Chemical Analysis of Non-antimicrobial Veterinary Drug Residues in Food

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Chemical Analysis of Non-antimicrobial Veterinary Drug Residues in Food

Edited by Jack F. Kay, James D. MacNeil, and Jian Wang

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This book is dedicated to the memory of Dr. John J. O'Rangers Jr., 11 June 1936–20 January 2013.

John was internationally well known and respected in the field of veterinary drug residue chemistry and international regulations. Both Dr. James MacNeil and Dr. Jack Kay were honored to have known and worked with him over many years and also to call him a friend. Developing international cooperation and understanding was a cornerstone of John's view of life and work ethic, regardless of the more politically opinionated views held by some. Many international developments in this field and friendships are the result of the work John conducted behind the scenes to break down barriers. He truly was one of a kind and his passing leaves us all impoverished.

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Preface

Food safety continues to be a topic of great interest to consumers and is frequently a topic for media discussion. However, what is not routinely reported is the vast effort by many national, regional, and international bodies and scientists – both governmental and independent – to ensure that food production and trade do not place consumers at risk while ensuring a continuous supply of wholesome food.

A key aim of regulating the use of veterinary drugs is to ensure that authorized products are used responsibly in animals and that their residues in food of animal origin do not pose unacceptable health risks to consumers. To assist in this process, robust and validated analytical methods to detect a wide range of potential residues in food matrices are required.

The earlier volume in this series, *Chemical Analysis of Antibiotic Residues in Food*, was published in 2012 and set out in detail how drug safety is considered and limits are set for their residues in foods. It also described how residue monitoring programs are established and checked to ensure sound results are generated to inform necessary regulatory actions to protect consumers. These topics are generic and apply equally to antibiotics and other veterinary drug classes. The companion volume to this current book also provided detailed information on analytical methods for antibiotic residues.

The purpose of this current book, *Chemical Analysis of Non-antimicrobial Veterinary Drug Residues*, is to update readers on developments in technology and approaches since the publication of the earlier volume. It also seeks to expand the coverage of veterinary drug residues to all other key areas of veterinary drug treatments, thus providing a comprehensive two-volume set for reference and training purposes.

The main themes of the book include detailed discussions on emerging technologies (Chapter 2); high resolution mass spectrometry and related techniques (Chapter 3); hormones and β-agonists (Chapter 4); anthelmintics (Chapter 5); sedatives and tranquilizers (Chapter 6); pyrethroids, carbamates, organophosphates, and other pesticides used in veterinary medicines (Chapter 7); non-steroidal anti-inflammatories (Chapter 8); dyes (Chapter 9); and developments in the validation of multi-class multi-residue methods and related quality control/quality assurance issues (Chapter 10).

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The editors and authors of this book are internationally recognized experts and leading scientists with extensive personal experience in preparing food safety regulations and/or in the chemical analyses of veterinary drug residues in food of animal origin. This book offers a valuable and up to date view of the science in this area. It has been deliberately written and organized to complement and update where necessary the information contained in the earlier companion volume. The editors hope that this volume completes and addresses the need for readers from regulatory backgrounds and analytical laboratory staff to have a cutting-edge reference and training resource for the residues of all veterinary drug residues in food of animal origin.

26 August, 2016

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Dr. James D. MacNeil received his Ph.D. from Dalhousie University, Halifax, NS, Canada in 1972 and worked as a government scientist until his retirement in 2007. From 1982 to 2007 he was Head, Centre for Veterinary Drug Residues, now part of the Canadian Food Inspection Agency. He has served as a member of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), co-chair of the working group on methods of Analysis and Sampling, Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF), is the former scientific editor for "Drugs, Cosmetics & Forensics" of J. AOAC Int., worked on IUPAC projects, has participated in various consultations on method validation, and is the author of numerous publications on veterinary drug residue analysis. He is a former General Referee for methods for veterinary drug residues for AOAC International and was appointed scientist emeritus by CFIA in 2008. He holds an appointment as an adjunct professor in the Department of Chemistry, St. Mary's University, Halifax, Canada, and has served as a part-time consultant to the JECFA Secretariat of the Food and Agriculture Organization of the United Nations since 2012.

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1

Basic Considerations for the Analyst for Veterinary Drug Residue Analysis in Animal Tissues

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1.1 Introduction

It is not sufficient to be expert in the techniques applied in an analytical method to produce a meaningful result when applying a method for the analysis of veterinary drug residues, as is the reality in many other types of chemical analysis. The analyst must also have a sufficient understanding of the nature of the targeted veterinary drug residues to ensure that the method used is fit for the purpose. That is, the method used should be developed and validated for an appropriate concentration range for the right analyte and should be directed at a matrix where residues are likely to be found. In addition, the analyst might reasonably be expected to provide advice on the significance of the results generated with respect to regulatory limits to clients with limited scientific knowledge.

In this chapter, we discuss some of the terminology that is commonly applied in veterinary drug residue analysis, as well as some of the basic information on pharmacokinetics, metabolism, and distribution that help with direct choices of analyte and matrix and that also inform the interpretation of analytical results. We also briefly review the common national and international approaches to the regulation of veterinary drug residues in foods and the establishment of maximum residue limits (MRLs).

1.2 Pharmacokinetics

The term pharmacokinetics is used to describe studies related to quantitative changes in the concentrations of an administered drug in a body over time. Basic parameters associated with a dose are C_{max}, the maximum concentration attained

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following the receipt of a dose of a drug, and $t_{1/2}$, the half-life of the drug in the body. These may be determined in the blood or in specific tissues. For the residue analyst, some knowledge of these factors is required to help target analysis at a matrix where residues are likely to be found and to interpret the significance of a residue finding. If the half-life ($t_{1/2}$) of a drug in a body fluid or a tissue is measured in minutes or a few hours, there is very probably little to be gained by testing that matrix for residues in an animal slaughtered days or weeks after the drug administration.

The means by which a drug is administered may influence the pharmacokinetics. Veterinary drugs may be available in a variety of formulations, which include injectables, feed additives, sprays, pour-ons, and dips. Injections may be via routes which included intravenous, intramuscular (i.m.), subcutaneous (s.c.), and intramammary. In some cases, the injection may lead to the occurrence of a depot at the injection site, with a low rate of absorption, leading to the presence of significant residues at the injection site for an extended period. The residues at the injection site will not be representative of residues found in muscle tissue away from the site of injection. Thus, a finding of high residue concentrations in muscle tissue, for example, should lead the analyst to suspect that the tissue analyzed may be from an injection site, and therefore additional analyses should be conducted on muscle samples from other parts of the carcass or lot before concluding that the initial results are truly representative.

For example, the 47th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommended MRLs of 10 μg/kg for doramectin residues in beef muscle.[1] It also noted that residues were slightly higher in the muscle from cattle given an s.c. dose when compared to cattle which received treatment by i.m. injection. In addition, after 35 days withdrawal, residues in muscle were < 3 and < 2 μg/kg from the s.c. and i.m. treatment groups, respectively. However, injection site muscle from these animals contained 930 μg/kg (s.c. group) and 177 μg/kg (i.m. group) at 35 days post-treatment. The committee in recommending MRLs for doramectin in cattle noted that high concentrations of residues may remain at the injection site after treatment according to approved uses. In adopting the MRL recommendations, the Codex Alimentarius Commission (CAC) included a note with the MRLs for beef muscle and fat that there was a potential that residues of doramectin in excess of the MRLs could persist at injection sites following recommended treatment.[2]

Subsequently, in reviewing data for the use of doramectin in the treatment of pigs, the 52nd Meeting of the JECFA recommended an MRL of 5 μg/kg in pork muscle, based on twice the limit of quantification (LOQ) of a method judged to be suitable for routine regulatory use.[3] In a depletion study reviewed by the 52nd Meeting of the JECFA, pigs were treated by i.m. injection at 1.25 times the recommended dose and subjected to a 28-day withdrawal period, as per the approved use from a Codex Alimentarius member state.[3] No quantifiable residues were detected in "normal" muscle tissue, meaning that residues in the muscle tissue should be below this limit if the drug is used according to the

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established Good Veterinary Practices (GVP). The committee again noted that higher concentrations could be found in the injection site tissue from pigs. A finding of residues in excess of the MRL for doramectin in muscle or fat may therefore mean that the tissue sample is from a site of injection and does not represent the residues present in "normal" muscle or fat. Such a finding indicates that additional sample material should be obtained to determine if the initial sample analyzed was truly representative of tissues from the animal or lot. Thus, knowledge of the pharmacokinetics and depletion of a drug is required when interpreting the results of analysis.

1.3 Metabolism and Distribution

The term metabolism refers to the chemical processes which occur in a living organism and which can transform an administered drug into other chemical compounds, while the term distribution refers to the manner in which residues are distributed to different tissues and body fluids. Knowledge of these elements is critical to determining the nature of the residues which should be determined by a method and the matrix or matrices in which these residues are most likely to be found.

This brings us to two fundamental terms frequently used in the analysis of veterinary drug residues: the marker residue and the target tissue. The CAC has defined the marker residue as the "residue whose concentration decreases in a known relationship to the level of total residues in tissues, eggs, milk or other animal tissues."[4] CAC guidelines for the design and implementation of a program for the control of veterinary drug residues in foods note that the marker residue "may be the parent drug, a major metabolite, a sum of parent drug and/or metabolites or a reaction product formed from the drug residues during analysis" and that "the parent drug or the metabolite may be present in the form of a bound residue which requires chemical or enzymatic treatment or incubation to be released for analysis."[5] The target tissue is usually "the edible tissue in which residues of the marker residue occur at the highest concentrations and are most persistent." Knowledge of the appropriate marker residue and target tissue is usually obtained from controlled studies to investigate the metabolism and distribution of residues of a drug following administration to an animal species. For veterinary drugs which have been reviewed by the JECFA as part of the process of the development of international standards (MRLs) through the CAC, monographs detailing the pharmacokinetics, metabolism, distribution, and depletion studies may be found on the Food and Agriculture Organization (FAO) JECFA website.[6]

It was common practice in most countries until about 2000 to monitor nitrofuran use by testing for parent compounds, although it had been shown in the 1980s that these compounds were rapidly metabolized, as noted in a JECFA review of residues of furazolidone,[7] and that monitoring for parent compounds was therefore highly unlikely to produce positive results. However, when methods

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became available to monitor for bound residues of the metabolites of these compounds, the use of which had been banned in food-producing animals in most countries, detection of use became practical and positive results were reported.[8] This provides an example of the importance of identifying the appropriate marker residue. Some drugs, such as lasalocid sodium[9] and ractopamine hydrochloride,[10] are administered as salts but are rapidly transformed to the free parent drug (lasalocid or ractopamine) on injection, and it is the free parent drug, not the salt, which is the appropriate marker residue. Other drugs are rapidly transformed into new active substances immediately following injection. The organophosphate trichlorfon is used orally or topically to treat parasites in various animal species. Following administration, it is rapidly transformed to the insecticide dichlorvos, and it was noted in the JECFA evaluation that trichlorfon is "metabolized so extensively and rapidly that the ratio of marker residue to total residues cannot be defined."[11] However, despite the extensive metabolism, it was determined by JECFA that trichlorfon parent drug was the most appropriate marker residue.

Metabolism can also convert parent drugs into metabolites which may prove to be better marker residues for use of the compound. For example, the anthelmintic drug monepantel, which belongs to the amino-acetonitrile derivative class and is used for control of intestinal nematodes in sheep, is extensively metabolized, with monepantel sulfone identified as the major metabolite found in tissues and blood.[12] Monepantel sulfone has therefore been identified as the preferred marker residue for analysis of edible tissues. Other drugs, such as diclazuril, an anticoccidial drug, show no significant metabolism and the administered parent drug is the designated marker residue.[13]

There are also examples where extensive metabolism occurs and results in the same residues being observed from the administration of different drugs, with the benzimidazole group of drugs being a primary example. Administration of fenbendazole, oxfendazole, or febantel leads to the formation of common metabolites, with the result that the marker residue for these compounds has been identified as "the sum of the three principal metabolites (fenbendazole, oxfendazole and oxfendazole sulfone) calculated as oxfendazole sulfone equivalents."[14] In this case, a method targeting only the individual parent compounds is not consistent with the marker residue as defined by the CAC for international trade.

Information from residue depletion studies is also useful to the analyst in providing interpretation of results obtained from an analytical method. Indeed, knowledge of residue depletion and distribution may help the analyst identify a spurious result, perhaps from contamination of a sample or the presence of injection site material in a sample. As an example, there are veterinary drugs which, if administered according to label instructions, should result in no detectable residues in the muscle or perhaps other tissues, even when analytical methods are used capable of detecting residues in the low μg/kg range. The example of doramectin residues has already been cited in Section 1.2,[1] but other examples may easily be found. For the anti-parasitic compound cyhalothrin, a synthetic pyrethroid used for the control of ectoparasites, it was observed that

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there should be no detectable residues in the liver, kidney, or muscle, based on methods with a limit of detection of 3 – 5 μg/kg.[15]

Knowledge of the metabolism can also enable the analyst to distinguish between residues resulting from treatment with a drug and post-mortem contamination of tissues or fluids. For example, malachite green, which has been used as an antifungal agent in aquaculture, is widely used as a dye for paper, textile, and leather products.[16] Although use of malachite green is prohibited in aquaculture, residues have been reported in regulatory samples analyzed in numerous jurisdictions. Malachite green is extensively metabolized, and typical findings for incurred residues include both parent compound and the primary metabolite, leucomalachite green. A finding of malachite green residues without evidence of metabolism should therefore be investigated as potentially from sample contamination. The authors are aware of a case in which residues of malachite green parent compound were detected in a retail sample of salmon, yet an investigation demonstrated that there was no use of this prohibited drug at the aquaculture site from which the salmon originated. The nature of the residues was considered suspicious, as only the parent drug and none of the major metabolite, leucomalachite green, were present in the material. Further investigation determined that the source of the malachite green residues was transfer from the dye in a paper towel used on a weighing scale at the retail source.

There can be situations where targeting the conventional marker residue or an edible target tissue is not the optimal approach to the detection of a drug use, particularly when dealing with a non-approved or banned use. We will see some examples of this in subsequent chapters, such as the designation of retinal tissue as the most appropriate tissue for the detection of the use of banned β-agonist drugs. Other circumstances may require targeting residues at the injection site to confirm a prohibited or non-approved use, as discussed in the chapters dealing, respectively, with the analysis of hormones (Chapter 4) and sedatives (Chapter 6). For example, the administration of testosterone to veal calves did not result in residues in muscle tissue which exceeded the normal range, but targeting the presence of testosterone propionate in injection sites confirmed that non-approved treatment of these animals had occurred.[17] Again, knowledge of the behavior of a drug following administration is a key element in selecting the appropriate marker residue and target tissue to achieve the objectives of the analysis using a method which is fit for purpose.

1.4 Choice of Analytical Method

In the subsequent chapters, we will deal with methods of analysis for a wide range of veterinary drugs, most of which have approved uses, some of which have not been approved for use in some countries, and the rest of which have been legally prohibited from use in food-producing animals in a number of countries. For the drugs which fall in the latter category, the Codex Committee on Residues of

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Veterinary Drugs in Foods (CCRVDF) has recently adopted a process to deal with "Risk Management Recommendations for Residues of Veterinary Drugs for which no ADI and/or MRL has been recommended by JECFA due to Specific Human Health Concerns."[18] This identifies drugs which have been evaluated by JECFA and are not considered as safe for use in food-producing animals due to concerns about potential risk to consumers from the resultant residues in foods. It includes compounds such as chloramphenicol and the stilbenes, which have been banned from use in food-producing animals in many countries, and is published with the list of approved MRLs for residues of veterinary drugs in foods, updated by the CAC after new recommendations are formally approved.[2] We can therefore identify four situations for which an analyst may need to choose an appropriate analytical method and demonstrate that the method is "fit for purpose":

- *Enforcement of an MRL (or tolerance) which has been established by the national government and/or the CAC for the approved use of a drug in one or more animal species*. This requires a method validated for the determination of residues over a concentration range which includes the MRL in appropriate tissues or other food matrices designated for analysis.
- *Determination of residues resulting from extralabel use of a veterinary drug*. This situation occurs when a veterinarian prescribes the use of a drug which is approved for use in other species to a species for which there is no formal approval. A number of countries permit such use under veterinary discretion but require that the veterinarian takes measures to prevent residues which could pose a risk to the consumer. Equally, when dealing with imported samples, situations may arise where the exporting country has an approved use, but that use is not required in the importing country. As with the enforcement of MRLs, there typically will be an existing MRL for the residues in tissues, milk, or eggs from another relevant species or MRLs for the use in the exporting country which may be accepted by the responsible authority for which the analysis is conducted. These would be the target range for the method, as in the aforementioned situation.
- *Determination of residues resulting from the non-approved use of a veterinary drug*. In this situation, there is no "target value" established by an existing MRL, so the method selected is usually chosen on the "as low as you can go" basis. Typically, this may be achieved by including residues of the drug in a screening method with an appropriately low limit for detection, quantification, and identification of the residues, with the objective of preventing use of a non-approved drug in food-producing animals.
- *Determination of residues resulting from the use of a banned veterinary drug in food animals*. This case may be similar to the situation for non-approved use, unless there is a formal minimum required performance limit (MRPL), such as are required for residues of banned substances (e.g., chloramphenicol at 0.3 μg/kg and nitrofuran metabolites at 1 μg/kg for all) by the European Commission Decision 2003/181/EC.[19] The requirement then is that methods

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used must be capable of detecting, quantifying, and confirming the identity of residues at the MRPL (when available) or at the lowest concentration which can be achieved with the available equipment and technologies. The expectation would be that any banned drug would be detected at concentrations of 1 – 2 μg/kg or lower, given the current state of the art.

1.5 Importance of Regulatory Limits

An understanding of regulatory limits, the terminology used, and the scientific basis of these limits is important for an analyst in ensuring that analytical methods used are fit for purpose and also to provide a critical evaluation of analytical results. As discussed earlier, the performance requirements of methods and even the type of method selected should be based on the regulatory requirement, which typically involves determination of compliance with a regulatory limit or observance of a prohibition on the use of a substance in food-producing animals. The two types of regulatory limits typically related to the application of analytical methods for veterinary drug residues in foods are termed maximum residue limits or tolerances.[20] The MRL is the regulatory limit used by the CAC and most Codex member states, while tolerances are used as the regulatory limits in the United States of America. Both regulatory limits are derived from the acceptable daily intake (ADI), established from a toxicological evaluation of the drug, but using different assumptions of potential consumer exposure. Thus, while an MRL and a tolerance may be based on a common ADI, the numerical values assigned to the MRL and the tolerance may differ.

1.5.1 Derivation of the Acceptable Daily Intake

Both national authorities and the CAC, which establishes standards for international trade, rely on a common approach to the determination of the ADI. In the case of a national authority, this responsibility usually falls within the government department or agency responsible for health and health protection, such as the United States Food and Drug Administration (USFDA), which is part of the Department of Health and Human Services in the United States, or the Australian Pesticides and Veterinary Medicines Authority, which reports to the Australian Minister of Agriculture. Regionally, there are authorities such as the Directorate Health and Consumers (SANCO) of the European Commission which establish standards applicable within member states of the European Union. Internationally, the CAC establishes safety standards for residues of veterinary drugs in foods, and these are the standards which are most likely to prevail in cases of international dispute at the World Trade Organization (WTO).

The process leading to the establishment of international standards by the CAC is similar to the process used by regional or national authorities, in that the first step is the establishment of an ADI. The CAC has, within its structure,

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various committees with specific areas of responsibility, including the CCRVDF. The responsibilities of the CCRVDF are found in the CAC Procedural Manual.[21] These include the determination of priorities for the consideration of residues of veterinary drugs in foods, the recommendation of MRLs for veterinary drug residues in foods, the development of codes of practice, and the consideration of methods of analysis and sampling for veterinary drug residues in foods. Under the risk analysis policy for CCRVDF contained in the Procedural Manual, the CCRVDF commissions the JECFA to conduct a risk assessment of each veterinary drug identified on a priority list established by the CCRVDF. The outcome of the risk assessment conducted by the JECFA is an ADI, when sufficient scientific information is available, with MRL recommendations for consideration by the CCRVDF.

The JECFA is an independent scientific committee[22] which meets as needed, typically once every 12–18 months, to conduct the assessment of veterinary drugs identified for review by the CCRVDF. The committee consists of members with expertise in toxicology, appointed by the World Health Organization (WHO), and members with expertise in drug residues and/or drug residue analysis, appointed by the FAO. These experts are selected from rosters of independent experts maintained by the two host organizations. Information considered by JECFA is provided in the form of dossiers of proprietary information from the companies which manufacture the drugs, supplemented by information which may be provided by national authorities and information obtained by the experts from a search of the peer-reviewed scientific literature. The information provided by the companies includes not only information on the product ingredients, formulations, and usage but also the detailed toxicological and residue studies required by national authorities for review to establish regulatory limits for these substances. All proprietary information provided to JECFA is considered confidential, but data provided in these dossiers is summarized with the expert analysis and published in toxicological monographs by the WHO[23] and residue monographs by the FAO[24] as well as being further summarized in the reports of the JECFA Meeting.[25]

The ADI is derived from an examination of both long-term and short-term studies of acute and chronic toxicity, supplemented by any information which may be available from human studies for drugs used both in human and veterinary medicine. Information on the experiments typically required by regulatory authorities may be found in a series of guidelines issued by the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) which may be accessed on the VICH website.[26] These include the following:

- VICH GL22 – Studies to evaluate the safety of residues of veterinary drugs in human food: Reproduction testing
- VICH GL23 – Studies to evaluate the safety of residues of veterinary drugs in human food: Genotoxicity testing

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- VICH GL28 – Studies to evaluate the safety of residues of veterinary drugs in human food: Carcinogenicity testing
- VICH GL31 – Studies to evaluate the safety of residues of veterinary drugs in human food: Repeat-dose (90 days) toxicity testing
- VICH GL32 – Studies to evaluate the safety of residues of veterinary drugs in human food: Developmental toxicity testing
- VICH GL33 – Studies to evaluate the safety of residues of veterinary drugs in human food: General approach to testing
- VICH GL36 – Studies to evaluate the safety of residues of veterinary drugs in human food: General approach to establish a microbiological ADI
- VICH GL37 – Studies to evaluate the safety of residues of veterinary drugs in human food: Repeat-dose chronic toxicity testing

The selection of an appropriate end-point on which to base the ADI is determined after a review of all relevant toxicological information. Typically, the end-point selected is that which provides the most conservative end-point, that is, the end-point which provides the highest standard of protection to consumers. For hormonally active veterinary drugs, such as zeranol, the end-point typically chosen is a "no hormonal effect level."[27] For antibiotics, the end-point typically is based on a minimum inhibitory concentration, provided that this leads to a lower ADI than would be derived from chronic or acute toxicity studies. Most other veterinary drugs have the ADI established from chronic toxicity data, although there are a few for which the ADI is based on acute toxicity studies, such as ractopamine hydrochloride.[28]

The ADI is not the toxicological, hormonal action or microbial action end-point that is selected, but is derived from that end-point.[29] Typically, the toxicological end-point is derived from experiments in laboratory animals, adjusted by a safety factor. A multiplication factor of 10 is usually applied to allow for differences in response between the test animal species and humans. An additional multiplication factor of 10 is then applied to allow for differences in response within the human population. Another additional factor of up to 10 may also be applied to allow for any uncertainties associated with the data. As an example, the safety factor applied by JECFA in establishing the ADI for flumequine was 1000, as the study from which the toxicological end-point was derived was of short duration and there was "a lack of histochemical characterization of the foci of altered hepatocytes."[30] The ADI is defined by the CAC as an estimate of "the amount of a veterinary drug, expressed on a body weight basis, that can be ingested daily over a lifetime without appreciable health risk."[4, 31] The estimate is based on a body mass of 60 kg, which is used to represent the average body weight of a consumer over their lifetime.

1.5.2 Derivation of the Acute Reference Dose

The WHO defines an acute reference dose (ARfD) as "the estimate of the amount of a substance in food or drinking-water, expressed on a body weight basis that

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can be ingested in a period of 24 hours or less without appreciable health risk to the consumer."[29] It is derived from toxicological experiments in a similar manner to the ADI, except that in this case the focus is on acute, as opposed to chronic, response. Procedures used for the establishment of the ARfD for pesticides followed by the Joint FAO/WHO Meeting on Pesticide Residues (JMPR) are described in Environmental Health Criteria 240, a publication of the WHO,[29] and similar procedures have recently been applied by the JECFA in the evaluation of the veterinary drugs ivermectin and zilpaterol hydrochloride,[32] incorporating a new estimate of consumer exposure, the global estimate of acute dietary exposure (GEADE), proposed by a recent expert consultation.[33]

1.5.3 Derivation of Maximum Residue Limits

MRLs are not in themselves "safety limits," in the sense that any exposure to a residue above the MRL poses a severe risk to a consumer. In the system used by the CAC to establish MRLs for veterinary drug residues in foods and similar systems used by national and regional authorities, the MRL is derived from the depletion curve by choosing a timepoint at which the proposed MRLs, when incorporated into a model diet calculation, yield a resultant theoretical exposure that does not exceed the ADI. The model diet used in the standard calculation is considered conservative and to provide additional protection to the consumer. The following assumptions are made in the exposure calculation[33]:

- The animal-derived foods eaten by each consumer on a daily basis will all be from animals that have been treated with the veterinary drug for which the MRLs are being established.
- All of these foods will be from animals for which the minimum withdrawal or withholding period established under the conditions of use on the label has been observed.
- Each consumer will eat each day a diet which includes 300 g of muscle tissue, 100 g of liver, 50 g of kidney, 50 g of fat, and 1.5 kg of milk for drugs approved for use in both meat and dairy animals. The residue concentrations used in the exposure calculations are those associated with tissues from whichever food species contain the highest residues at the timepoint for which MRLs have been established. When a drug is approved for aquaculture use, the 300 g of muscle tissue in the exposure calculation may come from fish. When the drug is approved for use in laying hens, the exposure calculation is expanded to include 100 g of eggs. In addition, if a drug also has approval for use in honey production, 50 g of honey is added to the exposure calculation. There is also an assumption that all residues are of the same toxicity as the parent drug unless some of the metabolites can be demonstrated to be of no toxic concern. The typical exposure calculation therefore includes a factor to convert marker residue to total residues.

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In the JECFA approach, the representative concentration of residue to be used in the estimated daily intake (EDI) calculation for each food item is the median residue determined in the depletion experiment at the timepoint for which the MRLs are derived.[34] When data are insufficient to calculate median residue concentrations, the MRL value is used in the intake calculation, which is then referred to as the theoretical maximum daily intake (TMDI). Some regional and national authorities prefer to use the TMDI for calculation of the potential intake as it is a more conservative approach and will usually provide a higher estimate of potential intake than the EDI. The MRLs are typically derived from the upper tolerance limit (UTL 95/95) of the residue concentration determined from the depletion curve at a timepoint where the potential intake by a consumer will be below the ADI. When tissues contain no quantifiable (or detectable residues), MRLs recommended by the JECFA are typically based on 2× the LOQ of an analytical method that is considered suitable for regulatory use. Similar approaches are used by national/regional regulatory authorities.

When a substance is used both as a pesticide and as a veterinary drug, the initial evaluation is conducted by JECFA or by the JMPR, another independent scientific committee which is jointly administered by the FAO and the WHO.[34] The first committee to conduct an evaluation will typically establish an ADI which will be used in subsequent evaluations by both committees, unless the basis for the toxicological evaluation differs for the two uses. For example, the JMPR established an ADI for horticultural use of abamectin which included consideration of a toxic photodegradation product, but subsequently established a different ADI for the use of abamectin as a veterinary drug after discussions with the JECFA because the degradation product was not formed in such uses.[35] The JECFA will also consider exposure from horticultural use of such substances in conducting dietary exposure assessments associated with the veterinary use.[34]

In the establishment of MRLs, only a fraction of the ADI is represented by each food for which an MRL has been assigned, based on the relative distribution of the residues across the various foods represented in the model diet used in the exposure calculation. In addition, the MRLs established by the CAC are based on GVP, defined as "the official recommended or authorized usage including withdrawal periods, approved by national authorities, of veterinary drugs under practical conditions."[4] Thus, the exposure calculation may yield a result well below the ADI, particularly for drugs which are rapidly metabolized and result in very low residue concentrations in foods.

Typically, depletion is determined in two types of experiments: one using a radiolabeled preparation of the drug and the other using the unlabeled drug. The requirements for these experiments are described in two VICH guidelines.[26] These are as follows:

- VICH GL46 – Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Metabolism study to determine the quantity and identify the nature of residues

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- VICH GL48 – Studies to evaluate the metabolism and residue kinetics of veterinary drugs in food-producing animals: Marker residue depletion studies to establish product withdrawal periods

Both experiments should be conducted at the dosage and under the conditions of use which represent typical field use. The studies with the radiolabeled drug are used to determine the relationship between the marker and total residue and also to provide the total concentration of residues in each tissue, milk, or eggs at the timepoint corresponding to the withdrawal time. In some cases, total residues are known in the muscle from the radiolabel study, where the detection limit may be 1 μg/kg, while there are no detectable or quantifiable residues of the marker detected. MRLs for those foods where no marker residue has been detected may be established based on the LOQ of an analytical method considered suitable for routine regulatory use. For example, only traces of ractopamine total residues were detectable in the muscle and fat of pigs administered with radiolabeled ractopamine hydrochloride at 12–24 hours after last administration, using an analytical method with a detection limit of 20 μg/kg.[28] In studies with unlabeled drug, marker residue was detected at 5 μg/kg in muscle and 1 μg/kg in fat at no withdrawal, but marker residue was not detectable in muscle and fat at 2 days withdrawal or longer times. The MRLs for muscle and fat were therefore recommended based on the method LOQ of 5 μg/kg, with the MRL being set at 2 × LOQ (10 μg/kg) for muscle and fat.

1.5.4 Derivation of Tolerances

Tolerances are the regulatory limits established by the USFDA for residues of veterinary drugs in foods. They also are derived from the depletion data, similar to MRLs, but the dietary exposure assumptions on which the tolerances are based differ from those used in the establishment of MRLs. Once the ADI has been established, the potential sources of exposure to veterinary drug residues in food are considered. As in the procedure described earlier for the derivation of MRLs, the USFDA considers that consumers will eat more muscle tissue than organ tissue and accordingly uses the same quantities of muscle, liver, kidney, and fat in assessing potential exposure that are used in the derivation of MRLs.[36] The same factors are applied across all species, as it is assumed that the typical consumer will only eat a full portion of meat from a single species at any given meal. It is also assumed that a full portion of eggs (100 g) will be consumed in addition to the muscle or organ tissue on any given day. For milk, a consumption factor of 1.5 l/day is estimated, equivalent to the 1.5 kg/day estimate used in the model diet for derivation of MRLs for the CAC.

The next step involves considering the consumption factors in the light of the approved uses. When a product is approved for use in both beef cattle and dairy cattle, for example, one-half of the ADI is typically reserved for edible tissues and one-half for dairy products. When approved uses include laying

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hens in addition to other animals producing edible tissues, one-fifth of the ADI is reserved for eggs. The tolerances or "safe concentrations" are then derived from the applicable fraction of the ADI for the food and the consumption factor. Where circumstances warrant, alternative consumption factors may be used, or the tolerance may be reduced to reflect the residues that should be associated with the approved use of the drug. The end result is that while MRLs and US "safe limits" are in most cases derived from a common ADI, the processes generally lead to US "safe limits" which are different in value from the MRLs established by the CAC, the European Union, or national authorities which use the MRL approach to regulation of residue concentrations in foods. This is not to imply a difference in the standard of consumer protection, but rather reflects some procedural differences in the exposure estimates. The same depletion data are used in both models. For example, while the USFDA has established a tolerance of 25 ppb (25 μg/kg) for residues of melengestrol acetate (MGA) in edible tissue of treated animals,[37] the MRLs established for MGA residues by the CAC[2] are 1 μg/kg for muscle, 10 μg/kg, for liver, 2 μg/kg for kidney, and 18 μg/kg for fat, based on the differences in the dietary intakes used in the estimate of exposure.

1.6 International Obligations for Regulatory Analytical Laboratories

Many laboratories undertaking regulatory testing for veterinary drug residues in foods are engaged in the testing of products which are either imports from other countries or are domestic products which may be exported. Under procedures and guidelines which may be referenced in disputes referred to the WTO, the CAC has approved a guideline for the settling of disputes between member states over analytical results.[38] The guideline deals with three major concerns: the accreditation status of the testing laboratory, the validation of the analytical method(s) used, and the availability of sample material for further testing, if requested. These guidelines should be considered as simply representing best practices which should be followed by any laboratory that claims competence in a field of testing and not as a set of rules for elite laboratories.

1.6.1 Laboratory Accreditation

The guideline begins with the assumption that the testing laboratories involved will be in compliance with the CAC *Guidelines for the Assessment of the Competence of Testing Laboratories Involved in the Import and the Export Control of Food*.[39] This guideline established four principles which should be met by regulatory laboratory testing imported and/or exported products for compliance with regulatory standards. Such laboratories should:

- Be accredited under the general criteria of ISO/IEC-17025, *General requirements for the competence of testing and calibration laboratories*[40].

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- Participate in appropriate proficiency testing programs, when available, and these proficiency programs should comply with the requirements of *the International Harmonized Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories*.[41]
- Apply analytical test methods validated according to the criteria established by the CAC, which, in the case of methods for veterinary drug residues in foods, is CAC/GL 71-2009, *Guidelines for the design and implementation of national regulatory food safety assurance programme associated with the use of veterinary drugs in food producing animals*.[5]
- Use established internal quality control procedures consistent with the *Harmonized Guidelines for Internal Quality Control in Analytical Chemistry Laboratories*.[42]

Compliance with these criteria does not ensure that all test results issued by such a laboratory are correct. However, it does ensure that the laboratory has procedures in place to ensure that the performance of test methods used and the analysts using them has been demonstrated and that procedures are in place which should detect errors which may occur. Such assurances cannot be provided when laboratories use methods that are selected and applied without demonstration that these methods are "fit for purpose" and will provide consistent results. Equally, such assurances cannot be provided if there are no requirements that the analysts using the methods have demonstrated competency in the techniques used and in the performance of the specific method on materials that are representative of typical samples and that have been provided blind to the analyst.

1.6.2 Validation of Analytical Methods

On the issue of method validation, CAC/GL 70-2009 requires that, in case of dispute, a laboratory should be able to provide information on the validation of the method or methods used in the testing, including any method-specific sample handling and preparation procedures.[38] For laboratories dealing with the analysis of residues of veterinary drugs in foods, the primary authoritative references for guidance on method validation should include CAC/GL 71-2009,[5] which provides the criteria and guidance to be followed by laboratories conducting official analyses in member states of the Codex Alimentarius. Additional guidance is provided in 2002/657/EC, the official requirement for validation of analytical methods for veterinary drug residues in foods established by the European Commission, which applies to laboratories conducting official analyses in Member States of the European Union and also to laboratories conducting tests for products exported to countries within the European Union,[43] and also the general guidance on single laboratory method validation from the International Union of Pure and Applied Chemistry (IUPAC).[44] For laboratories developing analytical methods to be used in support of the approval of new animal drugs,

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guidance on method validation procedures for such methods and definitions for terminology are contained in several VICH guideline documents[26]:

- Validation of analytical procedures: Methodology, VICH GL2 (Validation methodology)
- Validation of analytical procedures: Definition and terminology, VICH GL1 (Validation definition)

In the United States of America, guidance on the validation of regulatory analytical methods used in the analysis of food and feeds for veterinary drugs has been provided by the USFDA.[45]

Method validation requirements are discussed in detail in Chapter 10.

1.6.3 Consistent Use of Terminology

Another area to which consideration must be given in documenting the validation of analytical methods and ongoing monitoring of the performance of analytical methods is the terminology used when discussing or reporting the parameters used in method performance assessment. For laboratories involved in the import/export testing of foods for veterinary drug residues (or other analytes), a primary source of definitions for the terminology to be used in describing method performance is the *CAC/GL 72-2009, Guidelines on analytical terminology*, issued by the CAC.[46] The definitions cited in the Codex guideline are primarily drawn from the relevant standard issued by the International Organization for Standardization[47] and from the *International Vocabulary of Metrology*.[48]

One term used in many published reports on method performance that is not used consistently is "sensitivity," which is defined in CAC/GL 72-2009[46] as the "Quotient of the change in the indication of a measuring system and the corresponding change in the value of the quantity being measured," as defined in the *International Vocabulary of Metrology*.[48] Simply put, this definition means that the sensitivity relates to the calibration curve and the ability of a method to discriminate between concentrations (i.e., the difference in concentration of analyte in a sample that can be measured using the method). However, the term sensitivity is also frequently used in describing the performance capabilities of analytical instruments and has come to be used synonymously with terms such as limit of quantification and/or limit of detection by many authors. This probably occurs most frequently in the reporting of analytical methods used in mass spectrometry. The editors recognize that there are some differences in practice, and therefore the term "sensitivity" is used in Chapter 3, which deals with current developments in high-resolution mass spectrometry, to refer to the capability of a mass spectrometry-based detection system to detect and quantify small amounts of analytes within a complex sample matrix, consistent with usage in the current literature dealing with such methods. The term sensitivity also is used differently when referring to the performance of screening tests, where typically it refers to "the lowest concentration at which the target analyte may be reliably

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detected within defined statistical limits."[5] Otherwise, the term sensitivity is used in this book consistently with the definition in the *International Vocabulary of Metrology*.[48] This also points out the need when developing a method validation protocol for a laboratory and reporting on method performance parameters to include a clear statement of the definitions of the terms being used and their source.

1.6.4 Sample Handling and Retention

A knowledge of the stability and behavior of residues in food matrices received for analysis is fundamental to good analysis. For veterinary drug residues in foods, samples typically are collected on farm, at point of processing, at port of entry, or at retail. It is therefore important that there are clearly defined procedures for collection, packaging, shipment, and handling on receipt of sample materials. These procedures should ensure the integrity of the sample material, both by preserving the sample from degradation and by protecting the sample from contamination or tampering. Analysis of a contaminated or degraded sample is not only a waste of resources, but may result in false-positive or false-negative results which can either lead to unnecessary investigations (false positives) or cause exposure of consumers to potentially harmful residues (false negatives). This is also an area over which laboratories may have little or no control until the sample material is received, so at a minimum it is important that the laboratory has clearly defined sample acceptance criteria which must be met before a sample is accepted for analysis. The sample acceptance criteria would typically include:

- *Quality of documentation*: The source of the sample material, time of collection, specific identifiers such as sampling plan number, and name of the sample collector should be included.
- *Integrity of packaging*: The sample material should be in an appropriate package which is sealed and contains the required documentation. In the case of legal or official samples, a chain of custody should be demonstrated.
- *Integrity of sample material*: The sample material should show no obvious signs of degradation, decomposition, or external contamination. Typically, tissue samples are frozen prior to shipment and should be frozen on receipt.

A laboratory should have and follow written, auditable procedures for sample receipt, sample acceptance, sample handling, and storage prior to analysis and sample handling, storage, and disposal subsequent to analysis. Different storage criteria will generally apply if samples have been shown to contain residues in excess of regulatory limits. CAC/GL 70-2009 requires that a portion of the original sample material received by the laboratory should be retained for further analysis in the case of dispute.[38] Specifically, it is recommended that the original sample received at the laboratory should be "split into three essentially identical parts for the purposes of primary analysis and for confirmatory analysis (reserve

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samples)." In the case of dispute, the reserve sample should be made available for independent testing, if requested.

1.6.5 Confirmatory Analysis

There is often confusion in the terminology used by analysts between "identification" and "confirmation." An analytical method, such as LC-MS or LC-MS/MS, typically provides both "identification," based on the presence of a minimum number of characteristic ions or ion transitions, and "confirmation," again based on the same characteristic transitions. However, you cannot "identify" and "confirm" in the same analysis. As noted by an ASMS expert working group, there is confusion in the literature over the term "confirmation," as it is used in some instances to denote "verification of a prior test" and in others to refer to "verification of the presence of a suspect compound."[49] The report from this group also notes that it is very difficult to prove with absolute certainty that the signals obtained from an unknown are from a specific compound, based on a comparison of the signals from a standard of that compound, as there is always a finite possibility that the observed signals are "from some hitherto unknown compound or phenomenon." That is, the confidence that can be placed in the confirmation relates to the selectivity of the method used. Guidelines for confirmation of pesticide residues issued by the CAC state that there are generally two phases to the multi-residue methods typically used in pesticide residue analysis, screening, and confirmation.[50]

We recommend that the term confirmation should be used only when referring to the process of verifying (confirming) a previously obtained analytical result. This means that the confirmation process is conducted using a second test portion of the original sample material, which is extracted and analyzed separately from the original test portion from which a result is to be confirmed. The purpose of the confirmation may be to confirm the identity and/or to confirm the quantity of analyte detected in the initial analysis. For regulatory purposes, it is generally accepted that for substances with an MRL or other established regulatory limit, confirmation is required for both the compound identity and the quantity present. For substances which are legally banned from being present in foods, confirmation of the presence (identity confirmation) may be the primary requirement, although the amount present is usually of interest to regulatory authorities both for assessment of consumer exposure and as a source of potential information on the use pattern of the prohibited substance.

The preferred techniques for confirmation in most regulatory laboratories today involve mass spectrometric techniques, typically MS/MS or high-resolution MS combined with gas or liquid chromatography. The preference for such techniques is that they combine information from two analytical techniques: the retention time from the chromatographic separation and the structural information from the mass spectrometric measurement. The mass spectral information, using either multiple characteristic ions or multiple reaction monitoring (MRM)

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transitions when low mass-resolution mass spectrometry techniques are applied, or accurate mass measurements from high mass-resolution mass spectrometers, greatly increases the analytical selectivity and therefore the confidence in the validity of the confirmation.

It has been noted in several guidance documents produced by Eurachem that there is sometimes a confusion between the terms "repeatability" and "confirmation."[51, 52] The distinction made is that repeatability deals with the ability to obtain the same result from replicate analyses, while confirmation requires the use of several different analytical techniques. This distinction has become somewhat blurred as techniques such as LC-MS/MS have come into routine use in regulatory laboratories in the past decade. Prior to the common availability of mass spectrometers as detectors for chromatographic techniques, a standard approach to confirmation in residue analysis involved the use of chromatographic columns of different polarities, the use of different detectors, and the preparation of characteristic derivatives of the analyte which had different separation and detection properties from the original target compound. This approach automatically required the analysis of multiple test portions to meet the requirement that different analytical techniques should be applied. Thus, an initial analysis for a residue of a pesticide or veterinary drug or for a contaminant might involve a quantitative analysis by gas chromatography with electron capture detection or liquid chromatography with UV or fluorescence detection, followed by a subsequent confirmatory analysis using GC-MS or LC-MS. Such an approach is still valid and is applied when the initial method targets a single analyte or a small number of related analytes.

However, the approach has changed with the now routine use of LC-MS/MS and LC-HRMS instruments as primary analytical instruments for multi-residue methods. In the current approach for a multi-residue analysis, the initial analysis may target only a single ion or MRM transition, usually the most abundant, to detect the possible presence of a particular analyte. When the analysis is conducted without inclusion of a calibration curve for that compound, the method is used in a screening mode to detect the presence of any targeted analytes above a known minimum concentration. Typically, some representative standards would be included in QC materials spiked at the minimum concentration to verify performance. The same method may next be applied with inclusion of appropriate standard curves for any analytes detected to provide a quantitative result. In addition, when using low mass-resolution MS or MS/MS detection, additional ions or ion transitions may be monitored to improve the quantification and/or to confirm the identity of the detected compound. When a high mass-resolution mass spectrometer is used as the detector, more accurate mass measurement is used to provide the confirmation. Either technique improves the method selectivity and thereby provides greater statistical confidence in the confirmation.

The criteria which are considered acceptable for regulatory result confirmation are contained in a number of guidance documents.[5, 42, 49, 50, 53] In general, these criteria require comparison of the information obtained from the unknown

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detected with the information obtained from a chemical standard. The retention times should match within specified limits for the chromatographic separations, and there should be matching ions or MRM transitions in equivalent relative proportions. The measurements on the reference standard should be made at the same approximate concentration as the unknown to reduce the risk of concentration or matrix effects affecting the results.

1.6.6 Quality Assurance Measures

Quality assurance measures in a residue control laboratory typically include procedures for method validation, verification of instrument performance, documentation of analyst qualifications, documentation of routine quality control, procedures for investigation of anomalous results, and documentation of such investigations, as well as a quality manual which describes roles and responsibilities.[40] These requirements were discussed in some detail in the companion volume Chemical Analysis of Antibiotic Residues in Food.[54] The same principles and approaches recommended for antibiotics are also generally applicable to the analysis of other veterinary drug residues and therefore have not been repeated in this publication. However, some relevant principles for application to multi-residue methods are included in Chapter 10.

1.6.7 Proficiency Testing

This topic was also covered in some details in the companion volume[54] and will not be repeated in detail here. However, some discussion on the challenges associated with the preparation and analysis of appropriate PT materials for use in multi-residue methods is contained in Chapter 10. Analysts should be aware of the expectation that they will participate in appropriate PT material exchanges when available. An appropriate PT material is one which is representative of analytes and matrices typically analyzed in your laboratory. Participation in PT exchanges which are not representative of typical sample materials and analytes is not recommended, as it can not only bias the outcome of the exchange for laboratories routinely involved in such analytical work but also provide a false impression on the competencies of your laboratory. A simple approach to determining when a PT round is appropriate for participation by your laboratory would be if it includes analytes and matrices which are included in the scope of a method for which you have received or are seeking accreditation.

1.6.8 Reporting of Results

CAC/GL 70-2009,[38] *Guidelines for settling disputes over analytical (test) results*, is the guidance document approved by the CAC for procedures to be followed in disputes between member states over analytical results. In addition to laying out requirements for making sample material available and documentation

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of validation of methods, proficiency testing results, and quality assurance measures, this also provides recommendations on the reporting of sample results. On this issue, the guidance states that quantitative analytical results should be reported as "$X \pm 2u$" or "$a \pm U$." The guidelines defines "X" as "the best estimate of the true value of the concentration of the measurand," "u" as "the standard uncertainty," and "U" as "equal to $2u$," "the expanded uncertainty." It further states that "$X \pm 2u$" represents "a 95% level of confidence where the true value would be found" and that "U" or "$2u$" is "the value which is normally used and reported by analysts and is referred to as the measurement uncertainty." Another CAC guideline, *CAC/GL 54-2004*, *Codex Guidelines on Measurement Uncertainty*, recognizes that there are a number of accepted approaches for estimation of the measurement uncertainty.[55]

In addition, when reporting the results, two other important issues should be considered: analytical recovery and significant figures.[38] While some regulatory authorities require that all results reported should be corrected for recovery, others prefer to report uncorrected values. This is perhaps less of an issue when laboratories routinely use methods based on internal standards, as this approach provides a recovery-corrected result, based on the internal standard. Whether the results reported are corrected for recovery, this information should be documented and should be available to laboratory clients or in case of a dispute on the results. It is also important that the analyst appreciates if the relevant regulatory limits were set on the basis of analytical results corrected or uncorrected for analytical recovery as this will affect any advice given. The CAC guideline for settlement of trade disputes requires that it should be stated whether or not the reported results are corrected for recovery, the procedure used to correct for recovery, and the actual method of recovery.[38] The CAC has adopted a guideline for recovery correction, CAC/GL 37-2001, *Harmonized Guidelines for the Use of Recovery Information in Analytical Measurement*,[56] based on the guidance developed by the IUPAC.[57]

On the subject of significant figures, CAC/GL 70-2009 states that laboratories should provide "information necessary to interpret the results," which specifically includes "the number of significant figures."[38] In the authors' experience, significant figures are too often not properly considered when reporting analytical results, whether in laboratory reports or papers in the literature. This unfortunately seems to be a result of the reporting of computer-generated results or the outputs from calculators without a consideration of the meaningfulness and reliability of numbers several places after the decimal when reporting results in μg/kg or ng/kg. Quite often, computer programs or calculators will yield results to two or more places after the decimal, depending on the criteria established for the calculation. It is then the role of the analyst to proactively translate the number generated by the calculator or computer into a meaningful and defensible analytical result. The analyst should consider at least two factors in determining where rounding of the result should occur, irrespective of the inclusion of measurement uncertainty in the result. First, the sensitivity

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of the method of measurement should be considered, that is, the ability of the measurement to distinguish between concentrations of the analyte. It is nonsensical to report results which are beyond the measurement capability of the measurement system used and are a professional disservice to the client. A simple approach recommended two decades ago to determine where rounding should occur in the reporting of an analytical result is to base the rounding on the method precision – rounding off so that the last digit reported is the first in which there is uncertainty.[58] For example, if method precision is 10% (i.e., 10 μg/kg ± 1 μg/kg), a calculated result of 9.67 μg/kg should be reported as 10 μg/kg since the precision indicates uncertainty at concentrations of 1 μg/kg.

1.7 Conclusions

To be an effective analyst of record for the analysis of veterinary drug residues in food, it is not sufficient to be expert in one or more analytical techniques or in the performance of validated analytical methods for such residues. An analyst of record, the analyst who signs the laboratory report for the client, must also have the necessary knowledge and experience to provide interpretation of the results. As we have noted in various sections of this chapter, this requires knowledge of the conditions of use of veterinary drugs; the nature of the residues formed; typical depletion patterns, including the tissues that most likely contain residues; the persistence of these residues and their nature (e.g., free or bound); and the basic knowledge to recognize a test result which requires further investigation. We hope the contents of this chapter and those which follow in this book will be a useful source for such knowledge.

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2

Emerging Techniques in Sample Extraction and Rapid Analysis

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2.1 Introduction

With advances in generic and minimalist extraction and sample clean-up procedures in the past decade, veterinary drug residue methods have expanded rapidly from procedures with only a few analytes to methods that include a wide range of veterinary residues, pesticides, and chemical contaminants in foods. This chapter is focused on emerging techniques applied to the analysis of non-antimicrobial veterinary drug residues in foods, with an emphasis on literature published from 2010 to 2015. In many cases, the techniques described have also been applied to the analysis of antimicrobials and to larger multi-residue analyses that include a wide variety of drug classes from both classifications. Liquid chromatography (LC) with mass spectrometric analysis is the main analytical platform for multi-residue veterinary drug analytical methods as most compounds are readily ionizable. Specialized techniques with greater selectivity have also emerged to improve residue quantification at low concentrations and minimize matrix interferences from complex animal-derived foods. This is particularly true for some of the non-antimicrobial classes of veterinary drugs included in this book where illegal drug use is regulated and residues are monitored at very low concentrations. As some techniques have been designed to be highly selective, they will clearly not be applicable to a wide range of analytes even with further development; yet, many offer advantages of simplicity, matrix reduction, and speed that are important considerations for regulatory analysis.

This chapter is divided into sections based on chemical and physical extraction procedures to separate analytes from the bulk sample, solid and liquid extraction techniques (sample clean-up) to separate analytes from co-extracted matrix components, and emerging analytical techniques for fast and direct analysis of extracts. The companion volume to this book provides excellent reviews of

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mass spectrometry and bioanalytical assay methods.[1] Chapter 3 of the current volume is dedicated to high-resolution mass spectrometry. As such, details on immunoassay sensors and mass spectrometry detection are not included in this chapter. Rather, we have focused on current state-of-the-art practices and new developments for extracting and isolating analytes, as well as techniques to quickly introduce these analytes for mass spectrometric detection. For example, many current veterinary drug residue analysis methods are designed with features to enhance protein precipitation or phase separation or to physically separate analytes from matrix based on molecular size or solubility at low temperature. Efficiency in solvent extraction continues to evolve with enhanced mixing efficiency using ultrasound and microwave radiation assistance. In other areas of research, environmentally friendly extraction solvents such as ionic liquids and pressurized solvents have been incorporated into sample extraction. New solid sorbent materials have been designed to aid the separation of targeted analytes from complex biological matrix components to reduce analytical interferences, while online sample preparation continues to play a role to automate clean-up prior to analysis. Selective molecular recognition agents and nanomaterials have been tested in emerging sorbent materials and techniques, and an assortment of micro-extraction techniques have been developed to separate and concentrate drugs from bulk matrix extracts. Another area of growth is in rapid analysis techniques where chromatography has been improved with core–shell columns or is eliminated entirely in favor of direct sample analysis by mass spectrometry. Ion mobility techniques have been applied to enhance analyte detection post-ionization by exploiting differences in compound structures to filter out interfering matrix components or isobaric contaminants. Individually, or used in combination, recent developments provide chemists with many opportunities to further expand the field of veterinary drug residue analysis in food.

2.2 Sample Extraction

Sample extraction is an important first step for chemical analysis, especially for veterinary drug residues in foods, where the sample matrix is complex with high content of proteins, fats, and/or sugars. Advances in mass spectrometry instrumentation have enabled sensitive and selective residue analysis, with streamlined extraction protocols for faster analyses of constantly increasing numbers of compounds detected per method. Over the past decade, veterinary drug residue analysis has shifted from multi-residue methods for several drugs in a single class to broad methods that combine in excess of 100 veterinary drug residues from many drug classes into one analysis. Common modern approaches to veterinary drug residue analysis are focused on simplified sample extraction procedures with minimal sample clean-up. Whereas in the past, multiple extraction steps and complex sample clean-up procedures were the norm, now sensitive and selective instrument techniques are largely used to overcome matrix background interference

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which may accompany simple sample processing procedures. Isolation of single drug classes was formerly based on separation by chemical properties (eg., pK_a/pH) and sample clean-up techniques selected by specific chemical interactions. Analytical extraction strategies to isolate many compounds from a variety of chemical classes cannot easily be optimized toward specific chemistries, but must allow extraction of analytes with many different chemical properties.

2.2.1 Solvent Extraction and Protein Precipitation

Animal products are composed of fats and proteins. To effectively separate veterinary drug residues from these larger sample components, a variety of techniques have been used to precipitate proteins and remove fats from samples. For multi-class analyses, simple solvent extraction is commonly used, often based on acetonitrile, which is also useful for precipitating proteins in dairy and tissue samples with few additional processing steps. After initial solvent extraction, additional sample clean-up techniques based on sorbent or liquid extraction may still be needed to further reduce the sample matrix; however, multi-analyte methods which target the recovery of a broad scope of analytes by their nature limit the possibilities for how much additional clean-up can be applied.

Universal extraction strategies have been proposed for hundreds of veterinary residues in a variety of animal-based food matrices.[2] A few examples of generic solvent extraction techniques are provided as examples of the development of this approach. Mol et al. validated an ultrahigh performance liquid chromatography (UHPLC)–MS/MS method for 86 veterinary drug residues from numerous drug classes in milk, muscle, egg, honey, and feed matrices.[3] Various extraction solvents and clean-up methods were tested, and the final method was based on a simple solvent extraction with acetonitrile and 1% formic acid, centrifugation, and filtration. Robert et al. used pure acetonitrile to precipitate proteins in samples of milk, egg, honey, and meat in an analytical method for >160 veterinary drugs.[4] Simple techniques to prepare milk extracts for drug residue screening were developed based on protein precipitation with acetonitrile, acidification, and isolation of analytes using ultracentrifugation and molecular weight cut-off filters.[5, 6]

It has been observed that higher recoveries can be achieved for polar drug residues when acetonitrile and water mixtures are used for extraction.[7] Methods developed by Lehotay et al. for over 100 veterinary drugs in cattle muscle and kidney are based on extraction with 4:1 acetonitrile/water, clean-up with dispersive C_{18} sorbent and/or hexane defatting, and filtration.[8–10] Biselli et al. homogenized chicken muscle with acetonitrile, succinate buffer (pH 4), EDTA, and sodium chloride.[11] After the samples were centrifuged, the acetonitrile was evaporated, and extracts were reconstituted and filtered to determine 84 veterinary drugs from antibiotic, imidazole, and triphenylmethane dye drug classes. Storey et al. developed a similar method applicable to fish and shrimp to simultaneously extract antibiotics, triphenylmethane dye, and hormone compounds for a simple regulatory screening method.[12] The extraction was based on a complex solvent

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mixture designed to stabilize the dye compounds, but required only a few steps; after mixing and centrifugation, the acetonitrile was removed, evaporated, and filtered. Kaufmann et al. discussed efficient protein precipitation methods for extraction of analytes from tissue (muscle, liver, kidney, fish) and honey samples including precipitation techniques based on acetonitrile, trifluoroacetic acid, metals, ammonium sulfate, and dyes.[13] Merits of each were compared and applied to the analysis of over 100 veterinary drugs from many classes.

2.2.2 Phase Separation by Salt-Induced Partitioning

Liquid–liquid partitioning induced by salts is commonly used to reduce the water content of acetonitrile extracts and drive residues into the organic phase. This is the basis for the popular QuEChERS method for Quick Easy Cheap Effective Rugged and Safe sample extraction.[14] In QuEChERS, a mixture of salts is added to the acetonitrile/water extract to induce phase separations. Sodium chloride is commonly used to separate the phases, magnesium sulfate to dehydrate the organic phase, and a variety of buffering salts can be added to optimize pH conditions of the mixture. The technique has varied and evolved since its introduction and has been applied to many different chemical analyses.[15] QuEChERS has been the focus of many studies for veterinary drug residue extraction methods from a variety of matrices including milk,[16] milk and liver,[17] chicken muscle,[18] eggs,[19] milk,[20] shrimp,[21] milk, liver, and pork,[22] milk and honey,[23] and urine from cattle,[24] to name a few.

Recently, ammonium salts were suggested as alternatives for phase separation.[25] Ammonium salts are more volatile than sodium salts, thus causing less deposition on the mass spectrometer ionization source. Nanita et al. optimized a method for pesticides in beef, milk, egg, and other agricultural and biological matrices by extracting matrix with acetonitrile and aqueous ammonium chloride.[25] González-Curbelo et al. also found ammonium salts with formate and acetate anions to be promising in the QuEChERS extraction method as they offer buffering capabilities and have significantly lower boiling points than ammonium chloride and decompose into non-corrosive products.[26] Kaufmann et al. introduced a salting-out procedure for veterinary drugs in milk based on ammonium sulfate followed by clean-up with supported liquid extraction (SLE) on diatomaceous earth columns.[7] Ammonium sulfate was also used by Wang et al. followed by solid-phase extraction (SPE) clean-up.[27]

2.2.3 Phase Separation by Low-Temperature Partitioning

Phase separation has also been induced by lowering the temperature of acetonitrile/water extraction solutions. Low-temperature partitioning has been used in combination with liquid–liquid extraction to retain extracted analytes in the organic liquid phase while allowing interfering matrix components (e.g., fats, proteins) to congeal or partition at low temperature into a separate phase.

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Goulart et al. applied low-temperature partitioning to the analysis of pyrethroid compounds in milk from cows that had been doused with deltamethrin or cypermethrin to kill ticks.[28] In this method, milk samples were extracted with an organic solvent at room temperature, shaken, then placed in a freezer at −20 °C for 24 hours. Fat, aqueous, and protein components of the milk froze, while the pyrethroids remained in the liquid organic solvent layer and could be easily removed for analysis. Low-temperature partitioning has been used for pyrethroid extraction from other animal-based matrix samples as well including pork, poultry, cattle, sheep, and game muscle.[29] Rübensam et al. applied low-temperature partitioning to the analysis of five avermectin compounds and moxidectin in milk samples using acetonitrile, sodium chloride, and a 12 hour temperature reduction to −20 °C.[30] A similar technique was applied to prepare beef samples for these macrocyclic lactones.[31] In 2011, Lopes et al. introduced fast partitioning at very low temperatures for the extraction and clean-up of sulfonamide antibiotics in pork liver.[32] This technique was expanded as a general extraction/clean-up procedure to determine 34 antibiotics and benzimidazoles in pork muscle.[33] Pork muscle was extracted with acetonitrile, homogenized, and then centrifuged. Centrifuge tubes were then plunged into liquid nitrogen for 15 seconds. The liquid organic layer was withdrawn, evaporated, and reconstituted for analysis. This simple procedure required approximately 25 minutes to complete and was subsequently applied to milk.[34]

Zhan et al. applied low-temperature partitioning to acetonitrile extracts of infant formula, pork, and beef to determine β-agonists, thyreostats, azoles, sedatives, steroids, dyes, coccidiostats, anthelmintics, and non-steroidal anti-inflammatory drugs (NSAIDs), in addition to several classes of antibiotics, pesticides, and contaminants.[35, 36] Recently, Xie et al. published a method for the analysis of compounds from 17 different classes of veterinary drugs, pesticides, and contaminants in milk, cheese, and yogurt samples.[37] Samples were extracted with 1% acetic acid in an acetonitrile and ethyl acetate mixture and then kept at −80 °C for 30 minutes to solidify fats and aqueous sample components. The organic layer was collected, evaporated, reconstituted in methanol and water, and further cleaned up using an Oasis HLB SPE cartridge prior to analysis with LC–MS/MS. Dasenaki et al. reported an analysis of 115 drugs from 20 drug classes from milk, butter, egg, and fish using a simple ultrasonic extraction of the matrix at 60 °C with a mixture of aqueous formic acid and EDTA, methanol, and acetonitrile, followed by low-temperature partitioning of the fats and proteins at −23 °C for 12 hours.[38] A final hexane extraction was applied prior to LC–MS/MS analysis.

2.2.4 Physical Separation by ultra-filtration

Centrifugal ultra-filtration with molecular weight cut-off filters has also been used to reduce matrix interference in food extracts by separating low molecular weight drug residues from larger proteins and matrix components. Filtration

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devices are available commercially (Merck Millipore) with porous membranes that are designed to pass molecules with size below molecular weight cut-off limits at 30,000, 10,000, and 3,000 kDa. High-speed centrifugation is required to assist penetration of the lower molecular weight compounds through the membrane. This technique has been applied as a simple clean-up technique for several multi-class drug residue analysis methods. In all cited examples, formic acid-acidified acetonitrile extracts were applied to the ultrafiltration device and separation was assisted by centrifuging samples at $17{,}000 \times g$. Turnipseed et al. used 30,000 kDa molecular weight cut-off devices to further reduce matrix following SPE clean-up of milk extracts.[39] In later studies, researchers noted improved sample clean-up for milk extracts using 3,000 kDa filters without SPE.[5,6] Centrifugal ultra-filtration devices were also applied to clean up extracts for multi-residue analysis of frog legs and fish.[40] In this case, the 30,000 kDa device provided superior extract clean-up compared to smaller cut-off sizes and SPE cartridge clean-up.

2.2.5 Sample Extraction with Green Chemistry Techniques

2.2.5.1 Pressurized Liquid Extraction

While extraction procedures based on acetonitrile are by far the most common used in modern veterinary drug residue analyses,[2] efforts have been made to reduce the use of organic solvents. Pressurized liquids or supercritical fluids can enhance and accelerate the extraction efficiency of analytes based on unique solvation properties of these compressed fluids.[41–43] Veterinary drug residue extraction using pressurized water as a solvent was explored for efficient extraction of antibiotics in beef samples.[44] Lower solvent consumption and faster, more efficient extraction were reported for the analysis of 21 benzimidazoles in liver and muscle samples of swine, cattle, sheep, and chicken using pressurized liquid extraction (PLE) with acetonitrile and hexane.[45] PLE was also applied to the determination of glucocorticosteroids in swine, cattle, and sheep muscle.[46] Tao et al. extracted malachite green, crystal violet and their leuco metabolites from salmon and shrimp samples using PLE.[47]

2.2.5.2 Room Temperature Ionic Liquids

Room temperature ionic liquids (RTILs) have been developed recently as alternatives for liquid extraction solvents, as well as for micro-extraction techniques and chromatographic mobile phases. Whereas salts are ionic compounds with high melting temperature, ionic liquids are composed of a bulky asymmetric cation with an alkyl side chain and an anion. The structure and asymmetry of the cation prevent ordered packing of the ions allowing the salt to remain in the liquid phase at and below room temperature, while the structure and properties of the anion determine the solubility of the salt.[48] As miscibility and solvation properties can be dramatically varied by interchanging cation, anion, and length of the alkyl chain, ionic liquids can be designed for specific applications. In general, they have

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low volatility, can interact with polar and non-polar analytes simultaneously, and can be used as liquid-phase extractants and immobilized on sorbent supports to assist extraction.[49] They are also generally considered to be solvents causing little environmental harm. An excellent review article was recently published by Poole and Lenca on properties and applications of RTILs.[50] Applications for RTILs in veterinary drug residue analysis are typically based on micro-extraction techniques that are discussed in detail in later sections.[51]

2.2.5.3 Ultrasound-Assisted Extraction

Regardless of extraction solvent selected, many studies have demonstrated enhanced extraction by increasing the contact of the extraction solvent with the matrix through ultrasound-assisted extractions (UAE). The application of ultrasonic radiation can induce a variety of chemical and physical effects in samples, extraction solvents, and sorbents, largely due to acoustic cavitation where microbubbles in the liquid phase expand and collapse.[52] Micro temperature and pressure changes can disrupt cells and enhance mass transfer of analytes into the extraction solvent, while turbulence and swelling of the matrix permit greater diffusion of the solvent into the matrix.[52] Ultrasonic radiation has been used in a number of recent studies.[53] In some cases, ultrasound enhances the extraction of a solid sample with a liquid solvent, and in others, it is required for thoroughly dispersing solid sorbents and small-volume liquid extracting phases in the emerging techniques described in later sections. Boscher et al. used ultrasonic radiation to assist the extraction of veterinary drug residues from several classes from pig, cow, and lamb feeds.[54] Feed samples were blended with methanol, acetonitrile, McIlvaine buffer, and EDTA and then extracted in an ultrasonic bath for 15 minutes; following extraction, the extract was cleaned up with dispersive primary secondary amine (PSA) sorbent. In this study, UAE was found to provide similar extraction yields to PLE; however, the faster extraction time and ability to extract many samples simultaneously in the ultrasonic bath resulted in a more efficient process using UAE. Fernandez-Torres et al. combined extraction using an ultrasonic immersion probe with enzymatic digestion to accelerate the extraction of antibiotics and metabolite residues from four drug classes in fish and mussel tissues.[55] Following irradiation, analytes were extracted into dichloromethane and concentrated prior to HPLC analysis. Magiera et al. applied UAE to fish extraction for several classes of drugs commonly found in waste waters, including NSAIDs.[56] Solvent, pH, liquid volume, irradiation time, temperature, and power were optimized in this study. Porto-Figueira et al. introduced a micro-QuEChERS method applied to the extraction of zearalenone from cereal grains.[57] In this procedure, extraction was carried out with 0.3 g of cereal matrix, 0.7 ml of acetonitrile, 0.2 g of QuEChERS salts, 5 minutes of ultrasonic irradiation, and dispersive clean-up with magnesium sulfate, C_{18}, and PSA. While this application does not fall into the category of veterinary drug residue analysis, the finding that ultrasonic mixing was critical for extraction

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efficiency is notable since QuEChERS extractions do have general applicability for food analysis.

2.2.5.4 Microwave-Assisted Extraction

In another low environmental impact chemistry technique, microwave-assisted extraction (MAE) is based on using microwave energy to rapidly heat the sample and extraction solvent to accelerate partitioning of analytes into the extraction solvent. When MAE is applied to samples in closed systems, extraction solvents can be quickly heated above their boiling temperature to achieve simultaneous high-pressure extractions. The dielectric constant of the solvent determines the amount of microwave radiation it will absorb, and solvents can be selected based on this property to tune the extraction temperature profile relative to the sample matrix temperature.[58] Dynamic MAE was developed to continuously pump solvent through a microwave-heated sample to prevent analyte degradation from overheating and to assist with the removal of analytes from the sample.[59] Wang et al. applied dynamic MAE to the extraction of steroid hormones from fish tissue.[60] Fin-fish, shrimp, and squid muscle were blended with alumina and then extracted first with portions of acetonitrile and then with water flowing through the extraction cell while applying microwave heating to the sample. Once the extract was collected, ammonium acetate was added to the collection vial to induce phase separation and the acetonitrile phase was collected, evaporated, reconstituted, and filtered for LC–MS/MS analysis. A limit of quantification (LOQ) of 0.1–0.5 μg/kg and greater than 78% recovery (< 8% RSD) were achieved by this simple method. Other applications to food sample extraction were noted.[60]

2.3 Extract Clean-up with Solid-Phase Sorbents

Physical extraction of an analyte from matrix components based on solubility, partitioning, and molecular size may not be enough to permit sensitive and selective analysis of veterinary drug residues. Additional sample clean-up techniques based on sorbent or liquid extraction may still be needed to further reduce the sample matrix and concentrate analytes prior to analysis. For both multi-class and single-class veterinary drug residue analytical methods, SPE remains a common approach to clean-up sample extracts.[2] Solid sorbents can be used in formats ranging from packed cartridges and columns, loose material to be dispersed in liquid extracts and solid samples, and as a solid framework to support liquid extractions. These variations in solid-phase format are described later. In the subsequent section, the discussion is focused on the properties of the sorbent materials that are used to isolate veterinary drug residues from matrix based on functional chemistry, molecular recognition and size, and incorporation of nanomaterials and magnetic features.

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2.3.1 Solid-Phase Extraction Formats

2.3.1.1 Cartridge SPE

In traditional practice, SPE material is packed into a cartridge and sample extracts are applied to the sorbent to selectively adsorb and retain the analytes of interest while allowing matrix components to pass through and be discarded. The sorbent can be washed with a weak solvent to remove additional matrix and the analytes are then eluted with a stronger solvent. Alternatively, cartridges can be used in flow-through mode where a sample extract in a strong solvent is applied to the sorbent column such that the analytes remain dissolved in the solvent but matrix components are retained by the SPE material. In the flow-through mode, the SPE cartridge serves as a chemical filter and the filtrate is collected for analysis in a single step process. For some analyses, solid sorbents used for SPE cartridge clean-up are also useful in alternative online or dispersive formats, where advantages of one format over another depend on the particular application. Some of the different chemistries of sorbents commonly used for SPE in veterinary drug residue analysis are discussed in Section 2.3.2.

2.3.1.2 Online Cartridge SPE

Cartridge SPE can be coupled directly to the analytical platform for online sample clean-up. With column switching valves, sample extracts are introduced onto an extraction column to separate analytes from matrix, and then the desired extracted analytes retained on that column are eluted directly onto the analytical column. Online SPE has been used to analyze avermectin residues in milk,[61] a variety of antibiotics and triphenylmethane dyes in shrimp,[62] and for albendazole and metabolites in crab tissue.[63] Li et al. recently developed an online SPE method based on a polymeric monolith column for the retention of avermectins from beef and milk samples,[64] Automated sample clean-up and analysis were completed within 15 minutes per sample, and the SPE monolith column was reused for hundreds of samples without loss of performance. Various online sample clean-up techniques were recently reviewed by Barreiro et al.[65]

2.3.1.3 Turbulent Flow Clean-up

Turbulent flow chromatography (TFC) is another online sample clean-up procedure where analytes are separated by physical size characteristics as well as adsorption to the sorbent. In TFC, the small (e.g., $< 5\,\mu m$), uniform, spherical column packing material commonly used for HPLC is replaced with large (e.g., $50-100\,\mu m$), non-uniform particles. The size and uniformity change allows an increase in column flow rates (1.5–5.0 ml/minute) which, in turn, generates areas of laminar flow around the packing material and areas of turbulent flow within the remaining interstitial spaces of the column. Large molecules tend to remain in the fast-moving turbulent flow areas and are quickly eluted through the column while small molecules tend to remain in the slower laminar flow areas surrounding the packing material particles. This allows the small molecules to diffuse into and out

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of the packing material, which increases their retention and leads to separation of molecules by size. Once the large molecules have been sent to waste, the retained small molecules are then eluted off the turbulent flow column directly onto an analytical column for analysis. Theoretically, this technique would allow for the injection of various types of samples with little or no sample preparation.[66]

This technique has been applied to veterinary drug residue analysis in a variety of animal-based matrix types. Stolker et al. investigated TFC for online sample clean-up of target compounds from a variety of drug classes in milk samples.[67] Aguilera-Luiz et al. used this sample clean-up technique in the analysis of 40 antibiotics, imidazothiazoles, avermectins, and benzimidazole residues from honey samples.[68] Lafontaine et al. reported on an automated TFC analysis method for ractopamine in beef with a 30 minute total sample preparation time and an LOQ of 0.3 µg/kg.[69] Zhu et al. recently reported a TFC procedure for 88 veterinary drugs from antibiotic, benzimidazole, sedative, and hormone drug classes in milk samples that were initially ultrasonically extracted with acetonitrile and Na_2EDTA, centrifuged, and filtered.[70] The online purification and analysis procedure required 39 minutes per sample and permitted detection limits ranging from 0.2 to 2.0 µg/kg and 63–117% recovery (<20% RSD).

2.3.1.4 Dispersive SPE

In addition to cartridge and column techniques, solid sorbents can also be dispersed directly in the sample or sample extract to remove matrix components from the extract prior to analysis. Dispersive SPE (dSPE) is often used in combination with QuEChERS type procedures to further clean up acetonitrile extracts by mixing with a portion of bulk sorbent material. Centrifugation and filtration assist in separating the purified supernatant from the bulk sorbent material prior to instrumental analysis. Reversed-phase C_{18} sorbents are commonly used for dSPE in animal drug applications in conjunction with QuEChERS extractions for large multi-residue methods that include non-antimicrobial compounds. An LC–MS/MS method for veterinary drugs (antibiotics, anthelmintics, tranquilizers, β-agonists, etc.) in beef muscle utilized end-capped C_{18} as a dSPE sorbent.[8] Kang et al. tested several dSPE sorbents in a method for quantification and confirmation of 100 veterinary drugs including benzimidazoles, β-agonists, hormones, and tranquilizers in milk powder.[71] Based on an evaluation of analyte recoveries and the extent of sample clean-up, a C_{18} sorbent was selected to purify the milk powder extracts prior to LC-QTOF-MS analysis.

Recently, Han et al. combined dSPE, centrifugation, and filtration steps by weighing dSPE material directly into the lower section of a filter vial.[72] A portion of organic extract was mixed with the sorbent, and then the filter vial plunger was depressed to collect sorbentless extract in the upper portion of the vial, ready for use in an LC autosampler (Figure 2.1). The feasibility of this technique was demonstrated for the analysis of pesticides and environmental contaminants in shrimp. Schneider et al. used filter vial dSPE to clean up beef tissue extracts in a multi-residue method for veterinary drugs.[9] Beef muscle was extracted

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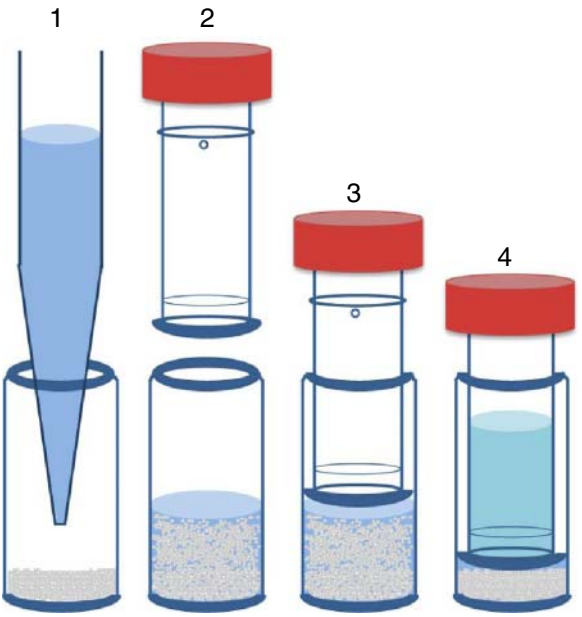


Figure 2.1 Filter vial dispersive solid-phase extraction. Source: Han 2014[72] Reproduced with permission of Elsevier.

with 4:1 acetonitrile/water, shaken, and centrifuged, and then a 0.4 ml portion of the supernatant was pipetted into a filter vial (0.2 µm PVDF) containing 25 mg of C_{18} sorbent for clean-up. Extracts were directly analyzed from the upper portion of the filter vials by LC–MS/MS for 129 residues from a wide variety of antibiotic, anthelmintic, growth promoter, anti-inflammatory, and other classes of veterinary drugs.

2.3.1.5 Matrix Solid-Phase Dispersion

In matrix solid-phase dispersion (MSPD), the sorbent material is thoroughly mixed with the bulk sample to disperse the sorbent and disrupt the sample. The sorbent/sample mixture can then be packed into a column from which analytes are eluted from the solid phase using a suitable solvent. Capriotti et al. have described recent advances in extractions with MSPD for food analysis.[73] Zhao et al. used MSPD with Florisil® and sodium sulfate sorbents to extract cypermethrin from carp tissues.[74] In this simple procedure, the fish tissue was mashed with the solid sorbents, packed into a column, then cypermethrin eluted with ethyl acetate/petroleum ether. Bittencourt et al. described a method for veterinary drug residue extraction where muscle from cattle and poultry was mixed with sand to disrupt cells.[75] Small volumes of EDTA (250 µl) and methanol (600 µl) were added to the solids and the sample was extracted with vortex and ultrasonic mixing, then centrifuged to yield an 800 µl aliquot for additional protein precipitation.

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2.3.1.6 Supported Liquid Extraction

Supported liquid extraction (SLE) is a type of liquid–liquid extraction, where the liquid phase containing the analytes (the donor phase) is spread out and held by a solid sorbent, and transfer of analytes into the extracting liquid phase (the acceptor phase) occurs as the acceptor solvent elutes the sorbent column. Typically, a liquid aqueous phase is adsorbed onto a column of diatomaceous earth to form a solid gel-like donor phase with large surface area. The column is eluted with an organic acceptor solvent and analytes are transferred from the donor into the acceptor phase along the highly dispersed supported liquid–liquid interface. SLE has advantages for reducing matrix interference for LC–MS/MS analysis when compared to protein precipitation techniques for drug analysis in plasma.[76] Akre et al. applied SLE to extract steroid and resorcylic lactone analytes from an aqueous urine hydrolysis mixture into organic solvent for further sample clean-up.[77] Recently, Kaufmann developed a salting-out SLE sample clean-up procedure (SOSLE) for milk matrix for the analysis of numerous classes of veterinary drug residues including antibiotics, imidazoles, tranquilizers, β-agonist, and others using HRMS analysis.[7] In this method, salt was used to separate the aqueous and acetonitrile phases of a milk extract. The aqueous phase containing the polar analytes was immobilized into the pores of the diatomaceous earth sorbent. The acetonitrile phase was also added to the sorbent column to transfer less polar analytes and initiate column elution. Additional volumes of acetonitrile were used to fully elute all analytes from the column. Compared to ultra-filtration, SPE, and QuEChERS, SOSLE was found to generate high recoveries and low signal suppression.

2.3.2 Solid-Phase Sorbent Chemistry

2.3.2.1 Sorbents for SPE and dSPE

SPE sorbents typically consist of either reversed-phase materials such as C_{18} or C_8, normal-phase sorbents including silica possibly with bonded cyano or amine groups, or ion-exchange materials. Historically, SPE materials were based on silica, but now polymeric materials (e.g., cross-linked styrene–divinylbenzene) are very common. Polymeric materials may contain both polar (hydrophilic) and nonpolar (lipophilic) moieties in order to absorb a broad spectrum of compounds. Smaller sorbent pore size has been noted to assist the physical separation of drug residue analytes from higher molecular weight matrix components.[13] In addition, several SPE chemistries can be used simultaneously by combining cartridges in tandem or by purchasing mixed mode SPE formats to optimize sample clean-up. Other chemistries that are available for SPE include graphitized carbon, Florisil®, and alumina, among others. In place of particulate-based sorbents, monolithic materials based on a single polymeric structure provide numerous possibilities for generating specific chemical properties and are finding applications as SPE sorbents and for chemical separations as the rigid and porous structure can enhance flow rates and mass transfer.[78]

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One novel type of SPE chemistry that has been applied to isolating veterinary drug residues from complex matrices is sorbent bonded with phenyl boronic acid (PBA). PBA selectively binds molecules containing diols. This was used by Berendsen et al. to isolate the antiviral compound ribavirin from chicken muscle.[79] Sin et al. also used PBA SPE in a method to isolate florfenicol amine from fish tissue extracts and observed lower matrix effects compared to procedures using cation-exchange SPE or SLE.[80]

Recently, there has been an increase in new commercially available solid sorbent materials, both in the cartridge form and as dispersive materials that are designed to remove phospholipids and other fats from food matrices prior to residue analysis. Phospholipids are well known to cause ion suppression in electrospray LC–MS. The chemistry of these sorbents is often proprietary, and published examples of their application to veterinary drug analysis are limited as the products are relatively new. However, it is expected that their use will become more widespread, so a brief description of some of these materials is provided here.

Z-Sep is a zirconium oxide-based product (Supelco) that has been effective in removing some fats from sample extracts. For example, Geis-Asteggiante et al. found Z-Sep products to be an effective dSPE sorbent to remove matrix components and isolate many veterinary drug residues from beef muscle.[8] However, some classes of drugs (tetracyclines, quinolones, macrolides) had significant loss of recovery as they were retained on the zirconia product. Prime HLB is a new variation of a hydrophilic–lipophilic polymer-based material introduced by Waters Corporation that more effectively retains phospholipids with increased sample flow through. Enhanced Matrix Removal (EMR) is a recently developed dSPE material (Agilent) designed to trap aliphatic lipid chains from sample extracts without capturing analytes. The EMR material has been demonstrated to minimize matrix interferences following a simple acidified acetonitrile extraction of beef liver in the LC–MS/MS analysis of 30 veterinary drugs including anthelmintics, sedatives, NSAIDs, a β-agonist, and an antithyroid drug.[81]

2.3.2.2 Molecular Recognition Based on Molecularly Imprinted Polymers

Molecularly imprinted polymers (MIPs) are polymeric materials that contain selective biomimetic cavities to adsorb specific molecules. These materials are prepared by incorporating the compound of interest or a structurally similar template molecule into the polymerization process. A functional monomer is selected with properties such that it will surround, bind, and later release the template molecule. A cross-linker is added to impart structural stability to the template/functional monomer complex.[82] The template molecule is then removed, leaving a cavity with highly specific recognition sites which are complementary in shape, size, and functional group to the target compound. The specificity of binding site can be manipulated to allow binding of one specific compound or to a group of compounds. Use of a homologous compound as the

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template can avoid incomplete removal of the template molecules and eliminate background effects and carryover.[83, 84]

The versatility of MIP materials allows them to be used in many different types of analyses such as the determination of the coccidiostat ethopabate in chicken.[85] In this example, an ethopabate imprinted MIP was produced using a methacrylic acid monomer, and the MIP sorbent was packed into an SPE cartridge. Chicken muscle was extracted with acetonitrile, and then the filtered supernatant was loaded on the conditioned MIP SPE cartridge. Ethopabate residues in the eluate were determined by GC-FID and gave good accuracy and precision (87% ± 3%), with an LOQ of 0.32 μg/l.[85] Selectivity was determined by analyzing structural analogs of ethopabate, which showed little or no retention on the column.

A somewhat less selective MIP capable of binding several compounds with similar structures can be developed by careful choice/preparation of the template molecule. An example of this can be observed in the development of a MIP for nine pesticides, where the template was not one of the analytes of interest but a molecule that possessed structural similarities common to all the target pesticide compounds.[86] The structure of the test pesticides matches the shape of the prepared MIP to varying degrees; therefore, they will bind/adsorb to the MIP in varying degrees. MIP materials have been developed for SPE of several veterinary drugs and drug classes including metabolites of carbadox and olaquindox in animal muscle,[87, 88] triphenylmethane dyes and metabolites in fish and shellfish,[89–91] β-agonists in ham sausage and pork,[92, 93] estrogens in fish,[94] and nitroimidazoles in egg and chicken muscle.[95] MIPs have also been used as sorbents in MSPD applications for steroids in goat milk[96] and clenbuterol[97] and olaquindox[98] in chicken muscle.

MIP materials can be incorporated into many extraction platforms including packed columns for HPLC,[99] coatings for electrophoresis capillaries,[100] SPE sorbents,[101] as well as stir bars,[102] fibers,[78] and membrane materials[103] used in micro-extraction techniques. MIPs can be used singularly or in combination with other extraction techniques such as MIPs with restricted access materials (RAMs).[104] Advances in MIP materials, preparation methods, and incorporation into different extraction and analytical platforms have been recently reviewed for food analysis applications.[105] Many MIPs are prepared within research laboratories, but a limited number are available commercially (e.g., Biotage, Sigma Aldrich/Supelco, AFFINISEP), or can be fabricated upon request (MIP Technologies, AFFINISEP).

2.3.2.3 Molecular Recognition Based on Aptamers

In addition to MIPs, affinity capture solid phases have been developed based on antibody immunosorbents and aptamers. Aptamers are synthetic single-strand oligonucleotides of DNA or RNA (approximately 20–100 base pairs) that are designed to fold into shapes that allow highly specific binding with target analytes.[106] Whereas production of antibodies can be complex, expensive, and

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require animal use, aptamer development is relatively simple and customizable by a standardized process called SELEX.[107] In SELEX, target analytes are incubated with a large library of oligonucleotides and the sequences that show favorable binding are retained, amplified, and tested for a next round of selection. The process is repeated for a number of cycles to obtain the best-fit oligonucleotide sequence for target analyte binding. Stead et al. developed a method for malachite green and leucomalachite green extraction from fish using a 38 base pair RNA aptamer to selectively isolate the dye from the fish extract and permit a simple fluorescence analysis of the bound MG–RNA complex.[108] In this method, salmon and trout were extracted with acidified acetonitrile, and the extract oxidized to convert the leuco metabolite to the cationic dye form. The extract was then cleaned up by SPE with Oasis® MCX sorbent, eluted from the cartridge, evaporated, and reconstituted in buffer. The buffer extract was incubated with the aptamer for 20 minutes and malachite green determined by very simple measurement of the fluorescence signal from the aptamer-bound residue. Aptamer sequences can be chemically synthesized and modified in various ways to link the receptors to solid sorbents (oligosorbents). Aptamers have been developed for several veterinary drugs and immobilized for use by a number of techniques as described in recent reviews.[106, 109]

2.3.2.4 Restricted Access Materials

Solid phases based on restricted access materials (RAMs) are also available for online/direct injection procedures to adsorb low molecular weight analytes from complex matrices. RAM columns use hydrophilic/hydrophobic, ion exchange, and/or size exclusion mechanisms to separate large hydrophilic molecules from smaller hydrophobic molecules.[110] By restricting the types of compounds that can penetrate the adsorption sites on these porous sorbents, RAMs can reduce or eliminate sample preparation procedures. The hydrophilic phase on the surface of the RAM particle and the small pore size restricts the access of large molecules (such as proteins) to binding sites on the inner surface of the RAM particle pores (Figure 2.2). The small analyte molecules can pass through the outer phase and bind to the inner hydrophobic pore surfaces of the RAM particle. As a result, protein molecules quickly pass through the column while compounds of interest are retained on the inner adsorptive sites. The retained compounds are released from the RAM sorbent by increasing the organic content of the mobile phase. The eluted compounds are then directed to an analytical column for further separation or directly to a detector depending on the type of RAM column and analytical system used. There are numerous RAM column manufacturers with packing materials covering a range of retention properties to suit various applications (e.g., Regis Technologies, Shodex, Merck). RAMs can be categorized into two types,[111] those with internal surface phase (ISP) materials or semipermeable surface phase (SPS) materials. In the ISP type, the outer surface of each porous packing material particle is coated with a passive non-retentive moiety while the inner surface of each pore is lined with functional groups. With SPS,

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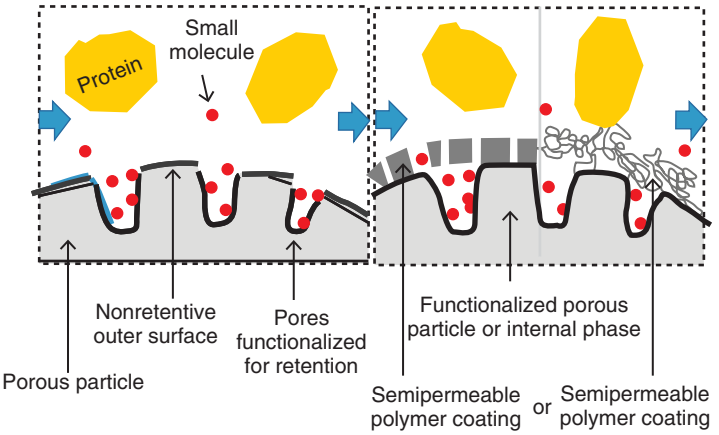


Figure 2.2 Representation of restricted access material particle. Source: Yang 2013[111]. Reproduced with permission of John Wiley.

both the internal pores and the outer surface are functionalized (Figure 2.2).[111] RAM columns can be further divided into system configurations based on single column flow-through or dual column load/back flush designs. In the former, the sample is injected onto the RAM column in a high aqueous environment which flushes large molecules (proteins) to waste, a switching valve is then activated directing flow to the detector, and the organic content of the mobile phase increases to elute the compounds of interest. This type of analysis usually employs an ISP type RAM column and requires only one pump and switching valve. A dual column load/back flush type system also loads the sample onto the RAM column in a high aqueous mobile phase and then reverses the flow to elute small hydrophobic compounds onto an analytical column for separation and subsequent detection. This approach requires two pumps and one switching valve. The use of either RAM column type can isolate and concentrate analytes in the sample. As an example, an alkyl-diol-silica C_4 load/back flush RAM column was used for the determination of benzimidazole and its metabolites in milk.[112] In this method, the sample was prepared using protein precipitation followed by centrifugation, and then 50 µl of the resulting sample was loaded onto the RAM column and back-flushed onto an analytical column for separation and subsequent detection/quantification by LC–MS/MS. Recoveries were between 82% and 117% with CCα and CCβ values of 3.3 and 5.7 µg/kg, respectively. RAM materials can also be incorporated into other off-line extraction platforms such as stir bars,[113] dispersive sorbents,[114] and SPE cartridges.[115] In the latter report, for example, Wang et al. described a novel ISP type RAM material for SPE of drugs in milk. Experiments were performed to test the ability of the RAM to both exclude bovine

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serum albumin and lysozyme proteins and retain small drug molecules. Data indicated that on average approximately 94% of the proteins were excluded by the RAM column, while recovery of the drugs was > 96% (< 16% RSD).

2.3.2.5 Nanomaterials

Nanomaterials are those consisting of nanoscale (1 – 100 nm) particles or materials with nanoscale features (e.g., pores, embedded materials). Nanomaterials have interesting chemical and physical properties compared to non-nanomaterials, and these features can be exploited to enhance chemical separation and analysis.[116]

2.3.2.5.1 Metal/Metal Oxide Nanoparticles Noble metal nanoparticles have long been known to enhance the optical detection of chemical residues based on their unique properties.[117] For example, gold nanoparticles have been incorporated into a variety of analyses and sensors to enhance sensitivity for screening of veterinary drug residues. Though chemical sensors cannot be covered in detail here, a few recent examples of signal enhancement by gold nanoparticles have been reported for electrochemical sensors for diethylstilbesterol,[118] clenbuterol,[119] and β-agonists;[120] time-resolved fluorescence immunoassay for diethylstilbesterol;[121] and surface-enhanced Raman analysis of triphenylmethane dyes[122, 123] and β-agonists.[124] Gold nanoparticles have also been used to detect fluorescence quenching in the presence of clenbuterol residues.[125]

Metal oxide nanoparticles have also been used in separations. For example, terbium oxide nanoparticles were used in a dual sample preparation and enhanced residue screening application by direct chelation of lasalocid and salicylate residues followed by spectrophotometric determination of the Tb^{3+} luminescence.[126] This application provided a detection limit of 1 μg/kg in feed and egg samples. Iron oxide nanoparticles and other paramagnetic materials have been used extensively in magnetic separations described in Section 2.3.2.5.4.

2.3.2.5.2 Graphene Graphene is a planar sheet of carbon with single atom thickness. The material has a very large surface area with a high capacity for analyte adsorption through π – π interactions and other chemistries when the material is functionalized.[116] Functionalized graphene oxide materials have been developed for veterinary drug residue adsorption with sufficient wettability for use with food matrix extract solutions.[127] Graphene oxide nanosheets were used as a dispersive sorbent to quickly adsorb malachite green and crystal violet from aqueous solutions. The addition of sodium chloride caused the sorbent to aggregate for easy removal after centrifugation.[128] Chen et al. packed graphene oxide nanosheets into SPE columns to adsorb MG and LMG from fish extracts.[129] Graphene oxide has also been bound to silica particles and used as a sorbent for dSPE to extract diethylstilbestrol (DES) and dienestrol from water samples.[130] Graphene has been used in pipette tip extraction of antibiotics from milk samples after protein precipitation with lead acetate.[131]

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2.3.2.5.3 Carbon Nanotubes Carbon nanotubes (CNTs) are composed of a graphene sheet rolled into a tube in a single-walled (SW) configuration or as two or more tubes concentrically arranged as multi-walled (MW) CNTs. Like graphene, CNTs are hydrophobic materials with many applications for analytical sample preparation.[132] CNTs have a very high surface area permitting high adsorption capacity for analyte extraction, high inner volume permitting fast flow rates, fast sorption kinetics, easily modified surfaces to functionalize the nanotubes, and high stability.[133] Several excellent reviews describe recent developments in the preparation and the numerous applications of CNTs for sample preparation.[134, 135] For veterinary drug applications, CNTs have been incorporated into SPE materials to enhance residue binding and derivatized to enhance chemical interactions. Magnetic materials have also been bound to or encapsulated within CNTs to assist analyte separation and concentration.[133, 136, 137]

Su et al. used MWCNTs to adsorb hormones from butter samples.[138] MWCNT sorbent was ground with butter in an MSPD technique, then packed into a column, and eluted with ethyl acetate. The extracts were evaporated and derivatized for GC–MS analysis, generating recoveries >85% (<10% RSD) and detection limits ranging from 0.2 to 1.3 μg/kg for the eight hormones in this simple extraction method. Du et al. used MWCNTs as a dSPE sorbent to adsorb 10 β-agonist residues from pig urine.[139] After enzymatic hydrolysis in buffer, urine samples were pH adjusted to pH 10, vortex mixed with MWCNTs for 5 minutes, and centrifuged. The supernatant was discarded and the β-agonists desorbed from the MWCNT sorbent by mixing with an acidified water–methanol solution. Samples were fortified over the concentration range of 0.2–1.0 μg/l and the method met the necessary performance requirements. MWCNTs also have been used as a dSPE sorbent to extract resorcylic acid lactone residues from pig and poultry feeds.[140] Dry feed samples were extracted ultrasonically with acetonitrile and water and then centrifuged. The supernatant was diluted with water, and MWCNT sorbent was mixed with an aliquot for 2 minutes. As before, the sample was centrifuged, the supernatant discarded, and the MWCNT desorbed by vortexing with organic solvent (ethyl acetate). Six resorcylic acid lactones were determined at fortification concentrations ranging from 1.0 to 500 μg/kg, with good analytical performance for LC–MS/MS detection. Conveniently, in the aforementioned three reports, MWCNTs were purchased from commercial sources and a few different types of materials were compared to determine the best analytical performance.

2.3.2.5.4 Magnetic Materials for Solid-Phase Extraction Magnetic SPE (MSPE) is a technique whereby a magnetic micro-/nano-sorbent material is dispersed throughout an extract to adsorb analytes. After mixing, the sorbent can be easily and efficiently collected by holding a magnet to the side of the sample tube, thus isolating and concentrating the analytes for subsequent analysis. MSPE was originally demonstrated to concentrate crystal violet and malachite green from

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urine by stirring the samples with sorbent containing an affinity ligand bound to magnetite particles.[141, 142] In recent years, dispersive magnetic sorbents have been primarily based on materials characterized as coated magnetic nanoparticles (MNPs) and CNTs filled or linked to magnetic materials. There is great variety in the synthesis and chemical properties of these materials, but the incorporation of a magnetic feature imparts a similarity in experimental procedures.[133, 143] Gao et al. developed an MSPE sorbent based on polypyrrole-coated magnetite nanoparticles and used the material to bind estrogens and stilbenes directly from diluted milk samples.[144] In this procedure, 1 ml of milk was diluted to 10 ml with pH 10 phosphate buffer and mixed with 5 mg of the MNPs. An external magnet was applied to collect and hold the sorbent on the side of the sample tube while the milk sample was discarded. The sorbent was then washed with water and the analytes desorbed by mixing the sorbent with acetone. The MNPs were removed from the solution magnetically and the eluate was evaporated and reconstituted for LC–MS/MS analysis. This method provided limits of detection ranging from 0.06 to 0.22 μg/l for the stilbenes with recoveries of 94–108% (<20% RSD) at the 0.5 μg/l spiking concentration. Oleic acid-coated MNPs were prepared to extract leucomalachite green from carp.[145] In this method, fish muscle was first homogenized with McIlvaine buffer (pH 3) and acetonitrile, and the supernatant collected from the centrifuged extract. The magnetic sorbent was mixed with the extract and diluted with sodium chloride solution adjusted to pH 10. The MNPs were separated magnetically, washed, pH adjusted, and then eluted with acetonitrile. LMG in the final concentrated extract was oxidized to MG for detection by HPLC with diode array detection. Recoveries for spiked LMG residues over the range of 0.2–2 μg/kg were >80%, and analysis of positive samples yielded comparable results compared to a standard liquid–liquid extraction method. This research group applied a similar technique to extract clenbuterol from pork samples.[146] In that case, oleic acid- and undecylenic acid-coated magnetite nanoparticles were derivatized with a sulfonated polystyrene copolymer. Pork muscle was homogenized with acid and centrifuged. Magnetic sorbent was mixed with the neutralized extract. Following magnetic separation, washing, elution, drying, and reconstitution, clenbuterol was detected with a gold nanoparticle immunochromatographic assay with 93–98% recovery (RSD ≤ 13%) at 0.25–1.0 μg/kg concentrations. The sulfonated MNPs were also found to adsorb salbutamol, ractopamine, cimaterol, and several other β-agonists.[146]

MNPs have also been used to assist liquid phase separation by adsorbing a micro liquid extraction phase that contained the analytes of interest. For example, Li et al. used barium ferrite MNPs to bind an ionic liquid phase containing pyrethroids extracted from diluted honey samples.[147] Acetonitrile was used to desorb the ionic liquid and pyrethroids from the MNPs for subsequent analysis. In another method, diatomite-bound maghemite MNPs were used to capture an anionic surfactant phase containing malachite green residues extracted from fish.[148] The surfactant–nanoparticle conglomerate was collected

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magnetically, the surfactant layer was separated with ethanol and ultrasonic mixing, and MG was measured spectrophotometrically directly in the surfactant.

Carbon-based nanomaterials have been modified with MNPs to generate new materials for sample extraction. Magnetic sorbent coated with graphene oxide and titanium dioxide was used in a microfluidic device to concentrate estrogens from milk samples prior to HPLC detection.[149] A magnet was used to fix the material in the narrow polymethacrylate channels of the device, and the high adsorption capacity of graphene oxide assisted the adsorption process. Ding et al. combined CNTs with MNPs to prepare a new sorbent consisting of an aggregated tangle of the two materials, described as a magnetic nano/micro carbon composite.[150] The composite was used to bind estrogens from milk extracts (initially acidified, diluted, and centrifuged) and magnetically separate the residues for analysis. With this magnetic composite, hexestrol at a concentration of 0.020 μg/l was extracted with 98% recovery (9% RSD, interday) with an LOQ of 0.007 μg/l.[150]

MNPs have been derivatized with molecular recognition features for selective extraction. Hu et al. incorporated magnetite particles into a MIP polymerization process to create a magnetic β-agonist selective sorbent.[151] This was used to extract ractopamine and other compounds from pork muscle and liver extracts with 0.5–1.0 μg/kg detection limit and 80% or higher recovery. A novel MNP sorbent was prepared incorporating a molecular recognition feature for metronidazole.[152] Rather than in typical MIPs where the molecular template is embedded and distributed within a polymer network, the sol–gel process used in this preparation resulted in binding sites to be imprinted on the surface of silica-coated MNPs for greater accessibility of the target analyte and faster mass transfer. Using this sorbent, metronidazole (17–170 μg/l) was extracted from milk and honey samples with >85% recovery.

Commonly used SPE materials have also been incorporated into MNPs. Silica-coated magnetite nanospheres were functionalized with methacrylic acid–ethylene glycol dimethacrylate copolymer to extract residues of benzimidazoles and metabolites from pork muscle and liver.[153] Recoveries were >80% (<15% RSD) and detection limits 1–10 μg/kg. Magnetic silica nanospheres have also been derivatized with C_{18}/C_8 functionality to extract phenicol drugs from fish extracts.[154] Reyes-Gallardo et al. tested the combination of embedding cobalt ferrite nanoparticles into a sulfonated polymeric network of several commercially available sorbent copolymers to prepare magnetic sorbents for residue analysis.[155] Final testing was based on the OASIS® MCX material for extraction of nitrophenols from aqueous samples. The potential development of magnetic sorbents based on polymeric sorbent materials commonly used for veterinary drug residue extraction may provide exciting future applications.

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2.4 Micro-extraction Techniques for Solvent and Sorbent Extraction

Liquid and solid micro-extraction techniques are used to remove analytes of interest from sample extracts and concentrate them into volumes of 100 µl or less. These techniques find applications in veterinary drug residue analysis as targeted residues are typically in the low µg/kg concentration range. Larger volumes of extraction solvents are commonly used in veterinary residue work to ensure thorough mixing of tissue portions so that a representative quantity of drugs can be extracted. With micro-extraction, analytes of interest can be transferred from the bulk extract into a much smaller volume of an immiscible solvent that is withdrawn from the bulk sample for analysis or into a solid support such as an SPME fiber, stir bar coating, or a carbon nanotube for subsequent analysis.

2.4.1 Solvent Micro-extraction

Solvent micro-extraction has been used to extract and concentrate samples of veterinary drug residues and other contaminants from animal matrices as well as a variety of other food, beverage, and environmental samples. Methods are usually divided into two categories where the extracting solvent is either in direct contact with a bulk liquid sample or protected by a membrane.[156] Exposed-solvent methods are further divided into two general categories where analytes are extracted into a small volume of immiscible solvent that is either suspended as a droplet into a sample (e.g., single drop micro-extraction (SDME)) or highly dispersed through the sample and later condensed (e.g., dispersive liquid–liquid micro-extraction). Membrane-protected techniques are based on extracting analytes into the inner cavity (lumen) of a hollow fiber (hollow fiber micro-extraction). Recent examples of these micro-extraction techniques applied to veterinary drug residue extraction are provided.

2.4.1.1 Single Drop Micro-extraction

In single drop micro extraction (SDME), an aqueous sample or sample extract is stirred, while a droplet (e.g., 1–2 µl) of an immiscible solvent is suspended from the tip of a syringe needle into the aqueous phase. After an exposure period, the solvent droplet is pulled back into the syringe needle where it can subsequently be dispensed for instrumental analysis. Sekar et al. extracted monensin from a 20 ml urine sample by suspending a 1.5 µl droplet of chloroform/toluene from a syringe.[157] Raterink et al. demonstrated that crystal violet could be extracted from an aqueous solution into a single drop using a three-phase electroextraction procedure.[158] Crystal violet was in the lower aqueous solution (donor phase), an immiscible organic solvent chemical filter solution was layered on top, and an aqueous droplet (acceptor phase) was suspended into the organic layer from a conductive pipette tip. An electric field was applied between the donor and acceptor solution to drive crystal violet through the organic phase and into the

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acceptor droplet. Williams et al. noted that SDME produced variable results when volatile solvents were used as the acceptor and microbubbles formed in the suspended drop.[159] They introduced a technique to deliberately form a large bubble within a suspended solvent drop, named bubble-in-drop (BID) SDME, and found that analyte enrichment and sensitivity were enhanced by the increase in surface area of the inflated droplet. BID-SDME was applied to the determination of stilbenes in urine from cattle.[160] In this method, 5% sodium chloride was added to diluted urine and the sample was pH adjusted to 3.5. A 5 µl micro syringe was loaded with 1 µl of a 3:1 chloroform/toluene solvent mixture, and then the plunger pulled up to collect a 0.5 µl air bubble. The syringe was inserted into the aqueous sample and the plunger depressed to form an air-filled droplet submerged in the aqueous sample. After 20 minutes static equilibration, the solvent droplet was retracted into the syringe and directly injected into a GC–MS. The method yielded linear results for DES and hexestrol from 0.05 to 10 µg/l, with reported LODs of 0.03 µg/l and below.[160]

2.4.1.2 Dispersive Liquid–Liquid Micro-extraction

Dispersive liquid–liquid micro-extraction (DLLME) is a technique where a micro volume of a water-immiscible extraction solvent is mixed with a water-soluble disperser solvent, and then the mixture is rapidly injected via syringe into an aqueous sample to form an emulsion. The sample is centrifuged and the nonsoluble extraction solvent sediments as a droplet that can be withdrawn by syringe for analysis. Traditional DLLME works best for less polar analytes, as they favorably partition into the hydrophobic extractant phase. Macrocyclic lactones were extracted from milk and infant formula samples with DLLME after protein precipitation with aqueous TCA.[161] In this method, the aqueous supernatant was separated from the milk solids, blended with sodium chloride, and diluted with water (10 ml volume). A mixture of 200 µl chloroform (extraction solvent) in 2 ml acetonitrile (dispersion solvent) was rapidly injected into the aqueous sample. The emulsion was mixed and centrifuged, and the chloroform extract was withdrawn from the bottom of the tube with a syringe for subsequent evaporation, reconstitution, and analysis of the avermectins and moxidectin compounds by LC–MS. Analyte enrichment factors ranged from 65 to 200, with analyte recoveries between 90 and 105% and detection limits below 1 µg/kg. Honey analysis is also amenable to DLLME where sample preparation of honey samples often includes dilution with water. Yang et al. extracted organophosphorus pesticide residues from diluted honey into 30 µl of chlorobenzene, which was removed for GC analysis.[162] The method relied on vortex mixing and addition of Triton X-114 surfactant to enhance the emulsification in the viscous honey mixture.

Veterinary drug residues are usually extracted from tissue and fluid matrix into nonaqueous solvents. DLLME has also been used as a technique to purify and concentrate drug residues prior to analysis. For example, stilbenes were extracted from canned food products (i.e., meats, beverages) using a QuEChERS extraction and then concentrated by DLLME.[163] In this case, 2 ml of the acetonitrile-based

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QuEChERS extract was used as the dispersant and mixed with 200 µl of carbon tetrachloride as the extractant. The mixture was rapidly dispersed into a 5 ml aliquot of water, then sonicated to increase the emulsion, and centrifuged to sediment the stilbene-rich carbon tetrachloride phase. Ju et al. performed a DLLME sample clean-up of fish extracts for triphenylmethane dye analysis with a fluorescence detection method.[164] Shrimp and carp muscle were extracted with acetonitrile and alumina. A portion (2 ml) of the extract was reduced with sodium borohydride to convert the cationic dyes malachite green and crystal violet to their lipophilic leucobases. Extraction solvent (400 µl chloroform) was mixed directly with the acetonitrile extract (dispersion solvent), immediately followed by rapid addition of a 5 ml aliquot of acetate buffer to form the emulsion. The mixture was centrifuged and the leucomalachite green and leucocrystal violet analytes were deposited in the bottom of the tube in the dichloromethane phase, which was withdrawn for analysis. Alshana et al. applied this technique to clean up and concentrate NSAID residues from acetonitrile extracts of milk, yogurt, and cheese, by mixing a portion of the acetonitrile with chloroform and rapidly injecting the mixture in water.[165]

DLLME can also be performed using lower density extraction solvents that do not sediment or easily disperse in the aqueous sample extract, but rather float on top of the sample. In some cases, the extracting solvent is a solid at low temperatures enabling a solid droplet to be easily removed from the sample surface when samples are chilled. For low-density solvents, rapid dispersion of the disperser–extractant mixture will not form an emulsion throughout the sample, and it is necessary to assist the emulsification by adding surfactant or applying ultrasonic or vortex mixing techniques to ensure there is adequate contact between the immiscible phases for analyte partitioning.[166]

DLLME was used with surfactant to concentrate benzimidazole analytes from milk extracts.[167] Fat and proteins were precipitated from milk samples by mixing samples with zinc sulfate and then adding acidified acetonitrile. Samples were centrifuged and filtered, evaporated, and redissolved in water before DLLME with 1-octanol as the extraction solvent and acetonitrile modified with 0.5% Triton X-114 emulsifier as the disperser. The method yielded residue concentration factors of 21–38 for mebendazole, albendazole, and fenbendazole with limits of detection below 10 µg/l and residue recovery greater than 80%.

These researchers applied a similar technique with ultrasound-assisted emulsification rather than surfactant emulsification to the extraction of benzimidazole residues from egg samples.[168] Fats and proteins were removed from homogenized egg using a QuEChERS-like extraction with acidified acetonitrile and magnesium sulfate, followed by dichloromethane extraction, centrifugation, and filtration. Sample filtrates were diluted with water, and the organic solvent was removed by evaporation. DLLME was achieved by rapidly injecting 1-octanol extraction solvent in methanol disperser into the aqueous solution. Samples were ultrasonicated to enhance the extraction solvent dispersion and then centrifuged to collect the extractant phase for subsequent HPLC-DAD analysis. Using this technique,

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four benzimidazoles were recovered from chicken and duck egg samples with a limit of detection below 15 µg/kg and accuracy in the range of 74–112% with <12% RSD. Estrogenic compounds were extracted from deproteinated, salt saturated, milk samples using 2-dodecanol as the extractant and nitrogen gas bubbling through the mixture to assist equilibration.[169] Dodecanol was solidified at low temperature and collected after removing the aqueous phase and salts from the bottom of the sample tube.

