).

42.4.6 *Glycyrrhiza glabra* (Licorice)

According to the *European Pharmacopoeia*, only *G. glabra* roots can be used as drug; in the *Chinese Pharmacopoeia*, three species (*G. uralensis*, *G. glabra*, and *G. inflata*) are listed as possible sources for licorice. Triterpene saponins are among the characteristic constituents of the plant and responsible for its sweet taste. Considering the three used species, more than 20 different saponins, all of oleanane type, have been identified thus far, with glycyrrhizic acid (syn. glycyrrhizin) being the most known and studied one. Many of the pharmacological properties of licorice (antiulceric, anti-inflammatory, and hepatoprotective, to name a few) are explainable by this compound and the corresponding aglycone glycyrrhetic acid [72].

In a recent review by Zhang and Ye [72], procedures to analyze the licorice are summarized. The authors state that, despite the number of identified saponins, nearly all reports describe the determination

of glycyrrhizic acid only, regardless of the method employed. This compound has been determined in plant material as well as multicomponent herbal preparations by using TLC (e.g., mobile phase: ammonia/water/ethanol/ethyl acetate = 1/9/25/65; detection: 254 nm [73]), HPLC-UV [74,75], HPLC-MS [76], and CE [77]. The majority of HPLC separations were performed on reversed-phase material (C-18 or C-8) using an acidic mobile phase. This is explainable by the fact that all of the licorice saponins are glucuronides, requiring an acidic mobile phase to suppress the ionization and increase the retention. The direct ultraviolet (UV) detection of glycyrrhizic acid at 254 nm is possible due to diene and carbonyl substructures in the molecule. In case the compound was detected by MS, usually ESI was used. In many CE studies, borate buffers were selected for the assessment of glycyrrhizic acid, most of the time permitting the determination of other compounds, such as flavonoids (e.g., liquiritin). A typical example for such an assay is the one published by Rauchensteiner et al. ([77], Figure 42.3). They obtained optimum results (baseline separation of five compounds including glycyrrhizic and glycyrrhetic acid in 15 min) by using a 70 mM sodium tetraborate solution with pH 9.22 as electrolyte, with applied voltage and capillary temperature set to 25 kV and 20°C, respectively. Recording the electropherograms at 254 nm, the limit of quantification (LOQ) for glycyrrhizic acid was found to be 5 μg/mL.

Among the few reports on the analysis of several triterpene glycosides is the one published by Ye and coworkers [78]. By means of LC/ESI-TOFMS, they studied a Chinese herbal formulation used in cancer therapy, which, among other constituents, contained *G. uralensis*. Separation conditions were as those described above, yet the MS system used facilitated the identification of a multitude of compounds including licorice saponins A3, B2, G2, and K2. This study emphasized on the optimization of MS parameters (Saponins were mainly identified in negative ionization mode.) and the discussion on fragmentation patterns. Thus, quantitative results or method validation were not presented.

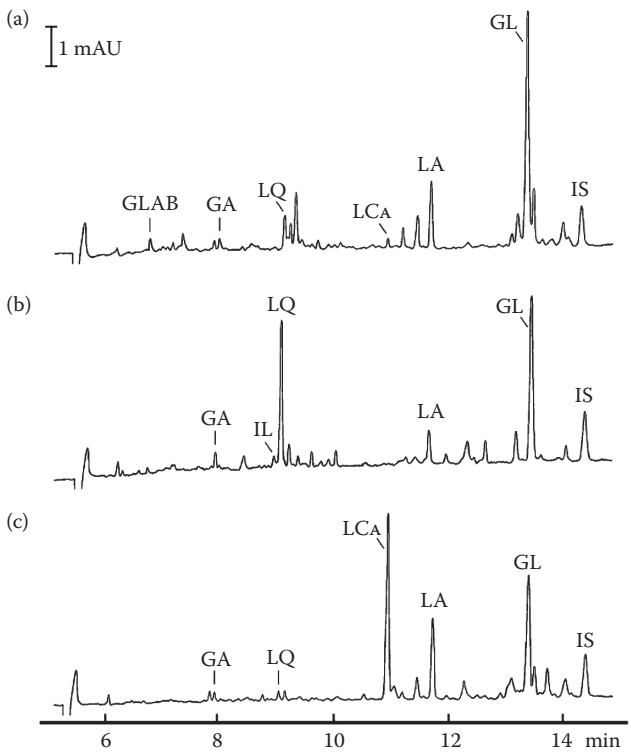

FIGURE 42.3 Analysis of *G. glabra* (a), *G. uralensis* (b), and *G. inflata* (c) by CE (GLAB: glabridin; GA: glycyrrhetic acid; IL: isoliquiritin; LQ: liquiritin; LCA: licochalcone A; LA: liquititin apioside; GL: glycyrrhitin; IS: internal standard). (Reproduced from Rauchensteiner, F. et al., *Journal of Pharmaceutical and Biomedical Analysis*, 2005, 38, 594–600. With permission.)