Ionic liquids (IL) have also been used as extraction solvents in DLLME experiments.[51] For example, 60 µl of 1-octyl-3-methylimidazolium hexafluorophosphate [C_8MIM][PF_6] extractant was dispersed with 200 µl of methanol into a 10 ml aqueous solution of honey to extract pyrethroid compounds.[170] The ionic liquid dispersion was assisted with ultrasound, and centrifugation was used to sediment the ionic liquid (IL) phase. The recovery of four pyrethroid compounds was 101–103% (<6% RSD) within the 1–200 µg/l calibration range, and LOD was reported as 0.2–0.4 µg/l. Crystal violet was extracted from salmon extracts using a dispersed ionic liquid extractant. In this method, 1-hexyl-3-methylimidazolium hexafluorophosphate [Hmim][PF_6] and 1-hexyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide [Hmim][Tf_2N] were dispersed in the aqueous extract by shaking samples warmed to 35 °C for 10 minutes.[171] In this system, [Hmim][Pf_6] was the extracting solvent and the presence of [Hmim][Tf_2N] served to increase the hydrophobicity of the [Hmim][Pf_6]. Once the ILs were well dispersed, the sample tubes were placed in an ice water bath for 10 minutes, reducing the solubility of the ILs and creating a cloudy solution. The samples were centrifuged to sediment the IL for removal and spectrophotometric analysis. In another technique, based on foaming properties of ILs, steroid hormones were extracted from water samples by forcibly bubbling nitrogen into a 160 ml aqueous sample to which 20 µl of 1-ethyl-3-methylimidazolium tetrafluoroborate [EMIM][BF_4] ionic liquid had been dispersed as the extracting solvent. The bubbling caused the analyte-rich IL to foam out of the reactor, through tubing to deposit onto an SPE cartridge, from which the analytes were eluted and then analyzed.[172] This technique was also applied to yogurt and milk samples.[173, 174]

2.4.1.3 Hollow Fiber Micro-extraction

Hollow fiber micro-extraction (HFME) is a method based on permeating a hollow fiber with a solvent to establish a supported liquid membrane within the pores of the fiber. The membrane is immersed in a nonimmiscible solvent containing the sample, and analytes are extracted into the fiber through the membrane. In a two-phase system, the fiber is filled with the same solvent contained in the pores of the fiber membrane. In a three-phase system, the fiber is filled with a third solvent, such that the solvent creating the liquid membrane in the fiber walls is immiscible with both the inner and outer solvents. Following system equilibration, analytes can be withdrawn from within the hollow fiber by syringe. Antifungal drugs clotrimazole and miconazole residues were concentrated from milk extracts using

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n-dodecane as the extracting phase (supported liquid membrane on the fiber) and acetonitrile as the acceptor solvent filling the lumen of the fiber.[175] HFME has also been used to extract estrogens and stilbenes from milk samples[176] and fish extracts.[177] NSAIDs were extracted from an aqueous fish slurry (pH 2) through a di-n-hexyl ether membrane into a fiber lumen containing carbonate buffer acceptor solvent at pH 9.[178] The extraction process can be accelerated by inserting an electrode into the fiber and applying a potential for electromembrane extraction (EME), as demonstrated by Ramos-Payán et al. for NSAID extraction.[179] Fibers modified with silver nanoparticles were found to enhance the electrical transport process through the fiber.[180]

Carbon nanomaterials have been incorporated into hollow fiber extraction techniques as well. Graphene dispersed in 1-octanol was used as the acceptor phase in a sealed fiber to extract phenylurea herbicides from milk samples.[181] Hollow fibers have also been modified with nanomaterial sorbents in a hybrid solid–liquid-phase micro-extraction technique. For example, CNTs were embedded within the pores of a hollow fiber to selectively extract DES directly from milk samples.[182] A similar technique was applied to extract NSAIDs from water samples.[183] In these methods, the nanotubes in the fiber serve as a solid-phase sorbent while the lumen of the fiber is filled with liquid-phase 1-octanol to assist analyte transfer to the nanotubes via liquid–liquid extraction. Es'haghi et al. filled the lumen of a fiber with a carbon nanotube 1-octanol dispersion and sealed the ends of the fiber with magnetic stoppers.[184] The device was then operated as a stir bar to extract brilliant green residues from fish pond water. A carbon nanotube 1-octanol technique was combined with EME for NSAID extraction from biological samples.[185]

2.4.2 Sorbent Micro-extraction

Solid sorbent-based micro-extraction techniques have been developed to miniaturize or incorporate small extracting phases into a variety of formats.[49] In some techniques, small portions of common SPE sorbents have been used to adsorb and remove analytes from bulk solutions for elution with microlitre volumes of solvents to minimize long evaporation times when concentrating extracts in traditional SPE. Pipette tip solid-phase extraction (PT-SPE), micro-extraction by packed sorbent (MEPS), and dispersive micro SPE (d-μ-SPE) techniques are examples of this approach. In other sorbent micro-extraction techniques, analyte adsorption occurs onto modified solid extracting surfaces including rods, fibers, monoliths, stir bars, thin films, membranes, and fabrics. Several reviews have been recently published on these topics.[186, 187] With some of the techniques amenable to 96-well plate simultaneous sample processing and/or automated analysis, sorbent micro-extraction has applications for rapid sample analysis as well.[65, 188] A few examples of sorbent-based micro-extraction applied to veterinary drug residue analysis are described in the following.

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2.4.2.1 Solid-Phase Micro-extraction

Solid-phase micro-extraction (SPME) is a highly developed extraction technique, with wide ranging research and numerous examples for environmental and biological analysis.[189, 190] In this technique, an SPME fiber is exposed to an analytical sample and analytes partition and concentrate into the coating of the fiber for later desorption and analysis. For example, Du et al. extracted β-agonist residues from pork muscle and liver with acetonitrile, then evaporated the solvent, redissolved the residues in phosphate buffer at pH 10, and immersed a polydimethylsiloxane/divinylbenzene fiber in the extract for 20 minutes.[191] Desorption of the fiber into acidified methanol and analysis by HPLC yielded recoveries of 80% and above (<10% RSD) with LODs ranging from 0.05 to 0.1 μg/l for clenbuterol, ractopamine, and salbutamol.

Recent applications for veterinary drug residue analysis include using monolithic polymer fibers for higher capacity adsorption and/or incorporating molecular recognition or nano features into the SPME fiber.[49] In-tube and in-tip SPME techniques have also been developed to increase adsorption capacity and automate the SPME process.[192] A silica SPME fiber coated with a 17β-estradiol template in methacrylate polymer as a MIP was used to extract estrogenic compounds from acetonitrile extracts of fish and shrimp.[193]

In other research, a methacrylic acid and ethylene glycol dimethacrylate copolymer monolith fiber was developed to extract 10 benzimidazole residues from extracts of milk, egg, chicken, and pork.[194] The recoveries ranged from 75% to 117% (<15% RSD), and detection limits ranged from 0.1 to 3 μg/kg for the different matrices with LC–MS analysis. A similar copolymer monolith was used to extract benzimidazole compounds from milk and honey samples for HPLC-DAD analysis.[195] This research group also developed a monolithic SPME method for extraction of stilbenes from milk samples using a fiber bundle of four monoliths made from an ionic liquid 1-allyl-3-methylimadazolium bis(trifluoromethylsulfonyl)imide and ethylene dimethacrylate copolymer.[196] The fiber bundle was immersed in a deproteinated, diluted milk sample with gentle stirring for 30 minutes. Extracted residues were desorbed into a small volume of methanol for analysis by HPLC-DAD. The method resulted in a 70–93% recovery (<8% RSD) of DES, dienestrol, and hexestrol residues from milk fortified at a concentration of 1 μg/l and a LOD of 0.1–0.3 μg/l.

2.4.2.2 Stir Bar Sorptive Extraction

Similar to SPME, stir bar sorptive extraction (SBSE) is based on immersing a solid support into a sample and partitioning analytes from the sample into the support. For SBSE, the support is the stir bar that mixes the sample and the analytes adsorb into the stir bar coating. Because the stir bar coating is thicker than that of an SPME fiber, SBSE typically provides higher extraction capacity. Commercial SBSE devices (Twister®, GERSTEL GmbH & Co. KG) are based on coatings with polydimethylsiloxane (PDMS), which have been widely used to extract less polar analytes, and newer coatings of polyethylene-glycol modified silicone and

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polyacrylate designed for polar analytes.[197] Larger surface area stirring disks and other devices (e.g., stir cakes) with an embedded magnet or devices intended to float in a stirred solution have also been introduced for sorptive extraction. As with SPME, major SBSE advances are in the expansion of coating materials to increase the capacity of adsorbed analytes and enhance partitioning.[197, 198]

Huang et al. have developed several polar polymeric monolithic coatings for SBSE including poly(methacrylic acid-3-sulfopropyl ester potassium salt-co-divinylbenzene) for nitroimidazole residues in honey[199] and poly(vinylphthalimide-*co*-*N*,*N*′-methylenebisacrylamide) for benzimidazoles in milk and honey.[200] In these experiments, SBSE was performed directly in diluted aqueous milk and honey samples without initial protein precipitation or defatting. Hu et al. developed stir bars by modifying PDMS with β-cyclodextrin and divinylbenzene in a sol–gel process to extract stilbene residues from aqueous-diluted acetonitrile extracts of pork and chicken.[201] To improve the extraction kinetics for stilbene and estrogen extraction, these researchers later developed a porous PDMS coating based on metal-organic frameworks that permitted lower limits of detection.[202] Fan et al. prepared ionic liquid bonded stir bars for the extraction of NSAIDs from milk and urine samples.[203]

MIPs have been incorporated in the SBSE coatings to extract β-agonist residues from pork, liver, and feed samples[204] and for DES and other estrogens from environmental samples.[205] Recently, Qiao et al. developed a sorptive extraction device based on an array of eight screen-printed electrodes that were coated with molecular imprinted film sheets bound to titania-coated magnetite nanoparticles.[206] This complex device permitted a rapid (15 minutes) and simple extraction of DES from pork and chicken extracts (acetonitrile extracts, evaporated and diluted in salt water) with >80% recovery (<8% RSD) and 0.5 μg/l detection limit with HPLC analysis.

2.4.2.3 Fabric Phase Sorptive Extraction

Fabric phase sorptive extraction (FPSE) was recently introduced as an SPE technique using a sol–gel sorbent incorporated into a small swatch of fabric as an extraction substrate.[207] FPSE was demonstrated for the extraction of NSAIDs from environmental waters using a polyethylene glycol sorbent on cotton substrate.[208] Karageorgou et al. prepared FPSE sorbents for a simple extraction of sulfonamide antibiotics directly from untreated milk samples.[209] In the extraction of milk, a 5 cm^2 piece of FPSE material was placed in a vial with 1 g of milk for 30 minutes with stirring. The FPSE material was removed and soaked in subsequent portions (250 μl) of methanol, then acetonitrile, for a total of 13 minutes elution time. The eluate was then filtered prior to HPLC–UV analysis. The method yielded accuracy and precision of greater than 90% recovery and <7% RSD.[209]

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2.5 Emerging Techniques in Liquid Chromatography

In the earlier companion volume,[1] fundamentals of LC were reviewed with an emphasis toward antibiotic residues in food. In particular, the advantages and application of newer types of column stationary phases including ultrahigh performance liquid chromatography (UHPLC) and hydrophilic interaction liquid chromatography (HILIC) were summarized. In this chapter, the focus is on innovations in LC columns and instrumentation with applications to methods for non-antibiotic animal drug residues.

2.5.1 Ultrahigh Performance Liquid Chromatography

The use of ultrahigh performance Liquid Chromatography (UHPLC), characterized by LC columns packed with sub-2 μm particles, has become standard laboratory practice.[210, 211] The first commercial instrument able to effectively operate at the higher pressures required by UHPLC (> 10,000 psi) was introduced in 2004 by Waters Corporation; today, UHPLC pumps are available from many vendors. The advantages of UHPLC have been reviewed and include more efficient separation, faster analysis times, and increased analyte sensitivity (better signal-to-noise ratio). Multi-class, multi-residue veterinary drug residue MS methods often utilize UHPLC separation.[4, 212, 213] Combined with selective MS data acquisition, UHPLC allows for the monitoring of many residues (>100) with an analysis time of 15 minutes or less. Although many of the compounds in these methods are antibiotics, anthelmintics, coccidiostats, NSAIDs, and thyreostats have been monitored as well. One reason for the successful expansion of UHPLC in residue testing laboratories has been the continued development of detectors (e.g., diode array, triple quadrupole, and time-of-flight MS[213] instruments) with fast enough data acquisition to adequately define narrow chromatographic peaks. Recent developments in UHPLC consist of increasing separation efficiency with elevated temperature, longer columns, and even smaller particle sizes. However, logistical challenges including the need for higher pressure pumps and carefully controlled temperature zones remain before these innovations can be put to practical use. UHPLC columns have also expanded beyond reversed-phase separation to additional types of chemistries such as chiral, HILIC, and size exclusion stationary phases.[210]

2.5.2 Core–Shell Columns

The use of LC columns packed with core–shell particles as an alternative to UHPLC columns is a trend that has grown significantly in recent years.[211, 214, 215] Core–shell LC particles consist of a solid core of silica surrounded by a porous chemically modified shell 0.2–0.8 μm thick. They can be manufactured with a very narrow size distribution range, typically 2.6–2.7 μm in diameter, although variations can range from 1.7 to 4.6 μm. These homogenous particles provide

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excellent separation efficiency because they can be packed efficiently, which minimizes chromatographic band broadening. The smaller volume of porous material also reduces the amount of partitioning time and analyte diffusion, and the columns can also tolerate higher flow rates than those packed with completely porous particles. The result is that core–shell LC columns can provide similar separation power as UHPLC with significantly lower back pressures. They may also be less likely to become obstructed with particulates as compared to UHPLC columns.

As core–shell columns have become more popular, many choices in manufacturers, stationary-phase modifications, and column dimensions have become available. One example of core–shell column separation is for the analysis of nitroimidazole residues by LC–MS/MS in several animal matrices.[216] In this method, five nitroimidazoles were separated in less than 3 minutes using a XB C_{18} core–shell column with protective butyl side chains designed to separate basic compounds and an isocratic methanol/0.1% formic acid mobile phase. Analysis of 20 coccidiostats at residue (carryover) concentration in animal feeds was achieved with a C_{18} core–shell LC column.[217] Core–shell columns are also being routinely utilized for multi-residue LC–MS/MS veterinary drug methods. One method describes the analysis of corticosteroids, anabolic steroids, and basic NSAIDs in milk and animal tissue by LC–MS/MS using a C_{18} core–shell column.[218] A rapid (8 minutes) ammonium acetate/acetonitrile gradient was capable of separating most of these analytes, but an extended (16 minutes) separation was needed to separate dexamethasone from its isomer betamethasone. Schneider et al.[9] used a C_{18} core–shell column and 0.1% aqueous formic acid/0.1% acetonitrile gradient for the rapid (10 minutes) separation of 131 residues representing 13 classes of veterinary drugs including thyreostats, β-agonists, coccidiostats, anti-inflammatories, and anthelmintics, in addition to many types of antibiotics.

2.5.3 Hydrophilic Interaction Liquid Chromatography

Hydrophilic interaction liquid chromatography (HILIC) has become a viable alternative for compounds that are too polar to retain on a reserved-phase stationary column, and this technology has been the subject of previous reviews.[1, 211, 219] Briefly, HILIC columns have a polar stationary phase (bare silica or silica with a polar bonded phase) that adsorbs water. Polar compounds will partition between this surface water layer and the LC mobile phase which consists of a mixture of acetonitrile/methanol and aqueous buffers. The mobile phase gradients with HILIC typically go from higher organic to more aqueous in order to elute more polar compounds. The chromatographic partitioning mechanisms with HILIC are complicated with hydrogen bonding, dipole–dipole, and/or ion-exchange interactions, so careful control of the pH and ionic strength of the mobile phase can be critical. Newer variations of HILIC include zwitterionic stationary phases, UHPLC, core–shell, and monolithic column formats.[219] A classic example of

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HILIC in drug residue analysis is for aminoglycoside antibiotics.[1] An example of a non-antibiotic animal drug residue method using HILIC chromatography is for the analysis of amprolium,[220] a quaternary ammonium salt effective for treating coccidiosis in poultry. In this study, a core–shell HILIC column and an isocratic mobile phase consisting of acetonitrile/50 mM (pH 4) ammonium formate buffer were used for the analysis of amprolium residues in eggs, feed, and chicken muscle with a triple quadrupole MS detector. Amprolium was also part of a multi-residue method that separated antibiotic residues isolated from chicken muscle with zwitterionic HILIC prior to MS/MS analysis.[221] Residues of carbadox and olaquindox in feed have been analyzed using a rapid (<2 minutes) UPHLC HILIC separation coupled to photodiode array detection.[222] A HILIC method developed for low μg/ml concentrations of NSAIDs in human plasma samples may have applications to residues in food.[223] In this LC–MS/MS method, a HILIC column with an aliphatic aminopropyl group bonded to silica and an acetonitrile/ammonium acetate buffer gradient separated 13 NSAIDs in approximately 3 minutes.

2.5.4 Other Emerging LC Techniques

A few additional emerging technologies in chromatography that are worth mentioning include monolithic columns[224] and microflow LC.[225] Although these technologies have not been tested extensively for the analysis of veterinary drug residues in food, they may have applicability in future analytical method development. The fact that LC using monolithic stationary phases or columns operating at low flow (5–200 μl/minute) can have significant advantages in terms of separation efficiencies and increased MS detection signal has been known for some time. Updated technologies, in terms of both column manufacturing processes and instrument detection capabilities, have led to a renewed interest in these techniques. Monolithic columns have a stationary phase comprised of a continuous piece of material rather than individual particles. They can be based on organic or inorganic (silica) polymers. The advantages, in terms of separation efficiency and permeability, are similar to those seen with core–shell LC columns. Applications of polymer monolithic columns to food analysis, including the analysis of antibiotic residues, have been reviewed.[1, 224]

Microflow LC combined with electrospray MS has the primary advantage of increasing sensitivity by generating smaller droplets with higher analyte ion concentration at the source interface.[225] More efficient UHPLC, core–shell, and monolithic LC columns allow for efficient separation of low concentrations of compounds at these flow rates. The increased sensitivity can also allow food extracts to be diluted further, which can significantly reduce matrix effects commonly seen with electrospray MS. A significant reduction in organic solvent use is also an advantage. Microflow LC has been applied to the analysis of over 90 pesticide residues in several food matrices.[225] Recently, the analysis of anabolic steroids in beef muscle using microflow LC combined with quadrupole

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time-of-flight MS detection was investigated by Vanden Bussche et al.[226] This group found that microflow LC could significantly increase the sensitivity for zearalanol compounds as compared to UHPLC when using electrospray ionization (ESI). However, estrogens did not respond well by ESI and required analysis using APCI with higher LC flow rates.

2.6 Direct Mass Spectrometry Analysis of Sample Extracts

In addition to emerging techniques allowing improvements in liquid chromatographic separation for mass spectrometric analysis, some researchers have departed from LC separation altogether and are advancing direct analysis approaches for veterinary drug residue analysis. Advances have been made by directly infusing sample extracts into a mass spectrometer for flow injection analysis and by a variety of mass spectrometry techniques featuring unique analyte desorption and ionization mechanisms. While complex food matrix analysis is expected to require some sample pre-treatment, direct analysis MS techniques all offer advantages of obtaining analytical results in the order of seconds, and when using automated systems, the corresponding benefit of screening hundreds of sample extracts in a short period of time.

2.6.1 Flow Injection Mass Spectrometry

Flow injection mass spectrometry (FI-MS/MS) was introduced in 2009 by Nanita et al. for the analysis of sulfonylurea herbicides and carbamate insecticides in water, corn, lemon, and pecan samples.[227] In this technique, Nanita et al. used a 1 m section of PEEK tubing with 0.13 mm inner diameter to connect the autosampler from a chromatography system directly to the ESI inlet of a triple quadrupole mass spectrometer. Sample extracts (1 µl injections) were analyzed at a rate of one sample every 65 seconds with a limit of quantification of 10 µg/kg. FI-MS/MS was applied to residue testing in animal products as well, where egg, milk, and beef samples were extracted with ACN and an aqueous solution of ammonium chloride to assist with phase separation, prior to centrifugation and filtration.[25] Extracts were diluted with ammoniated methanol and injected into a carrier of methanol mobile phase. Samples (1 µl) were directly injected into the electrospray source of a triple quadrupole mass spectrometer for analysis. Recoveries of the pesticides tested were typically greater than 80% in beef, milk, and egg with LOQs of 10–50 µg/kg in these animal matrices.

Mol and van Dam applied the technique to the direct analysis of very polar pesticides (cyromazine, fosetyl, paraquat, etc.) that are poorly extracted with ACN in a variety of agricultural and animal products, including milk and pig kidney.[228] Samples were extracted with water or acidic water and subjected to a variety of clean-up methods including dSPE, LLE, and ion-exchange cartridge

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clean-up. A simple dilution of the aqueous extract and filtration were comparable to the results achieved with more elaborate clean-up methods. Lehotay et al. applied FI-MS/MS to the analysis of veterinary drug residues in muscle and kidney from cattle.[229] Samples were initially extracted with acetonitrile and water and then supernatants filtered using filter vials with and without dSPE with C_{18}. Extracts were injected into a flow of formic acid-acidified aqueous acetonitrile through 1 m of 0.25 mm i.d. fluoropolymer tubing directly connected to the ESI source of a mass spectrometer. The veterinary drugs ($n = 135$) analyzed in the study included antibiotics as well as those from anthelmintic, flukicide, tranquilizer, NSAID, nitroimidazole, β-agonist, growth promoter (zilpaterol), thyreostat, and corticosteroid drug classes, including drugs such as ractopamine, carbadox, and MGA.

2.6.2 Direct Desorption/Ionization Mass Spectrometry

Ambient ionization techniques are based on a direct desorption and ionization of analytes from the surface or bulk of a sample. By definition, ambient ionization mass spectrometry should permit direct ionization of an unprepared sample.[230] For example, pesticide and fungicide residues have been identified from direct surface analysis of fruit and vegetable peels[231, 232] and from swabs of produce surfaces.[233] While pesticide residues typically are concentrated on the outer surface of agricultural products, veterinary drug residues are dispersed in trace concentration throughout the complex sample matrix. For this type of analysis, as is true for traditional LC–MS procedures, sample preparation is required to remove biological matrix components and/or to concentrate residues to render a sample suitable for the direct analysis.

Although extensive sample preparation defeats the intent of true ambient mass spectrometry, researchers have successfully applied ambient-like MS techniques to the analysis of veterinary drug residues in prepared sample extracts. We will include in our discussion techniques that permit either direct ionization under ambient conditions or desorption followed by ionization of extracted samples. While it may be improbable to consider direct analysis MS techniques to provide screening capability for hundreds of veterinary drug residues in a single sample as is the trend for current LC–MS/MS and LC–HRMS analytical methods, there is no doubt that analytical methods providing compound detection in the order of seconds can be useful for sample screening.[234]

Direct desorption/ionization MS methods are often divided into categories based on whether the desorption/ionization process is related to atmospheric pressure chemical ionization (APCI), where ionization results from interaction with a plasma created from an electrical discharge, or related to ESI, where a charged solvent spray is directed at a sample to desorb and ionize analytes.[235, 237] The two most well-known ambient ionization techniques are the APCI-related technique of direct analysis in real time (DART) and the ESI-related technique desorption electrospray ionization (DESI). Direct MS techniques have expanded

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rapidly since their introduction, and novel sources, advances, and applications are regularly developed with a range of reported desorption and ionization mechanisms. In addition to DART and DESI, contributions have been made to the literature for direct MS analysis of veterinary drug residues in sample extracts with desorption/ionization via atmospheric pressure solids analysis probe (known as ASAP), laser diode thermal desorption (LDTD), and paper spray (PS) ionization sources. Applications of these and other APCI- and ESI-related techniques are described in the following sections.

2.6.2.1 APCI-Based Techniques

2.6.2.1.1 Direct Analysis in Real Time (DART) DART-MS has been used to evaluate a wide range of analytes in various samples. In the DART source, a glow discharge plasma is created in a gas supply, resulting in a mixture of ions and metastable species that is heated and directed at a sample (Figure 2.3).[237, 238] The DART ion source can be directed at unprepared samples with many shapes, sizes, and physical properties, as well as used with various autosamplers designed for prepared liquid and solid samples.[239] Though there are many applications for food analysis using DART-MS,[236] reports of veterinary drug residue analysis are limited. Martínez-Villalba et al. validated a quantitative method for DART with high-resolution MS (single-stage Orbitrap™) analysis of anti-parasitic compounds including benzimidazoles in milk and coccidiostats in feed.[240] Both types of samples were prepared by a QuEChERS type process with acetonitrile (ammoniated for milk) extraction, salt-induced partitioning, and dSPE clean-up with magnesium sulfate, C_{18}, and PSA sorbents. Prepared extracts (5 µl) were sampled from glass rods in a Dip-It tip autosampler with a reported desorption time of 10 seconds. Detection of benzimidazole and coccidiostat compounds was made by accurate mass measurement of protonated or deprotonated molecular ions (sodium adducts for polyether ionophores), where mass differences were within 4 ppm of the exact mass. Fortified milk samples (100 µg/kg benzimidazoles) were initially analyzed with DART directly and also after a simple ACN extraction. In both cases, the analytes could not be detected in the presence of matrix.[240] With the addition of a QuEChERS clean-up, calibration linearity was achieved with matrix-matched calibration standards; low concentration calibrants (1 – 10 µg/kg) were detected and the recovery of 20 benzimidazoles fortified at 10 and 100 µg/kg ranged from 65% to 95% (RSD < 10%). For the coccidiostats, higher concentrations (1 and 10 mg/kg) of monensin, salinomycin, narasin, and robenidine were detected in QuEChERS extracts of chicken feed (>72% recovery, <10% RSD), but other tested compounds could not be detected even with this extent of sample preparation.

Doué et al. quantitatively determined anabolic steroid esters in commercially available internet products using DART with high resolution LTQ-Orbitrap MS analysis.[241] The oily samples were diluted by a factor of 1000, fortified with labeled internal standards, and analyzed in transmission mode from dried 5 µl aliquots of

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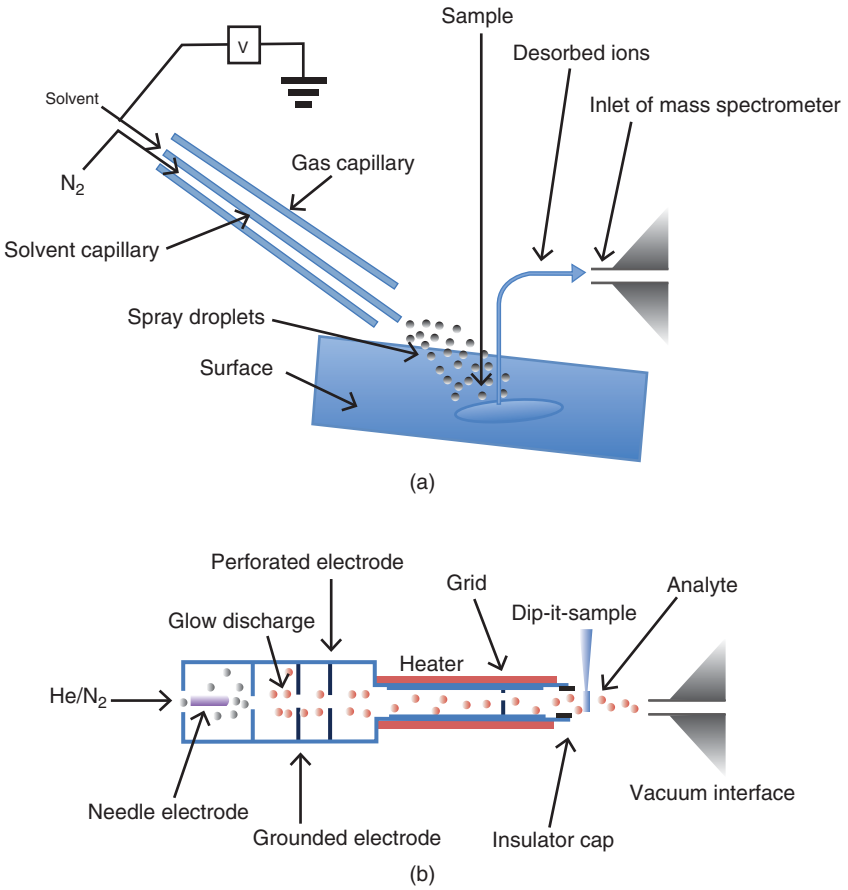


Figure 2.3 Direct analysis in real time (DART) and desorption electrospray ionization (DESI) sources for ambient mass spectrometry. Source: Farre 2013[78]. Reproduced with permission of Elsevier.

sample deposited onto a metal mesh surface. Twenty-one steroid esters of testosterone, estradiol, boldenone, and nandrolone were determined in this targeted analysis with a LOQ of 1 µg/l and sufficient linearity and repeatability for rapid regulatory analysis. The mass accuracy for the protonated molecular ions for the 21 esters was less than 3 ppm. Two product ions were also collected for each compound from collision-induced dissociation experiments each with a mass accuracy of 7 ppm or less.

2.6.2.1.2 Atmospheric Solids Analysis Probe (ASAP) ASAP is based on desorption/ionization from the surface of a glass capillary after introducing the capillary into a standard APCI source chamber.[242] Samples are prepared by dipping or

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pipetting a liquid sample onto a glass melting point capillary or rubbing the capillary across a solid sample. The capillary is inserted into the source housing and desorption is assisted by the flow of heated source desolvation gas directed at the capillary. Gas-phase molecules are then ionized by the plasma formed around a corona discharge needle.[242]

Wang et al. used ASAP-MS/MS to screen for 13 β-agonist residues in pig urine.[243] Urine was first subjected to deproteinization, pH adjustment, and incubation for 4 hours with β-glucuronidase/acyl sulfatase. Portions of urine were then cleaned up using MCX SPE cartridges to separate the β-agonist residues from urine matrix interferences. Following cartridge elution, solvent evaporation, and reconstitution of the dried extract, the glass sampling probe was dipped into the sample extract and immediately inserted into the source for desorption/ionization and MS/MS analysis of the target analytes. Detection limits ranged from 0.05 to 0.2 µg/l for the 13 β-agonist compounds tested and recoveries ranged from 59% to 80% (6–17% RSD) for urine spiked at a concentration of 0.2 µg/l. ASAP has also been used to identify steroid standards[244] and steroid esters in commercial formulations.[245] In these examples, little sample preparation was required owing to higher concentrations of analytes.

2.6.2.1.3 Laser Diode Thermal Desorption (LDTD) LDTD with APCI-MS/MS analysis is another separation-free analytical method for direct analysis of sample extracts.[246] In this technique, dried sample extracts are thermally desorbed from metallic surfaces of 96- or 284-well plates using an infrared diode laser (Figure 2.4). Gas-phase analytes are then carried in a gas stream toward a corona discharge needle for APCI ionization.

LDTD-MS/MS has been applied to the analysis of antibiotics in food matrix extracts including sulfonamides in milk[247] and quinolones in fish[248] with quantification concentrations in the order of 10 µg/kg achieved. For veterinary drug residue analysis in salmon, catfish, and shrimp, Lohne et al. extracted tissue with acidified water and then partitioned three quinolones into acetonitrile with addition of sodium chloride.[248] Concentrated extracts were deposited into the sampling plate and samples thermally desorbed with a 3 second diode laser program; 250 sample extracts were analyzed in less than 2 hours. Three MS/MS product ion transitions were collected and used for identification of each quinolone, and an internal standard was used to improve precision and linearity ($R^2 > 0.99$) in the desorption process. In salmon, catfish, and shrimp fortified at 10 µg/kg, quinolone recoveries were 77–102% (RSD < 20%), and method detection limits ranged from 2 to 7 µg/kg. LDTD-MS/MS analysis has also been applied to the determination of residue concentrations of steroid hormones in waste water, sludge, and plasma,[249–251] neonicotinoid insecticides in honey,[252] and veterinary drugs in pig manure.[253]

2.6.2.1.4 Other APCI-Based Techniques Desorption corona beam ionization (DCBI) is an APCI-related technique based on a flow of heated helium passing

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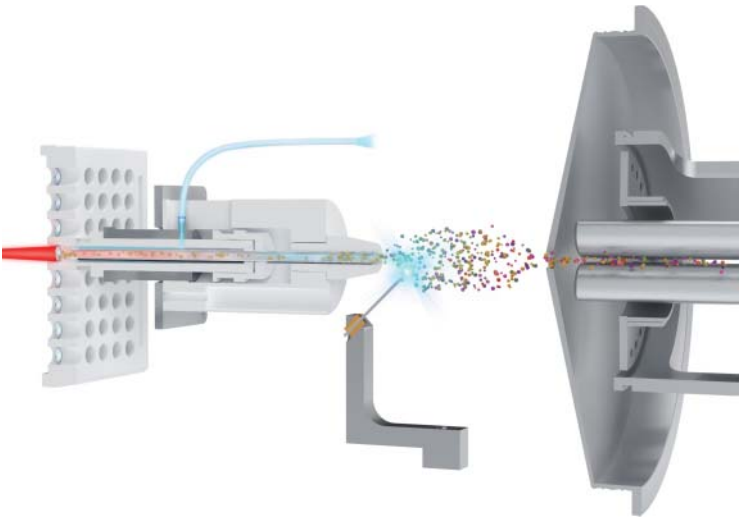


Figure 2.4 Laser diode thermal desorption source for rapid mass spectrometry. Source: Reproduced with permission from Phytronix Technologies Inc.; copyright 2013.

through a charged hollow needle electrode to form a visible narrow corona beam that extends out of the source and can be directed at a sample surface.[254] Standard solutions of clenbuterol, estradiol, acetaminophen, and pesticides, including deltamethrin and cypermethrin, were amenable to DCBI-MS analysis.[254] Huang et al. performed frontal elution paper chromatography on an extract of clenbuterol from pig feed samples to concentrate residue at the tip of a triangular paper substrate after elution, then applied the visible corona beam of a DCBI source to the tip of the paper.[255] This technique provided simultaneous sample clean-up and analyte concentration with a reported 2 mg/kg detection limit, recovery >70%, and RSD < 20%.

Other APCI-related techniques are based on dielectric barrier discharge ionization (DBDI) and low-temperature plasma. These are related techniques that generate low-temperature plasmas from an electrical discharge through a dielectric material.[235] Differences in the configuration of the electrodes and dielectric result in sampling from a fixed position glass surface for DBDI and a portable source probe for low-temperature plasma. Both DBDI and low-temperature plasma have been applied to drug residue analysis. Low-temperature plasma was used to directly detect human drugs of abuse (~5–100 µg/l) in diluted urine samples.[256] Gilbert-López et al. combined DBDI with diode laser thermal desorption to detect veterinary drug standards from a fixed glass sampling surface.[257] While high concentration standard solutions were tested (400 µg/ml), this approach could detect low vapor pressure antibiotics that had not been amenable to lower temperature plasma analysis.

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2.6.2.2 ESI-Based Techniques

2.6.2.2.1 Desorption Electrospray Ionization (DESI) In DESI, an electrospray nozzle is directed at a surface to spray a sample on a surface with charged droplets (Figure 2.3).[258] While the mechanism is multi-faceted, the charged solvent spray both desorbs and ionizes analytes from the surface.[235] DESI-MS has been applied to a large variety of compounds and matrix types. For veterinary drug residue analysis, several applications were reported in a 2011 review.[234] For example, clenbuterol was found to have a 1 μg/ml detection limit with direct DESI analysis of a urine sample, while urine first subjected to SPE clean-up produced an eluate that yielded a 2 μg/l detection limit.[259] Hormones in commercial preparations were also directly determined by DESI with QTOF-MS analysis.[260] More recently, DESI-HRMS was optimized to identify residues of nine coccidiostats, benzimidazoles, and macrolides in feed samples.[261] Though direct analysis of pressed feed pellets was attempted, degradation of the pellet surface resulted in MS contamination by sample dust, which made analysis of a solvent extract more practical. Thus, feed samples were extracted ultrasonically with an acidified acetonitrile/water solvent mixture, centrifuged, and then 2 μl of supernatant was spotted onto a substrate consisting of PTFE spots printed onto a glass slide. Compounds were desorbed/ionized from the substrate with an acetonitrile/water spray and detected with full scan spectra collected and isotopic cluster fit used for additional identification. For the optimized compounds, monensin $(M+Na)^+$, narasin $(M+Na)^+$, lincomycin, and tiamulin were identified in feed samples. Salinomycin, lasalocid, decoquinate, oxytetracycline, and doxycycline were also identified and trace residues of additional drugs were detected. For the identified compounds, the mass accuracy was 2.5 ppm or better and isotopic cluster fit was at least 80%.

DESI has also been used to image injection sites in cattle and identify anabolic steroid ester use.[262] Like other direct analysis techniques, residue identity confirmation cannot be based on the usual practice of product ion ratio (or mass error) coupled with retention time matching to an analytical standard as there is no analyte-specific temporal difference in the desorption/ionization process. To provide sufficient identification points to meet EU residue identification criteria,[263] three or four product ions were collected per steroid ester (at least 4.5 identification points), satisfying the compound identification requirement of four identification points based solely on ion ratios.[262]

Injection site analysis highlights the versatility of DESI-MS for analyzing a variety of sample configurations. This feature has been emphasized by several studies combining solid sorbent extraction or liquid micro-extraction formats with DESI-MS. While these are examples of human drug analyses, similar combinations of micro-extracts coupled with DESI techniques may someday be applied to veterinary drug residue analysis. DESI-MS was used to directly desorb/ionize steroids from an SPME fiber that had been used to extract a urine sample.[264]

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Dénes et al. adsorbed analytes onto C_{18} in a modified SPE cartridge with a polymer membrane frit flush with the exit of the cartridge.[265] The analytes were eluted toward the exit frit into a flow of a drying gas, which evaporated the elution solvent and crystallized analytes on the surface of the frit, where they were subjected to direct DESI-MS analysis. Rosting et al. used DESI-MS/MS to detect and quantify human drugs in urine, blood, and saliva samples that had been extracted by thin-film micro-extraction (TFME).[266] In this technique, drugs were partitioned from samples into a Teflon-supported hexadecane thin film, where they diffused through the porous Teflon™ membrane for DESI-MS/MS analysis from the opposite thin film (the side not in contact with the sample). TFME with a porous mixed-mode C_{18}/SCX sorbent blade was also coupled with DESI-HRMS analysis to extract and analyze targeted and non-targeted drugs (e.g., NSAIDs) from waste water.[267] This type of sorbent material is amenable to the extraction of many veterinary drug residues.

2.6.2.2.2 Paper Spray (PS) Unlike DESI, which is a combined ionization/desorption method, PS ionization is in a direct ionization category where ESI is generated directly from a solvent-wetted triangular paper substrate.[268, 269] Samples are deposited on the paper and the paper is positioned near the MS inlet. When a voltage is applied to the paper, charged droplets emerge from the triangular tip becoming the ESI source. With many possibilities to vary the chemical and physical properties of both the solid-phase substrate and the spray solvent, PS-MS has been reported for the analysis of a wide range of drug residues in blood, saliva, urine, and tissue extracts.[268] Zhang et al. used PS-MS to detect clenbuterol, terbutaline, salbutamol, and ractopamine residues in pork and beef extracts with a LOD of 1 µg/kg for pork and 5 µg/kg for beef.[270] The β-agonists were spiked into aqueous extracts of muscle and then sprayed from silica-coated paper with methanol/water and 3.5 kV voltage. While PS-MS was not applied to β-agonist residues extracted from muscle, the method demonstrated linearity ($R^2 > 0.99$) and RSD < 15% by selected reaction monitoring of $[M+H]^+$ without internal standard correction for clenbuterol standards in pork matrix.[270]

2.6.2.2.3 Other ESI-Based Techniques For ESI-related emerging techniques, probe electrospray ionization (PESI) is a direct analysis method that generates ions by dipping a solid metal needle into a liquid sample, positioning the needle in front of the mass spectrometer inlet, and applying a voltage to the needle to generate ESI directly from the liquid sample.[271] PESI was used to detect drugs of abuse from unprepared human urine, saliva, and plasma samples at concentrations in the range of 2–5 µg/l.[272] In an interesting combination of in-capillary solvent micro-extraction and direct electrospray of a liquid sample, Ren et al. extracted epitestosterone from a 5 µl urine sample in a pulled-tip glass capillary by mixing with 5 µl of ethyl acetate and 5 µl of hydroxylamine (to improve ionization of steroids) and directly ionizing/spraying the ethyl acetate phase.[273] Detection limits below 1.0 µg/l were reported for steroids in these micro samples.

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Other platforms have also been investigated with ESI-type desorption/ionization procedures. Rather than a solid needle, Hu et al. pioneered using a sharpened wooden toothpick as an ESI probe for direct ionization of tissue samples.[274] Deng et al. derivatized wooden toothpicks with sorbent to serve as solid-phase micro-extraction probes to extract and concentrate residues of fluorinated organic acids from acidified milk samples (probe immersion with 20 minutes of stirring), achieving >80% recovery of perfluorinated compounds in milk fortified with 0.1 μg/l concentrations.[275] Wang et al. extracted ketamine and norketamine directly from urine from a loaded C_{18} micro-extraction pipette tiph(ZipTip).[276] The washed pipette tip sorbent was connected to a glass barrel syringe filled with elution solvent, and high voltage was applied to the metal syringe needle to form ESI of eluted compounds through the pipette tip.

2.6.3 Direct MS Considerations for Regulatory Analysis

For all direct analysis techniques, the absence of a chromatographic retention time decreases the amount of independent data, or the number of identification points, available for absolute identification of analytes. Many of the aforementioned studies have considered how to overcome the loss of temporal resolution. In some cases, multiple product ion transitions were collected to increase the number of identification points.[248, 262] Lehotay proposed an identification system wherein four product ion transitions were collected for each analyte and identification was made based on the comparison of six ion ratios for analytes in samples versus reference standards.[229] Additional compound identification information can be provided by a thermal profile in direct MS techniques that are designed to induce a thermal desorption gradient to selectively desorb analytes at different temperatures[239, 254] or with the use of collisional cross section information from analysis coupled with ion mobility spectrometry (IMS).[277] Seró et al. applied isotopic pattern matching to identify compounds by DESI-MS analysis.[261] The collection of MS^3 spectra and HRMS data for molecular and fragment ions has also been suggested to increase identification accuracy.[234, 260, 278]

Despite these suggestions, many regulations require or suggest that a retention time is a necessary component of identification criteria for qualitative identification.[278, 279] As such, for qualitative identification in regulatory analysis, direct MS methods are currently best suited as rapid screening methods.[234] For quantitative analysis of veterinary residues for regulatory samples, none of the sample matrix background is removed chromatographically in direct MS techniques; therefore, signal suppression can be significant in these complex biological matrix samples and inconsistencies that may be inherent in the desorption or ionization process can lead to poor analytical precision.[239] Sample preparation can dramatically increase analytical performance (LOD, LOQ) as described in the aforementioned examples. For quantification, the use of calibrants prepared in matrix extract and the incorporation of internal standards are often necessary techniques used to reduce isobaric interference, normalize

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the desorption/ionization process, and increase quantitative precision.[245, 248, 280] Lohne et al. found that high efficiencies were obtained for quinolone extraction from fish, but two of three quinolone compounds required internal standard calibration to normalize the desorption/ionization process in LDTD-MS/MS.[248] Internal standard was added to the final residue reconstitution solution prior to depositing extract on the sample plate. In other studies, internal standard was added directly to the solid substrate.[281, 282]

2.7 Ion Mobility Spectrometry

IMS is a technique that separates gas-phase ions based on their ability to move in an electric field. Differences in charge, size, and shape of the ions affect their ability to accelerate in an electric field, collide with other molecules, and form clusters. Mobility differences can be exploited to distinguish how long it takes for a particular analyte ion to pass through a drift tube against a traveling voltage wave or how much offset voltage must be applied to allow an ion to pass through a region of pulsed electric field. Excellent reviews have summarized the principles of IMS and the various types of highly developed IMS systems for trace analyte detection.[283, 284]

For veterinary drug residue analysis in food, IMS systems are predominantly coupled with mass spectrometer systems. IMS is particularly useful for residue analysis in complex food matrices because it can act as a filter to permit the separation of isobaric compounds, either to distinguish two isobaric analytes or to isolate an analyte from interfering matrix components. IMS is also useful to introduce an orthogonal analyte identification feature, particularly for direct analysis MS experiments where compounds are not separated chromatographically. For this discussion, we limit our focus to two commercial IM-MS systems where the mobility separation is either in the source or integrated into the mass analyzer.

The first system is based on the SelexION (Sciex) differential mobility spectrometer (DMS), in which a pulsed electric field is applied as an ionized sample enters a parallel plate DMS cell placed just before the mass spectrometer inlet. A separation voltage waveform moves the analytes toward the plate electrodes while a compensation voltage is selected to permit the transmission of only the desired analyte. In this way, compensation voltage is used to selectively filter analytes with particular mobility.[285] Mobility through the DMS cell can be affected by increasing the clustering around analyte ions by deliberately introducing solvent modifiers into the gas flow to increase the possibilities for ion separation.[286]

In the second system, mass separated ions are passed through an ion guide cell composed of stacked ring electrodes placed prior to the ion detector.[287] Pulsed voltage waves travel from ring to ring to push the ions, slowing those with larger collisional cross sections.[284] This is the basis for the traveling wave (T-wave) ion mobility mass spectrometer (TWIM-MS) system developed by Waters Corporation. The TWIM-MS system allows collision cross sections to

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be determined from drift times, and these can be used as criteria for analyte identification.[283]

IMS has been coupled with drug residue analysis in a number of experiments relevant to testing of food and food-producing animals for residues of veterinary drugs. Silvestro et al. showed data for clenbuterol in diluted urine samples with and without DMS separation.[288] The urine matrix background was significantly reduced with DMS, resulting in lower signal-to-noise ratio for higher sensitivity analysis. In the preliminary work for stilbene residue analysis in salmon matrix, our laboratory has also observed matrix reduction.[289] Sniegocki et al. recently reported a LC-DMS-MS/MS method to determine metabolites of carbadox and olaquindox growth promotants in pig muscle.[290] Matrix effects were determined with and without use of the DMS cell, and significant improvement of signal-to-noise ratio was noted with the DMS. Ray et al. applied LC-DMS-MS/MS to reduce matrix background and separate isobaric analytes in the analysis of steroids in plasma and serum samples.[291] Beucher et al. used a Q-TWIM-TOF-MS system to analyze 30 β-agonists in urine, muscle, and retina from cattle.[277] The method was found to meet regulatory analysis criteria and was evaluated with respect to limit of detection, ability to increase the separation of co-eluted and co-detected ions, and the ability to gain identification information from obtained collision cross section data. Ion mobility has also been combined with direct MS techniques.[292] For example, LDTD was combined with DMS for sensitive testosterone analysis in plasma.[250]

2.8 Conclusions

Universal extraction methods continue to be developed and applied to sensitive and selective MS and HRMS analysis. The development of solvent systems geared toward partitioning analytes away from matrix components and new sample clean-up materials and techniques should continue to drive possibilities for universal analysis for a wide variety of drug residues and chemical contaminants in food. Selective (or broad) solid and liquid micro-extraction techniques coupled with fast chromatography and direct MS analysis methods add exciting directions for rapid screening of samples. The addition of ion mobility separation can enhance selectivity post-ionization when combined with direct MS techniques to reduce isobaric background interference and can also introduce another identification feature for qualitative analysis. These emerging techniques will continue to increase the scope of monitoring for veterinary drug residues, both in terms of types of analytes that can be detected and the number of samples that can be analyzed while maintaining the data integrity necessary to implement any regulatory action on non-compliant samples. Other advances in analytical methods currently being investigated in clinical and biological laboratories may eventually find applications in food testing with the overall goal of improving the safety and reliability of the global food supply.

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3

Capabilities and Limitations of High-Resolution Mass Spectrometry (HRMS): Time-of-flight and Orbitrap™

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3.1 Available Technology

Until relatively recently, high-resolution mass spectrometry (HRMS) was synonymous with Fourier Transform Ion Cyclotron Resonance (FTICR) or sector instruments. In the past decade, the importance of these two techniques has been greatly diminished, and their current use is restricted to a rather narrow field of analytical applications.

FTICR shows the highest resolving power and the best mass accuracies of all types of MS instruments. However, the strength of the required magnetic field (4–15 Tesla) generally demands the use of a superconducting magnet. The cooling requirement (e.g., liquid helium) and the cost and size of the instrument have restricted this technology to a few dedicated research laboratories. Furthermore, the technical complexity, the relative low scan speed, as well as a number of complex space charging issues, are the reasons why there are virtually no published routine applications of FTICR instruments. The availability of cheaper and simpler-to-use instruments, such as the Orbitrap™ systems (Thermo Scientific), has further restricted the use of FTICR to applications where ultrahigh resolution is required.

Sector instruments gained a wider acceptance than FTICR. Again the size of the instruments, the frequency of tuning, maintenance, and cost have been issues that have limited the expansion of their user base. The original strength of this technology (sensitivity and resolution) is currently insufficient to compete with modern Time-of-flight (TOF) or Orbitrap™ systems. However, sector instruments have found niche applications as GC-MS detectors operating in the selected ion mode. Until recently, legislation dictated the use of sector instruments in the highly regulated field of dioxin and dioxin-like PCB analysis. Such analytical work is now

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increasingly frequently done with GC-MS/MS (based on the European Commission Regulation No 589/2014[1]). A further example of the use of sector instruments has been the use for high-sensitivity detection of steroids within certain World Anti-Doping Agency (WADA) laboratories.

FTICR and sector instruments are still commercially available, but the number of companies developing and selling such instrumentation has decreased. The small user base is certainly a major obstacle for instrument producers to make the financial investments required to further improve hardware and, probably even more importantly, software. Currently, FTICR and sector instrument applications seem to be limited to high-sensitivity dioxin analysis, structural elucidation, and proteomics.

TOF and the more recently introduced Orbitrap™ mass analyzer have clearly overtaken FTICR and sector instruments, and virtually all scientific papers reporting the use of HRMS in the field of veterinary drug, mycotoxin, or pesticide multi-residue analysis rely on the use of these technologies. Further, while many of the original uses were for screening applications, more recently, their utilization has spread to both quantitative and confirmatory applications.

3.1.1 TOF

The proof of principle for the TOF technology is older than most other mass spectrometry technologies. However, a number of engineering and electronic innovations were required to create a commercially viable mainstream technology.[2] TOF relies on the accurate measurement of ion flight times. Ions of the same charge state can be accelerated within a well-defined electric field, where they gain identical kinetic energy. Hence, the velocity of the ions leaving the acceleration region directly depends on the mass of the ion (or more correctly, the mass-to-charge ratio). A sufficiently fast detector can be used to measure the arrival time and the intensity (number) of the accelerated ions at the end of a potential-free flight or drift tube. Low-mass ions (low m/z ratio) will arrive earlier than heavier ions (high m/z ratio).

Originally, TOF had the reputation of being an incredibly fast scanning technology, at the price of a poor mass resolving power. Therefore, the use of TOF was limited to high-speed applications where slower scanning instruments such as quadruples or sector instruments could not compete. In fact, the apparently high scan speeds are due to the ion flight times, which are well below a millisecond; in reality, hundreds of consecutive measurement cycles (transients) are summed to produce a single spectrum. Nevertheless, the spectra acquisition data rate can still exceed 100 Hz. The low resolving power of the first TOF instruments was caused by the inherent spread of the kinetic energies among the ions prior to the ion acceleration process. Furthermore, most ionization sources produce a continuous ion beam, while TOF relies on pulses of accelerated ion clouds. These limitations were successfully addressed by introducing the concept of orthogonal acceleration, Figure 3.1.

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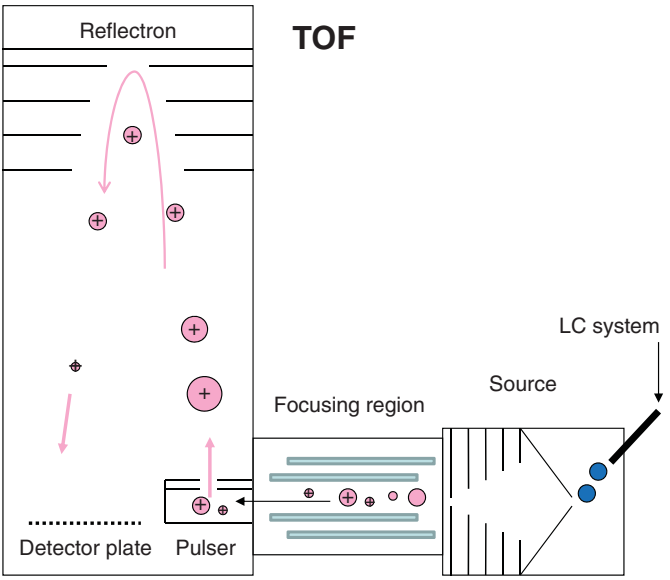


Figure 3.1 Schematic drawing of an orthogonal time-of-flight instrument.

Even a sharply focused ion beam will always show a kinetic energy distribution along the beam axis (*x* axis). However, due to the sharpness of the ion beam, ions possess no relevant kinetic energy, which is directed orthogonally to the beam axis (*y* axis). Therefore, the standard deviation of the orthogonal kinetic energy distribution is very low. This is shown schematically in Figure 3.2. A pusher, which accelerates ions orthogonally away from this beam, will therefore produce ion packets (dashes) with a very narrow spatial distribution. Even ensuring homogeneous acceleration and production of a point-like ion cloud does not resolve all band broadening effects. Hence, additional focusing concepts such as specifically designed electrostatic mirrors (reflectrons) were introduced to refocus the ions during their flight.

Further significant improvements were obtained by implementing the pusher and puller acceleration concept. The aim is not only to narrow down the orthogonal kinetic energy distribution, but also to extract a significant percentage of the ions out of the continuous ion beam. In early orthogonal TOF instruments, the percentage of ions samples was dictated by the duty cycle. Basically, a new ion packet can only be accelerated when the last (heaviest) ion from the previous push has reached the detection plate. This represents an engineering challenge, considering the long ion flight path and the size limitation of a pusher plate. While modern TOF instruments as shown in Figure 3.1 have a greatly improved duty cycle, an improvement in the efficiency with which the orthogonal beam is sampled is a field where further innovations are to be expected. For example, it would be desirable to have an ion optic device with the capability to slice the continuous

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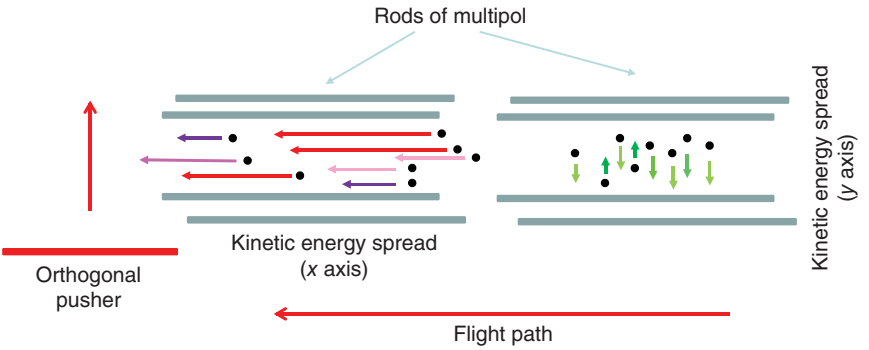


Figure 3.2 Spread of kinetic energy of transmitted ions.

ion beam into packets, which would need to be focused and stored prior to being accelerated into the pusher region. Last but not least, the release of these focused ion clouds needs to be synchronized with the orthogonal pusher frequency to obtain a near 100% sampling efficiency. Such systems need to maintain the lowest possible orthogonal kinetic energy spread and to avoid a horizontal TOF discrimination. An innovative concept to achieve this goal is the traveling-wave device introduced by Waters Scientific.

TOF detectors need an extremely fast response time in order to accurately monitor the flight time of the incoming ions. The speed required refers not only to the detector response, but also to the subsequent electronic signal amplification process. Older types of detectors such as the time-to-digital converter (TDC) were fast, but produced only a digital signal, showing the arrival time of a single ion. Hence, two simultaneously arriving ions will be detected as a single event. Furthermore, early TDC detectors had a significant recovery time, that is, the period of time required between the arrival of two ions in order for these to be detected as separate events. In effect, the first ion "blinds" the detector until it recovers. This means that when several ions arrive at the detector within the recovery time, only the first arriving ion will induce a detector signal. This has implications not only on the quantification but also on the measured flight time. As mentioned before, there is always a degree of band broadening within a cloud containing identical ions. The preferential detection of the ions that arrive at the detector first with those following immediately afterward possibly not being detected leads to an artificially high signal resulting from the first ions of a given mass arriving at the detector. In these circumstances, there will be a significant bias of the measured m/z value toward a lower value than the actual "accurate" masses. While this appears to be a very major problem, the concurrent arrival of ions is less frequent than it may be assumed, since a single scan representing large numbers of measured ions is actually produced from the summation of many cycles of the pusher (each referred to as a transient). Generally, hundreds or even thousands of transients are summed to produce a single spectrum; therefore, even

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a TDC detector device can produce quantitative data. Despite this, TDC technology has been gradually replaced by analog-to-digital convertors (ADC). These devices have a significantly larger dynamic range and have further undergone improvements regarding the speed (frequency) of acquisition.

Despite all these technological advances, detector saturation remains a relevant issue even for currently available TOF technology. Depending on the instrument used and the chosen mass resolving settings, symptoms of detector saturation may be observed. As mentioned, this is relevant not only for the quantitative information, but also for the qualitative data (deviation in measured accurate masses). Therefore, defining a very narrow mass window in data processing (to improve selectivity) may result in the distortion or even the loss of a high signal to exceed a certain intensity (saturation).[3]

The mass resolving power of TOF instruments has been improved to some 70,000 FWHM at $m/z > 800$ (e.g., Bruker maXis II or spiral TOF from JEOL), in part due to faster electronics but also by providing longer flight paths. Longer flight paths can be obtained either by the construction of physically longer flight tubes or by the use of reflectrons. Long flight tubes do not only require increased horizontal or vertical laboratory space but also demand a well-controlled temperature environment as thermal expansion of the flight tube affects ion flight time and with this the accurate mass measurements. Instrument producers are aware of this and are utilizing special metal alloys for the construction of their flight tubes to counteract such problems. Smaller instrumental dimensions are feasible when using reflectron types of devices. Reflectrons (ion mirrors) commonly consist of a number of stacked rings. The distance between the rings and the DC voltage applied to each ring is carefully adjusted. Hence, ions penetrating this space will encounter an electrostatic field, which slows them down and finally repulses them. Reflectrons also provide a degree of energy focusing not provided by linear flight tubes, resulting in refocusing ion clouds, and they also remove fast neutral species, which can contribute to the baseline. However, each manipulation of the ion flight path inevitably leads to the loss of ions and decreased transmission efficiency. Not all TOF instruments utilize reflectrons. Some commercial instruments permit the switching on or off of this feature to give the user the choice between sensitivity and resolution. The combined use of such technologies is the primary reason why currently available TOF instruments provide mass resolving powers of $\geq$30,000 FWHM at $m/z > 500$ and mass accuracies ≤ 2 ppm. This is a dramatic improvement when considering the sub-unit resolution of older, non-orthogonal acceleration TOF instruments.

While single-stage TOF instruments have been commercialized, hyphenated machines have been significantly better received within the analytical community. Currently, the most common configuration is the use of a quadrupole and collision cell in front of the TOF analyzer. However, at least one commercial instrument incorporating an ion trap device has been launched (LCMS-IT-TOF from Shimadzu). The use of a mass resolving device in front of the TOF is not only relevant for the generation of unit mass precursor selected product ion spectra.

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A quadrupole can also be used as a mass cut-off device to prevent the collection of ions beyond the mass range of interest, which prevents ions above the mass range of interest entering the TOF flight tube. The presence of high mass ions with long flight times prolongs the required TOF transient time and negatively affects the duty cycle. Based on currently published papers, most QTOF authors seem to utilize their instrument in such a mode, utilizing the Quadrupole as a wideband mass filter. Typically, the use of the quadrupole as a unit mass selecting device is restricted to applications where the confirmation of a finding or the highest possible sensitivity or selectivity is needed. It has to be added that the inclusion of a quadrupole in front of a TOF instrument can also have a negative impact on the results. The fragmentation process in the collision chamber may require a second ion beam focusing step, and as a result, the mass accuracy of the product ion spectra can be poorer than the spectra of precursor ions (no fragmentation involved).

Modern TOF instruments can produce mass accuracies of less than 1 ppm, a value that used to be restricted to FTICR instruments. However, such high performance requires the careful and constant recalibration of the instrument. Hence, many users feel more confident reporting a mass accuracy of 5 ppm.

A fluctuating power supply voltage and the thermal expansion or contraction of the flight tube are factors that directly affect the flight speed or the length of the flight path, leading to inaccurate mass assignment. To date, three ways have commonly been utilized to ensure mass axis stability. A common and technically simple approach is the continuous infusion of a lock mass that ensures the constant presence of a known mass peak. However, the infusion of a mass calibrant solution almost certainly results in the presence of other unwanted peaks related to impurities in the infusion solution. Furthermore, careful adjustment of the calibrant concentration is required to ensure an adequate signal in situations where a high matrix effect may suppress calibrant signals while avoiding contaminants from the calibration solution dominating the analyte spectra. A technically more complicated approach of switching between analyte spectrum and lock spray spectrum (using two spray devices and an oscillating baffle) has resolved these issues. The disadvantage of this approach is that at the point where the lock mass signal is measured (typically every 10–30 seconds), chromatographic data are lost. This loss of data can create problems when integrating chromatographic peaks.[4] Some commercially available instruments now show such improved mass axis stability that mass calibration can be performed immediately before and immediately after the chromatographic run. A potential issue with this approach is, depending on the instrument, accurate masses may only be available after the closure of an acquired data file. This is relevant if data-dependent scans triggered by accurate mass peaks are to be initiated (triggering on the fly).

Due to some of the issues highlighted earlier in this section, early QTOF instruments not only had a limited dynamic range but also poor sensitivity. As a consequence, they were seldom used in environmental and residue analysis, but of late, there has been a change regarding this situation. Significant improvements

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related to the duty cycle and the detector technologies have been achieved. Last but not least, modern interfaces, as those used in tandem quadrupole systems, have been incorporated into commercially available QTOF instruments. This includes ion funnel-like devices or multiple bore electrospray sources.[5] These combined improvements have made QTOF technologies a serious competitor for residue applications, which used to be the long-held and near-exclusive domain of tandem triple-quadrupole instruments.

Strengths of TOF instruments are as follows:

- Scan speed is generally higher than that of Orbitrap™.
- Good spectral quality.
- Large number of manufacturers developing and selling instruments.
- Good mass resolving power for high m/z ions and large molecules.
- Good sensitivity, which is partially due to the utilization of modern, high ion transmission interfaces (e.g., ion funnel).

Current limitations of TOF are as follows:

- Need for frequent (or constant) mass axis calibration.
- Resolving power is clearly below Orbitrap™ instruments. Further improvements may require longer ion flight paths. This will affect the dimension of the instrument or, in the case of multi-reflectron instruments, affect sensitivity or the duty cycle.
- Mass resolving power drops toward low m/z ratios. This can become a relevant issue when detecting very small molecules in complex matrices.
- TOF baselines are noisier than Orbitrap™, because ions that undergo decomposition during the flight time (e.g., metastable ions or labile adducts) can produce spurious signals.

3.1.2 Orbitrap™

This is the most recently introduced type of mass spectrometer, yet, the basic idea behind this technology can be traced back to 1923 and the so-called Kingdon trap,[6] which, while never commercialized, provided the concept of electrostatic orbital trapping. This finally led to the commercial introduction of the LTQ Orbitrap™ Classic instrument in the year 2005. Orbitrap™ and FTICR mass spectrometers share some common features in that in both cases, trapped ions are detected due to their motion within the trap inducing a sinusoidal wave in the detection circuitry. In both cases, the resultant signal, a mixture of all detected sinusoidal waveforms, is converted to a mass spectrum using the same deconvolution process (Fourier transform). Despite these similarities, the systems differ greatly in that in the Orbitrap™, ions are trapped within an electrostatic field,[7] while FTICR instruments trap ions in a fixed magnetic field. It is important to stress that the Orbitrap™ is not related to conventional spherical ion trap instruments or to the more recently launched linear ion traps. Both of these analyzers

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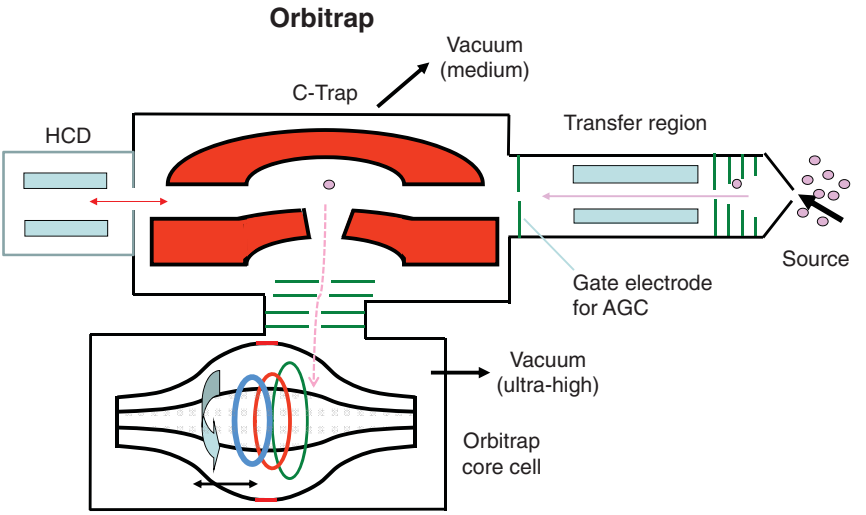


Figure 3.3 Schematic drawing of a modern Orbitrap™ instrument.

use a radio-frequency (RF) electrical field to trap ions; the method of detection is also completely different with ions of a specific mass-to-charge ratio being selectively emitted from an RF ion trap and detected using an electron multiplier.

The commercialized Orbitrap™ instruments consist of a central spindle-like electrode, which is positioned within a hollow, barrel-like outer electrode. The applied DC voltages and the particular geometric shape of the two electrodes create a quadro-logarithmic field. As a result, ions that are injected tangentially into the void space between the two electrodes can be trapped (Figure 3.3) with the ions showing a complex three-dimensional flight path. Successful trapping of ions requires a balance between the electrostatic forces (attraction between the ion and the oppositely charged central electrode) and the centrifugal forces encountered by the injected ions.

The introduction of ions into the Orbitrap™ cell represented a major engineering problem that had to be overcome. In an Orbitrap™ with a static electric field, the kinetic energy of ions introduced tangentially into the Orbitrap™ would prevent trapping, as they would fly past the central electrode and consequently hit the outer electrode. This issue was solved by quickly ramping the voltage applied to the central electrode; as a result, the trajectory of the ion cloud is altered to force all injected ions into an orbital rotation around the central electrode.

An important aspect of the design of the Orbitrap™ is that while the radial frequency of the trapped ions depends not only on the m/z ratio but also on the kinetic energy spread, this does not affect mass measurement. The unique geometrical shape of the void space between the two electrodes and the slightly off axis injection of the ions result in an electrostatic field, which accelerates ions toward the center of the Orbitrap™ cell. Hence, ions undergo harmonic axial

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oscillation; this is not dissimilar to the concept of orthogonal acceleration used in TOF instruments. However, the Orbitrap™, unlike the TOF instruments, does not require the application of an electrostatic pulse to induce an orthogonal (axial) acceleration of incoming ions. The combination of radial rotation and axial oscillation produces very complex ion trajectories. It is important to note that the axial oscillation frequency is strictly governed by the m/z ratio. In other words, initial kinetic energy distribution and other parameters do not affect the measured axial oscillation; this characteristic is the basis of the very precise mass measurement attained with the Orbitrap™.

Technological requirements for injecting ions into the Orbitrap™ were initially considered to be extremely daunting as ions have to be introduced in the form of a point-like ion cloud; failing to do so would prevent the initiation of concerted axial oscillations. To complicate matters, ions have to be transported from a relatively high-pressure area into an extremely low-pressure environment and disadvantageous time-of-flight discrimination can only be prevented by the use of a very short flight path. These challenges were solved by a number of innovative engineering solutions. The fast pulse-like injection of an ion cloud is currently achieved using a device called a "C-trap." The C-trap collects ions from the continuous ion beam (coming from the LC-MS interface), focuses them, and accelerates the resulting ion cloud into the core cell of the Orbitrap™. It is relevant to notice that the exit of the C-trap and the entrance of the Orbitrap™ are slightly off-line in order to prevent the entrance of neutral molecules.

The C-trap is not only responsible for the focusing of ions and injecting them into the core cell. The C-trap also has a second exit and entrance from where collected ions can be transferred into the higher-energy collisional dissociation (HCD) cell; ions fragmented within the HCD cell are sent back to the C-trap from where they are injected into the core cell. It is an interesting feature of the Q-Exactive™ (Thermo Scientific) that the instrument permits multiple fills. Hence, a number of different precursor ions can be sequentially isolated and fragmented. All fragments derived from the selected precursor ions are afterward injected as a single ion cloud into the core cell.

As alluded to earlier, ions are detected within the Orbitrap™ by the image current they introduce on the outer electrode. To this end, the barrel-like outer electrode actually consists of two parts, which are connected by a thin, electrically insulating section. The detected currents induced by the ions oscillating from the one section of the electrode to the other section of the electrode consist of the superimposed signals caused by all axially oscillating ions. Hence, a Fourier transform is needed to deconvolute the measured complex signal into the individual ion frequencies required to calculate the accurate masses of all the oscillating ions (time domain to frequency domain).

The mass resolving power of the analyzer is proportional to the number of monitored oscillations. This means the mass resolving power is lower for high m/z ions (oscillating slower) than for low m/z ions. This is the exact opposite of the situation with TOF measurements where longer flight times (higher m/z values)

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provide higher resolving power. As the resolving power of the Orbitrap™ is a function of scan time, the user is therefore free to select the desired mass resolving power without having to modify the hardware. The price that has to be paid for higher mass resolution is the limited number of data points per unit time, which is clearly important when very high efficiency chromatographic separations are employed. However, it is important to note that the sensitivity is nearly independent of the selected mass resolving power. This is markedly different from the situation with TOF instrumentation, where a higher data acquisition rate negatively affects either the sensitivity or the mass resolving power of the instrument. The upper mass limit of current Orbitrap™ systems varies upon the intended application. For example, the recently introduced Q-Exactive™ Focus, intended for small molecule analysis, has a mass range of 50–2000 Da while Q-Exactive™ Plus has an upper mass cut-off of 6000 Da. This represents a lower upper mass range than most TOF instruments, yet this is only relevant if the intended use of the instrument is for the analysis of large molecules (e.g., proteomics).

It should also be noted that for the Orbitrap™, there is an upper limit of mass resolving power, which is caused by the loss of ions and the dephasing of oscillations. Ions can be lost by collision with residual gasses while the dephasing is caused by imperfections of the injection step or geometry of the Orbitrap™ core cell. This does not represent a significant issue for typical small molecule analysis; for example, the current Q-Exactive™ instrument operating at a scan rate of 3 s^{-1} has a resolution of 70,000 at 200 m/z falling to 35,000 at 800 m/z.

Although the Orbitrap™ represents an extremely complex piece of technology, it is now available in the form of various reliable and robust analytical instruments. The user does not have to comprehend the mathematics of the Fourier transform process, and indeed, he or she does not even have access to parameters to control or influence this mathematical transformation. This makes the instrumentation available to a large user base. On the other hand, the lack of more in-depth instrumental control may be considered to be a limitation. The Orbitrap™ provides superior resolution ranging up to 500,000 FWHM,[7] which is significantly higher than available from the first generation of instruments. As mentioned before, such resolution will seldom be used for routine analysis because the data points obtained across a chromatographic peak may be insufficient for quantitative applications.

Probably, the most relevant limitation of the Orbitrap™ remains the ion capacity of the instrument. Current instrumentation permits the injection of a maximum of approximately 5,000,000 charges. While this is significantly more than conventional ion trap instruments can handle, it can be a limitation when full-scan data acquisition of a sample with a high matrix background is desired. The limiting factor seems to be the C-trap and not the Orbitrap™ measuring cell, this being indicated by the fact that the mass accuracy is virtually unaffected by the ion abundance.

Overfilling of the C-trap is prevented by the automatic gain control (AGC) feature for which a gate electrode installed in front of the C-trap is used to deflect

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the ion beam originating from the interface and transfer region. Setting appropriate gate electrode voltages will either permit or block the entrance of ions into the C-trap with the collection period or "injection time" controlled by the AGC algorithm. Depending on the total ion current, the injection time is regulated to obtain the correct trap filling (e.g., 5,000,000 charges). While this successfully prevents space charging effects within the C-trap and possibly the Orbitrap™ core cell, changing the injection time directly affects the measurement sensitivity. For example, longer time segments will be collected if the analyte is present in a low matrix sample such as a standard while shorter time segments will be sampled when the same analyte is present in a complex matrix sample. In order to account for the difference in injection time, peak areas are mathematically corrected by a multiplication factor, representing the modulation of injection time. However, there is no use in employing a multiplication factor if the number of analyte ions sampled falls below the ion detection threshold. In other words, the limit of detection (LOD) can be significantly poorer in matrix than in pure standard.[8] This problem has been partially resolved by modern Orbitrap™ technology, which is capable of trapping significantly more ions than the first generation systems and also by the use of a mass cut-off filter (quadrupole) to remove ions below and above the mass range of interest. The capability to remove heavy ions should resolve another problem related to the first-generation single-stage Orbitrap™ (Exactive™) for which it has been reported that high loads of multiple charged ions (e.g., proteins) prevent the collection and retention of lighter ions in the C-trap.[9]

There are also reports indicating that TOF spectral quality is superior to that obtained from Orbitrap™ systems[10]; this specifically refers to the measured isotopic ratio and its agreement with the theoretical isotope ratio. This is attributed to the fact that in the Orbitrap™, there seems to be a low abundance cut-off value below which no data are acquired or stored. Hence, the intensity of low abundance peaks (e.g., the second or third isotopic peak) is lower than the theoretically calculated abundances. This can become an issue when the elucidation of elemental composition is not based simply on the accurate mass of the most intense isotope but on accurate mass and ratios of the isotopes.

In common with the QTOF, a Q-Orbitrap™ can be used to produce precursor selected product ion spectra. The availability of product ion accurate masses ensures an extremely high selectivity, which far exceeds conventional QqQ performance. The quadrupole mass filter can also be used to select specific ions for accurate mass measurement; this accurate mass selected ion monitoring (SIM) acquisition provides high sensitivity and is particularly valuable for analytes where fragmentation is difficult or yields low intensity ions. On the other hand, additional QqQ experiments such as neutral loss or precursor ion scans can only be obtained by mathematical calculations and not by physical experiments.

Strengths of the Orbitrap™ technology:

- High mass resolving power.
- Low m/z analytes are extremely well resolved.

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- Excellent long-term mass axis stability regardless of environmental influences.
- Mass accuracy is independent of ion abundance.
- Equal mass accuracy for unfragmented and fragmented ions.
- Ion storage capability can be used to produce extremely intense product ion scans (Q-Exactive™).
- Fast positive–negative ionization switching.
- Multiplexing capability (Q-Exactive™).
- Ease of operation.

Weakness of the Orbitrap™ technology:

- Data acquisition speed can be a limitation when monitoring fast UHPLC peaks by using high mass resolution settings.
- Limited ion capacity.
- The scan range is limited to a factor of 15. In other words, if the low mass is set to 100 Da, than no masses beyond 1500 Da can be monitored.
- In-spectra dynamic range may be insufficient for some applications.
- A lower signal is frequently observed when measuring analytes present in a high background matrix than analytes present in clean standard solutions. This is due to the reduction of injection time, as caused by the AGC downregulating the ion injection time.
- Ion capacity issues are of even higher importance for single-stage Orbitrap™ (unwanted ions can fill a significant portion of the C-trap).
- TOF may produce higher resolutions for high m/z analytes or large molecules, for example, multiple charged proteins.
- Single vendor technology.

In general, TOF and Orbitrap™ are both well suited for residue analysis in complex matrices. This refers to the sensitivity and selectivity in terms of limit of quantification (LOQ) and/or LOD and mass accuracy. The high Orbitrap™ mass resolving power is a benefit when monitoring small molecules. On the other hand, TOF is superior when monitoring very narrowly resolved chromatographic peaks or multiple charged high m/z analytes.

3.2 Capabilities and Limitations of the Technology as Compared to LC-MS/MS (Tandem Quadrupole Mass Spectrometer)

There are still many LC-MS users who consider HRMS as a technology dedicated for research or structural elucidation. Such analysts conceive tandem quadrupole instruments as the pre-eminent tool for use for quantitative residue analysis, and until relatively recently, this was undoubtedly correct. HRMS is now a hot topic within scientific conferences reflecting intense interest in its application. Despite this, an examination of proficiency test reports reveals that HRMS is currently

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only used by a small minority of analytical chemists. Whether this reflects a reluctance to change technologies or financial constraints, there are a number of reasons why HRMS is currently and will even more seriously challenge the dominant position of QqQ instruments in future. For this to happen, there are issues that have to be understood and properly addressed in order to successfully use HRMS technology. Generally, HRMS is perceived to provide a number of unique capabilities, which include the suitability for semi-targeted or non-targeted work and the possibilities for the retrospectively review of the stored full scan data.

3.2.1 Selectivity

HRMS selectivity is governed by the mass resolving power of the instrument and the mass window applied to the data. The first parameter is given by the physical performance of the instrument, while the mass window can be freely defined by the user. Insufficient mass resolving power may result in the failure to resolve some analyte signals from the matrix, as can be clearly seen from Figure 3.4.

Applying narrow mass windows improves the selectivity[11] by reducing the number and intensity of matrix-related signals as shown in Figure 3.5. However, the selection of very narrow mass windows (e.g., ≤5 ppm) may lead to false-negative findings[3] because of isobaric interferences. In this instance, a co-eluting matrix compound with a nearly identical exact mass not physically resolved from the

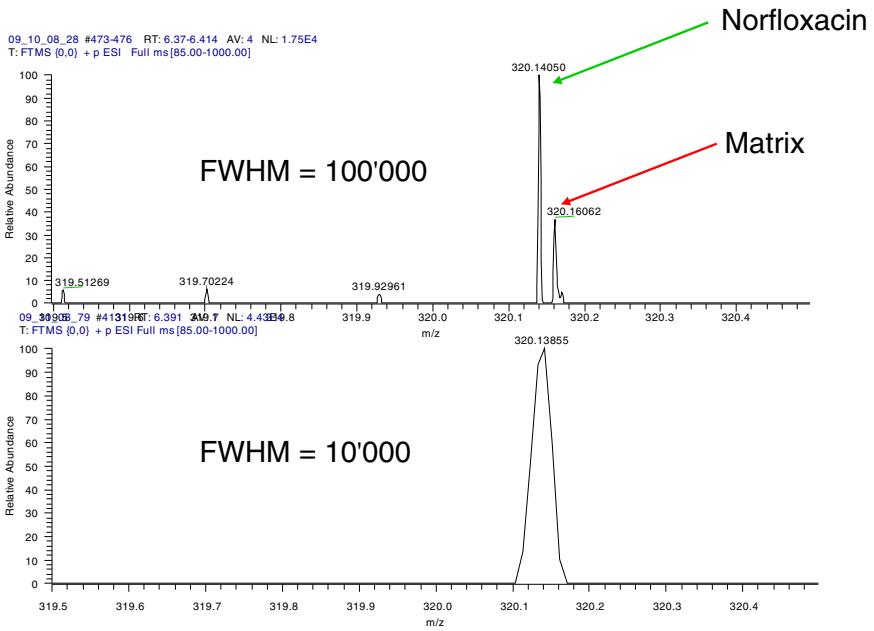


Figure 3.4 Effect on mass resolving power on the separation of exogenous from endogenous (matrix related) compounds.

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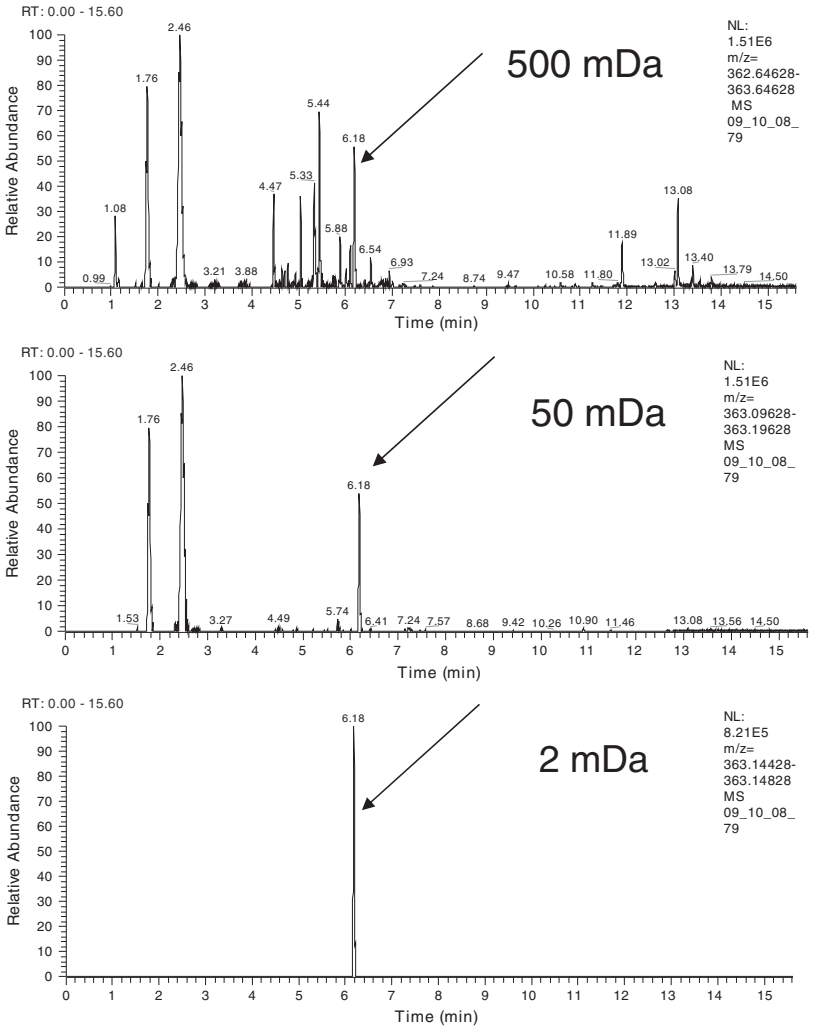


Figure 3.5 Effect of reducing the mass extraction window on the selectivity of detection (marbofloxacin in liver extract).

analyte ion can shift the apex of the analyte mass peak. Spectra are normally centroided (peak area determined and accurate mass assigned to peak center) during data acquisition or processing, which can lead to a slightly shifted centroid mass (see Figure 3.4). Depending on the narrowness of the user-defined mass window, the centroided peak may be located beyond the applied mass window. The result may be the complete absence of the analyte signal. Often, the application of overly narrow mass windows can be detected by distorted chromatographic peaks[3] (see

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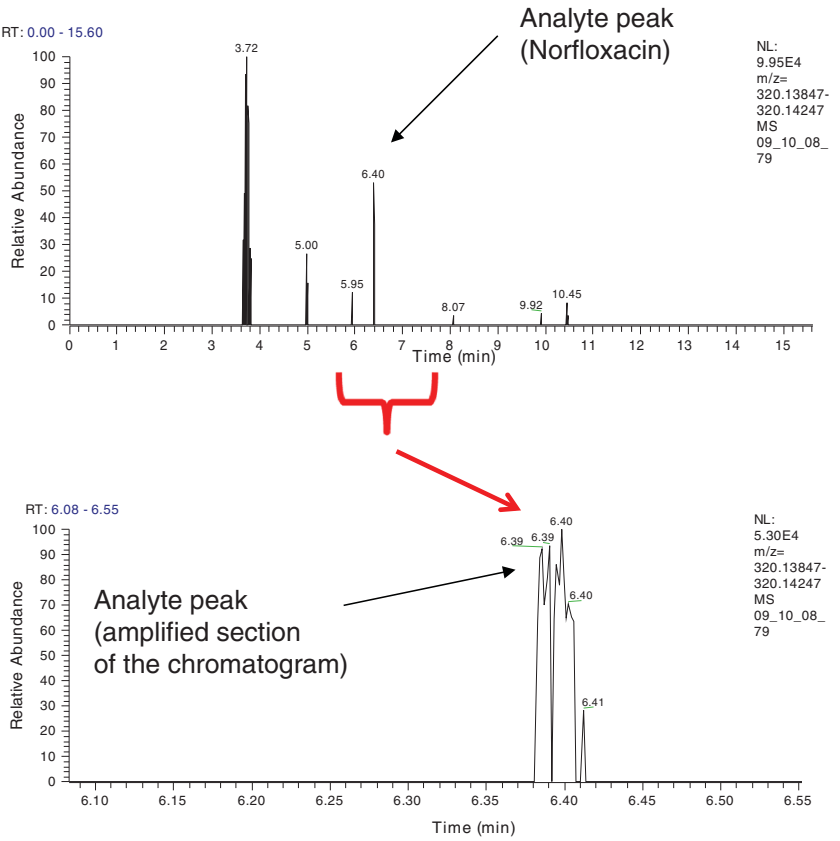


Figure 3.6 Distortion of a chromatographic analyte signal by applying a too narrow mass extraction window (norfloxacin with 10,000 FWHM and 2 mDa mass extraction window).

Figure 3.6, which shows the performance of a TDC TOF instrument). A number of authors have provided guidelines for selecting appropriate mass windows.[3, 11, 12]

However, there is no clear consensus. Appropriate mass windows depend on the mass of the analyte, the observed matrix interferences, the mass resolving power of the instrument, and the way data are acquired, processed, and stored (e.g., continuum vs centroid data).

In terms of selectivity and diagnostic power of the techniques, it is not easy to make a comparison between selected reaction monitoring (SRM) transitions (QqQ based) and HRMS. Some SRM transitions can be highly diagnostic while others (e.g., involving the loss of water) provide a significantly lower degree of selectivity. For the most part, the authors of a given study assessed that a particular (empirically determined) mass resolving power is sufficient for their application. A more comprehensive study investigated the occurrence of false positives, as caused by the injection of an analyte-free blank matrix.[13] Some 100 random SRM

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(for QqQ instruments) and accurate mass signals (for HRMS instruments) were studied. Using such a large number of monitored masses resulted in many traces containing chromatographic signals caused by unidentified matrix compounds (shotgun principle). Comparison of the number and intensity of the observed signals (false detects) leads to the conclusion that a mass resolving power of 50,000 FWHM corresponds to unit mass MS/MS selectivity. This conclusion has been confirmed by the use of more theoretically based approaches.[14]

3.2.2 Quantification

For the majority of applications in sports drug testing, detection of a prohibited substance using qualitative methods is sufficient. By contrast, techniques used in the field of food safety have to be quantitative. As a result, any new technology will be directly compared against the QqQ benchmark. For a number of reasons, the early HRMS instruments could not compete with QqQ in this respect. This refers to the sensitivity, dynamic range, and, last but not least, the availability of stable and user-friendly software for routine applications. However, this has changed significantly with the introduction of modern HRMS instrumentation that provide, among other benefits, a significantly extended dynamic range compared to early models. There have been a number of papers reporting a direct comparison of the quantification capabilities of HRMS versus QqQ.[15–19] The statistical data provided by these studies show that there is no relevant difference between the two techniques.

3.2.3 Sensitivity

Different definitions for the term "sensitivity" are used within the field of mass spectrometry. This includes the change in ion current per unit mass of sample flow (IUPAC Orange Book[20]) or the slope of the calibration curve (IUPAC Gold Book[21]). The IUPAC definition, while useful, relates more directly to the strength of signal generated by analyte rather than the detection capability of an instrument or method, particularly when dealing with a complex matrix. Within the mass spectrometry community, it is more common practice to report the LOQ or LOD. In order to provide a realistic comparison of instrumentation and methods based upon the available literature, it is necessary to use this more commonly applied concept of sensitivity. For this reason, within this chapter, the term "sensitivity" refers to the capability of a mass-spectrometry-based detection system to detect and quantify small amounts of analytes within a complex sample matrix (see Chapter 1 for further discussion of the term "sensitivity").

The first generation of QTOF instruments were appreciated for their ability to assist the identification of unknown compounds. However, their poor sensitivity and questionable mass axis calibration stability proved to be limitations, and as a result, they were seldom used in environmental or food chemistry. More recently, the situation has changed significantly. While the SRM mode of QqQ instrument

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still represents the most sensitive detection mode available, HRMS sensitivity, mass axis stability, and ease of use have improved such that it can be used now for almost any residue application. The sensitivity of response of current TOF and Orbitrap™ instruments is probably not significantly different.[22] Direct and meaningful comparison is difficult in that each type of instrument is equipped with a different interface, the contribution of which might be more significant than the difference between the detection technologies used. Furthermore, TOF sensitivity improves when the user is willing to accept a lower data acquisition rate. This is not the case for the Orbitrap™, where a lower data acquisition rate improves the mass resolving power but hardly affects the sensitivity. The result is that both technologies have their advocates and generalizations regarding the superiority of one technology as compared to another are highly application-dependent.

There are three important aspects to consider when making a sensitivity comparison between QqQ and HRMS instruments. Firstly, the SRM mode of QqQ instruments provides the highest sensitivity only when sufficiently long dwell times can be applied. In general, for multi-residue methods, the length of dwell time must be compromised, particularly when used with high-efficiency separation techniques. Due to this, monitoring 100 or more analytes may require the use of retention time window–based SRM acquisition in addition to short (low sensitivity) dwell times. The establishment and maintenance of these retention time windows are not only time-consuming but also error-prone.

Secondly, it is a common or, in some cases, the required practice, that a positive finding has to be confirmed by monitoring a second SRM trace (transition). While a second transition may be available, it is not uncommon for this to be significantly less intense than the quantification trace, a situation exacerbated by the need to include numerous other additional transitions. Hence, it is often the exception rather than the rule that a suspected low intensity peak can be confirmed by a second SRM transition. It is an advantage of HRMS instruments operated in full-scan MS mode that in many instances, additional diagnostic signals are available without significantly affecting the optimum instrument settings. This may be the presence of diagnostic adducts, ions resulting from fragmentation in the interface or characteristic isotope patterns. The presence and the relative ratio of one or more of these can provide useful additional data for identification or confirmatory purposes. Furthermore, where MS/MS must be applied to obtain the required selectivity, in the case of Q-HRMS instruments, all product ions are visible (full product ion spectrum). This is an important advantage over the single ion monitored in the conventional SRM trace. Modern Q-Orbitrap™ instruments even permit the prolonged collection and storage of precursor ions in the C-trap to produce high-sensitivity product ion scans. Last but not least, the accurate mass of a product ion provides a significantly higher selectivity than unit mass resolved (SRM based) product ions and increases the confidence in the analytical finding. While these are major benefits of Q-HRMS, it should be noted that TOF

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and Orbitrap™ instruments do not permit some typical and at times useful QqQ techniques such as neutral loss or precursor ion scan modes.

Thirdly, various groups have made a number of sensitivity comparisons between QqQ and HRMS.[16, 17] Some of these attempts have been criticized as they frequently involved the use of the latest HRMS instrument versus a significantly older QqQ instrument. Such a comparison is potentially misleading, considering the increase of QqQ detection sensitivity over the past decade.

It is probably safe to say that sensitivity was until recently a justifiable reason to buy a QqQ instead of a HRMS instrument. Considering the latest data provided by a number of independent research groups, HRMS sensitivity seems to be adequate for handling the vast majority of modern residue applications.

3.2.4 Validation of HRMS-Based Methods

While there is no fundamental difference between validation for QqQ and HRMS methods, some attributes of HRMS analysis require a slightly modified approach. Before initiating the validation process, the analyst must investigate the required mass resolving power and the acceptable mass extraction window for a HRMS method. Most authors of TOF-based methods use the highest available mass resolving power provided by their instrumentation and mass extraction windows of 5 ppm. Orbitrap™-based methods frequently use a mass resolving power of 50,000 or 75,000 FWHM. This is clearly below the maximum instrumental performance, but permits a data acquisition rate that is adequate for fast-eluting chromatographic peaks. Once decided, these parameters cannot be changed without undertaking a re-validation of the process. This is different from QqQ-based methods as these parameters are not available. It should be noted that in the case of full-scan (single-stage) HRMS methods, provided the acquisition parameters are not altered, additional analytes can be readily added and the validation is extended to cover these new compounds without affecting the initial validation. In the case of QqQ-based methods, addition of new analytes will in almost all instances impact the acquisition parameters applied and, as a result, the existing validation.

Another relevant difference is the frequently observed absence of any baseline noise in HRMS data as extracted mass traces based on narrow mass range windows often show the analyte peak seeming to rise directly out of a baseline-free chromatogram. This facilitates the integration of peaks; however, it can cause problems when determining the LOD, LOQ, or related parameters such as CCα or CCβ since the calculation of these values is based on the comparison of the intensity of the analyte signal versus the average detector noise. Some detection-based parameters can conventionally be calculated by use of linear regression and/or from signal-to-noise measurements. The regression approach relies on a number of assumptions, for example, the linearity of the calibration curve and the homoscedasticity of the data. It is common for multi-residue methods that compounds show different behavior, which can result in a mix of linear and non-linear

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calibration curves. The generation of non-linear curves may be due to analyte adsorption in the HPLC vial, degradation during chromatography, poor chromatographic peak shapes, or some analytes exceeding the linear range of the ion source/detector. As a result, regression-based calculations can produce very questionable results when a single approach is applied across several analytes. On the other hand, signal-to-noise based techniques require the presence of a measurable noise, which as mentioned before is often absent when using HRMS.

A solution to these problems has been suggested based upon calculating detection performance values using the standard deviation of low analyte spiking concentrations. In this approach, the LOD is reached when the relative standard deviation of repeated measurements approaches the absolute value of the measurement. It has been proposed that CCβ is reached when the relative standard deviation reaches a third of the measurement value established by repeatedly injecting fortified samples.[23] For this to be demonstrated, the fortification range should encompass the expected CCβ, Figure 3.7 illustrates this procedure. Testing the response of samples with a low concentration fortification is also an efficient tool to ensure the absence of isobaric interferences, which may result from the interaction of matrix components, an insufficient mass resolving power, and too narrow a mass extraction window.

While the lack of analytical noise may introduce the need for procedural differences between high- and low-resolution data, a more significant limitation is that the EU validation and confirmation guideline (2002/657/EC[24]) does not include sufficient information on criteria related to the use of modern HRMS.[11] This is, in part, a reason why there is some reluctance to use HRMS-based methods in a regulated environment such as the analysis of veterinary drug residues in animal-based food. The absence of suitable HRMS confirmation criteria in the guideline 2002/657/EC has been realized, and as a result, recently, there has been

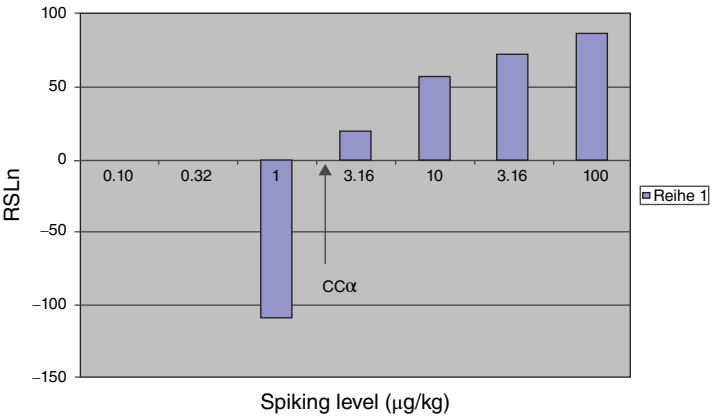


Figure 3.7 Determination of detection related parameters such as LOD or CCα in a detection-noise-fee environment.

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an increased effort to devise appropriate HRMS-based confirmation criteria.[25, 26] Analysts who do not have to comply with the EU validation guideline have been adhering to the recently updated Codex Alimentarius guideline for methods of analysis for veterinary drug residues,[27] which contains requirements for mass spectral confirmation similar to those found in 2002/657/EC.[24] Guidance on the use of mass spectrometric methods is also contained in another Codex Alimentarius Commission guideline for the analysis of pesticides,[28] but this guideline also precedes the development and use of current HRMS instruments.

3.2.5 Method Diagnosis Tools

Prior to the emergence of LC-MS, it was considered good chromatographic practice to monitor the dead volume signal, the height of the baseline, or the presence of unexpected peaks, all of which provided valuable diagnostic measures of the analytical performance. This has all been abandoned by relying on highly selective SRM traces, which reveal little other than the presence or assumed lack of the analyte. Issues that could affect the method performance such as an impure mobile phase, strongly bleeding analytical column, or a wrongly processed sample extract will not be detectable from the SRM of an analyte alone. As a result, assessment of the performance of an SRM method is heavily reliant upon the detection of internal standards. By contrast, the availability of HRMS full-scan data provides a wealth of diagnostic data unavailable when relying on QqQ instruments, to a point where HRMS can be used as a QqQ troubleshooting tool.

3.3 Analytical Methods for Veterinary Drug Residues

3.3.1 Initial Applications (Non-antimicrobial Veterinary Drugs)

The first generation of orthogonal acceleration TOF instruments with mass resolving powers of 10,000 FWHM have been used for the analysis of relatively simple matrices, an example being the screening of bovine urine samples for the presence of veterinary drug residues.[29] In this application, semi-quantitative trace concentration detection of more than 100 different veterinary drugs (primarily antibiotics, benzimidazoles, and tranquilizers) was achieved. The first quantitative and fully validated HRMS-based veterinary drug multi-residue method was published in- 2008.[4] The authors considered that their use of a TOF instrument with a resolution of 12,000 FWHM was the limiting factor for the quantification of low concentration analytes present in difficult matrices such as the liver and kidney. They concluded that the observed isobaric interferences could be reduced by the use of an instrument with a higher mass resolving power. Furthermore, the availability of a high chromatographic separation power (based upon sub-2-μm particulate stationary phase) was considered to be essential.

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There are currently only a few papers focusing specifically on the detection and quantification of non-antimicrobial veterinary drugs (e.g., benzimidazoles, coccidiostats, anthelmintics, non-steroidal anti-inflammatory agents, tranquilizers, or antiviral drugs) by the use of HRMS. However, such compounds were included in a number of multi-residue methods, which focused on a wider range of veterinary drugs (e.g., antimicrobial agents). Hence, such methods are also discussed in the following sub-sections.

Orbitrap™-based instrumentation appeared significantly later on the market. Furthermore, the first Orbitrap™ instruments were neither developed nor marketed for residue-based applications. More recently, the Orbitrap™ technology has become more affordable and the instrumental specifications have permitted their successful use in the field of routine residue analysis. This is reflected by the number of published scientific papers reporting the use of Orbitrap™ versus TOF.

3.3.2 Methods Limited to a Single-Drug Group

There are probably two major reasons why only a few HRMS-based methods (TOF as well as Orbitrap™) have been published for single (non-antimicrobial) drug groups. Firstly, HRMS users tend to cover as many analytes as possible by attempting to detect any exogenous compound, which might be expected in a particular sample and seldom limit their effort to monitoring only a particular family of compounds. Secondly, routine HRMS is still a relatively new and expensive technology, and it has not been the aim to replace well-designed LC-MS/MS methods by LC-HRMS equivalents. Also, as mentioned earlier, until recently, sensitivity and ease of use were further issues. In particular, LC-HRMS (e.g., first generation of QTOF instruments) methods showed significantly lower sensitivity than QqQ LC-MS/MS methods, provided the scope of the latter was limited to a relatively small number of compounds. As a result, there has been a lack of published HRMS methods developed for the analysis of sensitivity-critical single-residue applications; for example, chloramphenicol or the nitrofurans. This situation is likely to change in the foreseeable future as deduced from the most recent HRMS papers.

More recently, a limited number of publications reporting the quantification of single-drug groups by HRMS have been published. The capability of HRMS to perform quantitative work was shown for residues of anthelmintic drugs in milk and muscle tissues.[16] In this paper, the authors validated an extraction and clean-up approach based on the European Communities' validation concept (2002/657/EC[24]) by splitting the prepared samples and injecting them onto both an LC-Orbitrap™ (single stage operating at 50,000 FWHM) and a QqQ LC-MS/MS instrument. Virtually identical validation data were obtained from both technologies, and it was concluded that the HRMS is equally suited for quantitative work. In addition, higher sensitivity was obtained for some avermectins when using HRMS. This was explained by the fact that these analytes

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ionize in the electrospray ion source by the formation of sodium adducts, while only a low-abundance $[M+H]^+$ signal can be observed. Most QqQ methods are based on the $[M+H]^+$ ions as these tend to fragment readily. By contrast, the sodium adducts of most analytes show very poor fragmentation properties, resulting in relatively poor sensitivity. No such limitations exist for full-scan HRMS, since this technique permits the direct detection of the intense sodium adduct. It is also worth noting on a related point that modern Q-Orbitrap™ systems allow the isolation of multiple ions in small mass windows, so the resultant high-resolution SIM data can provide even higher sensitivity than full-scan HRMS but at the cost of the wide range detection that full-scan HRMS provides.

HRMS methods focusing entirely on antiviral agents and tranquilizers have also been published. One method used an Orbitrap™ instrument,[30] while another relied on a QTOF.[31] Both papers report good quantitative performance capabilities for monitoring residue concentrations.

3.3.3 Methods Covering Multiple-Drug Groups

Early reviews covering residue analysis of veterinary drugs recognized the potential of HRMS-based methods, but also stressed the limitations (e.g., sensitivity, selectivity, and dynamic range).[32] The availability of instruments with higher mass resolving power led to the development of a number of multi-residue methods for milk,[33, 34] as well as eggs and fish.[35, 36] Significant advances regarding the sensitivity and selectivity were reported following the introduction of higher resolving power Orbitrap™ instruments.[16, 30, 37, 38] Some recently published HRMS methods report the coverage of a wide range of veterinary drugs using Orbitrap™ [37–39] and QTOF[40, 41] instruments. A recent review discusses applications of HRMS methods to the analysis of veterinary drugs in aquacultured products.[42]

There are various reasons for analyzing multiple-drug groups by the use of a single analytical method, not least of which is the potential for higher efficiency and lower cost per analyte. In addition, it may be important to know if drugs have been utilized to treat a particular disease. However, drugs showing identical therapeutic effects may be structurally very different chemicals and, as a consequence, show a wide range of chemical or physical properties. As an example, coccidiostats are used to treat parasitic protozoa (coccidiosis), which can affect poultry, pigs, or rabbits. Drugs showing activity against protozoa can belong to the family of polyether ionophores (e.g., narasin, lasalocid), the triazines (e.g., toltrazuril), or organoarsenic compounds (e.g., roxarsone). Hence, the control of the use or abuse of coccidiostats requires the availability of analytical methods, which can detect and quantify analytes that are structurally widely divergent.

Furthermore, there is an increasing interest in multi-residue methods due to consumer expectations that food is not only free of specific compounds but free of any potentially harmful contaminant or residue at relevant concentrations. This

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may refer to the absence of not only veterinary drugs, but also pesticides, mycotoxins, or marine toxins, as well as environment-related contaminants.[25, 35, 38] The fulfillment of such expectations (non-targeted strategies) represents an enormous challenge for analytical chemists, and realistically, this aim is currently not achievable, yet the continuous evolution of analytical technologies permits the pushing of current boundaries toward that ultimate goal.

3.3.4 Method Components

3.3.4.1 Extraction and Clean-up

Ideally, extraction and clean-up methods should result in a significant concentration of the analytes of interest with highly efficient recovery present in a solvent that is applicable to the analytical method of choice. Furthermore, the final residue should contain the fewest possible number and lowest possible concentrations of interfering co-extracted materials. In reality, this represents an ideal that cannot be met, particularly where the desired end point is multi-analyte detection of a large number of compounds. A further complication is the wide range of matrix types, including solids and liquids, that may be presented to the analyst. Where the matrix is a solid or semi-solid, initial extraction to a liquid medium is frequently required prior to clean-up. As some separation of analytes and interference almost certainly occur, extraction may also be considered to be a part of the clean-up process.

The exhaustive extraction of drug residues requires the proper selection of suitable extraction solvents, and typically, the polarity of the solvent has to correspond to that of the analytes that are to be extracted. Furthermore, solvent polarities have not only to match the polarity of the analytes, the utilized solvent should also extract as little as possible of the bulk matrix compounds. When dealing with animal tissues, the matrix components that it is desirable to exclude are primarily fats and proteins. By contrast, the co-extraction of carbohydrates from honey represents the predominant matrix component of which the presence should be limited. When dealing with animal tissues or even blood, there are further complications. For example, some drugs can be bound to cell organelles, to cell walls, or even simply to proteins. A very significant issue is that such compounds may be easier to recover from spiked samples than from incurred samples, thus giving the impression of higher recovery than will in fact be the case in real-world samples. This represents a particular problem for proper method development, since there are only very few incurred samples available. Hence, it is virtually impossible to investigate the matrix–drug interaction for all possible analyte and matrix combinations.

Acetonitrile or acetonitrile–water-based solvent mixtures are popular for the extraction of a wide range of analytes. This is partially due to the fact that acetonitrile is a solvent of good intermediate polarity and also effectively precipitates

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dissolved proteins. This is in marked contrast to methanol, which extracts significantly larger amounts of endogenous matrix compounds from the samples. On the other hand, less polar solvents such as ethyl acetate, which efficiently partition proteins, co-extract lipids. A further complication is that a significant number of veterinary drugs contain nitrogen heteroatoms, which render these compounds basic. Such ionic interactions either require the use of counter ions or an extreme pH environment to improve extraction efficiency. Clearly, it is important to ensure that the extraction pH does not cause the degradation of acid or base labile analytes. Frequently, a readjustment of the pH value after the extraction and centrifugation step is required to limit the degradation of the most sensitive analytes.

There is a significant difference between clean-up techniques employed for single-compound or single-group versus true multi-residue methods. Single-group methods often rely on the use of a more or less selective clean-up strategy. This is often achieved by the use of a pH-controlled ion-exchange clean-up, which may be based on liquid/liquid extraction or more frequently the use of solid-phase extraction. Such steps not only produce cleaner but also significantly concentrated sample extracts. Clean extracts are stable, prolong the lifetime of analytical HPLC columns, reduce the downtime of the MS interfaces, and, probably most important, reduce the extent of signal suppression. Clean-up can be further improved by use of molecular imprinted polymers or antibody-based methodology. However, this significantly narrows the range of analytes that can be recovered and is not really consistent with multi-residue analysis. Modern multi-residue methods therefore use a minimum of sample clean-up. Techniques such as Quick Easy Cheap Effective Rugged Safe (QuEChERS) have been developed for the analysis of pesticides in fruits and vegetables.[43] Such approaches have also become popular for veterinary drug residue analysis in animal tissues.[38, 42] Major benefits are that the QuEChERS-based extraction and salting-out step recovers a wide range of analytes. Depending on the matrix, resins with specific modifications such as primary or secondary amine functionalities can be used to further remove interfering matrix compounds. However, such additives always have some effects on the recovery of particular analytes. QuEChERS is probably better suited for analysis of pesticides, rather than for veterinary drugs, as the latter are for the most part significantly more polar. Hence, polar veterinary drugs and even more polar metabolites of such drugs are often insufficiently extracted by the QuEChERS technique.

A popular clean-up method for multi-residue analysis is the use of reversed-phase solid-phase cartridges.[33, 38–42] This is now often done with polymeric materials as they do not contain unshielded residual silanol groups, which can introduce unwanted secondary interactions. Such polymeric materials are well suited to significantly reducing the abundance of co-extracted proteins and in general are more user-friendly than silica-based C_{18} materials as they are not prone to drying and the resultant phase collapse. On the other hand, loading of extracts free of organic solvents is required in order to prevent the

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breakthrough of polar analytes. Furthermore, the quantitative elution of some very non-polar analytes from the SPE cartridge can be problematic.

Clean-up often requires a solvent exchange or concentration step. This is not always straightforward, as the evaporation and reduction of the organic solvent content from the extract will reduce the solubility of both matrix interferences and the analyte in the extract. The precipitation of matrix-related compounds may block analytical columns while the precipitation of proteins can also result in the co-precipitation of analytes. Furthermore, some analytes are attracted to and efficiently bound by the glass wall of an extraction vessel. Analyte lost to the extraction vessel or via precipitated matrix material is frequently entirely lost or only poorly recovered. Hence, for some analytes, evaporation to dryness should be avoided, and in some cases, the addition of a "keeper" such as dimethyl sulfoxide has been used to combat such losses.[37] Other steps that may help in maintaining sufficient analyte recoveries include the use of silylated glass vessels or plastic equipment.

Despite these apparent complications, it should be remembered that currently, the most relevant and unpredictable source of issues or uncertainty in LC-MS(/MS) analysis is posed by signal suppression effects as caused by co-eluting endogenous matrix compounds. Hence, the efficiency of a clean-up should be assessed based not only on the recovery rates obtained for the analytes of interest but also on the extent and impact of signal suppression effects.

3.3.4.2 Separation

Regardless of the MS technology employed, good chromatographic separation improves the reliability and accuracy of mass-spectrometry-based detection. The use of flow injection as an inlet for mass spectrometers has been largely abandoned due to poor selectivity, sensitivity, and the matrix-dependent extent of signal suppression effects in atmospheric-pressure ionization MS interfaces. In addition, even high-resolution accurate mass instruments fail to provide fragmentation data capable of identifying close structural isomers. This is of particular importance in the area of steroid analysis where multiple isomeric compounds are encountered and chromatographic separation remains a primary requirement for the correct identification of an analyte. Therefore, rather than a move away from chromatography, there is currently a trend toward enhanced chromatographic separation power. This has been initiated by the availability of sub-2-µm particular chromatographic columns, which provided an increased separation power while maintaining or even shortening the chromatographic run times. The successful use of sub-2-µm separation columns required the introduction of dedicated ultra high performance liquid chromatography (UHPLC) systems. This refers not only to the required increased pump pressure but also to the reduction of detrimental void volumes within the flow path of the instrument. The introduction of core–shell column technology was initially intended to have sub-2-µm separation performance available for every LC system. However, sub-optimal separation performance could not be completely

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avoided because of the relatively high dispersion inherent to conventional HPLC equipment. The widespread availability of UHPLC systems has caused core–shell column producers to develop such columns for the exclusive use within UHPLC instruments. Hence, narrower 1.7 and even 1.3 μm core–shell materials have become available. The length and internal diameter of these columns result in back pressures of up to 1000 bar, which is close to the pressure limit of modern commercially available UHPLC systems.

The use of UHPLC is primarily intended to maximize peak capacity. The resultant decrease in spectral interferences caused by matrix compounds facilitates the detection and final confirmation of suspect findings. Furthermore, the narrower peaks obtained by UHPLC produce a better signal-to-noise ratio and therefore improve sensitivity. On the other hand, the reliable monitoring of very narrow chromatographic peaks requires mass spectrometers capable of fast scanning. In addition, to attain these very sharp peaks, it is normal to operate UHPLC systems at relatively high solvent flow rates, which further challenge the API interface even on modern LC-MS systems. The fact that UHPLC provides improved separation power has led to a somewhat questionable belief that UHPLC-based separations are less affected by signal suppression than conventional HPLC separations. Signal suppression is caused by co-elution of matrix compounds together with the analyte of interest. While it might be argued that narrower peaks reduce the likelihood of co-elution, it cannot be guaranteed that this is the case, and due to the reduced peak width, it might be expected that even greater signal suppression may result if a matrix compound still co-elutes with an analyte peak.

In addition to UHPLC, a number of advances have been made in the interfacing of nano bore and capillary LC to API LC-MS instruments. Although, in theory, these provide significant enhancement in sensitivity, in practice, these systems have found little application outside of research laboratories. A further area that has recently become available and that might have greater impact is the development of UPC2 or "convergence" chromatography. This technique merges UHPLC and supercritical fluid chromatography (SFC) and uses CO_2 as the primary mobile phase. Due to the very low viscosity and high diffusivity of supercritical fluids, very high-efficiency separations can be attained at relatively low pressure. In addition, the separations attained using UPC2 can be considered orthogonal to reverse-phase UHPLC and therefore provide truly alternative chromatographic solutions. The impact that this technology will make upon the field of residue analysis currently remains open to question. The need to operate UPC2 at high flow rates with closely regulated back pressure limits the proportion of the sample that can be presented to the mass spectrometer. Further, the very high separation power of the technique results in peaks that are even sharper than those encountered in UHPLC, further pushing the scan capability of modern mass spectrometers if the full separation capability of UPC2 is to be accessed.

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3.3.5 Residue Testing of Anabolic Steroids and Growth Promoters

Although there are many areas of overlap between sports drug testing and food residue analysis, one aspect that is unique to the latter is the requirement under some circumstances to undertake direct analysis of the product, be it milk, honey, muscle, or other tissues. In common with sports drug testing, there has been significant interest in the potential for accurate mass LC-MS for the analysis of growth promoters within residue analysis of meat. In a review of current approaches and future trends in the extraction of animal-derived matrices, the authors noted that in addition to generic sample preparation methods that can suffer from unwanted matrix affects, they expected a trend toward more selective methods for confirmatory analysis.[44]

Examples of a high-throughput UHPLC-QTOF MS method include a multi-analyte multi-class method for the detection, quantification, and confirmation of various classes of compounds including beta-agonists, most of which are banned in meat production as potential growth promoters in many countries (all such use is banned in the EU). Initial extraction of egg, milk, or meat was carried out with acetonitrile followed by solid-phase clean-up. The wide range of compounds successfully covered in relatively complex matrices may at least have been assisted by the long run time of 30 minutes, which would help separate isobaric interferences and minimize ion suppression affects.[35] A further example of the application of UHPLC coupled to a QTOF instrument to detect a range of steroids in muscle demonstrated how the QTOF allowed the use of accurate mass analysis to be carried out using either full-scan or accurate mass MS/MS acquisitions. Comparisons were carried out against both QqQ and Orbitrap™ instruments and the method was validated to EU requirements.[45, 46] It was concluded that QTOF-MS provided good quantitative and confirmatory performance using the TOF-MS/MS mode and that the full-scan TOF-MS mode provided potential screening for new designer drugs.

The potential of single-stage accurate mass detection for confirmatory analysis of anabolic steroids, again utilizing UHPLC, has also been studied. In this instance, analysis was carried out using an Orbitrap™ mass spectrometer running at 50,000 resolution, which was compared to tandem mass spectrometry for the confirmation of anabolic steroids in meat.[46] The authors concluded that the technique had great potential particularly for untargeted screening and detection of unknowns. For confirmatory analysis, they concluded that single-stage MS at 50,000 resolution had the potential to compete with triple-quadrupole instruments in terms of selectivity and specificity. Quantitative assays also showed good linearity and precision, but for some analytes, the system could not attain the required detection and quantification limits and, in this respect, was inferior to triple-quadrupole instruments for this application. While accurate mass single-stage MS may not always attain the performance available from triple-quadrupole systems, modern accurate mass MS/MS systems are capable of the detection of very low concentrations of target analytes. One example of this is

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demonstrated by a UHPLC–Orbitrap™ method for the detection of zearalenone in pig and chicken plasma.[47] The method demonstrated comparable detection capabilities to an LC–tandem MS analysis using a triple-quadrupole instrument.

Analysis of hair has been used in forensic and occupational drug testing areas for some time, largely due to the ability to provide extended detection periods (up to several months postdose), the relatively non-invasive nature, and ease of sample collection. The application to residue testing has been investigated for some time and was sufficiently mature to have been reviewed in 2006.[48] The majority of methods have been targeted using the highest sensitivity GC- and LC-MS (/MS) techniques, but accurate mass approaches have also been investigated. For example, UHPLC and Orbitrap™ full-scan HRMS at a resolution of 60,000 were used to detect steroid esters in bovine hair at low nanogram per gram concentrations.[49] The performance of the same Orbitrap™ system operating at 7500 resolution or a TOF system operating at 10,000 resolution was significantly reduced due to the inability to separate analyte ions from interfering signals. The fact that analysis of hair provided extended detection of the steroid esters is an important aspect of the method as the esters are entirely exogenous and represent clear evidence of the administration of steroidal growth promoters, even when the active steroid is in itself endogenous. While it is clear that as accurate mass systems become increasingly sensitive, they hold out the possibility of providing an extended detection period for a large range of compounds, the choice of matrix and analyte is equally important. This application demonstrates the potential for the detection of agents with the ability to improve performance (sports testing) or increase body mass (meat residue analysis) long after they are no longer detectable in more traditional matrices of blood, urine, and tissues.

There are a number of further cases where detection of anabolic agents in meat-producing animals is complicated by the endogenous nature of a steroid.[50] For example, the detection of boldenone (and its prodrug boldione) abuse in cattle has been complicated by the recognition that boldenone is also an endogenous steroid thought to arise through conversion of phytosterol precursors in feed via gut microflora. Direct detection of 17β-boldenone sulfate, a metabolite of administered boldenone/boldione, has been proposed as a potential differentiator and the application of accurate mass LC-MS using an Orbitrap™ to assist defining appropriate criteria for detection of abuse described.[51]

In a further example of studies related to endogenous steroids, accurate mass LC-MS has also been used in combination with *in vitro* methods to investigate the metabolism of the steroid prohormone DHEA.[52] In this example, liver slices were treated with DHEA and various techniques applied to monitor the effect. In addition to an examination of gene expression of the slices using transcriptomics, an androgen expression assay was used to monitor changes in androgenic activity with the metabolites responsible for the changes in androgen response being identified by UHPLC-TOF mass spectrometry. Androgen receptor assays

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have also been used to investigate designer steroids,[53] an application that will be discussed later in this chapter.

3.4 Doping Control

The use of accurate mass techniques for the detection of drugs in human and animal doping is an active area of interest and much work has been carried out in this field. This is especially true in the area of growth promoters, particularly androgenic anabolic steroids (AAS) and β2-agonists. These analytes represent areas where there is a significant overlap in interest between sports and residue testing, particularly in the area of equine sports testing where the synergies with residue testing and food safety are obvious. While the links to human sports testing may be somewhat more tenuous, the extent to which accurate mass MS is used in doping control should be considered indicative of the likely future direction of veterinary drug residue testing. For some years, an annual review of the field of human drug testing has been carried out by Thevis et al.[54], and this provides an excellent point to assess the increasing importance of accurate mass applications in this field.

While reviews of this nature are a good indicator of the interest expressed in a technique, they do not necessarily reflect the number of laboratories employing a technology. This is certainly true for animal sports drug testing where, from the authors' experience, the use of accurate mass techniques for screening for a range of drug types is now commonplace, yet there are relatively few publications in readily accessible peer-reviewed journals. The extent to which there is a good degree of common ground between doping control and veterinary drug analysis is shown by the publication of an excellent review of the application of accurate mass technologies in both fields.[55]

3.4.1 GC-HRMS

There are relatively few published applications of accurate mass GC-MS technologies to residue testing. However, in a related application, high-resolution accurate mass GC-MS has been applied for some time to the detection of steroids in human urine using sector instruments operated in SIM mode.[56] The technique has proven extremely sensitive, in part due to the improved signal-to-noise offered by the selectivity of the accurate mass platform, although the enhanced signal provided by sector instruments when compared to older quadrupole GC-MS systems is also a contributory factor. In a further example of the use of sector GC-MS, anabolic steroids and their esters were detected in hair samples following administration to the horse.[57] While the use of sector instruments for the GCMS analysis of AAS is well established, the high cost and relatively high degree of operator skill required have limited its application outside a small number of specialist laboratories.

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Other than sector instruments, the only other commonly available accurate mass analyzer routinely applied to GC-MS is TOF. A review of the application of the technique in the environment, food safety, and toxicology fields has recently been published.[58]

3.4.2 Accurate Mass LC-MS and LC-MS/MS in Doping Control

Following the introduction of reliable accurate mass LC-MS systems in recent years, there has been a marked move toward multi-class and multi-analyte test methods for sports drug testing similar to that seen in food residue analysis applications. Drivers for this have been the demand to incorporate more substances into screening methods and provide retrospective data analysis for untargeted analytes without sacrificing the low sample consumption and fast turnaround provided by modern triple-quadrupole instruments. Development that has made this feasible is the ability of modern mass spectrometers to provide the required scan speed and/or resolving power to cover hundreds of analytes per analytical run.[59]

While the sensitivity and scan speed of modern triple-quadrupole instruments have allowed a significant improvement in the number of analytes that can be covered using SRM experiments, the use of dedicated precursor/product-ion pairs only provides data for the targeted analytes. As a result, there has been a trend toward either non-targeted methods utilizing full-scan MS and accurate mass data acquisition or, where enhanced sensitivity is required for a limited number of analytes, combined targeted/non-targeted analytical methods. For the latter, LC-MS(/MS) approaches using hybrid instruments consisting of quadrupole or ion trap mass selection devices and TOF or Orbitrap™ analyzers have been used.[59–62]

A limitation of the use of LC-MS-based methods for the detection of AAS in both doping control and food residue testing is that many of the metabolites excreted in urine do not ionize well in their unconjugated state under atmospheric-pressure ionization conditions (Figure 3.8). One approach to circumvent this issue is the formation of derivatives with good liquid chromatography and mass spectrometry properties, for example, the use of oximes or Gerard's reagent P for keto steroids.[63] The latter reagent introduces a permanently charged quaternary ammonium ion, which provides significantly enhanced LC-MS sensitivity. In this instance, steroids were targeted using HPLC separation and a QTOF instrument. The use of accurate mass measurement improved selectivity despite the relatively modest resolving power of the instrument. In general, when applying accurate mass techniques to MS/MS experiments, lower resolution can be used as many of the potential isobaric interferences are removed by the first stage of mass spectrometry.

In the field of equine doping control, LC-HRMS is now widely used by a number of testing laboratories. Published examples include the analysis of 320 agents in equine plasma using an Orbitrap™ operated at 60,000 resolution following SPE

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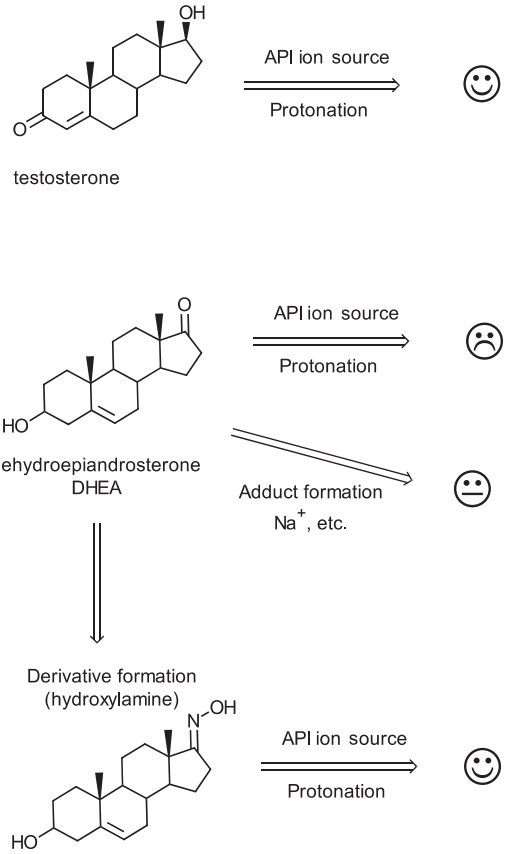


Figure 3.8 Steroid ionization under atmospheric pressure conditions, analytes lacking a 3-keto-4-ene in the A ring may require derivatization.

extraction and UHPLC separation.[64] The method was demonstrated to provide coverage for a wide range of drug types, most relevant in terms of potential application to veterinary drug residue analysis in foods being a number of anabolic steroids and β2-agonists. An interesting aspect of the method was the fact that two data processing approaches were taken, one to provide qualitative detection of drugs and a second to quantify 73 compounds via a single-point calibrator. Single-point calibration involves the extraction and analysis of a standard or standards at a single specific concentration analyzed within the analytical run rather than a calibration curve made up of multiple samples at different concentrations.

3.4.3 "Dilute and Shoot" with Accurate Mass LC-MS

The methods discussed so far largely relied upon extraction and concentration of the (urine) sample prior to injection. In addition, in most instances, enzyme hydrolysis steps were included prior to extraction. In practice, one of the main factors limiting the coverage of a HRMS-based method is the extraction method,

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and for truly generic methods, the simplest possible sample pre-treatment is desirable. As a result, a number of methods have recently been developed that rely on the so-called dilute-and-shoot approach. A recent review article focused in particular on forensic and clinical toxicology applications of this approach.[65]

The use of "dilute and shoot" has a number of advantages, particularly when used with HRMS, in that the simplicity of the sample processing method not only allows high throughput but also ensures that the maximum number of drugs and metabolites are presented to the mass spectrometer. When coupled with accurate mass instrumentation, this allows a very wide analyte coverage, as reflected in the increasing number of papers on this subject. Issues with the approach are that the sample presented to the instrument is very complicated and that ion suppression and isobaric interferences are likely the limiting factors. To an extent, these effects can be limited by extending the run time of the separation method, but this is at the cost of throughput. In the case of early applications of "dilute and shoot," the method was limited to sample types that contained relatively high drug concentrations. However, with developments in instrument sensitivity and resolving power, more sensitive assays are being developed.

A recent example of a "dilute-and-shoot" approach applied to doping control of anabolic steroids also has relevance to food residue analysis.[66] The methodology covered 21 AAS and respective metabolites in urine by targeting both unconjugated (12) and conjugated (eight glucuronic acid and one sulfate conjugate) analytes. LODs between 0.5 and 18 μg/l were accomplished with LC-HRMS by detection of the protonated ion or sodium, ammonium, or acetate adduct ions in positive ion mode or the deprotonated ion in negative ion mode. Compared to the commonly applied GC-MS(/MS) approaches, the method was technically simple and very rapid but failed to meet the required detection capability for all the targeted analytes. Despite this, direct analysis of steroid conjugates by LC-MS(/MS) provides a likely route to rapid analysis of this drug class reflected in the number of papers recently published on LC-MS(/MS) of steroids and steroid conjugates. Detection using full-scan HRMS for steroid conjugate detection would no doubt provide further advantages, although the lack of steroid conjugate standard materials currently represents a barrier to the development and introduction of such methods.

3.5 Accurate Mass MS in Research and Metabolism Studies

In addition to the increased routine application of accurate mass LC-MS, the availability and ease of use of high-resolution TOF and Orbitrap™ mass spectrometers have resulted in their wider application to research activities. Accurate mass analysis is well suited for use with *in vitro* metabolism techniques, and a

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number of examples of this application exist. Clearly, accurate mass data can provide increased structural information compared to low-resolution techniques. A further significant advantage is the fact that metabolites generated by *in vitro* techniques, which might not be observed in the target matrix, can still be incorporated into non-targeted full-scan acquisitions. An example of this is provided by a study of metabolism of a number of steroids using equine liver S9 fractions and/or microsomes, using LC-HRMS on an Orbitrap™ system.[67]

LC-HRMS on a Q-Exactive™ (i.e., Orbitrap™) has also been used to study the *in vitro* metabolism of zeranol.[68] The results were used to assist in determining if the presence of this semisynthetic estrogenic veterinary drug with growth-promoting properties was due to abuse or as a result of mycotoxin contamination of feed.

The detection of metabolites, which, although not necessarily the most concentrated at peak excretion, represent long-lived and therefore potentially more sensitive targets is becoming a common theme in human sports drug testing. Again, similar approaches may become increasingly important in food residue testing. Accurate mass LC-MS techniques can provide powerful tools for detecting these analytes at low concentrations. For example, metabolites of stanozolol, which provide long-term detection of this important anabolic agent, have been determined using UHPLC-MS/MS on a Q-Exactive™ instrument.[69] In this instance, N-glucuronide conjugates resistant to cleavage by β-glucuronidase were found in an elimination study and subsequently in several doping control urine samples. LODs between 5 and 25 ng/l were obtained, demonstrating the improvement in detection that can be obtained on modern accurate mass LC-MS/MS systems.

The *in vivo* characterization of urinary excreted metabolites of the selective androgen modulators (SARMs) S1, S4 (Andarine), and S22 (Ostarine) in the horse has been carried out using UHPLC separation and a QTOF accurate mass system.[70] The results indicated that the SARMs had a very high excretion rate in the horse and that the parent drug could only be detected for a very short time frame, if at all. The results highlight the need for detailed metabolism studies to support drug detection systems, whether for use in sport doping studies or in the detection of non-approved use of veterinary drugs.

3.6 Designer Drugs and Generic Detection Strategies

Thus far, doping control in general and human doping in particular have represented the area considered most at risk of the abuse of designer drugs. As a large number of designer anabolic agents and growth promoters have been developed and marketed, the potential for their abuse in food production cannot be dismissed. Much of the work related to doping control carried out in this area is therefore of potential interest and application in food testing.

The acquisition of full-scan accurate mass data is likely to represent the most flexible approach to the detection of designer drugs and the provision of generic

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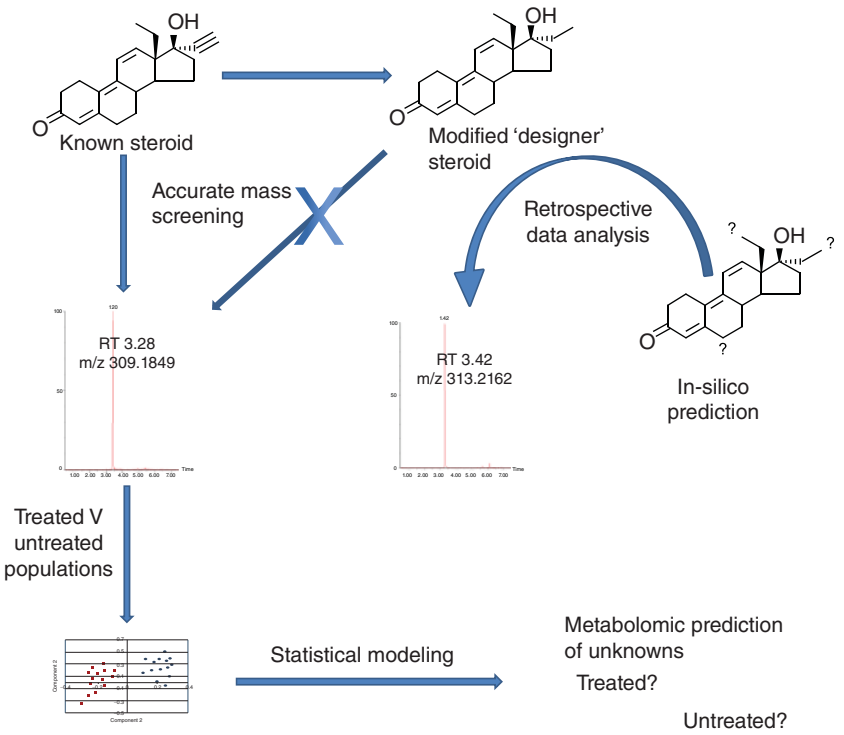


Figure 3.9 Accurate mass data and designer drugs, potential for direct analysis, or detection using either targeted or untargeted metabolomics.

detection strategies, as depicted in Figure 3.9. Various screening methods have demonstrated the ability of full-scan HRMS to detect all analytes with masses that fall within the acquired scan range and that are extracted/eluted by the analytical method. Based upon this ability, the use of retrospective data analysis has become a reality. Despite this, the detection of true "unknown" doping agents directly through the use of accurate mass acquisition has proved elusive. This situation reflects the difficulty of mining the very large accurate mass data sets to extract signals for all possible analytes. In effect, while the data may be present, we are currently limited by the ability of available software to identify novel or unusual signals that might represent new doping agents. Despite this, the power to detect multiple agents without preselection does make accurate mass LC-MS a very powerful tool for use in the detection of designer drugs, through coupling the technique with novel technologies used to detect the presence of designer drugs, searching for new agents using mass or fragmentation predictions, or in the area of metabolomics.

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Accurate mass LC-MS in combination with an androgen bioassay has been used to identify potential designer steroids.[54] The comprehensive detection capability of full-scan MS, the additional structural information provided by the ability to assign molecular/fragment ion formulae, and search an accurate mass database were vital attributes of the MS acquisition. In this case, the method was applied to a variety of herbal products and sports supplements, but in theory could be applied to other matrices. However, the approach is sufficiently involved that it could not be readily applied as a generic screening method. In an example of using predictive approaches, LC-MS employing TOF analysis and a modified software program was shown to support the detection of *in silico* predicted modifications (including oxidation, reduction, hydroxylation, etc.) of corticosteroids and anabolic–androgenic steroids in urine specimens.[71]

3.6.1 Metabolomics in Food/Residue Analysis

While the aforementioned represent valuable research into the detection of designer drugs and in particular novel steroids, the "omic" technologies represent the most active area of research into the detection of designer drugs. Equally or possibly more importantly, the "omic" approaches also have the capability to provide highly generic detection methodologies for drug classes based upon pharmacological activity rather than structure. A thorough review of the "omic" technologies in food technology has recently been published.[72] While interest in both proteomic and transcriptomic analysis remains, of late metabolomics has become the primary "omic" technique investigated in terms of application to residue analysis in food. This is also true for doping analysis, and there is a particularly strong overlap in the area of growth promoters and anabolic steroids. Recently, various reviews of the use of mass-spectrometry-based metabolomics have been published.[73–75] The earliest of these focused specifically on food sciences and included a useful description and appraisal of a metabolomic workflow, with sample preparation, including solid and liquid matrices, data processing, biomarker identification, and implementation of screening methods being described. A later review identifies the potential for the use of accurate mass LC-MS techniques for this application, particularly in instances where non-targeted approaches are used.[76] Interestingly, of the six applications of GC-MS or GC-MS/MS cited in this review, none utilized accurate mass techniques. The majority of the LC-MS(/MS) metabolomic studies, however, utilized accurate mass, no doubt due to the suitability of accurate mass data when applied to non-targeted studies.

In terms of applicability and preference between TOF and Orbitrap™ mass analyzers for metabolomic studies, TOF has the benefit of very fast scanning while Orbitrap™ can provide very high resolution and mass accuracy. A metabolomic study into the detection of anabolic steroid administration to the calf comparing HPLC coupled to an Orbitrap™ and UHPLC coupled to a TOF concluded that both technologies were appropriate for this application.[77] In general, the "omics"

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technologies attempt to cover the widest possible range of analytes. An interesting approach to extend the number and polarity of metabolites covered by metabolomic analysis in the bovine following use of anabolic agents has been carried out using a combination of C_{18} reverse-phase and HILIC separations.[78] Analytes were detected using HRMS and the combined data set used for multivariate statistical treatment. Separation between treated and untreated animals was obtained, leading the authors to conclude that metabolomics offered a powerful tool for detecting anabolic steroid abuse.

A number of other papers using LC-HRMS related to metabolomic analysis for growth promoters in food-producing animals have also been published, including studies on the serum of cattle following the administration of estradiol and progesterone.[79] The detection of naturally occurring steroids such as estradiol and progesterone represents a significant issue using direct detection of the administered product or its metabolites, as this normally requires the establishment of threshold concentrations. Due to variations in the population and the need to include uncertainty of measurement, individual thresholds are frequently established at concentrations that prove too high to provide adequate detection of abuse. The metabolomic approach detects the perturbation of a number of markers, often an endogenous steroid, which can provide improved detection of abuse.

In a further example, accurate mass UHPLC TOF metabolomics allowed detection of the administration of the prohormones DHEA and pregnenolone to the bovine.[80] Other articles related to the use of metabolomics to detect small-molecule growth promoters in meat-producing animals include detection of clenbuterol abuse in calves using LC-HRMS on an Orbitrap™ instrument.[81] An initial investigation of clenbuterol abuse in calves using accurate mass LC-MS techniques was able to highlight metabolic modifications in urine. These were built into a predictive model using chemometric tools. As the model detected changes in the metabolome due to the effect of the drug rather than the presence of the drug, this approach had the potential to detect other β-agonists, including cocktail administration. A more generic metabolomic analysis of cattle receiving various β-agonists and intended to provide coverage of designer drugs of this class as they become available has also been carried out recently using Orbitrap™ LC-HRMS.[82] The performance of the model generated from these studies was consistent with EU requirements for screening methods.[83]

It should be noted that metabolomic approaches are not limited to detection of the abuse of small molecules and accurate mass LC-MS methods have been developed to detect the administration of growth hormone to the horse using both Orbitrap™[84] and TOF.[85] The detection of growth hormone abuse represents a particularly difficult area in many species due to the very short detection period of the administered product and the fact that the high degree of homology between the growth hormones of different species further complicates direct detection.

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Based upon the increasingly robust methods developed and success of metabolomic methods intended to identify abuse of drugs, particularly growth promoters, in both sports and food residue testing, it would appear that these technologies are likely to have significant impact upon screening methods in the foreseeable future. Questions do remain as to how these approaches are likely to be incorporated into testing laboratories and their relevance to confirmatory methods where the availability of secondary information indicating misuse might not be sufficient to support the imposition of a sanction. However, both laboratories and regulators appear keen to embrace these new technologies; this bodes well for the wide application of the technology.

3.7 The Future of Accurate Mass Spectrometry in Residue Analysis

Thevis and Volmer have speculated that ideally advances in mass spectrometry would result in a single multi-purpose instrument providing accurate mass data at mass uncertainties of 1 ppm or less.[59] Further, they considered it should provide linked precursor/product ion formation to allow mapping of fragmentation reactions and it should be capable of fast scanning for optimum connectivity to high-resolution separations. In terms of current mass spectrometric technology applied to chromatographic separations, accurate mass applications are dominated by Orbitrap™ and TOF instruments, with Orbitrap™ instruments generally offering higher resolution at low mass than TOF, but this is generally at the expense of scan speed. TOF instruments, on the other hand, provide very fast scan speeds with sensitivity being sacrificed at higher scan speed. At present, it seems that these analyzers will dominate the affordable accurate mass instrument market for the foreseeable future. Although the possibility of alternative technologies making a significant impact cannot be ignored, few would have predicted the speed with which Orbitrap™ instruments were introduced and accepted. Assuming that these very different technologies maintain their pre-eminence, the question of which would come nearest to the "ideal" of high sensitivity, high mass accuracy, and fast scanning arises.

In addition to the improvements in the currently available instrumental attributes (resolution, mass accuracy, scan speed, etc.), other technologies that have the potential to enhance the application of accurate mass techniques are being introduced. One such area is the emerging ion mobility technologies, for which the availability of very fast scanning instruments is highly desirable. Ion mobility provides additional orthogonal separation based upon the migration of ions against a flow of inert gas such as nitrogen. Separation is extremely rapid and could result in even faster analytical methods. An area where access to novel and rapid separation techniques such as ion mobility could provide a significant benefit is in conjunction with the use of direct detection methods. The majority of the applications and approaches discussed in this chapter

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have utilized accurate mass techniques in combination with chromatographic separation of the analyte and matrix components. Some initial studies have investigated novel approaches to detection of food contaminants, particularly for solid matrices, by the use of direct ionization processes such as direct analysis in real time (DART) or direct electrospray ionization (DESI). Both techniques allow the direct ionization of analytes from a surface, and in one application, DART has been used to detect and quantify steroid esters directly from injection solutions using an Orbitrap™ operated at 60,000 resolution.[86] DESI has also been applied to the detection and profiling of steroid esters in injection sites, although in this instance, low-resolution MS/MS was used for detection.[87] Currently, the technology has limited sensitivity compared to LC-MS methods, but the rapidity and simplicity of the technology are likely to result in a high degree of interest in such approaches.

Arguably one of the most difficult areas to predict is the extent to which bioinformatics and *in silico* approaches will enhance the analytical capability offered by accurate mass systems. *In silico* metabolism has been used along with *in vitro* techniques to support the identification of designer drugs in human urine using accurate mass data.[88] Full-scan accurate mass provides an extremely rich data set and accurate mass databases are currently being created. The availability of comprehensive databases will represent an increasingly powerful tool. *In silico* software and powerful computers could be used to convert the increased information they offer into predictions of the analytical performance, for example, fragmentation or retention characteristics of unknown or predicted analytes. It is increasingly feasible that access to this information, the ready availability of ultrafast computers, and potential to develop novel algorithms coupled to ever more powerful accurate mass instruments have the potential to radically change the analytical landscape.

In terms of likely advances in the detection of anabolic steroids and with specific regard to the potential use of accurate mass LC-MS, an issue with many of the steroid metabolites encountered in sports testing or residue analysis is that once deconjugated (cleaved from the polar sulfate or glucuronic acid group), a large proportion of the resultant metabolites have limited capability for analysis using atmospheric pressure ionization techniques in their native form. Several researchers have investigated the use of derivatization techniques to induce ionization or direct fragmentation; the majority of these approaches have been targeted at QqQ applications. However, derivatization approaches should be equally applicable to accurate mass full-scan or accurate mass MS/MS technologies. It is also feasible that specific derivatives intended to move drug classes into more specific accurate mass areas could be developed, for example, by introducing several mass-deficient atoms such as fluorine. A further area of interest has been the direct analysis of conjugated steroids. These, due to the polar/ionizable nature of conjugate groups, readily ionize under atmospheric-pressure ionization conditions. In addition, conjugate group extraction generally involves fewer analytical steps and minimizes issues due to artifact generation during the

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deconjugation step. The two limiting factors to greater application of steroid conjugate analysis are arguably the limited availability of standards and, in the case of naturally occurring steroids, the need for rapid and efficient separation of isomers. A potentially interesting aspect of the latter is the potential of separation techniques such as UPC2 and ion mobility to assist in the rapid separation of isomers. Currently, the sensitivity and capital cost of these techniques represent barriers to more widespread use. This is likely to become less of an issue if they become mainstream techniques incorporated into more cost-effective platforms.

In general, given the rapid improvement in sensitivity, mass resolution and mass accuracy, and the increasing affordability of accurate mass instruments, it is difficult to envisage any situation other than their increasing application to detection of growth promoters and other drugs in sports and meat residue testing.

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4

Hormones and β-Agonists

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4.1 Introduction

There are many chemical residues that have the potential to cause adverse health effects, in humans and animals, which end up in our food chain. This includes contaminants such as pesticides and dioxins; processing contaminants such as substances migrating from packaging materials, for example, isopropylthioxanthone (2-ITX); and natural toxins such as myco- and phycotoxins. But it also includes a range of compounds used to treat animals for diseases or to increase the animal production. The latter two groups include veterinary drugs such as antibiotics and anthelmintics, and growth-promoting agents, frequently referred to as "growth hormones," including, among others, steroid hormones, stilbenes, and somatotropin (ST). β-Agonists, although not considered hormones, were introduced as veterinary medicines and growth promoters in the 1990s. However, the boundary is not strict, as antibiotics can be misused for growth promotion and several β-agonists and steroid hormones can be used as veterinary drugs.

The use of hormones in animal production goes back in time for decades. Stilbenes are non-steroidal synthetic estrogenic compounds with anabolic properties. The most representative stilbene is diethylstilbestrol (DES), an endocrine disruptor with carcinogenic properties and one of the first growth promoters used in veal production.[1] Thyreostatics or antithyroid agents are orally active compounds, which may be used as growth promoters in the so-called *finishing period* of cattle, approximately 4 weeks prior to slaughter. The weight gain is mainly due to an increased water absorption and accumulation in the gastrointestinal tract and water retention in edible tissues, so their effect is not anabolic. Thiouracil (2-thiouracil, TU) is a particularly strong drug and thus was

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one of the most frequently abused thyreostatic agents in cattle. These compounds are potentially harmful for humans (carcinogenic and teratogenic), and for this reason, they, together with the stilbenes, have been banned in the European Union (EU) since 1981 (Council Directive 81/602/EEC).[2] Anabolic steroids can be distinguished according to their chemical structure and origin (estrogens, gestagens, androgens, and corticosteroids). These compounds stimulate growth leading to improved feed conversion and gain in protein deposition. In this group, both natural compounds and synthetic derivatives are included. This group includes the natural hormones when exogenously administered. Zeranol, which belongs to the group of resorcylic acid lactones (RALs), was widely adopted as a growth stimulant with estrogenic activity in the EU in the past and still is in use in several counties worldwide. The application of these compounds has been forbidden in the EU since 1985.[3]

Although strictly speaking, not included in the same legislation, the use of ST, primarily developed to enhance milk production, is also banned in the EU.[4]

β-Adrenergic agonists are derivatives of catecholamines such as epinephrine and norepinephrine, whose structure is characterized by a six-membered aromatic ring, hydroxyl group linked to the β-carbon, positively charged nitrogen in the ethylamine side chain, and a substituent on the aliphatic nitrogen. This structure is common to all β-adrenergic phenethanolamines, with the exception of large groups on the aliphatic nitrogen present on the natural adrenergic neurotransmitters, adrenaline.

There are different views around the world on the use of hormones for growth-promoting purposes. As a consequence of this, a range of different legislations regulate their use, ranging from a complete ban in the EU[5] to the registration of several hormone-containing preparations for growth-promoting purposes or to increase milk production in dairy cattle in some other countries.

The long history of research on approaches for the analyses of hormones and β-agonists does not mean that there are no remaining problems to be solved. Specific challenges remain, including:

- adequate control methods for natural hormones;
- methods for protein hormones; and
- identification of new, not previously recognized, compounds.

The objective of this chapter is to provide updated information on contemporary methods for hormone and β-agonist analyses. The initial section deals with the classical approaches for the effective detection and identification of exogenous hormones. Even in this domain, significant developments are taking place. The focus of this section is on instrumental confirmatory methods. However, an equally important role is played by effect-based screening methods. In view of their importance for effective control, the subsequent section focuses on developments in this area. The next section of the chapter deals with specific problems related to control strategies for natural hormones. These include both the traditional and generally recognized natural hormones as well as a series of androgenic

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steroids that can be present in biological samples obtained from a series of species. The fact that natural background concentrations can be present strongly complicates the analyses.[6, 7] The final section of the chapter is dedicated to β-agonists. In view of the very different pharmacological and analytical aspects of the analyses of β-agonists, the information has been combined in a single section.

4.2 Advances in Classical Analysis of Exogenous Synthetic Hormones

Most of our current knowledge with respect to the analyses of hormones is based on the work on exogenous synthetic compounds. The complexity of the target matrices and the trace concentrations of these "hormones" require highly sophisticated analytical strategies combining both specificity and detection at very low concentrations. In this regard, mass spectrometry (MS)-based methods are always part of the strategy of choice for confirmatory processes. The application of MS in combination with gas chromatography (GC) or liquid chromatography (LC) is still considered the "gold standard" for analytical methods in residue analysis. Although GC continues to be used, recent and novel liquid chromatography–mass spectrometry (LC-MS) powerful combinations have shown great convenience and suitability as analytical platforms for "hormone" analysis. Advances in chromatography enable the development of rapid, highly efficient, and precise LC separations. The overwhelming popularity of electrospray ionization (ESI) over other types of ionization reflects improvements in source and probe design, not yet paralleled in other ionization options such as atmospheric pressure chemical ionization (APCI).

Other options such as *omics* approaches are becoming available for monitoring veterinary drugs, mainly as screening options and for biomarkers discovery. Some guidance has been published, such as the tutorial on mass-spectrometry-based metabolomics recently presented by Courant et al.[8] The focus of this part is on the main topical options for the analysis of exogenous synthetic hormonal compounds.

4.2.1 Multi-methods: Multi-residue Methods (MRMs) and Multi-class, Multi-residue Methods (MCMRs)

The large number of possible "hormone" residues to be monitored in food-producing animals requires the application of reliable, high-throughput, and efficient analytical methods. As a result, multi-residue methods (MRMs) and particularly multi-class, multi-residue methods (MCMRs) are a major trend in the field of residue control. Such methods allow the analysis of a high number of compounds in a single analytical run; hence, they save time, sample, and solvent use and also reduce costs. For that purpose, typically LC or GC instruments coupled to triple quadrupole (QqQ) MS, and more recently, with high-resolution

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mass spectrometry (HRMS) instruments, are preferred. Multi-residue analysis has more recently benefited from the advantages of full-scan operation mode of HRMS, as it provides high specificity without limiting the number of observed compounds. These MS analyzers, for example, time-of-flight mass spectrometers (TOF-MSs) or Orbitrap™, provide high specificity because of both high mass accuracy and high mass resolution and allow the reconstruction of mass chromatograms for a theoretically unlimited number of compounds in complex matrices. The development and application of extraction procedures, which are as generic as possible, are necessary, in order to widen the scope of the method. The different options of sample preparation for multi-methods include common solid-phase extraction (SPE) protocols, QuEChERS (standing for "quick, easy, cheap, effective, rugged, and safe" method) or even "dilute-and-shoot" methods.

4.2.2 Alternatives in Sample Preparation and Clean-up

Sample preparation before analysis remains a critical step due to the high content of potential interfering compounds in samples of animal origin. While classical SPE and liquid–liquid extraction (LLE) protocols are still used in "hormone" analysis (Table 4.1), several innovative strategies to improve and ease sample purification have grown in importance in recent years.

4.2.2.1 Generic

Blokland et al. applied a specific combination of classical SPE and LLE to extract three separate fractions of urinary (natural) steroid aglycones, sulfate, and glucuronide conjugates,[9] while Kaabia et al. subsequently combined two consecutive SPE extractions to develop a method for 23 steroids in plasma and urine of horses, performing the quantitative analysis with GC-MS/MS.[10] Either single SPE or a combination of different sorbents has been extensively applied for the analysis of steroids in bovine urine and tissues.[11–14] The usefulness of LLE, alone or in combination with other procedures such as SPE, is illustrated by several authors (Table 4.1).

De Clercq et al. used an LLE *tert*-butyl methylether preparative step for ultrahigh-performance liquid chromatography–high-resolution mass spectrometry (UHPLC-HRMS) analysis of glucocorticoids in urine.[15] In a similar way, ethyl acetate has been applied to extract TU from bovine, porcine, and ovine urine samples and subsequently analyzed by LC-MS/MS.[16] A simple extraction with acetonitrile, without any additional purification step, proved to be effective to develop a multi-class LC-MS/MS method for the analysis of more than 160 regulated or banned compounds residues in egg, honey, milk, and muscle samples.[17]

4.2.2.2 QuEChERS

To be able to develop wide-scope MRMs including compounds with a wide variety of physicochemical properties, generic sample preparation is required.

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Table 4.1 Summary of representative confirmatory methods based on LC and GC for the control of hormonal active compounds in food-producing animals in 2012–2014.

Compounds	Matrix	Sample preparation	Instrumental analysis	Comments	References
Stilbenes					
Diethylstilbestrol, dienestrol, and hexestrol	Milk (cattle)	Acetonitrile and sodium chloride extraction, followed by purification with PF SPE	Column: C_{18}; LC-MS/MS ESI (–)	Comparison to conventional SPE; more environmentally friendly	Hu et al.[23]
Diethylstilbestrol, dienestrol, and hexestrol	Canned foods, water, beverages	Acetonitrile and QuEChERS, followed by DLLME; derivatization TFA	GC-MS	Includes bisphenol A	Korolev et al.[24]
Diethylstilbestrol, dienestrol, and hexestrol	Edible fin-fish	Acetonitrile followed by silica SPE	Column: C_8; LC-MS/MS ESI (–)	Fish muscle with high and low fat content	Lohne et al.[25]
Diethylstilbestrol, dienestrol, and hexestrol	Milk (whole, semi and skimmed)	Acetonitrile with acetic acid, evaporation, reconstitution in water, and HF-LPME with 1-octanol	Column: C_{18}; HPLC-DAD in series with FD	Includes estriol, estradiol, estrone, ethynyl estradiol, and hydroxyestradiol	Socas-Rodríguez et al.[26]
Antithyroid agents					
Methimazole	(Hen) brain, liver, thyroid gland, plasma, egg, embryos	Homogenization in phosphate buffer; perchloric acid 50%; direct injection	Column: C_{18}; HPLC-UV	No extraction/preconcentration; derivatization only	Chwatko et al.[27]

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Table 4.1 (Continued)

Compounds	Matrix	Sample preparation	Instrumental analysis	Comments	References
2-Thiouracil	Bovine and porcine colonic suspensions	Phosphate buffer; iodobenzyl bromide derivatization; diethyl ether extract; silica SPE in dichloromethane/ cyclohexane (1:3, v/v)	Column: C_{18}; LC-MS/MS ESI (−)	*In vitro* study of endogenous formation after consumption of Brassicaceae crops	Kiebooms et al.[28]
2-Thiouracil, 6-methyl-2-thiouracil, 6-phenyl-2-thiouracil, 6-propyl-2-thiouracil, 2-mercaptobenzimidazole, Tapazole	Thyroid and muscle	QuEChERS with ethyl acetate	Column: C_{18}; LC-MS/MS ESI (+)	Quick; no derivatization	Lega et al.[19]
2-Thiouracil, 6-methyl-2-thiouracil, 2-mercaptobenzimidazole, 6-propyl-2-thiouracil	Bovine plasma	Methanol; phosphate buffer; iodobenzyl bromide derivatization; diethyl ether extraction; dried with sodium sulfate; silica SPE in dichloromethane/ cyclohexane (1:4, v/v); filtered 0.45 µm	Column: C_{18}; LC-MS/MS ESI (+)	Factorial effect analysis (storage, operator, cartridge batch, time sample preparation)	Schmidt[29]

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2-Thiouracil, 6-methyl-2-thiouracil, 6-phenyl-2-thiouracil, 6-propyl-2-thiouracil, Tapazole	Bovine milk	Methanol; petroleum ether extract eliminated; Britton–Robinson buffer; iodobenzyl bromide derivatization; diethyl ether extract; dry with sodium sulfate	Column: C_{18}; LC-MS/MS ESI (+)	Naturally occurring	Wozniak et al.[30]
Steroids					
Dexamethasone, cortisone, cortisol, 6β-hydroxycortisol	Bovine urine, liver, kidney, and muscle	Enzymatic hydrolysis (urine); C_{18} (and NH_2 for urine) SPE	Column: C_{18}; LC-MS/MS ESI (−)	Direct and indirect markers (urinary ratios)	Biancotto et al.[11]
Estradiol esters	Bovine hair	Methanol 50 °C overnight; NH_2 and SiOH SPE; dansyl chloride derivatization; hexane extract	column: C_{18} and Phenyl; LC-MS/MS ESI(+)	Dansyl derivatives	Bichon et al.[31]
17 natural steroids	Bovine urine	C_{18} SPE (hydrolysis and second SPE/LLE for conjugates fraction only)	GC-MS/MS	Separated measurement of steroid aglycones and glucuronide and sulfate conjugates; profiling approach	Blokland et al.[9]

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Table 4.1 (Continued)

Compounds	Matrix	Sample preparation	Instrumental analysis	Comments	References
Cortisol, cortisone, dihydrocortisone, prednisolone, prednisone, methylprednisolone	Bovine urine	LLE with MTBE	Column: C_{18}; UHPLC-HRMS	Study of long-term stability of natural and synthetic glucocorticoids	De Clercq et al.[15]
20 glucocorticoids	Bovine milk	Protein precipitation with acetone; enzymatic hydrolysis; C_{18} SPE; sodium carbonate and diethyl ether washing; SiOH SPE	Column: C_{18}; LC-MS/MS ESI (−)	Pending specific validation for quantification of regulated MRL compounds in milk (dexamethasone, betamethasone, methylprednisolone, prednisolone)	Deceuninck et al.[32]
Dexamethasone, flumethasone, cortisone, hydrocortisone, methylprednisolone, and prednisolone	Milk	MISPE	Column: C_{18}; LC-MS/MS ESI(+)	Two templates were tested	Díaz-Bao et al.[33]
Testosterone, estradiol, androstenedione, boldenone	Bovine urine	MISPE/SFC-MIP	GC-MS/GC-C-IRMS	Template: 17β-estradiol	Doué et al.[34]
Dexamethasone, prednisolone, prednisone, hydrocortisone, cortisone	Bovine urine, liver, kidney, and muscle	Enzymatic hydrolysis (plus proteolysis for muscle); (MTBE extraction and hexane defattening for muscle) HLB SPE	Column: C_{18}; LC-MS/MS AI (−)	Animal study: endogenous presence of prednisolone	Ferranti et al.[12]

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17β-Estradiol (exogenous)	Bovine urine	Enzymatic hydrolysis; SPE C_{18}; LLE NaOH/*n*-pentane+diethyl ether (aqueous and organic fractions); HPLC C_{18}; HPLC diol; derivatization	GC-MS/C/IRMS	Confirmation of exogenous estradiol on the basis of $^{13}C/^{12}C$ isotopic ratio	Janssens et al.[35]
23 steroids	Equine urine and plasma	Water dilution; SPE C_{18}; acidic methanolysis; $NaHCO_3$ + LLE hexane/ether; silica SPE; derivatization	GC-MS/MS	Nandrolone abuse in entire male horses: profiling	Kaabia et al.[10]
18 steroids (natural and synthetic)	Antler velvet	Methanolic extract + hexane (discarded) + water; alumina A SPE; C_{18} SPE; derivatization	GC-MS/MS		Lu et al.[36]
26 steroids (natural and synthetic)	Eggs (various species)	(Enzymatic hydrolysis for conjugates) acetonitrile; $ZnCl_2$; reconstitute in methanol + water; polymeric SPE	C_{18}; LC-MS/MS ESI (+) (−)	Pregnenolone into progesterone during enzymatic hydrolysis; PCA study of different eggs	Mi et al.[37]
35 endogenous steroids	Equine serum	Water-diluted serum; polymer based TFC online clean-up	TFC-LC-MS/MS heated ESI (+)(−)	Minimal sample prep.; 2D-LC	Moeller and Stanley[38]

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Table 4.1 (Continued)

Compounds	Matrix	Sample preparation	Instrumental analysis	Comments	References
6 gestagenic synthetic steroids	Kidney fat	Methanol; alumina clean-up; filter 0.2 µm	Column: C_{18}; LC-MS/MS ESI (+)		Rejtharová and Rejthar[39]
Prednisolone, prednisone, cortisol, cortisone	Bovine urine	Enzymatic hydrolysis; MTBE LLE; polymeric RP SPE	Column: C_{18}; UHPLC-QTOF MS	*In vitro* and stability studies; a prednisolone threshold is proposed	de Rijke et al.[40]
19 Steroids	Bovine urine	Enzymatic hydrolysis; C_{18} SPE; NH_2 SPE; filter 0.45 µm	Column: C_{18}; LC-MS/MS ESI (−)	Age and sex variations for natural compounds	Snoj et al.[13]
Stanozolol metabolites	Porcine, bovine and ovine urine	basify urine; polymeric strong anion SPE	column: C_{18}; LC-MS/MS ESI (+)	Simple and rapid clean-up; validated for three species	Tölgyesi et al.[14]
17α-Ethinylestradiol, 17α-estradiol, estriol, 17β-estradiol, estrone, 17α-hydroxyprogesterone, progesterone, medroxyprogesterone, norethisterone acetate	Bovine milk	HF-SEBLLME procedure; derivatization	GC-MS	Magnetic separation	Xu et al.[41]
RALs (including zeranol)					
Zeranol (α-zearalanol), zearalanone	Bovine muscle and liver	Enzymatic deconjugation; methanol; defat with hexane; ethyl acetate; amino SPE; filter 0.22 µm	Column: C_{18}; LC-MS/MS ESI (−)	Comparison to chemiluminescence enzyme immunoassay	Haiyang et al.[42]

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Zeranol (α-zearalanol), taleranol (β-zearalanol), zearalanone, zearalenone, α-zearalenol, β-zearalenol	Urine	Enzymatic deconjugation at pH 5.2 with glucuronidase, LLE diethyl ether, and SPE with C_{18} and NH_2 columns	Column: C_{18} ODS-3; LC-MS/MS ESI (−)	Integrated in Polish monitoring programs	Matraszek-Zuchowska et al.[43]
Zeranol (α-zearalanol), zearalanone, taleranol (β-zearalanol), zearalenone, α-zearalenol, β-zearalenol	Pig muscle	Enzymatic digestion β-glucuronidase/ sulfatase, extraction with diethyl ether, immunoaffinity clean-up	Column: C_{18}; LC-MS/MS ESI (−)	Includes chloramphenicol	Wang et al.[44]
Zeranol (α-zearalanol), zearalanone, taleranol (β-zearalanol), zearalenone, α-zearalenol, β-zearalenol	Feed	Acetonitrile/water (80:20, v/v), dSPE with MWCNTs	Column: C_{18}; LC-MS/MS ESI (−)	Optimized MWCNTs method, fast and suitable for routine	Ying et al.[45]
Multi-class, multi-residue methods (MCMRs)					
116 drugs (stilbenes, steroids, corticoids, RALs, β-agonists, and others)	Feed	Acidified acetonitrile extraction, double centrifugation, dilution with water	Column: C_{18}; UHPLC-QTOF MS ESI (−) (+)	Multi-residue and multi-class; very simple and straightforward method. Incurred samples analyzed	Boix et al.[46]
17 hormones: androgens, corticosteroids, progestagens, estrogens	Powdered ingredients derived from bovine milk	QuEChERS (modified)	Column: C_{18}; LC-MS/MS ESI (+)	Universal matrix matching approach based on whole milk	Ehling and Reddy[20]

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Table 4.1 (Continued)

Compounds	Matrix	Sample preparation	Instrumental analysis	Comments	References
21 growth promoters (gestagens, corticosteroids, RALs, stilbenes, steroids)	Bovine milk	Acetonitrile extraction and SPE with HLB	Column: C_{18}; LC-MS/MS APCI (−) (+)	Rapid and simple	Kaklamanos and Theodoridis[47]
Trenbolone, ethinylestradiol, zeranol (α-zearalanol), stanozolol, diethylstilbestrol, dienestrol, hexestrol, taleranol, zearalenone	Bovine urine	Enzymatic hydrolysis; HLB SPE; water + diethyl ether/petroleum ether; organic phase reconstituted in acetonitrile/water and filtered	Column: C_{18}; UHPLC-MS/HRMS (Q Exactive™)	tSIM/ddMS/MS acquisition mode, validated according to CD 2002/657/EC[7]	Kumar et al.[48]
87 drugs (steroid hormones, corticosteroids, RALs, stilbenes, and others)	Bovine urine	QuEChERS	Column: polar endcapped C_{18}; UHPLC-HRMS (Orbitrap Exactive™ at 50,000 FWHM) ESI (+) (−)	Wide-range screening banned drugs; library search	León et al.[21]
160 drugs (steroids and others)	Milk, egg, honey, meat	Acetonitrile only	LC-MS/MS	Qualitative screening only	Robert et al.[17]
120 drugs (thyreostats, steroids, and others)	Bovine kidney	Acetonitrile/water (80:20, v/v); hexane	Column: C_{18}; LC-MS/MS ESI (+)	Qualitative and quantitative	Schneider et al.[49]
34 anabolic steroids	Meat	Methanol; hexane; diethyl ether, SPE with Si and NH_2	Column: C_{18}; UHPLC-HRMS (Orbitrap Exactive™ at 50,000 FWHM) APCI (+) (−)	HRMS lower sensitivity compared to a QqQ method	Vanhaecke et al.[50]

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22 drugs (steroids and others)	Feed	Acidified acetonitrile; MCX SPE	Column: C_{18}; LC-MS/MS and HRMS (Orbitrap™) ESI (+)	Screening, confirmation, and quantification	Wang et al.[51]
Stilbenes, RALs (including zeranol)	Bovine, porcine, and poultry muscle	Water homogenate and ethyl acetate; QuEChERS: C_{18}, PSA and $MgSO_4$; methanol and defat with hexane; filter 0.2 µm	Column: C_{18}; LC-MS/MS ESI (−)	Estrogenic drugs; efficient separation in short run time	Wozniak et al.[22]
226 drugs (thyreostats, steroids, and others) and contaminants	Porcine and bovine muscle	EDTA and acetonitrile/ethanol; hexane. Two fractions: hexane (further extracted with acetonitrile and PSA-C_{18}) and acetonitrile–ethanol	Column-switching LC-MS/MS ESI (+) (−)	Requires two analytical runs	Zhan et al.[52]

DLLME, dispersive liquid–liquid micro-extraction; dSPE, dispersive solid-phase extraction; HF-LPME, hollow fiber liquid-phase micro-extraction; HF-SEBLLME, hollow fiber-based stirring extraction bar liquid–liquid micro-extraction; MIP, molecularly imprinted polymer; MISPE, molecularly imprinted solid-phase extraction; LLE, liquid–liquid extraction; MSPD, matrix solid-phase dispersion; MTBE, methyl *tert*-butyl ether; MWCNTs, multi-walled carbon nanotubes; PF SPE, packed-fiber solid-phase extraction; SFC-MIP, supercritical fluid chromatography with molecularly imprinted polymers; SPE, solid-phase extraction; TFC, turbulent flow chromatography; tSIM/ddMS/MS, targeted SIM data-dependent MS/MS.

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QuEChERS procedures are also frequently applied in multi-residue and multi-class methods for hormonally active compounds in food of animal origin. QuEChERS-like strategies found their origin in pesticide analysis,[18] and they comprise extraction with an organic solvent and phase separation with high salt content, in some cases followed by dispersive SPE. Lega et al. developed a method for determining antithyroid agents in bovine thyroid gland and muscle using QuEChERS extraction followed by LC-MS/MS.[19] Other researchers have also successfully demonstrated the suitability of this strategy for MCMRs.[20, 21] An illustrative example is provided of the use of QuEChERS to extract RALs and stilbenes from muscle tissue from different species, in order to reduce the number of steps and shorten the time of analysis, obtaining good recoveries and acceptable within-laboratory reproducibility.[22]

4.2.2.3 Molecularly Imprinted Polymers (MIPs)

Molecularly imprinted polymers (MIP) are synthetic polymers exhibiting specific cavities complementary to a template molecule (or a family of compounds). The most frequent application of these polymers is as selective sorbents for SPE, for the so-called molecularly imprinted solid-phase extraction (MISPE). MIPs are reusable sorbents, and they usually permit a fast one-step simple clean-up of complicated biological samples such as urine or milk. Díaz-Bao et al. developed a corticosteroid-specific sorbent by precipitation polymerization, and these materials were successfully applied to isolate several corticosteroids in milk samples.[33] In 2012, Doué et al. described for the first time a semi-preparative application based on MIP for isolation of urinary steroids prior to gas chromatography–combustion–isotope-ratio mass spectrometry (GC-C-IRMS).[34] Gañán et al. designed a molecularly imprinted polymer-matrix solid-phase dispersion (MIP-MSPD) preparative procedure to extract five steroids in goat milk.[53] These polymers usually result in an easy, fast, and efficient system for the extraction, avoiding almost completely the use of organic solvents.

4.2.2.4 Hollow-Fiber Micro-extractions and Similar Techniques

Some nearly solvent-free extraction methods have become popular, such as solid-phase micro-extraction (SPME) and liquid-phase micro-extraction (LPME). Socas-Rodríguez et al. applied a novel sample preparation protocol to isolate estrogenic compounds from milk-based materials on protein precipitation with acidified acetonitrile and hollow-fiber liquid-phase micro-extraction (HF-LPME) to further concentrate the analytes.[26] Using a similar approach, Xu et al. used a hollow-fiber-based stirring extraction bar as the stirring system and liquid–liquid microextractor of nine steroids in milk.[41] This micro-extraction has the advantages of both stir-bar sorptive extraction (SBSE) and HF-LPME, with the magnetic bar easily isolated from the matrix with an external magnet. A sensitive and cost-effective LC-MS/MS method for the determination of stilbenes in milk was recently developed using packed-fiber solid-phase extraction (PF SPE) with a cartridge containing electrospun polystyrene.[23]

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4.2.2.5 Dilute and Shoot

Along with QuEChERS, "dilute and shoot" (DS) is one of the most frequently reported generic sample-preparation methods. Thanks to the progressive improvement in instrument detection concentrations and mass resolution, DS in combination with LC has become more common in recent years.[54] Dilution can minimize the matrix effect, but can also complicate the detectability of the compounds. This is the reason why the majority of methods using DS still focus on highly ionizable compounds and with relatively high detection concentrations required. An additional advantage of this approach is the reduction of sample manipulations, thereby lowering the total uncertainty. Despite all these benefits, there are only a few examples of DS application to the analysis of hormones in farm animals, and the inclusion of a sample clean-up stage is still the more reliable approach. León et al. evaluated three different sample preparation procedures (DS, SPE, QuEChERS) for the multi-class screening of 87 banned and unauthorized veterinary drugs in bovine urine.[21] Although DS is a simple and attractive option, its effectiveness for banned substances at low concentrations seems to be very limited and QuEChERS was finally selected as the optimal approach. In the human anti-doping field, Tudela et al. successfully applied and validated a dilute-and-shoot liquid chromatography–high resolution mass spectrometry (DS-LC-HRMS) approach for the analysis of more than 30 anabolic compounds (conjugates and free steroids) in urine.[55] In this case, the sample preparation consisted of a simple and fast DS extraction with methanol. In a similar way, Boix et al. used acetonitrile extraction for the screening of 116 veterinary drugs in feed, using liquid chromatography–high resolution mass spectrometry (LC-HRMS), and evaluated its potential for quantitative analysis.[46]

4.2.3 Advances in Separation

With the introduction of fast separation techniques such as ultrahigh-performance liquid chromatography (UHPLC), many laboratories have chosen these instruments in combination with MS not only for confirmation but also for screening. UHPLC has contributed to the reduction in the time needed for residue analysis of complex samples. The small particle (sub-2 μm) column packing provides high peak capacity and chromatographic resolution with analysis times as much as 10 times shorter than conventional high-performance liquid chromatography (HPLC) methods. For this reason, the majority of the analytical methods currently developed for hormone analysis involve UHPLC separations, particularly for multi-methods. In addition to UHPLC, some innovative options have emerged in recent years.

4.2.3.1 Miniaturized Separation Techniques

Miniaturized separation techniques have emerged as environmentally friendly options for pharmaceutical and biomedical research[56] and in food analysis.[57] Nano-LC, microchip devices and nanocapillary electrophoresis are methods that allow the reduction of solvent consumption and waste generation and are easy

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to couple with MS instruments. Despite the advantages of these technologies, including enhanced separation and sensitivity, fast analysis, and reduced sample consumption, their use for the analysis of veterinary drugs has clearly been limited in comparison to HPLC and UHPLC.

4.2.3.2 Turbulent Flow LC

The use of turbulent flow chromatography (TFC) for on-line sample extraction allows the direct analysis of biological samples, reducing the overall analysis time compared to traditional off-line clean-up protocols. TFC is a semi-automated technique for sample preparation based on the combination of high flow rates inside a small column filled with large stationary-phase particles, creating a turbulent environment. TFC systems assembled on a two-dimensional LC system provide a powerful, high-throughput sample preparation alternative to SPE, LLE, and protein precipitation. An example of its potential for hormone analysis is the work of Moeller and Stanley, who successfully applied TFC for the direct analysis of 35 endogenous steroids in serum.[38]

4.2.3.3 Ion Mobility Spectrometry

The techniques of ion mobility spectrometry (IMS) are becoming more popular as they provide high specificity with low limits of detection by simplifying spectral data and reducing spectral noise. This separation occurs on the millisecond timescale, and it is based essentially both on the mass and on the charge of the analytes. There are few reports on the application of ion mobility to the separation of steroids in animal samples. Kaur-Atwal et al. successfully applied ultrahigh-performance liquid chromatography–ion mobility spectrometry separations combined with mass spectrometry (UHPLC-IMS-MS) and with tandem mass spectrometry (UHPLC-IMS-MS/MS) for the simultaneous determination of testosterone and epitestosterone glucuronides in human urine.[58] Studies on the applicability of a traveling-wave ion mobility (TWIM) device combined to MS in the analysis of selected nonderivatized and *p*-toluenesulfonyl isocyanate (PTSI) derivatives of the steroids estradiol, testosterone, and androsterone were described by Ahonen et al.[59] The proposed method finally included derivatization, as it improved the separation in the Intra Muscular (IM) cell. Traveling-wave ion mobility–mass spectrometry (TWIM-MS) can be performed on a millisecond scale and therefore can provide immediate separation, characterization, and quantification of α/β-steroids.

4.2.3.4 Techniques to Facilitate IRMS: High-Temperature LC, Two-Dimensional Chromatography, and Others

Generally, compound-specific isotope analysis of steroids is performed using GC combined with isotope ratio mass spectrometry (IRMS). GC-C-IRMS typically relies on extensive and time-consuming sample preparation such as LC fraction collection, in order to achieve the required baseline separation for the analytes of interest. Brailsford et al. introduced a microfluidic flow-splitting device to allow

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simultaneous acquisition of full-scan MS of IRMS peaks.[60] This two-dimensional GC approach may provide sufficient purification for IRMS investigation of testosterone abuse. Similarly, Tobias et al. indicated the potential of GC × GC separations to minimize sample preparation requirements for GC-C-IRMS, showing for the first time that synthetic steroid use is detectable without the need for extensive urine clean-up.[61] Additionally, they confirmed testosterone abuse in urine from an individual who was given a T-shot, fulfilling the World Anti-Doping Agency (WADA) criteria. Despite all these benefits, the authors suggested further refinement of this method and the use of a more conventional approach with LC clean-up to make sure that the presence of testosterone is abnormal. When IRMS is performed with GC, a derivatization of the steroids prior to the measurement is required. However, this is a laborious step that may alter isotopic signatures of the target analytes. To overcome this limitation, a new approach based on high-temperature liquid chromatography (HT-LC) has been proposed.[62] HT-LC is an alternative sample preparation that avoids GC derivatization during IRMS measurement of unconjugated steroids. A novel protocol based on MIPs was developed by Doué et al., using these polymers as stationary phase for semipreparative SFC, providing the required high degree of purity for IRMS.[34]

4.2.4 Advances in Detection

From the literature, it may be concluded that use of a LC–QqQ mass spectrometer is currently the preferred method for "hormone" analysis. However, a clear tendency toward the introduction of HRMS instruments has been observed in the past few years, even though they have not been widely applied in routine analysis. High resolving power and accurate mass measurements make HRMS instruments an attractive tool for identifying both targeted and non-targeted hormones in complex food matrices. When there is no *a priori* hypothesis of the presence of certain drugs, HRMS instruments can be used for nontargeted (or retrospective) screening for a wide range of residues. In theory, virtually an unlimited number of compounds can be simultaneously analyzed in full-scan mode, rather than preselected ion transitions corresponding to specific residues.[63] This approach represents a solution to the limitation in the number of analytes in common targeted MS/MS methods, with the possibility of post-acquisition reinterrogation of data and screening of unknowns.[50]

The development of criteria for the confirmation of identity using measured exact mass data is still in progress due to the recent emergence of this technique. The Commission Decision 2002/657/EC introduced a system of identification points (IPs) for MS detection.[7] A minimum of four IPs is required for Group A (banned) substances. Subsequently, Nielen et al. proposed additional LC-MS criteria to be implemented in the 2002/657/EC decision.[64] Based on this proposal, the resolving power for HRMS confirmation should be greater than 20,000, the mass accuracy lower or equal to 5 ppm, and two IPs will be earned per precursor or product ion. For the confirmation of unknown substances, it was proposed

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that the resolving power should be greater than 70,000 with 5 ppm mass accuracy. So far, several wide-range screening methods for hormones have been reported using HRMS.[21, 46, 51, 63, 65] In more advanced hyphenated techniques, fragmentation and high resolving power MS is achievable (LTQ-Orbitrap™, Q-Orbitrap™, QTOF MS). The quadrupole-Orbitrap (Q Exactive™) hybrid instrument was recently introduced, and only a few studies have been reported so far. This combination has great potential to avoid false results in food safety, as it features both the mass selection capability of quadrupoles and the high resolution of an Orbitrap. A few HRMS confirmatory methods for hormones in bovine urine were developed and validated according to Commission Decision 657/2002/EC,[7] achieving similar detection capabilities and enhanced selectivity compared to QqQ instruments.[15, 48] The quadrupole in Q Exactive™ acts as a filter to reduce ion suppression and targeted modes increase the signal-to-noise ratios. Kumar et al. concluded that targeted selected ion monitoring (SIM) data-dependent scan modes are the most suitable for residue analysis with HRMS instruments.[48]

4.2.4.1 Isotope-Ratio Mass Spectrometry (IRMS)

High-precision gas-IRMS is used for the measurement of differences in the stable isotopic abundances, as a ratio in the sample relative to a traceable standard. For carbon isotope ratio measurements, the $^{13}C/^{12}C$ ratio of a sample is measured and values are reported in δ notation with respect to an international standard, expressed in units of parts per thousand (‰). A distinction between endogenous steroids and exogenous homologs based on their carbon isotopic composition has been accepted as a confirmatory option by the WADA in the field of sport doping. In recent years, its capability has been also illustrated in the field of food safety. Janssens et al. showed that GC-MS/C/IRMS analysis of urine samples is also a powerful tool for the detection of steroid abuse in farm animals, especially in the case of estradiol.[35] However, this approach requires expensive equipment and time-consuming preparative steps prior to analysis, reducing its applicability to only a few laboratories. Therefore, the selection of suspicious samples through the application of more accessible screening methods is still pertinent.

4.2.4.2 Ambient Ionization Mass Spectrometry (AMS)

Since the pioneering introduction of the first ambient ionization source by Takáts et al. in 2004,[66] this area has undergone a rapid development in diverse areas employing LC. One of the merits of these methods is that they permit the rapid and direct measurement of the analytes on the sample surface, which is exposed to the ionization medium under ambient conditions and usually with no need of extraction and separation processes. Among the existing techniques, desorption electrospray ionization (DESI) and direct analysis in real time (DART) have become the most established. DESI shares characteristics with typical ESI sources in terms of enabling the analysis of substances over a large mass range while DART represents an APCI-related technique. This approach has also intrinsic

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limitations, for instance, the detection of a compound largely depends on the matrix (analyte *in* or *on* the sample) and the quantification abilities are limited.

In DESI-MS methods, the ionization is achieved by spraying the sample with electrically charged aqueous mist and sample-surface ions are transported to the mass spectrometer at atmospheric pressure. It is applicable to solid samples, including complex biological matrices, but also to other matrices (frozen solutions, liquids, adsorbed gases). In recent years, DESI has gained popularity for imaging MS applications, a technique that allows for the direct monitoring of the abundance and spatial distribution of chemical compounds over the surface of a tissue sample. The main advantage of DESI in comparison to the vacuum-operating sources matrix-assisted laser desorption/ionization (MALDI) or secondary ion mass spectrometry (SIMS) is the possibility of analyzing under ambient conditions and without (or with minimal) sample pre-treatment and/or matrix addition. DESI is able to give huge amounts of information correlated to the status of the sample, for example, in the surface of tissue sections. De Rijke et al. developed an imaging MS method with DESI for the direct detection of anabolic steroid esters in injection sites, performing also 2D and 3D profiling of the distribution of these compounds in the tissue.[67] Another example of the application of ambient techniques to the analysis of anabolic compounds in biological matrices is the strategy developed by Saha et al.[68] In this work, urine was directly and rapidly (1 minute) analyzed using Leidenfrost-phenomenon-assisted thermal desorption (LPTD), coupled to dielectric barrier discharge ionization (DBDI) MS in open atmosphere, detecting trace concentrations of steroids. In this technique, a liquid droplet containing the analytes is slowly evaporated on a heated metallic plate in front of the inlet of the mass spectrometer.

In DART, helium or nitrogen is used to produce excited metastable species by a corona discharge, which react with the ambient water and air to produce reactive ionizing species. A stream of heated nebulizing gas directs the ionizing species toward the sample where they ionize the analytes on the surface of the sample. In a recent article by Doué et al., the applicability of DART in combination with HRMS for fast identification and quantification of 21 anabolic steroid esters was demonstrated.[69] Apart from DESI and DART, atmospheric solid analysis probe (ASAP) also stimulated the development of new applications. Doué applied atmospheric solid analysis probe–mass spectrometry (ASAP-MS) to the analysis of anabolic steroid esters in oily commercial drug preparations.[70] This technique permitted the rapid identification and quantification of 21 selected compounds (based on testosterone, estradiol, nandrolone, and boldenone), allowing a rapid screening in only minutes and with minimal sample preparation. For the first time and thanks to isotope-labeled internal standards, ASAP-MS was used for quantification. Further identification was achieved using a triple-quadrupole–mass spectrometry (QqQ-MS) instrument.

4.2.4.3 Other Techniques

MALDI is a soft ionization technique applicable to the analysis of solids, requiring an exogenous matrix to aid in the desorption/ionization process and operating

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under vacuum. MALDI has been frequently combined with TOF MS instruments for the analysis of proteins and peptides or lipids from bacterial cells, classically combined with two-dimensional electrophoresis. The targets of MALDI-TOF MS analysis have evolved in recent years due to the development of new commercial MALDI matrices. Galesio et al. developed a database search engine, MLibrary, designed to help in the automated identification of androgenic anabolic steroids and metabolites in human urine when using MALDI-TOF MS.[71]

Solvent-assisted inlet ionization (SAII) is a recently developed ultrasensitive liquid introduction ionization method for MS, which ionizes small molecules, peptides, and proteins requiring no voltage or laser, achieving sensitivity that surpasses ESI. Ions are generated even using ultrapure water as solvent, within a heated inlet tube linking atmospheric pressure with the first vacuum stage of the mass spectrometer. The first experiments performed with SAII on an Orbitrap Exactive™ MS instrument suggested that it may surpass nanoelectrospray in detection limits but without the need for extremely low solvent flows.[72] The enhanced detection limits obtained using SAII as ionization method for the analysis of synthetic and natural steroids were recently demonstrated by Chubatyi et al.[73] The data were acquired using a QTOF and a high-resolution Orbitrap™ mass spectrometer, using only a simple Ziptip clean-up procedure without sample concentration. Their results on Orbitrap™ suggested that it is easy to implement SAII and that it may advantageously replace ESI, reaching exceptionally low limits of detection and quantification at low parts per 10^{12} (low parts per 10^{15} under infusion conditions).

4.2.5 Classic and New Analytical Matrices

An ideal analytical matrix should be non-invasive, inexpensive, easy to collect, stable and easy to store, resistant to biotransformation reactions, and with long-term retrospection capabilities. Most analytical methods for monitoring hormones in food-producing animals have been carried out targeting common matrices such as feed, urine, blood (serum and/or plasma), edible tissues, and even hair. Alternative specimens such as oral fluids (saliva), feces, or feathers have received lesser attention. Even though classic options are still preferred, a few examples of novel options have been published in recent years.

Milk is increasingly used in residue control for hormones as it has proved to be an adequate matrix to monitor the (ab)use of several hormonal compounds in bovine animals.[20, 30, 32, 47, 74] Recently, porcine saliva has been identified as a matrix with great potential for proteomics studies of animal health.[75] Oral fluids are non-invasive and have considerable potential for the development of new analytical methods, with reduced potential for adulteration, as it has been demonstrated for natural steroids abuse in the human antidoping field.[76] However, handling and analysis of saliva require particular attention, with recommended storage conditions at −20 °C or below and preferably analyzed within 24 hours of collection.[77]

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In any case, matrix storage conditions are also important to avoid transformation of natural compounds, which can result in misinterpretation of results.[12, 15]

In analogy to hair, feathers have been suggested as an alternative sample material. Evidence was found for the accumulation of tetracycline antibiotics in this matrix in an experiment with poultry.[78] However, their usefulness for monitoring other veterinary drugs has not yet been demonstrated. Eggs, fat, and even antler velvet are other unconventional matrices that have been used to quantitatively assess "hormones" in farm animals.[36, 37, 39] Due to its minimal invasiveness, simplicity, and speed, the potential of dried blood spot (DBS) sampling has also been explored and recommended as an advantageous technique in doping control analysis in humans, with detection limits in the low ng/kg range.[79] Along with the small sample volume, simplified storage and shipment conditions are great advantages of this kind of samples. However, sample preparation may be tedious and time-consuming and the required instrumental sensitivity is usually high.

4.2.6 Conclusions on Analysis of Exogenous Synthetic Hormones

Innovative new technologies, both in sample preparation techniques and in instrumentation, are continuously supporting the development of new and improved analytical methods. Overall, these result in the availability of a still increasing number of efficient and sensitive analytical methods.

4.3 Bio-Based Screening Methods for Steroid Hormones, β-Agonists, and Growth Hormones

In spite of the intrinsic value of chemical analytical methods, they have one serious drawback. When performed in their classical sense, they most often overlook new compounds and novel hazards ("you only find what you are looking for"). This is one of the reasons that the European Food Safety Authority (EFSA) argued in favor of a modernization of meat inspection across the EU, following a risk-based approach. In response to a question from the European Commission, EFSA has recommended improvements to meat inspection procedures to protect consumers from risks related to such hazards.[80] These include biological hazards (e.g., pathogenic bacteria) and chemical hazards. Three areas of chemical hazards were mentioned: residues of veterinary drugs (e.g., antibiotics), forbidden anabolic substances (e.g., anabolic steroids and β2-agonists), and other chemical contaminants (e.g., dioxins).

Bio-based screening methods offer the opportunity to overcome the present difficulties and shortcomings. However, bio-based screening methods are not able to identify the responsible compounds in non-compliant samples and are only of added value when combined with chemical analytical methods. Bio-based screening and chemical analytical confirmation are thus complementary. This section

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gives an overview of the available bio-based screening methods for the detection of hormones and β-agonists. Their main purpose is to identify samples that require additional chemical confirmation and their main advantage is their capability to detect unknown compounds, for example, designer steroids, and new risks caused by, for example, hormonally active compounds. This is especially useful in the control of the growth promoters, where there is a constant development toward novel compounds to circumvent control on the illegal application of such substances.[81, 82]

There are several bio-recognition principles. Roughly, these can be divided into binding assays and cell-based effect assays (bioassays). Binding assays include the binding of a compound to a specific transport protein, for example, the binding of thyroid hormones (THs) to transthyretin; or a specific antibody, for example, an enzyme linked immunosorbent assay (ELISA) for β-agonists; or a specific receptor, for example, the binding of testosterone to the androgen receptor (AR). Cell-based bioassays include proliferation assays, such as the proliferation of human breast cells in the presence of estrogens or transcription activation assays such as the AR CALUX®, a modified human U2-OS cell line that expresses a luciferase enzyme when exposed to androgens. A third bio-based option is to determine indirect effects of the administered compounds, that is, the use of biomarkers. This section gives an overview of the available bio-based screening methods for the detection of hormones and β-agonists, focusing on estrogens, androgens, progestogens, corticosteroids, thyroids, β2-agonists, and growth hormones (GH) (protein hormones).

4.3.1 Estrogens

4.3.1.1 Binding Assays for Estrogens

Over the past two decades, a panel of different *in vitro* assays has been developed for compounds with an estrogenic mode of action. *In vitro* estrogen receptor (ER) competitive binding assays have become well established and are extensively used to investigate ER–ligand interactions. ER competitive binding assays identify chemicals that have the potential to interact with the ER *in vitro* by measuring the displacement of a receptor-bound labeled molecule by a test compound, allowing the determination of the relative binding affinity of the test compound as an ER ligand. "Old-fashioned" radiolabeled binding assays have been very useful as fast-track assays for the prediction of endocrine drug activities. The rat uterine cytosol ER binding assay, currently listed as part of the United States Environmental Protection Agency (EPA) Endocrine Disruptor Screening Program Tier 1 screening battery, uses the ERs prepared from rat uterine cytosol and measures the displacement of radiolabeled 17β-estradiol.[83] As conducted, this assay utilizes all cytosolic ER subtypes that are expressed in this tissue, including ERα and ERβ. The binding affinities determined in the assay are thus not specific, and this assay still requires the use of animals as a source of the ERs. Competitive binding assays that use cytosol preparations also suffer

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from cross-talk caused by other nuclear receptors (NRs) and proteins present in the homogenate. Binding assays using pure recombinant receptor protein are not affected by this possible artifact and are animal-friendly and should thus be preferred.[84] However, ER binding assays only have the ability to determine if a chemical can interact and displace the endogenous hormone; they provide no information on whether a chemical will act as an agonist or antagonist at the receptor(s) to either activate or inactivate an estrogen-dependent response.

The latest assay based on ER binding is a protein array on the PamChip® plate format. It assesses the ligand-modulated interaction of ERα with coregulators on a PamChip® plate, consisting of 96 identical arrays, each array containing 155 immobilized NR co-regulator proteins (co-activators and co-repressors).[85] Although proven to be valid and of added value when used to determine the estrogenic properties of chemicals, as it is the only binding assay that is able to predict if a compound will act as an agonist or antagonist, the test is most probably not suited to test complex sample extracts. Even if it was, the technique is by far too expensive to analyze large numbers of samples in a daily routine setting.[86]

Binding assays based on sex hormone binding globulin (SHGH), the natural transporter of endogenous (steroid) hormones, are not specific, as sex-hormone-binding globulin (SHBG) binds estrogens and androgens. SHBG binding assays may, however, still be suited for the broad screening for the presence of estrogens and androgens, although many non-hormonal or non-relevant compounds with regard to legislation might also compete with steroids for SHBG binding and result in false positives. A study performed by Déchaud et al. showed that 4-nonylphenol and 4-tertoctylphenol, two alkylphenols used as surfactants in many commercial products, and bisphenol A and O-hydroxybiphenyl, widely used in the plastics industry, are able to displace estradiol from human sex-hormone-binding globulin (hSHBG).[87] Thus, there are probably many chemicals that alter SHBG binding. However, as SHBG binding assays have not been used for regulatory purposes in veterinary control or for analyses of complex environmental samples, it is difficult to establish to what extent this relates to relevant or nonrelevant compounds in real practice. Whether SHBG binding assays have clear advantages over ER binding assays is not clear, and until that time, SHBG binding assays are considered as less suited for veterinary residue control programs than competitive ER binding assays. However, it should be emphasized again that the SHBG binding assay is highly relevant and of added value in a test panel of *in vitro* assays for predicting the potential endocrine disrupting characteristics of chemicals *in vivo*. Moreover, if there are (unknown) compounds that have no affinity for the ER, but are able to release endogenous estradiol from SHBG, such compounds would be ideal (illegal) growth promoters that could only be detected with an SHBG binding assay.

Despite the great number of available immune-based methods, these types of binding assays are not relevant for broad screening, as specific antibodies that are raised against estrogens will only recognize one or a few estrogens. Due to the great variety of chemicals with estrogenic properties, immunochemical methods,

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such as the ELISA for 17β-estradiol and 17α-ethinylestradiol,[88] have the drawback that they are only able to detect structurally related compounds and are thus unable to detect the biological activity of unknown compounds and their metabolites. Moreover, these immune-based methods often suffer from matrix effects. This is in contrast to cell-based effect assays (bioassays) that are based on the molecular or cellular mechanism of action of estrogens.

4.3.1.2 Bioassays for Estrogens

Many reporter–receptor gene assays have been developed, using both yeast and mammalian cells. In principle, these assays can detect all relevant compounds. In contrast to competitive ER binding assays, these receptor–reporter gene bioassays can distinguish receptor agonists from antagonists, as receptor–reporter gene bioassays also include the transactivation and translation steps.

The first yeast estrogen bioassays, developed by Pham and O'Malley[89] and Routledge and Sumpter,[90] were based on the expression of ERα and β-galactosidase, the latter upon exposure to estrogens. These yeast bioassays are already considered as highly valuable for testing compounds based on their estrogenic properties and for the analysis of complex sample extracts. However, as these yeast bioassays make use of β-galactosidase, the addition of a substrate is needed and some substrates for this enzyme are estrogenic themselves.[91] A step forward was achieved with the development of a yeast estrogen bioassay based on the increased expression of yeast enhanced green fluorescent protein (yEGFP), which can be measured directly in intact living cells (no cell lysis and no substrate needed).[92] Overall, yeast estrogen bioassays have proven to be highly valuable for the determination of the estrogenic potency of compounds and complex matrices such as calf urine, feed, plant extracts, and environmental samples.[93–103] So far, the yeast estrogen bioassay expressing yEGFP has been shown to detect all compounds with known estrogenic properties (no misclassification) and was fully validated according the guidelines and criteria described in EC Decision 2002/657[7] and acquired an ISO 17025 accreditation status in the Netherlands for the analysis of both feed and calf urine.[104, 105] This assay also performed well in an inter-laboratory test; that is, it was successfully applied to calf urine samples in a ring test study, showing that the assay is easily transferable to other laboratories.[106] More recently, the assay was used on a herbal supplement for prostate function, showing that it was fortified with DES, causing gynecomastia in a male taking the supplement.[107]

The MVLN assay for estrogens was one of the first *in vitro* transcription activation bioassays based on a human mammalian cell.[108] Subsequent assays were also based on human breast carcinoma cells already expressing endogenous ERs and thus only required the introduction of an Estrogen Responsive Element (ERE)–reporter gene construct. Examples are the T47-D breast cancer cell-line-based ER CALUX test and the MMV-Luc assay.[109, 110] Other well-known tests are those based on human ovarian cell lines and Chinese hamster ovarian cell lines, BG-1 and Chinese hamster ovary (CHO) cells, respectively.[111, 112] These mammalian-cell-based assays express luciferase when exposed to estrogens and

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are more sensitive than their yeast-based counterparts using β-galactosidase, luciferase, or yEFGP. In addition, these mammalian-cell-based assays are already considered as highly valuable for testing compounds on their estrogenic properties. The BG1Luc ER TA test method, which has been validated by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATMs), and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), has also been validated under and according to OECD test guidelines (TG455).[113] However, examples of the use of mammalian cell-line-based assays for the analysis or screening of complex sample extracts are limited. An important disadvantage of these mammalian cell-line-based assays, compared to yeast-based assays, is the presence or induced expression of additional endogenous receptors, as this can lead to interference with the specific receptor response of the cell that is under investigation. The most recently developed mammalian-cell-based assay based on the CALUX technology does not suffer from this cross talk. This assay offers improved performance and is based on the human osteosarcoma (U2-OS) cell line that expresses no estrogen, androgen, progestagen, or glucocorticoid receptors (ER, AR, PR and GR).[114] Similarly to the yeast assays, both the reporter construct and the receptor were stably introduced into these human U2-OS cells. Mammalian-cell-based assays are more expensive than their yeast counterparts, are more difficult to grow, and need serum-enriched culture media to grow. The latter requirement was formerly problematic, as serum contains small amounts of steroids and other growth factors; the exposure of these cells to compounds and sample extracts must be performed in media with serum that is stripped from these steroids.[115] Currently, stripped serum is commercially available (although expensive). In addition, there is a disadvantage on using luciferase as a reporter, as several natural compounds have been shown to stabilize the luciferase enzyme and cause superinduction, with a potential risk of introducing false positives.[116] On the other hand, it should be mentioned that mammalian-cell-based assays that make use of the endogenous expressed receptor might be useful to detect compounds with an indirect effect that still involves the receptor, for example, when a compound upregulates the expression of ER. In general, both yeast assays and mammalian assays are able to detect known and unknown estrogens and antiestrogens, and both yeast assays and the latest generation of mammalian-cell-based assays are able to analyze complex sample extracts.

Cell proliferation is a process further down the mechanistic pathway than binding, transcription, and translation (expression of proteins). The E-screen is a proliferative assay based on the human MCF-7/BOS breast cancer cell line and has been used to determine the estrogenic characteristics of pesticides and alkyl phenols[117] and extracts of food samples.[118] Proliferation is measured by counting cells or nuclei. This test is also able to detect antiestrogenic activity by incubating test compounds in the presence of 17β-estradiol, measured by the inhibition of the proliferation caused by 17β-estradiol.[119, 120] An advantage, but at the same time a disadvantage, is that the assay is sensitive to the indirect effects caused

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by insulin-like growth factor (IGF), epidermal growth factor (EGF), and transforming growth factor β (TGFβ).[121, 122] The main disadvantage is that most cell lines, including MCF-7, also express androgen, progesterone, glucocorticoid, and retinoid receptors. This may compromise drawing straightforward conclusions from the assay results when testing compounds for antiestrogenicity or when testing complex mixtures or complex sample extracts for estrogenicity, which are able to activate these receptors, as it has been shown that androgens, progestins, and glucocorticoids can antagonize estradiol-induced cell proliferation. The E-screen is thus cross-talk sensitive. Furthermore, proliferative responses can only be determined after a number of days, resulting in a test that is not very rapid.[123] In a recent study by Wang et al., the proliferative responses of four different cell lines derived from three estrogen sensitive tissues, that is, breast, uterus and ovary, were compared in order to determine which cell line most accurately predicted the estrogenic effect (uterotrophic assay) observed *in vivo*.[119, 120] In that study, the E-screen was found to give the best results, but all four cell lines were suited to test compounds for their estrogenic properties. However, their use in veterinary control, for example, for urine and feed samples, is rather limited and not very successful.

While binding assays determine the affinity of a compound for the ER, the cell-based bioassays determine estrogen activity and the latter are thus very suited to uphold the European Union's ban on hormones, as this ban prohibits all substances having hormonal action. Receptor-based transcription activation bioassays are privileged because they are fast, easy, suited for high-throughput purposes, and cheap compared to the proliferation assays. The yeast yEGFP estrogen bioassay, for example, was shown to be as suited as GC/MS analysis for the detection of estrogens in calf urine.[124, 125]

4.3.2 Androgens

4.3.2.1 Binding Assays for Androgens

The first *in vitro* AR competitive binding assays that have been developed are based on the isolation of the AR from animal tissue and the use of radiolabeled ligands. An example is the assay described by Bauer et al., using a cytosolic preparation of calf uterus as AR source and radiolabeled dihydrotestosterone ([^{3}H]-DHT).[126] Just as for the estrogens, binding assays using pure recombinant receptor protein do not suffer from cross talk due to the presence of other nuclear hormone receptors and are animal-friendly. An example of such an animal-friendly AR competitive binding assay is described by Freyberger et al., using a recombinant fusion protein of the rat AR, containing both the hinge region and ligand binding domain fused to thioredoxin, and [^{3}H]-R1881 as the radiolabeled ligand.[127]

As for the estrogens, the latest assay based on AR binding is a protein array on the PamChip® plate format. It assesses the ligand-modulated interaction of AR with coregulators on a PamChip® plate consisting of 96 identical arrays,

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each array containing 155 immobilized NR co-regulator proteins (co-activators and co-repressors). However, although valid, the technique is too expensive for the analysis of samples in a daily routine setting, but it is of added value for the characterization of chemicals (mode of action research) and for lead finding in the pharmaceutical industry. The array on the PamChip® plate format works for all NRs, for example, estrogen, androgen, glucocorticoid, and peroxisome proliferator-activated receptors.[85, 86, 128, 129]

Binding assays based on SHGH, the natural transporter of the endogenous estrogens and androgens, are not specific and might result in substantial amounts of false-positive outcomes compared to competitive ER and AR binding assays when applied in a veterinary control program. However, that has not been established as yet and if there are (unknown) compounds that have no affinity for the AR, but are able to release endogenous testosterone from SHBG, such compounds would be ideal (illegal) growth promoters that can only be detected with an SHBG binding assay. In addition, the SHBG binding assay is highly relevant and of added value for predicting endocrine disruption *in vivo*, as compounds that are able to displace estrogens and androgens from SHBG will have great effects on the homeostasis, as estrogens and androgens bound to SHBG are inactive, while their free forms are already active at low concentrations. Danzo showed that nonylphenol reduced the binding of dihydrotestosterone (DHT) to hSHBG by 70% and hexachlorocyclohexane by 20%, and also *o*,*p*′-dichlorodiphenyltrichloroethane (DDT) and pentachlorophenol resulted in a statistically significant 20% inhibition of DHT binding to hSHBG[130]. So far, these and other data suggest that there is a great overlap between ER and AR binding on the one hand and SHBG binding on the other hand, which is also expected, as SHBG is the natural transporter of estrogens and androgens. In the study described by Aqai et al., recombinant human sex-hormone-binding globulin (rh-SHBG) was used in a competitive assay with labeled 17β-testosterone-d_3, which was measured in a 96-well plate format with LC-MS.[131] Suspect screened sample extracts of dietary supplements were confirmed with a chip-UHPLC (nanoTile™)-Q-time-of-flight MS system. The authors describe it as a generic steroid-binding assay and claim that this technique is suited for the detection and identification of unknown designer steroids; that is, it can be used for high-throughput screening of androgens, estrogens, and gestagens in dietary supplements to fight doping. However, (pro)gestagens and (gluco)corticoids either do not or only minimally bind to SHBG. Moreover, when critically compared to the outcomes of a previously performed study with a yeast androgen bioassay that also successfully identified the responsible anabolic compounds in these dietary supplements, that is by bioassay-guided fractionation LC-MS/MS analysis,[132] the latter combination of the cell bioassay and LC-MS/MS analysis is simpler and less expensive.

In 2009, Mooney et al. assessed whether the determination of the SHBG binding capacities in serum as a biomarker of a hormone treatment in adult heifer animals was effective and concluded that this biomarker assay had potential to identify

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illegally treated animals, particularly those exposed to androgens.[133] However, when reading the report, it becomes clear that this was a rather optimistic conclusion, as no change in binding capacities was observed following the administration of estradiol to adult male animals. Moreover, the reduced "SHBG binding capacities" did not seem to result from the possibility that exogenously administered compounds were competing directly for steroid hormone binding sites of circulating SHBG, but rather by reducing hepatic SHBG synthesis (i.e., lower concentrations of SHBG). Moreover, "SHBG binding capacities" might also vary or change with animal sexes, species, diets, exposure to environmental pollutants, or by animal illness. Overall, SHBG as a biomarker does not seem to be very promising for veterinary control purposes.

Binding assays based on specific antibodies against testosterone are not relevant for broad screening. Due to the great variety of chemicals with (anti)androgenic properties, immunochemical methods are only able to detect structurally related compounds and are unable to detect all compounds that have affinity for the AR. An example is the antibody-based indirect competitive ELISA method for detecting testosterone as developed and validated by Zhang et al. in 2014.[134] This icELISA is able to detect low concentrations of testosterone in spiked bovine samples, that is, muscle, liver, and kidney, but the affinity of the monoclonal antibody (mAb) for other androgens was not determined. The validated test is only proven to be useful to detect testosterone, that is, the antigen used to produce the mAb. There are many other ELISAs to detect androgens, for instance, the test strip described by Ploum et al. for the detection of nortestosterone residues in urine samples.[135] This test strip enzyme immunoassay could be performed within 60 minutes and was able to detect 5 μg/l of nortestosterone in urine.

4.3.2.2 Bioassays for Androgens

In vitro cell-based bioassays are widely used to characterize the androgenic properties of chemicals. They were mainly developed for lead finding approaches in the pharmaceutical industry and to test environmental pollutants for their potential ability to alter normal hormone function *in vivo*. A lot of designer steroids have been synthesized and many chemicals, that is, nonylphenol, bisphenol A, and pesticides, are now classified as endocrine-disrupting chemicals (EDCs). The most commonly used yeast-based bioassay for androgens is the assay developed by Death et al. in 2005, using a *Saccharomyces cerevisiae* that expresses the human AR and β-galactosidase upon activation of the hAR.[136] Another yeast androgen bioassay is described by Michelini et al.; this yeast expresses luciferase upon exposure to androgens and shows an EC_{50} for testosterone of 10 nM.[137] This assay was used to screen human serum samples, and the authors claim their assay is superior to conventional assays for steroid hormones based on immunological detection.[138] Both the luciferase and β-galactosidase enzyme-based assays may have issues due to artifacts, for example, if test compounds inhibit enzyme activity or stabilize the enzyme.[116] A change for the better was achieved with the development of a yeast androgen bioassay based on the increased expression of

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yEGFP, as this reporter is superior to β-galactosidase and luciferase, because it does not need the addition of an enzyme substrate and because it can be measured directly in intact living cells (no substrate and no cell lysis needed), making this yEGFP yeast androgen bioassay cheaper, quicker, and easier. However, this yeast androgen bioassay is less sensitive, EC_{50} for 17β-testosterone 50 nM, compared to the yeast-cell-based bioassay expressing the β-galactosidase enzyme, that is, EC_{50} for 17β-testosterone 5 nM.[139] Still, this yeast yEGFP bioassay is sensitive enough for control purposes and was the first bioassay shown to be able to detect the designer steroid tetrahydrogestrinone (THG) in human urine samples.[82] Moreover, this yEGFP yeast androgen bioassay showed its added value compared to an LC-MS/MS analysis, when it was used for the screening of dietary supplements on the presence of anabolic substances.[132] In the latter study, 18 different dietary supplements that had previously been analyzed with LC-MS/MS were screened with this yeast androgen bioassay. While LC-MS/MS showed 11 samples positive for androgens, the bioassay showed two more positives in the seven that were negative by LC-MS/MS. Subsequent bioassay-guided fractionation LC-TOF MS analyses identified 1-testosterone in one supplement and 4-androstene-3β, 17β-diol, and 5α-androstane-3β,17β-diol in the other. These anabolic steroids were missed by the LC-MS/MS analysis, meaning that the chemical analytical method alone gave over 15% of false-negative outcomes (2 out of 13). This yeast androgen bioassay expressing yEGFP was fully validated according to the guidelines and criteria described in EC Decision 2002/657[7] and acquired an ISO 17025 accreditation status for the analysis of both feed and calf urine.[140]

Prohormones with an androgenic mode of action, for example, dehydroepiandrosterone (DHEA), are not active in this yEGFP yeast androgen bioassay, but by introducing a metabolic activation step, DHEA was activated and could be detected.[141] The *in vitro* activation of inactive "mother" compounds was further developed, resulting in protocols for the *in vitro* activation of prohormones (e.g., DHEA and pregnenolone), hormone esters (e.g., estradiol benzoate and testosterone decanoate), and conjugated hormones (e.g., genistin). Herbal mixtures and sport supplements were screened using the yeast yEGFP bioassay in combination with these *in vitro* protocols to activate proandrogens, androgen esters, and conjugated androgens. Samples screened positive were then analyzed by UHPLC-TOF MS and led to the positive identification of nortestosterone, phenylpropionate, testosterone cyclohexanecarboxylate, and methyltestosterone in herbal supplements.[142] These *in vitro* protocols to activate inactive compounds have also been combined with the yeast estrogen bioassay expressing yEGFP and can be combined with all other binding and bioassays as well.

A common feature of mammalian cell-based bioassays, including luciferase, Secreted Embryonic Alkaline Phosphatase (SEAP), and green fluorescent protein as reporter enzyme or protein, is their high sensitivity, resulting in lower EC_{50} values when compared to yeast-cell-based bioassays.[143] However, especially in the case of *in vitro* transcription activation assays for androgens, the lack of known endogenous receptors in yeast is a big advantage compared to mammalian

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cell lines, as androgen responsive elements (AREs) can also be activated by the progesterone and glucocorticoid receptor (PR and GR) and the latter receptor is normally expressed in all mammalian cell types. This resulted in mammalian bioassays that are not specific for androgens, but also respond to progestagens or glucocorticoids, such as the AR CALUX® based on the T47-D human breast carcinoma cell line. This T47 AR-CALUX® expresses luciferase when exposed to androgens, but it also responds to progesterone and the synthetic glucocorticoid dexamethasone.[144] The TM-Luc T47-D assay is based on a similar human breast cell line and suffers from the same drawback.[145] It showed maximum responses with 100 nM of the androgenic compound 17β-trenbolone, but also with 100 nM progesterone. Moreover, mammalian cells express a range of androgen metabolizing enzymes including aromatase, 5α-reductase, 17β-hydroxysteroid reductase, and 3α-hydroxysteroid reductase, which can alter the potency of the test compound, either activating or deactivating it.[146] The benefits of the metabolizing enzymes are that they may allow for prohormones to be detected and offering insight into how a complex extract may behave *in vivo*. However, the metabolic capacity of mammalian cells is limited and affected by the type of host cell, as most cell types express only a few of the metabolizing enzymes and at different concentrations. Moreover, the metabolic capacity might be influenced by the passage number of the cell line, as some cells are reported to switch off expression of some metabolizing enzymes during *in vitro* culture. Thus, *in vitro* metabolism in cultured mammalian cells does not reflect *in vivo* metabolism. To date, the literature reports ambiguity with EC_{50} sensitivities and specificities, most likely due to these metabolizing effects.[114, 147–150] Only the mammalian AR bioassay based on the human U2-OS cell line is as specific as the yeast-based bioassays for androgens. In order to fully exclude the cross-talk by the PR and GR, normally present in almost all mammalian cell lines, this latest AR CALUX® test, such as the new ERα CALUX, is based on the U2-OS cell line that expresses none, or only very low concentrations, of the endogenous ER, AR, PR, and GR. This new AR CALUX® bioassay was shown to be specific and sensitive and useful for the detection of androgenic steroids abuse in doping control.[151, 152]

One of the first proliferation assays used to study androgen action was based on a human LNCa-FGC prostate cancer cell line. It turned out that this cell line showed an enhanced estrogen binding due to a point mutation in the androgen binding domain. The group of Soto developed a so-called A-screen for detecting androgenic activity based on cell proliferation.[153–155] For this test, the same MCF7 cells as used for the E-screen were modified and transfected with the human androgen receptor (MCF7-AR1 cells). However, just as the E-screen, the test has some drawbacks due to possible cross talk and the duration of the assay. While the E-screen is used worldwide, use of the A-screen is less common.

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4.3.3 Metabolic Profiling Assay to Detect Abuse of Estrogens and Androgens

Administration of exogenous steroids induces temporal modifications of endogenous steroid profiles, which can be detected and used for pinpointing misuse.[156] In this case, the animal itself can be regarded as the bioassay. It has been shown that the administration of testosterone or one of its precursors to humans alters the concentrations of steroids excreted in urine.[157] The best known example is the alteration of the testosterone:epitestosterone ratio, enabling detection of testosterone abuse by athletes. However, metabolism in cattle differs from humans and profiles such as those established for humans are not available for cattle.[158] Therefore, a novel approach, based on the total measurement and profiling of natural circulating hormones in bovine urine, was developed.[159] In circulation, these natural hormones are present at different concentrations and can be classified as precursors, active steroids, or phase I and II metabolites. This is a dynamic system and homeostasis is reached as a balance between the rate of steroid hormone synthesis and the rate of metabolic inactivation or elimination of these natural hormones. Administration of exogenous hormones will induce disruptions by changing the processes of hormone synthesis and hormone elimination, resulting in modified concentrations of circulating natural hormones and modified concentrations excreted in urine. A databank was created, containing urine profiles of both treated and untreated animals.[9] Dedicated statistical tools based on multivariate techniques were applied to handle these large datasets. A model was built for the detection of exogenous natural hormone abuse using Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), and this model enabled discrimination between a normal and a treated population. Unknown samples are projected in this model, samples of untreated animals will be projected in the untreated group, and in case of a sample of a treated animal, it will be projected in one of the treated groups. The model was validated and applied to samples originated from treated and untreated herds, and all samples were classified correctly.

4.3.4 Progestagens and Glucocorticoids

4.3.4.1 Binding Assays for Progestagens and Glucocorticoids

There are several PR and GR competitive binding assays. Scippo et al. used a human PR that was produced in a genetically modified bacteria in combination with tritium-labeled progesterone ([^{3}H]-progesterone) to investigate the endocrine-disrupting properties of various chemicals.[160] Attardi et al. used recombinant human progestin receptors (hPR-A and hPR-B) isolated from cytosolic preparations of genetically modified Sf9 insect cells in combination with [^{3}H]-progesterone to study the properties of bolandiol.[161] In the same study, a commercially available purified recombinant human GR was used in combination with [^{3}H]-dexamethasone, while in a previous study, Attardi et al. used cytosolic preparations from uterus or thymus of immature rabbits,

for PR and GR competitive binding assays, respectively, to study the binding characteristics of several pharmaceuticals.[162]

As described for the estrogens and androgens, the PamChip® peptide array on the 96-well plate format is an option to study the cofactor requirement of ligand-induced activation of the human progesterone receptor (hPR) and human glucocorticoid receptor (hGR).[85, 86, 128, 129]

Progestagens and (gluco)corticoids do not bind to SHBG. Progestagens are mainly bound to serum albumin and to a lesser extent by transcortin, and (gluco)corticosteroids are mainly bound by transcortin, also known as the (gluco)corticosteroid binding globulin (CBG). Neither serum albumin nor transcortin competitive binding assays have been used for the detection of progestagens. However, a transcortin competitive binding assay was set up to measure cortisol in plasma over 40 years ago. Horse serum was used as the source of transcortin and [^{3}H]-corticosterone or [^{3}H]-cortisol as the labeled ligand.[163, 164] In 1978, Stahl et al. used this transcortin competitive binding assay for the routine measurement of cortisol in plasma, urine, and amniotic fluids.[165] Ever since, the use of these assays has been rather limited and ELISAs are preferred to determine cortisol in plasma and urine. One of the latest ELISAs described for the detection of progesterone makes use of a mAb in combination with a hormone releasing peptides (HRP)–enzyme conjugate and has been used to determine progesterone concentrations in human serum.[166] This direct competitive ELISA with a simple HRP enzyme assay is user-friendly compared to earlier immunoassay techniques that have been developed for the measurement of progesterone, for example, radioimmunoassays (RIAs), chemiluminescence immunoassays (CLIAs), time-resolved fluorescence immunoassays (TRFIA), and fluorescence polarization immunoassays (FPIA). One of the most recently described ELISAs for the detection of cortisol is described by Nadendla et al., using a commercial kit for the determination of cortisol in saliva samples.[167]

4.3.4.2 Bioassays for Progestagens and Glucocorticoids

There are several *in vitro* transcription activation assays for the detection of progestagens and glucocorticoids. The yeast progesterone assay developed by Chatterjee et al. provides a sensitive, fast, and user-friendly progesterone receptor transactivation assay.[168] It uses a recombinant yeast, *S. cerevisiae*, which is modified to express the hPR and a progesterone response element (PRE) driving the expression of yEGFP when these cells are exposed to progestagens. This yeast progesterone assay and the latest PR CALUX test, based on the U2-OS cell line,[169] are the state of the art with respect to the determination of the progestogenic properties of chemicals and the analysis of complex matrices such as food, feed, and environmental samples. A similar observation is valid for the (gluco)corticosteroids. Here a recombinant yeast cell was constructed by Bovee et al., expressing the human glucocorticoid receptor alpha (hGRα) and a green fluorescent reporter protein in response to glucocorticoids.[170] Both the receptor construct and the reporter construct were stably integrated into the

yeast genome. The correct and specific functioning of this yeast glucocorticoid bioassay was studied by exposures to cortisol and other related compounds and critically compared to the latest GR CALUX® bioassay, based on the human U2-OS bone cell line. Although less sensitive, the new yeast glucocorticoid bioassay showed sensitivity toward all (gluco)corticoids tested and revealed similar relative potencies as obtained with the GR CALUX® bioassay. Hormone representatives for other hormone NRs, such as 17β-estradiol (E2) for the ER, 5α-dihydrotestosterone for the AR, and progesterone for the PR, showed no clear agonistic responses. Both assays are also suited to test complex sample extracts and are the state of the art with respect to the determination of the corticosteroid properties of chemicals and the analysis of complex matrices. When applied to the detection of (gluco)corticoids in feed, the GR CALUX® was validated and used for regular monitoring purposes.[171]

Effects of (pro)gestagens are often studied with endometrial cells, as this is the main target of endogenous progesterone. However, only one proliferation assay to determine the (pro)gestagenic activities of compounds is described.[172] This proliferation assay is based on human endometrial endothelial cells (HEECs), and it shows that the proliferative responses are rather poor. Moreover, proliferation of HEEC was only significantly affected by a low dose of estradiol (10 nM) and not by progesterone (100 nM). Other hormones such as ethinylestradiol and levonorgestrel decreased proliferation, and the decreased proliferation observed with several EDCs was most often due to cytotoxicity; that is affected HEEC cell viability as was established by vital staining with propidium iodide and Hoechst 33258.[172]

There is no specific proliferation assay for (gluco)corticoids. Activation of the GR is often associated with a decrease in proliferative responses (growth arrest) and an increase of adipogenesis.[173, 174] This is the reason that corticosteroid therapy, for example, dexamethasone, is or may be useful to treat certain cancers[175]; however, this is highly dependent on the cell type, as dexamethasone can also enhance the growth of cancer cells.[176]

4.3.5 Thyreostatics

So far, attention on the endocrine activity of chemicals has largely been focused on estrogen and androgen disruption. This is in contrast to the number of chemicals listed on the Toxnet hazardous substance databank (HSDB) that lists 376 chemicals for estrogen, 147 chemicals for androgen, and 895 chemicals for thyroid activity.[177] This is a cause for concern as altered TH concentrations have the ability to cause severe adverse effects, such as decreased fertility and retarded development (especially of the bones and the brain). Changes in TH concentrations are also directly related to changes in cardiac output, heart rate, and systemic vascular resistance.[178–180] Given the complexity of the *in vivo* hypothalamic–pituitary–thyroid (HPT) axis, it is impossible to discuss

all relevant binding and bioassays. This part will therefore mainly focus on the compounds that are of veterinary concern, the thyreostatics.

Two binding assays, one based on thyroxine-binding globulin (TBG) and the other based on recombinant transthyretin (rTTR), developed by Marchesini et al., were tested for their ability to detect known thyroid disruptors such as halogenated phenols, halogenated bisphenols, bisphenol A, and 3,5-dichlorobiphenyl and its hydroxylated metabolite 4-hydroxy-3,5-dichlorobiphenyl.[181, 182] The TBG-based assay was only sensitive toward the TH thyroxine (T4), and the rTTR-based assay was sensitive toward several compounds. Jomaa et al. investigated the *in vitro* effect of 11 thyroid-active compounds, known to affect pituitary and/or thyroid weights *in vivo*, on the proliferation of GH3 rat pituitary cells in the so-called T-screen, and of FRTL-5 rat thyroid cells in a newly developed test denoted as "thyroid-stimulating hormone (TSH)-screen."[180] Pituitary cell proliferation in the T-screen was stimulated by three compounds, namely thyrotropin-releasing hormone (TRH), triiodothyronine (T3), and thyroxine (T4), while *in vivo* T4, propylthiouracil (PTU) and aminotriazole (3-AT) caused an increase in relative pituitary weight. Thus, T4 was the only compound for which the effect on *in vitro* cell proliferation correlated with an increase in pituitary organ weight. As to the newly developed TSH-screen, 2 out of 11 compounds tested had an effect, namely TSH-induced and T4-antagonized FRTL-5 cell proliferation, and these effects correlated with *in vivo* changes induced by these compounds on thyroid weight. Altogether, the results indicated that most of the selected compounds affect pituitary and thyroid weights *in vivo* by modes of action different from a direct thyroid hormone receptor (THR)- or thyroid-stimulating hormone receptor (TSHR)-mediated effect on cell proliferation. Moreover, these assays did not detect effects of PTU and the other thyreostatics (antithyroid drugs) tested, that is, methimazole (MMI), ethylene thiourea (ETU), and the herbicide 3-AT. For monitoring and enforcement purposes, a screening assay should at least be able to detect thiouracil (TU), methylthiouracil (MTU), PTU, and MMI. It should be noted that MMI is also known as thiamazole or Tapazole (TAP). However, it was not really surprising that the binding assays and bioassays described earlier do not detect these thyreostatics, as these antithyroid drugs decrease serum concentrations of THs by inhibiting thyroperoxidase (TPO), a critical enzyme in TH synthesis.[183–185] Despite the fact that these assays are relevant and of added value in a test panel of *in vitro* assays for predicting the potential endocrine disrupting characteristics of chemicals *in vivo*, a TPO enzyme assay is needed for the enforcement on the abuse of thyreostatics. The problem is that there is no commercial source of a functional TPO enzyme, and isolating the enzyme from fresh animal tissue is the only option. Such a TPO enzyme assay is able to detect known TPO inhibitors and, in addition, would be able to detect other designer thyreostatics that also inhibit TPO. However, enzyme assays are often only sensitive in the μM range, while a nM range would be required in order to test for the presence of these compounds in urine samples when dealing with sample volumes of 0–10 ml. It

is thus expected that such an enzyme assay would not be suited in real practice for urine samples (EU CRL recommended concentration for control purposes of 10 µg/l for thyreostatics – see Section 4.4.2.1), but would only be of added value for testing feed samples or preparations for the presence of these oral active thyreostatics.