42.4.7 *Hedera helix* (Common Ivy)

Despite the well-studied pharmacological properties of common ivy and its medicinal relevance (A monograph of *Hederae folium* can be found in the *European Pharmacopoeia*.), only few analytical procedures for the assessment of saponins in this plant have been reported. These oleanolic acid or hederagenin derivatives occur natively as bisdesmosides (hederacosides, syn. hederasaponins), but the sugar residues attached to position C-28 are already cleaved under mild conditions (e.g., overnight in aqueous solution), and respective monodesmosides (hederins) are formed [3].

For *H. helix*, the *European Pharmacopoeia* requires the confirmation of identity by TLC and a quantitative HPLC assay; for both, hederacoside C is used as reference [73]. TLC separations are performed on normal-phase material (silica gel), using a mixture of formic acid (FA), methanol, acetone, and ethyl acetate (4/20/20/30) as the mobile phase; detection is achieved with ethanolic sulfuric acid. The described HPLC procedure is based on C-18 material and a mobile phase comprising water and acetonitrile (both with phosphoric acid); hederacoside C is quantified at 205 nm. Besides, a few similar TLC assays [79,80], only two procedures with increased selectivity have been published to date. Maffei Facino et al. [81] quantified three saponins (α-hederin, and hederacosides B and C) in plant material and commercial products by direct MS analysis, Crespin and colleagues [82] reported on an HPLC-ELSD assay for the determination of six saponins (hederasaponins C, D, B, and respective hederins). These authors developed a simple water/acetonitrile gradient for analysis on a Bondclone C18 column, enabling the excellent separation of the six markers in <20 min. With a content of 7.7%, hederasaponin C was found to be the major saponin in the sample investigated.

42.4.8 *Primula veris* (Cowslip), *Primula elatior* (Oxlip)

Like common ivy leaves, the roots of both *Primula* species (also known as primrose) are found in many herbal preparations used for the treatment of respiratory tract problems (cough, asthma, bronchitis, or catarrh). Secretolytic and secretomotoric triterpene glycosides such as primulasaponins I and II were identified as the major bioactive constituents, but as for common ivy analytical methods for their determination are scarce. Apart from a rather unspecific TLC assay in the respective monograph of the *European Pharmacopoeia* [73], only one selective method can be found in literature. Müller et al. presented an HPLC-UV-ELSD method that permitted the simultaneous assessment of major saponins and phenolic glycosides in primula root [83]. For optimum results, they employed a Synergi Fusion RP column from Phenomenex and a mobile phase comprising 0.025% trifluoroacetic acid (TFA) in water and 5% ACN in methanol. Within 55 min, the percentage of the organic phase was gradually increased from 15% to 95%, with the phenols being detected at 210 nm (e.g., LOD of primulaverin 22 ng on-column), and the saponins monitored by ELSD (e.g., LOD of primulasaponin II 34 ng). For confirmation of peak identity, MS experiments in negative APCI mode were performed. The saponin patterns of *P. veris* and *P. elatior* showed distinct differences, so that both species could easily be differentiated.

42.4.9 *Panax* Species (Ginseng)

With ginseng root being one of the most important oriental medicines, it is not surprising that a multitude of studies has dealt with the pharmacology, analysis (Table 42.3), and large-scale production of this herbal drug. But it is important to know that the term "ginseng" comprises not only one but several *Panax* species of medicinal relevance, including *P. ginseng* (Korean or Asian ginseng), *P. quinquefolius* (American ginseng), *P. notoginseng* (Tienchi or Sanchi), and *P. japonicus* (Japanese ginseng). The major active ingredients, triterpene glycosides called ginsenosides, are found in all of them; even species-specific saponin patterns have been observed. Up to now, more than 80 ginsenosides are known, with the majority being based on two dammarane-type aglyca, 20-(*S*)-protopanaxadiol and 20-(*S*)-protopanaxatriol [84]. Six derivatives (ginsenosides Rb_1, Rb_2, Rc, Rd, Re, and Rg_1) account for ~90% of the total ginsenosides in the drug; thus they are commonly selected as quality markers [85]. Processed or unprocessed ginseng is mostly used as adaptogenic. Other biological effects reported for ginsenosides are immunoregulatory, hepato-protective, anti-inflammatory, and anticancer properties [86].