4.3.6 β-Agonists

4.3.6.1 Binding Assays for β-Agonists

Besides the AR, the β2-adrenergic receptor is the main target and receptor type that is responsive to anabolic agents that are illegally used in sports doping and legally or illegally, depending on national regulations, in meat production. Agents such as clenbuterol, mabuterol, salmeterol, and ractopamine are the best known examples.[186] Boyd and coworkers developed and validated a competitive β2-adrenergic receptor binding assay for the detection of a broad range of β-agonists in feed.[81] The β2-adrenoceptor was isolated from cultured cells and solubilized. This solubilized receptor was found to be highly stable when stored at −80 °C. The method was validated according to EC Decision 2002/657[7] and proved capable of detecting 250 ng clenbuterol equivalents per gram of sample. This is well below the quantities normally associated with β-agonist medicated feeds. The β2-adrenoceptor used in the study only failed to bind the compound zilpaterol, raising doubts as to whether this compound is a true β2-adrenergic drug. However, Nielen et al. used this competitive β2-adrenergic receptor binding assay and reported the finding of a previously unidentified β2-agonist.[81]

Many immunoassays have been developed for the screening of β-agonists: for example, the on-site tube enzyme immunoassay developed by Haasnoot et al. in 1996.[187] In this on-site tube test, a mixture of antibodies raised against clenbuterol and salbutamol was used, resulting in a test that showed activity toward a whole range of β-agonists. In the case of β-agonists, antibody-based tests are suited for broad screening, as many β-agonists have similar structures, that is, are structurally related, and will be recognized by the same antibody. Application of the on-site tube test to 269 bovine urine samples yielded positive results for all samples with concentrations of 3 µg/l clenbuterol and higher. Even lower concentrations, that is, 1 µg/l, could be detected in calf urine (due to lower matrix effects). As antibody-based tests are very well suited for the broad screening of β-agonists, these tests are still used in monitoring programs and enforcement approaches in the control of veterinary production. Actually, the new β-agonist discovered by Nielen et al. in 2003 was first detected by accident with an immunoassay designed for clenbuterol.[81] The β-adrenergic potency was subsequently confirmed by the radioreceptor assay, and the latter assay was used to identify this new β-agonist.[81]

Hair has been shown to be an excellent matrix for veterinary inspection for use of some β-agonists, as clenbuterol residues accumulate in hair, with for some compounds, a preference for the pigmented fraction.[188] Moreover, compared with other matrices, in spite of the need for some clean-up, hair is easy to sample and

analyze. When a simple digestion–extraction procedure for hair was combined with an ELISA sensitive for clenbuterol, it resulted in a fast screening procedure that was able to detect and track the illegal use of clenbuterol, bromobuterol, mabuterol, and mapenterol.[189] Subsequently, these tests have been used, and over time, the specified limits for the detection of these illegal drugs have been lowered. To improve the immunochemical screening of urine samples in order to detect lower concentrations of several β-agonists, Haasnoot et al. applied immunofiltration (IF) for sample clean-up in combination with a β-agonist ELISA.[190] Compared with the direct β-agonist ELISA, the IF clean-up resulted in 30 times lower limits of detection and the combination of IF with the β-agonist ELISA was found suitable for the detection of at least 10 β-agonists in urine. Currently, these kinds of competitive binding assays are the most suitable tests for detecting β-agonists in food samples.[191, 192] There is only one drawback to the use of immune-based methods for the detection of β-agonists. As zilpaterol structurally belongs neither to the group of the anilinic nor to the group of phenolic β-agonists, existing antibody-based screening methods may not successfully detect this or similar β-agonistic compounds. The competitive β2-adrenergic receptor binding assay described earlier is also not able to detect this compound.

4.3.6.2 Bioassays for β-Agonists

The development of a specific reporter gene assay for β-agonists is relatively very difficult, as the β2-adrenergic receptor is located in the outer cell membrane and has no direct interaction with the DNA. Upon binding of an agonist, these 7-transmembrane receptors activate an adenylate cyclase via a G-protein. The subsequent formation of the intracellular adenosine 3′,5′-cyclic monophosphate (cAMP) signaling molecule is not specific enough and can be influenced by many other cell processes. Despite this potential cross talk, a large amount of cell-based tests for these so-called G-protein coupled receptors (GPCR) or membrane receptors (MRs) can be found, for example, the ALPHAscreen® or dissociation-enhanced lanthanide fluorescence immunoassay that directly measures the amount of cAMP.[193] Methods where the cAMP activates protein kinase A that phosphorylates the cAMP-responsive element binding (CREB) protein, which in turn binds a particular responsive element promoter setting of a reporter gene, are described as well.[194, 195] However, due to potential cross talk, the fluorescence or bioluminescence resonance electron transfer (FRET and BRET) techniques offer a more specific alternative to measure the activation of GPCR membrane receptors.[196, 197] Another example is the bioassay developed by Takeda et al., using CHO cells stably expressing E3-tagged β2-adrenergic receptors (E3-β2ARs).[198] The E3-β2ARs on these cells were stained with pH-dependent fluorescein (FL) and pH-independent tetramethylrhodamine (TMR). Upon activation of the E3-β2AR by a ligand, receptor internalization (i.e., translocation of the receptor from cell surface to intracellular regions) was automatically detected using a fluorescence image analyzer. Moreover, acidification in endosomes leading to a decrease of the signal of the pH-dependent TMR over time was

detected simultaneously. The extent of endocytosis was significantly dependent on the agonist used, indicating the presence of a biased signaling for endocytosis. In addition, the assay was shown to be able to detect receptor antagonists as well, by competitive inhibition of agonist-induced endocytosis. To date, this assay is the state-of-the art effect-based cell bioassay, for the detection of β2-agonists. It only requires normal cell culture facilities and a fluorescence image analyzer, although the latter is rather expensive.

4.3.7 Growth Hormones

GH, also known as ST, is a 22 kDa protein hormone, which is endogenously produced by the anterior pituitary gland. GH stimulates growth, lipolysis in adipocytes, and also growth of muscle and bone.[199] Release of GH by the anterior pituitary gland is triggered by growth hormone–releasing hormone (GHRH) and growth hormone–releasing peptides (GHRPs). GH release can also be repressed, for example, by somatostatin (SS) and insulin-like growth factor 1 (IGF-1). Releasing and repressing factors can operate in two ways; directly onto target tissues or indirectly by IGF-1 release from hepatic tissues. Use of GH in the dairy industry has been known since the 1930s,[200] but its use was limited for a long time, as crude extracts of several pituitary glands of slaughtered bovines were needed to treat a single cow. This changed in the 1980s, when biotechnology offered the opportunity to produce recombinant bovine somatotropin (rbST) in large amounts, resulting in a widespread use of GH to enhance milk production.[201] Recombinant forms of GH are now more widely used to increase food production. In cattle, it is used for growth and for enhancement of the lactating performance. In fish, GH is used to increase length and bodyweight.[202] Also noteworthy is the use of recombinant GH in equine sports doping. This use is intended to enhance the performance of horses, but as a result, GH might eventually end up in the food chain as well. These recombinant forms of GH are very similar to the endogenous forms, and this similarity makes it difficult to pinpoint the recombinant GH as exogenous and misuse. Several bio-based screening approaches to detect abuse of GH are discussed in the next section.

4.3.7.1 Recombinant Growth Hormone in Cattle

Recombinant bovine GH, or rbST, is widely used to enhance growth and the lactating performance of cattle. Worldwide legislation, however, is very different. In the United States of America, for instance, it is common practice to use rbST to increase milk production, while there has been a ban on rbST use since 2000 in Europe.[203] Sensitive methods to detect rbST abuse are needed in order to enforce this ban, but detection of rbST is hampered due to the similarity with the endogenous hormone, the low concentrations of rbST in serum, and strong fluctuations in natural bovine somatotropin (bST) concentrations. However, rbST-dependent biomarkers have a longer half-life and may offer

a promising alternative, as biomarkers have been used successfully to detect steroid abuse in cattle and in sports doping.[204–208] Several proteins of the GH/IGF-axis and of bone and soft tissue turnover are affected by (r)bST and can be used as biomarkers to detect the illegal administration of these GHs. Moreover, rbST administration induces an immunological response in dairy cattle, resulting in the formation of specific antibodies against these administered GHs.[209–211] Many bio-based screening assays to pinpoint rbST abuse have been developed and were reviewed by Ludwig et al. in 2014.[212] Immunoassays to detect single biomarkers related to (r)bST administration were developed on different platforms, for example, RIAs,[213–215] ELISA,[216–219] Western Blot (WB) techniques,[220, 221] and flow cytometric immunoassays (FCIA).[211, 222, 223] Although these techniques to detect single biomarkers work, more powerful methods are needed to prove abuse of (r)bST, that is, methods based on several biomarkers. Four candidate biomarkers, IGF-1, IGFBP2, osteocalcin, and anti-rbST antibodies, were selected and simultaneously analyzed in one serum sample.[224] The data obtained with a multiplex FCIA method based on these four biomarkers eventually indicated that a combination of only two biomarkers, that is, osteocalcin and anti-rbST antibodies, was powerful and sufficient enough to identify over 95% of the rbST-treated cows as truly positive in two independent *in vivo* studies in which cows had been treated this GH.[224] The same anti-rbST antibodies were also found in raw milk from the rbST-treated cows. The response in these milk samples was even detectable for at least 2 weeks after the last rbST treatment.[223] When analyzing tank milk samples from rbST-treated cows, also over 95% of the samples were identified as positive for rbST treatment.

4.3.7.2 Growth Hormone in Fish

In fish production, it has been shown that rainbow trout and tilapia injected with ST increase in length and body weight. As recombinant bovine and recombinant porcine somatotropin (rpST) can easily be obtained and also give growth effects in fish, rbST and rpST are the main forms used as GHs of fish. Fish show increased serum IGF-1 concentrations as well as a specific immunological response as a physiological response to exogenous rbST.[225, 226] Interestingly, the negative feedback loop of decreased endogenous ST, as observed in mammals, was not observed in trout and tilapia.

In trout, changed IGF-1 concentrations were determined by RIA and specific endogenous trout anti-rbST antibodies were found by WB analysis.[225] In tilapia, anti-rbST antibodies were detected by ELISA.[226] These altered protein concentrations are promising candidate biomarkers for the detection of rbST treatment of fish, but these biomarker-based methods have not specifically been developed for fish and are not yet validated. Until now, control of the use of rbST in aquaculture has been based on feed and water analysis for the presence of these GHs and does not yet include routine control of rbST administration to fish.

4.3.7.3 Growth Hormone in Horse

Administration of equine somatotropin (eST) to horses is expected to influence the concentrations of the same proteins as observed in human and cattle. Proteomic analysis indeed revealed increased IGF-1 and IGFBP3 concentrations after eST administration, which are therefore promising biomarkers.[227] Specific antibodies produced by the horse upon injection of eST were analyzed by ELISA and by a surface plasmon resonance (SPR)-based biosensor.[227] Ultimately, radio immunoassay quantifications of IGF-1 and IGFBP3 concentrations were implemented in routine horse racing doping control, whereas it is suggested to use anti-eST antibodies as an additional biomarker when results of IGF-1 and IGFBP3 analysis are doubtful.

4.3.8 Conclusions and Future Developments in Bio-Based Screening Methods

The following conclusions may be drawn regarding the current situation and likely future developments in this field of testing for hormones:

- ER, AR, PR, GR, and β2-adrenergic receptor competitive binding assays offer good opportunities for the screening of estrogens, androgens, progestagens, glucocorticoids, and β-agonists.
- Steroid hormones and several other classes of compounds, such as pharmaceuticals and contaminants, can alter endogenous hormone concentrations by affecting the steroid biosynthesis *in vivo*. These effects can be detected by metabolite profiling methods as described by Blokland et al., generating fingerprints that are capable to detect all compounds that disrupt the homeostatis.[9] Specific profiles were generated to pinpoint external administration of steroid hormones.
- Immune-based binding assays are less suited for broad screening, as they only detect a single compound or structurally related compounds. However, in the case of β-agonists, antibody-based tests are suited for broad screening, as many β-agonists have similar structures, that is are structurally related, and will be recognized by the same antibody. These immune-based tests for β-agonists are still used in monitoring programs and enforcement approaches. The great advantage of immuno methods is their simplicity and their possibilities for on-site testing.
- Cell-based effect assays (bioassays) are superior for broad screening compared to binding assays based on receptors, transport proteins, or antibodies.
- For estrogens, androgens, progestagens, and glucocorticoids, the receptor-based transcription activation bioassays are the most suited. Both mammalian-cell- and yeast-based bioassays are able to detect both agonist and antagonist activity.
- A sensitive TPO enzyme assay is needed for the enforcement on the abuse of thyreostatics. Such a sensitive assay has not been described yet.

- With respect to the veterinary drug control, future developments should be focused on the quick identification and confirmation of non-compliant bio-screening results[132, 228].
- More attention should be given to compounds that might alter the expression of steroid receptors, as upregulation of these receptors might also lead to growth promotion. The biomethods described in this section do not allow the detection of such compounds, and new bioassays based on cells expressing endogenous receptors are needed to detect such compounds.
- Several biomarker-based assays were developed for detection of GH misuse in dairy cattle. The multiplex screening assay using four biomarkers was shown to be very reliable and able to correctly predict 95% of treated samples.
- In aquaculture, although biomarker-based methods are not specifically developed for fish, biomarker-based methods can also be used for the detection of GH abuse, but until now, control has only focused on rbST analysis in water and feed.
- In horse racing doping control, a radio immunoassay to detect increased concentrations of IGF-1 and IGFBP3 biomarkers upon treatment with GHs is implemented. When results of IGF-1 and IGFBP3 analysis are doubtful, it is recommended to use anti-eST antibodies as an additional biomarker to detect eST misuse.

4.4 Natural Hormones

This section focuses on analytical methods for the detection of natural hormones. The use of some of these compounds, such as 17β-testosterone and 17β estradiol, for growth-promoting purposes is legalized in many countries, but banned in the EU and some other countries. It is recognized that both on the legal and on illegal markets, a variety of synthetic hormones that are not naturally present in animals are also available. Examples of compounds registered in some countries for growth promotion are zeranol, trenbolone, and altrenogest. In addition to these compounds, there are numerous synthetic hormones for which there is a history of abuse for growth promotion for sports-doping purposes. Well-known examples are methyltestosterone, ethynylestradiol, and stanozolol. However, the contemporary analytical challenges are not with these compounds but with those compounds where a natural background can be present.

It has been known for years that natural hormones are administered, whether legally or illegally, to cattle. Several synthetic natural hormones, frequently as esters, were identified in seized preparations. Natural hormones can be present in biological samples: edible tissues, serum or plasma, fat, hair, and skin, and the analytical challenge is to elucidate their origin. The presence of the natural hormones in a sample can be the result of any (combination) of the following specific situations:

- Endogenous production as part of the normal physiology of the animal species concerned
- Endogenous production under specific circumstances: for example, pregnancy, injury, or stress
- Production within the sample due to the presence of specific bacteria or enzymes
- Production in the sample through instability, heat temperature, light, moisture, and so on

Due to this variety of sources, the steroids that need to be considered as "natural" or "semi-natural" also include compounds such as nortestosterone and boldenone.

These sources must be discriminated from the presence following treatment of the animal by injection, orally through feed or drinking water, implantation under the skin or dermally (pour-on), or a range of combinations of the aforementioned (Figure 4.1).

The fact that multiple explanations can be given for the presence of a range of compounds in a biological matrix severely hampers the possibility of enforcement of the ban on their use in meat production. The usual approach to detect the abuse of banned substances, in which biological samples are semi-quantitatively tested for the presence of a banned substance or a marker metabolite ("classical model"), cannot be directly applied in case a natural background concentration can be present. Even when this background concentration is relatively low, the

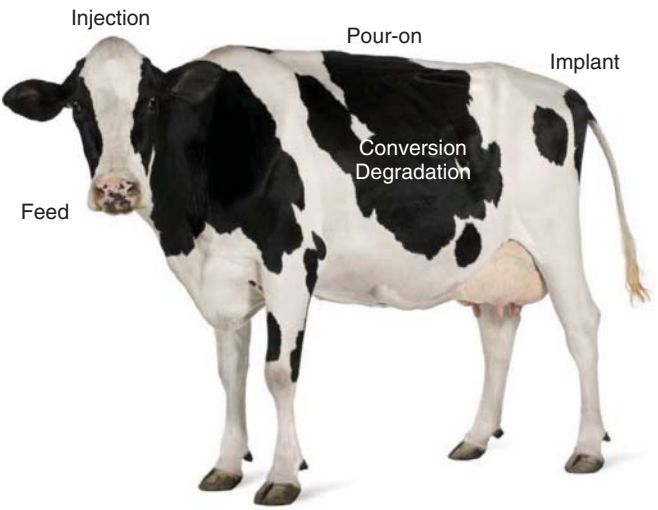


Figure 4.1 Possible sources of exogenous natural compounds can be from feed, injection (steroids or somatotropin), pour-on application, or conversion or degradation of compounds in the gastrointestinal tract.

mere fact that the presence of a particular analyte may be due to general or specific physiological conditions cannot be excluded. This then requires a different analytical testing strategy for exogenous compounds. The classical testing model comprises two steps: a screening procedure focusing on selecting suspect samples while avoiding false-negative results, followed by a confirmatory step focusing on the unambiguous confirmation of the identity of the analyte. Screening for the abuse of (semi)natural hormones can follow a similar approach since also in this case the outcome of the screening procedure is either a result "compliant," providing no indication of treatment with a banned substance, or "suspect," where test results indicate that such treatment cannot be excluded. The definition of "suspect," however, is slightly different in this case. In the classical model, "suspect" means that there are indications that the target analyte is present in the sample tested. When testing for natural hormones, "suspect" means that there are indications that the animal from which the sample was taken may have been treated with a banned substance. This difference has significant impact on the complexity of the confirmatory procedure to be applied. So far, only two approaches for confirmation are available: the direct detection of the steroid as ester (application form) or by the use of GC-C-IRMS (see Section 4.2.4).

The following sections summarize the current scientific knowledge that is available on the "semi-natural" occurrence of hormonal active compounds. Screening and confirmation methods for different classes of "natural hormones" will be discussed depending on administration route, for example, conversion during digestion, contaminants from feed, illegally administrated, and protein GHs.

4.4.1 Natural Compounds Formed During the Digestion Process

It is known that several steroids are excreted in bovine feces, and it has been demonstrated that, under the influence of certain conditions, such as the presence of certain bacteria, these steroids can be converted to other steroids. Ultimately, this can cause false-positive results in monitoring programs.

4.4.1.1 Prednisolone

Prednisolone, a corticosteroid with glucocorticosteroid activity, is used for the treatment of a wide range of inflammatory and autoimmune conditions. The use of corticosteroids in livestock is regulated in the European Union for therapeutic purposes. Prednisolone is part of registered veterinary drugs also containing amoxicillin, a clavulanic acid for intramammary administration. The maximum residue limit established in the EU for therapeutic use of prednisolone in bovine animals is 4 μg/kg in muscle and fat, 10 μg/kg in liver and kidney, and 6 μg/kg in milk (EU Commission Regulation 37/2010[229]).

The illegal use of corticosteroids as growth promoters cannot, however, be excluded. Corticosteroids can prolong the effect of growth-promoting substances, such as anabolic steroids and β-agonists, in the last weeks before slaughter.[230–232] In recent years, low concentrations of prednisolone in bovine

and equine urine have been reported.[233, 234] Because prednisolone differs in structure from cortisol by only one double bond, the formation of prednisolone could resemble the process described for the formation of boldenone from testosterone in the presence of feces in the urine sample.[235, 236] The possibility of *in vivo* formation of prednisolone from endogenously present cortisol, under a variety of conditions, has indeed been reported.[237–241]

From these and numerous other studies, it can be concluded that prednisolone is found in bovine urine from animals of different ages, in both beef and dairy cattle, and also in porcine and equine urine samples, although to date, only a few reports have appeared for pigs and horses. A relation between prednisolone in urine and fecal contamination has also been provided, with *in vitro* proof, possibly indicating that the formation of prednisolone was caused by bacterial transformation due to bacteria from feces or soil. Leporati et al. proposed 20β-dihydroprednisolone as a biomarker to detect illegal use.[242] After intramuscular administration of prednisolone acetate, this metabolite was detected for 6 days at concentrations up to 27 µg/l in urine. In addition, 20α-dihydroprednsiolone, 6-β-hydroxy-prednisolone, and 20β-prednisone were found but concentrations were lower.

After administration of prednisolone to bovines, prednisolone and a small amount of prednisone were detected. Recent studies from a Belgium group showed the presence of prednisone in calves and 20β-dihydro-prednisolone in adult cows.[243]

4.4.2 Feed-Related Compounds

Some growth-promoting substances can be formed by fungi, which grow on crops eaten by cattle, or may be present in the feed cattle eat. In this section, two of these examples are discussed.

4.4.2.1 Thiouracil

Pinel et al. showed in a single animal experiment that there was a link between the cruciferous diet and findings of thiouracil in urine.[244] The origin of a positive thiouracil could therefore be due to natural contamination. Concentrations in urine did not exceed 10 µg/l. As early as 1941, Kennedy showed that cruciferous and brassicaceous vegetables contain substances called goitrogens, which impair iodine uptake by the thyroid and consequently inhibit the conversion of T4 to T3 (triiodothyronine).[245]

Vanden Bussche et al. showed that thiouracil was found in untreated animals in porcine, bovine, and ovine species and also in a dog and in human volunteers.[246] In these animal species, the concentrations did not exceed 10 µg/l. Evidence was also provided that endogenous plant compounds can be converted by myrosinase enzymes into thiouracil, which can be the reason for the thiouracil findings in these animal species. Vanden Bussche et al. also developed a method for analysis

of feed and food for thiouracil.[247] This method includes an enzymatic step for the conversion of glucosinolates using the myrosinase enzyme.

Currently, reliable and sensitive methods are available for the control of residues using LC-MS/MS and are sensitive enough with regard to the recommended concentration set by the EU Reference Laboratories in the Guidance paper of 2007 at 10 μg/l.[248]

4.4.2.2 Zeranol

Zeranol is prepared commercially from zearalenone, one of a number of structurally similar toxins produced by *Fusarium* spp. Zeranol and zearalenone are known to give identical metabolites explaining the fact that these metabolites, including zeranol itself, can also occur naturally in the urine of deer, goats, sheep, ovine, bovine, and horses and in bovine bile following metabolism of *Fusarium* spp. toxin. Figure 4.2 shows the metabolism of RALs.[249, 250]

Detection of RALs is based on gas chromatography–mass spectrometry (GC-MS)[251] or LC-MS/MS.[252] Matrices used are urine, muscle, liver, kidney, hair, and milk.[253–256] Sample clean-up consist of LLE,[257] SPE, or immunoaffinity column (IAC) extraction.[232] Several fully validated methods capable of analyzing all six compounds with decision limits (CCα) below 1 μg/l in urine have been published. In addition to detecting all six targeted RALs, it possible to include them in a targeted MRM including other compounds.

In 2004, an EU project was finalized with the goal to develop methods to discriminate between abuse of zeranol and the presence of residues resulting from the consumption of contaminated feed containing mycotoxins. The outcome of this project was a statistical model based on the metabolite pattern using concentrations of all RALs present in urine.[253, 258] Discrimination between natural contamination and abuse is based on differences in the metabolite pattern of α/ß-zearalanol (zeranol and taleranol) plus zearalanone versus α/ß-zearalenol plus zearalenone. When the combined concentrations of α/ß-zearalanol plus zearalanone are higher than those of α/ß-zearalenol plus zearalenone, this is an indication of illegal use. This model was extensively validated for urine from cattle and is used in routine residue control programs in case of a non-compliant finding. Although well validated, the model has no legal basis. It is a screening tool, and the result can help in deciding if a non-compliant finding requires a follow-up action or that the non-compliant finding was due to ingestion of mycotoxin contaminated feed. The model is only applicable for urine samples. There are no methods available that can discriminate abuse from contamination for other matrices or animal species.

The metabolism of zearalenone in pigs is described in a study of Zöllner et al.[259] In this study, it was found that the metabolism is identical to the one depicted in Figure 4.2. After feeding zearalenone-contaminated feed to the pigs, some trace amounts of zeranol and taleranol were found in urine. Samples of the liver and meat were also analyzed, and neither zeranol nor taleranol and zearalanone were

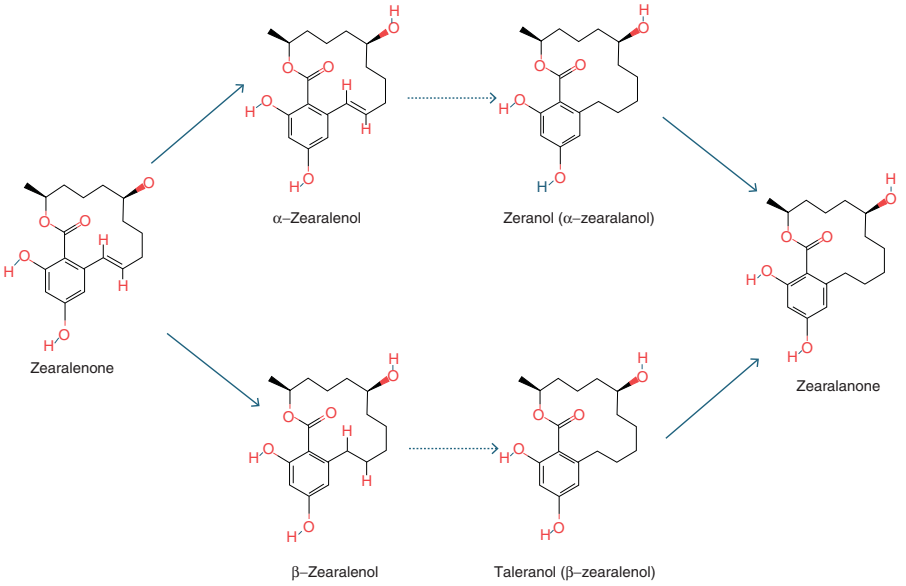


Figure 4.2 Metabolism of RALs, dashed arrows represent conversion of the toxins toward zeranol.

present in any of the liver samples. In the muscle samples, trace amounts of all RALs were detected.

4.4.3 The Natural Hormones 17β-Estradiol, 17β-Testosterone, and Progesterone

The fact that natural hormones are present in almost all matrices, and the complicated relations between the different steroids, has hampered the development of a definitive approach for control. Figure 4.3 shows the relation between the different natural hormones.

Notwithstanding the large number of published studies, it is concluded that there are currently only limited technical possibilities to prove abuse of natural hormones on the basis of analyses of biological samples for the parent compound

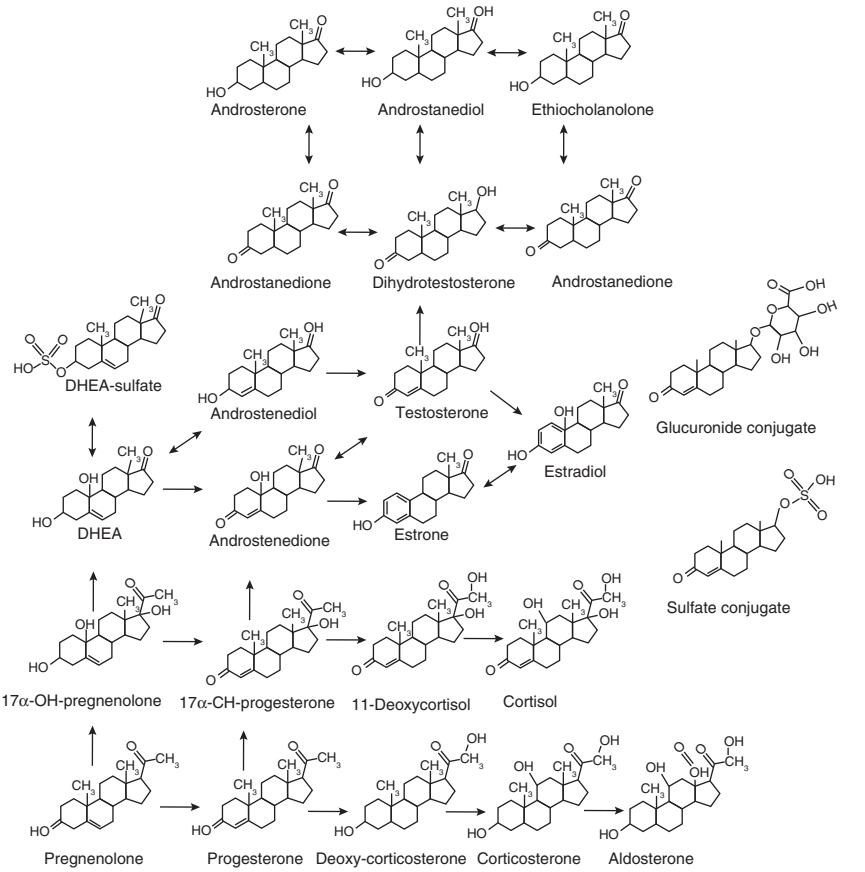


Figure 4.3 Steroidogenesis, the steroid pathway showing relations between individual steroids.

or its metabolites that go beyond an initial screening. The only approaches available are the direct detection of the hormones in the form of their esters and the use of isotope-ratio GC-C-IRMS.

For screening methods, immunoassays are popular, which are frequently based on methods originally developed for diagnostic purposes in human medicine. Screening animals on the basis of histological examination of selected tissues is among the oldest approaches used in residue testing. In spite of the complexity of interpretation, there is still potential for screening, mainly for male veal calves, where changes in the histology of the prostate can provide useful information.[260–262] Biomarkers are substances that can be used to identify specific animals, for example, animals treated differently from corresponding control animals. Regal et al. published a metabolomic study in which the influence of a treatment with estradiol or progesterone on the LC-HRMS total ion chromatogram was determined.[263] This untargeted approach resulted in sets of potential biomarkers for both treatments. The structure of these biomarkers remains to be elucidated, and the discriminating power of these biomarkers is being evaluated. Transcriptomics-based biomarker approaches based on RNA sequencing form a novel approach. In a study, in 40 selected heifers, a set of 20 was significantly regulated.[264] With principal component analyses, it was possible to discriminate animals treated with a combination of trenbolone acetate and 17β-estradiol from a control population.

A relatively new approach for screening is the use of steroid profiling. Targeted analysis for single compounds does not provide discriminative information due to the very small changes in mass concentration resulting from treatment. However, individual steroids are part of a complex physiological system balancing between the different active steroids, their precursors, and metabolites (Steroidogenesis). The hypothesis for screening methods based on steroid profiling, which implies the quantitative analyses of a large number of individual compounds, is that the combined effect on all the individual compounds provides a diagnostic tool for detecting abuse. Anizan et al. published a study in which the excretion of 25 known conjugated compounds (phase II metabolites) before and after administration of androstenedione was followed.[265] Blokland et al. published a study on the effect of treatment with several natural hormones on the steroid profiles for 17 steroids, aglycones, glucuronides, and sulfates.[9] Multivariate statistical analyses showed that the model could be used to classify animals into a treated or untreated group. Both studies show the potential of steroid profiling as a promising strategy to determine whether bovine animals have been treated with (natural) hormones or not.

All of these approaches are indirect. None of the methods provide an indication of the presence of a specific compound of which the mass concentration and identity can be subsequently confirmed during confirmatory analyses. Confirmatory methods for natural hormones or their markers must fulfill two separate conditions under EU regulations. As for all banned substances, their identity must be confirmed on the basis of criteria laid down in Commission Decision 2002/657.[7]

However, the compound(s) determined, the profile established, or biomarker(s) detected must be sufficiently diagnostic to "prove" abuse with a natural hormone.

The detection and confirmation of steroid esters in biological samples is one of the oldest analytical approaches for proving abuse of (natural) hormones. However, for many years, its applicability was limited to the analyses of alleged injection sites. Based on the meat inspection during slaughter, veterinarians were able to detect possible locations at which substances were injected. These tissues were removed from the carcass and extracted. Only limited extract purification was necessary in order to detect the steroid esters as such, using techniques such as HPLC combined with diode array detection (DAD).[266] For decades, however, the detection of intact hormone esters remained limited to these application sites.

In recent years, the analysis of intact hormone esters gained renewed interest based on new knowledge concerning the incorporation of steroid esters in hair and the increased analytical possibilities to detect and confirm very low concentrations of steroid esters in serum. Many scientific papers have described the detection of doping agents, therapeutic compounds, drug abuse, and tobacco residues in human hair samples. In 1994, several authors reported the possibility of using hair analysis for the detection of anabolics and β-agonists in animals, followed by studies in which the detection of steroid esters in hair was described.[31, 188, 267]

Hair has proven to be a suitable matrix for screening assays for β-agonists (see Section 4.3.6.1), but it also can be an important matrix for testing for abuse of natural steroids. Gray et al. published a method for 18 steroid esters in equine mane hair.[268] However, the use of hair for the direct analysis of steroid esters so far has been limited. Part of the reluctance to use hair as an analytical matrix is due to the risk of external contamination of the animal. A very recent study showed that, at least for clenbuterol, the chances of such (accidental) external contamination are very limited.[269]

In a recent paper, a new UHPLC-MS/MS method was described allowing the detection of steroid esters in serum of breeding (bovine) and racing (equine) animals.[270] The time during which the steroid esters can be detected in the blood, after intramuscular injection, depends on two factors: the time needed for the steroid ester to reach the blood stream and the efficiency of the esterase activity present in the blood for the specific steroid ester. The next critical parameter is the sensitivity of the analytical method. In order to allow the simultaneous detection of estrogenic and androgenic esters, it was necessary to derivatize the hydroxyl group at position A3 of estradiol and related structures through dansylation. This derivatization step has a positive influence on the detection limit, which was 0.02 μg/l for most estradiol esters, with the exception of estradiol decanoate (0.1 μg/l). For the androgens testosterone and nandrolone, the limit of detection (LOD) ranged from 0.020 to 0.050 μg/l. After intramuscular injection of 17β-estradiol-benzoate, the maximum concentration in serum was reached after 9 days. The release of the esters is more rapid for shorter chain than for longer or aromatic ester chains, explaining their shorter detection time window.

IRMS is a versatile application to determine the isotopic composition – usually expressed as a ratio – of a wide range of materials and compounds. IRMS is suitable to determine isotope ratios of the lighter elements, which include bioelements such as carbon ($\delta^{13}C$), nitrogen ($\delta^{15}N$), hydrogen ($\delta^{2}H$), oxygen ($\delta^{18}O$), and sulfur ($\delta^{34}S$). Because isotope ratios provide crucial information about fundamental processes and the patterns that emerge from these processes, IRMS is used in many research fields.

Applications of IRMS within agricultural research are mainly aiming at gaining a better understanding of complex biochemical processes such as nutrient cycling, nutrient uptake, and metabolic processes – for example to optimize human and animal diets – whereas applications in food science are mostly related to food safety and authenticity. Due to innovations and improvements of the IRMS technique, the list of applications is growing steadily, now also including distinguishing natural (endogenous) and synthetic (exogenous) hormones in sportsmen and farm animals, for which compound-specific isotope analysis is required.[35, 271, 272]

Even though IRMS (see Section 4.2.4) is considered a suitable confirmatory technique, its discriminating power has a statistical basis and depends on the observed actual differences in isotope ratios of the endogenous and synthetic form. Dietary factors no doubt are an important parameter. However, it is demonstrated that this basis can be strong enough to reach the reliability needed for confirmation.

4.4.4 Nortestosterone

Among the compounds used for growth promotion, 17β-19-nortestosterone (17β-NT), also named nandrolone, which belongs to the group of androgenic anabolic steroids, has traditionally taken a prominent place. As is the case for many other anabolic steroids, it is usually administered in esterified form, for example, via an implant in the ear or via an intramuscular injection (Figure 4.4).

The most commonly known ester is 17β-NT-phenylpropionate or nandrosol, its main (bovine) metabolite is 17α-NT. Reports on the natural occurrence in biological samples originate already from the 1980s, and since then, a large number of

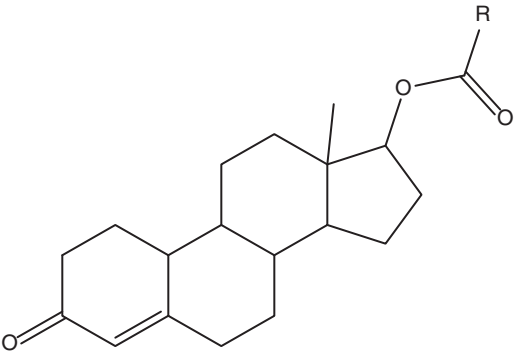


Figure 4.4 Structures of NT esters. NT-acetate: R = CH_3, NT-benzoate: R = phenyl, NT-cypionate: C_2H_4-cyclopentyl, NT-hemisuccinate: CH_2-succinyl, NT-decanoate: R = C_9H_{19}, NT-undecanoate: R = $C_{10}H_{21}$, NT-laureaat: $C_{11}H_{23}$, NT-phenylpropionate: R = C_2H_4-phenyl, NT-propionate: R = C_2H_5.

studies were published on the natural occurrence of nandrolone and its metabolites in a variety of species.

For urine analyses, several methods are available, based on GC-MS(MS) or LC-MS(MS) for nortestosterone (NT) and its metabolites. Most methods are multi-analyte methods including a number of steroids. Various LC-MS/MS methods have been published for analysis of intact phase II metabolites in urine [273] and for nortesterone esters in hair.[274] For milk, Kaklamanos et al. published a multi-analyte method for steroids[47]. For screening of meat samples, methods with low detection limits are available using ELISA[275, 276] or LC-MS/MS.[277] In the human sports-doping field, a number of methods have been published using IRMS to distinguish endogenous nortestosterone and metabolites from synthetic nortestosterone, such as the method by De la Torre et al., 2011.[278] This method is not (yet) routinely used in residue analysis.

17β-NT is known to occur naturally in urine of boars and stallions,[279–281] while its main bovine metabolite, α-nortestosterone (17α-NT), occurs naturally in pregnant cows and neonatal calves.[282] Furthermore, 17β-NT and metabolites, including 17α-nortestosterone, have also been detected in matrices of untreated animals from various species, including ovine,[283] caprine,[284] and cervine.[285] In cattle, 17α-nortestosterone is the major metabolite. 17α-NT was also found in bile, in an experiment in which nandrosol was administered to calves, with α-NT concentrations in bile being higher than in urine.[286] In bovine hair, only the intact esters were present after administration.[287]

Different research groups have performed studies to find new marker metabolites. For calves (both male and female), it was shown that after intramuscular administration of 17β-NT-laureate (NT-laureate - Nortestosterone laureate), profiles of 17β-NT metabolites in urine, the estranediol isomers 5α-estrane-3β,17β-diol (ABB), 5β-estrane-3α,17β-diol (BAB), 5α-estrane-3β,17α-diol (ABA), 5α-estrane-3α,17β-diol (AAB), and 5β-estrane-3α, 17α-diol (BAA), deviate from the normal urine metabolite profile in the bovine population. ABA was determined as the main metabolite while in non-treated cows, 17α-NT was the major metabolite.[288]

For calves (both male and female), it was shown that after intramuscular administration of 17β-NT-laureate, profiles of 17β-NT metabolites in urine, the estranediol isomers 5α-estrane-3β,17β-diol (ABB), 5β-estrane-3α,17β-diol (BAB), 5α-estrane-3β,17α-diol (ABA), 5α-estrane-3α,17β-diol (AAB), and 5β-estrane-3α,17α-diol (BAA) (see Figure 4.5), deviate from the normal urine metabolite profile in the bovine population for nortestosterone. ABA was determined as the main metabolite while in nontreated cows, 17α-NT was the major metabolite.

The relation between the natural presence of 17α-NT and 17β-NT and acute injury was established in slaughtered male cattle (bulls and steers).[289] There was no evidence of abuse at any of the farms involved and the phenomenon occurred in four different regions of the EU. The relationship of release of DHEA in response to the stress of the injury was tested by intravenous administration

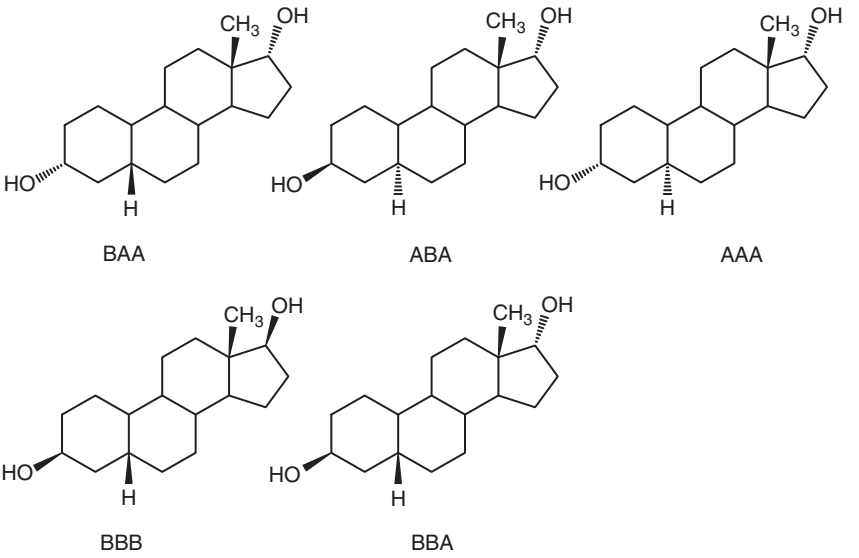


Figure 4.5 Chemical structures of the NT metabolites: estranediol isomers.

of DHEA to two normal steers, and 17β-NT was confirmed in the urine of one steer.

Ventura et al. showed in an animal experiment that after intramuscular administration of 17β-nortestosterone-laurate to male pigs (barrows), the main metabolites were 17β-nortestosterone-sulfate, free noretiocholanolone, nor-epiandrosterone, 5β-estrane-3α,17β-diol, and 5α-estrane-3β,17β-diol.[290] In untreated barrows, only 17β-norandrosterone and estrone were found. Scarth et al. determined the biomarker noretiocholanolone in the free fraction of urine from boars and gilts.[291] Threshold values of >7.5 µg/l for boars and 19.2 µg/l for gilts were determined.

In pigs, high concentrations of 17β-NT are observed in boars, but only low concentrations in barrows and gilts. In practice, however, high concentrations of 17β-NT are occasionally found in urine from barrows. This can be due to cryptorchid animals, that is, those having one testicle in the abdomen that produces hormones. In a small percentage of female pigs (sows), low concentrations of 17β-NT can also occur.[280] An animal experiment with nandrosol has shown that administration of nandrosol indeed causes a growth-promoting effect in boars.[287] It was also shown that nandrosol was present in hair of boars treated with nandrosol. It is probable that other nortestosterone esters that might be illegally used can be observed in the hair of the treated animals.

Van Hende et al. analyzed the urine of four ewes at different stages of pregnancy and the amniotic fluid of one ewe for the presence of 17α-NT; the urine of four pregnant animals was found to contain concentrations ranging from the LOD to above 2 µg/l.[285] Clouet et al. also analyzed urine samples of pregnant sheep for

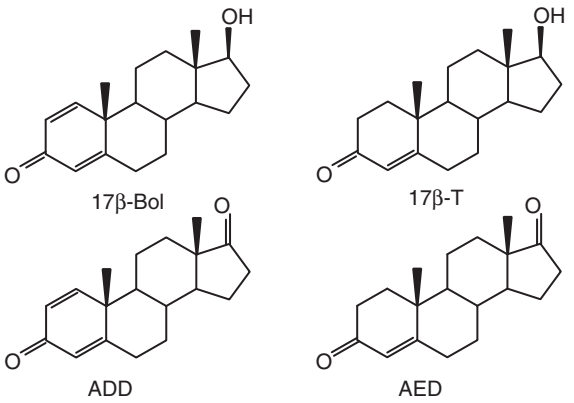


Figure 4.6 Structures of 17β-boldenone (17β-Bol) and 17β-testosterone (17β-T) (above) and of androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (AED).

17α-NT.[283] Detection occurred at different stages of pregnancy. Small concentrations (<0.5 μg/l) were found during the first 4 months of pregnancy and increased until parturition, particularly during the last month of pregnancy.[292]

Urinary metabolites of 17β-nortestosterone in the horse were investigated in detail by Teale and coworkers[293] and Houghton et al.,[279] who reported that 17β-nortestosterone was mainly excreted as 5α-estran-3β,17α-diol sulfate and glucuronide in horse urine. Therefore, 5α-estran-3β,17α-diol was selected as the target metabolite to determine 17β-nortestosterone abuse.

Steroid profiling of urine and serum samples proved to be a good tool to predict nandrolone abuse in race horses.[10] Statistical processing of the collected data permitted the establishment of statistical models capable of discriminating control samples from those collected during several months following administration.

4.4.5 Boldenone

17β-Boldenone (17β-Bol), also denoted as 1-dehydrotestosterone or androsta-1,4-diene-17β-ol-3-one, is a steroid with androgenic activity that differs in structure from 17β-testosterone (17β-T) by only one double bond at the 1-position. Important steroids closely related to 17β-Bol and 17β-T are the 17β-boldenone epimer, that is, 17α-boldenone, androsta-1,4-diene-3,17-dione (ADD), and androst-4-ene-3,17-dione (AED). These two di-keto substances, ADD and AED, are precursors of 17β-Bol and 17β-T, respectively, in humans and different animal species. Their chemical structures are shown in Figure 4.6.

17β-Bol, esters of 17β-Bol (e.g., undecylenate ester), and ADD are for sale as anabolic preparations. 17β-Bol improves the growth and feed conversion of cattle and therefore might be abused to achieve more efficient meat production. Boldione (ADD) is sold on the Internet for use by bodybuilders as a product with an even greater anabolic potency than 17β-Bol itself. De Brabander et al. published a review paper on the possible origins of boldenone.[294] Regarding the use of

the marker metabolite 17β-boldenone-glucuronide in bovines for regulatory purposes within the EU, the outcome was as follows[295]: "*The Commission presented the outcome of the meeting of 30 September 2003 on the control of boldenone in calves. The group of experts concluded that only 17 beta boldenone conjugates in urine of young calves could be used as a proof of illegal treatment, provided that some specific sampling procedures were applied to avoid faecal contamination. The Committee endorsed these conclusions to be used as guidance for control of boldenone by the Member States (MS).*"

For 17β-boldenone-glucuronide, a recommended concentration for control in urine of 1 µg/l was set. Findings of 17α-boldenone higher than 2 µg/l have to be investigated further.

For urine analyses, several methods are available, based on GC-MS/MS or LC-MS/MS for boldenone and metabolites. Most methods are multi-analyte methods, which include a number of other steroids. In LC-MS analyses, there are also methods published on analysis of intact phase II metabolites.[296, 297] For matrices such as hair, LC-MS/MS methods are available, which detect boldenone esters.[298] IRMS is used in the human sports-doping field to distinguish endogenous boldenone and metabolites from synthetic boldenone.[299]

Boldenone is metabolized into a number of metabolites depending on the species. Le Bizec and coworkers found nine metabolites in urine after administration of boldenone through several routes, boldenone esters, and boldione with 17α-boldenone being the marker metabolite in bovine urine.[297, 300] Unfortunately, these metabolites were also found in animals known not to have been treated. Looking at phase II metabolites, Le Bizec showed that 17β-boldenone was the only metabolite present as a sulfate conjugate in urine of treated animals. In 2009, Destrez et al. published a paper on direct measurement of this sulfate conjugate.[297] Blokland et al. proposed the 6-hydroxy-boldenone-sulfate as marker metabolite.[235] Studies on human volunteers showed that after administration of 17β-nortestosterone, it was excreted as a conjugate in urine.[301] Galletti et al. described the *in vivo* formation and excretion of metabolites of 17β-boldenone and 17β-boldenone itself in urine after an oral administration of 17β-boldenone to human volunteers.[302]

In vitro and *in vivo* metabolisms of 17β-boldenone were investigated by Van Puymbroeck et al.[303] The main metabolite produced by microsomes was ADD, while in the isolated hepatocytes, 6-OH-17β-boldenone and 6-OH-ADD were identified. This research group also examined the excretion of 17β-boldenone in a calf and a cow.[304] The main metabolite found in urine was 17α-boldenone. Several reduced, oxidized (such as ADD), and hydroxylated metabolites were also found. Feces samples were investigated for the presence of 17α-boldenone and 5β-AED, which does not naturally occur in bovines, and for other reduced metabolites. No 17β-boldenone was detected in bovine feces. Feces samples showed a different metabolite profile in comparison with urine samples. No hydroxylated or oxidized products were found. In the urine of a male calf treated intramuscularly with 200 mg 17β-boldenone-undecylenate, 17α- and

17β-boldenone and also ADD and 5β-AED were identified. In the second experiment, a mature cow was treated intramuscularly with 700 mg free, unesterified 17β-boldenone. The metabolic profile in urine was comparable. In addition to 17α- and 17β-boldenone, 5β-AED was found at concentrations comparable with 17β-boldenone, while ADD was found at lower concentrations. The differences in the excretion profile in feces were considerable. 17α -Boldenone and 5β-AED were the most predominant metabolites, whereas 17β-boldenone and ADD were not detected.[305]

Different hypotheses were postulated and tested on the possible origin of boldenone. Involvement of microorganism from feces was studied *in vitro* and *in vivo*. Also models using shrimp, *Neomysis integer*, were used. In nearly all cases using this model, 17β-boldenone could be measured after exposure to 17β-testosterone.[306] In the Netherlands, France, Italy, and Belgium, studies were performed on metabolism end excretion. Boldenone was found in high concentrations in several matrices by Nielen et al.[298] Conversion of phytosterols into 17β-boldenone was postulated and checked by Poelmans et al.[280] and Verheyden et al.[307] Verheyden also postulated formation of boldenone by maggots and moulds on feed. In the intact male horses and in pigs, boldenone is probably formed through aromatization of estrogens in the testis. Poelmans also demonstrated the presence of boldenone in pig testes.[280]

Draisci et al. analyzed urine samples for boldenone, epiboldenone, and androsta-1,4-diene-3,17-dione by LC-MS from 25 untreated animals.[308] Boldenone (limit of quantification, LOQ 0.2 μg/l), epiboldenone (LOQ 0.5 μg/l), and androsta-1,4-diene-3,17-dione (LOQ 0.2 μg/l) were not detected above the LOQ in any of the urine samples from the untreated animals.

Pompa et al. studied the concentrations of boldenone, epiboldenone, androsta-1,4-diene-3,17-dione, testosterone, and epitestosterone in the urine, skin swabs, and feces of Friesian calves and also assessed the effect of drying the feces on the resulting steroid concentrations in feces.[309] In urine, LODs for all steroids were 0.1 μg/l, and in feces, LODs for all steroids were 0.5 μg/l. Boldenone, epiboldenone, and androsta-1,4-diene-3,17-dione in urine were not detected in any of the samples from 10 calves. Boldenone was detected in feces sampled directly from the rectum (rectal feces) in all the calves at concentrations ranging from 28 to 89 μg/kg. Epiboldenone was not detectable in rectal feces from six calves and was detected at concentrations between 2.6 and 5.9 μg/kg in the samples from the other four animals. Androsta-1,4-diene-3,17-one was not detected in the rectal feces from nine calves while one calf had 21 μg/kg. Results from feces scraped from the skin, feces taken from the stall floor, and feces stored for up to 13 days at room temperature in a cowshed showed that the concentrations of all steroids increased significantly (but variably) over time. This is especially true of epiboldenone and androsta-1,4-diene-3,17-dione, which by day 13 of storage are present in high concentrations, while boldenone was reduced to not detectable by day 13. This study exemplifies the need for avoiding

fecal contamination of urine during sampling and to ensure swift storage and analysis of any samples taken.

Studies of the 17β-boldenone metabolism by Le Bizec and coworkers proposed that a 17β-boldenone-sulfo conjugate is a marker for distinguishing naturally formed and exogenously administered boldenone.[297, 300] In the same period, the EURL performed studies and proposed 6β-hydroxy boldenone as a marker.[235] Studies in humans show boldenone sulfate as a marker for exogenous administration.[310]

4.4.6 Protein Hormones

Protein hormones form a special class of compounds with respect to the potential abuse for growth-promoting purposes. Substances mentioned in the WADA 2015 prohibited list include GH, GHRH, Ghrelin, and GHRPs, IGF-1, metabolic modulators such as insulin, and gene doping.[311] For each protein hormone, both biomarker-based screening methods and current confirmatory methods will be discussed.

4.4.6.1 Insulin

Insulin is mentioned in the WADA prohibited list as a metabolic modulator.[311] It is released as a response to rising blood-glucose concentrations. Insulin is known to inhibit protein degradation and facilitates transport of glucose and amino acids into cells where it enhances protein synthesis and, for muscles in particular, storage of glycogen. This will enhance muscle growth, and therefore, misuse of insulin can be expected both in sports and also in food-producing animals. Moreover, insulin acts synergistically not only with other proteins such as IGF-1 and GH, leading to combined misuse,[312] but also with dexamethasone.[313] In food-producing animals, until now, no biomarker-based studies have been conducted to detect insulin misuse. Biomarker-based studies for detection of insulin misuse were performed in humans,[314, 315] and due to similar mammalian physiology, similar biomarkers are expected in other species. Biomarkers used for detection of insulin use in human are mature insulin and C-peptide, a fragment of proinsulin. Mature insulin and C-peptide are produced in equal molarities during insulin production. Misuse is considered when the ratio of mature insulin and C-peptide is altered, as investigated by Abellan et al.[316] Another biomarker-based approach is the detection and quantification by immunochromatography and LC-MS/MS of three insulin degradation products in urine.[317] Different abundances of these three fragments were found after insulin administration; however, no conclusions were drawn as to if they can serve as candidate biomarkers.

Confirmation of insulin misuse in sports drug testing is generally based on immunoaffinity purification, chromatographic retention times, and low- or high-resolution MS and MS/MS spectra.[318] Characteristic MS/MS product ions can aid the unambiguous confirmation of insulin analogs and are particularly

useful to differentiate between compounds with identical molecular weight such as human insulin and insulin lispro.[319]

4.4.6.2 Insulin-Like Growth Factor-1 (IGF-1)

IGF-1 mediates growth-promoting actions of the growth hormone and influences the IGF-1/GH axis. IGF-1 is not species-specific and is similar for human, horse, and bovine. It is therefore probable that the same methods can be used for endogenous IGF-1 detection. IGF-1 itself can be measured with immunological screening methods after acid–ethanol precipitation using RIA[320] and after an acid–SDS pre-treatment using a flow cytometric immunoassay.[222] In both methods, acidification is used to free IGF-1 from its complex with IGFBP3 and ALS, the abundant form in circulation, prior to detection. For screening purposes, biomarkers can also be measured. Candidate biomarkers for IGF-1 abuse are IGF-2, IGFBP2, and ALS. After administration of an IGF-1/IGFBP3 cocktail to recreational human athletes, these biomarkers were validated in their blood.[319]

Similar strategies may also be used for MS analysis for detection of IGF-1. Several methods were described in recent reviews to measure endogenous IGF-1 by LC-MS/MS (302).[319, 321] Synthetic analogs of IGF-1 with prolonged action have been developed and are available for use. They have extended, deleted, or substituted amino acid residues, including des1-3-IGF-1, R^3-IGF-1, and Long-R^3-IGF-1.

4.4.6.3 Growth Hormone

GH, also called ST, is an endogenous hormone, which has the stimulation of the postnatal growth and development of bone and soft tissue as its main function. Furthermore, it has several actions in the protein, carbohydrate, and fat metabolism.[322] GH is secreted in the anterior pituitary by cells called somatotrophs. GH synthesis is increased by a specific hypothalamic GHRH and is downregulated by SS, its hypothalamic antagonist. Both GHRH and SS are neuropeptides that are released by the hypothalamus into the hypothalamic–hypophyseal portal system where they can attach to somatotroph cells and induce GH secretion.[323] After GH is released by the pituitary gland, it will travel through the blood circulation and will eventually reach a target organ. In the target organ, somatomedins can be formed, which are growth factors. These growth factors are triggered by its binding to GH, which will induce growth-promoting substances in the organ. For instance, the liver as target organ produces somatomedins called IGF-1. IGF-1 proteins released from the liver enter the blood circulation and stimulate the secretion of SS from the hypothalamus (IGF/GH axis). In this way, a negative feedback loop for GH is maintained.[324]

The availability of rbST preparations has made its use for zootechnical purposes interesting. The main zootechnical use is to increase milk production in dairy cows. As previously noted (see Section 4.3.7.1), the use of rbST has been banned within the EU since 2000.[203] To monitor the use of rbST, several

methods are available for indirect biomarker-based detection. A fourplex FCIA detects IGF-1, IGFBP2, osteocalcin, and rbST-induced antibodies as biomarkers.[223] Furthermore, also an ELISA method for serum[217] and an FCIA method for milk[223] for rbST-induced antibodies were described. For direct rbST detection, an ELISA is described; however, it not yet able to reach the required sensitivity.[325]

For serum analysis of rbST, two confirmatory methods were described, with differentiation on the sample preparation, followed by trypsin digestion and LC-MS/MS measurements. Sample preparation used by Le Breton et al.[326] was a precipitation technique and Smits et al.[327] used an affinity purification technique. Both enabled measurement of two transitions of the rbST-specific peptide, with the difference that Smits et al. were able to measure rbST in the serum for a longer period of time.

A complicating factor is the fact that different rbST preparations, coming from different commercial sources, can have small differences in N-terminal amino acid composition. Methods based on biorecognition techniques and MS must take this into consideration.

In addition to the availability of rbST, rpST is also marketed for zootechnical purposes. The main use is for finishing treatment between 28 and 42 days prior to slaughter. The main compound used, marketed under the name Reporcin, is identified as methionyl porcine ST. Since the early 1990s, rpST has received little attention from the analytical community, with studies focusing on the influence of amino acid composition of the feed on growth and the muscle–fat tissue distribution.[328]

4.4.6.4 Growth Hormone Secretagogues

Ghrelin and its synthetic mimetics, GHRPs, are small peptides that have an important function in enhancing GH secretion via the GH secretagogues receptor 1a.[329] Since the early 1980s, attempts were made to synthetically develop GHRPs, as they were seen as potential enhancers for GH secretion for GH deficiency treatment. Conformational studies were needed to develop *in vivo* active GHRP; the first successful GHRP able to induce GH release activity was a hexapeptide named GHRP-6. More GHRPs were developed with slightly different structures or changes in amino acid sequence. The GHRP structures were selected to act on particular receptors of the hypothalamus and pituitary.[330] As Ghrelin and GHRPs stimulate GH release from the pituitary gland, GH blood concentrations are elevated and similar biomarkers as in GH use can be expected. Clinical studies indeed showed similar biomarkers to be effected. Increased GH concentrations were shown after treatment with GHRP-2 or ghrelin, which also increased IGF-1 and IGFBP-3 concentrations.

Several LC-MS methods for GHRP detection in plasma and urine have been developed and described in the literature. An overview provided by Van den Broek et al.[319] is shown in Table 4.2.

Table 4.2 The main characteristics of reported LC-MS assays to identify and/or quantify GHRPs in urine and plasma.

	Analyte									
	GHRP-1	**GHRP-2**	**AA-3**	**GHRP-4**	**GHRP-5**	**GHRP-6**	**Alexamorelin**	**Hexarelin**	**Ipamorelin**	**References**
Method	50 µl human plasma + nonanalogous IS → PP (ACN) + UPLC-HRMS									[331]
Recovery (%)	×	89	×	×	×	×	×	88	×	
LOD (ng/ml)	×	10	×	×	×	×	×	10	×	
Method	5 ml human urine (pH < 3) + GHRP-2* → SPE (HLB) + UPLC-MS									[332]
Recovery (%)	×	84	101	×	×	×	×	×	×	
LOD (ng/ml)	×	0.05	0.02	×	×	×	×	×	×	
Method	1.5 ml human plasma + GHRP-6* → PP (cold acetone) + nano-LC (monolith)-HRMS									[333]
Recovery (%)	×	×	×	×	×	~60	×	×	×	
LOD (ng/ml)	×	×	×	×	×	5	×	×	×	
Method	2 ml human urine (pH 6–8) + GHRP-4* + AA-3* → SPE (MCX) + UPLC-HRMS									[331]
Recovery (%)	56	94	62	65	79	45	63	50	94	
LOD (ng/ml)	0.50	0.20	0.25	0.20	0.25	0.30	0.25	0.30	0.25	
Method	1 ml human urine (pH 5–7) + GHRP-4* + AA-3* → SPE (MCX) + nano-UPLC-HRMS									[331]
Recovery (%)	56	94	62	65	79	45	63	50	94	
LOD (ng/ml)	0.010	0.002	0.005	0.005	0.005	0.010	0.010	0.010	0.010	
Method	2 ml equine plasma + nonanalogous IS → SPE (MAX) + nano-UPLC-HRMS									[334]
Recovery (%)	78	88	×	×	×	72	×	78	79	
LOD (ng/ml)	<0.05	<0.05	×	×	×	<0.05	×	<0.05	<0.05	

GHRP 1: Growth Hormone releasing Peptide 1, GHRP 2: Growth Hormone releasing Peptide 2, etc., AA-3: metabolite of GHRP 2
Source: Based on work by the chapter coauthors published in Van den Broek et al.,[319] copyright Wiley 2014.

As the GHRPs are degradable, methods used should be highly sensitive. Therefore, screening for metabolites can be an interesting approach to detect GHRPs.[332, 335] An assay that achieves LODs in the low ng/l range has recently been developed for urine samples.[336]

4.4.7 Future Perspectives (Natural Hormones)

The control of abuse of natural (protein) hormones will remain one of the challenges for the years to come, and a multidisciplinary approach will be necessary to answer all the current questions, which include questions concerning endocrinology, physiology, kinetics, and analyses. Further "omics-based" approaches will be developed for control, primarily for screening purposes. Their application in confirmatory analyses will also be studied. However, it will be necessary to collect extensive biological data before statements such as "beyond reasonable doubt" can be made on the basis of such techniques. Therefore, the identification of highly selective markers and the development of more traditional confirmatory techniques will remain essential for a long time.

4.5 Control for Synthetic β-Agonists: Screening and Confirmatory Methods

4.5.1 Basic Information on Nature and Regulatory Controls

The group of β-adrenergic agonists represents dozens of compounds, among which clenbuterol has been the most studied. The main β-adrenergic agonists for residue control are shown in Table 4.3.

Internationally, legislation with respect to the use of β-adrenergic agonists is diverse. Within the EU, their use is prohibited, with a small exception for specific cases in which the use of clenbuterol is allowed, for which MRL values have been established[229] or isoxsuprine for treatment of horses, for which no MRL is required. Ractopamine hydrochloride is registered for growth-enhancing purposes in many countries, including the United States of America and Canada. This means that in some regions, residue control needs use methods allowing the detection at nationally or regionally set limits, such as those established by the Codex Alimentarius Commission.[337] which are, for example, set at 10 μg/kg for muscle (cattle, pig), whereas in other regions, a zero tolerance must be monitored. This has offered some important challenges for method harmonization. For zilpaterol hydrochloride, some countries have set a MRL, and a Joint FAO/WHO Expert Committee on Food Additives (JECFA) recommendation on MRLs for cattle tissues (muscle, liver, and kidney) was recently made to the Codex Committee on Residues of Veterinary Drugs in Foods (CCRVDF) for consideration.[338]

Biological activity of β2-adrenergic agonists is attributed to the six-membered aromatic ring, which may be substituted with hydroxy groups, halogens, amines,

Table 4.3 Most relevant β-agonists for residue control.

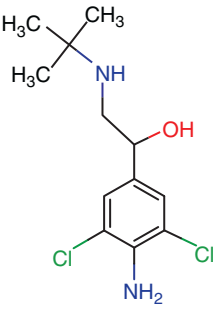
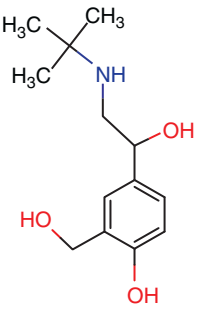
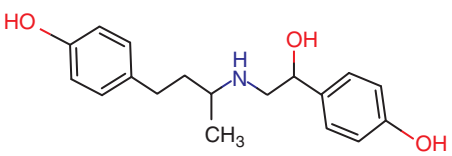
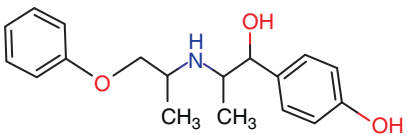
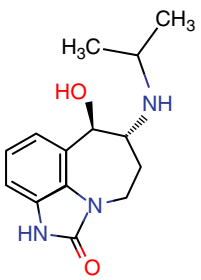
Name	Molecular formula	Molecular weight	Chemical structure
Clenbuterol	$C_{12}H_{18}Cl_2N_2O$	277.19	
Salbutamol	$C_{13}H_{21}NO_3$	239.31	
Ractopamine	$C_{18}H_{23}NO_3$	301.38	
Isoxsuprine	$C_{18}H_{23}NO_3$	301.38	
Zilpaterol	$C_{14}H_{19}N_3O_2$	261.15	

Table 4.4 Adrenergic receptor types, subtypes, and their effects in organism.

Adrenergic receptor type	Adrenergic receptor subtype	Effect
α-AR	α_{1A}	Maintenance of vascular basal tone and arterial blood pressure
	α_{1B}	Regulation of blood pressure and glucose homeostasis
	α_{1D}	Regulation of contraction vascular smooth muscle
	α_{2A}	Regulation of long-lasting fall in blood pressure
	α_{2B}	Regulation of short hypertensive phase
	α_{2C}	Implicated in modulation of behavior and memory
β-AR	β1	Responsible for positive inotropic and chronotropic effects
	β2	Smooth muscle relaxation
	β3	Regulation of lipolysis and thermogenic effects
	β4	Regulation of lipolysis

Source: Based on information contained in Badino et al.[340]

hydroxymethyl groups, cyano groups, or their different combinations. At the same time, the chemical substitution (especially halogen) related to the aromatic ring significantly affects the excretion half-time extension of β2-adrenergic agonists in mammals as well as their affinity for binding to the receptor.[339]

4.5.2 Mechanism of Action

Table 4.4 shows the different adrenergic receptor types, subtypes, and their effects in organism.[340]

β-Adrenergic receptors in mammals include β1-, β2- and β3-adrenergic receptor subtypes. These receptors are present in all tissues related to growth, including skeletal muscle and adipose tissue and β4-adrenergic receptors that are possibly present in some parts of the cardiovascular system and adipose tissue.[341] Representation of individual receptor subtypes is different in each tissue or organ and is dependent on the animal species. Certain tissue has primarily represented one subtype of β-adrenergic receptor.[342, 343]

β2-adrenergic agonists in the body act through a series of biochemical reactions (Figure 4.7) induced by binding of these substances for specific β2-adrenergic receptors located on cell membranes in the tissues of mammals.[344, 345]

The signal transduction involves activation of the receptor/adenylate cyclase system, production of the second messenger cAMP, further amplification via an enzyme cascade, and activation of protein synthesis (anabolic effect) as well

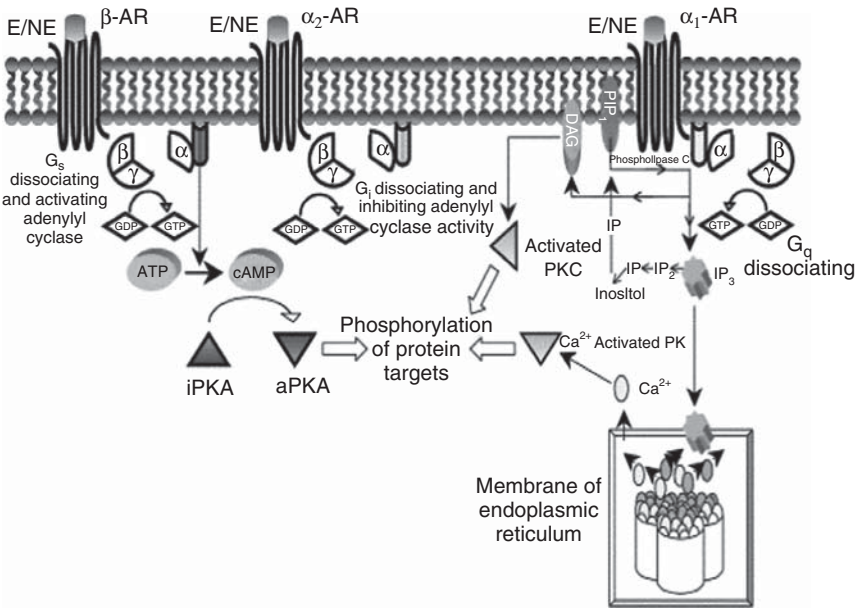


Figure 4.7 The mechanism of converting the signal from the β-adrenergic receptor (βAR). Source: Badino 2005[340] Reproduced with permission of Elsevier.

as inhibition of protein degradation (anticatabolic effect). Studies indicate that β-agonists may also stimulate the muscle directly.[347]

4.5.3 Therapeutic Use and Abuse

β2-adrenergic agonists have been used for more than 30 years in human medicine for the treatment of chronic bronchitis, chronic obstructive pulmonary disease, and asthma because of their relaxing effect on muscles.[348, 349] In veterinary medicine, these substances have an important use for therapeutic purposes as bronchodilators and tocolytic agents. However, long-term use of β2-adrenergic agonists can result in desensitization caused by the decrease in receptor number.[350–353]

Applied in higher doses, these substances can act as growth promoters for meat production in many animal species[354] resulting in a significant increase in lean body mass and a decrease in the amount of body fat, better utilization of food, and increased growth of animals.[355, 356] Application also is considered to result in better sensory properties of meat, with smaller portions of fat and greater proportion of muscle tissue.[357–359] This is not the case with clenbuterol-treated meat, as this treatment results in less marbling in muscle, a lower quality grade[360], and toughness.[361] Since 1984 to date, numerous studies of anabolic effect of β-agonists in animals of high economic interest, such as poultry, pigs, sheep, and cattle, have been conducted.[362, 363]

Clenbuterol was initially developed as a long-acting β2-adrenergic agonist for the treatment of respiratory diseases and other diseases, and later generations of β-agonists have been developed with structural differences that result in shorter elimination half-life and activity, and low oral action potential in animals for meat production. In addition to clenbuterol, data show the occurrence of many short-acting substances in meat sampled at the market, such as salbutamol, ractopamine, cimaterol, zilpaterol, terbutaline, and mabuterol, and the achievement of increasing the share of meat protein and reducing fat content for about 40%.[230] Parameters that generally have an influence on the result of treatment of animals for meat production are availability of dietary protein, duration of treatment, dosage, species, age, weight, and genetics.[346]

4.5.4 Absorption and Elimination

Only a few studies were published in the open literature on absorption and elimination of β-adrenergic agonists, mainly for clenbuterol[364] and ractopamine.[365, 366] Studies pointed to β-agonists having high oral bioavailabilities, long plasma half-lives, showing that relatively slow rates of elimination have high oral potencies in humans.[339] These substances are well absorbed orally, but have low systemic availability due to extensive first-pass sulfation. Plasma protein binding of most β-agonists is negligible, and there is substantial extravascular distribution of the administered dose. Elimination of intravenous drug is predominantly renal, whereas oral doses are mostly eliminated by biotransformation. Renal clearance correlates with creatinine clearance; therefore, dose reduction should be considered if renal function is impaired, such as in the elderly or in cardiac failure.[367] The elimination half-life of most β-agonists is relatively short, and pharmacokinetics is independent of dose and duration of treatment. The general pattern evident from studies is that halogenated β-agonists have longer plasma half-lives than the β-agonists bearing hydroxyl groups on their aromatic rings. Study results showed that ractopamine and clenbuterol concentrations in urine vary greatly during oral treatment for 28 days, with maximal concentrations recorded on day 25 and day 20, respectively.[368] Also, application of a 10-fold higher clenbuterol and salbutamol dose resulted in blood concentrations three- to fourfold higher for clenbuterol and two- to threefold higher for salbutamol, indicating a different release rate of these two β-agonists. Clenbuterol and salbutamol concentrations were significantly higher in the plasma than in the serum, suggesting the plasma to be a more suitable matrix for the determination of β-agonists in blood.[369]

4.5.5 Bioavailability and Residues

The total clenbuterol residues in pigs that received feed containing 1.0 mg/kg [^{14}C]clenbuterol HCl were found to be highest in the liver at zero withdrawal, whereas kidney contained residues about one-third of those in liver.[370] Muscle

and adipose tissue contained residues in quantities of approximately 18% of those found in the kidney and of approximately 7% of those found in the liver.[370] The author reported the highest post-treatment clenbuterol concentrations to be found in the lungs at zero withdrawal, slightly higher than the concentrations in liver. Concentrations of parent clenbuterol, the marker residue, were <LOQ (0.25 μg/kg) after 7 days of withdrawal in muscle and fat, while concentrations of marker residue in the liver, kidney, and lung were approximately 2.3, 0.4, and 1.2 μg/kg, respectively. In the liver, as a food tissue and regulatory matrix for the control of clenbuterol abuse, the maximum allowed concentration of 0.5 μg/kg (cattle) was achieved by clenbuterol depletion from liver tissue on day 14 and in the kidney on day 7 after treatment withdrawal.[371]

The highest accumulation of clenbuterol and salbutamol was observed in pigmented tissues, such as the eye and the hair.[372–374] The eye was shown to be the tissue in which clenbuterol remains in the highest concentrations for the longest periods of time, which makes the eye an excellent target tissue for the monitoring of illegal substance use. Several authors have reported that eye fluids and tissues may contain concentrations of β-agonists in orders of magnitude higher than those found in the liver, which is to be attributed to the substantial number of β-receptors harbored by the eye.

As a proportion of the administered dose, concentrations of salbutamol and terbutaline residues were lower than clenbuterol residues. When one considers the 2- to 20-fold greater dose, residues of parent ractopamine in the liver and kidneys of swine were proportionally lower than the residues of clenbuterol, terbutaline, or salbutamol in chickens.[375] Ractopamine depleted rapidly from internal tissues, with mostly no detectable residues on day 8 after withdrawal in the kidney, liver, heart, and brain.[376] The highest ractopamine concentration after treatment using anabolic dose was recorded in the lungs, with significantly higher concentrations in the period of 30 days after withdrawal, suggesting that depletion of ractopamine from the lungs occurs at a much slower rate than its depletion from other internal tissues.[377] Data pointed to high affinity of ractopamine for binding to the pigmented segment of the eye[378] and especially in hair, thus supporting the use of pigmented tissues as matrices in the regulatory monitoring of this β2-adrenergic agonist.

4.5.6 Determination in Biological Materials

4.5.6.1 Sample Preparation Techniques

As mentioned previously, the possible matrices to target for analyzing for residues of β-agonists include plasma, urine, muscle, and liver as well as hair and retina, so there is a necessity to develop extraction and purification procedures for these quite diverse matrices. In the group of β-adrenergic agonists, there are a large number of compounds that can be divided into phenolic (salbutamol) and anilinic (clenbuterol) types of compounds, in regard to the substitution of the aromatic ring.[379] Different chemical properties of these two types of

analytes present the main challenge in sample preparation. The metabolism of β-agonists after administration includes formation of different metabolites, such as sulfate and glucuronide conjugates. To detect the parent compound, it is necessary to perform an acidic/basic or enzymatic hydrolysis. The use of β-glucuronidase/arylsulfatase from *Helix pomatia* for enzymatic digestion is suitable for urine samples and also for liver, kidney, and muscle.[380] For hair, as a novel matrix in β-agonists determination, basic digestion with sodium hydroxide was found to be sufficient to release the compounds,[381, 382] but protease digestion can be also used. After the deconjugation (hydrolysis), the next step in sample preparation is extraction of target compounds.

In the analysis of β-agonists, a range of extraction procedures are available, including LLE, SPE, dispersive liquid–liquid micro-extraction (DLLME), immunoaffinity (IA) extraction, and MIP. LLE with solvent has a major disadvantage due to the loss of phenolic compounds that are more polar than anilinic ones, so development of a method for a broad range of β-agonists can result in poor recoveries for salbutamol-like compounds. The most widely used method for extraction of β-agonists is SPE on different sorbent types, such as the SPE method for extraction of seven β-agonists from liver and urine on C_{18} sorbent. Nielen et al. developed a method for extraction of 22 β-agonists from urine, liver, feed, and hair on mixed-mode sorbent (combination of C_8 that provides reversed-phase interactions and benzenesulfonic acid that acts as strong cation exchange).[381] Another method that utilizes an SPE cartridge with a cation-exchange mechanism was developed for the determination of β-agonists in animal feed and drinking water.[383] The extraction was done on a polymeric cation-exchange sorbent, and the method was validated for seven β-agonists including clenbuterol and ractopamine. In their method for analysis of 16 β-agonists in the liver, kidney and muscle, Shao et al. also used extraction on cation-exchange sorbent but with additional sample pre-treatment on Hydrophilic Lipophilic Balanced (HLB) cartridges.[380] Introduction of the SPE extraction with cation-exchange mechanism resolved some issues that were present in the methods with C_{18} or C_8 as a sorbent.

As enzymatic digestion with *H. pomatia* requires an acidic pH, β-agonists are protonated and thus poorly retained on reversed-phase sorbents. In addition to classic SPE, dispersive solid-phase extraction (dSPE) was also used for their extraction in a method that uses multi-walled carbon nanotubes (MWCNTs) as the reversed dispersive SPE sorbent.[384] The method was optimized for extraction of 10 β-agonists from swine urine.

Several new extraction techniques have been developed in the past few years. Molecularly imprinted SBSE, with ractopamine as template molecule, was introduced as a method for extraction of β-agonists from the muscle, liver, and feed.[385] In 2013, Huang et al. developed a new sample preparation format, derived from SBSE, named stir-cake sorptive extraction (SCSE) on monolithic material, which enables the extraction, clean-up, and enrichment in one step.[386] Poly(4-vinylbenzoic acid-divinylbenzene) was used as the monolithic material,

and the method was optimized for eight β-agonists with different structural characteristic (phenolic and anilinic). Another method that uses the molecularly imprinted technique, dummy molecularly imprinted microspheres (DMIM), was developed for extraction of clenbuterol and clorprenaline from urine.[387]

In addition to the aforementioned methods, several methods were developed for single-analyte analysis (i.e., clenbuterol or salbutamol). For extraction of clenbuterol from chicken muscle, Qiao and Du developed a molecularly imprinted matrix solid-phase dispersion (MI-MSPD) procedure,[388] while Liu et al.[389] used a combination of SPE combined with ultrasound-assisted DLLME for extraction of clenbuterol from swine tissues. A single-analyte extraction procedure also was developed by for salbutamol in swine tissues, utilizing an IAC for selective salbutamol extraction.[390]

4.5.6.2 Screening Methods

Binding assays for β-agonists have been discussed in detail in an earlier section of the chapter (Section 4.3.6.1). The most widely used screening methods for β-agonists initially were RIA, but ELISA represents the main type of screening method now in use. Recently, a hapten microarray based on indirect competitive immunoassay was developed for three β-agonists (clenbuterol, salbutamol, and ractopamine).[391] Currently, there are also a large number of methods based on different types of sensors. These sensor-based screening methods include electrochemical sensors, biosensors, and immunosensors.[392–399] Although these methods have low detection limits and are sufficiently sensitive, the main disadvantage is that they are optimized for a single analyte, which makes their usage as screening tests for a large number of compounds inadequate.

4.5.6.3 Current Approaches in Analysis of β-Agonists

For the determination of β-agonists in biological material samples, many HPLC, LC-MS, LC-MS/MS, GC-MS, and GC-MS/MS methods were developed in the last 5 years. High sensitivity and selectivity along with lack of derivatization highlighted LC-MS/MS as the leading analytical technique in the analysis of β-agonists. In addition, introducing the UHPLC instruments in analysis resulted in shortening the run times and consequently higher throughputs. Despite the many advantages of LC-MS/MS-based methods, there were two problems associated with the use of this technique: matrix effects in general and the ion suppression phenomenon.[400] Improved sample preparation, including introducing the MIP as a sample preparation technique and usage of appropriate isotope-labeled internal standard, overcomes the two problems mentioned earlier.

As urine is one of the matrices of choice for the detection of the use of β-agonists, and it can be taken from living animals, several methods were developed for urine samples. A method for a single analyte, ractopamine, was developed and validated for both bovine and sheep urine.[401] Validation demonstrated good method characteristics, with a LOQ at 0.25 μg/l and satisfactory

linearity, precision, and accuracy. The authors also compared free and total ractopamine by analyzing the samples with and without a hydrolysis step (β-glucuronidase). Results revealed that hydrolysis is necessary for quantification of the total ractopamine residue. However, methods to be used to enforce legislation on set MRL values for ractopamine have to take into account that the MRL is set on the free or non-conjugated form. For such methods, hydrolysis should not be performed as this could give an inflated result, which could lead to legislative action when the sample is actually in compliance with limits. Validation of an LC-MS/MS method for determination of 10 β-agonists in swine urine resulted in CCα values ranging from 0.03 to 0.10 μg/l and CCβ values from 0.07 to 0.17 μg/l.[384] Other multi-methods developed for the determination of β-agonists in urine samples have been described.[402–404] The authors introduced Orbitrap™ mass analyzers, high-resolution instruments, in β-agonist analysis, which resulted in detection limits at ng/l concentrations. In addition to the use of these high-resolution instruments, the sample preparation was improved by use of MIP SPE or TurboFlow™ clean-up strategy.

Besides urine, possible matrices for testing of β-agonist abuse are liver, kidney, and muscle. A multi-method for determination of 16 β-agonists in pig liver, kidney, and muscle by LC-MS/MS was developed by Shao et al.[380] The method has shown good performance with CCα values ranging from 0.02 to 0.79 μg/kg and CCβ values ranging from 0.04 to 1.62 μg/kg and was considered suitable for monitoring of β-agonists abuse. A LC-MS/MS method for determination of clenbuterol in muscle by means of QTrap™ mass spectrometer reported by Pleadin et al. has a LOD of 0.1 μg/kg, sufficiently low to render the method suitable for determination of clenbuterol residues in muscle.[405]

Besides the urine samples that can be taken on farms from living animals, and liver and muscle samples taken from slaughtered animals, hair has many advantages as a matrix in monitoring of β-agonist abuse. In a study on non-lactating cows that were treated with six different β-agonists, the analysis of hair and urine samples was performed by UHPLC-MS/MS, and the concentrations of different β-agonists were compared between matrices at different withdrawal times.[406] The results from a study conducted on pigs, which utilized an UHPLC-MS/MS method for determination of ractopamine in hair, have also been reported.[407] The method was validated using a matrix-comprehensive in-house validation protocol based on factorial design. The method showed relevant CCα and CCβ values of 2.53 and 2.98 μg/kg, respectively. The results revealed that ractopamine accumulates in hair while residues were found in pig's hair eight days after withdrawal at a concentration of 8.77 ± 1.13 μg/kg. Another method for detection of clenbuterol in hair used HPLC coupled with a QTrap™ mass spectrometer with ESI.[382] Results of method validation have shown an acceptable LOQ, set at 0.5 μg/kg for both black and white hair.

In addition to hair, as pigmented matrix, another pigmented matrix that can be used for detection of β-agonists is retinal tissue. The results from a study performed on pigs treated with clenbuterol were reported by Pleadin et al.[408] A quantitative screening ELISA method was utilized to determine clenbuterol residues in retinal tissue of treated pigs and revealed that clenbuterol persists in this matrix for 45 days after treatment withdrawal. A UHPLC-MS/MS method for detection of ractopamine in retinal tissue of treated pigs has also been reported.[378] The results indicated a high accumulation affinity for ractopamine binding to retina despite the low dose of treatment applied.

A possible approach to monitor for abuse of β-agonists in animal production is to analyze feed and drinking water as potential media for administering β-agonists to farm animals. A LC-MS/MS method for analysis of seven β-agonists in both feed and drinking water has been reported, which may be used in such investigations.[383] The method has shown CCα values ranging from 0.06 to 0.12 μg/l for drinking water and 0.46 to 0.87 μg/kg for animal feed. All other validation parameters met the criteria set in Commission Decision 657/2002,[7] demonstrating that the method can be used for monitoring of β-agonists in these matrices.

Current trends in screening methods for β-agonists are focused on development of different sensor-based methods that are suitable to detect compounds at trace concentrations. The main problem rising from these types of methods is that they target only a single analyte, which is insufficient to screen a large group of quite diverse compounds such as β-agonists. Current research approaches in confirmatory analysis of β-agonists are focused on including more analytes in one method, meaning developing multi-methods. Another aspect is lowering of the detection limits by improving the sample preparation step, which is critical in LC-MS/MS analysis of polar compounds such as β-agonists due to the ion suppression, and utilizing more sensitive instruments.

4.5.7 Future Perspective (β-Agonists)

In the past few years, the number of β-agonist compounds available has increased while the crackdown on banned β-agonists led to the emergence of some new compounds. One of the new synthesized compounds is phenylethanolamine A, for which abuse in livestock production was observed in China.[409–411] In addition to newly synthesized compounds, in the field of β-agonists, there is a problem of possible usage of cocktails containing very low doses of different compounds that makes the detection quite difficult.[412] In recent years, new approaches based on untargeted and global measurements are emerging. One of these approaches is metabolomics, which is based on detecting small molecules and excluding large ones (i.e., proteins).[263] By collecting the data characteristic of a certain matrix, a "fingerprint" can be achieved for this matrix. For data collection in metabolomics,

different analytical techniques can be utilized, including GC-MS, LC-MS, and nuclear magnetic resonance (NMR). LC or GC coupled to mass spectrometric detectors has an advantage over NMR due to the higher sensitivity. As for confirmatory methods, LC-MS has several advantages in comparison to GC-MS as there is no need for a derivatization step and the thermal stability of the targeted compound is not an issue. With respect to the mass detectors, high-resolution equipment such as Orbitrap™ should be the choice due to the high specificity and sensitivity.

With these demanding analytical techniques, there is also a need for powerful software that can process the large amount of data collected. A metabolic approach, based on LC coupled with high-resolution MS, was developed to screen cattle urine for evidence of clenbuterol abuse.[412] Although this is only a beginning in the field of β-agonists analysis, it is a valuable start to the introduction of the metabolic approach in this field. Future research could focus on other matrices, such as liver, muscle, or hair. In the future, this approach of untargeted analysis could be an excellent tool to screen for abuse of the β-agonist group of compounds in food animal production.

When setting up the different screening and confirmatory methods for analyses of β-agonist residues, the fact that β-agonists can be used for purposes other than growth promotion must be taken into account. Some countries have authorized the use of specific β-agonists, including ractopamine hydrochloride and zilpaterol hydrochloride, as growth promoters. In 1999, the USFDA authorized the use of ractopamine hydrochloride as feed additive for finishing pigs with zero-day withdrawal time.[413] However, ractopamine is not authorized for use in the EU (and many other countries including China and Russia), and according to Council Directive 96/22/EC,[4] it is prohibited to import farm animals and their meat from third countries if β-agonists have been administered (except in therapeutic purposes for calving cows, foaling horses, and companion animals).[414]

MRLs for use of clenbuterol in cattle and horses were adopted by the Codex Alimentarius,[337] but due to the potential abuse of clenbuterol, MRLs are only recommended when associated with approved therapeutic use. The Codex Alimentarius Commission has also adopted MRLs for ractopamine hydrochloride residues in cattle and pigs, ranging from 10 μg/kg in muscle and fat, 40 μg/kg in liver, to 90 μg/kg in kidney.[337] In addition to ractopamine, another β-agonist, zilpaterol hydrochloride, was authorized as growth promoter for cattle in South Africa and Mexico[415] and, more recently, in other countries. The use of zilpaterol hydrochloride was reviewed by the 78th Meeting of the JECFA in 2013, which requested additional information, particularly with respect to analytical methods.[416] Additional data were provided to the 81st meeting of JECFA, which has recommended MRLs for zilpaterol hydrochloride, as previously noted.[338] These recommendations will be considered at Step 3 in the 8-Step Codex process at the next meeting of the CCRVDF, scheduled to be held in autumn 2016.[417]

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5

Analysis of Anthelmintic and Anticoccidial Drug Residues in Animal-Derived Foods

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5.1 Introduction

Animal-based food production systems are faced by a number of parasite challenges that may be treated through prophylactic and therapeutic administration of pharmaceutical substances to control infection.[1] The anthelmintics are the most important group of anti-parasitic substances and are also probably the most widely used veterinary drugs because they are used as herd treatments rather than for treatment of individual animals.[2] Anthelmintics are used to control a range of parasitic infections, including roundworms (nematodes) and flatworms (tapeworms and flukes) in food-producing animals.[3, 4] These substances are mainly used in extensively produced animals such as cattle,[5] dairy cows,[6] and sheep[7] that are exposed to parasites during grazing.

The anticoccidials or antiprotozoan agents are the second most important group of anti-parasitic drugs.[8] They are used to treat different microscopic parasitic infections such as coccidiosis, which is a disease that is normally associated with intensively reared species, in particular poultry.[9] Coccidiosis affects livestock at early stages of life and older animals are less susceptible to infection. Broilers have to be administered with preventative doses of anticoccidials in feed during their growth because it is generally not economically viable to treat this disease following infection. Coccidiosis also affects ruminants, particularly lambs and calves, which can be infected during housing.[10]

Some of these substances exhibit undesirable toxicological effects, such as teratogenicity, hepatotoxicity, or neurotoxicity, at high doses in laboratory

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animals.[11–13] Extensive regulatory controls are currently in place in many jurisdictions to protect the consumer through toxicological risk assessment of veterinary drugs, setting of maximum residue limits (MRLs), and licensing of products, which includes the establishment of withdrawal periods and the labeling of veterinary medicinal products.[13] Despite these regulations, monitoring of foodstuffs is a necessary requirement to verify that MRLs are not breached or that products are not administered except as approved to food-producing species.

Consequently, there is a growing need for complex analytical methods that will provide more cost-effective analysis of a wide range of residues of anti-parasitic agents in food. Such methods should be capable of measuring residues accurately and precisely at concentrations at and around the regulatory control limits. In addition, they should be capable of detecting low concentrations of residues in food, which is important for monitoring of anti-parasitic usage in different species and detecting illegal usage of substances, as well as for assessment of consumer exposure. Residues of anti-parasitic drug can be analyzed using such techniques as high-performance liquid chromatography (HPLC) coupled with ultraviolet (UV) detection or fluorescence (FL) detection.[11–13] However, liquid chromatography coupled with mass spectrometry (LC-MS) is the current method of choice for the determination of residues of anti-parasitic drugs.[14–17]

The focus in this chapter is on the current analytical approaches for the analysis of anthelmintic and anti-parasitic drug residues in food of animal origin, with an emphasis on LC-MS(/MS) methods because the use of this technique provides for more efficient analysis of these substances in food of animal origin than methods based on the techniques which were in routine use prior to the availability of this technology. The authors acknowledge that a number of reviews have been published on the analysis of residues of some of these drugs, particularly the benzimidazoles,[12] macrocyclic lactones[11, 18], and anticoccidials.[13, 19] These papers should be read for additional details on these drugs and methods for their analyses.

5.2 Chemistry and Mode of Action

5.2.1 Benzimidazoles

The benzimidazoles are heterocyclic molecules that contain benzene and imidazole rings in their structures (Figure 5.1). They can also include different functional substituents such as aromatic, thiazole, and alkyl side chains. Many benzimidazoles contain carbamate and/or sulfide substituents that play a key role in their efficacy and metabolism. Benzimidazole metabolism can be complex, which means that both parent drugs and metabolites have to be monitored in food.[12] It has been shown that the prodrug netobimin metabolizes to albendazole, which has three major metabolites: albendazole sulfoxide, albendazole

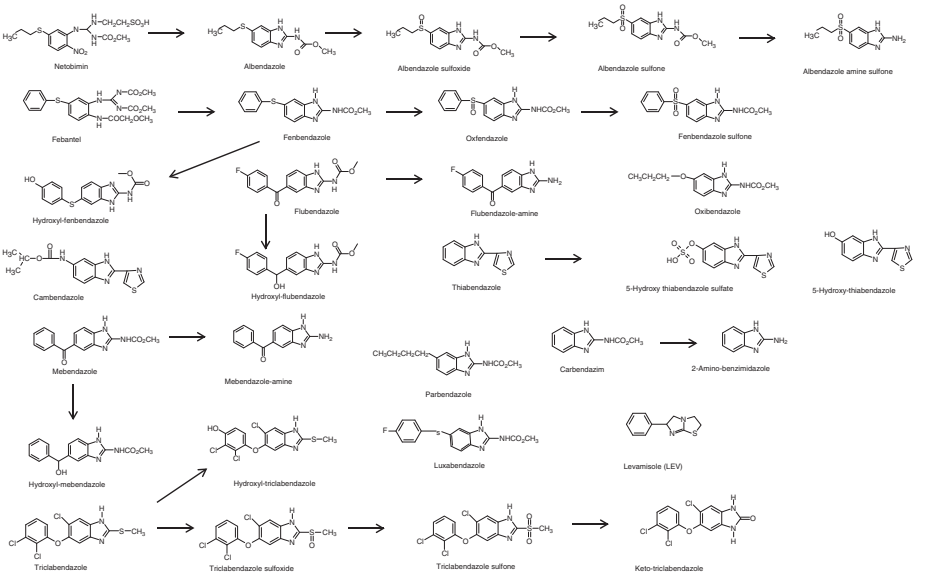


Figure 5.1 Chemical structures of benzimidazoles, benzimidazole metabolites, and levamisole.

sulfone, and albendazole amine sulfone.[20] Similarly, the prodrugs febantel and fenbendazole are metabolized to form oxfendazole and fenbendazole sulfone,[21] the main metabolites found in tissues as a result of hydroxylation of the phenyl ring, degradation of the carbamate to the amine, and oxidation of the sulfur.[22] Triclabendazole (TCB) undergoes oxidation to form triclabendazole sulfoxide (TCB-SO) and triclabendazole sulfone (TCB-SO_2), which can be derivatized to keto-triclabendazole (keto-TCB) which can be used as a marker residue for determining residues of triclabendazole.[23] The metabolism of triclabendazole is qualitatively similar in the liver of cattle and sheep and includes two pathways: a rapid oxidation of the methylthio group to the sulfoxide and then a slow oxidation to the sulfoxide, plus 4-hydroxylation of the dichlorophenoxy ring.[24] Hydroxylated metabolites of fenbendazole and triclabendazole are available but have not been included in the current residue definitions for the MRLs established for residues of the parent drugs in food.

The risk assessment by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) which recommended the MRLs adopted by the Codex Alimentarius[25] expressed them in terms of oxfendazole sulfone equivalents, the sum of what were identified as the three major metabolites – fenbendazole, oxfendazole, and oxfendazole sulfone.[26] The same residue definition was used in the evaluation of fenbendazole and related compounds by the Committee for Veterinary Medicinal Products (CVMP) in 1997[27], and the residue definition was recently affirmed by European authorities to be the "sum of extractable residues which may be oxidized to oxfendazole sulfone."[28] No hydroxyl-fenbendazole was detected in samples of milk and serum from cows which received fenbendazole by oral routes of administration (in suspension or in pellets) from a study evaluated by the 38th Meeting of the JECFA.[22]

The situation is somewhat different for triclabendazole. The risk assessment by JECFA which led to the current Codex MRLs for triclabendazole considered the total residues to be of potential toxic concern and therefore converted the marker residue concentration to total residue concentrations in the dietary exposure assessment, thus including any hydroxyl metabolite(s) in the exposure assessment which is the basis for the MRLs.[29] A conversion of marker residue to total residue was also applied in the evaluations conducted for establishment of MRLs in the European Union (EU) for tissues[30] and milk.[31]

Mebendazole (MBZ) can be hydrolyzed to form mebendazole-amine or can undergo reduction to form hydroxyl-mebendazole.[32] The evaluations by the CVMP identified two major metabolic pathways in their evaluation of MBZ.[33, 34] Reduction of the keto group resulted in the formation of methyl[5-(1-hydroxy-1-phenyl)methyl-1*H*-benzimidazol-2-yl]carbamate as the major metabolite from *in vitro* experiments using subcellular liver fractions (rat, dog, goat, sheep, horse, and cattle).[33] The second pathway involved carbamate hydrolysis to (2-amino-1*H*-benzimidazol-5-yl)phenylmethanone identified in *in vitro* experiments using horse and human hepatocytes.[34] These metabolites were also found in tissues from horses, sheep, and goats treated with MBZ in other

studies included in the evaluation by the CVMP.[33] The major metabolite identified in horse tissues was 2-amino-1*H*-benzimidazol-5-yl)phenylmethanone, while methyl(5-(1-hydroxy,1-phenyl)methyl-1*H*-benzimidazol-2-yl)carbamate was the major metabolite in tissues from sheep and goats, resulting in the definition adopted for the marker residue being the sum of the parent compound and the two metabolites.[34]

Flubendazole undergoes a similar metabolic pathway to MBZ to form flubendazole-amine and hydroxyl-flubendazole.[35–37] A number of drugs such as oxibendazole, parbendazole, and thiabendazole (TBZ) are no longer widely used. The major metabolite of TBZ is 5-hydroxy-thiabendazole (5-OH-TBZ), which occurs in the form of 5-hydroxy-thiabendazole sulfate in milk.[38] Carbendazim is a fungicidal agent and is metabolized to 2-aminobenzimidazole.[12]

The benzimidazoles are one of the most widely used groups of anthelmintics because of their broad-spectrum activity.[39, 40] TBZ was the first benzimidazole drug and was the first broad-spectrum anthelmintic. The success of this drug subsequently led to the development of over a dozen structurally related benzimidazole drugs and prodrugs, with the newer benzimidazole drugs showing broad-spectrum anthelmintic activity at lower doses. Benzimidazoles execute their effect by selectively binding to the β-tubulin of nematodes, thereby disturbing the formation and functions of microtubules. Early studies identified that flubendazole and MBZ cause infrastructural alterations to the intestinal and tegumental cells of nematodes and cestodes. The interruption of microtubule formation can have strong effects on mitosis, motility, and transport. Due to the crucial role that microtubules play in many cellular processes, in all eukaryotes, their inhibition/destruction eventually leads to the death of the organism.[39]

5.2.2 Imidazothiazoles

Imidazothiazoles exert their effect on the nervous system of the intended parasite, mainly as acetylcholine agonists.[41] Tetramisole, the first imidazothiazole anthelmintic drug to be introduced onto the market, was first used in the 1960s.[42] Tetramisole is a racemic mixture of two optical isomers, both in equal amounts, *S*(−)tetramisole (L-tetramisole, levamisole) and *R*(+)tetramisole (D-tetramisole). Anthelmintic activity resides almost solely in levamisole, the L-isomer.[43] The development of formulations using only the L-isomer allowed the doses to be halved and safety to be improved.[41] Levamisole shows a broad spectrum of activity against gastrointestinal and lung nematodes in a range of species. The chemical structure of levamisole is shown in Figure 5.1.

5.2.3 Tetrahydropyrimidines

Morantel, oxantel, and pyrantel are tetrahydropyrimidine drugs that are active against gastrointestinal nematodes (Figure 5.2).[44] They act selectively as agonists at synaptic and extrasynaptic nicotinic acetylcholine receptors (nAChRs) on

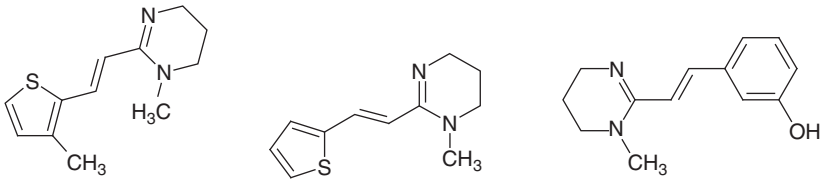


Figure 5.2 Chemical structures of tetrahydropyrimidines.

nematode muscle cells, resulting in spastic paralysis.[41] The tetrahydropyrimidines share similar structural properties with acetylcholine, which allows them to mimic its behavior within the cell. They show low toxicity because they act uniquely on nematode nAChRs.[39]

5.2.4 Organophosphates

Organophosphates such as coumaphos and haloxon were designed as broad-spectrum parasiticides with anthelmintic and insecticidal action (Figure 5.3).[45, 46] They are selective organophosphorus anticholinesterases, blocking acetylcholinesterase enzymes in parasites and resulting in the buildup of the neurotransmitter acetylcholine.[39, 41] The blockage of acetylcholinesterases occurs through the phosphorylation of esterification sites, which causes cholinergic nerve transmission to be blocked, leading to paralysis of the parasite. As acetylcholinesterase enzymes are also present in host animals, the mode of action adopted by these drugs can also lead to toxicity within the host animals. Consequently, endectocides are now more widely used.[44] See Chapter 7 for further discussion of organophosphates and other pesticides used in veterinary treatment of food-producing animals.

The JECFA has conducted risk assessments on two organophosphates which were registered for veterinary use in a number of countries, phoxim

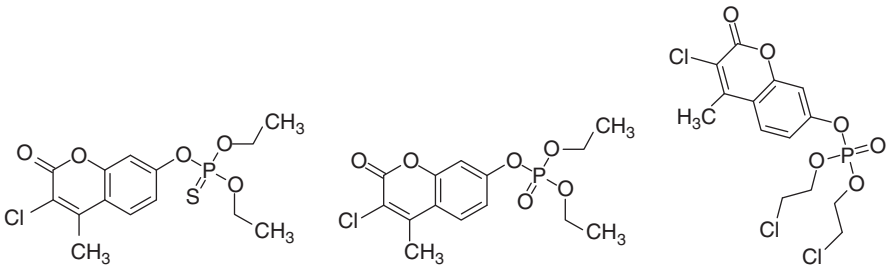


Figure 5.3 Chemical structures of some organophosphate anthelmintic agents and metabolites.

(diethyl *O*-(α-cyanobenzylideneamino)thiophosphate)[47, 48] and trichlorfon (dimethyl (2,2,2-trichloro-1-hydroxyethyl)phosphonate), which is also known as metrifonate.[49] Trichlorfon is metabolized in the treated animal to dichlorvos. Phoxim has been evaluated for use in the treatment of pigs, sheep, and laying hens by the CVMP.[50]

5.2.5 Flukicides

The flukicides include a range of drugs that are used primarily to control liver fluke in animals. They include some benzimidazoles (netobimin, albendazole, and triclabendazole), salicylanilides, substituted phenols, and clorsulon. The salicylanilides and substituted phenols are also known as proton ionophores, or protonophores,[51] as they play a major role in oxidative phosphorylation uncoupling. The salicylanilides include the drugs clioxanide, closantel, dibromsalan, niclosamide, oxyclozanide, rafoxanide, resorantel, and tribromsalan (Figure 5.4). Oxyclozanide also shows excellent activity against the rumen fluke.[52, 53] Once absorbed by the host animal, these compounds bind strongly to plasma proteins, as they are highly lipophilic, which facilitates the shuttling of protons across membranes.[41, 52]

The substituted phenols include bithionol, bromophen, hexachlorophene, niclofolan, and nitroxynil, which are highly toxic and have low margins of safety in target species. Nitroxynil is the only substituted phenol that is currently licensed for treatment of fluke infections in the EU.[54] Clorsulon is a flukicide that is used in combination products with ivermectin.[55] Its chemical structure is similar to 1,3-diphosphoglycerate, which allows it to competitively inhibit 3-phosphoglycerate kinase and phosphoglyceromutase in the glycolytic pathways of fluke.[41] This results in the selective inhibition of glucose utilization through blocking the oxidation of glucose to acetate and propionate.

5.2.6 Macrocyclic Lactones

The macrocyclic lactones comprise two drug groups, namely, the avermectins and the milbemycins.[11, 18] Both subgroups contain a 16-membered macrocyclic ring with a spiroketal group and a benzofuran ring. The avermectins can contain monosaccharide or disaccharide functional groups, but milbemycins lack the saccharide functional group (Figure 5.5). The macrocyclic lactones show broad-spectrum activity against both nematodes and arthropods and are often referred to as endectocides. They do not show any activity against trematodes and cestodes. Consequently, they are sometimes combined with other anthelmintic drugs to extend the spectrum of activity.[11] The macrocyclic lactones evoke their effects through binding to glutamate-gated chloride channel (GluCl) receptors. This results in paralysis of pharyngeal pumping and paralysis of body-level motility.[56] The inhibition of pharyngeal pumping is an intrinsic part of the nematode feeding process. These GluCls are encoded in the genome of

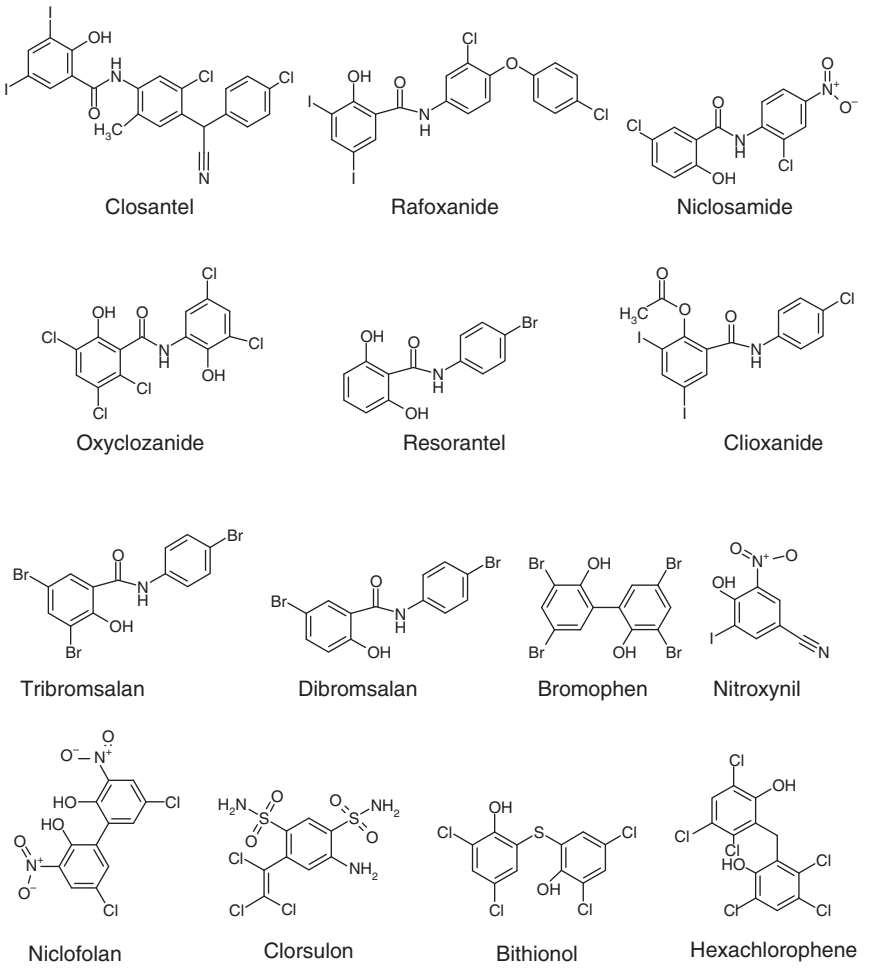
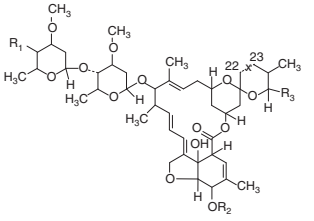


Figure 5.4 Chemical structures of some flukicide drugs.

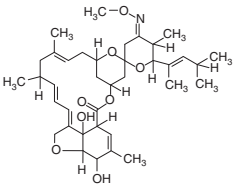
every member of the phyla *Nematoda* and *Arthropoda* so far examined, but are generally absent (in terms of recognizable homologues) from genomes of species not grouped in these phyla.

5.2.7 Other Anthelmintic Drugs

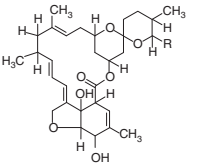
The amino-acetonitrile derivatives (AADs) are a new anthelmintic group that have been developed to combat anthelmintic resistance.[57, 58] Monepantel is an AAD that is rapidly metabolized to a sulfone metabolite, which has been identified as the marker residue in edible tissues (Figure 5.6).[59] Monepantel contains aryloxy and aroyl functional groups attached to an amino-acetonitrile



Avermectins

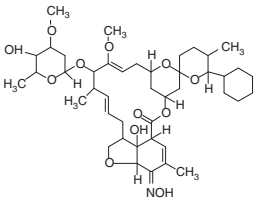


Moxidectin

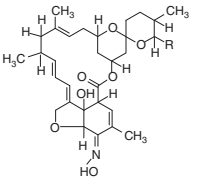


Milbemectin A3 (R = CH_3) and A4 (R = C_2H_5)

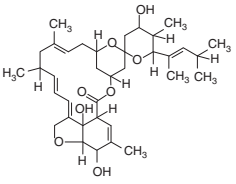
Avermectin	R_1	R_2	R_3	C_{22}–x–C_{23}
Abamectin B_{1a}	OH	H	$CHCH_3CH_2CH_3$	–CH═CH–
Doramectin	OH	H	C_6H_{11}	–CH═CH–
Emamectin benzoate B_{1a}	$C_6H_5COOHCH_3NH$	H	$CHCH_3CH_2CH_3$	–CH═CH–
Eprinomectin B_{1a}	$NHCOCH_3$	H	$CHCH_3CH_2CH_3$	–CH═CH–
Ivermectin B_{1a}	OH	H	$CHCH_3CH_2CH_3$	$–CH_2—CH_2–$



Selamectin

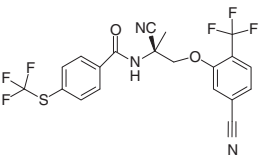


Milbemycin oxime A3 (R = CH_3) and A4 (R = C_2H_5)

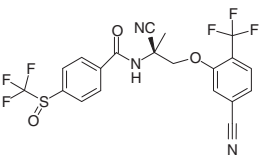


Nemadecti

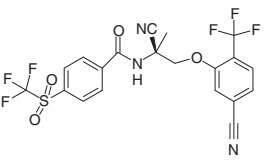
Figure 5.5 Chemical structures of different macrocyclic lactone molecules.



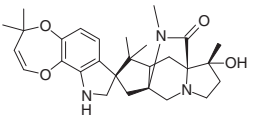
Monepantel



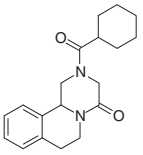
Monepantel sulfoxide



Monepantel sulfone



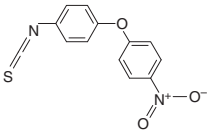
Derquantel



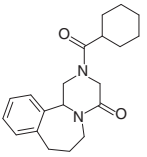
Praziquantel



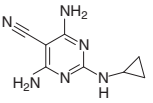
Piperazine



Nitroscanate



Epsiprantel



Dicyclanil

Figure 5.6 Structure of miscellaneous anti-parasitic drugs and metabolites.

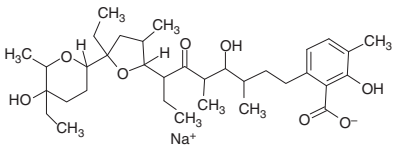
core and has broad-spectrum anthelmintic activity, even against the multi-drug resistant nematodes.[57, 58, 60] In addition, monepantel has low mammalian toxicity toward the host and laboratory test animals.[58] As a result of developing a better understanding of disease processes in animals, drug design has moved toward targeting specific mechanisms in targets. This design process has been undertaken with the aim of interrupting specific biochemical pathways in targets through various enzymes or receptors. AADs act as cholinergic agonists, as seen with tetrahydropyrimidines and imidazothiazoles.[58] The AADs, however, have a novel mode of action, in that they target a nematode-specific group of nAChR subunits. The binding of monepantel to the nematode-specific receptors results in the hypercontraction of body wall muscles, eventually leading to irreversible paralysis of the nematodes.[51, 58]

Derquantel is another relatively new anthelmintic drug that belongs to the spiroindol class and is a semi-synthetic compound derived from paraherquamide.[61] Derquantel interferes with β-subtype nAChRs and acts as an antagonist by inhibiting the 45-pS channels, leading to a flaccid paralysis of nematodes.[51] A number of other anthelmintic drugs are licensed in minor species or for specialized applications, including piperazine,[62] dicyclanil,[63] and praziquantel.[64] Two other drugs of this class, epsiprantel[65] and nitroscanate,[66] are used in companion animals.

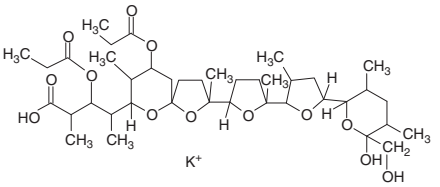
5.2.8 Ionophores

Lasalocid, maduramicin, monensin, narasin, and salinomycin are the most widely studied ionophores.[19] Another ionophore, semduramicin, is licensed as an anticoccidial in some countries, including the USA,[67] but not in the EU. Laidlomycin is a new ionophore that is used as a feed enhancer in cattle to improve production performance.[68] Nigericin is an ionophore that is not used in animal production but is frequently used as an internal standard for analytical methods.[69] The ionophores are relatively large molecules that are lipophilic in nature and have the capacity to complex different alkali metal ions (Figure 5.7).

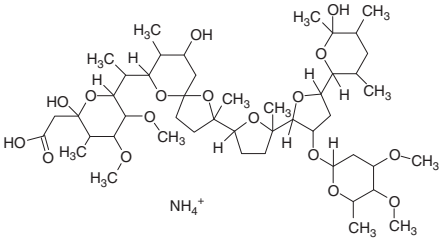
The ionophores have a very general mode of action based on the complexing of various cations,[13] such as Na^+, K^+, and Ca^{2+}. Individual ionophores differ from each other in their affinity toward different alkali metal ions. Most ionophores form complexes with monovalent cations (e.g., Na^+ and K^+), while lasalocid can form complexes with divalent cations,[19] such as Mg^{2+} and Ca^{2+}. All of the carboxylic ionophores have a similar mechanism of action based on the disruption of the intracellular cationic balance, causing Na^+ to increase and K^+ to decrease. The rate of Na^+ ion influx generally exceeds the Na^+/K^+-ATPase pump capability, which results in an influx of Cl^- to maintain the ionic balance.[70] This subsequently leads to absorption of water, causing swelling of the parasite until it eventually bursts.



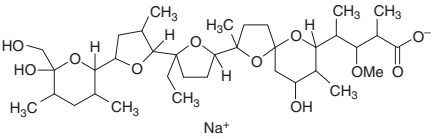
Lasalocid



Laidlomycin

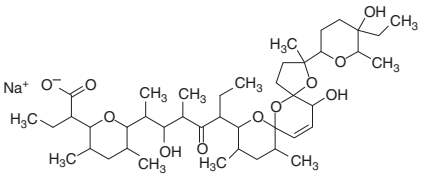


Maduramicin

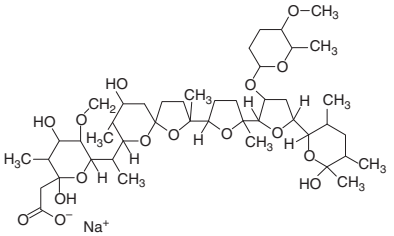


Monensin

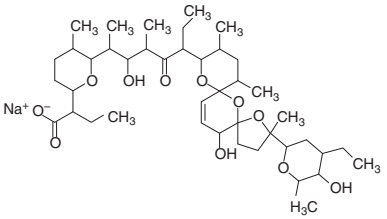
Figure 5.7 Chemical structures of ionophores.



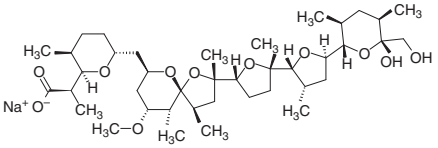
Narasin



Semduramicin



Salinomycin



Nigericin

Figure 5.7 (*Continued*)

5.2.9 Chemical Anticoccidials

The chemical anticoccidials can be sub-divided into quinolones, pyridones, alkaloids, guanidines, thiamine analogues, and triazine derivatives.[71] Residues of these substances can be difficult to measure in food because of their diverse chemical properties, which often requires special chromatographic separation protocols.[13] There are at least 12 chemical anticoccidials licensed within the EU and a number of other countries, and these are shown in Figure 5.8.

In recent years, there has been a trend to extend residue surveillance to include a wider range of residues of anticoccidial or antiprotozoan substances. Many of these are older drugs that are not widely used (they are not currently approved for use in food animals in the EU) but may still be useful for treating specific protozoan diseases. It can be seen that some of these drugs, such as aklomide, nitromide, and dinitolmide (zoalene), are low molecular weight compounds that are likely to be difficult to analyze using tandem mass spectrometry (MS/MS) (Figure 5.9). Suramin is a particularly interesting antiprotozoan agent that contains eight benzene rings and six sulfur groups that also has proven, in the authors' experience, to be challenging to analyze by LC-MS/MS.

Chemical anticoccidials have different modes of action but often act on developmental stages of the parasites. The modes of action are more specific than those of ionophores, such as inhibition of a number of biochemical functions including thiamine uptake (amprolium), mitochondrial energy production (clopidol, buquinolate, decoquinate, and nequinate), and inhibition of nucleic acid synthesis (ethopabate).[71] The first broad-spectrum anticoccidial, nicarbazin, is thought to inhibit succinate-linked nicotinamide adenine dinucleotide reduction, energy-dependent transhydrogenase, and the accumulation of calcium in the presence of adenosine-5′-triphosphate.[71] Toltrazuril (TOL) primarily affects the respiratory chain and the enzymes involved in pyrimidine synthesis.[72]

5.3 Legislation

As stated previously, even though anthelmintics tend to be more toxic toward parasites than mammals, residues of these drugs, which could pose a threat to human health, should not be found in food products intended for human consumption. According to the EU Regulation (EC) No. 470/2009, residues of pharmacologically active substances mean "all pharmacologically active substances, expressed in mg/kg or μg/kg on a fresh weight basis, whether active substances, excipients or degradation products, and their metabolites which remain in food obtained from animals."[74]

To ensure the continued safety of food, governmental bodies and international organizations, such as the EU[73, 74] and the Codex Alimentarius Commission,[26] have established MRLs for these products in foodstuffs. A MRL is the maximum concentration of residue that is permitted in a food product, which has been

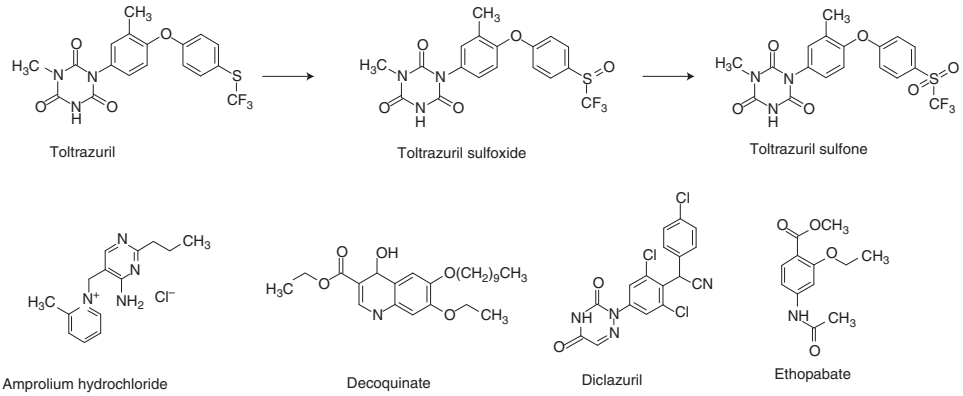
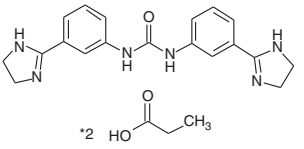
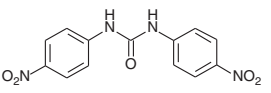


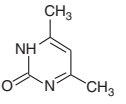
Figure 5.8 Structures of chemical anticoccidials (and some of their metabolites) that are licensed as veterinary drugs,[74] feed additives[75], or pesticides[76] in the EU.



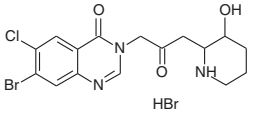
Imidocarb dipropionate



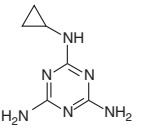
4,4′-Dinitrocarbanilide



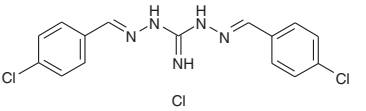
2-Hydroxy-4,6-dimethylpyrimidine



Halofuginone hydrochloride



Cyromazine



Robenidine

Figure 5.8 (*Continued*)

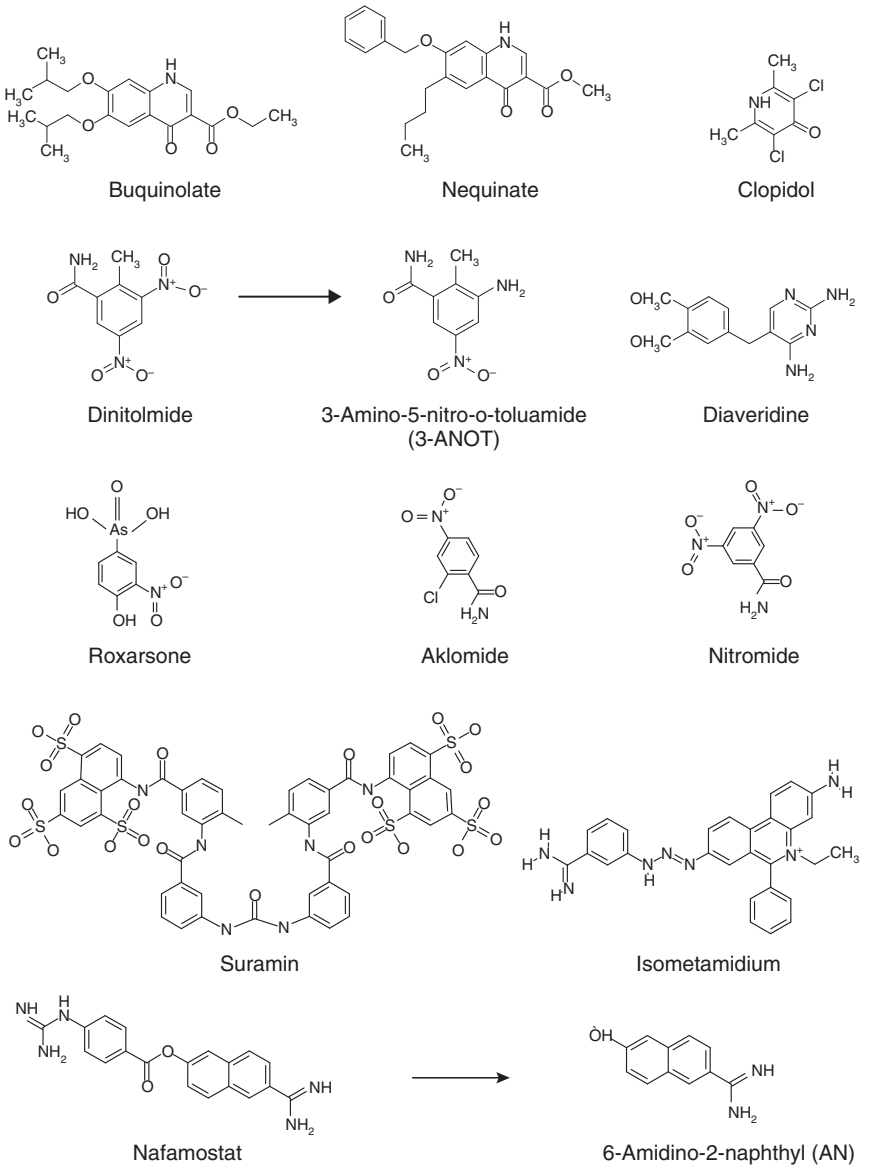


Figure 5.9 Structures of chemical anticoccidials that are not licensed in the EU.

derived from an animal administered or exposed to a veterinary product or biocidal product.

The use of medicines to treat animals is strictly controlled within many countries and requires a withdrawal period to be implemented. This is the time that elapses from the last dose administered to the animal and when the residue concentration within the animal's tissues (liver, kidney, etc.) and products (milk, eggs, etc.) is lower than or equal to the legislated MRL. Neither the animal nor its products can be used for human consumption until such a time as this withdrawal period has passed.

Using the EU as an example of a typical regulatory approach, the European Council Regulation (EC) No. 470/2009[73] lays out the procedures leading to the establishment of MRLs within the EU. European Commission Regulation (EC) No. 37/2010[74] sets out tables for those residues accepted for use within the EU and the marker residues to which these MRLs apply. MRLs appear in this regulation in two tables: Table 1[74] listing substances which can be used as veterinary medicines in food-producing animals with MRLs where necessary and Table 2[74] listing substances which cannot be used as veterinary medicines in food-producing animals such as chloramphenicol. The MRLs established in the EU for anticoccidial drugs are summarized in Table 5.1.

In terms of anthelmintics approved for use in the EU, these drugs are mainly licensed for treatment in cattle and sheep, primarily because these species are more susceptible to parasites through exposure during time spent on pasture. A few products such as albendazole, levamisole, flubendazole, and fenbendazole are licensed for avian and swine treatment. In terms of goats, horses, and game animals, very few products are licensed for use, and MBZ, monepantel, and TBZ are mostly used in goats. For this reason, the anthelmintic MRLs listed in Table 1 of the EU regulations[74] are mainly aimed at ruminants and swine, with flukicides predominantly licensed for use in cattle and sheep. Certain drugs such as levamisole, abamectin, ivermectin, and doramectin have not been licensed for treatment of animals during lactation. The MRLs established for anthelmintics under Commission Regulation 37/2010/EC are listed in Table 5.2.[74]

In general, approved usage of such drugs varies from region to region and country to country, depending on the types of livestock used in food production, production practices, and types of pests that must be managed. For example, usage of albendazole was considered so diverse that the Codex Alimentarius Commission has established MRLs applicable to "all species".[26]

Outside of the EU, the USA has established tolerances for the use of veterinary drugs in food and food commodities, which are stated in the Code of Federal Regulations under Title 21, "Food and Drugs."[77] Within Canada, Health Canada is responsible for setting out MRLs for residues of drugs in food of animal origin, both imported to and from Canada, under the Food and Drugs Act and Regulations.[78] MRLs for drugs approved for use in a number of other countries, including Australia,[79] India,[80, 81] China,[82] and Russia,[83] are also available.

Table 5.1 Tolerances and limits for anticoccidial residues in edible tissues.

Substance	Species	Tolerance limits (µg/kg)					
		Eggs	Milk	Liver	Kidney	Muscle	Fat
Lasalocid sodium[a)–d)]	Py	150		300	150	60	300 skin/fat
	NT		1	50	50	5	5
	B[e)]			100	20	10	20
Narasin	CF			50	50	50	50
	NT	2	1	50	5	5	5
Salinomycin sodium[b), c)]	CF			5	5	5	5
	RF						
	NT	3	2	5	2	2	2
Monensin sodium[a)–c)]	CF,CL,T			8	8	8	25 skin/fat
	B		2	50	2	2	10
	NT	2	2	8	2	2	2
Semduramicin[b)]	CF						
	NT	2	2	2	2	2	2
Maduramicin[b), c)]	CF			150	100	30	150
	T						
	NT	2	2	2	2	2	2
Robenidine[b), c)]	CF			800	350	200	1,300 skin/fat
	T			400	200	200	400 skin/fat
	RFB			200	200	100	100
	NT	25	5	50	50	5	50 skin/fat
Decoquinate[a), b)]	CF			1,000	800	500	1,000
	B[e)], O[e)]	No MRL required					
	NT	20	20	20	20	20	20
Halofuginone[a), b)]	CF, T						
	B[e)]			30	30	10	25
	NT	6	1	30	30	3	3
Nicarbazin[b), c)]	CF			15,000	6,000	4,000	4,000 skin/fat
	NT	100	5	100	100	25	25
Diclazuril[a)–c)]	CF, T			1,500	1,000	500	500 skin/fat
	RFB			2,500	1,000	150	300
	R,P	No MRL required					
	NT	2	5	40	40	5	5

(*continued*)

Table 5.1 (*Continued*)

Substance	Species	Tolerance limits (μg/kg)					
		Eggs	Milk	Liver	Kidney	Muscle	Fat
Amprolium[a)]	Py	No MRL required					
Imidocarb[a)]	B		50	2,000	1,500	300	50
	O[e)]			2,000	1,500	300	50
Toltrazuril[a)]	AMFPS[e)]			500	250	100	150
	Py[f)]			600	400	100	200

Source: Clarke 2014.[13] Reproduced with permission from Elsevier.
Key: AMFPS, all mammalian food-producing species; B, bovine; CF, chickens for fattening; CL, chickens reared for laying; NT, non-target species (species which unintentionally contains coccidiostat residues due to consumption of contaminated feed); O, ovine; P, porcine; Py, poultry; RF, rabbits for fattening; RFB, rabbits for fattening and breeding; R, ruminants; T, turkey.
a) Commission Regulation No. 37/2010.
b) Commission Regulation No. 124/2009.
c) Community Register of Feed Additives.
d) Commission Implementing Regulation No. 86/2012.
e) Not for use in animals from which milk is produced for human consumption.
f) Not for use in animals from which eggs are produced for human consumption.

5.4 Sample Preparation Protocols for Anti-parasitic Agents in Food Matrices

5.4.1 Selective Sample Preparation Procedures for HPLC-UV/FL Methods

Anti-parasitic drug residues can be extracted from biological matrices using different techniques including simple solvent extraction and liquid–liquid extraction (LLE) prior to HPLC analysis. LLE is commonly applied for the selective isolation of basic drugs through partitioning of residues from an aqueous solution into an immiscible organic solvent following adjustment of the pH to alkaline conditions.[84] Solvent extraction with acetonitrile is frequently used because it can selectively isolate drugs with diverse physicochemical properties while supporting protein precipitation and fat removal.[85] Several reviews[11, 12] have discussed methods which improved sample clean-up through the introduction of solid-phase extraction (SPE) for anti-parasitic agents such as the benzimidazoles,[84] levamisole,[86] macrocyclic lactones,[87–89] ionophores,[90] and chemical anticoccidials.[91–93] SPE is advantageous because it can be automated to allow unattended sample clean-up, which can be beneficial if large sample numbers have to be processed. Dowling et al.[94] developed an automated SPE method for 12 benzimidazoles in bovine liver. The method had a throughput of

Table 5.2 Maximum residue limits for anthelmintic veterinary drugs listed under Council Regulation 37/2010/EC.[74]

Veterinary Drug	Marker Residue(s)	Species	Maximum residue limit (µg/kg)					
			Muscle	Liver	Egg	Fat	Milk	Kidney
Abamectin	Abamectin B_{1a}	B &	NL	20	NL	10	NL	NL
		O	20	25	NL	50	NL	20
Albendazole, Albendazole sulphoxide, Netobimin	Sum of albendazole sulphoxide, albendazole sulphone and albendazole amino sulphone expressed as albendazole	All ruminants	100	1000	NL	100	100	500
Clorsulon	Clorsulon	B	35	100	NL	NL	16[a)]	200
Closantel	Closantel	B	1000	1000	NL	3000	45[a)]	3000
		O	1500	1500	NL	2000	45[a)]	5000
Derquantel	Derquantel	O	2	20	NL	40	NL	5
Doramectin	Doramectin	AMFPS	40	100	NL	150	NA	60
Emamectin	Emamectin B_{1a}	Fin Fish	100 µg/kg in muscle and skin in natural proportions					
Eprinomectin	Eprinomectin B_{1a}	B, Cp & O	50	1500	NL	250	20	300
Fenbendazole, Oxfendazole, Febantel	Sum of extractable residues which may be oxidised to fenbendazole sulphone	AFPS, except fish	50	500	1300	50	10	50
Flubendazole	Sum of flubendazole and amino-flubendazole expressed as flubendazole.	Py & P	50	400	NL	50	NL	300
	Flubendazole	Py			400			
Ivermectin	Ivermectin H_2B_{1a}	AMFPS	30	100	NL	100	NL	30
Levamisole	Levamisole	B, O, P & Py	10	100	NL	10	NL	10
Mebendazole	Sum of mebendazole, amino-mebendazole and hydroxyl mebendazole expressed as mebendazole	O, Cp & Eq.	60	400	NL	60	NL	60

(*continued*)

Table 5.2 (*Continued*)

Veterinary Drug	Marker Residue(s)	Species	Maximum residue limit (µg/kg)					
			Muscle	Liver	Egg	Fat	Milk	Kidney
Monepantel	Monepantel sulphone	O & Cp	700	5000	NL	7000	170	2000
Morantel	Sum of residues which may be hydrolysed to N-methyl-1,3-propanediamine and expressed as morantel equivalents	All ruminants	100	800	NL	100	50	200
Moxidectin	Moxidectin	B & O	50	100	NL	500	40	50
		Eq	50	100	NL	500	NL	50
Nitroxinil	Nitroxinil	B & O	400	20	NL	200	20	400
Oxibendazole	Oxibendazole	P	100	200	NL	500	NL	100
Oxyclozanide	Oxyclozanide	All ruminants	20	500	NL	20	10	100
Piperazine	Piperazine	P	400	2000	NL	800	NL	1000
		C	NL	NL	2000	NL	NL	NL
Praziquantel	Not applicable	O & Eq	No MRL Required					
Pyrantel	Not applicable	Eq	No MRL Required					
Rafoxanide	Rafoxanide	B &	30	10	NL	30	10[b)]	40
		O	100	150	NL	250	10[b)]	150
Thiabendazole	Sum of thiabendazole and 5-hydroxy thiabendazole	B & Cp	100	100	NL	100	100	100
Triclabendazole	Sum of extractable residues that may be oxidised to ketotriclabendazole	All ruminants	225	250	NL	100	10	150

Key: NL = Not listed, AMFPS = All mammalian food-producing species, AFPS = All food-producing species, B = Bovine, C = Chicken, Cp = Caprine, Eq = Equidae, O = Ovine, P = Porcine and Py = Poultry

a) Provisional MRL expired 1 January 2014

b) Provisional MRL expires 31December 2017

38 samples per week with a single analyst, which was limited by the long HPLC run time that was required. The macrocyclic lactone residues are most often extracted from meat and milk using acetonitrile, followed by clean-up using C_8 or C_{18} SPE.[88] In contrast, liver samples require a two-step approach involving two SPE cartridges packed, respectively, with deactivated alumina and C_{18}.[87] In general, it is preferable to use a gradient elution for HPLC when analyzing the macrocyclic lactones in liver tissue samples because non-polar matrix peaks can frequently be retained on the analytical column.

5.4.2 Selective Sample Preparation Procedures for LC-MS and LC-MS/MS

5.4.2.1 Anthelmintic Drug Residues

Sample preparation procedures employed in LC-MS analysis are generally less complicated than those used for HPLC-based methods owing to the selective and sensitive nature of these detection systems. However, LC-MS/MS is susceptible to matrix effects that can enhance or suppress analyte ionization, which can affect the quantification of analytes and method precision. It is important to evaluate matrix effects during method development so that this effect can be reduced or compensated. Alternatively, this effect can be negated through the use of matrix-matched calibration approaches or through the addition of stable isotopically labeled internal standards. It will be seen from this section that many early methods included a limited number of anti-parasitic drugs or structurally similar compounds. Consequently, more selective sample preparation procedures were applied in early methods to reduce matrix effects and contamination of the LC-MS source. Later, through the development of more robust, faster scanning and sensitive LC-MS/MS instrumentation, less selective procedures have been applied.[17]

Cannavan et al.[95] developed an early procedure for the isolation of TBZ and 5-OH-TBZ from animal tissues based on LLE into ethyl acetate at pH 7. Extracts were further purified on cyano SPE columns prior to LC-MS analysis. Recoveries for TBZ and 5-OH-TBZ were in the range 96–103% and 70–85%, respectively. Cherlet et al.[86] isolated levamisole from various animal tissues with hexane/isoamyl alcohol prior to clean-up using strong cation exchange SPE. It was found that accuracy and precision in LC-MS/MS analysis could be improved using the more selective ion exchange clean-up. De Ruyck et al.[35] adapted the method developed by Wilson et al.[84] for the analysis of benzimidazole anthelmintics by liquid chromatography (LC) to the analysis of flubendazole, hydroxyl-flubendazole, and flubendazole-amine residues in egg and poultry tissues (liver and muscle) prior to LC-MS/MS analysis. Recovery of analytes ranged between 77% and 95%. The same group later adapted this procedure to the analysis of MBZ, hydroxyl-mebendazole, and mebendazole-amine in sheep muscle.[32] Balizs[96] extracted residues of 15 benzimidazoles from muscle tissue from pigs, cattle, and sheep, cattle liver, and egg yolks using ethyl acetate and purified the extracts on styrol-divinylbenzene SPE cartridges prior to

LC-MS/MS. Analyte recovery was in the range of 36–117% but was lower for fenbendazole at 8%. Limits of quantification (LOQs) reported for most benzimidazoles were <10 μg/kg.

De Ruyck et al.[97] reported the extraction of residues of levamisole and seven benzimidazoles from milk samples with ethyl acetate under alkaline pH conditions. Analytes were separated on a C_{18} HPLC column with gradient elution and detected by MS/MS using positive electrospray ionizations (ESIs) with multiple reaction monitoring (MRM). Recovery was in the range of 79–110%. The method was validated according to the criteria in 2002/657/EC[98] and used in the monitoring program in Belgium for detection of veterinary drug residues in raw farm cow's milk. Jedziniak et al.[99] developed a method which included 19 benzimidazoles and levamisole in bovine milk using LC-MS. Samples were initially extracted into ethyl acetate, followed by liquid–liquid partitioning using hexane and acidified ethanol. This method also was validated according to the EU criteria.[98] Xia et al.[100] applied Oasis® MCX SPE clean-up for isolating benzimidazole residues from milk samples, followed by analysis using UHPLC-MS/MS. Analyte recovery ranged between 80% and 101%, with LOQs from 0.1 to 1.0 μg/l.

The flukicides are mostly acidic drugs, which allow alternative procedures to be employed based on anion exchange SPE clean-up systems. Caldow et al.[101] extracted phenolic and salicylanilide anthelmintics from bovine kidney using 1% acetic acid in acetone and purified samples using mixed-mode anion exchange SPE prior to analysis. Analytes included nitroxynil, oxyclozanide, rafoxanide, closantel, ioxynil, niclosamide, salicylanilide, and 3-trifluoromethyl-4-nitrophenol (TFM), with recovery typically in the range of 65–81%. The method was validated according to EU criteria.[98] Devreese et al.[102] recently reported the isolation of closantel residues from milk samples using acetonitrile/acetone (80/20, v/v) with SPE clean-up on Oasis® MAX columns. Analytes were separated on a C_{18} column using gradient elution with 1 mM ammonium acetate in water and acetonitrile and detected by MS/MS, with a linear range from 10 to 2000 μg/kg and LOQs of 1 and 10 μg/kg for bulk milk from cattle and sheep, respectively. Sakamoto et al.[103] reported a method using LC-MS/MS in which residues of bithionol, bromophen, nitroxynil, oxyclozanide, and tribromsalan were extracted from acidified milk samples with ethyl acetate. Following evaporation, residues were resuspended in acetonitrile, defatted with hexane, and separated on a C_{18} column using a 0.1% formic acid–methanol mobile phase, with detection by MS/MS. Recovery of the analytes was in the range of 83–97%, with limit of detection (LOD) of all these compounds in milk reported to be 0.1 μg/kg.[103] Turnipseed et al.[104] extracted four macrocyclic lactone residues from milk samples with acetonitrile and purified the sample extracts using C_{18} SPE prior to LC-MS/MS. Analytes included in the method were the avermectins ivermectin, doramectin, and eprinomectin and the milbemycin moxidectin. The research included an investigation into the ionization response of these compounds using atmospheric pressure photoionization (APPI) compared either atmospheric pressure chemical ionization (APCI), a

combination of APPI and APCI, or electrospray and demonstrated that the response observed using the different ionization protocols varied with the compound and the mobile phase used for LC separation.

Alternative techniques have been developed for isolating anti-parasitic agents from animal tissues including pressurized solvent extraction[105] and supercritical fluid extraction (SFE).[106, 107] Chen et al.[105] extracted 11 benzimidazoles and 10 metabolites of albendazole, fenbendazole, and MBZ from muscles and livers of swine, cattle, sheep, and chicken using acetonitrile/hexane as the extraction solvents for accelerated solvent extraction (ASE). Analysis of extracts by LC-MS/MS used separation of analytes on a C_{18} column by gradient elution with a mobile phase consisting of acetonitrile and 5 mmol/l formic ammonium and detection in the positive ion mode using MRM. Mean analyte recoveries ranged between 70% and 93%, with LOQs from 0.02 to 0.5 µg/kg.

Danaher et al.[106, 107] successfully developed methods for the extraction of benzimidazole and macrocyclic lactone residues from liver tissue using SFE. In the initial method, following extraction using supercritical carbon dioxide, residues of eprinomectin, moxidectin, abamectin, doramectin, and ivermectin extracted from pork and sheep livers were derivatized using methylimidazole, trifluoroacetic anhydride, and acetic acid and analyzed by high-performance liquid chromatography with fluorescence detection (HPLC-FL).[106] Subsequently, a method using SFE was developed for the extraction of residues of 10 benzimidazoles from livers of sheep, cattle, pigs, and poultry.[107] The SFE sample extracts were acidified and then purified using a strong cation exchange SPE cartridge, followed by analysis using HPLC-DAD. However, SFE is not widely used, in part due to low sample throughput for these substances and instrumentation cost.

Kinsella et al.[108] developed the first multi-residue method that analyzed for a wide range of flukicides, macrocyclic lactones, benzimidazoles, and other anthelmintic residues in one test. Milk and liver samples were extracted using a QuEChERS protocol that entailed the use of acetonitrile and $MgSO_4$ and NaCl to induce separation of acetonitrile and aqueous phases. The extract was then purified through C_{18} dSPE (Figure 5.10) with $MgSO_4$ and analyzed by LC-MS/MS. The method was somewhat remarkable because recovery for the majority of analytes included in the analysis was >80%, with LOQ of 5 µg/kg for all analytes except dichlorvos (LOQ of 10 µg/kg). This method was validated according to EU criteria.[98] Whelan et al.[109] enhanced the sensitivity of the Kinsella et al.[108] method through the introduction of a concentration step, while using dimethyl sulfoxide as the keeper solvent for milk analysis, and analysis by UHPLC-MS/MS (see Figure 5.10). Samples were first extracted into acetonitrile using $MgSO_4$ and sodium chloride to induce liquid–liquid partitioning followed by dispersive SPE for clean-up, followed by concentration of the extract into dimethyl sulfoxide.

For MS/MS analysis, the method employed rapid polarity switching in ESI, so that from a single injection both positively and negatively charged ions were detected in a 13-minute run time. The dimethyl sulfoxide keeper solvent was the

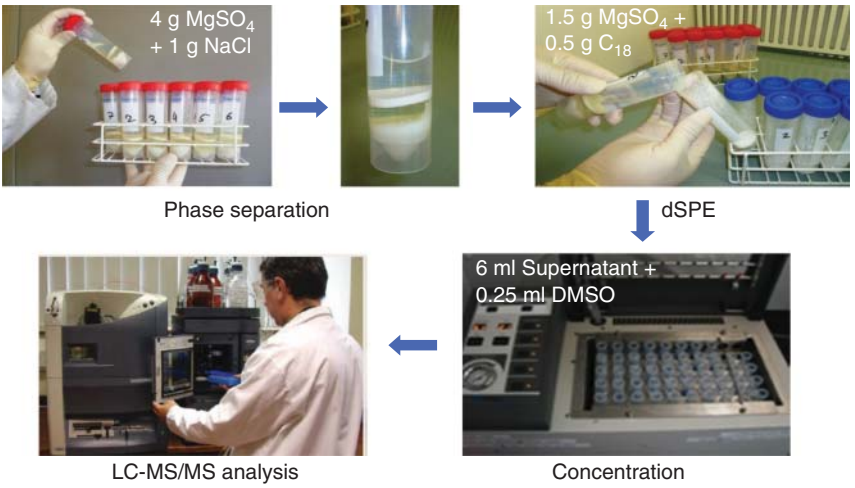


Figure 5.10 Overview of modified QuEChERS sample preparation procedure for the determination of anthelmintic drug residues in milk samples.

key to providing high analytical recovery for the albendazole, fenbendazole, and some flukicide residues. This UHPLC-MS/MS method was later adapted by the same group to anthelmintic analysis in liver tissue from cattle.[110] Both methods were validated according to the criteria of 2002/657/EC.[98] The method was later extended to include triclabendazole[23] and monepantel[60] marker residues in animal tissue and milk.

Rúbies et al.[111] recently reported the application of QuEChERS for the preparation of residues of ivermectin, abamectin, emamectin, eprinomectin, doramectin, and moxidectin in food for determination using LC-MS/MS and also explored the use of a hybrid Q-Orbitrap™ instrument. The LC-MS/MS procedure was capable of detecting residues of these drugs at 2.5 μg/kg in meat samples, enabled the high throughput of samples, and was validated according to EU criteria.[98]

5.4.2.2 Anticoccidials

Blanchflower et al.[112] extracted lasalocid from eggs using acetonitrile at an acidic pH and partitioning into *n*-hexane/toluene prior to LC-MS analysis. The method was later modified to isolate monensin, salinomycin, and narasin from muscle, liver, and eggs, with methanol substituted as the extraction solvent.[113] Yakkundi et al.[114] later developed a rapid procedure for the isolation of 4,4′-dinitrocarbanilide (DNC) residues from eggs and liver. Samples were extracted with acetonitrile prior to LLP clean-up to remove non-polar matrix interfering components. Yakkundi et al.[115] also found that addition of a tryptic digestion step was required to isolate halofuginone residues from eggs and liver. The digested samples required a complex isolation procedure based on LLE, LLP, and automated SPE clean-up on Oasis HLB cartridges. Martínez-Villalba

et al.[116] extracted TOL marker residues from meat samples with acetonitrile and purified samples by C_{18} SPE prior to analysis. Olejnik et al.[117] reported an intensive clean-up procedure for selective isolation of 12 anticoccidials from using combined alumina and Oasis HLB SPE clean-up.

More recent methods for anticoccidials do not generally include LLE steps because of the requirements for generic clean-up procedures and the improvements in LC-MS/MS detection systems. Matabudul et al.[118] developed a generic extraction protocol for isolating anticoccidial residues from egg and liver prior to LC-MS/MS. Samples were mixed with Na_2SO_4, extracted with acetonitrile, and passed through SPE silica cartridges. The method was initially applied to lasalocid, monensin, narasin, and salinomycin, and recoveries from fortified sample materials were >98% and >93% for egg and liver, respectively. The method was later extended to the analysis of ionophores and DNC in liver[119] and provided a significant improvement in sample throughput compared to previously published methods, in that an analyst could typically process 30–40 samples in a day.

In 2004, Dubois et al.[120] made a significant advance in anticoccidial analysis by showing that the Matabudul et al.[118] method could be extended to the analysis of nine anticoccidials in eggs and muscle. The procedure has since been applied by a number of different groups for anticoccidial analysis in animal tissues and eggs. Rokka and Peltonen reported the determination of residues of lasalocid, monensin, narasin, and salinomycin in eggs and poultry tissue.[121] Galarini et al.[122] later adapted the method of Matabudul et al.[118] to the determination of residues of lasalocid, maduramicin, monensin, narasin, salinomycin, semduramicin, decoquinate, diclazuril, halofuginone, nicarbazin, and robenidine in poultry eggs using extraction with acetonitrile, delipidation with hexane, and clean-up on a silica SPE cartridge. Both methods[121, 122] were validated according to 2002/657/EC.[98] Yue et al.[123] subsequently applied the method as reported by Galarini et al.[122] to the determination of residues of 5 ionophores and 15 chemical anticoccidials in poultry matrices. Shao et al.[124] mixed egg and meat samples with Na_2SO_4 and extracted 14 anticoccidial residues with acetonitrile prior to LC-MS/MS analysis. These authors omitted the silica SPE clean-up step used by other groups taking what is frequently described as a "dilute-and-shoot" approach. Method recoveries ranged from 78% to 125%, but due to matrix interferences, the method only had applications in screening and not quantification.

Other methods have reported that anticoccidial residues could be analyzed in eggs and tissue samples without the need for sample clean-up. In 2003, Mortier et al.[125] reported a method for detecting five anticoccidials (diclazuril, dimetridazole, halofuginone, nicarbazin, and robenidine) in eggs based on acetonitrile extraction without clean-up ("dilute and shoot"). Similarly, in 2009, Dubreil-Chéneau et al.[126] extracted residues of 10 anticoccidials (diclazuril, halofuginone, lasalocid, maduramicin, monensin, narasin, nicarbazin, robenidine, salinomycin, and semduramicin) from egg samples with acetonitrile and

selectively resuspended extracts in sodium acetate/acetonitrile (50:50, v/v) prior to LC-MS/MS analysis.

Moloney et al. developed a method for isolating residues of 20 anticoccidials from eggs and poultry muscle.[127] A number of extraction procedures were evaluated including QuEChERS with different SPE clean-up sorbents. However, it was found that recovery of ionophores was not satisfactory using QuEChERS. It was also determined that bonded silica sorbents strongly retained the ionophores, while the bare silica clean-up used by Matabudul et al.[118] was found to provide negligible clean-up. Consequently, egg and tissue samples were simply extracted using acetonitrile and selectively resuspended in acetonitrile/water. A comprehensive ion suppression study was carried out with the method, which identified some shortcomings particularly for the imidocarb. However, this issue has since been addressed through the inclusion of an isotopically labeled internal standard. The method has also been further enhanced through the use of more selective transitions for halofuginone. This work also brought to light the difficulty of applying developed anticoccidial methods to a range of matrices. The application of this method to liver matrices was also investigated, but it was determined that this was not achievable due to the large ion suppression effects that were observed. Clarke et al.[8] later adapted the method developed by Moloney et al.[127] to the analysis of the same 20 anticoccidials residues in milk, duck, and non-avian muscle. The muscle tissues were extracted using the procedure of Moloney et al.,[127] but a simple QuEChERS extraction was applied to milk samples, which included an alkaline pH adjustment.

QuEChERS has been used by some other groups for anticoccidial sample preparation. Stubbings and Bigwood developed a modified QuEChERS method for isolating ionophores and nicarbazin from chicken muscle.[128] The group modified the existing QuEChERS method through extraction with Na_2SO_4 followed by the adjustment of sample pH through the use of acetic acid (1%) in acetonitrile. Samples were purified using aminopropyl dSPE, which gave better recovery for the anticoccidials. Wang et al.[129] also used a modified QuEChERS for measuring concentrations of residues of cyromazine and melamine in egg and chicken muscle. Samples were extracted using acidified acetonitrile and purified dSPE using graphitized carbon black, followed by LC-MS/MS analysis with chromatographic separation on an anion exchange LC column. Nakajima et al.[130] developed a modified QuEChERS extraction procedure for isolating a number of coccidiostat and anti-parasitic drug residues from muscle (beef, pork, chicken) and eggs. The authors reported that using acetonitrile containing 0.1% formic acid and <5 g of sodium chloride greatly enhanced analyte recovery.

A method for the determination of residues of six ionophores (lasalocid, maduramicin, monensin, narasin, salinomycin, and semduramicin) in raw, UHT, pasteurized, and powdered milk, validated according to 2002/657/EC,[98] has recently been reported by Pereira et al.[131] The method includes a QuEChERS-based extraction and clean-up, with determination using LC-MS/MS. Recoveries of 93% and

113% were achieved within the range required for monitoring compliance with regulatory limits in the EU.

A method for the determination of residues of narasin and monensin in beef, pork, and chicken tissues using LC-MS/MS has been validated through the Official Methods process of AOAC International. The method uses nigericin as an internal standard, with extraction using *iso*-octane/ethyl acetate (9:1) and clean-up on a silica SPE cartridge. An initial single-laboratory validation (SLV), which included the analysis of incurred tissues and milk, demonstrated recoveries of 86–103% for narasin and 89–105% for monensin, with a LOQ of 0.8 μg/kg for most tissues, with the exception of chicken fat (LOQ = 4.0 μg/kg).[132] A subsequent collaborative study with 10 participating laboratories which included the analysis of blind duplicates of five incurred residue materials for each analyte provided results which met the AOAC acceptance criteria, with the result that the method was recognized with "Final Action" status by the Official Methods Board of AOAC International.[133] A method for nicarbazin residues has also been undertaken to develop an AOAC Official Method. An SLV study was reported on the evaluation of a method using LC-MS/MS for the determination and confirmation of the marker residue, DNC, in chicken liver, kidney, muscle, skin with adhering fat, and eggs.[134] Test portions of sample material are mixed with anhydrous sodium sulfate and then extracted with acetonitrile, mixed, then centrifuged, filtered, and diluted with acetonitrile for analysis. The method, which uses DNC-d_8 as an internal standard and was validated to a LOQ of 20 μg/kg, was accorded First Action Official Method status by AOAC International on 7 May 2013.

5.4.3 Multi-class Sample Preparation Procedures

Multi-class sample preparations have been developed that include >200 drugs and have been helped greatly through the improvements in analytical instrumentation. The sensitivity of newer instruments allows more dilute sample extracts to be injected onto systems, which can negate sample matrix effects. Yamada et al.[135] reported one of the earliest multi-class methods while attempting to establish a method for residues of 130 veterinary drugs, including benzimidazoles, ionophores, and miscellaneous other anthelmintics and coccidiostats in muscle tissues. Samples were mixed with Na_2SO_4 and extracted with acetonitrile/methanol. Extracts were defatted with *n*-hexane before analysis by LC-MS/MS. Recoveries ranged from 70% to 110% for 111, 122, and 123 residues in beef, pork, and chicken muscle, respectively. Peters et al.[136] developed a method for the isolation of >100 veterinary drug residues from animal tissue based on extraction with acetonitrile/water and Strata™-X SPE clean-up.

Zhan et al.[14] developed a method that was capable of isolating 225 veterinary drug residues and other contaminants from milk. The method, which included 14 benzimidazoles, 12 anticoccidials, and 7 anthelmintic drugs, included an extraction procedure in which raw milk samples were mixed with EDTA-Na_2

and ethanol/acetonitrile (1:5, v/v). The use of ethanol prevented phase separation that was observed when only acetonitrile was used. A subsequent precipitation step was added to remove water-soluble matrix components and salts. Sorbent clean-up steps were excluded because they led to the loss of hydrophobic drugs, tetracyclines, and β-lactams. Zhan et al.[15] later extended their research to include a method for the determination of residues of 226 veterinary drugs and other contaminants in porcine and bovine muscle. In this work, additional clean-up steps were introduced into their earlier method, including a hexane LLP step, cold temperature treatment at −40 °C (2 hours) to remove lipid, and a dSPE step. Mean analyte recoveries ranged between 62% and 139%.

Geis-Asteggiante et al.[16] developed a multi-class method for the detection of >100 veterinary drug residues in bovine muscle using UHPLC-MS/MS analysis. A range of sorbent clean-ups were examined in order to ensure that matrix interferences were kept to a minimum. Sample extraction used acetonitrile/water to ensure that highly polar molecules were not lost and clean-up was carried out using C_{18} dSPE. The method was found suitable for use to screen for residues of 113 analytes and to quantify 87 of these residues, out of the 127 tested, through the application of this procedure. More recently, Schneider et al.[17] developed a streamlined method for the analysis of veterinary drug residues in bovine muscle, including benzimidazoles, avermectins/milbemycins, and a variety of anthelmintics and coccidiostats. In this procedure samples were extracted using the procedure described by Geis-Asteggiante et al.,[16] but clean-up was carried out using an in-vial dSPE step (Figure 5.11). The implementation of the method

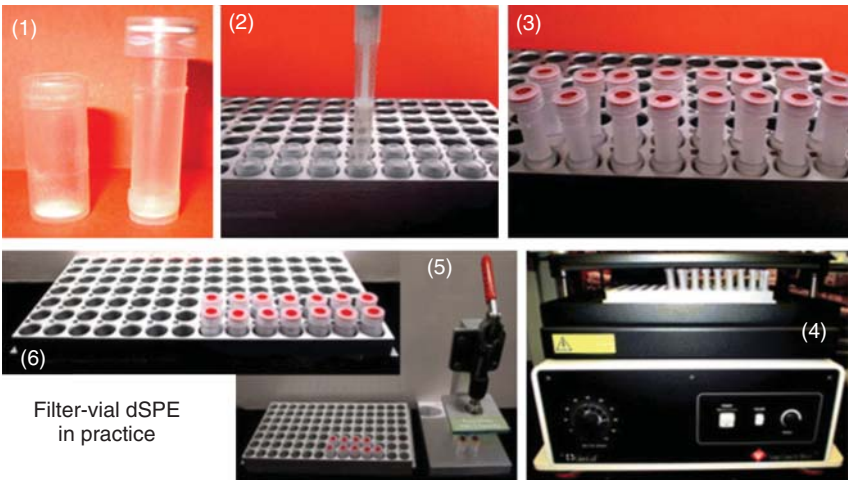


Figure 5.11 Steps of the filter-vial dispersive-SPE process: (1) 25 mg C18 contained in shell portion of the filter vial, (2) addition of 0.4 ml of the extracts, (3) partial capping of the vials, (4) shaking of the tray for 30 s, (5) pressing of the vials, and (6) final extracts ready for analysis. Source: Schneider 2015.[17] Reproduced with permission from Springer.

was facilitated through the use of a core–shell C_{18} column for chromatographic separation with determination using a QTrap™ hybrid MS/MS.

A multi-class method developed by Wei et al.[137] and validated according to 2002/657/EC[98] provides screening for residues of 128 veterinary anti-parasitic drugs and metabolites in meat (chicken, pork, beef). The main families of veterinary drugs included are the avermectins, benzimidazoles, ionophores, as well as various anthelmintics and coccidiostats. A QuEChERS approach is used for sample extraction and clean-up, followed by LC-MS/MS analysis.

5.5 LC-MS and GC-MS Detection of Anti-parasitic Agents in Food

5.5.1 Benzimidazole and Levamisole

LC-MS/MS has many advantages over HPLC-UV for analysis of benzimidazole and levamisole residues. In general, simpler sample preparation procedures can be applied with LC-MS/MS as a result of its selectivity, which allows twice the number of samples to be analyzed in a batch.[94, 108–110] In addition, contemporary LC-MS/MS can provide LOQs at least 50 times lower than methods using HPLC-UV.[94, 108–110] The inclusion of metabolites in the marker residue definitions of several benzimidazole drugs can require that individual metabolites have to be quantified below the MRLs.[12, 97, 108, 109, 138] This is especially an issue in the analysis for residues of such drugs in milk, where MRLs are as low as 10 µg/kg for authorized use of veterinary drugs. Consequently, LC-MS/MS has become the method of choice for analysis of these substances in complex food samples.

Early methods for the analysis of benzimidazole residues were mainly based on single quadrupole MS analyzer systems equipped with thermospray or APCI probes. Data were acquired in selected ion monitoring (SIM) mode as $[M+H]^+$ or $[M+H]^-$ diagnostic ions. An example of such methods was one developed by Blanchflower et al.,[139] who reported a thermospray LC-MS method for measuring fenbendazole and oxfendazole residues in liver and muscle samples from sheep. A subsequent publication from the same laboratory reported the development of a quantitative screening method using the same instrumentation for the determination of residues of TBZ and its metabolite 5-OH-TBZ in beef muscle, liver, and kidney.[95] TBZ and 5-OH-TBZ were monitored in SIM mode as their $[M+H]^+$ ions. Deuterated thiabendazole (TBZ-d_4) was used as an internal standard to improve accuracy and precision. Confirmation was based on analysis using an APCI probe, which produced four major ions for TBZ and 5-OH-TBZ.

Single quadrupole instruments fitted with ESI have also been used by other groups. Takeba et al.[85] developed a method for simultaneous analysis of residues of TCB, TCB-SO, and TCB-SO_2 in milk samples through the monitoring of their $[M-H]^-$ ions. The method LODs range between 4 and 6 µg/kg. Jedziniak et al.[99] reported a comprehensive LC-MS screening method for analyzing 19

benzimidazoles and levamisole in bovine milk. It was shown that TCB residues could be detected at lower concentrations when analyzed as negative ions. As a result, a rapid polarity switching was included in the method to allow analysis of positive and negatively charged analytes from a single injection. The single quadrupole LC-MS system was equipped with a multimode ion source, which allowed the comparison of ESI and APCI sensitivity. One ion was monitored for the majority of analytes, except for TCB, TCB-SO, and TCB-SO_2 for which two ions were used. This screening method enabled the analysis of anthelmintics in milk samples at concentrations above 5 μg/kg.

Around the year 2000, MS/MS in the form of triple quadrupole (QqQ) and ion trap mass spectrometers (IT-MS) started to become more widely available in residue control laboratories. Balizs[96] developed a multi-residue method for measuring residues of 15 benzimidazoles in swine muscle using LC-QqQ. One product ion was selected for each benzimidazole analyte. In this publication, Balizs highlighted the potential for cross-talk effects in the analysis of fenbendazole, oxfendazole, and febantel, which all produce a common product ion (m/z 159). LOQ ranged between 5 and 30 μg/kg in muscle. Cherlet et al.[86] developed a quantification method for levamisole in porcine tissue using an LC-MS/MS equipped with APCI interface. The LOQs of the method were 5 μg/kg (kidney) and 50 μg/kg (muscle, fat, and skin).

De Ruyck et al.[35] reported an LC-MS/MS method for measuring flubendazole and its metabolites to 1 μg/kg in egg and meat tissues. The method was used to monitor the depletion of flubendazole and metabolites in eggs and muscle following treatment. The same group later developed a multi-residue LC-MS/MS method for seven benzimidazoles (TBZ, oxfendazole, oxibendazole, albendazole, fenbendazole, febantel, triclabendazole, and levamisole) in milk.[97] Chen et al.[105] developed a method for the detection of 21 benzimidazole residues (parent drugs and metabolites) in the muscles and livers of swine, cattle, sheep, and chicken. This method employed LC-MS/MS operating in the ESI positive mode for all analytes, with separation on a C_{18} column by gradient elution using acetonitrile and 5 mmol/l ammonium formate as mobile phase. The precursor ions were chosen through their $[M+H]^+$ ions, and MRM was employed to confirm the presence of residues through two product ion transitions. This method produced LOQs between 0.02 and 0.5 μg/kg and LODs ranging between 0.01 and 0.2 μg/kg for the benzimidazoles, with recoveries ranging from 70% to 93%.

Xia et al. reported a UHPLC-MS/MS method for the determination of 13 benzimidazoles in milk.[100] This method again used a mass spectrometer equipped with an ESI interface for positive mode detection. The LODs and LOQs of the method ranged from 0.01 to 0.5 μg/l and 0.1 to 1.0 μg/l, respectively. UHPLC-MS/MS detection has also been employed with APCI for the determination of residues of 19 benzimidazoles in milk samples within a 7-minute run time.[140] Different ionization probes were evaluated in this work, including ESI, APCI, and APPI. APCI was found to be the best option, mainly for keto-TCB, providing detection at concentrations of 20 times lower than ESI. This paper also

investigated the use of an ion trap analyzer but found that the QqQ offered better sensitivity for the analytes. Polarity switching was also employed to combine the analysis of negative and positive ions. Keto-TCB and TCB showed significantly better ionization in negative mode. LOQs ranged from 0.6 to 3 μg/kg.

Cai et al.[141] established a method for detection of TCB, TCB-SO_2, TCB-SO_2, and keto-TCB at low concentrations in cattle and goat muscle, liver, and kidney. This method enabled LODs of 0.25–2.5 μg/kg in muscle and 1–10 μg/kg in the liver and kidney. Whelan et al.[23] also published work on triclabendazole residues (parent drug and metabolites) based on the use of a modified QuEChERS approach for extraction and clean-up, followed by determination using an LC-MS/MS employing positive mode ESI. The authors used trifluoroacetic acid as a mobile-phase additive to promote the production of positively charged ions. The method was validated according to 2002/657/EC guidelines,[98] with decision limits (CCα) calculated to be in the ranges of 251–287, 255–291, and 10.9–12.1 μg/kg for liver, muscle, and milk, respectively.

5.5.2 Macrocyclic Lactones

The macrocyclic lactones (avermectins and milbemycins) are one of the most important groups of anthelmintic drugs because of their broad-spectrum activity against internal and external parasites. Many of the macrocyclic lactones do not have MRLs set for milk, and the *as low as reasonably achievable* (ALARA) approach is used in many laboratories for their analysis. These drugs can be detected after derivatization using HPLC-FL to 0.1 μg/kg.[11] However, LC-MS/MS is required when combined multi-residue chemical analysis of macrocyclic lactones with other anthelmintic drugs is required.

The LC-MS/MS analysis of macrocyclic lactone residues in food was reviewed in 2012.[18] The macrocyclic lactone drugs are probably the most difficult of the anthelmintic drugs to analyze because of their susceptibility to form sodium clusters, particularly in the presence of acidic mobile-phase additives that are often used in LC-MS/MS. The sodium cluster ions of abamectin, doramectin, ivermectin, and moxidectin have been shown to be very stable, and even when a high collision energy is applied, only a few characteristic fragment ions are produced with low yield.[142] However, the inclusion of ammonium acetate or ammonium formate in the mobile phase promotes the formation of more suitable $[M+H]^+$ or $[M+NH_4]^+$ ions.[18] Turnipseed et al.[104] evaluated the suitability of APCI, APPI, and ESI ionization probes for analyzing macrocyclic lactones using ion trap MS detection. It was found that negative ion APPI and positive ion APCI produced best ionization techniques. However, APCI allowed for the detection of the macrocyclic lactones at lower concentrations in milk, that is, 5 μg/kg. Generally, the most commonly applied ionization mode used for the analysis of macrocyclic lactones in LC-MS/MS methods is ESI^+ mode.[18]

Kaufmann et al.[143] compared a single-stage Orbitrap™ high-resolution mass spectrometer (HRMS) with QqQ mass spectrometry (MS/MS) for macrocyclic

lactone analysis. The study showed that the precision of the two instruments was similar, but significantly higher sensitivity was achieved for avermectins with the Orbitrap™-based detection. It was concluded that analytes with poor fragmentation properties (e.g., sodium-cationized molecules) can be more easily quantified by single-stage HRMS than by QqQ instruments. Rúbies et al.[111] reported the application of a Q-Orbitrap™ hybrid instrument for the analysis of macrocyclic lactone residues in meat following QuEChERS sample preparation. The group utilized target SIM data-dependent MS/MS mode for the determination of ivermectin, emamectin, eprinomectin, doramectin, and moxidectin. These HRMS instruments, such as Orbitrap™ and time-of-flight, represent a significant advance from the previously preferred QqQ instruments using ESI and APCI. The use of such a hybrid Q-Orbitrap™ system provided sufficient sensitivity to enable confirmation of analytes at 0.5 μg/kg, thus providing a potential new area of research for veterinary drug analysis.[111]

5.5.3 Flukicides

The flukicides include a range of drugs such as albendazole, triclabendazole, clorsulon, closantel, niclosamide, nitroxynil, oxyclozanide, and rafoxanide. Few LC-MS/MS papers were published for the analysis of flukicide residues in food matrices until the late 2000s because most work until then focused on the benzimidazoles, levamisole, and the macrocyclic lactones. Takeba et al.[144] published a method in 1996 for measuring five flukicides in milk using HPLC coupled with a dual-electrode coulometric detector. The LOD of the method ranged between 4 and 20 μg/l. Blanchflower and Kennedy[145] reported one of the earliest LC-MS methods for the measurement of a flukicide, namely, nitroxynil, in different animal tissues. Caldow et al.[101] reported the first multi-residue LC-MS/MS method for analyzing phenolic and salicylanilide flukicides in bovine kidney. The analytes included nitroxynil, oxyclozanide, rafoxanide, closantel, ioxynil, niclosamide, salicylanilide, and TFM. The flukicides were monitored in ESI negative mode using two product ions formed by fragmentation of the $[M-H]^-$. The CCα and CCβ (detection capability) measurements for licensed anthelmintics in bovine kidney were determined at 0.5, 1.0, and 1.5 times the MRL, while those residues with no MRL were determined at 50, 100, and 150 μg/kg. Sakamoto et al.[103] later developed a multi-residue method for the determination of residues of five flukicides – bithionol, bromophen, nitroxynil, oxyclozanide, and tribromsalan – in milk using LC-MS/MS. All analytes were detected in negative ESI mode and LODs were 0.1 μg/kg.

5.5.4 Other Anthelmintic Drugs

Kinsella et al.[60] worked on the determination of a new anthelmintic in 2011, namely, monepantel, and its sulfone metabolite in milk and muscle tissues. LC-MS/MS was the detection method of choice with ESI in negative mode

employed for residue ion detection of two daughter transitions. This was the first paper published on the detection of these new anthelmintic drugs in goat's milk and goat muscle and was successfully single-laboratory validated using the 2002/657/EC criteria.[98]

5.5.5 Multi-residue Methods That Combine Different Anthelmintic or Drug Groups

Kinsella et al.[108] reported a comprehensive LC-MS/MS method for analyzing residues of 37 anthelmintic drugs in milk and liver. The procedure combined the analysis of benzimidazoles, macrocyclic lactones, flukicides, coumaphos, coumaphos-oxon, dichlorvos, haloxon, levamisole, and morantel in one assay. Two injections were required to achieve sensitive detection of 8 negatively and 29 positively ionized analytes. The LOD of the method was 5 μg/kg for all analytes except dichlorvos, for which the LOD was 10 μg/kg. Whelan et al.[109] subsequently improved the Kinsella et al.[108] method through the application of a more modern UHPLC-MS/MS system, which had a detector with rapid polarity switching that allowed both positive and negative charged ions to be detected from a single injection (Figure 5.12). The final validated method was one of the most sensitive methods for anthelmintic detection in milk with CCα of 0.14 – 1.9 μg/kg for unlicensed substances. Kinsella et al.[110] subsequently adapted this method to the analysis of anthelmintic residues in bovine liver.

5.5.6 Ionophore Anticoccidial Agents

The ionophores produce intense positive ions in ESI, but MS spectra are complex because of the ability to form different adducts including $[M+H]^+$, $M+NH_4]^+$, and $[M+Na]^+$.[13] Ionophores can be easily fragmented to produce suitable ions for confirmatory purposes. However, product ions need to be carefully selected in order to avoid non-specific losses such as 18 Da, which is a common product ion with these compounds. Research by the authors has found that low non-target concentration limits set by the EU can be a challenge to reach in multi-residue methods using older less sensitive instruments.[146] Maduramicin and semduramicin are two ionophores that are particularly challenging to detect in multi-residue methods.

Many of the early methods for anticoccidial analysis were developed for detecting monensin, salinomycin, narasin, and lasalocid. Blanchflower and Kennedy[112, 113] developed early methods for analysis of ionophore residues in eggs and poultry tissues at concentrations ≤1 μg/kg using LC-MS. These authors found that complex mobile phases in some cases were required for suitable chromatography and sensitivity. Volmer and Lock[147] reported a rapid LC-MS/MS method for analysis of salinomycin, monensin, lasalocid, and narasin in pet food samples. Analytes were separated in <4 minutes using gradient elution and detected in positive ESI. Mobile-phase additives, solvent composition, and pH

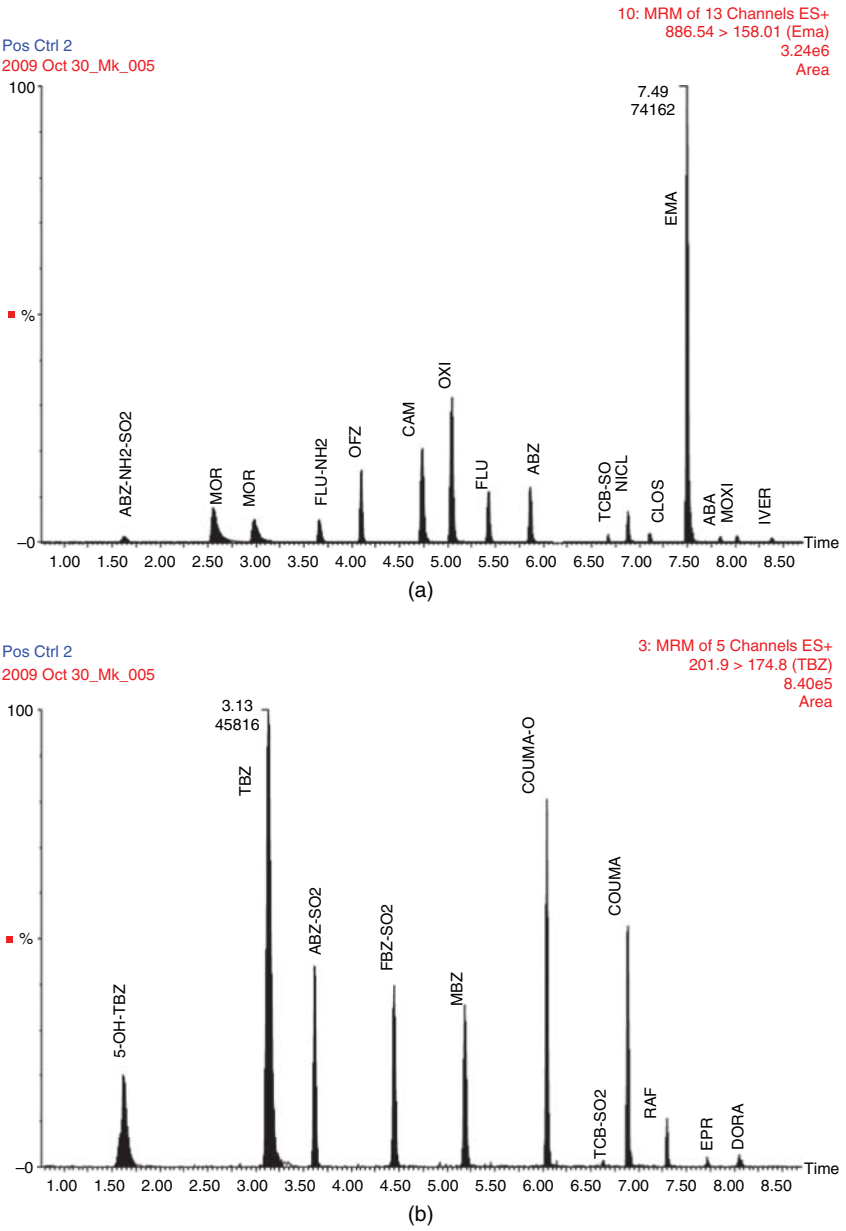


Figure 5.12 Overlay of analytes (a–c) at concentration of 2 μg/kg (OXY, CLOR, BITH, and MOR were 4 μg/kg) and selected internal standards (d–e). Source: Whelan 2010.[109] Reproduced with permission from Elsevier.

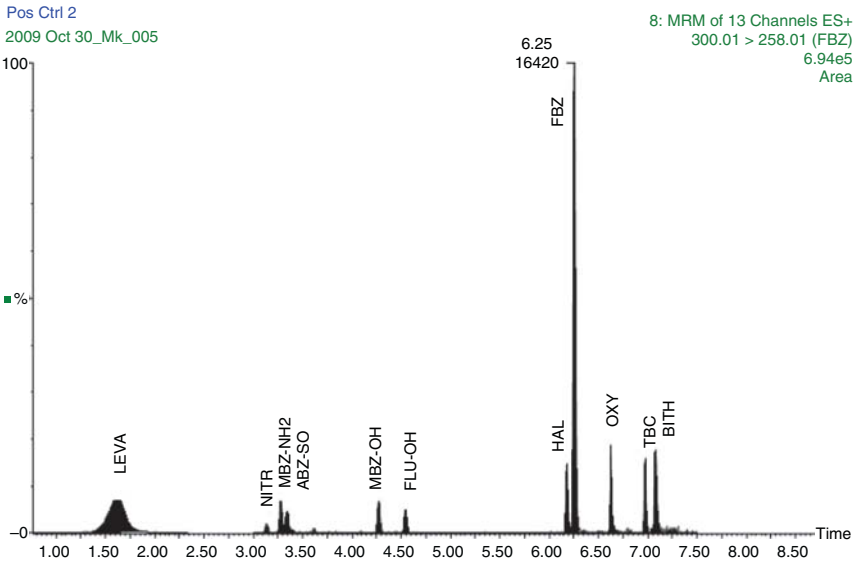


Figure 5.12 (*Continued*)

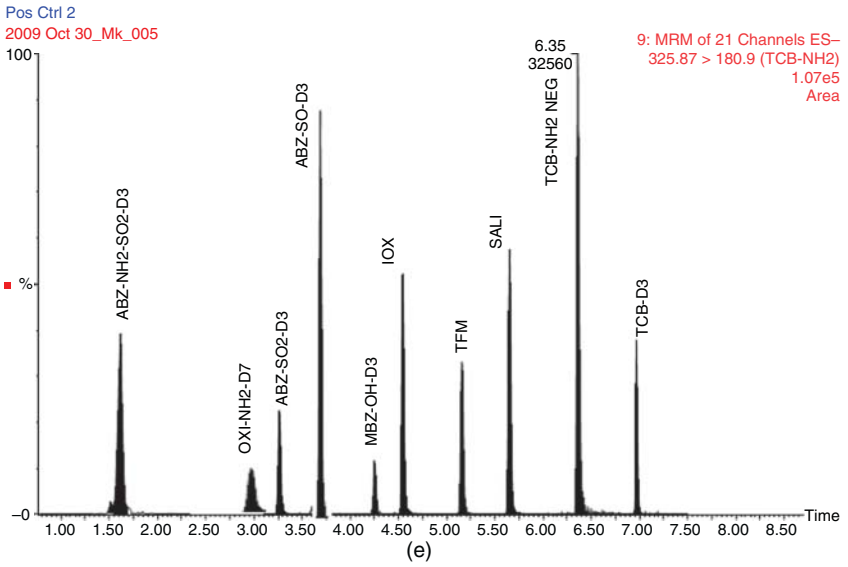


Figure 5.12 (*Continued*)

were carefully optimized for separation and MS detection. Ionophores were analyzed as their sodium adducts, which were found to be present in the solvents. Since 2000, many more methods have been reported in literature for the analysis of ionophores in various animal tissues and eggs.[69, 118, 121, 132, 133, 148–151]

5.5.7 Chemical Anticoccidials

The chemical anticoccidials cover a diverse range of synthetic chemicals with differing physicochemical properties that are challenging to analyze by LC-MS/MS. Some of these substances, such as amprolium and cyromazine, are highly polar and require more specialized chromatographic separations, including ion pair chromatography[152, 153] or hydrophobic interaction liquid chromatography (HILIC).[154, 155] Additionally, mobile-phase additives need to be carefully considered. These compounds ionize to form $[M+H]^+$ or $[M-H]^-$ species, which can be fragmented in lower-energy CID experiments to produce satisfactory ions for chemical confirmation. However, there are some exceptions including TOL, toltrazuril sulfoxide (TOL-SO), and toltrazuril sulfone (TOL-SO_2), which are difficult to fragment following ESI. Some groups have addressed this problem through the application of APCI to produce different precursor ions that can be fragmented more easily.[116, 156] Additionally, the products ions for halofuginone need to be carefully selected because this molecule produces an intense fragment ion with loss of H_2O, which is not selective and cannot be seen in complex matrices.[127]

Since the late 1990s, several LC-MS methods have been published for the determination of chemical anticoccidials. Most early methods were more specific methods for single molecules or structurally similar compounds. Nicarbazin is probably the most widely studied synthetic anticoccidial agent, and many groups have developed methods for the determination of this substances in animal tissue and eggs. Blanchflower et al.[157] developed an early LC/APCI-MS method for measuring the two active components of nicarbazin, namely, DNC and 4,6-dimethyl-2-hydroxypyrimidine, in eggs. The two respective analytes were detected as negative and positive ions from the same injection using polarity switching and could be quantified to 10 µg/kg. Yakkundi et al.[114] later developed an isotopic dilution assay using DNC-d_8 for measuring DNC in eggs and poultry liver by LC-MS/MS in ESI negative mode. DNC was monitored in negative mode using two transitions m/z 301 → 137 and m/z 301 → 107. As previously discussed, an LC-MS/MS method for the determination and confirmation of nicarbazin residues, expressed as DNC, in chicken liver, kidney, muscle, skin with adhering fat, and eggs has more recently been developed and proposed for AOAC Official Method status.[134] The method also uses DNC-d_8 as internal standard and monitors the two transitions m/z 301.0 → 136.7 and m/z 301.0 → 106.9 in negative ion mode.

In subsequent years, method development has focused on newer anticoccidial drugs or substances that are not licensed in the EU. Hormazabal et al.[158] developed an early LC-MS method that combined the analysis of TOL, TOL-SO_2, and flunixin in one test. Analytes were separated on a monolithic C_{18} column in an 8-minute run time. The LOQ was 2 µg/kg for TOL-SO_2 and 5 µg/kg for all other analytes. Mulder et al.[159] subsequently used LC-MS/MS to investigate the depletion of TOL and its metabolite TOL-SO_2 residues in eggs following treatment. TOL-SO_2 was found to be the major and most persistent metabolite in eggs. Ai et al.[160] developed a method for the analysis of diclazuril, TOL, TOL-SO, and TOL-SO_2 in egg and meat. These authors found that fragmentation of TOL marker residues was not ideal using a QqQ instrument operating in ESI. TOL, TOL-SO, and TOL-SO_2 were monitored using the transitions m/z 424 → 424, m/z 440 → 371, and m/z 456 → 456, respectively. These transitions do not strictly satisfy confirmatory analytical criteria outlined by the EU for group B substances.[98] Martínez-Villalba et al.[116] used LC/APCI-MS/MS method for analyzing TOL, TOL-SO, and TOL-SO_2 in poultry and porcine meat. This group found that these compounds underwent in-source fragmentation through an unusual electron capture dissociation mechanism. Novel precursor ions were observed for TOL and TOL-SO, [the $M-CF_3]^-$ ions, while for TOL-SO_2, the $[M-CHF_3]^-$ ion was observed. An LC-MS/MS method was subsequently established using highly selective selected reaction monitoring (H-SRM), which increased sensitivity and selectivity. The LOD of the method was 0.5 µg/kg (TOL and TOL-SO_2) and 5.0 µg/kg (TOL-SO). Mortier et al.[161] reported an LC-MS/MS method for diclazuril in poultry tissues and feed using electrospray negative ionization. Analyte was separated on a C_{18} column using a binary gradient

comprising water and acetonitrile prior to MS/MS detection. The decision limit (CCα) of the method was 0.5 μg/kg for poultry meat. Diclazuril was monitored as the $[M-H]^-$ ion, but only one major product ion was produced. Consequently, the chlorine isotopes were selected to satisfy EU identification point criteria. The method was tested on incurred breast muscle, thigh muscle, and liver samples from chicken administered with diclazuril in a residue depletion experiment.

A number of different groups have reported methods for analysis of halofuginone residues in eggs, meat and plasma using LC-MS/MS.[115, 162, 163] Yakkundi et al.[115] found that halofuginone could be monitored as the $[M+H]^+$ ion at m/z 416 and four product ions were produced at m/z values of 398, 138, 120, and 100 and proposed a tentative fragmentation pathway for halofuginone. The authors used a mobile phase comprising methanol/water containing 0.5% acetic acid for analyte separation and positive ion electrospray MS/MS for determination. The method was demonstrated through fortification studies to be able to measure halofuginone residues to at least 5 and 15 μg/kg in chicken eggs and liver, respectively. Mortier et al.[162] developed an LC-MS/MS method for detection of halofuginone residues in eggs using a binary gradient mobile phase consisting of water and acetonitrile, both containing 0.1% formic acid, for separation on a C_{18} column. This group also used positive ESI and monitored the $[M+H]^+$ ion at m/z 416 but utilized only two product ions, m/z 120 and m/z 100, in their method. It was found that concentrations as low as 2 μg/kg could be detected using a very selective clean-up that included a deproteination step using acetonitrile and clean-up on an immunoaffinity column.

A few single-residue methods have been reported in the literature for the detection of residues of clopidol in food of animal origin. Pang et al.[164] analyzed clopidol residues on a C_{18} column using a mobile phase of acetonitrile/water (20:80, v/v) without any mobile-phase additives and detection by single quadrupole MS. Clopidol was monitored using the ion at m/z 190. The LOQ of the method was 10 μg/kg in chicken muscle, liver, and kidney. A version of this method using the same clean-up and chromatographic separation, but with UV detection at 270 nm, was also developed by this group and successfully demonstrated for the analysis of chicken muscle in a collaborative study which included 18 laboratories, with acceptance as an AOAC Official Method (First Action) in April 2003.[165]

Wang et al. analyzed cyromazine and melamine residues in eggs using LC-MS/MS.[129] Detection used the positive ESI mode with selected reaction monitoring (SRM) mode of $[M+H]^+$ at m/z 167 and two product ions, which were generated with m/z values of 85 and 68. The LOD of the method was 1.6 and 5.5 μg/kg, respectively.

Amprolium, arprinocid, buquinolate, decoquinate, diaveridine, ethopabate, imidocarb, nequinate, and robenidine are chemical anticoccidials that are sometimes included in multi-residue methods. Few single-residue methods have been reported for these drugs, but analysis of these substances is generally straightforward by LC-MS/MS. A range of other anticoccidial agents are no longer widely used in animal production because more useful drugs have been

produced or the compounds were only ever used to treat particular protozoan infections in the tropics. Examples of such drugs include isometamidium, nafamostat, nitromide, roxarsone, and zoalene.

Li et al.[166] found that isometamidium residues could be measured to 5 μg/kg in bovine fat, liver, kidney, and milk using LC-MS/MS. Isometamidium was monitored as the $[M+H]^+$ at *m*/*z* 460, which produced two fragment ions at *m*/*z* values of 298 and *m*/*z* 313. Few methods have been published in the literature for nafamostat, which is a high polar drug that poses chromatographic challenges. This drug is also hydrolytically unstable because of its ester moiety, which requires hydrolyzing agents to be avoided during sample preparation. Cao et al.[167] reported a method for the quantification of nafamostat mesilate and its major metabolite 6-amidino-2-naphthol in human plasma using LC-MS/MS. Nafamostat was analyzed on a C_{18} column using a mobile phase consisted of methanol/water/formic acid (15:85:0.001, v/v/v) with ESI. MS tuning experiments showed that two protonated species were produced for nafamostat, at $[M+H]^+$ and $[(M+2H)]^{2+}$ with *m*/*z* values of 348 and 174.55, respectively. The $[(M+2H)]^{2+}$ ion was the most abundant ion in the acidic mobile-phase conditions and was selected for monitoring. The MS spectra of the 6-amidino-2-naphthol metabolite was more straightforward to interpret because this substance formed the $[M+H]^+$ species at *m*/*z* 186.9.

Zoalene is not frequently included in LC-MS/MS methods because it is poorly ionized in API, and many methods used in EU laboratories do not include this substance because it is no longer licensed or used in the EU. Wu et al.[168] found that zoalene (dinitolmide) and its 3-amino-5-nitro-*o*-toluamide (3-ANOT) metabolite could be separated on a C_{18} UHPLC column using a binary gradient comprising of 0.01% formic acid in water and acetonitrile with a 5-minute run time. Ionization of these compounds in ESI was non-ideal, and polarity switching was required for zoalene and 3-ANOT, which were monitored as the $[M-H]^-$ and $[M+H]^+$ ions, respectively. Two product ions were selected for each analyte following CID experiments. It was found that 3-ANOT required formic acid in the mobile phase to be efficiently ionized. In contrast, zoalene ionization was more intense using 20 mM ammonium acetate, but mobile-phase conditions had to be compromised to accommodate 3-ANOT in the analysis. The LOQ of the method was 25 and 50 μg/kg for zoalene and 3-ANOT in chicken tissues, respectively.

5.5.8 Applications of GC-MS

GC-MS is not widely used for the analysis of veterinary drug residues but can be advantageous if a molecule cannot be detected at the required concentrations by LC-MS/MS or if fragmentation of the parent drug does not satisfy confirmatory analysis criteria. In such cases the introduction of a derivatization step and GC-MS is attractive. He et al.[169] developed a GC-MS method for the determination of clopidol in chicken muscle. Following extraction and clean-up, clopidol was reacted with Sylon BFT™ to produce a trimethylsilyl derivative, which was

detected in SIM mode at m/z values of 191, 248, 263, and 265. The LOQ of the method was 1 μg/kg. Fang et al.[170] also used GC-MS to analyze clopidol in chicken muscle following derivatization with Sylon BFT™. The authors found that negative chemical ionization (NCI) was more selective and sensitive than electron impact (EI) for clopidol analysis. The SIM mode was performed at m/z values of 156, 158, 191, and 193.The LOD and LOQ were 0.1 and 0.5 μg/kg, respectively.

Cyromazine is another anticoccidial agent that can be analyzed by LC-MS or GC-MS. It is frequently analyzed with the contaminant melamine. Zhu et al.[171] derivatized cyromazine and melamine with *N*,*O*-bis(trimethylsilyl) trifluoroacetamide prior to GC-MS analysis. The LOQ of the method ranged between 5 and 10 μg/kg.

5.5.9 Multi-residue Anticoccidial Methods

In recent years, many groups have implemented multi-residue LC-MS/MS methods for the analysis of anticoccidial residues. This strategic shift has been facilitated by the widespread implementation of LC-MS/MS instruments in residue surveillance laboratories. In addition, the establishment of EU maximum limits for 11 anticoccidials in non-target species and matrices, which are difficult to efficiently achieve without LC-MS/MS, has been a contributing factor. Some early multi-residue methods were limited in scope to about five anticoccidials.[119, 121, 125, 172] However, the basic sample preparation approaches adopted in these methods have been adapted into other anticoccidial methods.

Dubois et al.[120] established an early method using LC-MS/MS for residues of nine anticoccidials, which included lasalocid, monensin, narasin, salinomycin, maduramicin, diclazuril, halofuginone, DNC (nicarbazin marker residue), and robenidine in muscle and egg. Two product ions were identified for each analyte and the method was validated to 2002/657/EC guidelines.[98] CCα and CCβ were found to range from 0.07 to 0.6 μg/kg and 0.2 to 1.0 μg/kg, respectively. Dubreil-Chéneau et al.[126] validated a LC-MS/MS method for 10 anticoccidial residues (diclazuril, halofuginone, lasalocid, maduramicin, monensin, narasin, DNC, salinomycin, and semduramicin) in eggs. The method included the addition of diclazuril-bis, DNC-d_8, and nigericin as internal standards and was validated in conformance with the criteria in 2002/657/EC.[98] The CCα values for this method ranged from 0.27 to 0.98 μg/kg. Anticoccidials were monitored in MRM mode as $[M+H]^+$ (halofuginone and robenidine), $[M-H]^-$ (diclazuril), and $[M+Na]^+$ (ionophores). Both of these methods used a mobile phase containing formic acid.

Olejnik et al.[117] developed an LC-MS/MS method for 12 coccidiostats in chicken liver. This application is unique because most groups select muscle tissue for the analysis of anticoccidial so as to avoid ion suppression effects encountered in the liver. The method included all six ionophores (lasalocid, monensin, narasin, salinomycin, maduramicin, and semduramicin) and five chemical anticoccidials (decoquinate, diclazuril, halofuginone, robenidine, and DNC) listed in the

European Commission Regulation 124/2009/EC.[173] The method also included the compound clazuril. The method required each sample to be injected twice to allow the analysis of negative and positively ionized analytes, which were separated using gradient elution with acetonitrile (mobile phase A), methanol (mobile phase B), and 0.01 M ammonium formate buffer, pH 4.0 (mobile phase C). The method LODs and LOQs were 1 and 2 μg/kg, with the exception of lasalocid.

Shao et al.[124] developed a rapid multi-residue LC-MS/MS method for the determination of 14 anticoccidials in eggs and poultry. Most analytes were monitored using an ESI source in positive mode, with the exception of DNC and diclazuril, which were evaluated in negative mode. The method LOQ ranged from 0.1 to 0.2 μg/kg. The authors concluded due to the large interferences observed in sample extracts that this procedure was only suitable for screening purposes. Galarini et al.[122] developed a method for the determination of 11 anticoccidials in eggs using LC-MS/MS. The analytes were identified and quantified using an LC-MS/MS system operating in positive and negative ESI. The method was validated for the confirmation of regulated anticoccidials in eggs at the legislated limits in the EU with CCα ranging from 2.2 to 174 μg/kg, depending on the analyte.

A method reported by Moloney et al.[127] using UHPLC-MS/MS method was validated[98] for the determination of residues of 20 anticoccidials to 1 μg/kg in egg and avian muscle. Analytes were acquired using ESI, and a polarity switching approach was adopted to combine the analysis of negatively and positively ionized analytes in one injection. The analytes were separated on a C_8 BEH UHPLC column (50 mm × 2.1 mm) using a binary gradient comprising of mobile phase 0.1% formic and 0.1% formic acid in acetonitrile (Figure 5.13). LOQs of 1 μg/kg were achievable for all analytes except imidocarb and DNC which were 10 μg/kg and for TOL and its sulfoxide and sulfone metabolites for which the LOQ was 50 μg/kg. Clarke et al.[8] subsequently adapted the method to analyze residues of anticoccidials in milk, duck muscle, and non-avian muscle. CCα values for muscle tissue ranged from 2.2 μg/kg for clopidol to 122 μg/kg for TOL-SO. CCα values obtained from the validation of the method for milk ranged from 1.1 μg/kg arprinocid, nequinate, and lasalocid to 27 μg/kg for TOL. The authors found that the quantification of halofuginone residues in equine and ovine tissues was non-ideal and needed to be improved. In addition, the method did not provide confirmation for TOL and its metabolites as only the precursor ion was monitored.

Yue et al.[123] also reported a method using LC-MS/MS for the determination of 20 anticoccidial residues in chicken muscle. The method was developed for quantitative purposes and included five ionophores (lasalocid, maduramicin, monensin, narasin, and salinomycin) and 15 chemical coccidiostats (amprolium, clopidol, DNC, ethopabate, halofuginone, clazuril, nequinate, decoquinate, zoalene, diclazuril, nitromide, TOL and metabolites, and aklomide). Anticoccidial residues were separated on a C_{18} BEH UHPLC column using a binary gradient comprising of 0.005 mM ammonium acetate and 0.05% formic acid in water and

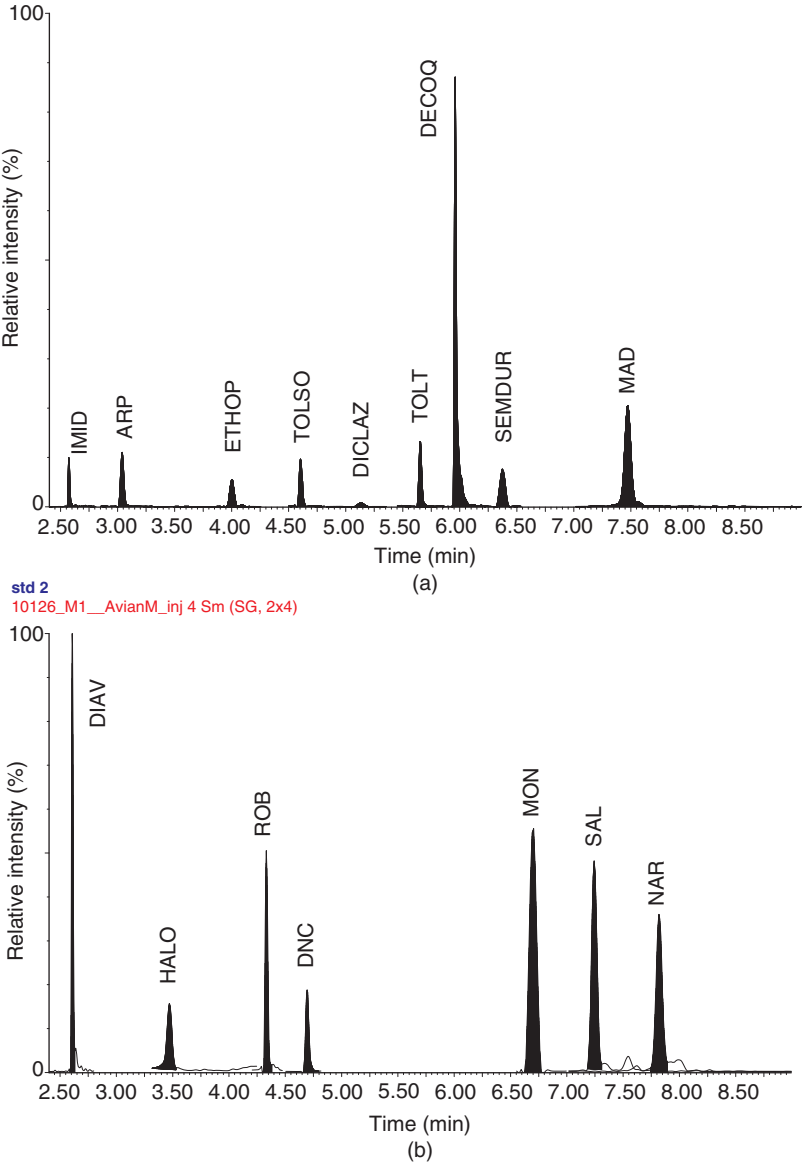


Figure 5.13 Overlay of LC-MS/MS chromatograms for all 20 coccidiostat analytes (a–c) at concentrations of 2.5 µg/kg (HALO at 10 µg/kg; DNC and IMID at 25 µg/kg; TOL, TOL-SO, and TOL-SO_2 at 50 µg/kg) and internal standards (d), in a fortified avian muscle sample. (Abbreviations: IMID, imidocarb; ARP, arprinocid; ETHOP, ethopabate; TOL-SO, toltrazuril sulfoxide; DICLAZ, diclazuril; TOL, toltrazuril; DECOQ, decoquinate; SEMDUR, semduramicin; MAD, maduramicin; DIAV, diaveridine; HALO, halofuginone; ROB, robenidine; DNC, 4,4′-dinitrocarbanilide; MON, monensin; SAL, salinomycin; NAR, narasin; CLOP, clopidol; TOL-SO_2, toltrazuril sulfone; LAS, lasalocid; LAID, laidlomycin). Source: Moloney 2012.[127] Reproduced with permission from Elsevier.

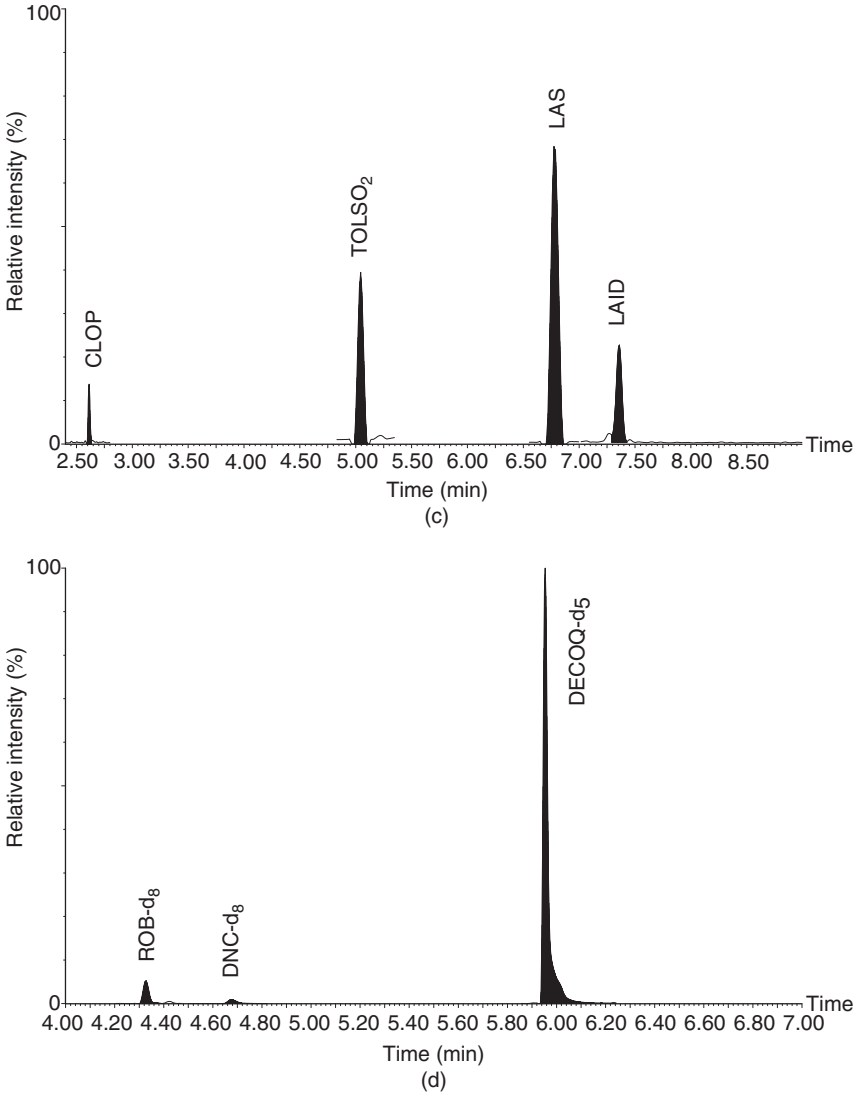


Figure 5.13 (*Continued*)

methanol in a 15-minute injection cycle. The LOQ of the method ranged between 5 and 50 µg/kg for the various analytes. Nász et al.[174] reported an LC-MS/MS which used both positive and negative ionization for the determination of residues of 10 anticoccidials (decoquinate, diclazuril, halofuginone, lasalocid, maduramicin, monensin, narasin, DNC, robenidine, salinomycin, and semduramicin) in milk and processed dairy products. The LOQ of the method ranged from 0.01 (diclazuril and maduramicin) to 1 µg/kg for (DNC, halofuginone, and robenidine).

5.5.10 Multi-class Methods

Sample preparation for multi-class, multi-residue methods is discussed in more detail in Chapter 2, while additional information on the use of various types of contemporary mass spectrometers can be found in Chapter 3. The focus in this section is on the methods which include some anticoccidial and anthelmintic compounds.

In recent years, many research groups have developed the so-called multi-class, multi-residue LC-MS methods that allow the analysis of different drug groups together in one assay. Many of these procedures now include >100 residues, but earlier multi-class methods were generally limited to substances that ionized in positive mode. A number of early multi-class methods were developed that combined anticoccidial with antibiotics analysis in eggs[149] and shrimp.[156] Jestoi et al.[175] developed a method that allowed the analysis of the ionophores lasalocid, maduramicin, monensin, salinomycin, and narasin and emerging *Fusarium* mycotoxins of concern, beauvericin and enniatins, in poultry meat and liver.

More extensive methods were developed by other groups for the detection of veterinary drug residues. Stolker et al.[176] and Peters et al.[136] both published work on a multi-class screening method for residues of approximately 100 veterinary drugs in food matrices (milk;[176] egg, fish, and meat[136]) using liquid chromatography with time-of-flight mass spectrometry (LC-TOF-MS). The method includes benzimidazoles, pyrimidines, and anticoccidials, as well as antibiotics, sulfonamides, tranquilizers, and non-steroidal anti-inflammatories. LC-TOF-MS analysis was achieved using ESI in positive mode for all analytes. The $[M+H]^+$ ions were monitored and identification of residues was based on retention times and accurate mass relative to the internal standards. Kaufmann et al.[177] similarly reported a multi-class UHPLC-TOF-MS method for the determination of ~100 veterinary drug residues in meat, which included 18 anti-parasitic agents. Deng et al.[178] more recently reported a method using UHPLC-QTOF-MS for the measurement of residues of 105 veterinary drugs, including benzimidazoles, in meat, milk, and eggs. Ortelli et al.[179] later reported a more extensive TOF-MS screening method that was capable of detecting residues of 150 veterinary drugs and their metabolites in milk. The method analyzed for a range of anti-parasitic drug residues including 25 anthelmintic drugs, 8 nitroimidazoles, and 2 anticoccidials. The majority of analytes were detected as their $[M+H]^+$ ions except for the some avermectins, which were monitored as their $[M+Na]^+$ ions. LODs for the majority of residues were between 0.5 and 25 μg/l and below the MRL for the majority of analytes. It is highlighted that the sensitivity of the method for the avermectins was ≥10 μg/l, which is less sensitive than many published methods. The method validation was conducted using an "in-house" procedure based on the criteria contained in 2002/657/EC.[98]

The improvement in scanning speed and sensitivity of lower-resolution tandem mass spectrometers (QqQ and QTrap™) instruments in recent years has allowed

many groups to develop comprehensive multi-class methods that provide detection at lower concentrations than HRMS applications. Geis-Asteggiante et al.[16] developed a reliable multi-class UHPLC-MS/MS method for residues of 113 veterinary drugs in bovine muscle. The method, which included 40 anti-parasitic drugs belonging to the anthelmintic and nitroimidazole groups, exploited rapid polarity switching capability to allow the inclusion of eight analytes that showed better sensitivity in negative ionization mode. The ruggedness of the procedure was tested through various UHPLC-MS/MS parameters, and it was found that the method could be used to screen for 113 analytes, with the ability to quantify 87 analytes, in a single analysis. Zhan et al.[14] developed a method for the detection of 255 veterinary drug residues in milk using UHPLC-MS/MS. The instrument used was more sensitive and had a faster scanning capability compared to the instrument used by Geis-Asteggiante et al.[16] and included approximately 50 anti-parasitic agents from different drug classes. Samples were extracted using a single sample preparation procedure but required three different UHPLC-MS/MS runs to allow sensitive detection of residues. Two run acquisitions were made in positive ionization mode for polar and more non-polar drugs, while negatively ionized analytes were injected separately. The LODs of the method ranged from 0.05 to 10 μg/kg.

Dasenaki and Thomaidis[180] recently published a method for the determination of residues of 115 veterinary drugs and pharmaceuticals, from more than 20 classes, in butter, milk powder, fish, and egg. Included within this work were 10 benzimidazoles and 4 other anthelmintics. LC-MS/MS was employed along with ESI in both positive and negative modes to accommodate the number of analytes involved within the analysis. Unlike some other methods discussed previously, this method did not employ rapid polarity switching. Two separate runs were used for positive and negative ionization in MRM to reduce the workloads on the spectrometer and data system. The presence of residues was confirmed through the monitoring of two daughter transitions for each analyte, to provide quantitative abilities, and produced LODs and LOQs for this method ranged from 0.008 μg/kg, in butter, to 3.15 μg/kg, in egg. The procedure was validated based on EU guidelines[98] and successfully applied in proficiency test samples.

Some groups have investigated the use of LC coupled with quadrupole linear ion trap (QTrap™) MS for veterinary drug residue analysis in food matrices. This technology offers similar quantitative capability to QqQ instruments but provides additional full product ion scan capability, which can help to reduce the likelihood of false-negative and false-positive results. Biselli et al.[181] developed a multi-residue method using an LC-QTrap™-MS for the determination of 84 veterinary drug residues in chicken muscle. The method included 19 benzimidazoles, levamisole, and 8 nitroimidazole drugs in the analysis. The LOQs of the method ranged between 2 and 48 μg/kg. Schneider et al.[17] developed a method for the detection of residues of 131 veterinary drugs in bovine muscle tissue, also using a state-of-the-art LC-QTrap™-MS system. This group was able to greatly reduce sample preparation time by eliminating the need for sample concentration

and through the utilization of a filter-vial dSPE step (see Section 5.4.3). Nakajima et al.[130] developed a multi-class LC-MS/MS for analyzing residues of 43 veterinary drugs, including sulfonamides, quinolones, coccidiostats, and anti-parasitics in eggs and muscle tissue. The LC separation was achieved using a binary gradient comprising mobile phase A: formic acid (0.1%) ammonium acetate (10 mm) in water and mobile phase B: acetonitrile. Using this approach, macrocyclic lactone residues were eluted in 95% acetonitrile and detection sensitivity was satisfactory. It is of interest that the authors used HPLC rather than UHPLC conditions with a flow rate of 0.3 ml/minute and a run time of 18 minutes, which facilitated the use of polarity switching in the method.

It can be seen that the inclusion of a wide range of anthelmintic and anticoccidial residues in multi-class veterinary drug residue methods is difficult due to a number of factors. Firstly, many laboratories prefer to inject positively and negatively ionized analytes separately because of the polarity switching capabilities and scanning speeds of the instrumentation available in their laboratory. Polarity switching for many LC-MS/MS instruments is typically ≥20 milliseconds, which greatly reduces the number of data points and thus transitions that can be acquired at a given time. In recent years, some instrumentation vendors have developed instruments with ultra-fast switching capabilities that can switch between positive and negative polarity in 5 milliseconds. It is expected that other vendors will follow this trend in the future with upgraded instruments. The application of ultra-fast switching technology should facilitate the inclusion of negative ionized analytes in more multi-class methods.

Secondly, multi-class LC-MS/MS mobile-phase conditions generally are the result of a compromise to get the best sensitivity for the overall group of analytes. It is generally found that compounds which provide a poorer response under these conditions, particularly the macrocyclic lactones and some anticoccidial agents, are excluded from these methods. The macrocyclic lactones require the mobile phases containing ammonium acetate or formate to produce the $[M+H]^+$ or $[M+NH_4]^+$ ions to suppress the formation of $[M+Na]^+$, which allow the fragmentation of product ions for confirmatory purpose.

5.6 Conclusions

In recent years we have seen an expansion of analytical methods for the monitoring of anti-parasitic agents in food. This is due mainly to the development of rapid sample preparation techniques, including robust SPE and QuEChERS procedures. Variations of these sample preparation protocols have been introduced, and many laboratories now use such methods routinely for veterinary drug residue analysis. Since the mid-2000s, chromatographic separation technology has also been significant improved, which led to improvements in chromatographic separations, shorter injection cycles, better retention time reproducibility, and enhanced detection at low concentrations. During this

time HILIC separation technology has become available to allow more polar anti-parasitic agents to be analyzed by LC-MS/MS without the need for using ion pair reagents such as heptafluorobutyric acid. Consequently, difficult polar drugs such as the amprolium and cyromazine can now be analyzed routinely in laboratories with short changeover times between methods.

The sensitivity of MS instrumentation has improved over 100-fold in the past 5 years. This may lead to the application of more rapid sample preparation procedures in routine laboratories. This in turn should support the inclusion of more anti-parasitic agents in multi-class analytical methods. Obviously, many early multi-class methods did not include residues of many key anti-parasitic drugs, including the macrocyclic lactones and ionophores. In addition, negatively ionized analytes were often seen as a challenge and excluded from methods for convenience. In the future, it is likely that it will become possible to monitor for anti-parasitic agents in foods using two multi-residue methods based on different separation techniques. It is suggested that HILIC separation technology will be used for the analysis of polar drugs, while other drug residues can be monitored using reversed-phase separations.

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6

Sedatives and Tranquilizers

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6.1 Introduction

Over the past decades, intensive farming has significantly increased the meat production capacity in many countries. However, these improvements have hads many drawbacks, such as increased vulnerability to diseases and stress associated with more intensive farming practices and transport of animals. In veterinary practice, sedatives and tranquilizers are administered for a variety of reasons, of which the main are sedation prior to handling, examination, or treatment of the animal or to sedate an animal prior to transportation. The creation of fewer but larger slaughterhouses in recent years has prolonged transport from the farm to the abattoir and thus created a situation where the use of sedatives and tranquilizers to reduce the problems caused by the stress associated with transport and holding prior to slaughter may be considered necessary.[1–3] Recent literature also reports on their illicit use as feed additives to slow down the metabolic processes and to reduce animal activity aimed to enhance the growth rate and to improve milk yield.[4–8]

Sedatives and tranquilizers are used to control stress in food-producing animals and facilitate their adaptation to a stressful environment.[9] Use of these substances, which has become generalized since the 1970s, may result in residues in the injection sites and edible tissues of food-producing animals, which may represent a potential risk to the consumers. For example, the 38th Meeting of the Joint FAO/WHO Expert Committee on Food Additives (JECFA), in its review of propionylpromazine, noted that "the Committee was aware that propionylpromazine

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is used in circumstances in which the consumer will be exposed to residues of the drug that may be capable of exerting a pharmacological effect".[10] For this reason many countries have carefully regulated their use in veterinary medicine. The use of certain substances (derived from phenothiazine) is totally prohibited, while other compounds (butyrophenones and β-blockers) are regulated through the establishment of acceptable daily intakes (ADIs), supported by maximum residue limits (MRLs).

6.2 Classification and Representative Compounds

Sedatives and tranquilizers belong to the group of substances which are weaker inhibitors of the central nervous system. Regarding their mode of action, these substances are divided into two groups. One group works primarily through the somatic part of the central nervous system and is therefore closer to hypnotics. The second group is more effective with respect to sedation, and the related effects appear to be more on the vegetative functions of the brain. The first group is called hypnotic sedatives, while the other is called tranquillant sedatives.[11]

Regarding their chemical structure, sedatives and tranquilizers belong to four families: butyrophenones, phenothiazines, benzodiazepines, and imidazopyridines.[12] The sedatives and tranquilizers most commonly used in veterinary medicine are azaperone, carazolol, acetylpromazine (acepromazine), chlorpromazine, propionylpromazine (propiopromazine), promazine, promethazine, triflupromazine, xylazine, diazepam, and haloperidol.[13–15] Their chemical structures are presented in Figure 6.1.

Acetylpromazine, chlorpromazine, propionylpromazine, promazine, promethazine, and triflupromazine belong to the phenothiazine group of sedatives. The sedative action is related to the broad activity these drugs exhibit in the blocking of many cell membrane receptors, as noted in the JECFA evaluation of chlorpromazine.[16]

6.3 Use of Sedatives and Tranquilizers to Prevent Stress Syndrome during the Transport of Pigs to Slaughter

Sedatives and tranquilizers are used to minimize death and injury during transport and to reduce stress.[17] Stress is especially noticeable in pigs which have been bred to give lean meat. By facilitated introduction of these highly productive breeds and crossbreeds of pigs, there is prior to slaughter a particular risk of stress that causes the so-called porcine stress syndrome (PSS). The stress situation triggers in the animals vegetative and psychosomatic reaction, followed by tachycardia.[18] The consequence of the sudden death of the pigs, premature glycolysis, and accelerated acidosis is the incidence of a poor-quality meat that

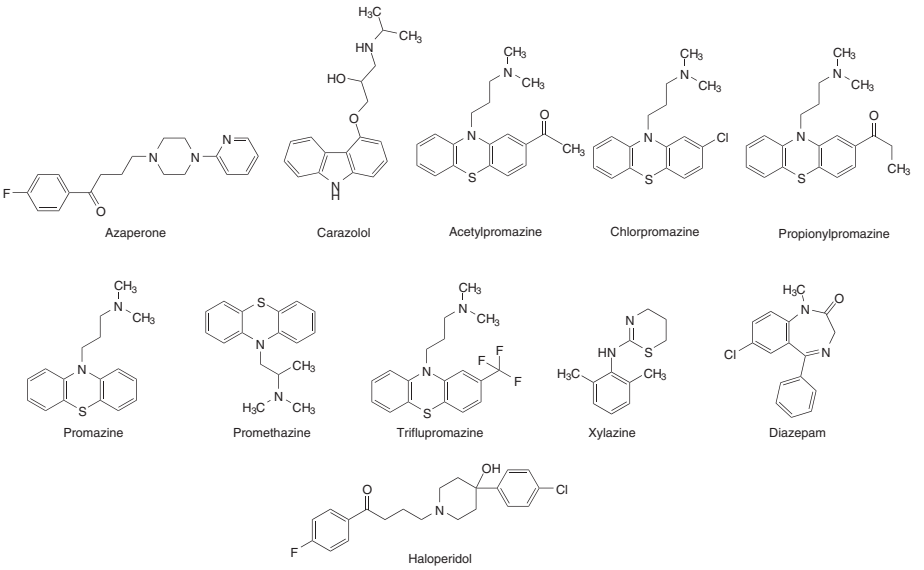


Figure 6.1 Chemical structures of selected sedatives and tranquilizers.

is pale, soft, and exudative (PSE meat) that is unattractive for consumers.[19–23] Consequently, such use of sedatives and tranquilizers is most likely to result in residues entering the food chain.

In the 1980s in Germany (the former Federal Republic of Germany, FRG), approximately 1% of slaughter pigs (360,000 animals) were considered susceptible to PSS.[24] To resolve the PSS problem, three alternatives were considered:

1) Breeding animals resistant to stress, because stress syndrome is genetically related
2) Improvement of the conditions of transport (use of elevators, ventilation, etc.)
3) Use of sedatives and tranquilizers

The effect of azaperone in preventing mortality of slaughter pigs during transport to the slaughterhouse from farms and prevention of deterioration in the quality of meat was studied in Belgium in 1970 by testing of animals of 95% of the Belgian Landrace breed.[25] The study involved 11,416 pigs, of which 4150 were administered intramuscularly 1 ml of Stresnil®. The mortality rate and the number of emergency slaughters were as much as five times lower ($p < 0.001$) in the treated animals than in the untreated animals, while meat of the treated animals also had a highly statistically lower temperature immediately post-slaughter ($p < 0.001$). Consequently, meat of the treated animals was of evidently higher quality compared to the control animals.

In the beginning of the 1980s, a comparative experiment using slaughter pigs of the Pietrain breed, which were also very susceptible to the stress syndrome, was conducted in the United Kingdom.[26] It had been shown that the use of carazolol to block the β-adrenergic receptors in stressful conditions (4 hour transport to the slaughterhouse, approximately 150 km) significantly improved ($0.001 < p < 0.005$) the quality of meat, which at 15 minutes after slaughter had a lower temperature on average (−2.1 °C), compared to the control group. Due to the lower loss of muscle glycogen and limited formation of lactate, the value of pH was 0.71 units higher at 45 minutes after slaughter.

In Italy, during the movements of pigs in stalls, a carazolol therapeutic dose of 10 μg/kg b.w. decreased symptoms of stress syndrome (tachycardia and dyspnea) for 75% of the treated pigs.[18] During transport in the summer, the mortality rate in the control and treated groups was 1.01% and 0.33%, respectively.

6.4 Sedatives and Tranquilizers with an Approved Veterinary Use in Food-Producing Animals

Note: The European Agency for the Evaluation of Medicinal Products (EMEA), referred to in some regulatory decisions discussed in this chapter, is now the European Medicines Agency (EMA). The acronym for the agency as it existed at the time of a regulatory decision, as it appears in the decision document cited, is used when reference is made to decisions of this agency (EMA or EMEA, as appropriate).

6.4.1 Azaperone

Azaperone, 1-(4-fluorophenyl)-4-[4-(2-pyridinyl)-1-piperazinyl]-1-butanone, is a butyrophenone neuroleptic drug with sedative and antiemetic effects, being the pharmacologically active substance of medicines named Stresnil® or Suicalm®.[11]

6.4.1.1 Indication, Dosing, and Withdrawal Period

Pigs are administered with azaperone for the prevention of stress at transport, for reduction of aggression, for calming before diagnosis examinations and minor surgical interventions, and in obstetrics at heavy and painful birth. Azaperone is administered by deep intramuscular (i.m.) injection, preferably behind the ear at usual recommended doses ranging from 0.4 to 2 mg azaperone/kg b.w., depending on the indication.[27] Subcutaneous administration in adipose tissue is ineffective, while intravenous (i.v.) administration can cause excitation. The dose for transport is 0.2 – 1 ml Stresnil®/20 kg b.w. It is noted in the residue monograph prepared by the 43rd JECFA that residues of azaperol and azaperone at the injection site may exceed the MRL for pig muscle for up to 7 days.[28] Therefore pigs may be slaughtered for human consumption only after 7 days from the last treatment, as stated by the International Summary of Product Characteristics, but may vary upon a national registration.[29] The evaluation by European Union (EU) authorities noted that due to "the high and more persistent concentrations of azaperone and azaperol in the injection site", azaperone was not recommended for use prior to transport of pigs to slaughter.[27] It has been found that azaperone yields a violet-blue color in fat tissues exposed to a wavelength of 360 nm from an ultraviolet (UV) light, so that the high concentrations in injection sites can be detected, which can be particularly convenient as a simple screening test in the slaughterhouses.[30]

In addition to pigs, azaperone, in some cases in combination with other drugs, is used in the treatment of horses and wildlife, including elephants,[31] deer,[32, 33] gazelles[34], and rhinoceros.[35]

6.4.1.2 Absorption, Distribution, Biotransformation, and Excretion

Azaperone has been evaluated at four meetings of the JECFA,[36–39] which also produced three residue monographs,[28, 40, 41] and by two summary reports of the EMEA.[27, 42] Heykants et al. reported a study in 1971 in which rats were treated subcutaneously at a dose of 1 mg tritium-labeled azaperone/kg b.w. [43] Azaperone was predominantly excreted in feces (75%) and less in urine (25%). At 48 hours after administration, 90% of the drug was excreted, and after 4 days no residual radioactivity was found in the animals. Of the injected azaperone, 13% was excreted unchanged, mainly in the feces. The major metabolic pathway (about 50% of the administered dose) was oxidative N-dearylation resulting in 4′-fluoro-4-(1-piperazinyl)-butyrophenone which metabolized to its N-acetyl derivative. A minor metabolic pathway was oxidative N-dealkylation, resulting in β-(*p*-fluorobenzoyl)propionic acid, being then rapidly metabolized to *p*-fluorophenylacetic acid and its glycine conjugate *p*-fluorophenaceturic acid.

The metabolic *in vitro* pathway of azaperone in rats and pigs demonstrated similar metabolites in both species, although in significant relative amounts.[40] The main metabolic pathways were reduction of the butanone to azaperol, oxidative N-dearylation, hydroxylation of the pyridine ring, and oxidative N-dealkylation (rats). The results demonstrated that kidney is the target tissue in terms of total residue as well as for assayable residue at times between 1 and 24 hours after dosing. It should be noted that the quantities of various metabolites were likely to be different *in vivo*.

In 1976 Rauws et al. reported the discovery of the main (reduced) metabolite of azaperone named azaperol, which was first isolated from the injection site in pigs and confirmed by both mass selective and infrared spectrometry.[44] Its chemical name is α-(4-fluorophenyl)-4-(2-pyridinyl)-1-piperazine butanol. In its pharmacological action, azaperol is fourfold less strong than azaperone, while in lowering body temperature, its activity is lower by as much as 30 times.[44] However, because its concentrations in the treated organisms exceed the concentrations of the parent substance and as it is eliminated more slowly, it is thus a limiting factor in determining the residues of azaperone.[44–46] In rats, after i.v. administration, the elimination half-life of azaperol in the liver was 45 minutes, and in the kidneys and the brain, it was 15 minutes.[44]

Extensive biological experiments using slaughter pigs in the mid-1980s in Germany (former FRG) were carried out by Arneth.[47] Two groups of five animals each were administered with azaperone intramuscularly at a dose of 0.4 mg/kg b.w. and were slaughtered after 2 and 4 hours. After 2 hours, the highest concentrations of azaperone and azaperol were found in the injection site, at 43,000 and 10,000 μg/kg, respectively, which reflected mostly non-metabolized parent substance. The second highest residue concentrations of azaperol and azaperone were found in the kidneys, at 130–320 μg/kg and 22–80 μg/kg, respectively, confirming the kidneys as the matrix of choice for routine residue monitoring and control. Azaperol and azaperone contents in the muscle tissue of chops and thighs were 13–50 μg/kg and 9–20 μg/kg, respectively. Due to greater lipophilicity, azaperone dominated in fatty tissues, with concentrations ≤158 μg/kg. High concentrations were also found in the urine even 2–3 days after the administration of the drug. Concentrations of azaperol and azaperone in tissues and body fluids from animals slaughtered after 4 hours after administration of azaperone were on average considerably lower, reflecting the rapid elimination of residues from the organism.