TABLE 42.3

Analytical Procedures for Assaying Triterpene Glycosides in *P. ginseng*

Analytes	Method	Separation Conditions	Detection	Val.	Appl.	Reference
Ginsenosides Ro, Rb_1, Rb_2, Rc, Rd, Re, Rf, Rg_1, Rg_2, and F_{11}	2D-TLC	Silica–gel, chloroform/MeOH/water (1), butanol/ethylacetate/water (2)	Vis	—	S, SA	[88]
Ginsenosides Rb_1, Rb_2, Rc, Rd, Re, and Rg_1	HPTLC	Silica–gel, 1,2-dichloroethane / ethanol/methanol/water	UV	1–4	S, SA +	[89]
Protopanaxadiol, protapanaxatriol	GC	HP-1 MS-fused silica column	MS	1,2	S, SA	[90]
11 Ginsenosides	HPLC	YMC-Pack ODS-AQ303, 10 mM K-phosphate (pH 5.8), and ACN	UV	1–4	S, SA +	[91]
13 Ginsenosides (e.g., Rb_2, Rb_3, Rd, Rh_1, Rg_2, 20R-Rg_2, Rg_3, and Rh_2)	HPLC	Ultrasphere C18, water, and ACN	UV	2	S, SA +	[92]
Ginsenosides Rg_1, Re, Rb_1, Rc, Rb_2, Rb_3, and Rd	HPLC	Zorbax Extend C18, water, and ACN	ELSD, CAD	1–4	S, SA +	[93]
12 Ginsenosides (e.g., notoginsenoside R_1 and pseudoginsenoside F_{11})	HPLC	Zorbax ODS C18, water, and acetonitrile	ELSD	1–4	S, SA +	[94]
9 Saponins, flavonoids and polyacetylenes	HPLC	Prevail C18 Rocket, water, and acetonitrile	APCI-MS, UV	1–4	S, SA +	[95]
23 Ginsenosides	UPLC	Acquity BEH C_{18}, water, and ACN (both with 0.05% formic acid)	ESI-TOF-MS	2	S, SA	[96]
Ginsenosides Rb_1, Rb_2, Rc, Rd, Re, Rf, and Rg_1	CE	100 mM Sodium tetraborate (pH 10) with 80 mM sodium cholate	UV	1–4	S, SA	[97]
Ginsenosides Rg_3, Rk_1, Rg_5, and F_4 (isolation)	HSCCC	Dichloromethane/methanol/water/isopropanol (6/6/4/1)	ELSD	—	SA	[98]

Note: Appl., application; S, standard; SA, sample; +, quantification; Val., validated (1, sensitivity; 2, specificity; 3, accuracy; 4, precision).

A simple colorimetric assay for the determination of total ginsenosides in ginseng roots has been described by Zhang et al. [87]. It is based on the reaction of sapogenins, which were obtained by acidic hydrolysis, with vanillin. The resulting products can be monitored at 560 nm. More specific investigations are possible by TLC. Just to name two out of many respective examples, a two-dimensional TLC procedure developed by Lui and Staba permitted the differentiation of Asian and American ginseng based on the occurrence of ginsenoside Rf [88], Vanhaelen-Fastré´s method facilitated the quantitative determination of six ginsenosides by HPTLC [89]. They extracted the compounds of interest by partitioning an ethanolic plant extract between *n*-butanol and water, and assayed the butanol soluble fraction on silica plates using a quaternary mobile phase (1,2-dichloroethane/ethanol/methanol/water = 56.8/19.2/19.2/4.8). Ginsenosides were visualized by treatment with vapors of thionyl chloride, and quantified by a densitometric evaluation of the results at 275 nm (Figure 42.4). This method was shown to be sensitive (LOD: 10 ng/10 mm) and precise ($\sigma_{rel} \leq 3.3\%$ for total saponins). Besides, the already mentioned analysis of genuine glycosides in ginseng by GC [24], this technique has mostly been employed for the quantification of protopanaxadiol and protopanaxatriol [90]. Both were obtained by oxidative alkaline cleavage using sodium methanolate; then they were converted to trimethylsilyl-derivatives, and finally analyzed by GC-MS.