Six hours post-injection of azaperone to pigs at a dose of 0.25 mg/10 kg, the sums of azaperone and azaperol concentrations were below their respective EU MRLs in the muscle, but at this time their concentrations in liver and kidney remained near or three- to four-fold above the EU MRL value.[3] Two hours post-injection, the azaperol/azaperone ratio was 2.3 in the muscle and kidney and 15.4 in the liver tissue.[48] The JECFA warned that concentrations of azaperone and azaperol residues at the injection site can exceed the MRL set for the muscle tissue during a 7-day withdrawal period.[28, 37] It was also stated by the EMEA that high and more persistent concentrations of azaperone and azaperol in the

injection site contra-indicate the use of azaperone for transport of pigs to the slaughterhouse.[27]

In horse urine, two hydroxylated major metabolites were identified as 1-(fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanol, designated as 5′-hydroxyazaperol, and 1-(fluorophenyl)-4-[4-(5-hydroxy-2-pyridinyl)-1-piperazinyl]-1-butanone, designated as 5′-hydroxyazaperone.[49] Both metabolites were eliminated in urine as glucuronic acid conjugates, and the presence of 5′-hydroxyazaperol was confirmed in the horse urine which was collected for 24 hours following i.v. azaperone administration. Following i.m. administration of azaperone to a horse, two N-dealkylated metabolites, that is, *N*-despyridinylazaperol and *N*-despyridinylazaperone, and a low concentration of azaperone were detected in the unhydrolyzed urine. Six metabolites – hydroxyazaperol, two hydroxyazaperones, azaperol, *N*-despyridinylazaperol, and *N*-despyridinylazaperone – were detected in the hydrolyzed urine extracts.[50] Three glucuronide-conjugated azaperone metabolites – hydroxyazaperol glucuronide, hydroxyazaperone glucuronide, and azaperol glucuronide – were detected in the urine.

6.4.1.3 Subacute and Acute Toxicity, Mutagenicity, and Carcinogenicity

Short- and long-term toxicological studies were carried out with azaperone in rats (dose of 40 mg/kg b.w./day) and dogs (20 mg/kg b.w./day), where in both types of animals passive behavior was observed.[36] In the course of 13 weeks, the body weight of the male rats was significantly reduced, while at doses above 10 mg/kg b.w./day, alkaline phosphatase had been increased. In the case of oral dosing at a concentration of 1600 mg/kg of food during 15 weeks, cholesterol decreased for both sexes of rats, while urobilinogen in males and urinary creatinine in females increased, allied with enlargement of the liver and pathological changes on the pituitary and sex glands. After 6 – 12 months of such "therapy", the brain increased in size and lipoid pneumonia appeared.

In dogs, occasional salivation was observed at a dosage of 5 mg/kg b.w./day, with enlarged liver and mammary glands in females.[36] In humans, the dosage at a concentration of about 0.1 mg/kg b.w./day did not result in any clinical effects after 2 ½ months, although dizziness appeared at a 10-fold higher dose.[36] Estimated LD_{50} value in rats was at oral and i.v. administration of 245 mg/kg b.w. and 28 mg/kg b.w., respectively.[36] Azaperone is not considered mutagenic due to the negative results in the *in vitro* tests (Ames test with *Salmonella typhimurium* and gene mutation test with mouse lymphoma cells) and the *in vivo* tests (micronucleus test with rats and dominant lethal test with mice). However, with the microsomal tests on salmonella (*S. typhimurium*), its weak mutagenic activity was demonstrated in only one laboratory,[51] but was not confirmed later in other laboratories.[27] In view of the negative results in mutagenic tests and as its chemical structure does not possess any structural alerts, no carcinogenicity studies were performed on azaperone.[27]

6.4.1.4 Embryotoxicity and Teratogenicity

Embryotoxicity and teratogenicity studies have been performed with azaperone in mice, rats, hamsters, and rabbits.[36] In the first three species, the dosing of azaperone during pregnancy at a concentration of about 40 mg/kg b.w./day resulted in a lack of ossification of limbs, while reduced pup body weight and decreased survival rate during lactation were observed in all of the species studied.

6.4.1.5 Acceptable Daily Intake (ADI)

The JECFA concluded at the 50th meeting (1999) that the pharmacological effects of azaperone are the most relevant for determining an ADI value.[38] An ADI of 0–6 μg/kg b.w. was established, based on an oral no-observed-effect level (NOEL) value of 630 μg/kg b.w., for neurobehavioral effects in dogs after oral administration and a safety factor of 100. The ADI value, established by the EMEA, is 0.8 μg/kg b.w., based on an NOEL value of 80 μg/kg b.w. for norepinephrine antagonism following single subcutaneous administration to rats, and is equivalent to 48 μg for a 60 kg person.[27]

6.4.1.6 Marker Residue and Target Tissues

In the evaluations of azaperone by the JECFA, it was recommended that either the liver or kidney could be used as a target tissue for a national monitoring program, as similar concentrations of residues were found in both tissues.[28] Lower concentrations of residues were observed in muscle and fat. The MRLs recommended by the JECFA were expressed as the sum of the residues of azaperone and azaperol.

6.4.2 Carazolol

Carazolol is structurally analogous to the catecholamines (adrenaline and noradrenaline) and is a nonspecific β-adrenergic receptor blocking agent, being a pharmacologically active ingredient of a medicine named Suacron®. At the time of the JECFA evaluation published in 1991, the chemical names listed for carazolol were 4-(2-hydroxy-3-isopropylamino-propoxy)-carbazole, 1-(carbazole-4-yl-oxy)-2-hydroxy-3-isopropylamino-propane, and 1-(4-carbazol yloxy)-3-(isopropylamino)-2-propanol,[52] while the current IUPAC name is 1-(9*H*-carbazol-4-yloxy)-3-(propan-2-ylamino)propan-2-ol.[53] Carazolol forms reversible bonds with β-receptors; however, it does not induce adrenergic effects, and it impedes the actions of the catecholamines in times of stress by saturating their sites of operation.[54]

6.4.2.1 Indication, Dosing, and Withdrawal Period

In veterinary medicine, carazolol given by i.m. injection to pigs is indicated in stress-inducing situations.[54, 55] Carazolol is used to relieve the stress of parturition, reduce the incidence of mastitis, metritis and agalactia syndrome, to prevent frenzy during mating, and to alleviate tachycardia.[56] In human medicine it is used for the treatment of angina pectoris, disturbances of the cardiac rhythm, and heart

attacks.[57] Administration to pigs is done intramuscularly at a dose of 10 μg/kg b.w. behind the ear. In adult animals, the recommended dose of Suacron® is 1 ml/50 kg b.w., although in piglets a higher dose is administered (1 ml/20 kg b.w.). Carazolol can also be administered to cattle by i.m. or endovenous route.[58] The withdrawal period for pigs varies from 0[58] to 12 hours.[59] The withdrawal time for cattle is one day for meat and 12 hours for milk.[58] Despite a zero withdrawal time prior to slaughter, there are unconfirmed reports of black market preparations being used which may explain the regular findings of carazolol residues. This is of concern since carazolol is also used in human medicine and thus its residues in foods may pose a hazard to human health.[56]

6.4.2.2 Absorption, Distribution, Biotransformation, and Excretion

Carazolol has been evaluated at three JECFA meetings,[60–62] which also produced two residue monographs,[52, 63] and by four summary reports of the EMEA.[54, 55, 64, 65] Biological experiments were reported in rats, rabbits, dogs, pigs, cows, and humans.[66] After intraperitoneal administration of ^{14}C-labeled carazolol at a dose of 0.5 mg/kg b.w., maximum tissue concentrations were found in rats after 15 minutes in the liver, kidneys, and lungs. After 48 hours, 93% of carazolol was excreted, 48% and 45% in the feces and urine, respectively. In rabbits, following an oral dose of 10 mg/kg b.w., the maximum concentration in serum was measured 1 hour after dosing, with depletion half-life of 20 hours, while about 60% of carazolol was excreted in the urine. In dogs, after an oral dose of 50 mg/kg b.w., the depletion half-life of carazolol in plasma was between 20 and 30 hours, while after 48 hours, 45% of the substance was excreted in the feces and urine.[60] In the urine of dogs administrated intravenously with 10 mg ^{14}C-labeled carazolol, the parent compound and five metabolites were identified by mass spectrometry (MS), resulting from such oxidative reactions as desamination, hydroxylation, and glucuronidation.[57]

The metabolism of carazolol in cattle is comparable to that in pigs and humans. Parent carazolol is the primary residue of toxicological concern and the designated marker residue, as the metabolites of carazolol possess no pharmacological activity.[64] Among the metabolites of carazolol studied (amine, lactate, diol, acetate, 4-hydroxycarbazole, and glucuronides), only carazolol amine has pharmacological efficiency on β-blocking receptor sites, being however 10 times less powerful than the parent substance.[67] Some biological experiments on slaughter pigs were carried out in Germany (former FRG). Among these, six pigs, approximately 80 kg b.w., were injected with carazolol 2 hours before slaughter at a dose of 10 μg/kg b.w., and their liver, kidney, and muscle were then analyzed.[68] The maximum residue content of free carazolol was found in the liver, between 3.5 and 6.9 μg/kg, with slightly lower concentrations found in the kidneys, that is, between 3.4 and 5.4 μg/kg. The lowest residue concentrations were found in the muscle, 2.7 and 3.0 μg/kg (only two samples were analyzed). The authors noted that different residue concentrations of carazolol in tissue resulted from an uneven distribution as well as sampling part of the organ or muscle tissue. Some authors have

demonstrated by the hydrolysis of tissues and urine that the glucuronide metabolites of carazolol are practically absent.[68, 69] Rattenberger et al. experimented with three treated and one control pig with body weights of approximately 90 kg.[69] Treated animals were intramuscularly injected with 3 ml of Suacron™ (1.5 mg of carazolol). Maximum residue concentrations of carazolol were found in plasma and urine, respectively, 3 and 8 hours after the administration at concentrations of 2.14 and 6.09 μg/l. Carazolol was detectable in plasma and urine up to 8 and 24 hours after administration, respectively, while in one animal, it was detected even after 32 hours at a concentration of 0.25 μg/l.

Carazolol is eliminated very rapidly in all tissues following post-intramuscular injection at the recommended dose rate of 10 μg /kg b.w.[56] The highest mean ± S.D. concentrations ($n = 2$) were detected in pigs at 1 hour post-injection in the kidney (10.84 ± 1.3 μg/kg) and muscle (3.59 ± 0.2 μg/kg), which were less than the respective MRLs. Two hours post-injection to pigs at the same dose, the residue levels were below the MRL in the muscle, liver, and kidney.[3, 48] The JECFA recommended MRLs for carazolol based on the concentrations determined at 2 hours after administration in pigs. The mean residue concentrations at this timepoint were 1.8 μg/kg for muscle, 9.9 μg/kg for liver, 6.9 μg/kg for kidney, and 1.8 μg/kg for fat and skin.[61] However, the JECFA also observed that concentrations of carazolol residues at the injection site may be present in excess of the ADI, noting that the ADI is based on acute pharmacological effects. In milk from treated cattle, carazolol residues were quantifiable only in samples collected at the first milking after treatment. At 12 hours post-treatment, the carazolol concentrations had depleted to <0.5 μg/kg.[64]

6.4.2.3 Subacute and Acute Toxicity, Mutagenicity, and Carcinogenicity

Short-term exposure of female rats to 400 mg/kg of carazolol in their diet resulted in a significant reduction in body weight due to reduced food consumption, although animals recovered after 4 weeks.[66] Rats that received treatment over a period of 1 year, regardless of the sex, exhibited a decrease in body weight at higher doses (up to 1800 mg/kg of diet), together with an increase in the relative weight of the heart, kidneys, ovaries, and testes.[66] The acute toxic dose of carazolol in rats was 80 mg/kg b.w., 40,000 times higher than a dose of 2 μg/kg b.w., which produced effects on β-blocking receptor sites after oral administration.[18]

A decrease in body weight was also observed in female dogs following an experimental dose of 10 mg/kg b.w./day over 14 days, allied with the changes in the blood composition, including reduced hemoglobin content and increased sedimentation, glucose, cholesterol, and alkaline phosphatase, all of which demonstrate the hepatotoxicity of the substance.[66] After a 1-year treatment of dogs with carazolol at a dose of 60 mg/kg b.w./2 times a day, an increase in weight of the liver, kidney, and testes was observed.[66]

In humans with chronic bronchitis, carazolol caused strong disturbances of the respiratory system after a single oral dose of 0.7 mg/person, while side effects were noticeable already at a dose of 0.1 mg/person.[60] In the *in vitro* tests (Ames test

with *S. typhimurium)* and in various *in vivo* cytogenicity tests, no evidence for genotoxicity of carazolol was found[65]. No carcinogenic activity was observed in an experiment conducted in rats.[60]

6.4.2.4 Embryotoxicity and Teratogenicity

Experimental results were inconclusive. While in an experiment in rats even at a dose of 100 mg carazolol/kg b.w./day there was no evidence of teratogenicity, another experiment carried out at a dose of 30–60 mg/kg b.w./day up to day 22 after fertilization demonstrated increase of the placenta, reduced ossification of the fetus, and a reduced number of live-born pups in the second and third generation.[60] When rabbits received carazolol at a dose of 100 mg/kg b.w./day between 7th and 19th day of gestation, placental and fetal weights were reduced, resulting in dead and undeveloped pups.[66] Information also revealed one fetus with spondylosis syndrome and one with scoliosis.

6.4.2.5 Acceptable Daily Intake (ADI)

An ADI value of 0–0.1 μg/kg b.w. was established by the JECFA, based on a NOEL of 0.5 μg/kg b.w. for reduced respiratory function from a clinical study in patients suffering from either chronic bronchitis or asthma.[61] The same ADI value of 0.1 μg/kg b.w., that is, 6 μg/person, was also established by the EMEA, considering a theoretical excess of the ADI by 16.7% in the case of pig meat and cow's milk being combined in the total food basket.[55] Both the JECFA[61] and the EMEA[55] noted that the concentration of carazolol at the injection site may exceed the ADI value, which is based on the acute pharmacological effect of carazolol.

6.4.2.6 Acute Reference Dose (ARfD) and Residues at the Injection Site

The 10th Session of the Codex Committee on Residues of Veterinary Drugs in Foods, meeting in 1996, in discussing the issue of residues of veterinary drugs at injection sites, stated as a goal an assurance that elevated residues at injection sites would not pose health risks to consumers and requested further consideration of this issue by the JECFA.[70] In response, the 52nd Meeting of the JECFA discussed the issue with respect to carazolol residues at the injection site and established an acute reference dose (ARfD) of 0–0.1 μg/kg b.w. for carazolol,[62] based on the reduction in respiratory function observed in human subjects with chronic bronchitis or asthma, applying a safety factor of 5. The JECFA used residue concentrations found at the injection site 2 hours post-injection in pigs to assess the potential intake of such residues which might occur if a pig were injected with carazolol shortly before transport and if slaughter were to occur within 2 hours of administration of the drug. The data from injection sites at 2 hours post-injection were chosen as the next data were for 12 hours post-injection. Using the data for 2 hours, the JECFA calculated that consumption of 300 g of injection site muscle containing 60 μg/kg of carazolol residues would result in an intake of 18 μg, three times the ARfD, and therefore recommended that carazolol should not be administered to prevent stress during the transport of animals to slaughter.[62]

6.4.2.7 Marker Residue and Target Tissues

Data provided to the JECFA for the evaluation of carazolol residues in pigs indicate that the tissue in which residues are highest and most persistent, other than the injection site, is the kidney and that the appropriate marker residue is the parent drug.[63]

6.4.3 Xylazine

Xylazine, a clonidine analogue, is an α_2-adrenoceptor agonist which exhibits action on the presynaptic and postsynaptic receptors of the central and peripheral nervous systems.[71] It is usually formulated as the hydrochloride salt, *N*-(2,6-dimethylphenyl)-5,6-dihydro-4*H*-1,3-thiazin-2-amine hydrochloride, and is the active ingredient in products such as Rompun®, Anased®, and Chanazine®[72] and is available for use in both companion animals and some food-producing animals.[11]

6.4.3.1 Indication, Dosing, and Withdrawal Period

Xylazine hydrochloride is available as a 2% injectable solution and also as "a dry substance with solvent for i.v. or i.m. injection".[73] Xylazine may also be administered orally or by subcutaneous injection.[74] In veterinary medicine, xylazine is used as an anesthetic premedication, as a sedative, as a muscle relaxant, and as an analgesic, with typical doses of 0.016–0.3 mg/kg b.w. in cattle and 0.6–1.0 mg/kg b.w. in horses. It is also used in the veterinary treatment of wild animals.[75] Cattle appear to be the most sensitive species and therefore require a lower dose than other animals in which this drug has been used.[11] Depletion of residues is rapid, and no withdrawal time has been specified in the EU.[73,76] It is noted in the EMEA Opinion of 1999 that it is unlikely that food-producing animals treated with xylazine hydrochloride would be sent for slaughter "during or immediately after treatment",[73] while the EMEA Opinion of 2000 further notes that the residues of xylazine found in "cattle-derived food" the first day following administration were at concentrations well below those which could raise concerns for consumer exposure.[76]

6.4.3.2 Absorption, Distribution, Biotransformation, and Excretion

Studies in rats indicated that nearly 100% of a dose of xylazine is rapidly absorbed, with rapid distribution into tissues.[73] The terminal half-life of xylazine after i.v. administration was reported in the evaluation by the JECFA to be 25 minutes in sheep, 36 minutes in cattle, and 50 minutes in horses, with half-life after i.m. administration being 22 minutes in sheep and 58 minutes in horses.[74] This rapid elimination was attributed to metabolic clearance in the evaluations by the EMEA[73] and JECFA.[74]

Metabolic studies in rats, horses, and cattle demonstrated a rapid and extensive metabolism.[73] In rats treated with ^{14}C-xylazine, excreted radioactivity was found primarily in the urine, associated with polar conjugates which were resolved as five major metabolites following deconjugation by enzyme hydrolysis. These

metabolites included products of the hydroxylation of the phenyl ring and products resulting from opening of the thiazine ring by oxidation. A qualitatively similar pattern of metabolites was found in the horse. A study in cattle isolated 10 metabolites, of which 5 were considered major metabolites. These were identified using LC-MS as glucuronide conjugates of xylazine hydroxylated in the phenyl ring, plus conjugated and/or unconjugated derivatives formed by opening of the thiazine ring through oxidation. The most abundant metabolite was identified as either 2(3-hydroxy-2,6-dimethylanilino)-4-oxo-5,6-dihydro-1,3-thiazine or 2(4-hydroxy-2,6-dimethylanilino)-4-oxo-5,6-dihydro-1,3-thiazine or a mixture of these two compounds. No residues of parent drug or 2,6-xylidine were detected in the urine.

In a study in which horses were treated with xylazine, the major metabolites identified in the hydrolyzed urine were 2-(4′-hydroxy-2′,6′-dimethylphenylamino) -5,6-dihydro-4*H*-1,3-thiazine, 2-(3′-hydroxy-2′,6′-dimethylphenylamino)-5,6-di hydro-4*H*-1,3-thiazine, *N*-(2,6-dimethylphenyl)thiourea, and 2-(2′,6′-dimethyl phenylamino)-4-oxo-5,6-dihydro-1,3-thiazine.[77] The same metabolites were also produced when xylazine was incubated with rat liver microsomes. The major metabolite produced *in vitro* by rat liver preparations was found to be *N*-(2,6-dimethylphenyl)thiourea, formed by opening of the thiazine ring. Following a single-dose i.v. administration of xylazine at approximately 1 mg/kg b. w. to two horses, xylazine was observed to be rapidly metabolized within 1–3 hours of administration.[78] Seven metabolites were identified in urine from the treated horses. The major metabolite, 4-hydroxy xylazine, which is regarded as the long-term metabolite of xylazine in the horse, was detectable for 25 hours post-treatment. In this study, 2,6-dimethylaniline was detected for the first time as a metabolite in the horse.

Xylazine hydrochloride was administered by i.m. injection at 0.35 mg/kg to 13 steers and 10 lactating dairy cows, following which tissue and milk samples were analyzed using gas chromatography (GC) with nitrogen and phosphorus detection to determine concentrations of xylazine and the metabolite 2,6-dimethylaniline.[79] Concentrations of xylazine and 2,6-dimethylaniline in tissues were below the limit of quantification (LOQ) of 10 µg/kg within 72 hours following treatment, while residues in milk were below the LOQ at 12 hours post-treatment.

More recently, the *in vitro* metabolism of xylazine was investigated using rat liver microsomes with five primary metabolites being observed.[80] The main metabolite was identified as a product of the oxidation of the thiazine moiety (*m*/*z* 235). The other metabolites included *N*-(2,6-dimethylphenyl)thiourea (*m*/*z* 181) and three hydroxylated metabolism products with *m*/*z* 237.

6.4.3.3 Subacute and Acute Toxicity, Mutagenicity, Embryotoxicity, Teratogenicity, and Carcinogenicity

Data reviewed by the EMEA included acute toxicity data from non-GLP studies for mice, rats, cats, and dogs, repeat-dose studies in dogs (conducted prior to

GLP), a GLP-compliant study of embryotoxicity and teratogenicity in rats, and mutagenicity studies.[73] No specific data on carcinogenicity were provided for review, but the EMEA determined that these were not necessary, based on the negative results of the mutagenicity studies.

6.4.3.4 Acceptable Daily Intake (ADI)

The use of xylazine in food-producing animals is restricted or prohibited in many countries. In the USA, for example, xylazine was not approved for use in food-producing animals due to concerns that it is potentially carcinogenic, including evidence that the 2,6-dimethylaniline metabolite is carcinogenic in rats.[81] The 47th JECFA meeting evaluated xylazine and did not establish an ADI as it concluded that the metabolite, 2,6-xylidine (2,6-dimethylaniline), was "genotoxic and carcinogenic".[71] A review of 2,6-xylidine by the International Agency for Research on Cancer concluded that this compound is "potentially carcinogenic to humans".[82]

The review of xylazine by the EMEA, however, received data from additional studies which were considered to have addressed the concerns regarding the potential carcinogenicity of 2,6-xylidine, as this metabolite was not detected in urine and tissues from cattle treated with the drug. In addition, no metabolites which would result from cleavage of the thiazine and the phenyl ring or from the decomposition of the thiazine ring were observed in tissues from cattle administered with xylazine hydrochloride.[73] The studies available to the EMEA were considered insufficient to enable the establishment of either a toxicological or a pharmacological ADI. Available data indicated that the threshold dose for the appearance of pharmacological effects is 170 μg/kg b.w. in humans, while acute toxic effects in humans are observed at doses of 700 μg/kg b.w. and above. Based on the limited veterinary use of this drug in cattle, its rapid elimination, and the low probability that cattle treated with xylazine would be sent to slaughter while residues were present, the EMEA determined that MRLs were not required for the use of this drug in cattle. Subsequently, the EMEA extended the use of xylazine to include the treatment of dairy cattle.[76] No MRL has therefore been required for xylazine hydrochloride in cattle and horses, as specified by the requirements of Commission Regulation (EU) No. 37/2010 on pharmacologically active substances and their classification regarding MRLs in foodstuffs of animal origin.[46]

6.4.3.5 Marker Residue and Target Tissues

In its evaluation of xylazine, the 47th JECFA meeting was unable to identify a suitable marker residue and target tissues due to insufficient information being provided on the metabolism and depletion.[74] Total residue data evaluated by the JECFA indicated that the highest total residues were in the injection sites, kidney and liver, but the nature of these residues was not further investigated in the studies evaluated by the JECFA. As previously noted, the subsequent evaluation by the EMEA included data in which both total residues and residues of parent

drug were available, and the EMEA concluded that due to the rapid depletion of residues following treatment, MRLs were not required. The EMEA therefore did not designate a marker residue or target tissues.[73] Based on the available information, the authors suggest that monitoring for xylazine residues, if required, should use parent compound as the marker residue and target injection sites or kidney as the most likely tissues in which residues might be detected.

6.5 Sedatives and Tranquilizers without an Approved Veterinary Use in Food-Producing Animals

6.5.1 Chlorpromazine

Chlorpromazine, 3-(2-chlorophenothiazin-10-yl)-*N*,*N*-dimethyl-propan-1-amine, is used mainly as an antiemetic in the treatment of dogs and cats and may also be used as a preanesthetic drug.[11] It is not normally used in the treatment of horses due to potential adverse effects. Chlorpromazine was evaluated by the 38th Meeting of the JECFA in 1990, which was unable to establish an ADI due to "a general lack of relevant toxicological information".[16] Based on the information available at the time of this evaluation, the JECFA recommended that this drug should not be used in food-producing animals.

A subsequent review of chlorpromazine was conducted by the EMEA in 1996.[83] The limited residue data provided to the EMEA were considered insufficient, and the lack of toxicological information identified by the JECFA was not addressed in the submission to the EMEA. As a result, the EMEA was unable to establish an MRL and warned that residues of chlorpromazine pose a potential health risk to consumers. The EMEA therefore recommended that chlorpromazine should be prohibited from use in food-producing animals. Chlorpromazine has been listed among prohibited substances in Table 2 to the Commission Regulation (EU) No. 37/2010.[46]

6.5.1.1 Subacute and Acute Toxicity, Mutagenicity, Carcinogenicity, and Genotoxicity

Acute toxicity studies evaluated by the 38th Meeting of the JECFA indicated that the LD_{50} following i.v. injection of chlorpromazine ranged from 16 mg/kg b.w. in rabbits to 30 mg/kg b.w. in dogs and approximately 50 mg/kg b.w. in mice and rats.[84] A short-term toxicity study in guinea pigs cited in the JECFA toxicology monograph reported that some inflammation and hemorrhage were observed, as well as the presence of fibrous adhesions to the peritoneal surface.[84] No data were available for review by the JECFA from long-term toxicity or carcinogenicity studies. Effects observed in humans receiving long-term treatment with chlorpromazine include abnormal pigmentation of the skin when it is exposed to sunlight, epithelial keratopathy, and opacities in the cornea and in the lens of the eye.

Chlorpromazine gave positive results in a number of genotoxicity assays reviewed by the JECFA,[84] including tests using human lymphocytes and an Ames test using *S. typhimurium*. A more recent review concluded that phenothiazine and its derivatives, including chlorpromazine, have photomutagenic properties but do not exhibit genotoxicity under the standard tests for mutagenicity.[85] It was also observed that there were method-related inadequacies in the various studies reviewed which led to conflicting results being reported.

A more recent study reported that in experiments using a cDNA microarray representing 1089 genes which are related to DNA damage and repair, metabolism, and other toxicologically important cell functions, chlorpromazine was not genotoxic.[86] Subsequently, a review of the results of genotoxicity and carcinogenicity assays conducted on 104 drugs used as antipsychotics and antidepressants concluded that there is a potential for genotoxic–carcinogenic effects to occur in humans resulting from the formation of *N*-nitroso compounds in the gut by the reaction of nitrosatable amine drugs.[87] Furthermore, this review noted for chlorpromazine that the product of its interaction with nitrite or the nitrosation reaction mixture in the gut produced genotoxic effects.

6.5.1.2 Reproductive Effects

Observations reported from reproductive studies in mice evaluated by the JECFA included a reduced number of pregnancies, reduced litter size, and lower weight gain during pregnancy.[84] In studies using rats, decreased weight of testes was observed in male rats treated with chlorpromazine, and effects were also observed in female rats during late stages of pregnancy. A study in which pregnant mice, divided into four groups, were injected once daily from the 6th to the 16th day of gestation with, respectively, 1.8 mg/kg b.w./day of chlorpromazine, 9.2 mg/kg b.w./day of chlorpromazine, 0.3 ml of cod-liver oil containing vitamins A and D (positive control), or 0.3 ml saline (negative control) demonstrated that there was a higher incidence of abnormal mouse fetuses in the groups treated with chlorpromazine, although this effect was also observed in the positive control group. Data from another study using rats evaluated by the JECFA indicated that chlorpromazine has an embryotoxic effect. The studies using rats reviewed by the JECFA showed other effects, including delay of ossification in newborn pups. A study in female rats had a NOEL for teratogenicity of 9 mg/kg b.w./day.

6.5.1.3 Absorption, Distribution, Biotransformation, and Excretion

Information available for evaluation by the JECFA suggested that chlorpromazine is metabolized in both the gut and the liver by processes which include oxidation, demethylation, hydrolysis, and conjugation with glucuronic acid, leading to formation of a sulfoxide metabolite.[84] The *N*-oxide metabolites were found to be reduced back to parent compound in a number of species, including humans. The elimination half-life was reported to be about 1.5 hours in goats, and the biological

half-life in dogs is approximately 6 hours, while in horses which received chlorpromazine by i.v. or oral routes of administration, metabolites were detected in urine for up to 96 hours .

6.5.1.4 Marker Residue and Target Tissues

Data evaluated by the JECFA[16] and the EMEA[83] were insufficient to identify a suitable marker residue and target tissues for chlorpromazine. The limited information available suggests targeting visible injection sites for parent drug or the analysis of extracts of kidney or liver following deconjugation of the sulfoxide metabolite.

6.5.2 Propionylpromazine (Propiopromazine)

Propionylpromazine, also known as propiopromazine, has been used as a tranquilizer to combat stress in both companion and farm animals, with the use in food-producing animals being primarily to reduce stress in pigs during transport to slaughter.[88] Information available to the 38th Meeting of the JECFA in 1990 indicated that propionylpromazine is extensively bound to tissue and to proteins and also that it accumulates in fat.[10] The JECFA Meeting Report noted that full information on distribution and metabolism was not available for this drug. In addition, the Committee noted that both short-term and long-term toxicity studies and studies of the effects on reproduction, mutagenicity, carcinogenicity, and immunotoxicity were not available and that no information on use of the drug in humans was provided. The evaluation of the toxicology of this compound by the JECFA determined that due to the absence of key pharmacological and toxicological information,[10] an ADI could not be established, and consequently no MRLs were recommended by the JECFA.

The limited data evaluated by the JECFA on the pharmacokinetics of propionylpromazine in the horse indicated a peak concentration in plasma at 30 minutes post-injection. In a metabolism study conducted using a single horse, parent drug and three metabolites – 2-(1-hydroxypropyl)promazine, 2-(1-propenyl)promazine, and 7-hydroxy-propionylpromazine – were identified in urine following enzyme hydrolysis.[89] A study in which pigs were given a dose of 0.5 mg propionylpromazine/kg b.w. found the highest residues in tissue from the neck area and in fat associated with the neck and kidneys, with lower residue concentrations in the back fat and belly fat than in the neck fat 2 hours post-treatment.[90] Residues were lower in the liver and much lower in the muscle samples, but residues at the injection site were as high as 863 µg/kg at 5 days post-injection. The study also identified the presence of the sulfoxide metabolite in some blood and tissue samples taken at 2 and 4 hours post-injection. However, a subsequent study demonstrated that the sulfoxide can be formed when propionylpromazine is exposed to light and air.[45] The JECFA therefore concluded that the sulfoxide found in the Arneth study might be an artifact and not a metabolite.[88] The limited residue data available to the JECFA included

results from a study which indicated that the highest residue concentrations in pigs were observed in injection sites, with the highest residue concentrations in other tissues being in the kidney.[91] No additional data have subsequently been provided for review by the JECFA.

6.5.2.1 Marker Residue and Target Tissues

Due to the lack of metabolic and total residue data, the JECFA was unable to identify a suitable marker residue for propionylpromazine and, in the absence of MRL recommendations, did not recommend target tissues for residue monitoring.[10] The limited residue studies and analytical methods available to the JECFA for their review in 1990 used parent drug as the marker residue. Available methods typically target the parent compound, and if propionylpromazine is to be included in a monitoring program for non-approved substances, the limited information available suggests that the tissue collected for analysis should include fatty tissues from likely areas where an injection might be administered and/or any visible injection sites. Injection sites containing promazines exhibit fluorescence under a UV lamp, being weak red brown in muscle and light yellow in the fat tissue.[30]

6.5.3 Acepromazine (Acetylpromazine)

Acepromazine (acetylpromazine), 1-[10-[3-(dimethylamino)propyl]phenothiazin-2-yl]ethanone, is the phenothiazine drug most commonly used in veterinary medicine.[11] It is available in liquid or tablet form under such product names as Vetranquil and Plegicil and is recommended for use in the treatment of cats, dogs, and horses. Dosages are also recommended for the treatment of pigs, cattle, sheep, goats, and rabbits.[11] There are no current approved uses for acepromazine in the treatment of food-producing animals in a number of countries, including the EU, the USA, and Canada.

The phenothiazines are extensively metabolized and exhibit extensive protein binding.[11] Metabolites are excreted primarily in the urine. Three metabolites were detected in urine from horses given acetylpromazine maleate.[92] These included an unconjugated metabolite, 2-(1-hydroxyethyl) promazine sulfoxide, and two conjugated metabolites which, after hydrolysis using β-glucuronidase/arylsulfatase, were identified as 7-hydroxyacetylpromazine and 2-(1-hydroxyethyl)-7-hydroxypromazine. The metabolite 2-(1-hydroxyethyl) promazine sulfoxide is quantifiable for up to 24 hours in urine from horses treated with acepromazine, compared to 3 hours for the parent drug, and was therefore recommended as an indicator for use of this drug in horses.[93]

There is little published information on the toxicity of acepromazine, such as short-term and long-term toxicity studies in laboratory animals. A review on the toxicity of phenothiazine and related compounds noted that the lipophilic nature of the phenothiazines and the formation of two redox systems among their metabolites can facilitate the occurrence of generalized macromolecular

disruption, with adverse reactions observed which included effects on blood elements, neuromuscular problems, and photosensitization.[94] However, a study of the inactivation of ϕX174 bacteriophages as a function of the irradiation time in the near-UV by phenothiazines demonstrated that while significant activity was demonstrated by promazine, chlorpromazine, and several other related drugs, no significant activity was observed with acepromazine.[95] Chlorpromazine, promazine, triflupromazine, and methoxypromazine exhibited two mechanisms for DNA breakage when photoexcited by near-UV irradiation, but acepromazine was not included in this study.[96]

6.5.3.1 Marker Residue and Target Tissues

Acepromazine has not been evaluated by the JECFA or the EMEA, so neither of these review bodies has recommended a marker residue or target tissue for acepromazine. Available methods typically target the parent compound, and if it is to be included in a monitoring program for non-approved substances, the limited information available suggests that the tissue collected for analysis should include fatty tissues, particularly those collected from likely areas where an injection might be administered and/or any visible injection sites. Based on the limited information available, the metabolite 2-(1-hydroxyethyl) promazine sulfoxide may be a suitable marker residue for the testing of liver tissue.

6.5.4 Diazepam

Diazepam (7-chloro-1-methyl-5-phenyl-3*H*-1,4-benzodiazepin-2-one) is a benzodiazepine drug which binds γ-aminobutyric acid receptors and is used for seizure control and as an anxiolytic.[97] Diazepam has been recommended for the treatment of seizures in dogs[98] and is used in the anesthesia of companion animals.[99] While there were some early investigations of the use of diazepam to reduce stress associated with handling and transport in pigs,[100, 101] the authors are not aware of any current registrations of the use of diazepam for such purposes. A recent international survey indicates, however, that it is widely used in the anesthesia of horses.[102] The common trade name for diazepam is Valium®.[103] Diazepam is listed in Schedule IV of the International Drug Control Conventions[104] and is available only under prescription in most countries.

6.5.4.1 Absorption, Distribution, Biotransformation, and Excretion

Studies have been conducted in a number of animal species on the metabolism of diazepam. In a recent study, it was demonstrated that the major metabolite of diazepam produced from microsomal reactions in horse liver is temazepam, but production of *p*-hydroxy diazepam in this system was much less than observed in rats.[105] A study using porcine hepatocytes with a flat membrane bioreactor produced oxazepam, temazepam, and desmethyldiazepam as major metabolites.[106]

Studies in mouse liver on the metabolism of diazepam indicated that the primary metabolite of diazepam, nordiazepam, is metabolized to the secondary

metabolite, oxazepam, which is then glucuronidated.[107, 108] Using this medium, the C3-hydroxylation of diazepam to temazepam, which can be N-demethylated to form oxazepam, was minimal. In another study using cynomolgus monkey hepatocytes, the major metabolites identified were *N*-desmethyldiazepam, temazepam, and oxazepam,[109] while a study to characterize the differences in metabolite profiles in hepatocytes isolated from Wistar rat, cynomolgus monkey, beagle dog, and human revealed that there were considerable differences in both the rates of formation and the profiles of metabolites produced from these species.[110] It was observed that the hepatocytes of all four species produced nordiazepam and temazepam as metabolites, with nordiazepam being the principal metabolite in the dog, monkey, and human cells. Oxazepam was a significant metabolite in the monkey, while temazepam occurred as only a minor metabolite produced by the beagle and human hepatocytes. The major metabolite produced by rat hepatocytes was 4′-hydroxy diazepam, which was further metabolized rapidly to glucuronides. The authors of this study stated that the metabolism using the hepatocyte cultures was the same as observed in *in vivo* studies in these four species, An earlier study using cultures of hepatocytes from rat, rabbit, dog, guinea pig, and human had also demonstrated that substantial differences exist in metabolism observed using hepatocyte cultures which correspond to known species differences in the metabolite profile of diazepam *in vivo*, with clearance of nordiazepam showing the most variation between species.[111] The differences observed in this study were attributed to the rate at which a metabolite was formed and the rate at which it was further metabolized.

In a comparative study of the pharmacokinetics of diazepam in human, dog, rabbit, guinea pig, and rat, diazepam and its major metabolite, desmethyldiazepam, were measured in blood and plasma following an i.v. bolus injection.[112] Concentrations of diazepam in plasma were observed to decline bi-exponentially in all five species, with a distribution half-life of 0.3 – 1.0 hours . In the horse, the elimination half-life of diazepam after a dose of 0.05 – 0.08 mg/kg was 7.5 – 13.2 hours , while the metabolite oxazepam, found in plasma, had an elimination half-life of 14 – 16.5 hours .[113]

6.5.4.2 Marker Residue and Target Tissues

No data were found for metabolism or pharmacokinetics of diazepam in cattle or sheep in the literature review conducted by the authors in preparing this chapter. While a study using cultures of pig hepatocytes indicated that oxazepam, temazepam, and desmethyldiazepam are major metabolites in pigs,[106] temazepam was the major metabolite produced from microsomal reactions in horse liver.[105] Comparative metabolism studies indicated that patterns of metabolites differ between species, and therefore, it is difficult to establish from the available data recommendations on a marker residue. The liver plays an important role in diazepam metabolism and therefore may be a suitable target tissue for a monitoring program. The available data suggest that residues are short-lived, with extensive metabolism in most species and the formation of

glucuronides as a final step in the metabolism. Based on the available information, a testing strategy for monitoring tissues collected at slaughter should therefore use a method which includes a deconjugation step and should target parent compound and the major metabolites found in studies in various species. Blood and plasma may be suitable matrices for testing of live animals for diazepam.

6.5.5 Haloperidol

Haloperidol, 4-[4-(4-chlorophenyl)-4-hydroxypiperidin-1-yl]-1-(4-fluorophenyl) butan-1-one, is a member of the butyrophenone group of drugs which exhibit antipsychotic effects in humans.[114] It has a relative high antipsychotic potency, such that 2–3 mg is equivalent to 100 mg of chlorpromazine.[115] It has been used in the sedation of wildlife during live capture[116–119] but is not approved for veterinary use in food-producing animals. However, it could potentially be used under veterinary prescription in the treatment of a food-producing animal under the extralabel policies on veterinary drug use in the EU[120, 121] and some countries, such as the USA.[122] If such use should occur, the veterinarian authorizing the use under extralabel use provisions is generally required to ensure that no residues are present when the animal goes to slaughter.

6.5.5.1 Absorption, Distribution, Biotransformation, and Excretion

An investigation of the distribution of haloperidol in rats and rhesus monkeys showed that the primary accumulation of this drug is in the lung,[123] while another early study in humans identified a metabolite, usually referred to as "reduced haloperidol", which is formed by the reduction of the carbonyl group to the alcohol, in the serum, liver, and urine.[124] In rats which received an i.v. injection of haloperidol, the elimination half-life was approximately 1.5 days, with 95% of dose excreted within 10 days.[125] Following i.m. administration, approximately 90% of the dose was excreted within 42 days. The major urinary metabolite of haloperidol identified in this study was *p*-fluorophenylaceturic acid, while the biliary metabolites were the glucuronide and sulfate of haloperidol. In rats administered with haloperidol at 5 mg/kg b.w., by oral and i.m. routes, maximum concentrations of ^{14}C-haloperidol in plasma were observed 1 hour post-administration and then decreased biphasically.[126] Tissues in which highest concentrations of the drug were found included the lung, liver, and kidney, with total residues detected in all tissues declining to less than 1 µg eq/g within 48 hours post-administration. Metabolites found in plasma included *p*-fluorobenzoylpropionic acid and *p*-fluorophenylaceturic acid, while the major urinary and biliary metabolites were *p*-fluorophenylaceturic acid and the glucuronide and sulfate conjugates of haloperidol. The distribution of metabolites was different in the liver, kidney, and lung.

A pyridinium metabolite of haloperidol was found in metabolic studies conducted *in vivo* and also found in urine and brain tissues from rats treated with

haloperidol.[127, 128] This metabolite, the 1,2,3,6-tetrahydropyridine derivative of haloperidol, was also found in NADPH-fortified metabolic incubation mixtures of haloperidol[129] and in samples of brain tissue, plasma, and urine from rats treated with haloperidol.[130] In a subsequent study, a butyrophenone quaternary pyridinium metabolite of haloperidol was detected in samples of liver and brain of rats treated with haloperidol, but the corresponding methyl quaternary pyridinium compound was not detected in these tissues.[131]

A study of the metabolism of haloperidol demonstrated that the formation of the metabolites *p*-fluorobenzoylpropionic acid, 4-(4-chlorophenyl)-4-hydroxypip eridine, and "reduced haloperidol" was catalyzed by hepatic microsomes.[132] It was also proposed that haloperidol undergoes dehydration to form its 1,2,3,6-tetrahydropyridine (HTP) analogue which is then further metabolized to the pyridinium metabolite, plus HTP *N*-oxide and its N-dealkylated product, 4-chlorophenyl-1,2,3,6-tetrahydropyridine. Compound identities were confirmed by comparison with synthesized standards using liquid chromatography (HPLC) and liquid chromatography–mass spectrometry (LC-MS). Three additional unidentified metabolites were found in microsomal metabolic incubations. Based on LC-MS analysis, it was proposed that one of these may be the oxygenated product of haloperidol, another the 2-pyridine analogue of haloperidol, while no structure was proposed for the third, a compound of neutral polarity. It has also been reported that haloperidol and its tetrahydropyridine dehydration product, 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-oxobutyl] -1,2,3,6-tetrahydropyridine, are metabolized *in vivo* in mice to several pyridinium metabolites, which include a pyridinium metabolite and the 4-(4-chlorophenyl)-1-[4-(4-fluorophenyl)-4-hydroxybutyl]pyridinium species.[133] The latter is the pyridinium species corresponding to "reduced haloperidol", which has been identified as a major circulating metabolite of haloperidol.[132] Based on data from atmospheric pressure ion-spray mass spectrometric analysis, the formation of fluorophenyl ring-hydroxylated derivatives of these two pyridinium metabolites was also proposed.

6.5.5.2 Marker Residue and Target Tissues

No data were found in the literature review conducted by the authors for metabolism or pharmacokinetics of haloperidol in cattle, pigs, horses, or sheep. Data from studies with humans and laboratory animal species indicate that haloperidol has an extensive metabolism, although the compounds that are typically targeted in available methods for monitoring haloperidol in human plasma are parent drug and the metabolite "reduced haloperidol",[134–137] while recent multi-residue methods for antipsychotic drugs, including haloperidol, target the parent drug.[138] It should also be noted that "reduced haloperidol" may potentially exist as chiral isomers.[139]

Based on the available information, monitoring of tissues for use of this drug in food-producing animals should probably focus on testing for the parent drug and selected metabolites, such as "reduced haloperidol" and

p-fluorobenzoylpropionic acid in liver or lung tissues. Live animal testing using plasma should probably target parent drug and "reduced haloperidol", while urine testing may better focus on parent drug and *p*-fluorophenylaceturic acid.

6.5.6 Ketamine

Ketamine, 2-(2-chlorophenyl)-2-(methylamino)cyclohexan-1-one, is a racemic mixture of *R*- and *S*- optical isomers, with the *S*-enantiomer having approximately four times the activity of the *R*-enantiomer against the target receptors.[140] Ketamine is considered one of the injectable anesthetics most frequently used in veterinary medicine,[141] with uses which included anesthesia for horses,[102] cats and dogs[142], and wildlife.[143] The authors are not aware of any current registrations of ketamine for the treatment of food-producing animals, so any such usage would be under extralabel provisions for use by a veterinarian when no suitable alternatives are available. In such cases, it is the responsibility of the veterinarian to ensure that no residues are present in animals which may subsequently be sent for slaughter.

6.5.6.1 Absorption, Distribution, Biotransformation, and Excretion

The metabolism of ketamine was examined in an *in vitro* system using microsomal preparations from rat liver.[144] Eight metabolites were identified, including six which had not been reported previously. The newly identified metabolites were products of the hydroxylation of the alicyclic ring of ketamine and of *N*-desmethylketamine (also known as norketamine). The results also indicated that 5,6-dehydronorketamine, identified previously as a major biotransformation product of ketamine in mammalian systems, is almost certainly a methodological artifact. In another study using rat, rabbit, and human liver microsomal preparations, norketamine was the major metabolite produced in all three preparations, with the 4-, 5-, and 6-hydroxynorketamines and possibly the 4- and 6-hydroxyketamines as minor metabolites.[145]

In dogs, the distribution half-life of ketamine was approximately 2 minutes, and the elimination half-life was approximately 61 minutes.[146] An investigation of the pharmacokinetics and metabolism of ketamine and its two major metabolites (norketamine and dehydronorketamine) was conducted using 10 horses undergoing airway surgery.[147] Concentrations of ketamine in plasma declined bi-exponentially with a rapid initial distribution phase ($t_{1/2}$ approximately 3 minutes), followed by a slower elimination phase with a half-life of approximately 65 minutes. Norketamine was detected at low concentrations in all samples, but dehydronorketamine was only detected in a few samples. In a more recent study, six healthy horses received a constant rate infusion of 1.5 mg/kg/h ketamine over a period of 320 minutes.[148] The mean concentration of ketamine in plasma during infusion was 235 ng/ml. The concentration–time curve post-infusion followed a two-compartment model for both ketamine and

norketamine, with ketamine half-lives of 2.3 and 67 minutes, respectively, for the initial and second phases of elimination, similar to those reported in the earlier study.

An investigation of the disposition and metabolism of ketamine administered to ponies demonstrated that metabolism is rapid, with both *R*-norketamine and *S*-norketamine detected in plasma samples taken within 1 minute of administration.[149] Concentrations of *S*-norketamine were higher than concentrations of *R*-norketamine in all samples taken 3 minutes or longer post-administration. Additional peaks were observed in the chromatograms produced by capillary electrophoresis which were attributed to enantiomers of metabolites of ketamine and norketamine, but these were not further identified. The results suggested that while the lipophilic nature of ketamine parent compound results in highest concentrations in fat, highest concentrations of metabolites, which are significantly more hydrophilic than the parent compound enantiomers, are found in the kidney. Metabolism occurs primarily in the liver and lung. Similar results were observed in an *in vitro* study using horse liver and lung microsomes where concentrations of *R*-ketamine exceeded those of *S*-ketamine at all time points.[141] It was also observed in this model system that concentrations of *S*-norketamine exceeded concentrations of the *R*-enantiomer in all samples in which concentrations were below C_{max}. These results suggested that stereoselective biotransformation of ketamine occurs in the liver and the lungs of horses.

Concentrations of ketamine and its metabolites in plasma were determined following administration of a single bolus-type i.v. dose of 10 mg/kg to 10 rabbits with normal renal function and to 9 rabbits with renal impairment.[150] The half-life of ketamine in plasma ranged from 0.74 hours in rabbits with normal renal function, but this increased to 2.6 hours in the animals with severe renal impairment. While concentrations of norketamine were similar in both treatment groups, the elimination kinetics of the dehydronorketamine were significantly different.

6.5.6.2 Marker Residue and Target Tissues

As ketamine is not registered for use in food-producing animals, no ADI or MRLs have been established by regulatory authorities, and therefore no target tissues or marker residue have been designated for enforcement of MRLs. Based on the available information, monitoring for residues of ketamine in tissues of food-producing animals should focus on testing of fat for parent compound or kidney for residues of the major metabolite, norketamine. As both parent drug and metabolites are optical isomers, analytical methods used should preferably be capable of providing a chromatographic separation of the optical isomers. The analyst should ensure that all isomers are captured in the chromatographic peak(s) which are used for quantification of ketamine residues.

6.6 Cocktails

When cocktails of two or more substances were injected, the behavior of individual compounds was unaffected, and elimination of each compound remained similar to that observed in animals treated with a single product.[48]

6.7 Issues of Environmental Contamination

There are a number of reports in the scientific literature from the past decade of the presence of diazepam as a contaminant in water and fish. In a study in which turbot (*Pleuronichthys verticalis*) caught in Southern California coastal waters were tested for the presence of 17α-ethynylestradiol, carbamazepine, diazepam, simvastatin, and oxybenzone, diazepam was detected in liver samples from all 10 fish at concentrations which ranged from 23 to 110 µg/kg.[151] Diazepam was also detected in water from the Douro River estuary in Portugal at concentrations up to 3.65 ng/l,[152] in surface waters from the Tagus River in Spain[153], and in the livers of turbot (*P. verticalis*) collected near four major waste water ocean outfalls in the Southern California Bight.[154] In a study of the occurrence of residues of illicit drugs and benzodiazepines in surface waters in the Madrid region of Spain, diazepam was found in all samples.[155, 156] Residues of diazepam have been detected in the freshwater invertebrate *Gammarus pulex*, and water samples collected from eight sampling sites on the River Thames[157] in the United Kingdom and in samples from two waste water treatment plants in New York state in the USA.[158] The evidence of widespread contamination of natural waters and fish which inhabit these waters suggests that monitoring for residues of diazepam as a contaminant (i.e., not as a residue resulting from veterinary use in aquaculture) may in future be a necessary element of the design of a residue monitoring program. There are no reports to date of which we are aware in which diazepam as a contaminant in drinking water or surface water has resulted in residues being detected in food-producing animals, but this may reflect a lack of testing for such contamination.

A recent study which investigated the fate of antianxiety medications, including acepromazine, azaperone, and xylazine, in incubated soil samples demonstrated that with the addition of powdered blood meal, commonly used as a fertilizer, dissipation of these drugs was enhanced.[159] Acepromazine and azaperone were strongly absorbed, with acepromazine showing an initial rapid depletion and azaperone being more persistent. Xylazine was persistent in soils under the conditions of the experiments, with low dissipation or degradation and a high leaching potential. The authors of this study concluded that there was a significant potential for leaching of xylazine from soils into surface waters and groundwater. A method to detect contamination of powdered blood meal used as fertilizer with antianxiety drugs has also recently been reported, but no contamination was detected in the four samples that were tested.[160] However, these studies do demonstrate the

potential for such drugs to become environmental contaminants of soil and water and to be introduced into food-producing animals from these sources.

6.8 Maximum Residue Limits (MRLs)

MRLs, established for compounds used as sedatives or tranquilizers by the Codex Alimentarius, the EU, and some of the world's largest food-producing/importing countries, are presented in Table 6.1. The MRL values for such drugs have been established for pigs, cattle, and horses, and the target matrices include the muscle, liver, kidney, fat/skin, and milk. MRLs have been established for azaperone (the marker residue is a sum of azaperone and azaperol) and carazolol (the marker residue is the parent substance), while the use of chlorpromazine has been prohibited in the EU.[46] MRLs have also been established by the Codex Alimentarius Commission for azaperone and carazolol, and Codex member states have been advised to prevent the use of chlorpromazine in food-producing animals by the Codex Alimentarius Commission.[161]

β-Blockers are also substances listed in the prohibited list of the World Anti-Doping Code by the World Anti-Doping Agency (WADA).[162]

6.9 Systematic Veterinary Control over Residues and Surveillance Studies

The results of the absorption, biotransformation, and excretion demonstrate, in the light of toxicological data, that there is a real risk of residues of sedatives and tranquilizers being present in animal-derived foods at concentrations which may exceed MRLs (or as residues of non-approved substances) as a result of failure with respect to withdrawal periods, illegal or unauthorized use, or potential for the administration of excessive quantities, any of which could present a health hazard for consumers.

Guidelines for the design and implementation of national regulatory food safety assurance program associated with the use of veterinary drugs in food-producing animals have been laid down by the Codex Alimentarius document CAC/GL 71-2009.[163] The EU has also established a framework for residue monitoring in animals and animal products, laid down by the Council Directive 96/23/EC,[164] where sedatives and tranquilizers are inserted into the Group B2d of Annex I and should be monitored in bovine, ovine, caprine, porcine, and equine animals. Regulatory authorities have developed analytical methods primarily for kidney since this tissue has the highest assayable concentrations of these substances at short withdrawal times, while the analysis of the injection site also offers the potential for residue determination. Based on the concerns expressed by the JECFA and the EMEA concerning residues of substances such as carazolol and

Table 6.1 Maximum residue limits (MRLs) of sedatives and tranquilizers (μg/kg), established by the Codex Alimentarius, the European Union, and some of the world's largest food-producing/importing countries.

Analyte	Animal species	Target tissues	Codex Alimentarius[161]	European Union[46, 227]	Australia[228]	Brazil[192]	Canada[229]	PR China[230]	India[231, 232]	Russian Federation[233]	USA[234]
Azaperone (marker residue is a sum of azaperone and azaperol)	Pigs	Muscle	60	100	200	—	—	60	—	60	—
		Liver	100	100	200	—	—	100	—	100	—
		Kidney	100	100	200	—	—	100	—	100	—
		Fat/skin	60	100	—	—	—	60	—	60	—
Carazolol	Pigs	Muscle	5	5	—	5	—	—	—	5	—
		Liver	25	25	—	—	—	—	—	25	—
		Kidney	25	25	—	—	—	—	—	25	—
		Fat/skin	5	5	—	—	—	—	—	5	—
	Cattle	Muscle	—	5	—	—	—	—	—	—	—
		Liver	—	15	—	—	—	—	—	—	—
		Kidney	—	15	—	—	—	—	—	—	—
		Fat/skin	—	5	—	—	—	—	—	—	—
		Milk	—	1	—	—	—	—	—	—	—
Acetylpromazine	Pigs	Kidney	—	—	—	10	—	—	—	—	—
	Cattle	Kidney	—	—	—	10	—	—	—	—	—
	Horses	Kidney	—	—	—	10	—	—	—	—	—

(Continued)

Table 6.1 (Continued)

Analyte	Animal species	Target tissues	Codex Alimentarius[161]	European Union[46, 227]	Australia[228]	Brazil[192]	Canada[229]	PR China[230]	India[231, 232]	Russian Federation[233]	USA[234]
Chlorpromazine	Pigs	Kidney	Serious health risk identified; residues should be prevented by not using in food-producing animals	Prohibited in all food-producing animals	—	10	—	Allowed to be used for treatment but not to be detected in food of animal origin	—	Residues should be prevented by not using in food-producing animals	—
	Cattle	Kidney	—	—	—	10	—	—	—	—	—
	Horses	Kidney	—	—	—	10	—	—	—	—	—
Ketamine	—	—	—	—	—	—	—	Allowed to be used but not required to develop a residue limit in food of animal origin	—	—	—
Xylazine	—	—	—	—	—	—	—	Allowed to be used for treatment but not to be detected in food of animal origin	—	—	—

chlorpromazine, a focus on the identification and testing of probable injection sites for residues of these substances may afford an additional layer of consumer protection.

Rauws and Olling reported the results of the testing of pig kidneys in the Netherlands in 1975, when 22 out of 27 random samples (81%) in the province of Noord-Brabant contained azaperol (at an average of 300 μg/kg) and azaperone (at an average of 120 μg/kg).[20] A study in Germany (former FRG) also demonstrated the incorrect use of neuroleptic preparations.[47] In 27 out of 380 pig kidneys (7%), sampled in the province of Baden-Württemberg, residues of azaperol (azaperone) were detected at concentrations ranging between 40 and 1260 μg/kg. In the mid-1980s, another study in Germany (former FRG) tested the liver and kidneys of 23 randomly selected slaughter pigs from different slaughterhouses, with residues of carazolol found in 22 samples.[165] Concentrations of carazolol found in the liver and kidneys ranged between 0.48 and 10.65 μg/kg and between 0.51 and 8.63 μg/kg, respectively. Although these concentrations did not exceed the MRL values, the results of the survey showed the widespread use of carazolol. More recently, the routine residue testing of pig and cattle kidney samples in Poland revealed one pig kidney with 37.5 and 6.2 μg/kg of azaperone and azaperol, respectively.[13] No notifications involving residues of sedatives or tranquilizers in animal-derived foods have been reported in the Rapid Alert System for Food and Feed (RASFF) of the EU for the period 2010 to the preparation of this chapter.[166]

In a Chinese survey which included local markets in Beijing municipality, chlorpromazine and diazepam were both found in five samples of pig muscle, with concentration ranges of 14.3–93.6 μg/kg and 6.1–41.3 μg/kg, respectively.[5] Haloperidol was detected in two samples of muscle at 8.5 and 0.5 μg/kg. From the class of β-blockers, only metoprolol was found in one pig sample at 3.5 μg/kg. In another study of retail samples in China, chlorpromazine was detected in one pig liver sample, taken at the local market, at a concentration of 0.11 μg/kg.[167]

Apart from the sedatives and tranquilizers discussed earlier in this section, other compounds appeared in illegal cocktails such as chlorprothixene and cyamemazine, not initially intended for food-producing animals.[48]

6.10 Analyte Stability

The stability of sedatives and tranquilizers in incurred kidney samples was demonstrated using incurred materials which, following initial analysis, were portioned into 5-g portions and then stored at different temperatures, which included −30, 4, and 20 °C in the dark.[14] Materials stored at −80 °C were used as a reference for the stability testing. Samples were analyzed after periods of 2 days; 1, 2, and 4 weeks; and 2, 6, and 12 months of storage. Results demonstrated that incurred residues in the sample material were stable when stored at −30 °C for at least 2 months. All analytes were stable in standard solution for at least 1 month

when stored at 4 °C in the dark. The sample extracts, kept in properly sealed HPLC vials in the refrigerator at 4 °C, were stable for a minimum of 2–3 days. The authors indicated that the effect of longer time storage of the incurred materials was being evaluated.

Analytes remained stable in edible swine tissue matrices for 2 and 4 days at room temperature and at 4 °C, respectively, and in solvent for 3 and 6 days at room temperature and at 4 °C, respectively.[167] Azaperone and azaperol were stable for 3 weeks in frozen spiked (homogenized) tissues at −20 °C.[168]

6.11 Analytical Methods for Determination of Residues

Many techniques are currently available for detecting the possible presence of sedatives and tranquilizers in biological samples in clinical and forensic toxicology and food of animal origin. Analytical methods for determination of these substances in food-producing animals and animal products serve as a useful tool for ensuring food safety in the national residue monitoring schemes as well as *in vivo* research on their pharmacological effects and tissue metabolism.

A review of the "traditional" chemical methods for the determination of these residues shows that the first "boom" in the development of analytical methods for these substances was at the end of the 1980s, with methods developed by researchers mainly in the former FRG[67, 169–171] and the Netherlands[45, 172–174], that is, in the countries in which the regulatory authorities were at that time apparently the most concerned about the use or potential abuse of these substances. The authors analyzed for individual substances and used the so-called multi-analyte methods which typically covered the whole group of sedatives and tranquilizers. This latter approach meant savings in time, chemicals, equipment, and financial resources.

The second analytical "boom" was post-2000, with the accelerated use of mass spectrometric detection. Liquid chromatography or gas chromatography coupled with single or tandem mass spectrometry (LC-MS(/MS) or GC-MS(/MS)) has become the most effective residue technique as it has the great advantage of providing information on the molecular structure of the analytes, enables study of metabolism and pharmacokinetics, and is also highly selective and sensitive to fulfill the regulatory requirements. In the twenty-first century it has, therefore, become a technique of choice as it provides the advantages of time-savings, convenience, and rapidness. Moreover, the introduction of new hyphenated MS techniques like time-of-flight (TOF) or Orbitrap™ has opened new strategies in the development of very selective and accurate multi-class multi-residue (MCMR) methods for simultaneous determination of more than 100 compounds in complex biological matrices (see Chapter 3 for a full discussion of contemporary high-resolution mass spectrometry (HRMS) techniques).

A summary of the analytical methods for determination of sedatives and tranquilizers in food-producing animals and derived products published after the year

2000 and including also some earlier selected analytical methods is presented in Table 6.2.

6.11.1 Matrices

The target tissue (matrix) for the determination of residues of sedatives and tranquilizers is predominantly the kidney, where it has been shown that the residue content is typically the highest, although most analytical methods should also be capable of determining these substances in liver and muscle tissues. Analytical methods have been used for the analysis of pig tissues, especially for pig kidneys.[175] This is quite reasonable, based on the most common use, available pharmacokinetic parameters, residue depletion, and monitoring data. A testing method for the determination of azaperone and azaperol in the kidneys of various animal species including horse was also developed,[176] while cattle tissues (e.g., liver) were also targeted for testing.[177] Aoki et al. tested for azaperone and azaperol in a variety of pig, cattle, and poultry matrices (kidney, liver, muscle) and also included milk, eggs, and salmon muscle tissue.[168]

The urine of various animal species has also been used for testing as it is a very convenient sample that is easily obtained from both live and slaughter animals, it is a somewhat cleaner matrix than, for example, offal tissues, and it does not require time-consuming homogenizing steps.[8, 178, 179] It is also used as the target matrix for doping control in horses.[180]

Sedatives and tranquilizers have been determined in milk and dairy products[15] and were also included as a class group in the comprehensive MCMR testing method for milk developed by Stolker et al.[181] Sedatives and β-adrenergic blockers were determined also in blood meal, a matrix being used in the Republic of Korea as an environment-friendly agricultural material but which had not previously been included in relevant studies regarding the presence of veterinary drugs residues.[182]

6.11.2 Extraction from the Matrix

The extraction and the purification of samples are based on the fundamental characteristics of sedatives and tranquilizers, which typically contain two or three nitrogen atoms. In aqueous solutions they are therefore in the charged (protonated) form of salts or esters and as such easily transfer from the organic phase to an aqueous phase. In an alkaline environment their dissociation is minimized, and therefore they are highly soluble in the non-polar solvents. Acetonitrile has been the extraction solvent most commonly used because of its high ability to precipitate proteins, to denature enzymes, and to produce an acceptable concentration of matrix-related co-extractives in the sample extract.[12, 68, 182, 183] Diethyl ether,[56, 184, 185] petroleum ether,[91] *tert*-butyl methyl ether,[186, 187] toluene,[188, 189] dichloromethane,[49] chloroform[190], and acetone[191]

Table 6.2 Current (published since 2000) and selected earlier analytical methods for determination of sedatives and tranquilizers in food-producing animals and derived products.

Analytes	Matrix	Instrumentation	Detection			References
			AZN, AZL	CAR	Others	
CAR	Muscle, liver, kidney (pig)	HPLC-FL		LOD: 0.33 µg/kg		Rudolph and Steinhart[68]
AZN, AZL, CAR, PP (ACP, CLP, XYL, HAL)	Muscle, liver, kidney (pig)	TLC-UV	LOD: AZN, AZL 50 µg/kg	LOD: 125 µg/kg	LOD: PP 25 µg/kg	Haagsma et al.[45]
CAR, ACP, AZN, AZL, CLP, HAL, PP, XYL	Kidney (pig)	HPLC-UV/FL	LOD: AZL 2 µg/kg (FLD), AZN 1 µg/kg (UV)	LOD: 0.3 µg/kg (FLD)	LOD: ACP, HAL 2 µg/kg, PP, XYL 4 µg/kg, CLP 6 µg/kg	Keukens and Aerts[173]
AZN, AZL, CAR, XYL, HAL, ACP, PP, CLP	Kidney (pig)	HPLC-DAD	LOD: AZN, AZL <1 µg/kg	LOD: <1 µg/kg	LOD: XYL, HAL 2.5 µg/kg, ACP, PP 5 µg/kg, CLP 10 µg/kg	van Ginkel et al.[174]
		TLC	LOD: AZN, AZL 1 µg/kg	LOD: 0.3 µg/kg	LOD: XYL 3 µg/kg, HAL 6 µg/kg, ACP, PP, CLP 0.3 µg/kg	
AZN, AZL, CAR, CLP, PRO, PP, TFP, XYL	Kidney, liver, muscle (pig, cattle)	GC-TID, HPLC-FL (CAR)	LOD: AZL 30 µg/kg, AZN 10 µg/kg	LOD: 0.04 µg/kg (FLD)	LOD: CLP 3 µg/kg, PRO, PP 7 µg/kg, TFP 6 µg/kg, XYL 200 µg/kg	Vogelgesang[170]

AZN, AZL, CAR, ACP, CLP, PP	Tissues, urine	HPLC-UV/FL	Not stated	Not stated	Not stated	Arneth[199]
AZN, AZL, CAR, XYL, HAL, ACP, PP, CLP	Kidney, liver (pig)	HPLC-ED	LOD: AZN, AZL 2 µg/kg	LOD: 2 µg/kg	LOD: 2 µg/kg	Rose and Shearer[195]
AZN, AZL, CLP, ACP, PP, HAL, XYL	Urine	GC-MS	LOD: AZN 5 µg/l, AZL 50 µg/l		LOD: CLP, ACP, PP 5 µg/l, HAL 10 µg/l, XYL 20 µg/l	Olmos-Carmona and Hernández-Carrasquilla[178]
AZN, AZL, CAR	Kidney, muscle (pig)	LC-MS/MS	LOQ kidney: AZL 3.51 µg/kg, AZN 1.76 µg/kg; LOQ muscle: AZL 0.73 µg/kg, AZN 2.55 µg/kg	LOQ kidney: 1.23 µg/kg; LOQ muscle: 0.13 µg/kg		Fluchard et al.[1]
XYL, CLP, ACP, AZN, AZL, PP, CAR	Kidney (pig, cattle)	LC-MS/MS	LOD: 1 µg/kg	LOD: 1 µg/kg	LOD: 1 µg/kg	Kaufmann and Ryser[2]
CLP	Muscle, urine	HPLC-UV			LOQ: muscle 50 µg/kg, urine10 µg/kg	Kožuh Eržen[198]
CAR	Muscle, kidney (pig)	RIA		LOD muscle: 0.93 µg/kg; LOD kidney: 1.47 µg/kg		Meenagh et al.[56]

(Continued)

Table 6.2 (Continued)

Analytes	Matrix	Instrumentation	Detection			References
			AZN, AZL	CAR	Others	
CLP, ACP, PP, AZN, AZL, CAR, XYL	Kidney, liver, muscle (pig)	LC-MS/MS	LOD: 10 µg/kg	LOD: muscle 1 µg/kg; kidney 5 µg/kg	muscle: CCα promazines, XYL 1.3 – 1.4 µg/kg; kidney: CCα promazines, XYL 2.8 – 3.1 µg/kg	Delahaut et al.[3]
PP, CLP, ACP	Liver (cattle)	HPLC-UV, HPLC-FL			LOD HPLC-UV: 31 – 45 µg/kg, LOD HPLC-FL: CLP 20 µg/kg, ACP 173 µg/kg, PP 102 µg/kg	Quintana et al.[177]
CLP, ACP, PP, AZN, AZL, CAR, XYL	Kidney, liver, muscle	LC-MS/MS	CCα muscle: AZL 56.1 µg/kg, AZN 56.3 µg/kg; CCα kidney: AZL 57.6 µg/kg, AZN 55.4 µg/kg	CCα muscle: 5.6 µg/kg; CCα kidney: 28.3 µg/kg	CCα muscle 0.3 – 1.1 µg/kg, CCα kidney 0.5 – 1.5 µg/kg	Delahaut et al.[48]
AZL, AZN, CAR, ACP, CLP, PP	Kidney (pig)	ELISA	CCβ: AZL 5 µg/kg, AZN 15 µg/kg	CCβ: 5 µg/kg	CCβ: ACP, PP 5 µg/kg, CLP 20 µg/kg	Cooper et al.[208]
MCMR for clean-up, including CAR, AZN, ACP, and HAL	Kidney (pig)	HPLC-UV	Not stated	Not stated	Not stated	Stubbings et al.[183]

4 compounds including DIA	Muscle (pig)	GC-MS			LOD: 2–5 µg/kg	Wang et al.[193]
MCMR, 130 compounds, including CAR, AZN, CLP, and XYL	Muscle (cattle, pig, poultry)	LC-MS/MS	LOQ: AZN 0.5–0.7 µg/kg	LOQ: 2 µg/kg	LOQ: CLP 0.7–0.8 µg/kg, XYL 1 µg/kg	Yamada et al.[220]
MCMR, 250 basic compounds, including ACP, AZL, HAL, PRO, PMT, PP, and XYL	Urine (horse)	LC-QLIT MRM, EPI	LOD: AZL 1 µg/l		LOD: ACP 10 µg/l, HAL/PRO 1 µg/l, PRO/PMT 100 µg/l, PP 1 µg/l, XYL 10 µg/l	Stanley and Foo[180]
AZN, AZL	Kidney (pig, cattle, poultry, horse)	HPLC-FL	LOQ: AZN 10 µg/kg, AZL 5 µg/kg; CCα: AZN 112 µg/kg, AZL 111 µg/kg			Cerkvenik-Flajs[176]
MCMR, >100 compounds, including AZL, AZN, PP, ACP, XYL, CLP, and CAR	Urine	UHPLC-TOF -MS	LOD: AZN 1.7 µg/l, AZL 3.8 µg/l	LOD: 0.7 µg/l	LOD: 0.5–1.7 µg/l	Kaufmann et al.[211]
CLP	Urine (pig)	HPLC-UV			LOQ: 20 ng/ml	Song et al.[4]

(Continued)

Table 6.2 (Continued)

Analytes	Matrix	Instrumentation	Detection			References
			AZN, AZL	CAR	Others	
MCMR, >100 compounds, including AZL, AZN, PP, ACP, XYL, HAL, CLP, and CAR	Milk	UHPLC-TOF-MS	CCβ: 1.4 µg/l	CCβ: 1.3 µg/l	CCβ: 1.2–2.9 µg/l, CLP: ND	Stolker et al.[181]
MCMR, >100 compounds, including AZL, AZN, PP, ACP, XYL, CLP, and CAR	Kidney, liver, muscle	UHPLC-TOF -MS	CCα muscle: AZL, AZN 110.9–125.4 µg/kg; kidney: AZL, AZN 132.5–161.9 µg/kg; liver: AZN 117.9 µg/kg	CCα muscle: 5.9 µg/kg; kidney: 1.9 µg/kg; liver: 16.1 µg/kg	CCα muscle: promazines, XYL 1.1–1.2 µg/kg; kidney: promazines, XYL, 1.2–1.6 µg/kg; liver: promazines, XYL, 1.2–1.3 µg/kg	Kaufmann et al.[221]
CLP, PP, ACP, TFP, PRO, AZN, AZL, CAR	Kidney (pig, cattle)	LC-MS/MS	CCα: AZN 105.5 µg/kg, AZL 121.4 µg/kg	CCα: 16.7–21.9 µg/kg	CCα 5.8–6.6 µg/kg	Mitrowska et al.[13]
19 β-Blockers, including CAR; 11 Sedatives, including AZN, XYL, CLP, ACP, and HAL	Kidney, liver, muscle (pig), muscle (cattle)	LC-MS/MS	LOQ: 0.5 µg/kg	LOQ: 0.5 µg/kg	LOQ: 0.5 µg/kg	Zhang et al.[5]
AZN, AZL	Kidney, liver, muscle, milk, eggs (pig, cattle, poultry, fish)	HPLC-UV; LC-MS	LOQ: 25 µg/kg (HPLC-UV)			Aoki et al.[168]

MCMR, 150 compounds, including CAR, PRO, TFP, and XYL	Milk	UHPLC-TOF		LOD: 0.5 µg/l; CCα: 0.3 µg/l	LOD: 1 – 5 µg/l; CCα: 0.8 – 10.7 µg/l	Ortelli et al.[215]
DIA, CLP, PMT	Liver, muscle (pig)	GC-MS			LOQ: 0.5 – 1 µg/kg	Cheng et al.[6]
AZN, CAR, CLP	Urine	HPLC-UV/FL	CCα AZN: 13.34 µg/l	CCα: 8.17 µg/l	CCα CLP: 5.86 µg/l	Sell et al.[200]
CLP, PMT, AZN, AZL, CAR	Kidney, liver, muscle (pig)	LC-MS/MS	LOQ kidney and liver: 0.4 µg/kg; LOQ muscle: AZL 0.4 µg/kg, AZN 0.2 µg/kg	LOQ kidney, liver, muscle: 0.4 µg/kg	LOQ kidney and liver: 0.4 µg/kg; LOQ muscle: 0.2 µg/kg	He et al.[167]
16 Sedatives and adrenergic blockers, including ACP, AZN, CLP, PRO, PP, TFP, XYL, and CAR	Powdered blood meal	Orbitrap™	LOQ: AZN 10 µg/kg	LOQ: 4 µg/kg	LOQ: 2 – 4 µg/kg	Choi et al.[182]
25 β_2-Agonists and 23 β-blockers, including CAR	Kidney, liver, muscle (pig)	LC-LIT-MS ($LC\text{-}MS^3$)		CCα MS^2: 0.9 – 2 µg/kg, MS^3: 0.15 – 0.33 µg/kg		Fan et al.[179]

(Continued)

Table 6.2 (Continued)

Analytes	Matrix	Instrumentation	Detection			References
			AZN, AZL	CAR	Others	
MCMR, 118 compounds, including AZN, XYL, CAR, ACP, HAL, PMT, PP, CLP, and TFP	Kidney (cattle)	LC-MS/MS	Not stated	Not stated	Not stated	Schneider et al.[217]
MCMR, 47 compounds, including AZN, AZL, CAR, PRO, CLP, ACP, PP, and TFP	Muscle	LC-MS/MS	LOD: 10 µg/kg	LOD: 10 µg/kg	LOD: 1 µg/kg	Galarini et al.[214]
CAR, AZN, AZL, ACP, CLP, PP, HAL, XYL	Kidney (pig)	LC-MS/MS	CCβ: AZN 115 µg/kg, AZL 130 µg/kg	CCβ: 28 µg/kg	CCβ: <5 µg/kg	Laza et al.[213]
MCMR, 47 compounds, including AZN	Muscle (cattle, pig, chicken)	IT-TOF MS	Validated at 30 µg/kg			Park et al.[219]
CLP, ACP, PP, AZN, AZL, CAR, HAL, XYL	Kidney (pig, cattle)	LC-MS/MS, QTrap	CCα: 56.5–57.3 µg/kg	CCα: 17.2 µg/kg	CCα: 3.24–6.32 µg/kg	Bock and Stachel[14]
20 Compounds, including HAL, PMT, CLP, and DIA	Milk, dairy products	UHPLC-TOF MS			LOQ: 1–5 µg/kg	Yan et al.[15]

25 β_2-Agonists and 21 β-blockers, including CAR	Urine (pig, human)	LC-LIT MS (LC-MS^3)		LOQ: 0.3 µg/kg		Fan et al.[8]
5 Sedatives (ACP, AZL, AZN, CLP, XYL) and 14 β-blockers, including CAR	Kidney (pig)	LC-MS/MS	Validated at 10 µg/kg	Validated at 10 µg/kg	Validated at 10 µg/kg	de Oliveira et al.[192]
15 Compounds, including XYL, AZN, CAR, HAL, PMT, ACP, PP, and CLP	Meat (sheep/mutton)	LC-MS/MS	LOQ: AZN 5 µg/kg	LOQ: 4 µg/kg	LOQ: 0.5–5 µg/kg	Wei et al.[12]

ACP, acetylpromazine (acepromazine); AZN, azaperone; AZL, azaperol; CAR, carazolol; CLP, chlorpromazine; DIA, diazepam; HAL, haloperidol; PMT, promethazine; PP, propionylpromazine (propiopromazine); PRO, promazine; TFP, triflupromazine; XYL, xylazine; MCMR, multi-class multi-residue.

were also used as extraction solvents. The analytes were usually extracted from alkaline matrix extract in the un-ionized form.

The majority of sedatives and tranquilizers possess an amino or hydroxyl group in their molecular structure and are thus combined with glucuronic acid or sulfuric acid *in vivo* in the process of metabolism in animals to form glucuronides and sulfates.[12, 179, 180] These bound analytes are dissociated and released by enzymatic hydrolysis prior to extraction.[8] Hydroxylated metabolites of azaperone in horse urine were released from their conjugate glucuronic form with β-glucuronidase/sulfatase, followed by addition of alkali and organic extraction.[49] The bound analytes were also dissociated from sheep muscle (mutton) and released by enzymatic hydrolysis before extraction. The recovery was thus increased to over 80% by adding β-glucuronidase/arylsulfatase to the alkaline extraction solvent compared to less than 50% by only using extraction solvent.[12]

To effectively achieve contact between sample and extractant and further disintegration, an Ultra-Turrax® (IKA Works GmbH & Co., Staufen, Germany) or similar homogenizers have been used.[192] Ultrasonication is also very effective for quantitative extraction[173, 193, 194]; moreover, it is capable of handling multiple samples simultaneously and excludes their mutual contamination. In addition, Ultra-Turrax® also demands the use of a clean dispersing rod for each sample extraction and therefore requires a time-intensive cleaning of the equipment. Use of an overhead shaker (Heidolph Instruments GmbH, Schwabach, Germany) was also reported as an efficient extraction technique.[14] In a method for the determination of residues of azaperone and azaperol in pig liver by GC-MS, the best homogenization of the samples was obtained by using solid CO_2 (dry ice).[194] Liver chunks were quickly ground, together with the pre-ground CO_2 into a fine powder. The CO_2 was then allowed to sublime at −20 °C in a freezer, leaving a fine powder of frozen liver.

6.11.3 Clean-up of Sample Extracts

In the "traditional" methods,[47, 195, 196] extraction of the sedatives and tranquilizers was usually followed by such classical separation procedures as liquid–liquid extraction (LLE) by acidifying the crude extract. Acidification enabled further purification with *n*-hexane[169, 170, 197] or petroleum ether,[196] which removed the non-polar lipid co-extractives. If the extract was then made alkaline, re-extraction with organic solvents such as diethyl ether or *tert*-butyl methyl ether was possible.[175, 197] For further purification of the extracts, columns packed with basic alumina,[44, 170] silica,[68, 189], or acidic Celite[190] sorbents have been used. Clean-up using the "manually" packed columns was the forerunner of the use of commercially prepared extraction columns for liquid–solid extraction, the so-called solid-phase extraction (SPE). In SPE, sample extract is absorbed on the activated sorbent of the extraction column on the basis of specific interactions, followed by washing of impurities and then elution of the target substance(s).

Depending on the polarity of the various sedatives and tranquilizers, the published SPE methods have used different types of sorbents, including polar silica,[45, 172] diol,[174, 175] and the essentially non-polar C_{18}[172, 173, 198] and C_8[198, 199] materials. The addition of a salt (e.g., NaCl buffer) to the extracts as first reported by Keukens and Aerts[173] has since been used in other methods to further increase the affinity of the sedatives and tranquilizers to the reversed-phase silica SPE columns to improve recovery and resolution of chromatograms.[178, 187, 194, 198]

It should be noted, however, that the use of SPE may result in the loss of the analytes during the sample loading step, especially for the highly hydrophilic analytes. This is of vital importance for the comprehensive screening methods, where all the possible analytes are required to be extracted from the samples without a significant loss.[15] The recovery of chlorpromazine in muscle thus was found to remain low due to interactions with the active silanol groups of the silica-based SPE sorbent.[198] Triethylamine (TEA) has also been added during SPE activation, sample addition, washing, and elution to prevent uncontrolled interactions of analytes with free silanol groups on the silica surface.[178] The use of a non-ionic resin-type EXtrelut® SPE cartridge (Merck, Darmstadt, Germany), which allows the application of an aqueous solution, can also reduce the loss of analytes from such interactions.[47, 188]

Other issues may cause problems in the analysis of these substances. For example, it was found that final re-suspension and extraction of urine samples were very efficient when using either carbonate–bicarbonate buffer[186] or *tert*-butyl methyl ether.[178] In order to prevent the adsorption of sedatives and tranquilizers onto glass surfaces which was reported in an early study,[91] it was demonstrated that washing glass prior to use with a basic solution,[68, 173] use of silanized glassware[91, 199], or use of plastic materials[12, 173, 176] addressed this issue.

6.11.4 Measurement Techniques

6.11.4.1 High-Performance Liquid Chromatography (HPLC)

Among the "traditional" chromatographic approaches, HPLC has been the dominant analytical technique used in the analysis for residues of sedatives and tranquilizers in samples from food-producing animals, with detection of target analytes by fluorescence or by their absorption in the UV range. The use of fluorescence detection eliminates interferences from biological matrices and thus allows greater selectivity and detection at lower concentrations.[68, 169–173] Two methods (from 1984 and 1992) also used electrochemical detection, which proved to be very selective and sensitive,[195, 196] as the interfering background in fluorescence detection of carazolol, which was mentioned in the literature in the same time period,[67, 199] was successfully removed by oxidation. Detection by consecutively connected electrochemical and fluorimetric detectors therefore appears to be an ideal approach when mass spectrometric detection is not available, particularly for confirmation of carazolol, although the recent literature provides no evidence of such applications.

A number of methods for either individual tranquilizers and their metabolites or multiple tranquilizers which use UV detection have been published, and it has been shown to be the better choice for determination of some of these analytes. For example, a method published in 1993 for the determination of xylazine and its major metabolite, 2,6-dimethylaniline, in cattle and pig kidney used HPLC with UV detection at 225 nm.[190] Quintana et al. subsequently demonstrated that fluorescence is not a good detection technique for acetylpromazine and propionylpromazine and that for these two analytes, UV detection is preferred.[177] Subsequently, a HPLC method was described by Sell et al., which coupled UV and fluorimetric detectors for the determination of azaperone, carazolol, and chlorpromazine residues in animal urine.[200] Two HPLC methods for determination of the residues of azaperone and azaperol have been published in the past decade, utilizing UV ($\lambda = 250$ nm)[168] and fluorescence detection ($\lambda_{ex} = 245$ nm, $\lambda_{em} = 345$ nm).[176] A method for the simultaneous determination of azaperone, acetylpromazine, carazolol, and haloperidol by HPLC-UV detection at 240 nm has also been described.[183]

The stationary phases in the analytical columns were reversed phase in all methods reported. The authors mentioned great difficulties which included peak tailing, column batch-to-batch irreproducibility, poor recoveries, and insufficiently resolved chromatograms. The latter issue was attributed to the irreversible retention of sedatives and tranquilizers as basic compounds by active silanol groups on the surface of the stationary phases.[178] It has also been reported that promazines in particular were strongly retained by the free silanol groups.[201] Therefore, stationary phases with well-deactivated silanol groups, for example, LiChrospher RP-Select B,[199, 201] SUPELCOSIL LC-ABZ,[202] or phenyl-[190, 196] or alkaline-tolerant silica RP columns,[2] have been used to overcome this problem. Trifluoroacetic acid (TFA) has also been added to the mobile phase.[187] In some cases, as reported for promazines, irreversible adsorption effects to the free silanol groups were too strong, even if deactivated stationary phases were used, so the detection was therefore performed by GC.[185]

As mobile phases with basic pH value can give very low retention with conventional silica-based columns, the mobile phases were generally isocratic or gradient mixtures of acidic buffers (acetate or phosphate) with acetonitrile in certain volume ratios[165, 187]: for example, a mixture of 0.05 M phosphate buffer, pH 4.5/MeCN/tetrahydrofuran (THF);[175, 176] a mixture of 0.05 M ammonium acetate, pH 6.5/MeCN/THF[203]; or a mixture of 0.01 M ammonium carbonate, 0.1 M tetraethylammonium chloride, MeCN, and methanol (MeOH).[183]

Overall, the two detectors used with HPLC for quantitative determination have been fluorescence and UV, with each offering advantages for the determination of certain sedatives and tranquilizers. While a combination of these two detectors may be a preferred choice when targeting multiple compounds (when a mass selective detector is not available), a choice should be made based on the target analytes if only one of these detectors can be used.

6.11.4.2 Thin-Layer Chromatography (TLC)

Thin-layer chromatography (TLC) was commonly used in the determination of residues of tranquilizers in the 1980s. For example, propionylpromazine was selectively determined in pig tissues by mono-dimensional development of TLC plates with detection using a densitometer in the fluorescence scanning mode.[91] Methods have also used two-dimensional panels[45, 174] which after a preliminary purification of samples allowed extreme sensitivity.[174]

TLC has also been used as a method to separate and purify residues of these substances for further analysis using more advanced analytical techniques. A method reported in the mid-1980s used TLC plates developed in seven solvent systems for the analysis of propionylpromazine and its metabolites in horse urine, supported by UV and nuclear magnetic resonance (NMR) spectrometry and GC-MS detection.[204] A semi-quantitative TLC screening method reported in 1996 to detect and isolate hydroxylated metabolites of azaperone in horse urine treated with β-glucuronidase/sulfatase provided further identification and confirmation with GC-MS analysis.[49]

No more recent methods were found in the literature search which indicated significant use of TLC in the analysis for residues of sedatives and tranquilizers in food-producing animals. However, the approach still can be applied in some circumstances, such as when access to more advanced techniques is limited or as a preparative tool for further analysis of residues of these substances.

6.11.4.3 Gas Chromatography (GC)

Prior to the widespread use of HPLC-based methods for the determination of residues of sedatives and tranquilizers and the development of LC column packings without active silanol groups on the surface of the stationary phases, methods using GC offered advantages for some residue control laboratories, particularly those already equipped with gas chromatographs for pesticide residue analysis. Methods for sedatives and tranquilizers based on GC primarily used selective types of detectors, such as alkali flame ionization and nitrogen–phosphorus detector (NPD) or thermionic ionization detector (TID), both being extremely sensitive to nitrogen,[170, 184, 185] or a flame photometric detector (FPD), fitted with a 394-nm filter, giving a specific response to sulfur compounds.[191] Both NPD and FPD were found to be especially appropriate for the determination of promazine and xylazine residues. Preparation of the samples in the GC methods was on average longer compared to other methods such as HPLC, mainly due to the derivatization step needed to overcome the thermal instability of the substances[47] and to enable the use of capillary analytical columns.[170] β-Blockers could be also determined in plasma, while samples were previously derivatized with the anhydride of heptafluorobutyric acid (HFBA). However, co-extracts in the liver and kidneys reduced the selectivity.[205] Contemporary GC methods generally use mass selective detectors, but today LC-based methods appear to be more commonly used for the determination of residues of sedatives and tranquilizers in samples from food-producing animals.

6.11.4.4 Immunochemical Methods

Immunochemical methods, either enzyme-linked immunosorbent assays (ELISAs) or radioimmunoassays (RIAs), allow efficient screening with single-analyte or multi-residue determination; however positive results have to be confirmed by a physicochemical method. ELISAs are widely used as high-throughput screening tests because they are highly sensitive, quick, relatively cheap, and reliable in their ability to distinguish between negative and suspected positive samples and they usually need a minimal sample clean-up.

Only a limited number of methods using an enzyme immunoassay (EIA) for determination of sedatives and tranquilizers were found in the literature. Over two decades ago, compound-specific EIAs using single antibodies have been reported for acetylpromazine and/or some of its metabolites in horse urine[206] and for carazolol in pig tissues and urine.[207] More recently, a rapid multi-analyte screening test for five veterinary sedatives and the β-blocker carazolol in pig kidneys by ELISA was developed by Cooper et al.[208] This test, reported in the literature in 2004, proved to be an efficient screening tool in the UK National Surveillance Scheme. Three antibodies were prepared and included in the screening test: against azaperol, carazolol, and propionylpromazine. The study revealed a significant difference in the cross-reactivity of the azaperol and propionylpromazine antibodies in buffer and sample matrix, arising from different affinities of polyclonal antibodies, depending on the temperature, pH value, and solvent. Azaperol antibody thus cross-reacted by 44.7% and 28.0% with azaperone in buffer and sample matrix, respectively, while propionylpromazine antibody cross-reacted with acetylpromazine and chlorpromazine in buffer by 63.1% and 56.2%, respectively, and in sample matrix by 24.9% and 11.7%, respectively. This demonstrated the necessity to perform immunoassays in the sample matrix, so that "real" conditions are taken into account, when characterizing antibodies.[208] The development of a highly sensitive, generic polyclonal antibody to the phenothiazines has been also reported in 2010.[209]

Among RIAs, a sensitive radioreceptor assay was developed for the determination of carazolol in pig blood plasma and urine in the 1980s.[69, 171] A rapid and sensitive radioreceptor assay was also reported in 2002 for the determination of carazolol in pig muscle and kidney using solubilized β_2-adrenoceptors isolated from a transfected cell line.[56] This assay also showed a potential affinity to several β-blockers, including labetalol, nadolol, pindolol, and propranolol, and a number of β-agonists. Minimal sample clean-up was required before application to the receptor assay.

Widespread use of such tests in residue control programs for residues of sedatives and tranquilizers in the testing of samples from food-producing animals has not been reported, with the exception of the screening test for five veterinary sedatives and the β-blocker carazolol in pig kidneys which was used in the UK National Surveillance Scheme.

6.11.4.5 Mass Spectrometry

A triple quadrupole (QqQ) MS has typically been used in contemporary analytical methods for these compounds, utilizing the selected multiple reaction monitoring (MRM) transitions, while use of high-performance liquid chromatography–linear ion trap mass spectrometry (LC-LIT MS) with consecutive reaction monitoring (CRM, MS^3) has also been reported.[8, 179] The use of an LC combined with a hybrid triple quadrupole/linear ion trap (QLIT) has also been reported, switching between MRM and enhanced product ion (EPI) spectral scan for data acquisation.[180]

The electrospray ionization (ESI) interface in a positive mode has been predominantly used, as the sedatives and tranquilizers which have the imine nitrogen group preferably form a stable $[M+H]^+$ ion. Therefore, the protonated $[M+H]^+$ molecular ions are selected as the precursor ions. For confirmation, two or three MRM transitions are used, while the most intense MRM transition is selected for quantification.[12, 167, 192] The atmospheric pressure chemical ionization (APCI) interface in a positive mode has also been used,[1, 3] while the negative APCI mode gave satisfactory results only for carazolol.[1]

In recent years, HRMS has been successfully applied for the determination of a large number of compounds and to control agricultural materials such as blood meal, derived from livestock industrial waste, which must be monitored for sustainable organic agriculture. Orbitrap™ MS was thus used to simultaneously determine 16 compounds.[182] Despite the multiple advantages of LC-HRMS, such as reduction of matrix effects, enlarged number of analytes in one run, confidence in identification, and post-acquisition data mining, this instrumentation has not yet been broadly adopted in routine residue control laboratories.[210]

TOF is even more powerful than triple quadrupole MS, especially in the case of the absence of analytical standards, as retention time, molecular weight, isotopic pattern, and drug group specific collision-induced (CID) MS (generic) fragments play a vital role in the identification of the analytes.[211] The high resolution of at least 10,000 full width at half maximum (FWHM) permits a significant selectivity in the resolution of chromatographic peaks from background interferences and therefore a high sensitivity gain.[181, 211, 212] An additional advantage of the TOF method is that not all compounds of interest have to be defined *a priori* as the collected full-scan spectra can be reprocessed and checked for the presence of the new compounds of interest.

GC-MS with or without derivatization with bis(trimethylsilyl)trifluoroaceta mide (BSTFA) to trimethylsilyl (TMS) derivatives before injection has also been used to detect sedatives and tranquilizers in urine and meat, which enabled direct structural information to reduce the risk of false-positive results.[187, 193] A GC-MS method was also developed to identify and confirm hydroxylated metabolites of azaperone in horse urine.[49] Both low- and high-resolution GC-MS were employed for the molecular structure identification, supported with NMR. While the data from LC-MS/MS and GC-MS/MS are usually scanned and collected in the MRM mode, using a specific acceleration voltage and specific

collision energy for each substance, the data for GC-MS determination were obtained in a selected ion monitoring (SIM) mode.[6, 178, 193]

As contemporary analytical methods can determine more and more compounds, MS resolution can be effectively supported by ultrahigh performance liquid chromatography (UHPLC). This low dead volume, high-pressure (1000 bar) LC equipment, using sub-2-μm particle size analytical columns, improves resolution while keeping good efficiency by maintaining or even shortening analytical run times.[12, 181] The use of UHPLC is especially powerful when biological extracts have to be screened because the additional LC selectivity compensates for the lack of selectivity in comparison with the MS/MS option of a QqQ MS.[181, 211]

Formic acid in water (e.g., 0.1%, v/v) as the mobile phase improves the protonation of target compounds during LC-ESI-MS/MS analysis by maximizing sensitivity and lowering tailing effect and was thus frequently used.[8, 167, 192] Other mobile phases used in LC-MS/MS included 0.01 M ammonium formate pH 3.8[213] and aqueous 0.1% acetic acid solution.[214] A mobile phase consisting of 0.1% formic acid/MeOH had even better chromatographic separation efficiency compared to 0.1% formic acid/MeCN due to the stronger elution capability of MeCN onto the C_{18} analytical column.[5] A mixture of TFA aqueous buffer (pH 3.5) and MeCN was also used as an LC-MS/MS mobile phase.[187] An alkaline MeCN gradient mobile phase provided a good chromatographic shape, and ammonia was chosen to render the mobile phase alkaline because of its volatility and better ESI response compared to the acidic mobile phases.[2]

The most important drawback of LC-MS/MS is ion suppression caused by the matrix effect of the non-drug-related co-eluting components in the original biological sample, which can have a significant influence on the quantitative analysis of sedatives and tranquilizers with LC-MS/MS[3, 12, 215] or GC-MS.[6] Matrix-matched calibration curves and stable isotopic internal standards have thus generally been used to overcome this problem. In addition, multiple MS detection, for example, using a linear ion trap spectrometer (LC-MS^3) with an ESI source in a positive mode, was observed to minimize ion suppression.[8, 179] A greater matrix effect was observed for liver and kidney compared to muscle, with more than 40% of ion suppression observed in a pig liver matrix, demonstrating a need for an additional clean-up step by SPE.[5] Extracts from pig kidney showed a slightly stronger suppression effect than extracts from cattle kidney, and chlorpromazine was the analyte most strongly affected, by 28%.[2] Significant matrix effect was also observed in the GC-MS analysis of urine for ketamine and haloperidol (both ion enhancement) and for xylazine and azaperone (both ion suppression).[178]

Methods to simultaneously detect the residues of more than 10 sedative and tranquilizer drugs in animal tissues are becoming more common, as discussed next. Wei et al. determined 15 analytes in mutton by LC-MS/MS,[12] while de Oliveira et al. developed a simultaneous determination of 5 sedatives and 14 β-blockers in pig kidney by LC-MS/MS.[192] High throughput screening of 20 tranquilizers in dairy products was developed by Yan et al.

using UHPLC-TOF MS.[15] Fan et al. developed methods for the simultaneous detection of 23 β-blockers, allied with 25 β_2-agonists, in pig tissues, including muscle, liver, and kidney,[179] and of 21 β-blockers, allied with 25 β_2-agonists,[8] in urine, respectively, by LC-LIT MS. Zhang et al. reported a rapid and comprehensive LC-MS/MS method for determination of 30 compounds (19 β-blockers and 11 sedatives) in animal tissues.[5]

MCMR methods are increasingly being used in regulatory monitoring programs and doping control due to their excellent analytical efficiency, with some such methods including a family of sedatives and tranquilizers. Galarini et al. simultaneously determined 47 basic drugs in muscle by LC-MS/MS, including 8 sedatives and tranquilizers.[214] Boison et al. developed and validated an LC-MS/MS method for detection and confirmation of 14 classes of veterinary drugs, encompassing over 100 compounds and their metabolites in fresh tissues and processed foods, including 9 tranquilizers.[216] Schneider et al. developed an LC-MS/MS method determining 118 analytes from 10 different classes and 135 analytes from 11 different classes in cattle kidney and cattle tissues, respectively.[217, 218] Park et al. reported an LC-IT-TOF MS quantitative method which screens simultaneously for 110 veterinary drugs in cattle, pig, and chicken muscle,[219] while Yamada et al. reported a simultaneous determination of 130 veterinary drugs in cattle, pig, and chicken muscle using LC-MS/MS.[220] Stolker et al.[181] and Ortelli et al.[215] reported methods which screened by UHPLC-TOF MS for more than 100 and 150 veterinary drugs and metabolites in milk, respectively. UHPLC-TOF MS was also utilized as a powerful tool for a multi-residue screening of about 100 veterinary drugs and their metabolites in urine and different meat matrices by Kaufmann et al.[211, 221] A rapid, selective, and robust direct-injection LC-QLIT MS method has also been developed by Stanley and Foo for simultaneous screening of more than 250 basic drugs in horse urine.[180]

6.11.5 Contemporary Sample Preparation Techniques

There is an increasing interest in more recently developed sample pre-treatment techniques, which may facilitate monitoring for residues in complicated biological matrices. A comprehensive review on the current trends in sample preparation for the isolation of veterinary drugs from foods, including sedatives and tranquilizers published in 2009, remains a good source of information.[222] The use of contemporary measurement techniques, such as LC-MS/MS, has simplified the purification of the crude sample extracts, which is thus often limited to evaporation, dilution, [48] and filtration.[13] For example, urine samples have been simply diluted and injected unfiltered into a UHPLC-TOF instrument.[211] However, there are a number of analytical challenges which have to be overcome in the MCMR methods (e.g., polarity, p*K* value) to isolate a wide range of residues from potential interferences which may be present in both simple and complex biological matrices.[183, 221]

6.11.5.1 Extraction

Contemporary analytical methods for the determination of residues of sedatives and tranquilizers still primarily use acetonitrile or acetonitrile/water extraction followed by SPE clean-up prior to the LC-MS/MS analysis.[5, 167] Acetonitrile has been the most commonly used organic solvent for extraction due to good extraction efficiency for a wide range of polarity of the sedatives and tranquilizers, being also supported by freezing of the crude extract at −20 °C to additionally precipitate the proteins and other interferences.[192, 200] Acetonitrile/water containing ammonium hydroxide and sodium chloride[213] and acetonitrile/water containing ammonium formate[219] were also used for extraction, as was methanol/water solution, which was followed by lipid removal using acetonitrile-saturated *n*-hexane.[217] Anhydrous sodium sulfate could also be used to dry the sample and improve the extraction efficiency of hydrophobic drugs (e.g., chlorpromazine, diazepam).[5, 167] A mixture of acetonitrile and methanol (95:5, v/v) has also been used for extraction of tranquilizers and other drugs from muscle followed by lipid removal with *n*-hexane.[220] Extraction with ammonia in ethyl acetate gave even better results and enabled also the removal of fats by *n*-hexane.[14] Sample extraction with acids, including milk by formic acid,[215] pig urine with perchloric acid[8], and pig tissues by trichloroacetic acid (TCA), which served as the extraction solvent and hydrolysis reagent,[179] has also been reported.

6.11.5.2 One-Step Sample Clean-up

Another tactic that has been used to reduce the quantity of the required test portion of sample material is integrating the sample preparation and chromatographic separation steps. A direct injection of the supernatant of enzyme hydrolyzed horse urine into the LC-MS/MS has been utilized to overcome the off-line sample pre-extraction, to reduce sample and solvent consumption, and to significantly increase the efficiency of the performance of the method, which is used for horse doping control.[180] Analytes were trapped using a short HLB® extraction column as a part of the HPLC system and were refocused and separated on a C_{18} analytical column.

Direct injection of crude sample extracts into the LC-MS/MS system by using an additional chromatographic separation may also be achieved by placing a Biomatrix® column upstream from the analytical column in order to eliminate most macromolecules in the sample extract. The ChromSpher Biomatrix® stationary phase consists of a combined phase: a hydrophobic part encapsulated by a hydrophilic outer layer. The latter rejects proteins by exclusion while drugs, because of their small size, can penetrate this layer and interact with the hydrophobic part, followed by entering the analytical column and mass spectrometer.[1, 3, 48]

Protein precipitation associated with ultra-filtration by the cut-off membrane of 3 kDa was combined with fast chromatography and enabled a simple, efficient, fast, and robust procedure for multi-class screening of more than 100 milk samples per day as reported by Ortelli et al.[215] The sample extracts were

even cleaner when compared to those obtained using SPE clean-up. The use of HybridSPE-Precipitation (HybridSPE™-PPT) has been also reported by León et al. as the only one-step clean-up technique before UHPLC-MS/MS, combining protein precipitation with the selectivity of the SPE for the selective removal of proteins and phospholipids from biological matrices.[210]

6.11.5.3 Multi-step Clean-up Techniques

Nitrogen-based functional groups of sedatives and tranquilizers can interact reversibly with hydrogen ions to form cations. Extracts containing these cations may be cleaned-up by SPE on cartridges packed with a cation-exchange sorbent, which may be either a bonded-phase silica or a polymeric resin. With the increasing challenges to achieve consistent and faster extractions, the frequently inconsistent and problematic performance of the conventional silica-based SPE technologies has led to the development and use of polymer-based sorbents, such as the strong cation-exchange (SCX) phase Strata-X™,[181, 200, 214] Bond Elut® of benzene sulfonate form,[183] or a hydrophilic and lipophilic balance-modified polymer Oasis® HLB,[1, 2] which gave more stable and improved recoveries compared to those obtained by silica-based C_{18} or NH_2 phases due to the absence of silanol groups.

Unlike classical silica-based SPE materials, the polymeric sorbents are stable under alkaline conditions, enabling the suppression of protonation of the analytes, particularly xylazine, and thus improving the recovery. Before loading onto the SCX phase, acetonitrile extracts were evaporated and then redissolved in an acidic solution to permit protonation of the analyte molecule.[214] Carazolol, for which recovery can be problematic by silica-based SPE clean-up,[183] was efficiently eluted from the polymeric SPE Oasis® HLB in a protonated form using acidified acetonitrile as an elution solvent.[2] A mixed SCX sorbent (Oasis® MCX) was also employed as it features two retention mechanisms (cation-exchange and reversed-phase) and can thus be manipulated very predictably, making the method selective, reproducible, simple, and fast, with acceptable recovery rates. This approach has been applied for determination of azaperone and azaperol in kidneys (pig, cattle, poultry, and horse),[176] residues of four sedatives in pig liver and muscle[6], and 23 β-blockers in pig kidneys, liver, and muscle, where a *n*-hexane wash step was also added into the SPE procedure.[179]

Specific adsorption materials, new molecularly imprinted polymers (MIPs), were also successfully used for pre-concentration and clean-up of sedatives and tranquilizers. MIP is a highly cross-linked polymer which possesses a cavity specifically designed in shape, size, and functional groups to retain the target molecule and thus has the ability to identify specific target compounds from a complex sample matrix. A MIP for chlorpromazine, for example, demonstrated specific selectivity, better separation efficiency, and higher recoveries compared to conventional C_{18} SPE sample preparation.[4] A previous extraction of urine with acetonitrile and a proper washing of the MIP sorbent with methanol

were still needed to assure optimal results, and some cross-selectivity toward phenothiazine analogues when extracting from urine was also observed. A MIP SPE for β-receptors was successfully used for the simultaneous clean-up of 21 β-blockers and 25 β_2-agonists in urine samples, which ensured a better clean-up than the normal SPE.[8] However, the multi-step washing procedures required were more time consuming than for normal SPE procedure.

A novel SPE using multi-walled carbon nanotubes (MWCNTs) was also developed by Wang et al. for determination of four benzodiazepine residues in pork.[193] Carbon nanotubes (CNTs) are hollow nanosized tubes that constitute a new structure of graphitic carbon with a large specific surface area and excellent adsorption ability, being significantly higher than that of C_{18} solid phase, especially for diazepam.

An analytical method, determining in total 16 sedatives and β-adrenergic blockers in powdered blood meal, was developed by Choi et al., employing accelerated solvent extraction (ASE) and dispersive SPE (dSPE) techniques.[182] The extraction conditions of ASE (extraction solvent, temperature, pressure, dispersant, assistant salts) were optimized, and additionally a combination of the C_{18} sorbent and primary secondary amine was used for dSPE to clean-up the extracts before injection into the Orbitrap™ mass spectrometer. dSPE with C_{18} sorbent has been successfully used in MCMR analytical methods.[218, 219] dSPE using a sorbent Celite® 545 was also used for the clean-up of 19 sedatives and β-adrenergic blockers in pig kidney by de Oliveira et al.[192]

Dimethyl sulfoxide (DMSO) was used to efficiently remove analytes adsorbed to the glass wall of the evaporation vessel and as a keeper in the final solvent exchange step, which was very beneficial for the recovery of promazine sedatives and tranquilizers in the MCMR method developed by Kaufmann et al.[221] The solution used for re-dissolving dry residues is also important in LC-MS/MS analysis for final protein and phospholipid precipitation. The experimental results showed that 2% ammonia in MeCN, which could achieve a sharp and narrow peak, was better than 0.1% formic acid in water, 0.1% formic acid in MeCN, or the combination of the two solutions.[167] The dry residue has also been dissolved in MeCN/0.025% aqueous diethylamine mixture (2:3, v/v) prior to HPLC-UV analyis,[168] in 0.027 M aqueous formic acid/MeCN (90:10, v/v) prior to LC-MS/MS analysis,[12] in MeCN + H_2O/formic acid (1000:2, v/v) prior to UHPLC-TOF MS,[181] in MeOH/0.1% formic acid (2:8, v/v) prior to LC-LIT MS,[8, 179], and in aqueous 0.1% acetic acid/MeCN (80:20, v/v) prior to LC-MS/MS.[214] After the organic acid precipitation, the average estimated ion suppression in MS detection was significantly lowered, for example, for carazolol in pig muscle samples by 281%.[179]

Final sample extracts were generally filtered using disposable sub-micron filters before injection into the instrumental system.[219]. Filtration using disposable microporous centrifugal devices was reported to make the extracts clearer and thus completely reduced the matrix effect.[13]

6.12 Performance and Validation of the Analytical Methods

The performance and validation of the majority of the current analytical methods for determination of sedatives and tranquilizers discussed in this chapter followed the EU legislation and standards, laid down by the Commission Decision 2002/657/EC,[223] which implemented the Council Directive 96/23/EC[164] concerning the performance of analytical methods and the interpretation of results. This was supported by the *Guidelines for the validation of screening methods for residues of veterinary medicines (initial validation and transfer)*, prepared by the EU Reference Laboratories.[224]

Another relevant method performance and validation model has also been laid down by the Codex Alimentarius Commission in the Codex Guideline CAC/GL 71-2009 for the design and implementation of national regulatory food safety assurance program associated with the use of veterinary drugs in food-producing animals.[163] The application of the Codex Alimentarius and EU guidelines is discussed in detail in Chapter 10.

The EU laboratories involved in the analysis of official samples should use screening and/or confirmatory methods of analysis that have been validated to demonstrate their fitness for the intended purpose.[225] Methods for substances with established MRL values (azaperone, azaperol, and carazolol) have been thus validated at concentrations around their MRL value (Table 6.1). The Codex guidelines also recommend that methods for substances with MRLs should be validated at concentrations which bracket the MRL.[163] In addition, as no MRL exists for the other sedatives and tranquilizers, such as the phenothiazine derivatives, their limits of detection (LODs)/decision limits (CCα) should be lower than the recommended concentrations (RC) defined by the EU Reference Laboratories, which are for kidney 10 μg/kg for chlorpromazine and 50 μg/kg for acetylpromazine, propionylpromazine, and haloperidol[226], and the detection limits and concentrations for validation of these analytes should be as low as possible. An RC has not been defined by the EU Reference Laboratories for xylazine and diazepam, and such limits have not been established in other countries; hence methods for these substances are typically validated at concentrations close to "zero" (i.e., at the lowest concentration which can be achieved with current methods and technology).

While validation of many of the methods presented in this chapter has been generally performed according to the "conventional validation procedures", described in the Commission Decision 2002/657/EC,[223] the method of Bock and Stachel is an exception.[14] In this method, an alternative multifactorial model of validation (including species, operator, duration of analysis, and storage) was used with the InterVAL software (QuoData GmbH, Dresden, Germany).

The criteria for the confirmation of the identity of drugs (mass resolution and mass accuracy) by novel comprehensive analytical techniques such as TOF MS

are not yet included in the EU guidelines (see Section 3.2.4 for further discussion of this issue). Thus, the reported methods were validated as quantitative screening methods, and the suspected samples had to be confirmed using QqQ MS/MS.[181, 219, 221]

Validation results in the analytical methods reviewed were generally suitable to meet the legislation, guidelines, or purpose expectations required by the EU and other authorities. However, sedatives and tranquilizers still present difficulties for analytical determination. In the MCMR method for determination of more than 100 drugs in milk, this group thus gave the worst validation results, as the accuracy, evaluated by the detection capability (CCβ), could not be established for chlorpromazine and RSD for propionylpromazine and acetylpromazine was very high, between 26% and 58%.[181] Promazines yielded recoveries of 18–61%, probably due to oxidation in acidic aqueous media.[214] Further improvements to include these analytes in multi-class methods may be expected in the coming years with the wider use of technologies such as TOF MS in combination with the newer techniques for extract clean-up.

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7

The Use of Pyrethroids, Carbamates, Organophosphates, and Other Pesticides in Veterinary Medicine

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7.1 Introduction

Insects have had an effect on the food industry since agriculture began. Perhaps the first recorded example is during the 10 plagues of Egypt when locusts destroyed the Egyptian crops.[1] The impact of insects on agricultural food production has been an ongoing problem for both cereal crops and the meat industry.