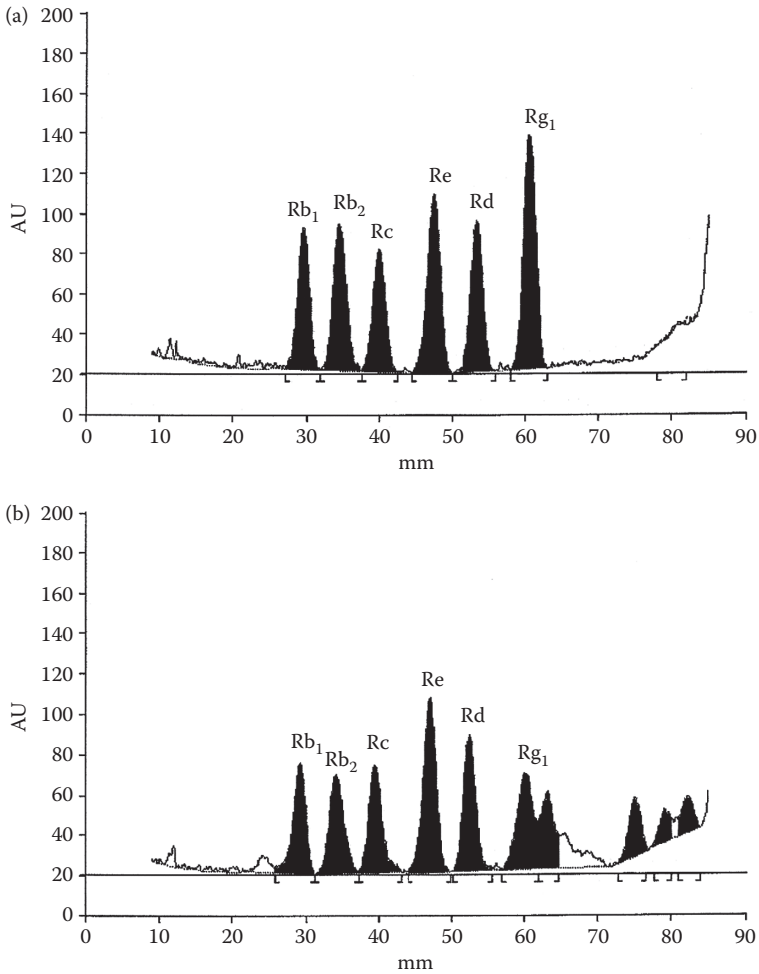

FIGURE 42.4 Separation of six ginsenosides by TLC (a) standard; (b) *P. ginseng* extract. (Reproduced from Vanhaelen-Fasteré, R., Faes, M., and Vanhaelen, M. *Journal of Chromatography A*, 2000, 868, 269–76. With permission.)

Thus far, most of the published manuscripts describe the determination of ginsenosides by HPLC [91–96], but the methodologies employed do not differ significantly from those already reported. Water/acetonitrile gradients were commonly used, sometimes with the addition of buffer or acid, and a reversed-phase stationary phase was selected for separation (Table 42.3). The saponins were assayed in diverse matrices (e.g., ginseng berries [92], leaves and roots [95], commercial products, and in biological fluids [86]), and detected either by UV at wavelengths between 195 and 205 nm, MS, ELSD or corona charged aerosol detector (CAD). The latter is an advancement of ELSD. Similar to ELS detection, the LC eluant is first nebulized. But in a second step, the analytes are charged by a corona discharge needle and the resulting ions are measured by an electrometer. Wang et al. compared the performance characteristics of CAD and ELSD for ginsenoside analysis [93]. In agreement to the manufacturers (ESA Biosciences) statement, they found CAD to be much more sensitive (LOD for ginsenoside Rg_1: 9.6 ng (CAD), 60.2 ng (ELSD)). Similar to ELSD, the CAD signal was not linear concerning the relation between peak area and concentration; only after a logarithmic conversion of the data, a linear trend was observed.

Due to the absence of chargeable groups in ginsenosides, not capillary zone electrophoresis but only MEKC is suitable for the separation of ginseng saponins; Glöckl et al. [97] have published a respective

method in 2002. For the separation of seven saponins, they employed a 100 mM borax solution adjusted to pH 10.0 as buffer; sodium cholate was added as detergent. Applied voltage, detection wavelength, and temperature were set to 30 kV, 200 nm and 30 C, respectively. Within an analysis time of 21 min, all compounds were resolved and could be identified in a ginseng extract (Quantitative results are missing.). An interesting approach for the isolation of ginsenosides was reported by Ha et al. [98]. These authors utilized HSCCC, an automated form of liquid–liquid partitioning, to obtain several pure ginsenosides occuring in steam treated *Panax* roots (red ginseng). Other, less common techniques for the analysis of ginsenosides include the use of near-infrared spectroscopy [99] or enzyme immunoassays using antibodies [100].

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