Several terms, including insecticide, pesticide, and acaricide, are used interchangeably to describe the chemicals used to eradicate various species of insects. In animal husbandry, the majority of pests causing concern are arachnids, with the majority of these being ticks and mites, such as *Boophilus microplus.* These insects are ectoparasites, meaning that they live on the skin but not within the body of domestic animals[2] and the chemical agents used to control them are correctly called ectoparasiticides. It has been reported that *B. microplus* could be the most economically important tick since it feeds off cattle in Australia and Central and South America as well as parts of Africa and Asia and transmits diseases such as *Babesia bovis, Babesia bigemina*, and *Anaplasma marginale*, which can often kill cattle.[3] The threat posed by ticks is taken so seriously that, in 2000, an 80 mile wide quarantine was put in place along the Mexico–USA border and the transport of cattle from Mexico was closely monitored to try to eradicate *B. microplus* ticks from the United States of America.[4] Pesticides are also used in veterinary medicine for the control of biting flies, which can affect production, and parasitic flies, which lay eggs under the skin of the animal (see Section 7.2.3).

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There are many different classes of pesticides, with new ones being introduced, and it would be impossible to mention them all. This chapter will focus on the pesticides currently used in food-producing animals, including pyrethroids, organophosphate pesticides (OPs), carbamates, and formamidines, and on their use in animal husbandry for the food industry, their chemical structures, mode of action, and methods of analysis published in the scientific literature.

7.1.1 History of Pesticide Use in Veterinary Medicine

Since about the seventeenth century, increasing industrialization meant that people traditionally employed in the agriculture sector were moving into industrial jobs, reducing the portion of the population involved in food production. With this came the concomitant demand for food as there was less time for producing home-grown crops, which put pressure on the farming industry to provide more food at a reasonable price. Factors such as these have driven the search for chemical control of pests. The use of pesticides in veterinary medicine has been of interest since the eighteenth century, when outbreaks of tick-borne diseases were recorded during the transport of a herd of cattle to Pennsylvania from South Carolina. Nearly all the native cattle along the route died, while those from the south remained healthy. The disease, which was called Texas fever and was believed to be due to bovine piroplasmosis, was first reported in 1906.[5] Similar events were observed in Australia when cattle were transported from Timor and Bali in the first half of the nineteenth century and in Africa as cattle were transported across the continent.[6] There are still outbreaks occurring around the world, especially in developing countries.[7] The economic benefit of controlling tick populations has driven both government and international animal health companies to invest in tick control programs with the concomitant need to develop effective chemical ectoparasiticides.[8] Pyrethrum, extracted from chrysanthemum flowers, is probably the first recorded chemical used to control insects. Records of its use date back to China in the first century A.D., with its use spreading slowly east toward Europe.[9] Its use is well documented, from use in medieval China and the Silk Route[10] to use as a constituent of louse powder in the Napoleonic Wars[11] to current use in household pesticides.[12] It is also one of the few compounds allowed to control pests in certified organic agriculture[9]. Apart from pyrethrum and other "folklore" remedies, the history of chemical control can be traced from the nineteenth century when treatments included smearing the legs and flanks of cattle with mixtures containing lard, kerosene, sulfur, fish oil, and cottonseed oil.[5, 13]

The first reported use of arsenic to control ticks on cattle in South Africa was in 1893, although it had been used to control ticks on sheep for a century before then,[14] and subsequently in Australia in 1896[15] where cattle were dipped in vats of arsenical solutions. The use of arsenic was very successful in the eradication of tick-borne diseases for many years, until it was noted that ticks were becoming resistant to this treatment[16] and other chemical treatments were developed. Many of the published papers dealing with the use of ectoparasiticides on animals are

from studies in South America and Africa, where tick and fly infestations are a common problem in herds grazed on open grasslands.

In Mexico, two of the most prevalent ticks are *Rhipicephalus microplus* and *Amblyomma cajennense.* A study in Mexico, where there is a tropical climate and cattle are extensively grazed for the beef and milk industries, described how tick control relies on the use of organophosphates (OPs), synthetic pyrethroids, and amidines, which are applied to the livestock by dipping, spraying, or pouring-on.[17] The authors noted that when *R. microplus* was subjected to chemical control, populations of *A. cajennense* replaced them. However, overuse and misuse of these treatments have led to resistance to almost all major classes of acaricides in some populations.[17, 18] The first report of *A. cajennense* resistance to organophophates was in 1986,[19] while in 2010 there were reports of populations of *A. cajennense* resistance to chlorpyrifos, coumaphos, and diazinon.[20] When Mexico held a national campaign to eliminate the *Boophilus* tick from 1974 to 1984, coumaphos was the only authorized acaricide.[19] Tick populations became resistant to coumaphos, so pyrethroids were introduced to control the OP-resistant ticks but resistance to pyrethroids emerged in the early 1990s. Deltamethrin resistance in *A. cajennense* has also been reported.[21] Amitraz was introduced in the mid-1980s as many tick populations became resistant to both OPs and pyrethroids, and it became increasingly popular as resistance to the other acaricides increased in the late 1990s. The first case of amitraz resistance was observed in 2001 in Tabasco, and since then more amitraz-resistant tick populations have been identified.[17, 22, 23]

Even though only certain pesticides are approved for use in aquaculture to treat sea lice, residues of other pesticides are often seen due to environmental contamination. For example, an OP, azamethiphos, and the benzoylphenyl urea insecticides diflubenzuron and teflubenzuron are used to treat salmonids for sea lice infestations; carbaryl and propoxur are carbamate insecticides approved for use in fish and shellfish colonies. Residues can accumulate in water and fish lipids and can be harmful to human health as well as other marine organisms and farmed species.

7.1.2 Development of Chemical Pesticides

Different classes of pesticides for both general and veterinary drug use have been developed since the mid-twentieth century and a comprehensive review is available in the "Compendium of Pesticide Common Names."[24] The classes which have been most used as veterinary drugs include organochlorine (OC) pesticides, synthetic pyrethroids, carbamates, formamidines, organophosphate pesticides, and, more recently, insect growth regulators (IGRs), which have a different mode of action compared to the more traditional pesticides (see Section 7.2). Each pesticide class has its uses with its different modes of action but also its drawbacks. Synthetic pyrethroids, organophosphate pesticides, carbamates, and formamidines all act by inhibiting acetylcholinesterase (AChE), while OC

pesticides act by inhibiting the sodium or calcium channels and IGRs inhibit the growth of chitin (see Section 7.3). OC pesticides were first used during the mid-1940s with the goal of controlling *Boophilus* ticks on cattle, but their usefulness was limited as ticks quickly became resistant to them and concerns were raised about their persistence in the environment and their toxicity. They have been largely withdrawn from the market in the United States of America and Europe, although some (e.g., lindane and methoxychlor) are still approved for use in developing countries.[25] For this reason, an overview of these compounds will be presented, but they will not be discussed in any depth in this chapter. Organophosphate pesticides were originally developed to control *Boophilus* ticks after they developed resistance to OC pesticides; while less persistent than OC pesticides in the environment, they are more toxic to vertebrates. They are chemically related to nerve gases with similar modes of action.[11]

Ectoparasite infestations tend to be seasonal. For example, flies are seen predominantly from late spring to early autumn, while tick populations increase in the spring and autumn, and lice and mites are more common during the autumn and winter months. Treatments can be targeted at anticipated times of peak activity as a means of limiting disease and parasite populations. The pesticides intended for veterinary use are available in several different formulations (pour-ons, spot-ons, dips, ear tags, sprays, etc.) and are generally broad spectrum so that they can be used to control different classes of insects and other classes of arthropods, including mites, ticks, and flying insects on crops and livestock.

7.2 Veterinary Drug Properties, Structures, and Regulation

While pyrethroids, organophosphates, and carbamates have very different structures, they all disrupt AChE production in slightly different ways, while OC compounds act differently (see Sections 7.1.2 and 7.3).

7.2.1 Organochlorines

OC pesticides can be divided into three types according to their chemical structures, resulting in slightly different modes of action (representative structures are shown in Figure 7.1):

- *Chlorinated ethane derivatives* such as dichlorodiphenyltrichloroethane (DDT), dichlorodiphenyldichloroethylene (DDE), dichlorodiphenyldichloroethane (DDD), dicofol, and methoxychlor open the sodium channels in neurons, leading to spasms and eventual death.
- *Cyclodienes*, including chlordane, aldrin, dieldrin, heptachlor, endrin, and toxaphene, inhibit the γ-amino butyric acid (GABA) stimulated Cl^- flux and interfere with the Ca^{2+} flux resulting in partial depolarization of the postsynaptic membrane.

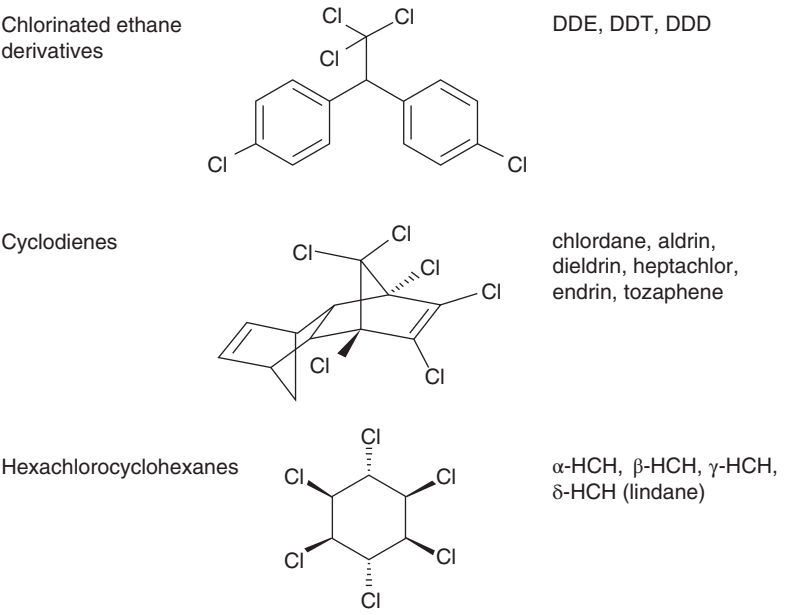


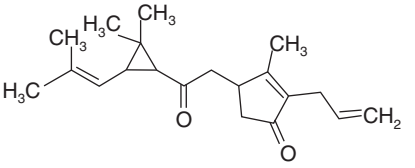
Figure 7.1 Organochlorine pesticide structures.

- *Hexachlorocyclohexanes* such as benzene hexachloride (BHC), which includes the γ-isomer lindane, one of the most widely used pesticides, interfere with the GABA neurotransmitter function by interacting with the GABA receptor–chloride channel complex.

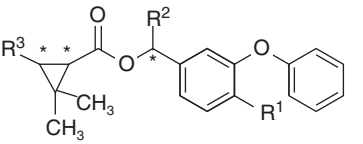
DDT and lindane were widely used in dip formulations for the control of sheep scab but have largely been replaced by the organophosphates and the synthetic pyrethroids.

7.2.2 Pyrethrins and Synthetic Pyrethroids

Pyrethrum is a mixture of Pyrethrins I, the naturally occurring esters of chrysanthemic acid, (pyrethrin I, cinerin I, and jasmolin I), and Pyrethrins II, the corresponding esters of pyrethrin acid (pyrethrin II, cinerin II, and jasmolin II). However, pyrethrum extract is unstable in sunlight and as such was not suitable for use as pesticide in the agricultural industry unless it was used with a synergist,[11] although it is still widely available for the worldwide domestic market in household insecticides, plant sprays, and garden products. Synthetic pyrethroids are based on the structure of Pyrethrin I and were developed as more stable alternatives for the agricultural industry. Pyrethroids are probably the largest and most widely used group of the pesticides, and as of 2009, over 1000 different pyrethroid compounds have been synthesized, with varying substitutions and corresponding activities[26] (Figure 7.2 shows some examples).

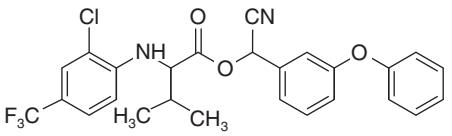


Pyrethrins

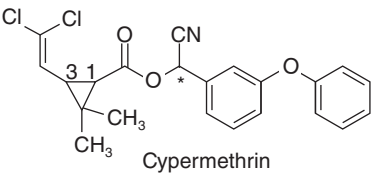


Pyrethroid base structure

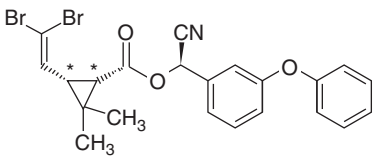
R1– H or F: R2 – CN or H; R3 – may contain halogens, aliphatic chains, or aromatic rings



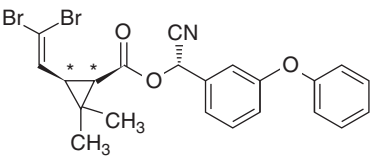
Fluvalinate



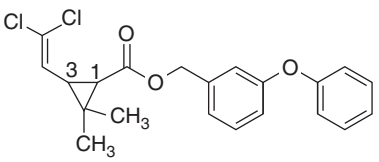
Cypermethrin



Deltamethrin_1R3SSaCN



Deltamethrin_1S3RRaCN



Permetrhin

Figure 7.2 Typical structures of pyrethrins and pyrethroids showing chiral centers.

Pyrethroids are effective ectoparasiticides, and a number of them are available in many countries in a variety of formulations with activity against biting and nuisance flies, lice, and ticks on domestic livestock. Their developmental history can be roughly separated into three or four generations depending on how the authors define them (Table 7.1). These generations show increasing insecticidal activity and stability in sunlight. The third-generation pyrethroids, including permethrin and fenvalerate, which proved to be virtually unaffected by sunlight, were effective for 4–7 days on crops and showed an insecticidal activity of 0.1 lb ai/A (pounds of active ingredient per acre) while the fourth-generation pyrethroids,

Table 7.1 Approximate dates for the pyrethroid generations.

Generation	First used	Pyrethroids	Notes
Pyrethrum	Used in early nineteenth century		Not effective for agricultural use Does not kill pests, just paralyzes them! Unstable in sunlight
First	1949	Allethrin	More active than pyrethrum Unstable in sunlight
Second	1962–1973	Resmethrin Bioresmethrin Tetramethrin Phenothrin	Unstable in sunlight Not suitable for use outdoors
Third	1972–1973	Permethrin, fenvalerate	Virtually unaffected by sunlight, effective 4–7 days on crops
Fourth	Late 1980s to present	Bifenthrin Cyhalothrin Cyfluthrin Cypermethrin Deltamethrin	Non-volatile, unaffected by sunlight effective up to 10 days after application

Further information can be found at
http://www.bbsrc.ac.uk/web/FILES/Publications/pyrethroid-timeline.pdf

including bifenthrin, cyhalothrin, cyfluthrin, cypermethrin, and deltamethrin, are even more effective, having an insecticidal activity of 0.01–0.05 lb ai/A, do not undergo photolysis in sunlight, and are non-volatile, so they can remain effective up to 10 days after application.[11]

Synthetic pyrethroids are considered broad-spectrum pesticides, and their applications are many and varied, including plant protection, as a pesticide/insecticide/acaricide for both farm and domestic animals and as a treatment for human clothing and bedding in very hot climates. Pyrethroids used in food animal production include allethrin, bifenthrin, bioresmethrin, cyfluthrin, cyhalothrin, cypermethrin, deltamethrin, fenvalerate (esfenvalerate), flucythrin, flumethrin, fluvalinate, fenpropathrin, phenothrin, resmethrin, tefluthrin, tetramethrin, and tralomethrin. Of these, the pyrethroids most commonly used in the cattle industry, cyfluthrin, cyhalothrin, deltamethrin, and permethrin,[27, 28] can be used in pour-ons, sprays, powders, dip tank, and ear-tag formulations. They are often mixed with other pyrethroids, pesticides, and/or synergists. Within the EU, the European Medicines Agency, or EMA, (formerly the

European Agency for the Evaluation of Medicinal Products, or EMEA*) has approved the use of permethrin,[29] deltamethrin,[30] cyhalothrin,[31] and cyfluthrin[32] for external applications on cattle, sheep, pigs, fin fish, goats, and poultry in the form of sprays, including udder sprays, powders, pour-ons, and ear tags. Dosage rates for each formulation are given in each EMEA monograph as well as specific instructions for each *cis*:*trans* ratio where applicable.

Pyrethroids, and especially cypermethrin, have been widely used for the treatment of sea lice in farmed salmon[33–36] even though fish are highly sensitive to pyrethrins and pyrethroids. For most fish, the 96 hour LC_{50}s are in the low μg/l for permethrin, cypermethrin, and fenvalerate.[37] Where pyrethroids have been metabolized by ester hydrolysis in soil and aquatic environments, the 96 hour LC_{50} values are much higher (> 300 μg/l) than that observed for the parent compound.[38]

The pyrethroid structure is complex, containing chiral carbons and optical isomers. They were originally synthesized by modifying the chrysanthemic acid moiety of pyrethrin I and esterifying the alcohol groups.[26] The structure of pyrethroids includes a central ester bond, a cyclopropane ring which contains two chiral carbons, and an alcohol moiety, which can also contain a chiral carbon (Figure 7.2). As a result, pyrethroids may contain up to three chiral carbons leading to as many as eight stereoenantiomers, and their biological activity depends upon the configuration of the asymmetric carbon centers within the molecules.[39]

The presence of two chiral centers in the cyclopropane ring results in two pairs of diastereomers, which are designated *cis* and *trans* based on the orientation of the C-1 and C-3 substitutions in the plane of the cyclopropane ring with the 1*R* (*cis*) conformations being considerably more toxic than the 1*S* (*trans*) isomers.[26, 28] For example, deltamethrin contains three asymmetric carbons and has eight stereoisomers. Of these, the 1*S*,3*S*,*S*αCN enantiomer is a very active insecticide, while its optical isomer, 1*S*,3*R*,*R*αCN, is inactive[40]; while permethrin has two asymmetric carbons and two sets of enantiomers, the insecticidal activity is associated with the (1*R*,3*S*)-*cis* and (1*R*,3*R*)-*trans* isomers.[40]

The chemical constituents of a pyrethroid can affect both the toxicity and the mode of action on a pest and the resultant symptoms of insect poisoning. Pyrethroids are classed as follows:

- *Type I*, which do not contain a cyano group (examples include permethrin and allethrin)
- *Type II*, which contain a cyano group and a 3-phenoxybenzyl alcohol derivative in the alcohol moiety (examples include fenvalerate, fluvalinate, and flucythrinate).

The cyano group is a chiral center in the compound and imparts a different mode of action to the compound resulting in different pyrethroid poisoning

* Where documents are referenced, the organization is identified as it appeared on the referenced document.

symptoms for each type. Compounds such as cyfluthrin, cyhalothrin, cypermethrin, and deltamethrin, which contain both a cyano group and a cyclopropane ring, possess three chiral centers and thus consist of eight possible isomers and exhibit toxicities which are usually a combination of those exhibited by Types I and II pyrethroids (see Section 7.3.1). Pyrethroids such as fenvalerate, fluvalinate, and flucythrinate containing a cyano substituent at the alcohol moiety (Type II pyrethroids) demonstrate differing toxicity based on the optical isomerism of the alpha (α) carbon where it has been demonstrated that the *S* conformation about the alpha carbon is considerably more toxic toward insects compared to the *R* conformation.[11] They may be formulated as racemic mixtures or as single isomers, and these isomers may have individual common names, where they are commercially important, that is, active pesticides. For example, the isomers of resmethrin are bioresmethrin and cisresmethrin and that of cypermethrin is α-cypermethrin. Some of these isomers are more commercially important than others.[41]

Clinical symptoms of pyrethroid poisoning are seen in both mammals and insects and include salivation, hyperexcitability, hyperesthesia, tremor and seizures, dyspnea, prostration, and death.[28] The symptoms can appear within minutes to hours of exposure, depending on the route of exposure, and generally last 2–3 days. Flumethrin was designed for application as a pour-on to treat cattle, but it has since been reformulated as a dip or spray. The pour-on formulation spreads rapidly on the skin and hair from the points of application and can control both one-host and multi-host tick species on cattle and is effective at relatively low concentrations,[42] with the *trans* flumethrin isomer being approximately 50 times more toxic to *B. microplus* than the other most toxic pyrethroids, deltamethrin and *cis* permethrin.[43]

7.2.3 Organophosphates and Carbamates

Organophosphate and carbamate insecticides are often considered together as they have similar modes of action. All organophosphate pesticides are esters of phosphorus, and there are six different sub-classes:

- *Phosphates*
- *Phosphonates*
- *Phosphorothioates*
- *Phosphorodithioates*
- *Phosphorothiolates*
- *Phosphoroamidates*

These have varying combinations of oxygen, carbon, sulfur, and nitrogen attached. These sub-classes are easily identified by their chemical names[11] (Figure 7.3).

An organophosphate moiety is a constituent of many compounds essential to life, including deoxyribonucleic acid (DNA) and ribonucleic acid (RNA).

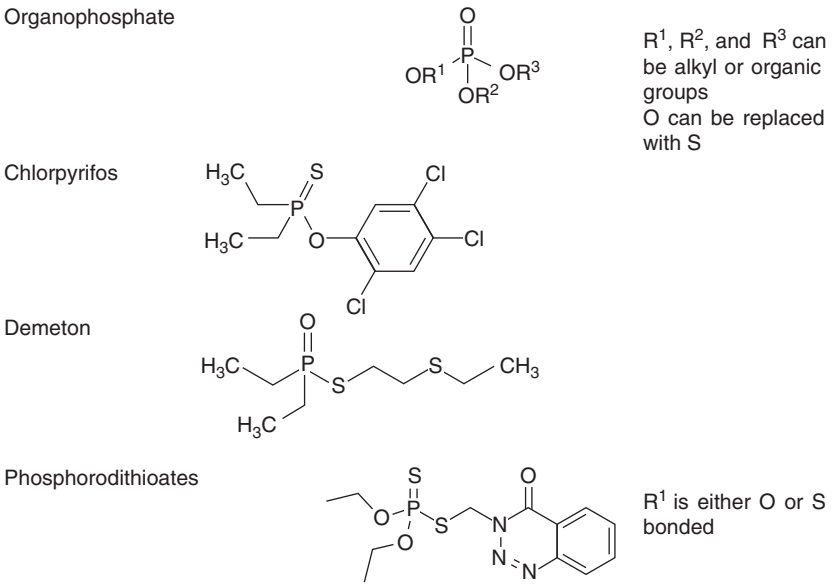


Figure 7.3 Structures of some typical organophosphates.

However, the name is usually taken to mean herbicides, insecticides, and nerve agents. For example, the insecticide diazinon, in addition to being toxic to humans and mammals, is considered as being highly toxic to birds, bees, and most other insects and also moderately toxic to fish and amphibians.[44] Although there are many organophosphate pesticides available, only a few are approved for use in veterinary medicine, where they are usually available as pour-ons or ear tags. Coumaphos, diazinon, dichlorvos, famphur, fenthion, malathion, trichlorfon, stirofos, phosmet, and propetamphos are approved for use as topical applications, while ear tags containing fenthion, chlorpyrifos, and diazinon are also available in some countries. The activity of each of these compounds depends on the formulation and the insect it is being used against. These insects include fly larvae, flies, lice, ticks, and mites on domestic livestock. In cattle, a number of compounds have been used for the systemic control of warble fly grubs and lice as pour-on applications or in hand sprays, spray races, or dips for tick control. Information on the toxicity of organophosphate insecticides is available from the United States Environmental Protection Agency (US EPA) at http://www.epa.gov/pesticides.

Coumaphos is the only acaricide registered in the United States of America for use in the Fever Tick Eradication Program, which is designed to eliminate re-infestations of *B. microplus* (Canestrini) and *B. annulatus* (Say)[45] and is also the only acaricide used in the dipping vats at livestock import facilities at the Mexico–USA border.

Carbamate insecticides are derived from carbamic acid and contain an ester functional group. Carbamate acaricides (carbaryl and promacyl) inhibit the target AChE but have much less mammalian and dermal toxicity.[6] They include aldicarb, carbofuran, carbaryl, propoxur, ethenocarb, fenobucarb, oxamyl, and methomyl. The synthesis and commercialization of carbamates began in the 1950s.[11]

There are over 50 carbamate pesticides known, which can be split into three classes:

- *Carbamate ester derivatives*, which are used as insecticides and nematocides.
- *Carbamate herbicides*, with the general structure shown in Figure 7.4 where R^1 and R^2 are aromatic and/or aliphatic groups. Examples include asulam, carboxazole, chlorprocarb, dichlormate, fenasulam, karbutilate, and terbucarb.
- *Carbamate fungicides*, which contain a benzimidazole group. Examples include benthiavalicarb, furophanate, iodocarb, iprovalicarb, picarbutrazox, propamocarb, pyribencarb, thiophanate, thiophanate-methyl, and tolprocarb.

Carbamates are generally applied directly to plants or soil and therefore enter the food chain directly via plants and plant products or by animals eating contaminated feed or plants. They are metabolized by plants via arylhydroxylation and conjugation or via hydrolytic breakdown by microorganisms, plants, and animals.[46] They are degraded in water and soil and do not persist in the environment, but can bioaccumulate in fish. Some are highly toxic to invertebrates and fish, while others are much less so. Interestingly, some common paralytic shellfish

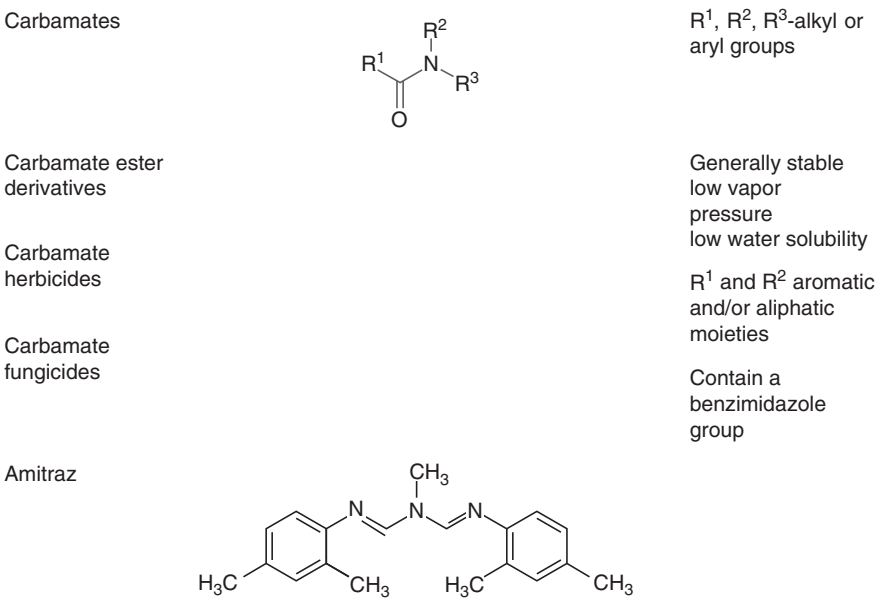


Figure 7.4 Structures of some typical carbamates.

toxins (saxitoxin and its analogues) contain the carbamate structure and exhibit the same toxic action.[47] In mammals, carbamates tend to be absorbed through the skin, mucous membranes, and respiratory and intestinal tracts, and the metabolites are generally less toxic than the parent compounds, although there are exceptions. They metabolize quickly so that accumulation is not a problem in most cases. Depending on the structure, hydrolysis follows two different routes. *N*-Methyl carbamates are hydrolyzed via an isocyanate intermediate, while *N*-dimethylcarbamates are hydrolyzed via the addition of a hydroxyl ion to yield an alcohol and an *N*-dimethyl-substituted acid. There is little information on the distribution of carbamates in organs and tissues in mammals, but residues have been reported in the liver, kidney, brain, fat and muscle, and in urine where excretion appears to be rapid.

Carbamates act by blocking the same enzyme (acetylcholinesterase) as organophosphate pesticides but, unlike organophosphates, they appear to cause a spontaneously reversible block on AChE without changing it. One of the most common carbamates is carbaryl, which has low mammalian toxicity but may be carcinogenic and is often combined with other active ingredients.

Unfortunately, because organophosphorus and carbamate insecticides are commonly used in agriculture to control pests on crops, livestock poisoning has occurred from both the mistaken addition of unused insecticide to feeds and animal access to materials that have been improperly disposed or to "empty" containers. The morbidity rate approximates a third of animals exposed and about half of those affected die.[48]

7.2.4 Formamidines

Formamidines are the amidine form of formic acid. The most commonly used of these substances are chlordimeform and amitraz, although amitraz is the only one used as an ectoparasiticide and is toxic to spider mites, ticks, and certain insects. When they were introduced, many insects were becoming resistant to the acaricides then in current use, so they were particularly effective against junior mites and other resistant species.[49] A review article by Taylor notes that amitraz is available as a spray, dip, or pour-on formulation against ticks, mites, and lice.[50] The usage of these types of amitraz formulations is common in tropical districts for the control of single-host and multi-host tick species on cattle. When used as a dip, it has to be stabilized at pH 12 using calcium hydroxide. Amitraz has also been used to control lice and mange in pigs and for psoroptic mange in sheep.[51]

Amitraz appears to act by inhibition of the enzyme monoamine oxidase and as an agonist at octopamine receptors.[50] Monoamine oxidase metabolizes amine neurotransmitters in ticks and mites, and octopamine is thought to modify tonic contractions in parasite muscles. Amitraz has a relatively wide safety margin in mammals; the most frequently associated adverse effect is sedation, which may

be associated with an agonist activity of amitraz on α_2-receptors in mammalian species.[52]

Amitraz has been used in the treatment of cattle against *B. microplus* (Canestrini).[45] In 1998, when its use in a dip was evaluated, the only amitraz-containing formulation available in the United States of America was a spray.[53] There have been reports that ticks detached themselves from the host 6 hours (*B. microplus*) or 7 hours (*Rhipicephalus appendiculatus*) after treatment with amitraz, but a longer detachment time was required for other ticks.[45] It was also noted that the rapid detachment of the North American strains of *B. microplus* was less pronounced than for those of other geographical regions. In a trial in South Africa where *Rhipicephalus (Boophilus) decoloratus* has shown resistance to both amitraz and cypermethrin, it was found that a change in application, from a pour-on to a spray dip, increased the efficacy of amitraz despite the observed resistance.[54] Amitraz has also been used in apiculture, where its recommended use is as a sustained-release strip containing 500 mg of amitraz suspended in the hive for the treatment of *Varroa* disease.[55]

7.2.5 Insect Growth Regulators

IGRs are a relatively new category of insect control agents. They do not kill the target parasite directly but interfere with growth and development. They are not usually suitable for the rapid control of established adult parasite populations but rather act mainly on immature parasite stages. They are particularly useful if a seasonal pattern of infestation can be predicted as they can be applied as a preventive measure at the correct time. They are widely used for blowfly control in sheep but have limited use in other livestock. The US EPA has labeled them as "reduced risk" as they target juvenile populations and are less harmful to beneficial insects. An added bonus seems to be that insects are less likely to become resistant to them.[11]

There are two types of IGRs:

- *Hormonal IGRs*. These work by inhibiting or mimicking the juvenile hormone which is involved in insect molting. These IGRs disrupt larval development by producing premature molting, resulting in a nonfunctional adult. If the IGR inhibits ecdysone, then the transformation of larval tissues into adult tissues is interrupted.
- *Chitin Inhibitors*. These prevent the formation of chitin, a carbohydrate required to form the exoskeleton. An insect will be unable to grow a new exoskeleton when it molts and will die. These are quicker acting than the hormonal IGRs but can affect arthropods and fish. Benzoylphenyl ureas are an example of chitin inhibitors. Benzoylphenyl ureas show a broad spectrum of activity against insects but have relatively low efficacy against ticks and mites. The exception is fluazuron, which has greater activity against ticks and some mite species.[56]

Benzoylureas are an example of IGRs and were first introduced in 1978. They exhibit herbicide, insecticide and acaricide activity but unlike other compounds considered here, they inhibit the chitin synthesis in the cuticle of the insect resulting in its rupture or death by starvation.[57] Their main use is the control of caterpillars and beetle larva. They have low toxicity for mammals but can be entrained in the food chain causing chronic exposure and long-term toxicity.[58] In Europe, diflubenzuron, lufenuron, and teflubenzuron have been licensed for use in salmonidae for the control of sea lice.[59–61] Benzoylphenyl ureas are highly lipophilic molecules, and when administered to the host, they build up in body fat, from which they are slowly released into the bloodstream and excreted largely unchanged.[50] Diflubenzuron and flufenoxuron are used for the prevention of blowfly strike in sheep. Diflubenzuron is available in some countries as an emulsifiable concentrate for use as a dip or shower. It is more efficient against first-stage larvae than second and third development stages of arthropods (instars) and is therefore recommended as a preventive measure, providing protection to crops and animals for 12–14 weeks. It may also have potential for the control of a number of major insect pests such as tsetse flies. Fluazuron is available in some countries for use in cattle as a tick development inhibitor. When applied as a pour-on, it provides long-term protection against the 1-host tick *B. microplus*.

7.2.6 Phenylpyrazoles and Neonicotinoids

Phenylpyrazoles, such as fipronil and ethiprole, are broad-spectrum parasiticides, acting on fleas, ticks, lice, and mites. They act by contact and inhibit GABA and seem to be more effective on invertebrates than vertebrates. In Latin America, their use is gaining popularity against horn flies and cattle ticks.[17] Phenylpyrazoles and neonicotinoids show similar toxicity and physiochemical profiles in that they bind to nicotinic acetylcholine receptors in the nervous systems in insects.[62] They are widely used in agriculture, but their use in livestock is limited. They are used in baits for the control of nuisance flies in stables, as pour-ons to control sheep lice, and as pour-ons to control horn flies and other flying species in cattle. They are also used in fish farming to control rice water weevil infestations in rice–crayfish rotation.[62]

7.2.7 Synergists

There are several compounds that are used as pesticides but do not fall into any of the aforementioned categories. For example, piperonyl butoxide (PBO), a methylenedioxyphenyl compound, is widely used as a synergistic additive in the control of arthropod pests, especially with natural pyrethrins. The ratio of insecticide to the synergist determines how potent the insecticidal activity is. As the proportion of PBO increases, the amount of natural pyrethrins required to produce the same level of kill decreases. It had already been shown that PBO

behaves as a synergist with pyrethroids containing a cyano group at a higher rate than those without a cyano group.[63] This suggests that the choice of pyrethroid to mix with a synergist is important. The insecticidal activity of other pyrethroids, particularly of knockdown agents, can also be enhanced by the addition of PBO. The enhancement of activity of synthetic pyrethroids is normally less dramatic.

However, the use of a synergist does not always improve the effect of pyrethroids, especially if some resistance to those compounds has been observed. For example, when *Haematobia irritans* began to display resistance to pyrethroids in Argentina, the use of PBO mixed with cypermethrin was suggested to increase efficacy against *H. irritans.*[64] The authors of this paper found that the resistance to cypermethrin was not improved by the use of PBO for this situation, but this does not mean that for populations where there is no record of resistance that it would not be successful.

It was thought that mixing two different classes of pesticides could improve the action of both, but instead the presence of organophosphates inhibits pyrethroid activity.[65] It was also noted that urinary excretion of unmetabolized pyrethroids was higher in sprayers using methamidophos/deltamethrin or methamidophos/fenvalerate mixtures when compared to the pyrethroid alone.[66] This may be because the OPs inhibit, or compete for, the carboxyesterases responsible for pyrethroid hydrolysis.[67]

7.2.8 Regulation and Maximum Residue Limits

Regulations enacted to safeguard the health of people often begin with a consideration of the food supply. These may vary from country to country, reflecting the local diets and lifestyles, but all have the same goals. For example, in 1996, the United States Congress passed the Food Quality Protection Act (FQPA), which, in part, amends the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA) and the Federal Food, Drug, and Cosmetic Act (FFDCA). It emphasized the protection of the food supply for children by determining aggregate dietary exposure to pesticides for infants and children. One of the requirements of the FQPA is that the US EPA must conduct dietary exposure estimates for risk analyses that include aggregate exposure from various sources and cumulative exposure from related chemicals that share a common mechanism of toxicity.[68]

As well as safeguarding the food supply, the approval of pesticides for use can depend on historical use. For example, in countries like Australia where resistance to DDT has been observed in *Boophilus* ticks, permethrin and fenvalerate have not been registered for use because cross-resistance to them was observed. Instead, the cyano-substituted pyrethroids deltamethrin and cypermethrin are registered in the hope that resistance to these pyrethroids can be delayed.[6]

In their residue control regulations for live animals and animal products, the European Union has included pesticides in Group B – veterinary drugs and contaminants. Carbamates and pyrethroids are listed as Group B (2) – other

veterinary drugs, while organophosphorus and OCs compounds are listed in Group B (3) – other substances and environmental contaminants.[69]

A maximum residue limit (MRL) is set after a rigorous scientific process to ensure that the maximum amount of residue expected to remain on food products when a pesticide is used according to label directions will not be a concern to human health.[70] A guidance document from the European Commission notes that MRLs are often mistaken for toxicological safety limits. In reality, MRLs define the maximum concentrations of a pesticide that should be present in a food commodity following use of that pesticide under the approved conditions of use. MRLs are intended to prevent "illegal and/or excessive use of a pesticide (e.g., to prevent damage to the environment or to the health of workers and bystanders) and to protect the health of consumers of the harvested products."[71]

In general, MRLs for pesticides are set for specific compound and food-type combinations and take into consideration many factors including climate, geography, topography, and water systems, as well as potential sources of exposure from various foods. For animal-derived foods, MRLs for pesticides evaluated for use as veterinary drugs are set based on depletion studies conducted under the approved conditions of use and are generally recommended for several edible tissues and products, depending on the species in question, such as muscle and organs for beef and skin and fat for poultry. Studies are also required to provide information on all types of residues formed such as free, bound, and conjugated residues.[72] These studies are usually conducted according to good laboratory practices (GLP)[73] using modern scientific methodology and follow Veterinary International Conference on Harmonisation (VICH) guidelines.[74]

At the international level, the Codex Alimentarius Commission (CAC) acts as the risk manager for the setting of MRLs. Draft MRLs are submitted by the Codex Committee on Pesticide Residues (CCPR) and the Codex Committee on Residues of Veterinary Drug Residues in Food (CCRVDF) based on scientific expert advice provided by the risk assessors, including the Joint Food and Agriculture Organization (FAO) of the United Nations/World Health Organization (WHO) Meeting on Pesticide Residues (JMPR) and the Joint FAO/WHO Expert Committee on Food Additives (JECFA), as discussed in Chapter 1. It has been agreed that JECFA and JMPR will coordinate the evaluation of MRLs for dual-use substances, that is, those with both pesticide and veterinary drug properties. Before a substance is submitted to a risk assessment for the establishment of Codex MRLs, the pesticide must be registered for use in a Codex Member State.[72]

The reader is directed to the following websites for more information on MRLs established by various authorities at the national and international level:

Codex – http://www.codexalimentarius.net/pestres/data/index.html
EU – http://ec.europa.eu/food/plant/pesticides/eu-pesticides-database/public/?event=homepage&language=EN

Canada – http://www.hc-sc.gc.ca/cps-spc/pest/part/protect-proteger/food-nourriture/mrl-lmr-eng.php
Australia – http://apvma.gov.au/node/10806

MRLs from the United States of America, as well as other issuing bodies around the globe, are available at http://www.GlobalMRL.com, where MRLs can be searched by commodity, compound, and market.

Many regulatory bodies issue default MRLs for pesticides, which may be applicable if a specific MRL has not been established. For example, the EU defines this in Regulation (EC) No. 396/2005 of the European Parliament and of the Council of 23 February 2005 on maximum residue levels of pesticides in or on food and feed of plant and animal origin and amending Council Directive 91/414/EEC.[75] As of November 2014, these are 0.01 μg/kg for the EU, Argentina, Iceland, Japan, Malaysia, Norway, and South Africa and 0.1 μg/kg for Canada and New Zealand.

One of the problems encountered in regulatory analysis when residues are observed is to determine whether the pesticide has been directly applied to the food commodity. This is especially true when very low concentrations are observed. The WHO has made recommendations on the reporting of low concentrations that fall between zero and the limit of detection (LOD) of a method, as many results are seen within this range. It is considered reasonable that if residue levels are below the LOD, then the said pesticide has not been used on that food commodity and the concentration is assigned as zero for the purposes of calculating the quantity of residue in food and estimating dietary exposure.[76] A more conservative approach has been taken for the evaluation of dietary exposure to veterinary drug residues in foods by the JECFA.[77] When there is sufficient data available, the Estimated Dietary Intake calculation is used by JECFA for veterinary drugs. In this calculation, the median concentration of the residue distribution at the time post-treatment at which the MRLs have been established is used in the intake estimate. When values from some animals in the study are below the limit of quantification (LOQ) or below the LOD, "half of the respective limit is used for the calculation of median concentrations of residues."[77]

7.3 Toxicology, Pharmacokinetics, and Metabolism

For pesticides used as ectoparasides for the treatment of food-producing animals, the active ingredients are typically applied in a variety of formulations on the skin of the animal. These include topical applications such as pour-ons, spot-ons, sprays, drench formulations, and dips, where the animal is submerged in a bath containing the active ingredient. Ear tags can also be used, although it has been shown that these only control pests around the face of the treated animal and the pesticides can be transferred to other animals in the herd by grooming other animals or by contact.[78]

The majority of pesticides used in veterinary medicine as ectoparasiticides to treat insects living on the skin of mammals or poultry are not absorbed dermally but can be absorbed through the gut and pulmonary membrane, giving rise to the concern that the widespread use of these compounds increases the risk of them entering the food chain by animals cleaning each other or by contamination of feed or water supplies.[79] They are lipophilic and are almost insoluble in water, so they tend to accumulate in the fatty tissues if repeat applications are used. For example, a report by Rothwell et al. suggests that treatment with a pour-on formulation of cypermethrin every two weeks leads to accumulation in fat, but treatment every three weeks does not.[80] They also report that residues tend to be higher in lean animals following the same treatment and that residue concentrations are higher in renal fat than those in subcutaneous back fat. This tendency of cypermethrin residues to accumulate in edible fatty tissue has been confirmed from the detection of persistent residues in livestock through the residue monitoring and control programs.[81]

7.3.1 Pyrethroids

Pyrethroids act on the nervous system, and it has been suggested that they alter the sodium channel dynamics in nerve tissues and polarize the membranes causing abnormal discharge in targeted neurons.[28] Type I pyrethroids, which contain a cyclopropane carboxylic ester structure but not a cyano group,[37] for example, permethrin, induce a tremor syndrome, which includes hyperexcitability, twitching, tremors, and prostration by prolonging the opening of the voltage-dependent sodium channel long enough to cause repetitive firing of action potentials.[82] Type II pyrethroids, which include a cyano group, for example cyfluthrin and λ-cyhalothrin, but may not contain the cyclopropane ring, induce choreiform movements of the forelimbs and trunk with tonic seizures (choreoathetosis), salivation. and seizure by holding the sodium channels open substantially longer than Type I pyrethroids to depolarize the membrane potential to the point where the generation of an action potential is impossible.[28] These differences in channel opening times may contribute to the differences in the two types of action of neurological toxicity after exposure. If formulations contain both Types I and II structures, a combination of symptoms is observed.[83] Lund and Narahashi[84] note that perturbation of the sodium channel function by pyrethroids is stereospecific, with the *cis* isomers being more toxic than the *trans* isomers. In mammals, the 1*S* isomers are not active (i.e., nontoxic) while the 1*R* isomers are active and therefore toxic. Anadon also suggests that pyrethroids may affect the chloride channels in the brain, nerves, muscle tissue, and salivary gland by decreasing the maxi chloride channel currents and having a synergistic action on the sodium channels, although only deltamethrin and fenvalerate seem to have this effect on chloride channels.[28] The direct action of pyrethroids on sensory nerve endings is more likely to occur after exposure to Type II than Type I pyrethroids. At relatively high concentrations, pyrethroids can also act on γ-aminobutyric

acid-gated chloride channels which may contribute to the seizures seen in Type II.[85]

Animal studies on small mammals have shown that the dermal absorption of pyrethroids is poor[86], but it was found that when they are ingested by rats, 14–70% of the dose of permethrin, deltamethrin, and λ-cyhalothrin can be absorbed, based on estimation of parent compound and metabolites in plasma, and once absorbed, the pyrethroids are rapidly distributed due to their lipophilicity.[87] One study found that elimination half-lives of permethrin in rats were greater in tissue than in plasma after oral administration and that the maximum levels of permethrin measured in all nervous tissues except medulla oblongata were higher than in plasma.[88] This suggested that permethrin accumulates in the nervous tissue, probably because of the lipophilicity of these compounds. They also found that the metabolites of permethrin, *m*-phenoxybenzyl alcohol, and *m*-phenoxybenzoic acid were observed in both plasma and all selected tissues for 48 hours after dosing, which suggested that a combination of metabolism by tissues and diffusion into tissues may occur in rats.

Pyrethroids are hydrolyzed in the gastrointestinal tract and, after absorption, are rapidly metabolized by the oxidation of methyl groups and aromatic rings, hydrolysis of the ester linkage, and conjugation reactions producing a wide range of metabolites. These are excreted in the urine as glucuronide, glycine, taurine, sulfate, and/or glutamate conjugates. There appears to be some stereospecificity in metabolism. The *trans* isomers are hydrolyzed more rapidly than the *cis* isomers, for which oxidation is the preferred mechanism.[89] Reactions take place in the liver, kidneys, and other organs and to a lesser extent in nerve tissues. These studies were performed on mice. There is a considerable reduction in toxicity by cleavage of the ester bond. The α-cyano group decreases the rate of hydrolysis of the ester bond, and the cyano group is converted to thiocyanate. Metabolism and excretion of pyrethroids seem to be faster in birds, especially after oral administration.

7.3.2 Organophosphates and Carbamates

The toxic properties of organophosphate compounds have been well known for many years, and their use as nerve gases in World War II has been documented.[90] After World War II, with the use of nerve gases on people declared illegal, research into their original intended use as pesticides again became very intense.

Organophosphate pesticides and carbamates act by inhibiting the action of AChE. For organophosphates this is irreversible, while for carbamates it appears to be a spontaneously reversible block on AChE without changing it.[51] They mimic the structure of acetylcholine and cause transphosphorylation of the enzyme, thus disrupting the nerve function in target organisms and leading to accumulation of acetylcholine at nerve terminals, causing subtle and long-lasting neurobehavioral impairment in humans and insects. This modified enzyme is

unable to break down the accumulating acetylcholine at the postsynaptic membrane leading to neuromuscular paralysis. In humans, other symptoms from these groups of compounds include abdominal cramps, nausea, diarrhea, salivation, dizziness, tremor, anxiety, and confusion.[91] Each organophosphate pesticide acts in a slightly different way so that there are many different possibilities for poisoning and targeting efficacy. They are known to degrade rapidly on exposure to air, water, and sunlight, making them less persistent than OC pesticides, although they are more toxic. In the tropical regions of Mexico, organophosphate pesticides are widely used to control dengue fever, but unfortunately, there are cases of careless use leading to cattle poisoning.[92] Where cases of pesticide misuse or abuse are suspected, rather than trying to determine the compound used, it is possible to determine the levels of cholinesterase biomarkers in red blood cells where ≥90% of the total cholinesterase activity has been observed.[92] The levels of these biomarkers are reduced when organophosphate (and other) pesticides are used, and a ≥ 20% decrease in blood AChE indicates exposure to organophosphate pesticides, while toxic effects are generally seen until ≥50% inhibition is seen. Through this approach, it was possible to demonstrate that consecutive treatments of organophosphate pesticides showed accumulative effects.

Fish are very susceptible to pesticide poisoning and as fish are a staple of the diet in developing countries, there is concern over the accumulation and persistence of pesticides in fish and the aquatic environment. Fish absorb pesticides by oral intake of contaminated water or feed or by absorption through the skin.[93] A recent paper suggests that monitoring biomarkers of pesticide exposure can be used to evaluate exposure levels. For organophosphates and carbamates, AChE and butylcholinesterase have been used as biomarkers.[93] The authors note the enzymes are activated when the organisms are exposed to toxic substances such as pesticides. The illegal use of pesticides in aquaculture has had devastating effects on the industry.[94]

7.3.3 Formamidines

There is very little information published on the mode of action and pharmacokinetics of the formamidines in food animals. Amitraz has been found to have complex pharmacological activity in mammals, which can be related to its ability to inhibit monoaminooxidase, block prostaglandin E_2 synthesis, produce a local anesthetic effect, and to stimulate α_2-adrenergic receptors.[52] It has also been shown to induce colic in horses and ponies.[52] One report considering the metabolism of amitraz in ponies and sheep notes that amitraz was hydrolyzed to its metabolite, 2,4-dimethylaniline, in both species.[95] The same study also demonstrated that amitraz was undetectable in sheep plasma after approximately 5 minutes but was persistent in ponies for at least 90 minutes. It is believed that the formamidines act by the activation of chlordimeform by N-demethylation *in vivo*. Strong evidence for this has been presented with the cattle tick, but

in other species such as mice, German cockroaches, or black cutworm eggs, N-demethylation is neither a strong activation nor a detoxication reaction.[49] No reports were found on the use of these compounds in aquaculture.

7.3.4 Insect Growth Regulators

The exact mode of action of the IGRs, including benzoylphenyl ureas, is not fully understood. They inhibit chitin synthesis but have no effect on the enzyme chitin synthetase. It has been suggested that they interfere with the assembly of the chitin chains into microfibrils. When immature insect stages are exposed to these compounds, they are not able to complete ecdysis and die during molting.[50] Benzoylphenyl ureas also appear to have a transovarial effect. Exposed adult female insects produce eggs in which the compound is incorporated into the egg nutrient. Egg development proceeds normally, but the newly developed larvae are incapable of hatching.[50]

7.3.5 Phenylpyrazoles and Neonicotinoids

Phenylpyrazoles are known to be GABA inhibitors. However, there is very little information on their metabolism in the target species although they are much less toxic to mammals than to insects.[96] Neonicotinoids act selectively on the insect central nervous system as agonists of the postsynaptic nicotinic acetylcholine receptors.[97] They are also much less toxic to mammals than insects. As with other compounds that act on the acetylcholine receptors, high levels can overstimulate and block the receptors leading to paralysis and death. AChE cannot break down neonicotinoids and their binding is irreversible.[11]

7.4 Analytical Methods

Products of animal origin are a group of food commodities characterized by a high fat content and are typically divided into four groups: (i) meat (muscle, liver, kidney, fat and offal), (ii) milk and milk products, (iii) eggs, and (iv) honey.[98] In this industrial and highly competitive age, organic pollutants, including pesticides, are widespread and can easily be entrained into animal food commodities, especially those with high fat content. Therefore, pesticides that can legitimately be used on crops can be present in the environment either by design or by accident and may be ingested by animals destined for the global food basket, although direct treatment of the animal remains the main source of pesticides in animals. These compounds then tend to accumulate in the fatty tissues and organs, including brain, lungs, and liver. Pesticides also accumulate in milk, which contains 4–6% fat, and milk products such as cream, which can contain up to 36% fat, where contamination may come from milking equipment, processing sites, or livestock areas. Even though the use of many OP and OC pesticides has been banned in the EU and severely restricted in many other parts of the world, residues are still found

in many animal products.[99] A review of the analytical methods used to determine pesticide residues in foods of animal origin from 1990 to 2010 was published in 2011.[100] Where examples are cited from the scientific literature, it should be noted that there are many others available. Table 7.2 lists some of the extraction and detection methods used in the analysis of veterinary drugs approved for use in food animals.

7.4.1 Detection Methods

Many pesticides are volatile compounds, so gas chromatography (GC) was for many years the analytical instrument of choice. The variety of detectors available, including electron capture detector (ECD), flame photometric detector (FPD), and nitrogen–phosphorus detector (NPD), gave a degree of selectivity and sensitivity. Many pyrethroids and OC pesticides contain electron-withdrawing halogens or groups, which make them amenable to determination by GC-ECD, while pesticides containing nitrogen or phosphorus are amenable to determination with NPD, and sulfur-containing compounds are amenable to determination by FPD. These techniques do not have sufficient attributes to provide unequivocal identification as a confirmatory method for the analysis of pesticides according to international guidelines[101, 102] or regulations such as those of the EU.[103] If confirmation is required, then mass spectrometric (MS) and/or tandem mass spectrometric (MS/MS) detection is required, as was demonstrated by Barbini et al., who screened for the presence of pyrethroids in meat using GC-ECD and used GC-MS for confirmation.[104] GC-ECD was compared to GC-MS (EI) for the analysis of four pyrethroids in pork muscle and pasteurized milk, but the claimed LOQ of 10 mg/kg for porcine muscle would not be considered suitable for many regulatory enforcement programs around the world.[105] Different ionization reactions occurring in the ion source such as negative chemical ionization (NCI) and electron ionization (EI) have been compared and contrasted for the analysis of pyrethroids,[106] while other papers have focused on the effects in the analyzer using ion-trap MS/MS.[107–109] While there is no record of these methods being used to determine pesticides used as veterinary drugs, they are included to complete the discussion. More than one class of pesticides have been determined in a single method[110] when GC-MS operated in the EI mode was used for the determination of organophosphate pesticides and pyrethroids in ground beef with claimed detection limits in the low μg/kg range. As well as comparing detection methods, dual column capillary GC has been used to determine eight organophosphate pesticides in milk and liver and muscle of wild boar.[111]

With the improvements in liquid chromatography instrumentation in recent years, these methods are gaining in popularity for the determination of pesticides, especially in multi-class methods where pesticides and veterinary drugs are often monitored in the same analysis. In the 1990s, many routine methods were based on the use of diode array detection (DAD) for a few compounds, but the majority of new methods use triple quadrupole mass spectrometry (MS/MS) or the

Table 7.2 Extraction and detection methods for pesticides approved for use in veterinary medicine.

Compounds	Matrix	Extraction methods	Detection methods	LOQ	References
Fenobucarb	Beef muscle	QuEChERS			Filigenzi et al.[112]
Pyrethroids	Meat		GC-ECD GC-MS		Barbini et al.[104]
Pyrethroids	Pork muscle Milk		GC-ECD GC-MS	10 mg/kg	Khay et al.[105]
Pyrethroids			GC-MS (EI) GC-MS (NCI)		Húšková et al.[106]
Pyrethroids Organophosphate pesticides	Ground beef		GC-MS (EI)	Low µg/kg	Stefanelli et al.[110]
Organophosphate pesticides	Milk Wild boar liver Wild boar muscle		Dual-column GC		Pagliuca et al.[111]
Benzimidazole carbamate	Beef liver Milk	MSPD, QuEChERS, LLE	Surface plasmon resonance Biosensor screening assay UHPLC-MS/MS	Below MRL	Keegan et al.[114, 115]
Carbamates Organophosphates	Whole milk Muscle tissue Liver tissue	QuEChERS	UHPLC-HRMS		Filigenzi et al.[112]

(*continued*)

Table 7.2 (*Continued*)

Compounds	Matrix	Extraction methods	Detection methods	LOQ	References
Organophosphate pesticides Carbamates Pyrethrins Pyrethroids Dithiocarbamates	Food basket survey	MSPD QuEChERS	LC-MS/MS LC-QTrap	10 µg/kg, OPs in food 5 µg/kg OPs, carbamates, and pyrethrins in water and dithiocarbamates	Chung and Chan[122]
Carbamates	Bovine milk	MSPD	LC-MS (ESI)	3–8 µg/kg	Bogialli et al.[125]
Carbamates Benzoylureas	Fish Shellfish	MSPD SPE	LC-MS/MS (ESI)	Below 10 µg/kg	Carro et al.[124]
32 pesticides	Fatty foods Milk Eggs	QuEChERS MSPD	GC-MS LC-MS/MS		Lehotay et al.[113]
Organophosphate	Milk	SPME	GC-MS GC-NPD	Low µg/l	Rodrigues et al.[126] Cardeal and Paes[127]
Organophosphate	Milk	LLE SPE	Dual column GC-NPD		Pagliuca et al.[118]
Organophosphate, carbamates, pyrethroids	Bulk and market milk		GC		Ciscato et al.[128]
Organophosphate N-methyl carbamate	Porcine tissue	MSPD	HPLC-DAD		Wang et al.[129]

Flufenoxuron Teflubenzuron	Whole milk Egg Beef	PLE LLE	LC ion trap in MS/MS mode	2–10 µg/kg	Brutti et al.[58]
Amitraz and its metabolite 2,4-dimethylaniline	Sheep liver Swine and beef liver	ASE	GC-MS GC-ECD	≤ 10 µg/kg	Yu et al.[131]
Multipesticide residues	Pork, beef, chicken, and fish	ASE GPC	GC-MS	Low µg/kg	Wu et al.[132]
45 pesticides	Chicken, pork, and lamb	ASE GPC	GC-MS/MS		Garrido Frenich et al.[133]
Organophosphates carbamates	Water Milk		GC-ECD		Fagnani et al.[135]
Aldicarb	Ground meat	Inhibition of acetylcholinesterase	Colorimetric acetylcholinesterase		Sabino et al.[137]
Carbaryl	Eggs	LLE and SPE	HPLC-FL	Below action limits	Schenck et al.[68]
Fonofos, diazinon, pyrazophos, pirimiphos ethyl, bromophos ethyl, chlorpyrifos methyl, and ethyl	Honey	SPME, QuEChERS, SPE, PLE			Blasco et al.[138]

various ion-trap technologies. These are often used in conjunction with ultrahigh performance liquid chromatography (UHPLC) to determine over 100 compounds of interest in food matrices.[112] In some cases, both GC and LC methods are used as they are seen to be complementary.[113]

In a very few examples, other detection techniques have been explored. Surface plasmon resonance (SPR) biosensor screening assay, which uses a polyclonal antibody raised in sheep against a methyl 5(6)[(carboxypentyl)-thio]-2-benzimidazole carbamate protein conjugate, has been assessed for the detection of 11 benzimidazole carbamate veterinary drug residues in liver and milk. Results obtained were confirmed by UHPLC-MS/MS methods, and detection limits below the MRLs for the tested compounds were claimed.[114, 115]

7.4.2 Extraction Methods

This discussion will focus on methods where foods of animal origin are included in the sample list of the method. Traditionally, analytical methods for animal-derived foods have tended to focus on separate groups of pesticides so that only a few compounds are monitored at a time. Although there is still a place for these methods, the trend is toward multi-class, multi-analyte methods with minimal sample preparation. The extraction methods used range from the very simple to the very complicated. Some are tailored to one compound, and some are more generic, allowing for the screening of many compounds and/or groups of compounds. The matrix may also play a part in the development of an extraction method. Common matrices are animal tissues including fat, liver, kidney, muscle, and milk. Plasma has also been analyzed, although this does not seem to be a common matrix for pesticide analysis.[116] Most extraction methods involve the use of liquid/liquid extraction and some form of solid-phase extraction (SPE), whether it is traditional SPE using columns or 96-well plates,[116] pressurized liquid extraction (PLE), matrix solid-phase dispersion extraction (MSPD), or quick, easy, cheap, effective, rugged, and safe (QuEChERS).[117] In the majority of published methods, two or more of these techniques are combined to produce clean extracts for analysis. There are many examples of liquid/liquid extraction followed by an SPE clean-up[111, 118] or MSPD followed by SPE.[119] In all cases, reported recoveries are acceptable, and LODs and LOQs are in the low μg/kg range.

7.4.2.1 Quick, Easy, Cheap, Effective, Rugged, and Safe (QuEChERS)

Echoing the need for faster, more economical analysis of a wide variety of contaminants in foods, QuEChERS methods are gaining in popularity. For the technique to be effective, there are three recommended choices of sorbent depending on the fat and color content of the sample:

1) If the fat content is expected to be greater than 5%, 900 mg anhydrous magnesium sulfate and 150 mg each of primary secondary amine (PSA) and C_{18} are used.

2) If the fat content is expected to be less than 5% and the extract is pale or colorless, then 900 mg anhydrous magnesium sulfate and 150 mg PSA are used.
3) If the fat content is less than 5% and intense color is observed, 900 mg anhydrous magnesium sulfate, 150 mg of PSA, and 45 mg of graphitized carbon black are used.

The buffer used for QuEChERS extraction can also have an effect on the extraction efficiency, and to this end, one paper described a method for fenobucarb analysis which compared unbuffered, citrate-buffered, and acetate-buffered QuEChERS extraction efficiencies in beef muscle.[120] While all gave acceptable results, the authors indicate that acetate-buffered extraction was preferred. While only one pesticide was considered in this paper, it would be interesting to see if a multi-class QuEChERS method would give the same result. QuEChERS was also used to determine plant alkaloids, carbamates and organophosphate pesticides and several types of veterinary drugs in whole milk, muscle tissue, liver tissue and corn silage. The authors claim that out of 118 compounds chosen for analysis, 86 were detectable at levels ranging from 0.001 to 5.0 μg/kg in all fortified replicates of at least one matrix. Although they did not attempt quantification, they claim that the method is suitable for screening.[112] A modified QuEChERS method was used to extract benzimidazole carbamate residues from milk. The authors use an MSPD extraction, followed by a QuEChERS extraction, and finally, a liquid–liquid extraction prior to SPR analysis.[114] The LOD was calculated at 2.7 μg/kg, and the mean recovery ranged from 81 to 116%. The method was compared to a UHPLC-MS/MS method using atmospheric pressure electrospray ionization in the positive ion mode for the analysis of milk samples, and it is suggested that this is still used for confirmation of any residues found.

One of the major advantages of a generic extraction method such as QuEChERS is that it has streamlined the analysis required for total diet surveys, which are a useful way of monitoring if chemicals used in the agricultural food system are entering the food chain. One of the most recent surveys considered pesticides in the Hong Kong food basket.[121] A total of 85 pesticides including 48 OPs, 20 carbamates, 15 pyrethrins and pyrethroids, and 2 dithiocarbamates were analyzed in 600 samples using methods described by Chung and Chan,[122] using a QuEChERS method and LC-MS/MS and LC-QTrap analysis. LODs and LOQs were reported as 2 and 10 μg/kg, respectively, in food; 1 and 5 μg/kg, respectively, in water for OPs, carbamates, and pyrethrins; and 1 and 5 μg/kg, respectively, for dithiocarbamates. No pesticides were seen in meat and meat products, although some were detected in fish and seafood.

7.4.2.2 Matrix Solid-Phase Dispersion (MSPD)

Matrix solid-phase dispersion (MSPD), in which the sample is ground with a solid support or SPE medium, is also growing in popularity.[119, 123, 124] A novel extraction method developed by Bogialli et al. exploits the changes in polarity observed at elevated temperatures by using MSPD with heated water as extractant and sand

as the support followed by acidification and filtration and analysis by LC-MS (ESI).[125] They observed recoveries of 76–104% with RSDs not larger than 8%, and LOQs were estimated to be between 3 and 8 μg/kg. It was noted that out of six carbamates, only carbaryl seemed to suffer from matrix interferences in the extraction.[125]

Fish tend to accumulate pesticides, and therefore, methods directed at fish are available. Homogenized fish and shellfish from aquaculture were ground with anhydrous sodium sulfate and C_{18} to provide a homogeneous mixture, which was placed into an SPE tube with silica as a clean-up agent. LC-MS/MS with ESI in both positive and negative modes was used for the analysis of azamethiphos, three avermectins, two carbamates, and two benzoylureas. LODs and LOQs were below 10 μg/kg, and recoveries ranged from 83.8 to 118.0%.[124] However, grinding each sample with the support medium until they are well mixed is labor intensive.

It is inevitable that when new extraction methods are published, they are compared to established methods. One such example compared QuEChERS and MSPD extractions for the determination of 32 pesticide residues in fatty foods, including milk and eggs. Extracts were analyzed using GC-MS and LC-MS/MS with the authors claiming that recoveries of the majority of semi-polar and polar pesticides in the presence of matrix were typically 100%, although the majority were between 70 and 120% in both methods. It was also noted that the recovery of nonpolar pesticides decreased as the fat content increased, a trend that was more pronounced in QuEChERS.[113]

Chung and Chan recently published a paper describing a fast sample preparation method for the determination of 98 organophosphate and carbamate pesticides residues in a diverse selection of food products including citric fruits, vegetables, tree nuts, eggs, dairy products, meat, poultry, edible oils, chocolate, coffee, beverages, seafood, and other matrices.[122] They performed an extraction of the homogenized sample in 1% acetic acid in acetonitrile with anhydrous magnesium sulfate, sodium acetate, and sodium chloride before transferring a portion of the supernatant to a QuEChERS tube for final clean-up before analysis by LC-MS/MS using positive electrospray ionization, which allows for the determination of 49 OPs, 24 carbamates, and their related substances. They state that satisfactory recoveries are obtained in fortified samples with no significant interference and a method limit of quantification of 10 μg/kg for all target analytes.[122]

7.4.2.3 Solid-Phase Micro-extraction (SPME)

Since pesticides are generally volatile, one of the methods of extraction and introduction to the GC is via solid-phase micro-extraction (SPME) in conjunction with headspace analysis, especially if a liquid matrix such as milk is used. The extraction involves the use of a fiber coated with an extracting phase. The sample is placed in a sealed vial, which is allowed to sit for a defined time to attain equilibrium between the headspace and the sample, after which it is transferred to a GC injection port, where the analytes are desorbed. Method parameters that should be optimized include fiber type and coating thickness, temperature,

extraction and desorption times, sample volume, effect of salt addition, and stirring velocities.[126] SPME has been used to extract organophosphate pesticides in milk with GC-MS[126] and GC-NPD for analysis.[127] The proposed methodologies were able to determine all of the pesticides with detection and quantification limits at the low μg/l levels for all compounds studied. It was also noted that residues of pesticides are not removed by boiling the milk.[127] A more complicated method for the extraction of organophosphorus pesticides from milk involved two liquid/liquid extractions followed by SPE with C_{18} and determination with a dual-column GC and NPD for both columns.[118] GC was also used in a survey of bulk and market milk samples in Brazil for organophosphates, carbamates, pyrethroids, herbicides, and fungicides.[128] While the authors did not find significant amounts of the 70 pesticides included in the method, they did find trace amounts of alpha-hexachlorocyclohexane (α-HCH) and both endosulfan isomers, all of which are banned for use in Brazil except for a limited number of applications for endosulfan.

It is relatively rare to see a report of a multi-class method where mass spectrometry is not the detection method of choice, so a paper published in 2009 which described the multi-residue determination of fluoroquinolones, organophosphorus pesticides, and N-methyl carbamates in porcine tissues using MSPD with C_{18} and sodium sulfate with an analysis using HPLC-DAD was unusual.[129] Only five compounds were considered, of which the only pesticides were dichlorvos and carbaryl. Recoveries of 60.1 – 107.7% were reported with LODs in porcine tissues between 9 and 22 μg/kg. A comparison with a method using SPE with C_{18} for the same analytes gave recoveries of 16.3% to 60.5%.

7.4.2.4 Pressurized Liquid Extraction/Accelerated Solvent Extraction (PLE/ASE)

Pressurized liquid extraction (PLE) or accelerated solvent extraction (ASE) is a widely used technique in pesticide analysis as it offers a rapid and effective clean-up method. It is similar to a Soxhlet extraction, except the solvent is used near its supercritical region where its extraction efficiency is higher. Advantages over Soxhlet extractions are that a smaller volume of solvent and a shorter extraction time can be used. The sample is normally mixed with an inert matrix to absorb any water present. For a review of ASE, which includes PLE, the reader is referred to an excellent review written by Sun.[130] Flufenoxuron and teflubenzuron were monitored in whole milk, rice, egg, lemon, lettuce, beef, and avocado using PLE with ethyl acetate, liquid – liquid extraction with methanol, and analysis using an LC ion trap in the MS/MS/MS mode.[58] The authors state that LOQs are between 2 and 10 μg/kg, which are below the MRLs. Matrix effects were reported to be more pronounced with the animal-based matrices. ASE has also been used to extract amitraz and its metabolite, 2,4-dimethylaniline, in food animal tissues using GC-MS and GC-ECD analysis.[131] ASE was achieved using a heating time of 2 minutes, static time of 2 minutes, pressure of 120 bar, temperature of 60 °C, 60 second purging with nitrogen, and water flushing with

50% of the cell size, recycled three times. 15 ml of extract was obtained at the end of each cycle, and 3 ml of this was mixed with 27 ml of NaOH (pH = 9.0) for SPE clean-up. Sheep liver was the matrix of choice for this study, although it was extended to swine and beef liver. Recoveries from fortified tissues were reported to be between 72.4% and 101.3% for GC-ECD and 77.4% and 107.1% for GC-MS with LODs ≤ 5 g/kg and LOQs ≤ 10 μg/kg.[131]

Wu et al. have also used ASE and gel permeation chromatography (GPC) to determine multi-pesticide residues in foods of animal origin with GC-MS detection.[132] They claim that 109 pesticides including isomers can be monitored. Target analytes were extracted from pork, beef, chicken, and fish using ASE extraction with acetonitrile, the residue solvent exchanged into cyclohexane:ethyl acetate for GPC clean-up. The organic mobile phase was cyclohexane:ethyl acetate, with a flow rate of 5 ml/minute and detection wavelength of 254 nm. The fraction from 8 to 20 minutes was collected, evaporated to a small volume, redissolved in acetonitrile and 0.2 g PSA added, followed by shaking and centrifuging. The final solution was amended with internal standard and GC-MS analysis was performed. LOQs were claimed to be at the low μg/kg level.

A comparison of Polytron™ homogenization, Soxhlet and ASE extractions was undertaken to determine the most efficient method for determining residues of 45 pesticides in chicken, pork, and lamb.[133] The final method extracted the pesticides into ethyl acetate using ASE followed by clean-up with GPC and determination with GC-MS/MS. Recoveries were in the range 70–90%.

7.4.2.5 Contaminated Feed

It has been noted that most food contamination issues start with contaminated feed and therefore this matrix is often monitored for the presence of chemical contaminants,[134] as it is also a source of pesticide contamination in food items of animal origin. Organophosphate and carbamate pesticides were found in 14 out of 48 feed samples and 6 out of 16 water samples tested from the Agreste region of Pernambuco in Brazil.[135] Some of the feedstuff samples were found to contain at least five compounds at levels above the pesticide residue level permitted in the EU. Meat from animals fed contaminated feed was tested using the extraction method described previously[111] and analysis was by GC-ECD. Milk samples from the same farms as the feed and water were collected to track the possible sources of milk contamination. Contaminated milk was found on farms where the contamination was observed. The authors note that Brazil does not have established limits for pesticides in milk, but that the values observed were lower than the MRLs established by the CAC for dimethoate, carbaryl, carbofuran, fenthion, coumaphos, and aldicarb.[136] The authors suggest that the inappropriate use of these compounds means that metabolites can be deposited in fat and muscle and can be found in milk. If an appropriate withdrawal period is not allowed between dosing and slaughter, then residues can also be found in milk and tissues.

7.4.2.6 Miscellaneous Methods

When food commodities are contaminated with visible "unknown" materials, then methods to detect the active ingredients of the contaminant are required. The need becomes even more urgent when human health is compromised. One such event was investigated in Brazil in 2010.[137] Rather than look for the specific suspected pesticides, the authors decided to monitor the inhibition of AChE by aldicarb and report results in aldicarb equivalents as this is the contaminant most commonly found in cases of poisoning. Between 2008 and 2010, 157 cases of aldicarb contamination in meat, coffee, milk, soup, juice, cookies and chocolate were reported in the state of Rio de Janeiro.[137] In this method, ground meat samples were mixed with water and an aliquot of the supernatant was extracted with methylene chloride followed by colorimetric AChE assay. The standard curve was constructed by extracting AChE from rat brains, and adding appropriate amounts of carbamate standard to the enzyme, adding color reagent, and measuring the absorbance at 412 nm. Recovery calculations were performed by fortifying meat samples before and after extraction with aldicarb and measuring absorbance as before. Quantification in real samples was performed using the aldicarb calibration curve constructed from fortified standards and results expressed as μg/g of aldicarb equivalents. While the method was able to determine AChE concentrations, it was not able to identify the carbamates responsible. The method was claimed to be rapid, precise, and accurate.

The pattern of deposition of pesticide residues in eggs is different than in tissue.[68] Individual egg yolks develop in the hen over a period of many months, so when the hen is exposed to drugs or pesticides, residues will be stored in egg yolks that are slated for ovulation days or weeks in the future and may be retained long after all of the drug or pesticide residue has been excreted from the rest of the animal. As with other animals, pesticides may be entrained into chickens as a result of being present in the feed or if the hens are directly treated with pesticides for external parasites, and thus pass into developing eggs. Carbaryl is used as a dust on chickens to control mites and eggs can contain measurable residues for weeks after treatment. Twenty-one carbamates were monitored in this method. Sample size is large (25 g) and solvent volumes are also generous (100 ml of acetonitrile) for a liquid–liquid extraction and SPE (aminopropyl) column, although these may be warranted to obtain the required limits of quantification. The final extracts were derivatized with *o*-phthalaldehyde for fluorescence detection. The authors found measurable levels of carbamates in eggs, but they were all below the action limits.

7.4.2.7 Honey

Honey is a foodstuff where the presence of insecticides can have a devastating effect. Not only can commercial crops become contaminated, but colonies of bees can be destroyed. A method developed by Blasco[138] dissolved honey in hot water and then compared four extraction methods to optimize the sensitivity and minimize interfering compounds in the extract before LC-MS/MS determinations.

They looked at SPME, QuEChERS, SPE, and PLE. The authors concluded that SPME returned the lowest CCα values, while QuEChERS returned the highest. The compounds considered were fonofos, diazinon, pyrazophos, pirimiphos ethyl, bromophos ethyl, and chlorpyrifos methyl and ethyl. They concluded that SPME cannot be used for quantitative work as the recoveries are not high enough.

Honey is a matrix very different from those normally thought of when veterinary drugs are applied. It is a complex matrix, and while many of the techniques described in preceding sections are also used on honey, it is sufficiently different that it warrants special consideration. A recent paper looks at the concentrations of 14 organophosphate pesticides in honey bees and hive matrices to assess their hazard to honey bees in Egypt.[139] A modified QuEChERS method was used to extract the pesticides and they were determined using LC-MS/MS. It seemed that more pesticides were detected more frequently in the summer months. The authors developed hazard quotients to assess the lethality of these compounds to bees and concluded that direct exposure and dietary exposure to OPs in honey and pollen pose little threat to the lethality of bees in Egypt.

A recent study in Belgium was conducted on honey bee wax combs from 10 hives for the presence of almost 300 OCs and OPs by LC-MS/MS and GC-MS/MS.[140] Pesticide residues were found in every sample, with traces of 18 pesticides found, ranging from 3 to 13 per sample. MSPD with mixed solvent elution (acetonitrile and ethyl acetate or dichloromethane) was used to extract organophosphate pesticides from raw propolis (bee glue – a resinous mixture that honey bees collect from tree buds, sap flows, or other botanical sources to seal unwanted open spaces in the hive).[141] Human et al. have investigated the effect of pesticides on honey bee larvae as they are less understood than for adult bees even though they accumulate in the hive.[142] They note that the mass of prepupae and white-eyed pupae was not affected by nicotine, neither was protein or lipid stores, although water content was affected. The authors attribute the absence of negative effects to detoxification mechanisms in developing honeybees, allowing them to resist both natural and synthetic xenobiotics.

In 2011, 61 honey samples were collected in four regions of Columbia.[143] Pesticides were extracted using liquid–liquid extraction followed by SPE on Florisil® columns. Detection was by GC-NPD/μECD. Pesticide residues were identified in 32 samples, with chlorpyrifos being the most common residue found, although only 4.9% of residues were above the MRLs established in Regulation (EC) No. 396/2005.[75]

7.5 Conclusion

The use of pesticides (ectoparasiticides) in veterinary drug medicines is well documented, and their use has been shown to be beneficial, both to the health of the animals and to the economy. Their use is more widespread in tropical

and semi-tropical climates where ticks, flies, and burrowing insects are more of a problem. Even though there is widespread use, they do not seem to present as residues in many of the edible meat products available for human consumption. Analytical methods used to monitor pesticides in veterinary applications include the use of both GC and liquid chromatography with MS detector systems, whether they be single quadrupole, triple quadrupole, or ion trap based. Detection limits are constantly being driven lower due to the developments of increasingly sophisticated instrumentation. Extraction methods are also generally becoming simpler so that many compounds or classes of compounds can be determined in one sample. However, this does not remove the need for more targeted analysis if suspect positive results are observed from the analysis of a sample.

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8

Non-steroidal Anti-inflammatory Drugs

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8.1 Introduction: What Are Pain Killers (Analgesics) and NSAIDs?

Pain killers are a group of drugs referred to as analgesics that help to reduce pain. There are various types of analgesics, for example, opioid (narcotic) analgesics such as morphine and codeine[1] and nonopioid (nonnarcotic) analgesics such as aspirin and ibuprofen. In this chapter, only one type of nonnarcotic analgesics, the non-steroidal anti-inflammatory drugs (NSAIDs), is considered.[2, 3] These drugs are also called non-steroidal anti-inflammatory analgesics/agents (NSAIAs) or non-steroidal anti-inflammatory medicines (NSAIMs). NSAIDs provide analgesic and antipyretic (fever-reducing) effects, and at higher doses, anti-inflammatory effects. The term "non-steroidal" is used to distinguish these drugs from steroids which also have anti-inflammatory (and many other) effects. The detailed function and use of NSAIDs has been comprehensively reviewed by Lees.[3]

Many anti-inflammatory drugs act by inhibition of prostaglandin (PG) biosynthesis.[3] Specifically, NSAIDs competitively inhibit cyclooxygenases (COXs), the enzymes that catalyze the synthesis of cyclic endoperoxides from arachidonic acid to form PGs.[2] COX-1 and COX-2 have been identified as the primary COX isoenzymes. COX-1 is expressed constitutively in many cells. It is important for the regular production of "homeostatic" PGs, which help regulate blood flow in the kidney and stomach. COX-1 is present in many tissues and cell

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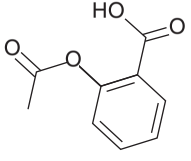
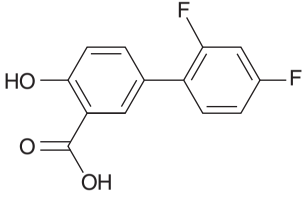
types, most notably in platelets, endothelial cells, gastrointestinal (GI) tract, renal microvasculature, glomerulus, and collecting ducts. Inhibition of COX-1 activity is considered a major contributor to NSAID GI toxicity. COX-2 is considered an inducible isoenzyme that plays a significant role in pain and inflammatory processes, though it may also be expressed normally in some tissues.

Nonselective NSAIDs will inhibit both COX-1 and COX-2 when used at the label dose.[3] Many older NSAIDs are nonselective COX inhibitors, the most prominent of which are salicylates (aspirin).[1] Ibuprofen, naproxen, ketoprofen (arylpropionic acids), indomethacin and sulindac (arylalkanoic acid derivatives), diclofenac, mefenamic acid and tolfenamic acid (anthranilic acid derivatives), and piroxicam (enolic acid) are also nonselective COX-1 inhibitors. Some can cause significant GI, renal, hepatic, or hematologic adverse events and may not be advisable for long-term use. The COX-2 selective (also known as COX-1 sparing) inhibitors are a newer class of NSAIDs. Examples of COX-2 selective NSAIDs that are licensed for veterinary use include meloxicam, etodolac, and "coxib" drugs such as firocoxib, robenacoxib, and deracoxib. The COX-2 selective NSAIDS often, though not always, have a greater margin of safety than COX nonselective NSAIDs.

The NSAIDs can be further classified on the basis of their chemical structure.[3] In general, NSAIDs consist of a carboxylic and/or phenolic functional group. Some NSAIDs contain polar groups that attach the planar aromatic moiety to an additional lipophilic group. As a result of this general common structural feature of the NSAIDs, they display a few common characteristics. They are all organic acids with pK_a values in the range 3–5. Most of them are carboxylic acids and, therefore, form salts easily on treatment with bases and ionize extensively at physiological pH. It is the presence of the acidic functionality that drives the COX inhibitory activity.[4] The acidic group serves as a major binding group with plasma proteins and thereby renders all NSAIDs highly bound to plasma proteins. The acidic group also provides a major metabolic site for conjugation. Hence, the major pathway of clearance for most NSAIDs is glucuronidation and inactivation followed by renal elimination. The NSAIDs will differ in their lipophilicities on the basis of the lipophilic nature of the aryl groups attached as well as any additional lipophilic moieties and substituents. They can be classified based on their chemical structure as shown in Table 8.1.

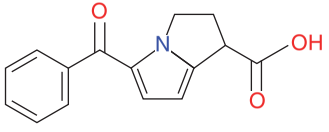
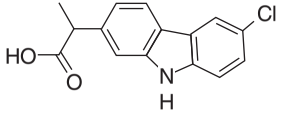
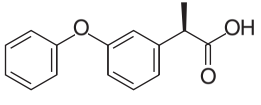
Although a wide variety of NSAIDs are currently available for human or veterinary use, only a few are licensed or commonly used in food-producing animals. Many NSAIDs are inappropriate to use in food-producing animals due to concerns over target animal safety, lack of efficacy, or drug residues. Other NSAIDs may have an inconvenient route of administration, short duration of effect, or prohibitive cost. The most common NSAIDs administered to ruminants (cattle, sheep, goats, etc.) include ketoprofen, flunixin, meloxicam, carprofen, tolfenamic acid, and aspirin. Ketoprofen, flunixin, tolfenamic acid, and acetaminophen/paracetamol are licensed for use in swine. NSAIDs licensed for use in horses include ketoprofen, carprofen, meloxicam, flunixin, vedaprofen,

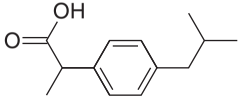
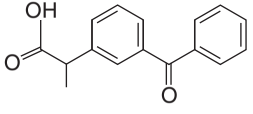
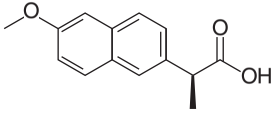
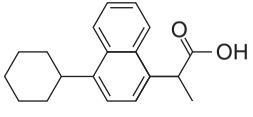
Table 8.1 Classification of the NSAIDs (number in table corresponds to subsection in text where compound is discussed).

Structure	Common name	IUPAC name	CAS Registry No.	Molecular formula	Molecular weight	pK_a
8.1.1 Salicylates						
8.1.1.1 Aspirin	ASA, Anacin, Bayer, Bufferin	2-Acetoxybenzoic acid	50-78-2	$C_9H_8O_4$	180.16	3.5
						
8.1.1.2 Diflunisal	Dolobid	2′,4′-Difluoro-4-hydroxy-3-Biphenyl carboxylic acid	22494-42-4	$C_{13}H_8F_2O_3$	250.20	2.69
						

(*continued*)

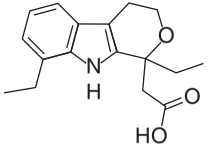
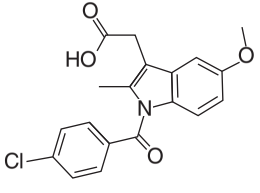
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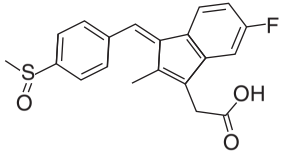
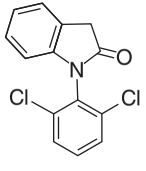
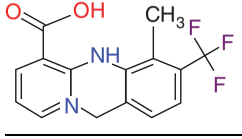
Structure	Common name	IUPAC name	CAS Registry No.	Molecular formula	Molecular weight	pK_a
8.1.2 Arylacetic acid derivatives: Pyrrole acetic acid derivatives						
8.1.2.1 Ketorolac 	Toradol	5-Benzoyl-2,3-dihydro-1*H*-pyrrolizine-1-carboxylic acid	74103-07-4	$C_{15}H_{13}NO_3$	255.27	3.84
8.1.3 Arylpropionic acid derivatives (profens)						
8.1.3.1 Carprofen 		2-(6-Chloro-9*H*-carbazol-2-yl)propanoic acid	53716-49-7	$C_{15}H_{12}ClNO_2$	273.71	4.42
8.1.3.2 Fenoprofen 		2-(3-Phenoxyphenyl) propanoic acid	31879-05-7	$C_{15}H_{14}O_3$	242.27	3.96

8.1.3.3 Ibuprofen 	Advil, Motrin, Nuprin	2-(4-Isobutylphenyl) propanoic acid	15687-27-1	$C_{13}H_{18}O_2$	206.28	4.91
8.1.3.4 Ketoprofen 		2-(3-Benzoylphenyl) propanoic acid	22071-15-1	$C_{16}H_{14}O_3$	254.28	4.45
8.1.3.5 Naproxen 	Aleve Naproxen	2*S*-2-(6-Methoxy-2-naphthyl) propanoic acid	2204-53-1	$C_{14}H_{14}O_3$	230.26	4.15
8.1.3.6 Vedaprofen 		2-(4-Cyclohexyl-1-naphthyl) propanoic acid	71109-09-6	$C_{19}H_{22}O_2$	282.38	4.74

(*continued*)

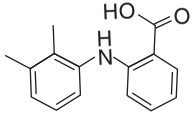
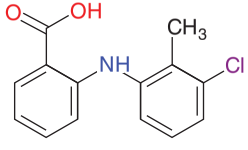
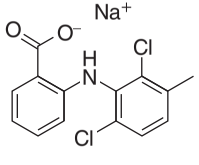
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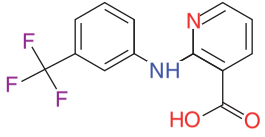
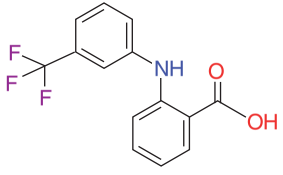
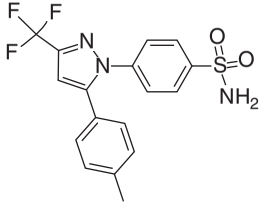
Structure	Common name	IUPAC name	CAS Registry No.	Molecular formula	Molecular weight	pK_a
8.1.4 Arylalkanoic acid derivatives						
8.1.4.1 Etodolac 		(1,8-Diethyl-1,3,4,9-tetrahydropyrano[3,4-b]indol-1-yl) acetic acid	41340-25-4	$C_{17}H_{21}NO_3$	287.35	4.7
8.1.4.2 Indomethacin 		1-(4-Chlorobenzoyl)-5-methoxy-2-methyl-1*H*-indol-3-yl] acetic acid	53-86-1	$C_{19}H_{16}ClNO_4$	357.79	4.5

8.1.4.3 Sulindac 	{(1Z)-5-Fluoro-2-methyl-1-[4-(methylsulfinyl) benzylidene]-1*H*-inden-3-yl}acetic acid	38194-50-2	$C_{20}H_{17}FO_3S$	356.41	4.7
8.1.5 *N*-Anthranilic acid derivatives or fenamic acid derivatives					
8.1.5.1 Diclofenac 	{2-[(2,6-Dichlorophenyl) amino]phenyl}acetic acid	15307-86-5	$C_{14}H_{11}Cl_2NO_2$	296.15	4.15
8.1.5.2 Flunixin 	2-{[2-Methyl-3-(trifluoromethyl)phenyl] amino}nicotinic acid	38677-85-9	$C_{14}H_{11}F_3N_2O_2$	296.24	5.82

(*continued*)

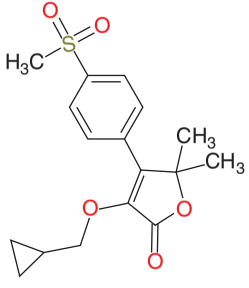
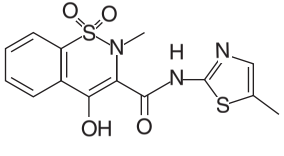
Table 8.1 (Continued)

Structure	Common name	IUPAC name	CAS Registry No.	Molecular formula	Molecular weight	pK_a
8.1.5.3 Mefenamic acid 		2-[(2,3-Dimethylphenyl) amino]benzoic acid	61-68-7	$C_{15}H_{15}NO_2$	241.29	4.2
8.1.5.4 Tolfenamic acid 		2-[(3-Chloro-2-methylphenyl) aminobenzoic acid	13710-19-5	$C_{14}H_{12}NO_2Cl$	261.70	4.3
8.1.5.5 Meclofenamic acid 		2-[(2,6-Dichloro-3-methylphenyl)amino] benzoic acid	644-62-2	$C_{14}H_{11}Cl_2NO_2$	296.15	3.8

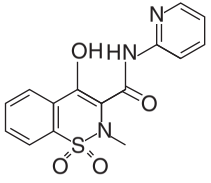
8.1.5.6 Niflumic acid	2-[3-(Trifluoromethyl) anilino]pyridine-3-carboxylic acid	4394-00-7	$C_{13}H_9F_3N_2O_2$	282.22	1.9
					
8.1.5.7 Flufenamic acid	2-{[3-(Trifluoromethyl) phenyl]amino}benzoic acid	530-78-9	$C_{14}H_{10}F_3NO_2$	281.23	3.88
					
8.1.6 Coxibs					
8.1.6.1 Celecoxib	4-[5-(4-Methylphenyl)-3-(trifluoromethyl)-1*H*-pyrazol-1-yl] benzenesulfonamide	169590-42-5	$C_{17}H_{14}F_3N_3O_2S$	381.37	10.7
					

(*continued*)

Table 8.1 (Continued)

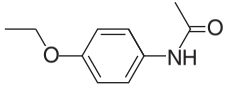
Structure	Common name	IUPAC name	CAS Registry No.	Molecular formula	Molecular weight	pK_a
8.1.6.2 Firocoxib		3-(Cyclopropylmethoxy)-5,5-dimethyl-4-[4-(methylsulfonyl)phenyl]-2(5*H*)-furanone	189954-96-9	$C_{17}H_{20}O_5S$	336.40	4.7
						
8.1.7 Oxicams or enolic acids						
8.1.7.1 Meloxicam	Metacam	4-Hydroxy-2-methyl-*N*-(5-methyl-1,3-thiazol-2-yl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide	71125-38-7	$C_{14}H_{13}N_3O_4S_2$	351.40	4.08
						

8.1.7.2 Piroxicam		4-Hydroxy-2-methyl-*N*-(2-pyridinyl)-2*H*-1,2-benzothiazine-3-carboxamide 1,1-dioxide-4	36322-90	$C_{15}H_{13}N_3O_4S$	331.35	6.3

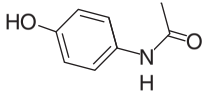


8.1.8 Anilides

8.1.8.1 Phenacetin		*N*-(4-Ethoxyphenyl) acetamide	62-44-2	$C_{10}H_{13}NO_2$	179.22	14.98

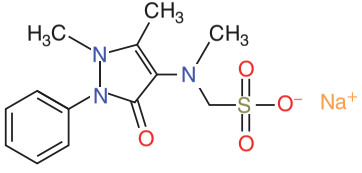
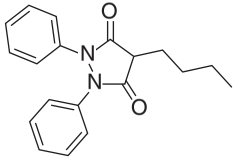
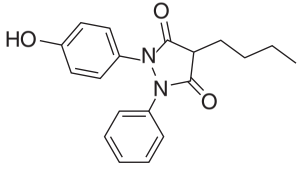


8.1.8.2 Acetaminophen	Anacin, Excedrin,Tylenol, paracetamol	*N*-(4-Hydroxyphenyl) acetamide	103-90-2	$C_8H_9NO_2$	151.16	9.38



(*continued*)

Table 8.1 (Continued)

Structure	Common name	IUPAC name	CAS Registry No.	Molecular formula	Molecular weight	pK_a
8.1.9 Phenylpyrazolones (pyrazolidinedione)						
8.1.9.1 Metamizole 	Dipyrone	Sodium[(1,5-dimethyl-3-*oxo*-2-phenyl-2,3-dihydro-1*H*-pyrazol-4-yl)(methyl) amino]methanesulfonate	50567-35-6	$C_{13}H_{16}N_3NaO_4S$	333.34	-1.2
8.1.9.2 Phenylbutazone 		4-Butyl-1,2-diphenyl-3,5-pyrazolidinedione	50-33-9	$C_{19}H_{20}N_2O_2$	308.37	4.5
8.1.9.3 Oxyphenbutazone 		4-Butyl-1-(4-hydroxyphenyl) -2-phenyl-3,5-pyrazolidine-dionehydrate (1:1)	129-20-4	$C_{19}H_{20}N_2O_3$	324.37	4.87

and phenylbutazone.[3] Note however that some of these NSAIDs (particularly phenylbutazone) are not to be administered to horses intended for food production.[5] Based on available information on approved uses in food-producing animals for this class of drug, NSAID use is uncommon in the poultry industry (see Section 8.4). No residues of NSAIDs authorized for use in dogs and cats (such as deracoxib, robenacoxib, etodolac, and tepoxalin) have been reported in regulatory testing of animal-derived foods, suggesting that these drugs are not commonly used in food-producing animals.

Additional information on use of NSAIDs in veterinary medicine may be found in the current edition of the Merck Veterinary Manual.[2]

8.1.1 The Salicylates

These are derivatives of 2-hydroxybenzoic acid. First discovered in 1838[6] following extraction of salicylic acid from willow bark, it was used medicinally as the sodium salt until 1853 when it was replaced by the acetylated derivative, acetylsalicylic acid (ASA) or aspirin.[7, 8] The therapeutic effect of the salicylates is enhanced by the esterification of the phenolic group and by substitution of the difluorophenyl (hydrophilic/hydrophobic) group at C-5 as in diflunisal. The salicylates are converted to salicylic acid after which they undergo a variety of secondary metabolic transformations that include conjugation with glycine to form salicyluric acid, ring hydroxylation, and carboxylic and phenolic conjugation with glucuronidase.

8.1.2 Arylacetic Acid Derivatives: Pyrrole Acetic Acid Derivatives

Ketorolac is an example of a pyrrole acetic acid.[9] It is unique in the sense that it does not carry any substituents on the benzylic group and is therefore not susceptible to the type of oxidation observed for other members of the group such as tolmetin[10] which has a methyl group on the benzylic group. Tolmetin is formulated for both oral and intramuscular administration. It has not only good oral bioavailability and primarily analgesic activity but also good anti-inflammatory and antipyretic activities. These drugs are available for use in humans by prescription, but no evidence of veterinary use in the treatment of food animals was found in the preparation of this chapter.

8.1.3 2-Arylpropionic Acid Derivatives (Profens)

These compounds, examples of which are carprofen,[11] ibuprofen,[12] ketoprofen,[13] naproxen,[14] and vedaprofen,[15] are often referred to as "profens" on the basis of the suffix of the prototype member, ibuprofen. They have the general structure Ar-CH(CH_3)-COOH rendering them all predominantly ionized at physiological pH; they are also more lipophilic than ASA or salicylic acid itself. The α-carbon in the profens is chiral, and the *S*-(+) enantiomer of the profens is the more potent

COX inhibitor. The α-CH_3 substituent in the profens increases COX inhibitory activity and reduces their toxicity. The profens undergo a metabolic inversion at the chiral carbon center that involves stereospecific transformation of the inactive *R*-enantiomer to the active *S*-form. This transformation is believed to proceed through an activated thioester intermediate.[16]

8.1.4 Arylalkanoic Acid Derivatives (Indene/Indole Acetic Acid Derivatives)

These are acetic acid derivative compounds in which the substituent at the 2-position is a heterocycle or related carbon cycle, examples of which are indomethacin[17] and sulindac.[18] The pyrroles discussed in Section 8.1.2 can also be considered to belong to this general classification group. Indomethacin contains a benzoylated indole nitrogen in which the methyl group at the 2-position of the indole ring is hindered and prevents free rotation about the C—N bond and thereby keeps the two aromatic rings in the right structural alignment for COX binding and therapeutic activity. Indomethacin is metabolized through glucuronidation of the carboxyl group to the phenol, along with demethylation and glucuronidation of the resulting phenol.[19] Sulindac, being a prodrug, metabolizes by reduction to the sulfide which is significantly more active than the parent drug.[20]

8.1.5 *N*-Anthranilic Acid Derivatives (Fenamic Acid Derivatives or Fenamates)

These are *N*-aryl-substituted derivatives of anthranilic acid, which is derived from salicylic acid, examples of which are diclofenac,[21] mefenamic acid,[22] and tolfenamic acid.[23] The acidic properties due to the carboxylic acid are retained for these compounds. While mefenamic and tolfenamic acids are derived from anthranilic acid, diclofenac is derived from 2-arylacetic acid. The most active anthranilates are characterized by the presence of small alkyl and/or halogen substituents at the 2′, 3′, and/or 6′ position of the *N*-aryl moiety. Among the disubstituted *N*-aryl anthranilates, the 2′, 3′ derivatives are more active suggesting that the 2′, 3′ position substituents act to force the *N*-aryl ring out of coplanarity with the anthranilic acid. Thus, it is this steric effect which enables the anthranilates to interact effectively at the inhibitory site on COX. Mefenamic acid undergoes benzylic oxidation of the ortho methyl group and ring oxidation followed by glucuronidation,[24] while diclofenac is metabolized by acyl-*O*-glucuronidation and oxidation of the aromatic rings.[25]

8.1.6 Coxibs or Cox-2-Selective Inhibitors

These compounds, examples of which are celecoxib[26] and firocoxib,[27] are diaryl-5-membered heterocycles. Celecoxib has a central pyrazole ring and two nearby phenyl substituents, one with a methyl group and the other a sulfonamide

group. The polar sulfonamide group binds to a distinct hydrophilic region that is present in COX-2 but not in COX-1.[28] Firocoxib has a central furanone group and one phenyl ring with a polar methylsulfonyl group.

8.1.7 Oxicams or Enolic Acid Derivatives (Prodrugs)

These compounds, examples of which are meloxicam[29] and piroxicam,[30] are characterized by the 4-hydroxybenzothiazine heterocycle. The 4-OH group stabilizes the enolate anion that is formed as a result of intramolecular H-bonding to the amide group and confers acidic properties to these drugs.[31] Because the chemical structure of piroxicam differs significantly from that of meloxicam, the drugs are metabolized by different routes. Piroxicam undergoes ring oxidation followed by glucuronidation, while meloxicam undergoes slow hydrolysis at the benzyl methyl group of the thiazole side chain.[4]

8.1.8 The Anilides

Phenacetin[32] and acetaminophen[33] are examples of simple anilide analogues with a hydroxy or an alkoxy group at position 4. They do not possess the typical carboxylic acid group functionality and are, therefore, considered to be neutral drugs in their acid–base functionality. Phenacetin undergoes oxidative-O-dealkylation to produce acetaminophen.[34]

8.1.9 Phenylpyrazolones

These compounds, examples of which are phenylbutazone[35] and oxyphenbutazone,[36] have the 1-aryl-3, 5-pyrazolidinedione structure. The acidity of phenylbutazone ($pK_a = 4.5$) is derived from the acidic hydrogen situated between the two electron-withdrawing carbonyl groups. Oxyphenbutazone is the hydroxylated metabolite of phenylbutazone and is a major metabolite found in the urine of horses[37] and cattle.[38]

8.2 Veterinary Drug Properties, Structures, and Regulation

Negative public perception associated with routine animal management practices, including dehorning and castration, is on the rise, with increasing calls to develop management practices that relieve pain and suffering in livestock. While there is no simple definition of pain, pain can be described as an unpleasant, sensory experience representing awareness by the animal to threat or damage to its tissues that elicits protective actions and results in learned avoidance. It may be acute, chronic, localized, generalized, emotional, physical adaptive, and/or maladaptive. In farm animals, pain is generally assessed in general body functions

such as reduced food intake, decreased production, and lameness.[39] Pain reduces performance, whether it is with milk yield or growth rate and therefore reduces farm income. Therefore, the goal of all farmers and their veterinary practitioners is to prevent diseases and injuries causing pain to farm animals. This can be achieved either through prevention of pain or through therapy to reduce pain.

NSAIDs are widely used to provide symptomatic relief in the treatment of both acute and chronic musculoskeletal inflammatory conditions, as well as pre- and postoperatively to mitigate surgical pain. The other major uses of NSAIDs in food animals include antipyretic effects (reduction of fever), antiendotoxin effects (particularly flunixin), ancillary therapy for other "noninflammatory" conditions such as diarrhea, and ancillary therapy for conditions with inflammatory components (such as respiratory disease and mastitis).[3]

8.3 Pharmacokinetics/Metabolism

In general, NSAIDs exhibit good bioavailability whether administered by the oral, intramuscular, or subcutaneous routes, although a delay in absorption may be observed in horses and ruminants after oral dosing.[40] Other typical characteristics include a high degree of binding to plasma protein, low volumes of distribution, limited excretion of the administered dose as parent drug in urine, differences in clearance and elimination half-life between species.

The pharmacokinetics (PKs) of NSAID can be highly variable, with extensive differences between compounds. For example, ketoprofen, a generally rapidly eliminated NSAID in most species, has an elimination half-life of approximately 30 minutes in cattle,[41] whereas the elimination half-life of meloxicam is longer than 24 hours in this species.[42] PK variability also occurs between species; the elimination half-life of ketoprofen is approximately 10 times longer in llamas than in cattle.[43] In some instances, even using the same compound in the same species may produce significantly different pharmacokinetics, depending on the age and health status of the animal.[44] Furthermore, NSAIDs which are racemic mixtures (such as the 2-arylpropionate NSAIDs ketoprofen and carprofen) can have different PK and pharmacodynamic (PD) properties for each enantiomer.[45] Therefore, studies involving chiral NSAID pharmacokinetics, dynamics, and tissue residues should include stereoselective analysis.[46]

Oral bioavailability of the NSAIDs is generally very high in most species, with the possible exception of ASA (Aspirin) use in ruminants.[47] Other NSAIDs commonly administered to food-producing species by the oral route include acetaminophen (paracetamol) in pigs,[48] and there is much research on the oral (extralabel) use of meloxicam in food-producing species. Many oral NSAIDs are licensed for use in horses, including phenylbutazone, flunixin, firocoxib, and vedaprofen. However, most NSAID formulations licensed for use in food animals are injectable (intramuscular, subcutaneous, or intravenous) formulations. It should be noted that some injectable NSAID formulations, such as flunixin, are

only to be administered by intravenous administration, and can cause injection site lesions when given by other routes of administration. This may result in prolonged drug absorption and result in non-compliant drug residues.[49–51]

Distribution of NSAIDs in veterinary species is highly variable. The newer-generation coxib NSAIDs (such as firocoxib) are lipid soluble and have a high volume of distribution. However, most NSAIDs have a lower volume of distribution ($V_D < 1$ l/kg) and are distributed mainly in plasma and extracellular fluid.[46] This may account for the low concentration of NSAID residues typically found in muscle and fat of most food animals. Many NSAIDs are extensively bound to plasma proteins, with values > 80% for ketoprofen, meloxicam, carprofen, flunixin, and phenylbutazone in most veterinary species.[46]

Most veterinary NSAIDs undergo extensive hepatic biotransformation by a variety of metabolic pathways, such as phase I cytochrome P450-mediated oxidation or hydroxylation (phenylbutazone and others[52]) and phase II conjugation (such as glucuronidation of acetaminophen[53]). Metabolism of ASA also occurs in other tissues, such as hydrolysis in the blood by plasma esterases.[54]

Elimination of NSAID metabolites commonly occurs by the renal route, whereas highly protein-bound parent compound has little glomerular filtration, and thus low urine concentrations are typically observed.[3] Many NSAID metabolites, as well as parent compound, are eliminated by biliary as well as renal routes. Therefore, kidney and liver are usually the tissues with the highest and most persistent NSAID residue concentrations and are the target tissues used by most regulatory agencies.

The elimination half-life is determined by the volume of distribution and the rate of clearance. Because these parameters are subject to high interspecies variance for NSAID compounds, the elimination half-life (and thus withdrawal period) of a compound can vary substantially between species. For example, injectable meloxicam (0.4 mg/kg) has an elimination half-life of only 2.5–6 hours in pigs and a corresponding withdrawal period of 5 days.[55] The same formulation has an elimination half-life of >24 hours in cattle, with a much longer 15-day withdrawal period.[55]

Some NSAIDs, such as meloxicam,[55] are excreted into milk in appreciable quantities and therefore may result in a withholding period for milk after drug administration to dairy cattle. Other NSAIDs like ketoprofen have little excretion in milk[56] and thus have no withholding time for milk when used in lactating animals.

The primary adverse events in the target species are GI (primarily ulcers and melena), renal (due to reduced renal blood flow, oxidative damage, or other mechanisms), hepatic, or hematologic (prolonged clotting times, blood dyscrasias).[3] The comparative toxicity of NSAIDs varies significantly between compounds, as well as between species. Almost all NSAID adverse events are dose dependent; therefore, the low residue concentrations encountered in food animal tissues are considered unlikely to pose a health risk to consumers, as recently noted in the addressing of consumer concerns in the European Union (EU) about findings of phenylbutazone residues in horse meat.[5]

8.4 Acceptable Daily Intake (ADI)

To date, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) has not established an acceptable daily intake (ADI) for any NSAIDs. As a result, no maximum residue limits (MRLs) for these substances have been established by the Codex Alimentarius Commission. ADIs established by various jurisdictions are as follows:

- Acetaminophen (paracetamol): A pharmacological ADI of 50 μg/kg bw was established by the European Medicines Agency (EMA), based on a lowest observed effect level (LOEL) of 5 mg/kg bw in human infants with a safety factor of 100.[48]
- ASA and related compounds: A pharmacological ADI of 8.3 μg/kg bw (0.5 mg/person) was established by the EMA[57, 58] for ASA, sodium acetylsalicylate, acetylsalicylic acid DL-lysine, and carbasalate calcium (ASAs).
- Carprofen: An ADI of 10 μg/kg bw (i.e., 600 μg/person) was established by the EMA, based on a no observed effect level (NOEL) of 1 mg/kg bw from a 2-year oral toxicity study in rats using a safety factor of 100.[59] Australia has established an ADI of 5 μg/kg bw based on the same data[60] but with a safety factor of 200.
- Diclofenac: A pharmacological/toxicological ADI of 0.5 μg/kg bw (30 μg/person) was established by the EMA, based on a LOEL of 0.1 mg/kg bw with a safety factor of 200.[61] Note that systemic formulations of this NSAID are no longer marketed in most jurisdictions, although topical formulations are available. The manufacture and sale of veterinary formulations of diclofenac were banned in India in 2006 due to concern over toxicity in nontarget species (vultures).[62, 63]
- Dipyrone (metamizole): An ADI of 10 μg/kg bw was established by the EMA, based on a pharmacological NOEL in mice of 10 mg/kg bw, with a safety factor of 1000.[64, 65]
- Firocoxib: A temporary toxicological ADI of 0.215 μg/kg (12.9 μg/person) was established by the EMA in 2006, based on a benchmark dose level (BMDL) of 0.043 mg/kg from a one-generation reproductive toxicity study in rats, with an uncertainty factor of 200.[66]
- Flunixin: A toxicological ADI of 6 μg/kg bw (360 μg/person) was established by the EMA, based on a NOEL of 0.6 mg/kg bw from a subchronic oral toxicity study in dogs, with a safety factor of 100.[67] An ADI of 0.72 μg/kg bw per day has been established by the United States Food and Drug Administration (USFDA) for the use of flunixin in food-producing animals.[68]
- Ketoprofen: A toxicological ADI of 1 μg/kg bw (60 μg/person) was established in Australia, based on a NOEL of 0.1 mg/kg bw with a safety factor of 100.[60] The EMA established a pharmacological ADI of 5 μg/kg bw based on a pharmacological NOEL of 50 μg/kg bw in humans, with a safety factor of 10.[56, 69]

- Meloxicam: A toxicological ADI of 1.25 μg/kg bw (75 μg/person) was established by the EMA based on a LOEL of 0.125 mg/kg bw for gestation length effects in rats, with a safety factor of 100.[70, 71]
- Tolfenamic acid: A toxicological ADI of 10 μg/kg bw (600 μg/person) was established by the EMA, based on a NOEL of 1 mg/kg bw from a 1-month toxicity study in rabbits with a safety factor of 100.[72] Australia has established an ADI of 5 μg/kg bw based on a NOEL of 0.5 mg/kg bw from a human study.[60]
- Vedaprofen: A toxicological ADI of 1.25 μg/kg bw (75 μg/person) was established by the EMA, based on a NOEL of 0.125 mg/kg bw from a 13-week dog study with a safety factor of 100.[73, 74]

There is one NSAID for which drug residues may be a particular human health concern. Phenylbutazone use in humans has been associated with blood dyscrasias (such as rare cases of aplastic anemia).[75, 76] It is likely that this outcome is dose dependent, but dose-independent (idiosyncratic) toxicity in some individuals cannot be ruled out.[77] Furthermore, as it is possible (though unlikely) that phenylbutazone may be mutagenic and/or carcinogenic, the EMA decided in 1997 that no ADI could be determined for residues of phenylbutazone, and therefore, no MRLs were established for this compound.[76]

In 2013, the detection of horse meat in food products labeled as beef and destined for human consumption raised concerns of public interest in the EU.[5] The potential hazard associated with the consumption of horse meat containing phenylbutazone and its metabolites was jointly assessed by the European Food Safety Authority (EFSA) and the European Medicines Agency (EMA).[76] The review noted that while available data on phenylbutazone are not conclusive that this compound is carcinogenic in humans, a threshold concentration cannot be established for the occurrence of phenylbutazone-induced blood dyscrasias in humans, a finding supported also in a recent review.[77] The outcome of an evaluation conducted by the CVMP in 1997, which resulted in the prohibition of the use of phenylbutazone in food-producing animals, was reaffirmed in the EFSA/EMA assessment, and measures were recommended to mitigate the risk that horse meat contaminated with phenylbutazone would enter the human food chain.[76]

It is also worth noting that other species can be susceptible to NSAID toxicity at residue concentrations far lower than required to affect mammals. This was clearly illustrated by the catastrophic plummet in the South Asian vulture population after ingestion of bovine carcasses containing residues of the NSAID diclofenac.[78–80]

8.5 Maximum Residue Limits/Tolerances

The administration of NSAIDs to food animals to treat and reduce pain presents its own challenges. In some jurisdictions, NSAIDs are approved for use in food

animals for alleviating pain in livestock. However, in the United States where NSAIDs are not labeled for pain relief, the use of any NSAID for pain relief constitutes extralabel drug use (ELDU) under the Animal Medicinal Drug Use Clarification Act (AMDUCA) of 1994.[81] ELDU is permitted for relief of pain in a food animal, provided that the following conditions are met:

- The drug administered is a USFDA-approved veterinary or human drug.
- It is administered by and/or under the supervision of a licensed veterinarian.
- There is evidence of a threat to animal health.
- It is not administered through the feed.
- The drug will not leave persistent residues in the food animal intended for human consumption.

MRLs for veterinary drugs have been determined by international and regional authorities as the maximum concentrations of residues to be legally permitted in or on a food. Where MRLs are defined for veterinary drugs used in food animals, a withdrawal period is also usually established. This is the time between the last dose given to the food animal and the time when the concentration of residue in the tissues (muscle, liver, kidney, skin/fat) or products (milk, eggs, honey) is lower than the MRL. Until the withdrawal period has elapsed, the animal or its products must not be used for human consumption.

In the EU, legislation establishes the procedures to be used in establishing MRLs for veterinary drug residues in foods.[82, 83] NSAIDs with MRLs (listed in Table I of EU regulations) such as carprofen can be used in cattle and horses[84]; diclofenac[61] and tolfenamic acid[72] can be used in cattle and pigs; flunixin,[67, 85] meloxicam,[86–88] and metamizole[65] can be used in cattle, pigs, and horses; and firocoxib[66] and vedaprofen can be used in horses.[74] Meloxicam has also been approved for use in rabbits and goats.[89] Two NSAIDS, ketoprofen[56, 69] and salicylates,[57, 58] do not require an MRL (listed in Table I of EU regulations) and can therefore be administered to the listed food-producing animals. Ketoprofen has been authorized in the EU for treatment of cattle and horses,[56] with an extension to use in pigs.[69] ASA and related substances were approved for use without a requirement for an MRL in cattle, pigs, and chicken,[57] with a subsequent extension to all food-producing species except fish.[58] A four-day withdrawal period has been established for use of ketoprofen in meat-producing cattle, horses, and pigs, but no withdrawal period has been established for use of ketoprofen in dairy cattle.[56] MRLs have been established for residues in milk for the authorized use of carprofen,[90] diclofenac,[91] meloxicam,[92] metamizole,[65] and tolfenamic acid[72] in dairy cattle. Dipyrone (metamizole) is authorized for use in cattle, including dairy cattle, pigs, and horses,[65] with MRLs established for edible tissues and milk.

MRLs established by several jurisdictions for residues of NSAIDs in animal-derived foods are provided in Tables 8.2–8.4.

Table 8.2 Maximum residue limits (MRLs) established for NSAIDs by the European Union.

		Maximum residue limit (µg/kg)											
NSAID	**Marker residue**	**Bovine**					**Porcine**				**Equidae**			
		Muscle	Kidney	Liver	Fat	Milk	Muscle	Kidney	Liver	Skin/fat	Muscle	Kidney	Liver	Fat
Acetaminophen (paracetamol)[48]		Not authorized					No MRL required				Not authorized			
Acetylsalicylic acid[57, 58]		No MRL required				Not authorized	No MRL required				No MRL required			
Carprofen[59]	Carprofen	500	1000	1000	1000	No MRL required					500	1000	1000	1000
Diclofenac[61, 91]	Diclofenac	5	10	5	1		5	10	5	1				
Firocoxib[66]	Firocoxib										10	10	60	15
Flunixin[67]	Flunixin + 5-Hydroxyflunixin	20	30	200	30	40								
Flunixin[85]	Flunixin						50	30	200	10	10	200	100	20
Ketoprofen[56, 69]	Ketoprofen	No MRL required												
Meloxicam[86, 89, 92]	Meloxicam	20	65	65		15	20	65	65		20	65	65	
Metamizole (Dipyrone)[65]	4-Methyl aminoantipyrin	100	100	100	100	50	100	100	100	100	100	100	100	100
Salicylic acid[57, 58]		No MRL required				Not authorized	No MRL required							
Tolfenamic acid[72]	Tolfenamic acid	50	100	400		50	50	100	400					
Vedaprofen[74]	Vedaprofen										50	1000	100	20

Table 8.3 Maximum residue limits (MRLs) established for NSAIDs by the Australian Pesticides and Veterinary Medicines Authority (APVMA).[93]

	Maximum residue limit (μg/kg)			
Tissue/milk	Flunixin	Ketoprofen	Meloxicam	Tolfenamic acid
Cattle meat		50	10	50
Pig meat			20	10
Cattle, edible offal		50		
Cattle meat (in the fat)	20			
Cattle liver	20		100	10
Pig liver			10	100
Cattle kidney	20		200	10
Pig kidney			10	10
Cattle milk		50	5	50

Table 8.4 Maximum residue limits (MRLs) established for NSAIDs by Health Canada.[94]

	Maximum residue limit (μg/kg)		
Tissue/milk	Acetyl salicylic acid	Flunixin (free acid)	Ketoprofen
Muscle of cattle	No MRL required	20	250
Liver of cattle		80	
Kidney of cattle		30	800
Fat of cattle		90	
Milk of cattle		6(5-Hydroxyflunixin)	50
Muscle of swine		20	100
Liver of swine		30	
Kidney of swine		30	500
Skin and fat of swine		40	

The USFDA has established tolerances for flunixin as follows[95]:
Cattle muscle: 25 μg/kg (as free acid); Cattle liver: 125 μg/kg (as free acid); Cattle milk: 2 μg/kg (as 5-hydroxyflunixin); Swine muscle: 25 μg/kg (as free acid); Swine liver: 30 μg/kg (as free acid).

8.6 Analysis of NSAID Residues in Food

The analysis of the NSAID residues in food is an increasingly important issue in food safety. Thus, the latest developments in this type of analysis with particular emphasis on detection by mass spectrometry (MS) are presented.

8.6.1 Single Analyte Methods

A literature search provided a selection of single analyte methods for various NSAIDS in biological matrices, including 3 methods for carprofen[96–98]; 4 methods for diclofenac[78, 79, 99, 100]; 18 methods for flunixin and its 5-hydroxy metabolite[101–118]; 1 method for flunixin with meloxicam,[119] 1 with ketoprofen,[120] and 2 with phenylbutazone[121, 122]; 5 methods for ibuprofen[123–127]; 5 methods for meloxicam[42, 128–131]; 1 method for mefenamic and flufenamic acid[132]; 1 method for metamizole[133]; 7 methods for ketoprofen and its metabolite[41, 43–45, 134–136]; 13 methods for phenylbutazone and its oxyphenbutazone metabolite[137–149]; 3 methods for salicylic acid and its metabolites[47, 150, 151]; and 3 methods for tolfenamic acid.[152–154] Most of the single analyte methods were developed for use in PK, PD, and tissue depletion studies for drug registration and licensing. Table 8.5 lists those methods which were applied to edible tissues or milk and for which basic method validation information, such as a limit of detection (LOD) or limit of quantification (LOQ), was provided.

8.6.1.1 Carprofen

Carprofen contains a single chiral center and therefore exists in two isomeric enantiomers, *S*-(+) and *R*-(−).[56] The veterinary product is the racemic mixture,[59] and it has been shown through laboratory animal studies that the PK of the enantiomers of 2-arylpropionic acids such as carprofen differ significantly as a result of differences in metabolism, excretion, and distribution.[45] Highest residues of carprofen are found in the liver and kidney of cattle and horses post-administration.[59, 84] Carprofen parent drug is the marker residue in animal tissues[84] and in milk from cattle.[90]

Chiral inversion of some 2-arylpropionic acids from *R*-(−) to *S*-(+) has been demonstrated in man and other species,[155, 156] and as a result it is important in studies that relate anti-inflammatory drug activity to PK disposition to determine the time-course concentrations of each enantiomer in biological fluids such as plasma. It is also important that in PK studies for carprofen, the parameters of each of the enantiomers are measured in each of the target species for which clinical use is intended. Studies have shown that unlike most 2-arylpropionic acids, the concentrations of the *R*-(−) enantiomer in plasma exceed those of the *S*-(+) enantiomer in horse and the magnitude of the difference increases with time; this difference has been demonstrated to be not due to chiral inversion.[96–98] Typically, 0.5–1.0 ml of animal plasma was fortified with a suitable internal standard. The sample was acidified with potassium phthalate buffer (pH 5.2) and extracted twice with diethyloxide. The crude extract was evaporated to dryness and derivatized with the chiral reagent L-leucinamide. The carprofen diastereomers were separated on a C_{18} reversed-phase column with gradient conditions at a flow rate of 1 ml/minute and detected at 300 nm. Total carprofen concentration was calculated by summation of the separate enantiomer concentrations.[98]

Table 8.5 Non-multiresidue methods for NSAIDs in animal-derived food matrices[a].

Compounds	Method	Species and matrix	LOD/LOQ/CCα/CCβ	References
Diclofenac	Optical biosensor	Milk (cattle)	LOD = 0.1 μg/l (0.1 μg/kg)	Rau et al.[100]
Flunixin	LC-MS/MS	Milk (cattle)	LOQ = 1 μg/kg	Feely et al.[102]
5-Hydroxyflunixin	LC-MS/MS	Milk (cattle)	LOD = 0.2 μg/kg; LOQ = 0.5 μg/kg	Ngoh et al.[103]
Flunixin	HPLC-UV	Muscle (cattle)	LOD = 6 μg/kg; LOQ = 15 μg/kg	Asea et al.[105]
5-Hydroxyflunixin	LC-MS/MS	Raw milk (cattle)	LOD = 0.2 μg/kg; LOQ = 1 μg/kg	Boner et al.[106]
Flunixin	LC-MS/MS	Liver, kidney, muscle, and fat (cattle)	LOD = 0.1 μg/kg (liver, kidney) LOD = 0.2 μg/kg (muscle, fat) LOQ = 0.3 μg/kg (liver) LOQ = 0.2 μg/kg (kidney) LOQ = 0.6 μg/kg (muscle) LOQ = 0.4 μg/kg (fat)	Boner et al.[107]
Flunixin and 5-hydroxyflunixin	LC-MS/MS	Milk (cattle)	LOD = 0.5 μg/kg (for both analytes) LOQ = 0.7 μg/kg (for both analytes)	Jedziniak et al.[108]
Flunixin and 5-hydroxyflunixin	CHARM and ELISA; UHPLC-MS	Milk (cattle)	LOD = 0.1 μg/l (5-hydroxyflunixin) LOQ = 0.2 μg/l (5-hydroxyflunixin)	Kissell et al.[109]
Flunixin and 5-hydroxyflunixin	UHPLC-MS	Milk (cattle)	LOD = 0.1 μg/l (for both analytes) LOQ = 0.2 μg/l (for both analytes)	Kissell et al.[110]

Flunixin	UHPLC-MS/MS	Liver, kidney, muscle (rabbit)	LOD = 0.3 μg/kg (liver, muscle) LOD = 0.8 μg/kg (kidney) LOQ = 1.0 μg/kg (liver, muscle) LOQ = 3.0 μg/kg (kidney)	Zhu et al.[113]
Flunixin	LC-MS/MS	Muscle (cattle)	LOD = 0.02 μg/kg CCα = 22.9 μg/kg CCβ = 27.3 μg/kg	Lugoboni et al.[114]
5-Hydroxyflunixin and meloxicam	HPLC-UV	Milk (cattle)	LOD = 0.5 μg/kg (5-hydroxy flunixin) LOD = 0.7 μg/kg (meloxicam) LOQ = 1.5 μg/kg (5-hydroxyflunixin) LOQ = 2.5 μg/kg (meloxicam)	Johannsson and Anler[117]
Flunixin and ketoprofen	LC-MS/MS	Milk (cattle)	CCα = 0.5 μg/kg (flunixin) CCα = 1.0 μg/kg (ketoprofen)	Daeseleire et al.[120]
Meloxicam	HPLC-UV[b)]	Milk (cattle)	LOD = 1.0 μg/kg LOQ = 2.5 μg/kg	Jedziniak et al.[128]

(*continued*)

Table 8.5 (Continued)

Compounds	Method	Species and matrix	LOD/LOQ/CCα/CCβ	References
Metamizole metabolites	LC-MS/MS	Muscle (cattle)	CCα = 113 CCβ = 139 μg/kg (for 4-methyloaminofenazone) CCα = 11.6 CCβ = 16.5 μg/kg (for 4-formylaminofenazone) CCα = 12.6 CCβ = 16.4 μg/kg (for 4-aminofenazone) CCα = 11.6 CCβ = 15.8 μg/kg (for 4-acetylaminofenazone)	Jedziniak et al.[133]
Phenylbutazone	LC-MS/MS	Milk (cattle)	LOD = 0.5 μg/l; LOQ = 2 μg/l	Thompson et al.[142]
Phenylbutazone	HPLC-UV	Muscle (cattle, pig, horse)	LOD = 3 μg/kg; LOQ = 10 μg/kg	Asea et al.[149]
Salicylic acid	LC-MS/MS	Muscle, kidney, liver, and skin + fat (chicken, pig)	LOD = 64 and 52 μg/kg (chicken liver and kidney, respectively) LOQ = 96 μg/kg (chicken liver and kidney) LOD = 23, 25, 9, and 32 μg/kg (porcine kidney, liver, muscle, and skin + fat, respectively) LOQ = 50 μg/kg (all porcine tissues)	Croubels et al.[151]

a) Includes only methods for which performance data such as LOQ were provided. LODs and LOQs have been rounded to the nearest whole integer or to one place after the decimal, as appropriate.
b) Multi-residue method used to analyze only meloxicam.

8.6.1.2 Diclofenac

The label-free optical biosensor method developed for the detection and quantification of diclofenac in bovine milk by Rau et al.[100] did not need sample preparation or clean-up, thus reducing the time and cost of analysis. This was possible because by utilizing an optimized surface modification and evaluation method, matrix effects were successfully prevented or circumvented. By obtaining a LOD of 0.1 μg/l (0.1 μg/kg), the capability of the developed biosensor was found to be comparable or better than those of standard detection methods. Moreover, the biosensor was able to detect diclofenac at the MRL (0.1 μg/kg) set by the EU. This was the first report of the successful quantitative analysis of diclofenac at regulatory limits in milk by using an optical biosensor.

An ELISA-based method has been used to detect and quantify residues of diclofenac in liver samples collected from livestock carcasses in India to monitor compliance with the national ban on the veterinary use of this drug.[99] The method detected diclofenac residues at concentrations of 10 μg/kg and was found to provide reliability as a screening test when compared with a liquid chromatography–mass spectrometry (LC-MS) (ESI) method. Further application of this test in routine monitoring of animal-derived foods has not been reported.

8.6.1.3 Flunixin and Its Metabolites

Free and total flunixin residues in animal tissues, plasma, and urine have been analyzed. Methods have also been developed to enable the study of the total depletion and metabolism of flunixin in dairy cattle as a requirement to provide data for drug registration.[101–118] Parent flunixin is the marker residue for tissues of cattle,[67] pigs,[67] and horses,[85] with highest residues found in cattle liver,[67] pig liver,[67] and horse kidney,[85] respectively, following drug administration. The marker residue for bovine milk is the metabolite 5-hydroxyflunixin.[67, 95] Methods for flunixin in tissues should therefore target the parent compound,[114] while methods applied to milk should determine residue concentrations of both flunixin parent compound and 5-hydroxyflunixin.[120]

Flunixin and its 5-hydroxyflunixin metabolite have been analyzed with and without hydrolysis. In general, tissue or biological fluid was homogenized with an organic solvent, typically acetonitrile, after basic or enzyme hydrolysis. Examples of this approach include the addition of 1.0 N NaOH to urine samples, with incubation for 1 hour at room temperature, to convert flunixin glucuronide to free flunixin,[116] or incubation of milk samples with β-glucuronidase, which led to the recovery of significantly higher residues of flunixin.[108] A study in cattle administered with ^{14}C-flunixin intravenously once daily for 3 days indicated that the ratios of marker residue to total residues at 2 days following the final dose were 0.3, 0.1, 0.3, and 0.25 for liver, kidney, muscle, and fat, respectively, while in a similar experiment with dairy cattle, the sum of residues of flunixin and 5-hydroxyflunixin accounted for 44% of the total residues at 36 hours following the final administration.[67]

The crude extract is typically subjected to clean-up by SPE, resolved under a reversed-phase gradient chromatographic condition, and analyzed by a suitable detection system. Zhu et al.[116] described a method for the determination of flunixin residues in rabbit tissue samples in which the samples were homogenized with acetonitrile, followed by a defatting procedure with *n*-hexane and purification using HLB SPE cartridges. The final extracts were analyzed by UHPLC-ESI-MS/MS using selected reaction monitoring (SRM). The LOQs were 1.0–3.0 μg/kg in the various tissues, with highest residues in edible tissues found in kidney 2 hours post-treatment.

8.6.1.4 Ibuprofen

Single analyte methods found in the literature search were for the determination of ibuprofen in biological fluids, such as plasma and urine. No single analyte methods were found for residues of ibuprofen in animal-derived foods. Following oral administration to humans, ibuprofen is completely absorbed and metabolized to three major metabolites, 2-[4-(2-hydroxy-2-methylpropyl)phenyl]propanoic acid (OH-ibuprofen, hydroxy ibuprofen), 2-[4-(2-carboxypropylpropyl)phenyl] propanoic acid (COOH-ibuprofen; carboxy ibuprofen), and ibuprofen glucuronide.[157] Incubation with a suspension of rat hepatocytes led to the production of two major metabolites, hydroxy ibuprofen and carboxy ibuprofen, which were the metabolites observed when metabolic studies were conducted *in vivo* in rats with ibuprofen.[158] Hydroxy ibuprofen exists as a pair of enantiomers, and because the formation of carboxy ibuprofen introduces a second chiral center, this results in four possible stereoisomeric products: *RS*- (*R*-propanoic acid, *S*- carboxy) *SR*-, *SS*-, and *RR* configurations. The conversion of *R*-(−)- to *S*-(+)-ibuprofen has been investigated in humans.[159] In rats, it has been shown by chromatographic analysis that *S*-ibuprofen metabolic inversion proceeds at a much slower rate than is observed for its R counterpart.[160] In developing methods for the analysis of ibuprofen, it is therefore important to recognize this metabolic behavior, and effort should be made to ensure that all stereoisomers are measured in order to be able to accurately determine the total concentration of ibuprofen in a biological fluid or tissue sample.

An acceptable method developed for urine analysis included adding 1 ml of urine to an equal volume of 6 M HCl and incubating for 30 minutes in a 90 °C water bath to ensure cleavage of ether and ester glucuronides.[123] After cooling the mixture for about 10 minutes and adding 40 μl of β-naphthoic acid as internal standard, each sample was extracted twice with 7 ml of methylene chloride by shaking for 20 minutes. The extract was centrifuged; the organic phases were combined and evaporated to dryness in a 37 °C water bath under a stream of nitrogen. The residue was reconstituted with 500 μl of freshly prepared 1,1′-carbonyldiimidazole in chloroform and allowed to stand for 10 minutes at room temperature to form the imidazole intermediates. Glacial acetic acid (10 μl) was added and allowed to react for 10 minutes; the derivatization reagent *S*-(−)-(α)-methylbenzylamine was added after mixing, and the samples were

allowed to react for 30 minutes to form the diastereomeric amides. The reaction mixture was washed with 0.5 M NaOH and rinsed with *n*-hexane (5 ml), and the chloroform hexane layer was removed and washed with 3 ml 1 M HCl. The organic layer containing the derivatized ibuprofen metabolites was evaporated to dryness under nitrogen and reconstituted in 250 µl water:methanol (62:38 v/v), and 30 µl aliquot was injected into the HPLC for analysis. HPLC analysis of the extract conducted on a chiral HPLC column showed that all six stereoisomers were chromatographically resolved and the total ibuprofen concentration was obtained from adding together the stereoisomeric hydroxy (*S*-hydroxyibuprofen, *R*-hydroxyibuprofen) and carboxy (*SS*-carboxyibuprofen, *RS*-carboxyibuprofen, *SR*-carboxyibuprofen, and *RR*-carboxyibuprofen) ibuprofen concentrations.

A more complex method applied to human urine used two HPLC columns, with initial separation on a Partisil® column, followed by fraction collection, evaporation of solvent, and reconstitution of the fraction containing the isomers and separation of the isomers on a chiral column (Chiralpak® AD CSP).[127] All six isomers could be quantified in a single chromatographic run.

In a method for the determination of ibuprofen and ibuprofen glucuronide in plasma, samples were deproteinated with acetonitrile, diluted with phosphate buffer, and cleaned up by solid-phase extraction using a C_{18} cartridge.[126] This method did not provide separation of the optical isomers. Another method for the determination of ibuprofen in plasma used reaction of the enantiomers with ethyl chloroformate and (*S*)-(−)-1-(1-naphthyl)ethylamine to form the naphthylethylamide derivatives of the isomers, which were subsequently separated using a C_{18} HPLC column.[125]

Based on the available information, a single analyte method for the determination of ibuprofen residues in animal-derived foods should probably focus on liver or kidney as a target tissue. While separation of the individual optical isomers may not be critical, such a method should probably include a deproteination step and a hydrolysis step to release residues present as the glucuronide.

8.6.1.5 Meloxicam

Meloxicam depletion from cattle tissues[70, 71, 86] and milk[92] has been reported in studies reviewed by the CVMP. Pharmacokinetics in plasma of pigs,[161–163] horses,[130] and llamas[131] has also been studied. The primary main metabolites of meloxicam identified in rats, mini pigs, and humans were a 5′-hydroxymethyl derivative and a 5′-carboxy metabolite.[164] Depletion data evaluated by CVMP for cattle indicated that at 2 days following administration, meloxicam residues were detected at concentrations of 570 µg/kg in liver, 534 µg/kg in kidney, 43 µg/kg in muscle, and 73 µg/kg in injection site tissue. At 8 days' withdrawal, meloxicam residue concentrations are typically below 10 µg/kg in muscle tissue and injection site tissues 8 days following administration, with residues in liver and kidney being, respectively, 22 and 25 µg/kg. Meloxicam parent drug accounts for 23% of the total residue in liver and 40% of the total residue in kidney at 4 days

withdrawal, the time point at which residue concentrations are expected to be below the MRLs established by the CVMP.[86] Data from a depletion study using ^{14}C-meloxicam reviewed by the CVMP demonstrated that meloxicam parent is approximately 75% of the total residues of meloxicam in milk.[92]

A multi-residue method that had been developed and validated for seven NSAIDs (carprofen, diclofenac, flunixin, phenylbutazone, tolfenamic acid, vedaprofen, and meloxicam) was used for the analysis of meloxicam residues in cow's milk.[128] To 5.00 ± 0.01 g of milk was added 5 ml of acetonitrile and 1 g NaCl, vortex mixed and centrifuged at 4500 × *g* for 15 minutes at −5 °C. The supernatant was transferred into a clean tube and evaporated to dryness under a gentle stream of nitrogen at 40 °C. The dried residue was reconstituted in 0.5 ml acetonitrile:methanol:0.05 M CH_3COONH_4, pH 5.0, [1:1:1(v/v/v)] solution and centrifuged at 4500×*g* for 5 minutes, and 50 µl was injected into the HPLC column for analysis by ultraviolet (UV) at 365 nm for meloxicam residues at and around its MRL of 15 µg/kg.

8.6.1.6 Mefenamic and Flufenamic Acids

A literature search revealed very little information on the use of mefenamic acid in veterinary medicine. For example, a method was reported in the late 1970s for the determination of mefenamic acid in horse plasma and urine,[165] but no reports were found on the application of this method (or other methods) in PK studies or depletion studies.

A simple luminescent method was recently reported for the simultaneous determination of mefenamic and flufenamic acids using direct fluorescence measurement.[132] The method uses lanthanide-sensitized luminescence, which provides an enhanced signal. Limits of detection were 3.7 and 14.6 µg/l for flufenamic and mefenamic acids, respectively. The method was tested in two different pharmaceutical preparations containing the analytes, obtaining recovery percentages close to 100%, and in human urine samples. No extraction or prior separation of the analytes was required for the urine samples.

A method has been reported for the determination of mefenamic acid in human plasma.[166] Following liquid–liquid extraction, samples are analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) using a C_{18} column and an APCI source. The method has an LOQ of 20 µg/l in human plasma, with a recovery of 73%, and was successfully applied in a PK study.

8.6.1.7 Metamizole

After oral administration, metamizole (dipyrone) is nonenzymatically hydrolyzed in the GI tract to 4-methylaminoantipyrene (4MAA), which is rapidly and nearly completely absorbed. 4MAA is further metabolized to 4-formylaminoantipyrene (4FAA) and 4-aminoantipyrene (4AA).[167] 4AA is acetylated to 4-acetylaminoantipyrene (4AcAA). The MRLs of 100 µg/kg established for dipyrone use in bovine, equine, and swine kidney, liver, and muscle tissues by CVMP identify 4MAA as the marker residue.[65] Methods developed

for the regulation of the use of metamizole in food animal production must therefore be able to detect and quantify the concentrations of metamizole in these matrices. In 2003, Jedziniak et al.[133] developed and validated a method for the four metabolites in bovine muscle tissues as follows: 2 g of minced bovine muscle tissue fortified with internal standard and metamizole was extracted with 10 ml of acetonitrile (8 ml) and 0.33 M acetate buffer (pH 5.0, 2 ml). The mixture was vigorously mixed and centrifuged at 1830×*g* for 10 minutes at 15 °C. The supernatant was passed through an Sep-Pak Alumina N and the collected in a tube. To 0.5 ml of the extract in the tube was added 0.5 ml of mobile phase (MeOH:ACN:0.01 M ammonium formate at pH 5.0 [8:2:90], filtered and analyzed by LC-MS/MS using a C_8 column and positive ionization.

8.6.1.8 Ketoprofen and Its Metabolites

Ketoprofen contains an aryl-deficient aryl substituent with carboxylic acid functionality and is therefore cleared, in part, as inactive acylglucoronides.[41, 43–45, 134–136] Ketoprofen also is a mixture of stereoisomers, and inversion of the *R*-enantiomer to the *S*-enantiomer has been observed in a number of animal species.[168, 169] In all species except the rat, it is metabolized to a carbonyl-reduced derivative, 2-(phenyl-3-α-hydroxybenzoyl) propionic acid.[56, 69] This is one of the few NSAIDs for which GC has been used in methods for its determination. To determine the concentration of ketoprofen in horse urine, 2 ml of urine was extracted with diethyl ether using flurbiprofen as an internal standard. The ether extract was evaporated to dryness and derivatized for gas chromatography–mass spectrometry (GC-MS) analysis, with reported recovery of 95% and an LOQ of 10 µg/l.[134]

Some examples of LC-based methods for the determination of parent ketoprofen were also noted. For example, ketoprofen enantiomers were determined in plasma and urine samples collected in a PK study using rats.[170] After initial extraction into isooctane–isopropanol (95:5) and washing with water, the extracts were extracted with chloroform and evaporated, and the residue was sequentially derivatized with ethyl chloroformate and L-leucinamide hydrochloride for HPLC analysis. The reported LOQ was 50 µg/l, with cov ≤10%. An HPLC method using a C_{18} column was also reported for analysis of samples of milk, and plasma collected from cows administered a single dose of ketoprofen (i.v. bolus).[41] The authors reported LOQs of 60 µg/l for plasma samples and 90 µg/l for milk.

More recently, a method using UHPLC-MS/MS was developed for use in the determination of ketoprofen in human dermal microdialysis samples.[171] As concentrations of drug collected in such samples are very low, typically in the µg/l or ng/l range and sample volumes are very small, a method is required which provides reliable results under such conditions, The method used a UHPLC column with 1.7 micron C_{18} packing and isocratic conditions. An LOQ of 0.5 µg/l was reported. While the sample preparation would not typically be sufficient for typical tissue samples analyzed in a regulatory program for veterinary drugs, the

UHPLC-MS/MS conditions presented could prove useful if development of such a method were required.

8.6.1.9 Phenylbutazone and Its Metabolites

A number of methods have been reported for the determination of phenylbutazone residues in PK studies (plasma and urine),[77, 137–141, 143–147] milk,[142, 148] and animal tissues.[149] The proton located on the carbon in position 4 of the pyrazolidine ring for PBZ has an acidic property that can form an enol with the group to form 4-butyl-5-hydroxy-1,2-diphenyl-1,2-dihydro-3*H*-pyrazol-3-one. This implies that PBZ can act as a very weak acid.[172] The pK_a of PBZ is 4.5 and exists in solution in three forms as the diketo, the enol, and a mesomeric anionic form. The primary state in solution is the diketo form, and conversion between the forms is slow. PBZ is metabolized in the liver and is oxidized to oxyphenbutazone (OXPBZ), γ-OHPBZ, β-OHPBZ, γ-ketoPBZ, and γ-dihydroxyPBZ.[173] It is, therefore, imperative that all methods designed for the analysis of PBZ take precautions to ensure that the analyte being measured is the stable form of PBZ and the methods should prevent oxidation of PBZ to the metabolites since solutions of PBZ are known to be unstable.[15, 61–74] It was noted in a paper published in 2004 that there were very few published methods for PBZ where precautions had been taken in the development of the methods to ensure that the analyte being measured is the stable form of PBZ.[149]

A more recent publication reported an LC-MS/MS method for the determination of PBZ and OXPBZ in plasma samples collected for post-competition monitoring of race horses.[137] Following the addition of H_3PO_4 (1 M), the plasma samples were extracted with methyl tertiary-butyl ether using thorough mixing and then centrifuged. The organic layer was removed, evaporated to dryness, and reconstituted in mobile phase. Samples were analyzed using an LC-MS/MS system, with separation on a C_8 column and determination using negative ESI mode. Analyte stability was tested under benchtop conditions to approximate typical times during which samples could sit in autosampler vials awaiting analysis and also under freeze–thaw conditions. The method was judged suitable to be used for screening, quantification, and confirmation of and OXPBZ in equine plasma samples. This method is probably more representative of the technology, which would be used in a regulatory laboratory testing for phenylbutazone and/or oxyphenbutazone residues in animal-derived foods if it was determined that a method targeting only these compounds was required. However, it is anticipated that the more extensive sample clean-up described in earlier methods applied to milk[142, 148] and animal tissues[149] would be required for such an application.

8.6.1.10 Salicylic acid and Its Metabolites

In the EU, the CVMP concluded that there was no need to establish an MRL for salicylic acid, sodium salicylate, aluminum salicylate, and basic and methyl salicylate. These drugs were therefore included in Table 8.1 of EU Regulation

37/2010.[83] In humans, salicylic acid is metabolized to various compounds, the most important of which are salicyluric acid, salicylic acid phenolic glucuronide, salicylic acid acyl glucuronide, and the oxidation product gentistic acid.[174, 175] Metabolism is similar in all species for which data are available, including humans, dogs, cats, pigs, cattle, horses, goats, rabbits, and chicken.[58] To be able to effectively study the biotransformation/metabolism of salicylic acid in animals, sensitive chromatographic methods able to detect the glycine, the glucuronide, and the ornithine metabolites must be available.[47, 150, 151] Croubels et al. developed a method to study the biotransformation of salicylic acid in plasma, excreta, kidney, skin + fat, muscle, and liver of pigeon and chicken by LC-MS/MS.[151] For tissue samples, about 1 g of minced muscle tissue was weighed into a 10 ml centrifuge tube. Fifty microliters of the 100 µg/ml internal standard was added, followed by 200 µl 1 M HCl and 6 ml ethyl acetate. The sample was extracted for 10 minutes by rotation, centrifuged for 10 minutes at 2500 rpm after which the organic phase was transferred into another extraction tube containing 3 ml of 0.1 M pH 9.2 carbonate buffer and extracted for 10 minutes and centrifuged at 2500 rpm for 10 minutes. The organic phase was discarded, and 60 µl of a 0.1 M HCl was added until the pH was 7, followed by clean-up of the neutralized sample on a preconditioned SAX SPE cartridge. Salicylic acid was eluted with 3 ml of a mixture of hexane/2% TFA in ethyl acetate (1/1, v/v), evaporated to dryness with nitrogen at 40 °C, and reconstituted in 1 ml 0.1% acetic acid in water:methanol (9:1, v/v) for LC-MS/MS analysis. An LOQ of 50 µg/kg was reported.

8.6.1.11 Tolfenamic Acid

MRLs have been established in the EU for residues of tolfenamic acid in cattle and pigs.[72] Studies have also been performed to determine the pharmacokinetics after intramuscular administration of tolfenamic acid in goats.[152] Concentrations of tolfenamic acid in calf[153, 154] and goat[152] serum, exudate and transudate were determined by an HPLC method with UV detection at 342 nm. A 0.5 ml serum or 0.1 ml exudate or transudate was acidified with 1.0 ml citrate phosphate buffer (pH = 3.0). Five milliliters of chloroform containing 0.5 µg/ml mefenamic acid (IS) was added and the contents mixed for 15 minutes and centrifuged at 2000×*g* for 20 minutes at 20 °C. Four milliliters of the organic phase was collected and evaporated to dryness under a stream of nitrogen at 50 °C. The residue was reconstituted in 150 µl methanol and dissolved by vortex mixing for 15 seconds. The components in the extract were separated on a C_{18} 7 µm LiChrosorb RP column with a mobile phase of 80% methanol:20% water, containing 0.001% perchloric acid at a flow rate of 1.0 ml/minute.[153]

Data provided to the CVMP demonstrated that in cattle the highest residues of tolfenamic acid are found in the liver, followed by kidney, muscle, and fat.[72] Almost all radiolabeled residues were recovered from tissues following treatment with β-glucuronidase. It was also noted that high concentrations of tolfenamic acid persist at injection sites. In animals which received two intramuscular injections of ^{14}C-tolfenamic acid at a dose rate of 2 mg/kg bw (48 hours between

injections), mean residue concentrations at the injection sites were in excess of 64,000 μg/kg 4 days after the second injection. A similar distribution of residues in the tissues was observed in pigs, with highest residues found in liver and lowest in fat. The data provided to the CVMP did not include residue concentrations in the injection sites in pigs.

Based on the information provided to the CVMP, inclusion of treatment with β-glucuronidase should be included in methods for tolfenamic acid residues in tissues. While liver is probably the preferred target tissue for monitoring programs, consideration should also be given to the potential need to target injection sites as part of a regulatory testing program.

8.6.2 Multi-analyte Methods

Table 8.6 lists the residue concentrations which can be monitored using the different multi-residue methods that have been used to simultaneously analyze more than one NSAID, as well as the matrices that have been analyzed. The extraction procedures of NSAIDs for various matrices and for different species of animals are summarized. The table is arranged according to the method of analysis, such as LC-MS/MS, LC-MS, GC-MS/MS, GC-MS, and other detection techniques. While the list is by no means exhaustive, it points out the relevant methods that can be used to support regulatory actions for the use of these NSAIDs in food animal production.

8.6.2.1 LC-MS/MS Methods

8.6.2.1.1 Methods for Animal Tissues Most of the LC-MS/MS methods reported for animal tissue analysis were developed after the year 2000, when LC-MS/MS instruments became more readily accessible.[176–184] A classic example of such a method was one developed for the determination of meloxicam, flunixin, carprofen, and tolfenamic acid using flunixin d_3 as internal standard.[181] To 1 g of liver or muscle tissue fortified with the analytes was added 5 ml 0.25 M HCl for overnight (16 hours) hydrolysis. Following hydrolysis 0.3 M sodium phosphate tribasic 12-hydrate solution was added. The mixture was neutralized at pH 7.1 ± 0.2 and extracted with ethyl acetate.

The residue was reconstituted in 50:50 formic acid 10 mM/methanol solution (mobile phase) and injected in the ion trap LC-MS/MS system.

A confirmatory method for the determination of residues of nine NSAIDs and one metabolite in animal muscles has been developed by Jedziniak et al.[182] The method was validated to Commission Decision 2002/657/EC criteria[205] using cattle muscle and was demonstrated as applicable to pig, horse, and chicken muscle. After enzymatic hydrolysis with β-glucuronidase, samples were extracted with acetonitrile and cleaned up using alumina and C_{18} SPE cartridges before determination by LC-MS/MS.

Table 8.6 Multi-residue methods for NSAIDs in biological matrices.

Compounds	Method	Species and matrix	LOD/LOQ; CCα/CCβ[a)]	Reference
120 veterinary drugs and metabolites including, among other drugs, quinolones, sulfonamides, tetracyclines, macrolides, nitrofurans, thyreostats, β-lactams, anthelmintics, β-agonists, tranquilizers, and the following NSAIDs (ketoprofen, oxyphenbutazone, flunixin, diclofenac, phenylbutazone, and tolfenamic acid)	LC-MS/MS	Kidney (cattle)	LOD ≤ 1 μg/kg for ketoprofen, oxyphenbutazone, and flunixin; LOD ≤ 5 μg/kg for diclofenac, phenylbutazone, and tolfenamic acid	Schneider and Lehotay[176]
Acetylsalicylic acid, ketoprofen, flunixin, tolfenamic acid, phenylbutazone, meloxicam	LC-MS/MS	Muscle (cattle)	CCα between 21 and 59 μg/kg and CCβ between 21 and 68 μg/kg for salicylic acid, flunixin, meloxicam, and tolfenamic acid; LOD ≤ 100 and 20 μg/kg for phenylbutazone and ketoprofen, respectively	Van Hoof et al.[177]
Naproxen, meloxicam, ketoprofen, flunixin, niflumic acid, carprofen, etodolac, mefenamic acid, tolfenamic acid, and vedaprofen	LC-MS/MS	Muscle, kidney (cattle)	LOQ between 0.4 and 51 μg/kg	Chrusch et al.[178]

(*continued*)

Table 8.6 (Continued)

Compounds	Method	Species and matrix	LOD/LOQ; CCα/CCβ[a)]	Reference
Acetaminophen, salicylic acid, ibuprofen, diclofenac, flunixin, 5-hydroxyflunixin, nimesulide, phenylbutazone, meclofenamic acid, tolfenamic acid, meloxicam, carprofen, ketoprofen, naproxen, and etodolac	LC-MS/MS	Muscle, milk (cattle)	CCα between 0.1 and 56 μg/kg for milk and between 0.2 and 652 μg/kg for muscle; CCβ between 0.2 and 196 μg/kg for milk and between 0.5 and 796 μg/kg for muscle	Gentili et al.[179]
Salicylic acid, loxoprofen, fenbufen, sasapyrin, nimesulide, etodolac, acetaminophen, aminoantipyrine, formylaminoantipyrine, tenoxicam, mepirizole, etoricoxib, acetophenetidine, piroxicam, benzydamine, ketorolac, indoprofen, rofecoxib, sulindac, tolmetin, firocoxib, flunixin, ketoprofen, meloxicam, naproxen, zomepirac, oxaprozin, nabumetone, indomethacin, and acemetacin	UHPLC-MS/MS	Muscle (pig)	LOD between 0.4 and 2.0 μg/kg; LOQ between 1.0 and 5.0 μg/kg	Hu et al.[180]
Carprofen, flunixin, meloxicam, and tolfenamic acid	LC-MS/MS	Muscle, liver (cattle, pig, horse)	CCα between 76 and 1090 μg/kg for liver and between 25 and 584 μg/kg for muscle; CCβ between 86 and 1193 μg/kg for liver and between 30 and 667 μg/kg for muscle	Igualada et al.[181]

Diclofenac, flunixin, ketoprofen, mefenamic acid, meloxicam, naproxen, oxyphenbutazone, phenylbutazone, tolfenamic acid, and carprofen	LC-MS/MS	Muscle (cattle, pig, horse, chicken)	LOD between 0.3 and 1.2 µg/kg; LOQ between 0.7 and 3.1 µg/kg	Jedziniak et al.[182]
Ketoprofen, naproxen, oxyphenbutazone, flunixin, meloxicam, carprofen, diclofenac, phenylbutazone, mefenamic acid, and tolfenamic acid	LC-MS/MS	Animal tissues (various tissues and species)	No LOD, LOQ, CCα or CCβ reported	Olejnik et al.[183]
Eighteen non-steroidal anti-inflammatory drugs (NSAIDs) in swine liver	UHPLC-MS/MS	Liver (pig); muscle, offal (various species); egg; milk.	LOD between 0.2 µg/kg and 10 µg/kg; LOQ between 1 µg/kg and 50 µg/kg	Peng et al.[184]
Carprofen, diclofenac, flufenamic acid, flunixin (5-hydroxyflunixin as marker metabolite in milk), ketoprofen, mefenamic acid, meloxicam, 4-methylaminoantipyrine (marker metabolite of metamizole in meat and milk), naproxen, niflumic acid, phenylbutazone (and metabolite oxyphenbutazone), ramifenazone, salicylic acid, and tolfenamic acid	LC-MS/MS	Meat, milk (cattle)	CCα between 0.5 and 579 µg/kg for meat; CCβ between 0.6 and 642 µg/kg for meat CCα between 0.1 and 55 µg/kg for milk; CCβ between 0.1 and 61 µg/kg for milk	van Pamel and Daeseleire [185]

(*continued*)

Table 8.6 (Continued)

Compounds	Method	Species and matrix	LOD/LOQ; CCα/CCβ[a)]	Reference
Carprofen, diclofenac, ibuprofen, ketoprofen, mefenamic acid, phenylbutazone, flunixin, hydroxyflunixin, tolfenamic acid, and meloxicam	LC-MS/MS	Milk (cattle)	CCα between 0.5 and 56 µg/l; CCβ between 0.80 and 72 µg/l	Dowling et al.[186]
Carprofen, diclofenac, mefenamic acid, niflumic acid, naproxen, oxyphenbutazone, phenylbutazone, and suxibuzone	LC-MS/MS	Milk (cattle)	CCα between 0.5 and 2.9 µg/l; CCβ between 0.8 and 4.9 µg/l	Dowling et al.[187]
Phenylbutazone, oxyphenbutazone, naproxen, mefenamic acid, vedaprofen, flunixin, 5-hydroxyflunixin, tolfenamic acid, meloxicam, diclofenac, carprofen, and ketoprofen	LC-MS/MS	Milk (cattle)	CCα between 0.1 and 63 µg/kg; CCβ between 0.1 and 76 µg/kg	Dubreil-Chéneau et al.[188]
Salicylic acid, naproxen, carprofen, flurbiprofen, ibuprofen, meclofenamic acid, niflumic acid, flunixin, 5-hydroxyflunixin, ketoprofen, suxibutazone, diclofenac, mefenamic acid, tolfenamic acid, phenylbutazone, and oxyphenbutazone	HPLC-DAD and LC-MS/MS	Milk (cattle, buffalo)	LOQ between 2 µg/kg and 15 µg/kg	Gallo et al.[189]

7 anti-inflammatory drugs, including 4 NSAIDs (meloxicam, 4-methyl amino antipyrine, tolfenamic acid, and 5-hydroxyflunixin)	LC-MS/MS	Milk (cattle)	CCα between 16 and 62 µg/kg; CCβ between 17 and 73 µg/kg	Malone et al.[190]
150 veterinary drugs and metabolites including avermectins, benzimidazoles, β-agonists, β-lactams, corticoids, macrolides, nitroimidazoles, quinolones, sulfonamides, tetracyclines, and the following NSAIDs (flunixin, ketoprofen, meloxicam, naproxen, and tolfenamic acid)	UHPLC-TOF-MS/MS	Milk (cattle)	CCα between 0.4 and 45 µg/l; CCβ between 0.6 and 52 µg/l	Ortelli et al.[191]
Paracetamol, phenacetin, 4-formylamino antipyrine, phenylbutazone, salicylic acid, piroxicam, meloxicam, ibuprofen, ketoprofen, naproxen, carprofen, vedaprofen, flunixin, diclofenac, mefenamic acid, meclofenamic acid, tolfenamic acid, rofecoxib, firocoxib, and celecoxib	UHPLC-MS/MS	Milk and dairy products (milk powder, yogurt, processed cheese, and milk beverage samples)	LOD between 0.03 and 0.3 µg/kg; LOQ between 0.1 and 1.0 µg/kg	Peng et al.[192]

(*continued*)

Table 8.6 (Continued)

Compounds	Method	Species and matrix	LOD/LOQ; CCα/CCβ[a)]	Reference
Carprofen, diclofenac, flunixin, ibuprofen, ketoprofen, mefenamic acid, meloxicam, naproxen, oxyphenbutazone, phenylbutazone, tolfenamic acid, metamizole, celecoxib, firocoxib, and rofecoxib	LC-MS/MS	Milk (cattle)	CCα between 0.1 and 55 µg/kg; CCβ between 0.2 and 65 µg/kg	Jedziniak et al.[193]
Salicylic acid, naproxen, carprofen, flurbiprofen, ibuprofen, niflumic acid, meclofenamic acid, ketoprofen, suxibutazone, diclofenac, mefenamic acid, tolfenamic acid, phenylbutazone, and oxyphenbutazone	LC-MS/MS	Serum, plasma (cattle, pig, rabbit, and horse)	No LOD, LOQ, CCα, or CCβ reported	Vinci et al.[194]
Ibuprofen, ketoprofen, diclofenac, and phenylbutazone	GC-MS/MS	Milk (cattle)	CCα between 0.6 and 2.7 µg/l CCβ between 1.0 and 4.6 µg/l	Dowling et al.[195]
Ibuprofen, naproxen, ketoprofen, diclofenac, flufenamic acid, tolfenamic acid, and meclofenamic acid	GC-MS	Milk (cattle)	CCα between 3.4 and 7.8 µg/kg; CCβ between 6.5 and 15 µg/kg	Arroyo et al.[198]

Salicylic acid, ibuprofen, ibuproxam, flurbiprofen, propyphenazone, niflumic acid, naproxen, flunixin, mefenamic acid, ketoprofen, tolfenamic acid, diclofenac, meclofenamic acid, phenylbutazone, oxyphenbutazone, indomethacin, and suxibuzone	GC-MS	Plasma, urine (horse)	LOD between <5 and 25 μg/l	González et al.[199]
Flurbiprofen, carprofen, naproxen, vedaprofen, 5-hydroxyflunixin, niflumic acid, mefenamic acid, meclofenamic acid, and tolfenamic acid	HPLC-FL	Milk (cattle)	LOQ between 0.3 and 20 μg/kg	Gallo et al.[200]
Ketoprofen, diclofenac, flunixin, and meloxicam	HPLC-UV	Muscle (sheep)	LOD between 5 and 10 μg/kg; LOQ between 15 and 30 μg/kg	Kang et al.[196]
Etodolac, naproxen, ketoprofen, flurbiprofen, and diclofenac	DLLME–FASS-CE[b)]	Milk (cattle) and dairy products (yogurt and white cheese)	LOD were between 4.8 and 12–13 μg/kg, between 3.0 and 9.7 μg/kg, and between 6.1 and 7.7 μg/kg for milk, yogurt, and white cheese, respectively	Alshana et al.[201]
Acetylsalicylic acid, salicylic acid, oxyphenbutazone, carprofen, niflumic acid, diclofenac, phenylbutazone, mefenamic acid, ketoprofen, propyphenazone, flunixin, and vedaprofen	HPLC-DAD	Plasma (cattle, pig, sheep, horse)	CCα between 42 and 184 μg/l; CCβ between 57 and 3600 μg/l	Gowik et al.[202]

(*continued*)

Table 8.6 (Continued)

Compounds	Method	Species and matrix	LOD/LOQ; CCα/CCβ[a)]	Reference
Five anti-inflammatory drugs including 3 NSAIDs (indomethacin, phenylbutazone, and oxyphenbutazone)	HPLC-UV	Serum (horse)	LOD between 250 and 500 µg/l; LOQ between 250 and 1000 µg/l	Grippa et al.[203]
Salicylic acid, ketoprofen, flurbiprofen, phenylbutazone, oxyphenbutazone, carprofen, ibuprofen, naproxen, niflumic acid, suxibutazone, diclofenac, mefenamic acid, and tolfenamic acid	HPLC-DAD	Serum, plasma (cattle, pig, horse)	No LOD, LOQ, CCα, or CCβ reported	Gallo et al.[204]

a) The editors have rounded results reported in the original publications to better reflect typical method precision at the concentrations reported, as follows: concentrations < 0.1 µg/kg (or < 0.1 µg/l) are given to two places after the decimal; concentrations > 0.1 µg/kg (or > 0.1 µg/l) and < 10 µg/kg (or < 10 µg/l) are given to one place after the decimal; concentrations > 10 µg/kg (or > 10 µg/l) are given as whole numbers. Readers are invited to read the discussion on this point in Chapter 1 to fully appreciate the appropriate reporting of significant figures.

b) Dispersive liquid–liquid micro-extraction combined with field-amplified sample stacking in capillary electrophoresis.

In 2008, a method was reported for nine NSAIDS (naproxen, meloxicam, ketoprofen, flunixin, carprofen, etodolac, mefenamic acid, tolfenamic acid, and vedaprofen) and 20 other veterinary drugs in which bovine muscle and kidney samples were allowed to react with 1% formic acid, buffered with TRIS (hydroxymethyl aminomethane) and digested overnight with protease (Type XIV).[178] The samples were loaded to C_{18} cartridges after two centrifugations with isopropanol and a mixture of water and hexane, respectively. Two additional cartridges (OASIS MAX and IRIS) were used, and the NSAIDs retained were eluted with 2% formic acid in ethyl acetate.

In 2012, Gentili et al. reported a method in which 15 NSAIDs in milk and muscle tissue samples were analyzed by first homogenizing the tissue with methanol, followed by two simple extractions – one with acetonitrile and the other one with acetone; the organic extracts were pooled together.[179] OASIS HLB cartridges were used to clean up the crude extract prior to analysis by LC-MS/MS. The method was validated according to the criteria of the Commission Decision 2002/657/EC[205] and applied in a small monitoring study.

In another method, the extraction of 30 NSAIDs from swine muscle was performed with acidic acetonitrile (acetonitrile:phosphoric acid, 80 + 1, v/v), in order to ensure that the recovery was not influenced by bound drugs.[180] Clean-up was achieved with Oasis HLB cartridges and elution with ammonium hydroxide – acetonitrile – methyl *tert*-butyl ether (5 + 95 + 1, v/v/v).

More recently, a method using LC-MS/MS has been described applicable to the determination of 15 NSAIDS and their metabolites in muscle and milk from cattle.[185] Analytical recoveries were from 81% to 114% for meat and from 79% to 118% for milk, with acceptable precision. The authors state that the method is in routine use for regulatory samples and that it was validated according to the criteria in Commission Decision 2002/657/EC.[205]

8.6.2.1.2 Methods for Milk A number of methods for the analysis of NSAID drug residues in milk using LC-MS/MS have been reported.[186–193] A rapid and accurate method for NSAID residues in milk was developed by Dubreil-Chéneau et al. with a simple liquid extraction with methanol followed by an evaporation step.[188]

Jedziniak et al. developed an extraction method for 19 NSAIDs and their metabolites from milk with acetonitrile and ammonium acetate.[193] The dry residue was reconstituted in mobile phase (MeOH – ACN 0.01 moles/l ammonium formate 0.25:0.75:9), after which samples for metabolite analysis were filtered, transferred to autosampler vials, and analyzed by LC-MS/MS. Samples for NSAIDs were subjected to clean-up with Sep-Pak NH_2 cartridge with an additional layer of sodium sulfate. Analytes were eluted with 5% formic acid in acetonitrile. DMSO was added and samples were analyzed by LC-MS/MS. The method was validated according to the criteria of Commission Decision 2002/657/EC.[205]

Dowling et al., after twice extracting the NSAIDs from milk with acetonitrile, added a mixture of ascorbic acid and hydrochloric acid to the extracts

and performed the solid-phase extraction by SPE (EVOLUTE ABN™ SPE cartridges) before LC-MS/MS determination.[186, 187] This method also was validated according to the criteria of Commission Decision 2002/657/EC.[205]

In 2008, Gallo et al. reported extracting 16 NSAIDs from cattle and buffalo milk with a solvent mixture of acetonitrile/methanol.[189] The crude extract was then loaded at atmospheric pressure onto a C_{18} SPE cartridge. The SPE was eluted with a mixture of hexane/diethyl ether and the eluate obtained evaporated to dryness. Malone et al. also extracted NSAIDs from milk samples with acetonitrile and sodium chloride followed by *n*-hexane.[190] The dried extracts were then reconstituted in acetonitrile:water (28:72, v/v) and injected into the LC system. The extraction procedure from bovine milk and muscle tissue was divided into two succeeding steps, one for deproteinization/extraction with organic solvent and the other for clean-up. Milk samples were vortexed with acetonitrile, centrifuged at low temperatures (in order to successfully remove the fat content of the samples), and finally concentrated under a gentle nitrogen stream prior to analysis by LC-MS/MS. These methods were validated to meet the requirements of Commission Decision 2002/657/EC.[205]

Dairy products were simultaneously deproteinized and extracted for nine sub-classes of NSAIDs with a solvent mixture of ascorbic acid buffer and acetonitrile–ethyl acetate by Peng et al.[192] The mixture was vigorously vortexed followed by a double centrifugation with the supernatants pooled and evaporated under a gentle nitrogen stream. No extra clean-up was performed in any matrix, although the authors suggest that the extraction procedure needs to be optimized by including a clean-up step or using other extraction solutions. LOQs reported were 0.10–1.00 μg/kg, with recoveries from analytes spiked into milk, milk powder, yogurt, processed cheese, and milk beverage which ranged from 61.7% to 117%. Relative standard deviations of less than 17.9% at the three spiking concentrations were tested, which were selected as 1, 10, and 100 times the LOQ for the analyte/matrix combination.

8.6.2.1.3 Methods for Other Biological Matrices Multi-residue methods have been reported for the analysis of animal serum and plasma. Vinci et al.[194] used a slightly modified version of the clean-up procedure reported by Gowik et al.[203] to optimize the recoveries of all the NSAIDs from bovine, pig, rabbit, and equine plasma and serum samples. A 5 ml test portion of the sample was centrifuged, and 500 ml of 1 mol/l HCl were added to adjust the pH of the separated supernatant to about 3. The solution was allowed to stand for 10 minutes at room temperature to denature plasma proteins and hydrolyze bound drug residues. After the addition of 25 ml ascorbic acid buffer, clean-up of the sample extract was achieved on a C_{18} SPE cartridge. After drying of the collected fraction, the residue was dissolved in 1 ml of LC-MS/MS mobile phase. In this study, two ion trap LC(ESI)-MS/MS methods were used to identify and confirm 14 NSAIDs belonging to different

sub-classes: salicylic acid, ketoprofen, carprofen, flurbiprofen, ibuprofen, and naproxen (as arylpropionic acid derivatives); suxibutazone, phenylbutazone, and its metabolite oxyphenbutazone (as pyrazolidinedione derivatives); diclofenac, mefenamic acid, meclofenamic acid, and tolfenamic acid (as anthranilic acid derivatives); and niflumic acid (as a nicotinic acid derivative). A complete study of the MS/MS fragmentation patterns of NSAIDs was also performed. This allowed the authors to choose the best optimum experimental conditions for the identification and quantification of NSAIDs by ion trap LC(ESI)-MS/MS. Positive ion mode was used for the determination of ketoprofen, suxibutazone, diclofenac, phenylbutazone, oxyphenbutazone, mefenamic acid, and tolfenamic acid and negative ion mode for the determination of salicylic acid, naproxen, carprofen, flurbiprofen, niflumic acid, ibuprofen, and meclofenamic acid. These methods were validated in-house according to Commission Decision 2002/657/EC[205] at above and below the action limit of 100 µg/l (50, 100, and 150 µg/l) indicated in the Italian National Residue Monitoring Programme. Recovery for each NSAID ranged between 72% and 101% from samples fortified at the three concentrations with within-day repeatability (coefficient of variability) ranging from 1% to 20%. Recovery for salicylic acid was lower at 48–69% at 100, 200, and 300 µg/l.

8.6.2.2 GC-MS/MS Methods

The only gas chromatography–tandem mass spectrometry (GC-MS/MS) method reported to date for the NSAIDs was developed and validated for the analysis of ibuprofen, ketoprofen, diclofenac, and phenylbutazone residues in bovine milk.[195] Following the extraction of milk samples with acetonitrile, sample extracts were purified on C_{18} solid-phase extraction cartridges. Aliquots were analyzed by GC-MS/MS with helium as carrier gas at a flow of 1.5 ml/minute. The NSAIDs were chromatographed on a column with a film thickness of 0.25 mm, a diameter of 0.25 mm, and a length of 30 m packed with SE-54/CP Sil 8-type material (Restek, Buchs, UK). The injector temperature was maintained at 275 °C, and it was a constant temperature splitless programmable temperature vaporizing (PTV) injection. The injection volume was 2 ml. The oven temperature was set at 100 °C for 1 minute and ramped to 300 °C in increments of 10 °C/minute. The oven temperature was held at 300 °C for 1 minute. The runtime was 22 minutes. The method was validated for bovine milk, according to Commission Decision 2002/657/EC.[205] The decision limits (CCα) were 0.59, 2.69, 0.90, and 0.70 µg/l, respectively, for ibuprofen, ketoprofen, diclofenac, and phenylbutazone, and detection capabilities (CCβ) of 1.01, 4.58, 1.54, and 1.19 µg/l, respectively, were obtained. The measurement uncertainty of the method was 17.8%, 80.9%, 28.2%, and 20.2% for ibuprofen, ketoprofen, diclofenac, and phenylbutazone, respectively. Fortifying bovine milk samples ($n = 18$) in three separate assays showed the accuracy of the method to be between 104% and 112%.

8.6.2.3 LC-MS Methods

A sensitive, selective, and accurate high-performance LC-APCI/MS assay for the determination of diclofenac sodium, flufenamic acid, indomethacin, and ketoprofen, either individually or in laboratory-made mixtures and pharmaceutical formulations, was developed.[197] The drugs were injected onto a Shim-pack GLC-CN column and were eluted with a mobile phase consisting of acetonitrile and 20 mM ammonium acetate solution (5:1 v/v)/pH 7.4 at a flow rate 1 ml/minute. The mass spectrometer, operated in the single ion monitoring mode, was programmed to admit the negative ions $[M-H]^-$ at m/z 295.9 (DIC), 280.1 (FLU), 355.8 (IND), and 252.9 (KET), respectively, with detection limits of 0.5–4.0 ng injected on column. This method was not applied to biological samples.

8.6.2.4 GC-MS Methods

8.6.2.4.1 Methods for Milk Arroyo et al. reported a method for the analysis of 7 NSAIDs, ibuprofen, naproxen, ketoprofen, diclofenac, flufenamic acid, tolfenamic acid, and meclofenamic acid in bovine milk.[198] After extraction from milk with a mixture of acetonitrile, NaCl, and *n*-hexane, the NSAIDs were derivatized to form ethyl esters. This was followed with a solid-phase micro-extraction (SPME) step, prior to injection in the GC-MS system. Three kinds of SPME fibers – polyacrylate (PA), polydimethylsiloxane/divinylbenzene (PDMS/DVB), and polydimethylsiloxane (PDMS) – were compared to identify the most suitable one for the extraction process on the basis of two steps: to determine the equilibrium time of each fiber and to select the fiber that provides the best figures-of-merit values. The best results were obtained with the PDMS fiber. Subsequently, eight experimental factors (related to the derivatization reaction and the SPME) were optimized by means of a D-optimal design that involves only 14 rather than 512 experiments in the complete factorial design. Owing to the fact that each analyte is unequivocally identified, a calibration model was not needed for each experimental condition.

8.6.2.4.2 Other Biological Matrices A method reported by González et al. was applicable to the detection of 17 NSAIDs in equine plasma and urine samples.[199] The analytes were extracted from the samples with diethyl ether at acidic pH (2–3). Urine extracts were washed with a saturated solution of $NaHCO_3$, while plasma extracts were treated with a solid mixture of Na_2CO_3 and $NaHCO_3$, after which the ether extracts were dried and then derivatized at 60 °C with methyl iodide in acetone in the presence of solid K_2CO_3. Recoveries for the target compounds from plasma ranged from 23.3% to 100%, while recoveries from urine ranged from 37.5% to 83.8%. Detection limits using selected ion monitoring (SIM) ranged from < 5 to 25 μg/l for both plasma and urine samples. The method was applied in doping control for horses.

Methods based on GC-MS have also been reported for use in toxicological screening for 25 NSAIDs in human urine.[206] In this method, a 2-ml test portion

of urine was mixed in a centrifuge tube with 2 ml of buffer (pH 12), followed by the addition of 6 ml of 1 M methyl iodide in toluene. The tube was closed and then shaken at 50 °C heating block for 30 minutes to form the methylated derivatives. Following centrifugation at 1500×*g* for 3 minutes, clean-up of the organic phase using an SPE (Diol) cartridge, collection, and drying of the eluate containing the analytes, the residue was reconstituted and injected into the GC-MS system, with detection using full spectra generated in the electron impact (EI) mode. The authors note that enzymatic hydrolysis of acyl glucuronides of the NSAIDs may be hindered because of acyl migration, described in an earlier publication.[207] The term "acyl migration" is used to describe the intramolecular transesterification which may occur at the hydroxy groups of the glucuronic acid, leading to the formation of glucuronide derivatives which may be resistant to treatment with β-glucuronidase. This paper references other contemporary methods for the determination of NSAIDs in samples undergoing clinical and forensic toxicology testing and in racetrack doping control.[208–211]

8.6.2.5 HPLC Methods

8.6.2.5.1 Methods for Animal Tissues A method developed for sheep muscle included residues resulting from treatment with flunixin meglumine, meloxicam, diclofenac sodium, and ketoprofen.[196] Ultrasonic–microwave extraction was used in this method, followed by analysis on a C_{18} HPLC column with UV detection at 255 nm. The authors reported LOQs of 15–30 µg/kg, recoveries from 65% to 100%, and relative standard deviations of less than 15%.

8.6.2.5.2 Methods for Milk Several HPLC methods have been described for the determination of NSAID residues in milk. Gallo et al. described a liquid chromatographic method using fluorescence detection for residues of nine NSAIDs (flurbiprofen, carprofen, naproxen, vedaprofen, 5-hydroxyflunixin, niflumic acid, mefenamic acid, meclofenamic acid, and tolfenamic acid) in bovine milk applicable with LOQs of 0.25–20.0 µg/kg, depending on the analyte.[200] The drugs were detected using their native fluorescence, using the characteristic excitation and emission wavelengths for each drug for confirmation. Alshana and coworkers reported a method for the determination of 5 NSAIDs in milk that offered numerous advantages compared with other conventional sample preparation, such as simplicity, low cost and ease of operation, and use of smaller quantities of organic solvents and high enrichment factors (42–229).[201] The method also provided a very short analysis time. After a sample clean-up by salting-out extraction, a dispersive liquid–liquid micro-extraction (DLLME) was performed with acetonitrile, coupled with field-amplified sample stacking (FASS) in capillary electrophoresis to achieve lower limits of detection, which ranged from 3.0 to 13 µg/kg for all the matrices analyzed. Materials analyzed included bottled milk, raw milk, yogurt, and white cheese. The combination of DLLME–FASS-CE was demonstrated to be a rapid and convenient method for the determination of NSAIDs in milk and dairy products.

8.6.2.5.3 Methods for Other Biological Matrices Other HPLC methods have been developed for the determination of NSAIDs in serum and/or plasma. Gowik et al. described a method using HPLC with photodiode-array detection (DAD) for the determination of residues of 12 NSAIDs in animal serum.[202] The method was validated using a variety of matrices. A multi-residue method using HPLC with UV detection at 254 nm included the determination of the NSAIDs phenylbutazone and oxyphenbutazone in horse serum.[203] Chromatographic separation used a C_{18} column, with an LOQ of 0.5 µg/l for phenylbutazone and 1 µg/l for its metabolite oxyphenbutazone. More recently, Gallo and coworkers described a method for 13 NSAIDs in serum and plasma from cattle, pigs, and horses.[204] After centrifugation, sample test portion were adjusted to pH 3 to denature proteins and hydrolyze any bound residues to free drug. After equilibration for 10 minutes at room temperature, samples were diluted with buffer, followed by clean-up on a C_{18} SPE cartridge. Eluates were evaporated to dryness under a nitrogen stream, and the residue was dissolved in 500 µl of methanol for HPLC-DAD analysis. Analytical recoveries were from 72.5% and 104.5%, except for salicylic acid, for which the recovery was from 36.3% to 54.9%.

8.7 Literature Reviews of Analytical Methods for NSAIDs in Biological Samples

Few reviews have been reported on the analytical methods for NSAIDs, particularly for residues in foods. A review by Gentili in 2007 on the use of LC-MS methods for analysis of residues of anti-inflammatory drugs, including NSAIDs, in animal-derived foods, discussed topics which included choice of MS ionization sources and analyzers, extraction procedures, and matrix effects.[212] More recently, Starek et al. reviewed the literature published in analytical and pharmaceutical chemistry journals for analytical methods which had been developed and used for the determination of some of the COX-2 inhibitors in bulk drugs, formulations, and biological fluids.[213] The review, which covered the time period from 1999 to 2011, revealed that over 140 analytical procedures including chromatographic, spectrometric, electrophoretic, and voltammetric techniques had been reported. The authors presented applications concerning the analysis of coxibs from pharmaceutical formulations and biological samples.

In 2012, Olives et al. published a review that reported that as a result of the widespread use of NSAIDs employed in both human and animal health care to reduce ongoing inflammation, pain, and fever due to their anti-inflammatory, analgesic, and antipyretic actions, these substances were being frequently found in the environment.[214] There was concern that this would lead to long-term exposure resulting in adverse effects on humans and wildlife. Therefore, it was important, the authors claimed, to develop analytical methods to detect and control the presence of these pharmaceuticals in very different kinds of samples, from urine, serum, or plasma, to river and waste water, sediments, or sewage

sludge, most of them having very complex matrices. Other problems to solve are the low concentration of the target analytes, the presence of a great number of potential interferences and, sometimes, incompatibilities with the detection systems. Consequently, the authors reported that sample pre-treatment was a very important step for NSAID determination. The authors reviewed the main extraction and clean-up procedures reported in the literature: ultrasonic extraction, Soxhlet extraction, pressurized liquid extraction, supercritical fluid extraction, microwave-assisted extraction, DLLME, hollow fiber liquid-phase micro-extraction, pressurized hot water extraction, solid-phase extraction, molecularly imprinted solid-phase extraction, and SPME. Analytical methods developed to quantify NSAIDs, including GC-MS, liquid chromatography with UV detection, diode array detection, fluorescence detection, and tandem MS, were discussed in this review.

A review by Maurer reported procedures used in the analysis of acidic drugs and/or metabolites relevant to clinical and forensic toxicology or doping control using techniques which included gas chromatography, GC-MS liquid chromatography, thin-layer chromatography, and capillary electrophoresis.[215] The review considered papers published in the period 1992–1998, covered matrices which included blood, plasma, serum, urine, vitreous humor, brain, liver, or hair) of humans or animals (horse or rat) and addressed various classes of drugs, including NSAIDs. Also in 1999, Hercegova and Polonsky reviewed methods for the determination of NSAIDs in biological fluids.[216] Analytical techniques covered in this review included CZE, HPLC, HPTLC, and GC-MS. Maurer subsequently reviewed the use of GC-MS with negative ion chemical ionization (NICI) in analysis of samples from areas that included clinical and forensic toxicology, doping control, and biomonitoring for substances that included drugs, pesticides, pollutants, and/or their metabolites.[217] The review covered English language papers from the period 1995–2000 and included methods applicable to matrices that included whole blood, plasma, urine, sweat, hair, bone, and muscle samples of humans and rats.

An earlier review by Davies in 1997 addressed the general principles that allow separation of chiral NSAID enantiomers and discussed both the advantages and disadvantages of the available chromatographic assay methods and procedures used to separately quantify NSAID enantiomers in biological matrices.[46]

8.8 New Developments in NSAIDs

A review of NSAIDs cannot conclude without a look at developments in the design and synthesis of new NSAIDs. A key objective is the development of NSAIDs that reduce the side effects associated with long-term use.[218] A primary intention is moving away from the alkyl, aryl, and propionic esters by masking the carboxylic acid with more elaborate conjugates or functional groups that contain carefully selected moieties.[20] The use of such additional moieties is intended to

improve the NSAID drug's solubility in water, release nitric oxide or hydrogen sulfide, or produce specific acetylcholinesterase inhibitory (AChEI) activity. These new NSAIDs, called prodrugs, are bioreversible derivatives of a drug that undergoes an enzymatic and or chemical transformation *in vivo* to release the active parent drug that can then deliver the pharmacological activity expected of the parent drug.[219] In most cases, prodrugs are synthesized by covalently linking the parent drug, itself with or without a carrier, to a pharmacologically inert group that is enzymatically or chemically cleaved after drug administration to release the parent drug. An example of a prodrug is sulindac,[18] which must first be converted to the active form by reduction to the sulfide. The active moiety is then able to inhibit cycloogenase. Promising classes of NSAID prodrugs include the nitric oxide-releasing NSAIDs (NO-NSAIDs),[220] NSAID prodrugs with anticholinergic or acetylcholinesterase inhibitory (AChEI) activity[221,222] and the phospho-NSAIDs.[223]

8.9 Conclusion

NSAIDS are one of the most commonly and frequently used classes of medications to reduce fever, pain, and inflammation. Because of the similar chemical structures, they exhibit similar chemistries in food animals and in humans and therefore are generally considered safe for use in food animals. For NSAIDs with a high margin of safety, it has not been necessary to set MRLs. In this chapter, we have considered the general pharmacology of NSAIDs, reviewed their basic PK and metabolic characteristics, and described and summarized analytical methods used to provide PK and residue depletion data. When methods are used to provide PK and depletion data information to assist with drug registration and licensing, any analytical method with demonstrated sensitivity, accuracy, and precision for the study of interest should be acceptable. However, when methods are used for regulatory monitoring to support national residue control programs, those methods should not only have been validated according to an internationally recognized protocol, such as 2002/657/EC[205] or the Codex Alimentarius Commission Guideline CAC/GL 71 – 2009,[224] but should also be conducted by analysts operating in an internationally accredited laboratory environment. Such methods must be rugged and "fit for purpose."

We have taken the opportunity in this chapter to emphasize that most regulatory laboratories today are investing their resources and efforts in developing and validating multi-residue analytical methods to take advantage of the availability of affordable hyphenated and tandem instruments and platform technologies such as LC-MS, GC-MS, LC-MS/MS, GC-MS/MS, LC-QTOF/MS, and LC-HRMS systems in order to increase laboratory efficiencies and reduce laboratory operational costs. This trend does not, however, exclude the use of such basic chromatographic detection technologies such as GC-FID, GC-ECD, and LC-UV/VIS for regulatory analysis where trace concentration requirements

are not critical needs, as long as these methods are supported by analytical methods that can provide confirmatory analysis of the identities of the drugs and/or analytes found.

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9

Certain Dyes as Pharmacologically Active Substances in Fish Farming and Other Aquaculture Products

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9.1 Introduction

The last 40 years have brought enormous changes to the aquaculture industry. The farming of fish and of seafood products has been continuously increasing from 3.9% by weight in 1970 to 36% in 2006 according to the World Health Organization (WHO) and Food and Agriculture Organization (FAO) of the United Nations.[1] The global trend of aquaculture development gaining importance in total fish supply has remained uninterrupted. Farmed food fish contributed a record 42.2% of the total 158 million tonnes of fish produced by capture fisheries and aquaculture in 2012 (Figure 9.1). This compares with just 13.4% in 1990 and 25.7% in 2000. Since 2008, Asia has been producing more farmed fish than wild catch, and its aquaculture share in total production reached 54% in 2012, when European production rose to 18% and other continents to less than 15%.[1] The 15 main producer countries accounted for 92.7% of all farmed food fish and seafood production in 2012. In the same period, there was a considerable intensification of seafood trading worldwide.

Fish is the main valued export commodity from the vast majority of the developing countries before coffee, natural rubber, cocoa, and sugar.[1] According to the seafood trade flows in 2010 from Natale et al.,[2] China appears as the major exporter to the rest of the world with also an increasing importance of Vietnam, Thailand, Chile, India, and Indonesia. China has also become the world's third largest importing country after the United States of America and Japan (Figure 9.2). The European Union (EU) is the largest market for imported fish and fishery products, and its dependence on imports is still growing. Such a food fish farming increase cannot be further intensified without controlling the zoosanitary aspects of this agri-food industry.

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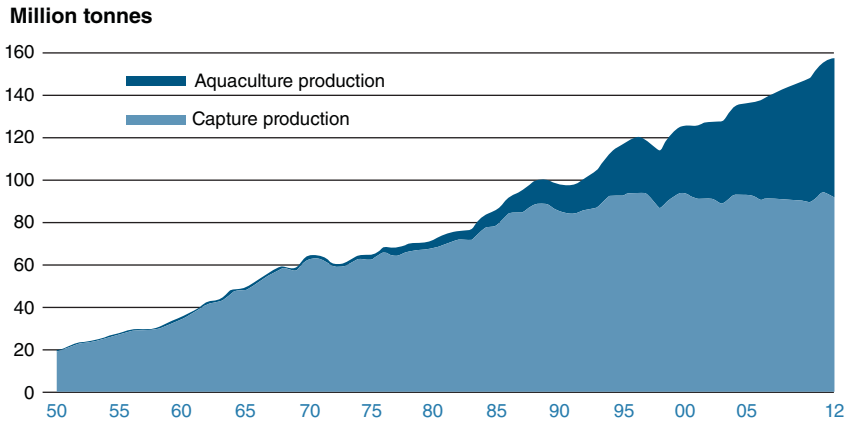


Figure 9.1 World capture fisheries and aquaculture production per year from FAO (2014).[1]

A considerable amount of food fish farming, 63% in 2012,[1] is now attributed to extensive and intensive freshwater inland aquaculture and also coastal brackish water ponds and shore-based mariculture. It is considered easy-to-establish aquaculture in developing countries. However, some technical barriers to trade, such as international standards and regional technical regulations in the importing countries aimed at protecting consumers from the presence of chemical residues and contaminants in traded seafood associated with intensive farming, may have significant impact on the efforts in these developing countries. For instance, disease problems have been reducing the farmed shrimp production and have forced farmers to introduce zootechnical practices and treatments to combat these diseases.

In contrast to the large therapeutic arsenal to fight against mammalian diseases, the use of pharmaceutical substances is rather limited in scope in fish and seafood farming, and it has always been basically limited to some anesthetic substances and to anti-infective and antimicrobial agents against parasitic and microbial diseases.[3,4] As a consequence, the unregulated use of dye chemicals from the family of the triphenylmethane dyes, malachite green (MG), a common commercial and inexpensive fabric dye, has developed and been used as a therapeutic multi-usage drug to globally reduce parasitic, microbial, and fungal diseases found in fish and seafood farming.[5] MG has, for instance, been used both prophylactically and in the treatment of fungal infections for fish and eggs for more than 80 years.[6] In the course of the 1980s, 1990s, and 2000s, many concerns were raised in regard to the toxicity of this substance, and different toxicological studies were carried out for MG and for some other similar dyes applied or potentially applied for their therapeutic qualities in fish farming. MG has now been banned in nearly all of the regions of the world, including North America and Europe,

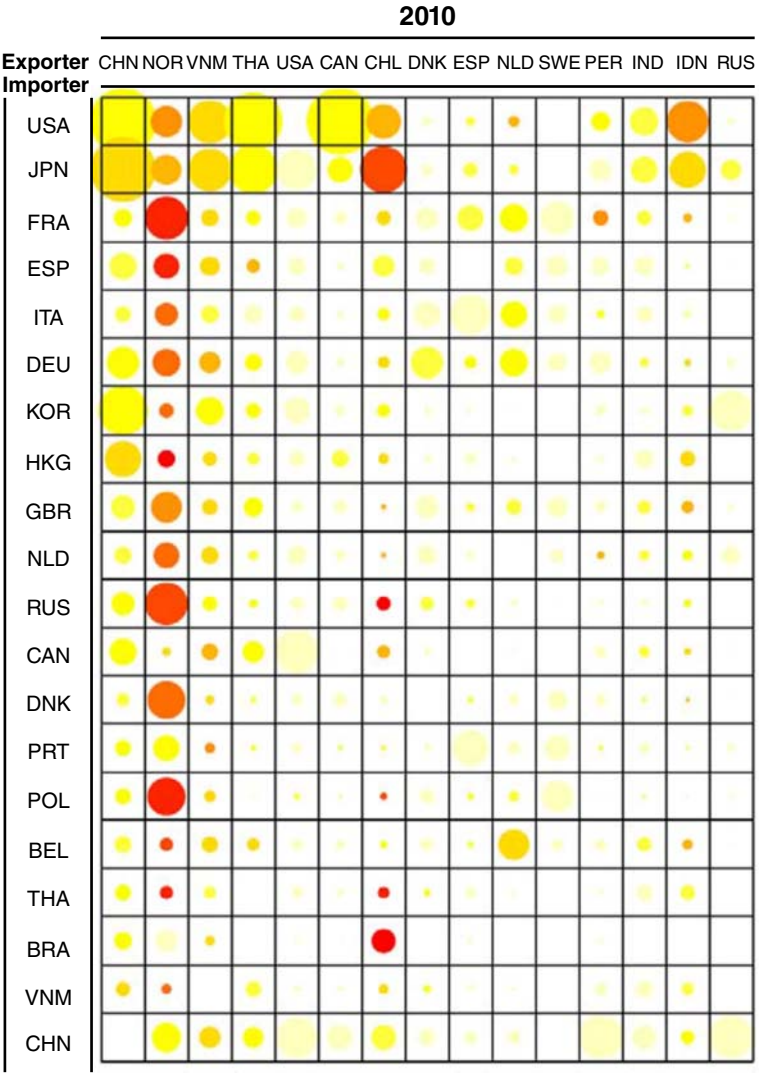


Figure 9.2 International seafood trade flows from the main importers and exporters by year and origin of production (aquaculture vs fisheries). The size of the circles is proportional to the value of the exports. The shading is based on the percentage represented by aquaculture of the total fish production in the exporting country in contrast to wild catch fisheries (10 equal intervals between 0% and 100%). The lighter the grey shading, the more aquaculture; the darker the grey shading, the more product is from wild catch fisheries. Source: Natale 2015.[2] Reproduced with permission from Elsevier. CHN, People's Republic of China; NOR, Norway; VNM, Vietnam; THA, Thailand; USA, United States of America; CAN, Canada; CHL, Chile; DNK, Denmark; ESP, Spain; NLD, Netherlands; SWE, Sweden; PER, Peru; IND, India; IDN, Indonesia; RUS, Russia; JPN, Japan; FRA, France; ITA, Italy; DEU, Germany; KOR, Southern Korea; HKG, Hong Kong; GBR, Great Britain; PRT, Portugal; POL, Poland; BEL, Belgium; BRA, Brazil.

but can still be present in various inappropriate fish farming practices around the world.

Recently, the Joint WHO/FAO Expert Committee on Food Additives (JECFA) has evaluated the risk for public health of the use of MG[7, 8] and crystal (gentian) violet (CV)[9, 10] in fish farming. The Codex Committee on Residues of Veterinary Drugs in Foods has recommended that competent authorities should not permit their use in food-producing animals including fish/seafood farming.[11, 12] This should therefore lead to an absence of detectable residues in products from this industry. However, they still appear to be present, probably because they are still widely used in the textile industry and elsewhere and are commercially available as inexpensive therapeutic chemicals for ornamental fish. In addition, the dyes are persistent in the sediment of water sources for aquaculture and will be absorbed and bioaccumulated in aquaculture tissues over time.[13] As a result of these assessments and recommendations, several countries and the EU since 2004 have assigned a specific food safety concern to these substances and mandated that they should be actively controlled in food products and food trading derived from the fish and seafood farming industry.

There have been trade issues associated with certain dye compounds used as veterinary medicines, particularly with MG and its chemically related congeners in aquaculture. This chapter is intended to review these pharmacologically active dyes from their chemistry and toxicological concerns to their regulatory monitoring in aquaculture products due to their undesirable presence in aquaculture-sourced foods.

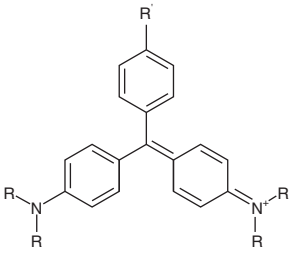
9.2 Therapeutic Applications and Chemistry of Certain Dyes Used in Fish Farming

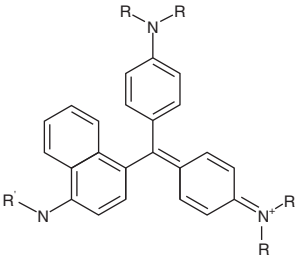
Dyes with pharmacological activity can be categorized into five chemical classes: triaryl(phenyl)methanes, phenothiazines, xanthenes, acridines, and azo compounds (Figure 9.3). In aquaculture, dyes are primarily used as a treatment for fungal and external parasite infections in fish and to protect incubating eggs from fungus. Many of the dyes described from these chemical classes have antiseptic, antimicrobial, or other medicinal properties with uses in veterinary and human medicine. Many also have unique affinities for binding to different cellular components rendering these therapeutic dyes excellent biological stains. Other dyes and pigment residues have been found in fish from environmental exposure to textile and manufacturing effluents[15] as well as from food additives intentionally added to color seafood products. For example, the carotenoid pigments canthaxanthin and astaxanthin are used as feed additives to redden the color of aquacultured salmon and trout flesh.[16] Though toxicity and safety concerns have led to restrictions and discontinuation of therapeutic dye treatments, the long history, efficacy, and ready availability of inexpensive dyes for infection control suggest that regulatory monitoring must continue.

9.2.1 Triarylmethanes

Triarylmethane dyes are cationic and have wide application as colorants for textiles, papers, plastics, and inks and are used as biological stains. These are characterized as the structurally simple triphenylmethane dyes and the more complex triphenylnaphthylmethane structures of the Victoria blue dyes, where one phenyl ring has been substituted with a naphthyl group (Figure 9.3). The triphenylmethane dyes have a long history of therapeutic use as fungicide and ectoparasiticide agents. Gentian violet was noted to have bactericidal properties in mammalian blood in 1913,[17] and it is effective as a human medicine for the treatment of fungal infections of candidiasis and thrush. In 1933, Foster and Woodbury[6] reported MG to be unusually effective for the treatment of

Triarylmethanes

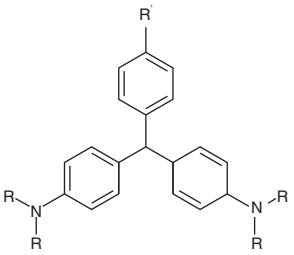




R = Me, R' = H	Malachite green
R = Et, R' = H	Brilliant green
R = Me, R' = NMe_2	Crystal violet
R = Et, R' = NEt_2	Ethyl violet
R = H, R' = NH_2	Pararosaniline

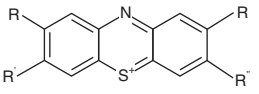
R = Me, R' = Ph	Victoria blue B
R = Me, R' = Et	Victoria blue R
R = Et, R' = Et	Victoria pure blue BO

Triarylmethane metabolites



R = Me, R' = H	Leucomalachite green
R = Me, R' = NMe_2	Leucocrystal violet

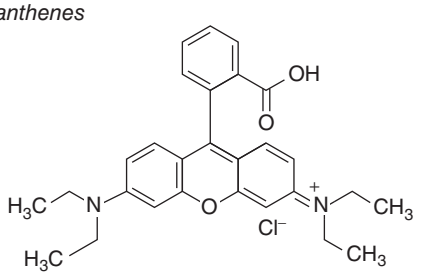
Phenothiazines



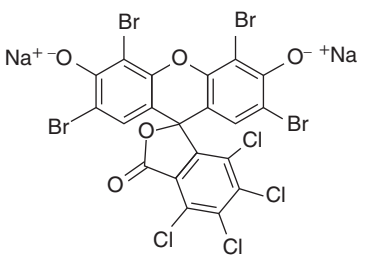
R = H, R', R' = NMe_2	Methylene blue
R = H, R' = NMe_2 R'' = NHMe,	Azure B

Figure 9.3 Structures of pharmacologically active dyes. Source: Tarbin 2008.[14] Reproduced with permission from Elsevier.

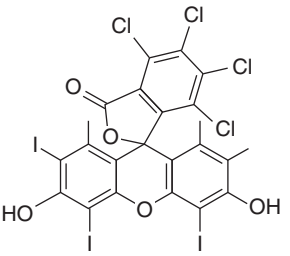
Xanthenes



Rhodamine B

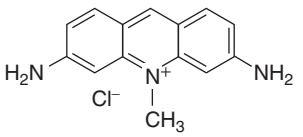


Phloxine B

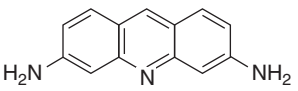


Rose Bengal

Acridines

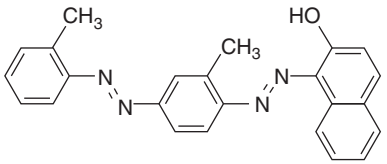


Acriflavine

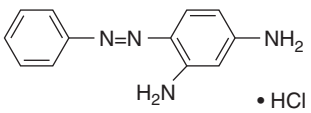


Proflavine

Azo Dyes



Sudan IV



Chrysoidine

Figure 9.3 (*Continued*)

fungus infections in trout, bass, and trout eggs. MG is considered to be the most effective antifungal treatment used in aquaculture.[18] Exposure bath treatments are effective for the control of the external protozoan *Ichthyophthirius multifiliis* in fish, and treatments of fish eggs with dilute MG effectively reduce fungal growth (e.g., *Saprolegnia*) and ensure viability of live eggs.[19] Other studies indicate additional members of the triphenylmethane class of compounds to

have similar antiseptic and antifungal properties. Alderman conducted *in vitro* studies of cultures of *Saprolegnia parasitica* against 11 triphenylmethane dyes and other compounds with antifungal properties.[20] Of the 40 compounds studied, the mercury-containing compound thimerosal and the triphenylmethane compounds MG, CV, and brilliant green (BG), monophenylrosaniline (Dahlia), and iodine green were the most effective. In a more recent study, the antifungal potency of MG, CV, BG, and methyl green was assessed against 36 different fungal strains and found to have comparable or greater activity when compared to antifungal reference standards.[21]

MG and metabolites are susceptible to oxidation/reduction and demethylation reactions in the presence of air and light. The MG cation has a pK_a of 6.9, and it slowly hydrolyzes to form an equilibrium mixture with the colorless carbinol base in aqueous solutions. Under acidic conditions (pH 3.5), only the cationic dye is present in solution. At pH conditions of 6.5, 7.0, and 9.0, after equilibration, the carbinol accounts for approximately 25%, 50%, and 100% of the material, respectively.[22] The less water-soluble carbinol has greater lipophilicity with higher potential than the cation to pass through cell walls.[23] After absorption, the compound is quickly metabolized to leucomalachite green (LMG). LMG is lipophilic and has a very long residence time in fatty muscle tissue. In a ^{14}C-labeled study of catfish treated by a 1-hour MG exposure bath, residues bioconcentrated in the catfish at higher concentrations than the exposure bath.[24] Immediately after exposure, LMG residue concentration was slightly higher than MG in muscle. After 14 days, MG had decreased to the method detection limit, while concentrations of LMG in muscle were more than 40 times higher. LMG was still quantifiable in muscle 42 days later. Demethylated metabolites of LMG were also identified in catfish muscle after treatment by MG exposure bath.[25] Metabolized LMG in fish muscle has been observed to oxidize back to MG when fish muscle is frozen.[26] The complex interconversions that these compounds undergo have led to a wealth of studies in the literature to better understand the chemistry of the triphenylmethanes in aquatic species.

CV and BG are other triphenylmethane dyes with similar properties to MG. CV is hexamethyl-*p*-rosaniline (Figure 9.3), whereas the similar dye product, gentian violet, is a mixture that is primarily composed of CV and also contains methyl violet, the pentamethyl-*p*-rosaniline compound. Leucocrystal violet (LCV) is the metabolic marker residue in fish after treatment bath exposure to CV. Thompson et al.[27] determined the concentration of CV and LCV residues in catfish muscle following the exposure to a 1-hour treatment bath of CV (100 µg/l). Catfish were then returned to a pond for withdrawal studies. One hour after exposure, a CV concentration of 0.5 µg/kg was determined, and residues of LCV in muscle were 12 µg/kg. CV concentration quickly dropped below the detection limit, while LCV was still present at a concentration of 3 µg/kg 79 days after the treatment bath. The predominance of the leuco metabolite was also noted after low-concentration exposure bath treatment (10 µg/l, 1 hour) of salmon and tilapia.[28] Chan et al. conducted depletion studies of CV and LCV in salmon.[29] One day after bath exposure

to CV, 98% of the residues were in the form of LCV, and this metabolite was detected in salmon as long as 91 days after exposure.

Data are more limited for the metabolism of BG, though this compound is also expected to metabolize to the leuco base in fish muscle. Leucobrilliant green (LBG) is readily oxidized to BG, limiting the stability of this compound and resulting in the lack of a commercially available standard. Andersen et al. fortified catfish muscle with LBG and found it to oxidize to BG during the extraction process.[30] Hurtaud-Pessel et al. identified both BG and LBG residues in samples of trout treated in a BG bath.[31] Immediately after bath exposure, BG and LBG residues were in equal proportion in the trout muscle. Two hours after exposure, the LBG residue concentration in muscle was two-thirds of the concentration of the BG residues. In another study from Schneider et al.,[32] LBG was not identified in incurred samples of salmon, catfish, and tilapia that had been exposed to a low-concentration bath (10 μg/l) of BG. These studies indicate that the parent dye is an acceptable marker residue to identify BG treatment, while regulatory testing for MG and CV must include the contribution of the leuco forms, which have greater stability and very long residence time in fish muscle.

The triarylmethane dyes Victoria blue B and Victoria pure blue BO were recently detected at low concentrations in one or two samples of wild freshwater eel, thought to be the result of dye effluents from textile plants.[15] Victoria pure blue BO residue was found in a sample of white fish as reported in the 2010 annual report of the European Rapid Alert System for Food and Feed (RASFF).[33]

9.2.2 Phenothiazines

Methylene blue (MB) is in the phenothiazine dye class of dyes. As the first synthetic drug, it has a long history and numerous applications for human and animal medical use. MB has been used in ruminant animals as an antidote against nitrate and cyanide poisoning.[34] In human medicine, it has been used to treat malaria, depression, and methemoglobinemia and is under current investigation to slow neurodegenerative disease.[35] In aquaculture, MB is effective as an antiseptic and disinfectant, with similar indications for use as MG against *I. multifiliis* and to protect fish eggs from fungal infestation, though with lower efficacy than MG.

Several studies noted that the uptake of MB residues into fish muscle was much lower than residues of triphenylmethane dyes under similar exposure conditions.[36, 37] In studies of catfish subjected to MB treatment baths, fish were exposed to 1 or 5 mg/l of MB for 1 hour. The average concentration of MB found in the muscle of these catfish was 10 μg/kg or less for the lower exposure group and 16 μg/kg for the higher exposure.[37] Like the triphenylmethane dyes, MB is expected to quickly metabolize to a colorless leuco form, though it may not be possible to stabilize and isolate the leuco form from the muscle.[38] Turnipseed et al. documented the instability of this compound in studies of incurred catfish

muscle, noting that leucomethylene blue (LMB) readily oxidizes back to MB.[37] In the metabolic process, MB may also lose one, two, or three methyl groups to form the demethylated azure dye metabolites or fully demethylate to thionine. Thionine was reported to be a protein-bound conjugate with a long residence time in milk from treated dairy cows.[34]

9.2.3 Xanthenes

Xanthene dyes consist of compounds such as fluorescein, rhodamine, and eosin. Compounds from this class are commonly used as fluorescent biological stains and as laser dyes. Rhodamine compounds and fluorescein have been used in tracer studies to monitor the flow of water in rivers and aquatic systems.[39] For example, these dyes were added to pesticide formulations used in sea lice treatment to follow the dispersion of pesticides to surrounding environmental waters.[40, 41] Some dyes from this class have bactericidal, insecticidal, or fungicidal properties.[42] Rhodamine B and the halogenated derivatives Rose Bengal and phloxine B showed antifungal action against *S. parasitica* in culture studies by Alderman.[20] Some dyes from this class act as photosensitizing insecticides. Xanthenes have been formulated for uptake by insects, where they are photoactivated by sunlight to form cytotoxic singlet oxygen and other reactive species.[43] The halogenated eosins (e.g., Rose Bengal, erythrosine, etc.) are effective in this regard. Phloxine B has been commercially developed as a photosensitizing insecticide used to control fruit flies in animal feed. Blair proposed the use of phloxine B to treat the protozoan infection *I. multifiliis* in fish.[44] In this application, phloxine B would be added to an aquaculture pond at night, absorbed by protozoa, and then activated by sunlight to generate free radical species to kill the protozoans. In another study, singlet oxygen produced from the irradiation of Rose Bengal was found to be effective against the virus responsible for white spot syndrome in *kuruma* shrimp populations.[45] Though there may be potential for xanthene residues to be present in seafood either by aquaculture or pesticide use or by the use of these compounds as color additives, reports of their identification in regulated products were not found.

9.2.4 Acridines

Acridine dyes were originally isolated from coal tars and were introduced as an antiseptic in 1912. Acridine dyes such as acriflavine, proflavine, and quinacrine have antiseptic properties with medicinal uses to treat malaria, sleeping sickness, and giardiasis.[46] Reported uses in veterinary medicine are the treatment of mastitis, urinary or enterobacterial infections, and parasite infections.[34] Though not as effective as MG, acriflavine is prescribed for use as a mixture with proflavine to treat external fungal infection in aquarium fish and to disinfect fish eggs.[47] Plakas et al.[47, 48] found acriflavine and proflavine to be poorly absorbed into the muscle

of catfish after bath treatment; Yu et al.[49] found similar results for trout. Glucuronosyl and acetyl conjugates were identified as the metabolites of proflavine in trout and catfish, yet the parent compounds were the primary residues present in muscle. The elimination half-life for catfish muscle was 1.5 days for proflavine and 5.3 days for acriflavine.[47] Residue concentrations in the skin remained largely unchanged 14 days after exposure bath treatment.

9.2.5 Azo Dyes

While many azo dyes are regulated in foods as illegal color additives (e.g., Sudan dyes), azo dyes such as Sudan IV (scarlet red) and Congo red are active against Gram-negative bacteria.[34] The azo dye chrysoidine was isolated in 1914 and found to have high bactericidal activity.[50] Chrysoidine was reportedly used to color lower-quality fish to look like more expensive yellowfin tuna.[51] Reyns et al. reported that chrysoidine has also been used illegally to disinfect fish skin and residues of this compound should be monitored to detect abuse.[52]

9.3 Toxicological Issues

The pharmacologically active dyes considered in this chapter are prohibited from use in food-producing animals due to their toxicity and potential to cause changes in genetic material. A number of studies have been conducted over several decades to classify the effects of these compounds on aquatic and mammalian species. Not every compound has been studied in depth, but similarities within the structures may be used as the basis to predict similar toxicological effects. In some cases, individual study results have been summarized in larger risk assessment (RA) evaluations. Comprehensive toxicological studies and summaries by the International Agency for Research on Cancer (IARC), the US National Toxicology Program (NTP), the JECFA, the European Food Safety Authority (EFSA), and the European Medicines Agency (EMA) are briefly summarized in the following text for specific classes of dyes.

9.3.1 Triarylmethanes

The health effect of MG has been studied extensively, with comprehensive toxicology review articles published,[53, 54] several major animal studies, and recent evaluations by international consortia. The toxicology and carcinogenicity of MG and LMG were investigated by the NTP and summarized in two technical reports.[55, 56] *In vitro* studies did not show either compound to be mutagenic.[55] However, 2-year feeding studies with rats and mice showed that MG caused an increase in tumor formation in female rats and that LMG was more potent, causing an increase in cancer in all rats and female mice[56, 57]. These results were consistent with other studies, where tumors were observed in *in vivo*

studies,[58] but *in vitro* assays with bacterial and human cell lines showed MG to be cytotoxic, whereas LMG did not cause mutations.[59, 60] *In vitro* studies indicated that mammalian and human intestinal microflora efficiently convert MG to LMG.[61] In the livers of treated rats, additional demethylated and N-oxide metabolic products were observed, indicating that *in vivo* enzyme activation may be necessary for more severe genotoxic or mutagenic effects.[62]

CV toxicology has also been reviewed.[63] Littlefield studied mice exposed to CV and determined a no-observed-effect exposure level that would prevent formation of liver tumors.[64] Safe doses were indicated to be 1–2 µg/kg. Like MG, human and mammalian intestinal microflora reduced CV to LCV in *in vitro* studies.[65] Genotoxic and mutagenic effects have been observed for other triarylmethane dyes as well. *In vitro* assays of BG, methyl violet, and Victoria blue indicated mutagenicity with fungal yeast cells.[66] Pararosaniline and other triphenylmethane compounds comprising magenta dye have been designated class 1 carcinogens by the IARC.[67]

Trout eggs and pregnant rabbits exposed to MG yielded significant abnormalities to the developing offspring.[68] Teratogenicity studies have been conducted for CV as well.[69] In fish, lethal concentration (LC_{50}) values have been determined for MG in different fish and range from 0.5 to 5.6 mg/l.[70, 71] For CV, LC_{50} was 0.2 mg/l.[71]

More recently, the JECFA evaluated the risk of using MG and CV in fish farming on public health.[8, 10] After reviewing studies on the genotoxic effects of these dyes and metabolites, the committee did not support permitting MG or CV use in food-producing animals and decided it inappropriate to establish acceptable daily intake (ADI) values for these compounds. Full toxicological evaluations on these compounds were published recently by the WHO.[7, 9]

9.3.2 Phenothiazines

In NTP studies,[72] MB trihydrate was found to be genotoxic in bacterial assays and to produce some evidence of carcinogenesis in male rats and mice. Anemia and a decreased ability of blood to bind oxygen (methemoglobinemia) were also observed in high-dose groups of rats and mice during the 2-year study. Reproductive toxicological effects have been noted as well.[73] The IARC provided a thorough summary of MB information and toxicological studies in the 2015 *Monograph.*[74] DNA damage from singlet oxygen or free radicals was observed when MB use was combined with white light photoactivation, but genotoxic effects have not been described for *in vivo* studies without photoactivation.[74] MB was designated as class 3, or not classifiable for carcinogenicity in humans[74]. The azure dye metabolites of MB were found to be mutagenic in bacterial assays.[72]

In a study of direct toxicity to fish, the 24-hour LC_{50} for MB fish exposure was 25 times higher than the more toxic MG (18 vs 0.6 mg/l).[71] MB has been studied extensively for use in human medicine. With human oral and intravenous

dosing at much higher than residue concentrations, some toxicity has been noted, particularly with respect to adverse effects in the blood.[35, 74] The EMA published a report on the safety of MB for use as a human drug to reverse methemoglobinemia from drug and chemical poisonings.[73]

9.3.3 Xanthenes

The toxicity of rhodamine dyes has been studied by the IARC and the NTP. The IARC[75, 76] reported that rhodamine B and 6G were carcinogenic to rats in subcutaneous exposure studies. The NTP[77] prepared a technical report based on rhodamine 6G feeding studies, where equivocal evidence of carcinogenicity was found in rats, but no evidence was found for mice. EFSA[78] concluded that rhodamine B is potentially genotoxic and carcinogenic. Rowiński and Chrzanowski[79] summarized differences in toxicity between two xanthene dyes used as aquatic tracers – rhodamine B and rhodamine WT – where the latter was designed to have lower biological adsorption and lower toxicity. In fairy shrimp, the 24-hour lethal concentration (LC_{50}) of rhodamine WT was approximately 200 times higher than for rhodamine B.

Phloxine B (D&C Red No. 28) has been approved in the USA as safe to use as a color additive for some cosmetic products and drugs.[80] Due to the potential of this and other halogenated fluorescein dyes (e.g., Rose Bengal) to form reactive oxygen species after the dyes are activated with light, additional toxicology evaluations have been performed to investigate genotoxicity after light exposure.[81] DNA damage has been reported for bacteria and human skin cell exposure to phloxine B and light from a fluorescent bulb.[81] Redness and swelling were observed after Rose Bengal application to damaged skin with exposure to visible light and sunlight.[81]

Toxicological effects in fish by xanthene dyes were described by Tonogai et al.[71] The LC_{50} for rhodamine B was 25 times higher than the more toxic MG (17 vs 0.6 mg/l), but rhodamine B had a much higher octanol–water partition coefficient suggesting better efficiency for permeating cell membranes ($K_{ow} = 74$ vs 5.6). Halogenated xanthene dyes were also evaluated in this study of Himedaka fish. LC_{50} values (24 hour) were 130, 280, 710, and 1000 mg/l for Rose Bengal, phloxine B, erythrosine, and eosin, respectively.[71]

9.3.4 Acridines

Available information on the acriflavine–proflavine mixture acriflavinium chloride was reviewed by the IARC[82] in 1977, though at the time there was not enough toxicological data available to draw conclusions about carcinogenicity. Proflavine salts were evaluated in 1980 and were observed to be genotoxic in viral and bacterial assay.[83] These planar compounds can intercalate between DNA base pairs and cause frame shift and other types of mutations.[84]

9.3.5 Azo Dyes

The IARC has reported on the carcinogenicity of several Sudan and azo dyes.[85] Sudan I was determined to be carcinogenic based on oral dosing studies in rats and genotoxic in *in vitro* studies.[86] By their structural similarity to Sudan I, other Sudan dyes are considered to be potentially genotoxic and carcinogenic.[78] Potentially carcinogenic aromatic amine metabolites are formed from the Sudan dyes when the azo bond is reduced by human intestinal microflora and liver enzymes.[87]

Chrysoidine was found to have high acute toxicity to fish with a 24-hour LC_{50} of 0.5 mg/l and was predicted to easily permeate gills based on a high octanol–water partition coefficient.[71] Bladder cancer in humans has been reported after long-term exposure to chrysoidine, though insufficient data are available to classify chrysoidine as a carcinogen (IARC class 3).[88] This dye was reported to be mutagenic to bacteria and to produce tumors and leukemia in mice.[88]

9.4 Regulatory Issues

To prevent the risk for human consumers from unexpected amounts of toxic chemicals possibly found in traded aquaculture products, a significant number of countries across the world have introduced regulations into their "food safety" laws. Toxicologically based limits called maximum residue limits (MRLs) have been set for approved drugs in seafood as well as in other food products from animal origin.[89–91] These MRLs are based on ADIs established after human food safety RAs.[92, 93]

Internationally, the WHO and the FAO have also derived such risk management (RM) recommendations (MRLs) acknowledged through the Codex Alimentarius General Standard for Food Additives (GSFA)[94] and posted in the GSFA database: http://www.fao.org/fao-who-codexalimentarius/standards/gsfa/en/. Over a period of more than 50 years, these internationally recognized MRLs have been derived for a certain number of food additives. This includes veterinary drug chemicals as a follow-up of the human food safety RAs and operated under the auspices of the WHO and FAO by means of the Codex Committee on Residues of Veterinary Drugs in Foods, acting as the risk manager, based on RAs prepared by an independent scientific committee, the JECFA.[95]

Regionally, many countries have aligned their food safety laws with the RA and RM recommendations of the internationally recognized WHO/FAO. This is the case for a majority of Asian, African, and Latin American countries. Moreover, a few countries, in cooperation with the WHO/FAO, have also implemented their own process of RA and RM by means of funding their own national Food Safety Agencies and collaborating with their government departments responsible for public health, agriculture, and fisheries.

For instance, in the USA, the Food and Drug Administration (FDA) is the regulatory body having the mandate for both RA and RM issues for veterinary drug use in seafood.[92, 96] For Canada, according to the *Food and Drug Act*, Health Canada through its Health Products and Food Branch (HPFB) is the administration concerned with both RA and RM for all food safety issues.[97, 98] In the EU, according to the General Food Law Regulation (EC) No. 178/2002, it is the Directorate-General of the European Commission for Health and Food Safety (DG-SANTE) that is in charge of the RM issues in coordination with the 28 EU Member States' regulatory competent authorities.[99, 100] In addition, the EMA[101, 102] and the EFSA[103, 104] are the two EU regulatory bodies in charge of the RA issues for residues of human and veterinary medicinal products and for all the other chemical residues and contaminants, respectively.

For Japan, the Pharmaceutical and Food Safety Bureau of the Ministry of Health, Labour and Welfare (MHLW) is the regulatory body in charge of both RM and RA issues.[105, 106] Since 1991, in Australia and New Zealand, there has been a bi-national food safety agency called the Food Standards Australia New Zealand (FSANZ) administration in charge of the joint Food Standards Code,[107, 108] which lists requirements for foods such as additives, food safety, labeling, and genetically modified foods. They share with the Australian Pesticides and Veterinary Medicines Authority (APVMA) the responsibilities for setting MRLs. All the RM issues in terms of enforcement and interpretation of the Code are the responsibility of the state and territory departments and food agencies within Australia and New Zealand.

For the Russian Federation, to enforce the federal laws on the quality and safety of food products and the sanitary and epidemiologic rules and regulations (SanPiN), the Federal Service for Surveillance on Consumer Rights Protection and Human Well-Being (Rospotrebnadzor)[109, 110] is the federal executive authority in charge of the RAs and other activities linked to the implementation of control and supervision in the sphere of sanitary and epidemiological well-being of the population. The Federal Service for Veterinary and Phytosanitary Surveillance (Rosselkhoznadzor)[111, 112] under the Ministry of Agriculture (MoA) is the federal organization of executive power, carrying out RM functions on control and supervision in the field of veterinary science including aquatic biological resources.

For China, the MoA[113, 114], the National Health and Family Planning Commission (NHFPC),[115] the General Administration of Quality Supervision, Inspection and Quarantine (AQSIQ),[116, 117] the State Food and Drug Administration (SFDA),[118] and the Commerce Department share the responsibilities for the food safety RM. However, the RA issues have been covered by the National Center for Food Safety Risk Assessment (CFSA)[119] since 2011.

When specifically looking at seafood safety and considering the veterinary drugs approved in aquaculture in the various regions of the world, it is obvious there are very few of these veterinary chemicals that have been effectively addressed with an RA to finally receive an official authorization with an MRL and consequently a registered use as a veterinary medicine treatment in aquaculture.

When the drug has not been approved after its RA or if a drug has not been assigned an MRL or ADI, then the substance is considered not safe at any concentration for humans and is prohibited from use in animal production. There is a "zero tolerance" concern for prohibited veterinary drugs in seafood, where "zero" is at or near the limit of detection of the analytical equipment in place for the official control. When referring to the specific internationally recognized RAs addressing the two triphenylmethane chemical products, MG[8] and CV,[10] these two substances have entered the group of non-authorized compounds to be avoided in food-producing aquaculture. The national/international regulations in place for these two pharmacologically active but undesirable substances in seafood are described in Table 9.1. Currently, the analytical - "zero tolerance" concentration in national seafood inspection programs for these two substances and for their respective leucobase metabolites ranges from 1 to 2 μg/kg, depending on the food safety RM enforced in the country of interest. Apart from these two substances, there is no other dye of concern in most of the official monitoring programs even though all are also considered undesirable. Most of the regulations across the world state that non-fully authorized drugs are thus prohibited for use in food-producing animals. However, recently the interest in other potential pharmacologically active dyes is starting to be addressed by several reference laboratories worldwide with the development of analytical methods for controlling other dye residues in seafood.[14] In the early 2010s, for instance, the competent authorities of a few Member States of the EU and the USFDA have started introducing analytical procedures capable of monitoring BG, Victoria blue, or MB in combination with MG and CV monitoring programs. In the EU, a new RA from EFSA is pending[120] for a set of aquaculture dyes with the objective of reconsidering the need to enforce new toxicologically based regulatory limits of action called Reference Point for Action (RPA). Also under consideration is an RM issue to generalize expanding the official monitoring for the presence of other dyes such as CV and BG at least.

9.5 Analytical Methods for Residue Control

Analytical methods to determine the presence of illegal pharmacological dyes in edible seafood products must meet a number of requirements for regulatory food control. Methods must be sensitive enough to permit residue detection at regulatory performance limits. Methods must be selective enough to provide adequate isolation of the dye residues from the complex and fatty fish matrix. Finally, methods must permit analysis of the correct metabolic marker for these dyes. Quantitative determination of residue concentration and the ability to confirm the identity of detected residues are important features of successful regulatory analysis, though these features are typically defined within the intended scope of the method, be it designed for rapid screening of many samples, accurate concentration determination, or identification with mass spectrometry.

Table 9.1 Overview of specific regulations for dyes in aquaculture products by countries.

Specific regulations for dyes in food products worldwide						
Country/institution		**Malachite green**	**Leucomalachite green**	**Crystal (gentian) violet**	**Leucocrystal (gentian) violet**	**Brilliant green**	**Leucobrilliant green**	**Other dyes potentially regulated: methylene blue, Victoria blue, etc.**
Codex Alimentarius (WHO/FAO)	RM[a)]	CAC-MRL-RMR 2-2015		CAC-RMR 3-2016 pending		Currently none		None
	RA[b)]	JECFA evaluation: 70th meeting (2008) JECFA		JECFA evaluation: 79th meeting (2015) JECFA				
	RVC[c)]	Malachite green not to be used in food-producing animals		Gentian violet not to be used in food-producing animals				
European Union (DG-SANTE; EMA; EFSA)	RM	Prohibited substance under Decision 2004/25/EC		Non-authorized substance in line with Decision 2004/25/EC[c)]		Non-authorized substance in line with Decision 2004/25/EC[d)]		None
	RA	New EFSA RA pending for RPA[d)]		New EFSA RA pending for RPA[d)]		New EFSA RA pending for RPA[e)]		
	RVC	Policy of "zero tolerance" with MRPL: 2 µg/kg		Policy of "zero tolerance"; detection capability recommended by EU CRLs: 2 µg/kg[f)]		Policy of "zero tolerance"; detection capability recommended by EU CRLs: 2 µg/kg[f)]		
United Kingdom (DEFRA; FSA)	RM	Prohibited substance under Decision 2004/25/EC		Non-authorized substance in line with Decision 2004/25/EC[d)]		Non-authorized substance in line with Decision 2004/25/EC [d)]		None
	RA	New EFSA RA pending for RPA[e)]		New EFSA RA pending for RPA[e)]		New EFSA RA pending for RPA[e)]		

	RVC	Policy of "zero tolerance" with MRPL: 2 µg/kg	Policy of "zero tolerance" with action limit: 0.5 µg/kg	Policy of "zero tolerance" with action limit: 0.5 µg/kg	
USA (USFDA)	RM	USFDA FFDCA Section 512 (21 U.S.C. 360b)	USFDA FFDCA Section 512 (21 U.S.C. 360b)	USFDA FFDCA Section 512 (21 U.S.C. 360b)	USFDA FFDCA Section 512 (21 U.S.C. 360b)
	RA	USFDA	USFDA	USFDA	USFDA
	RVC	Policy of "zero tolerance": detection capability required: 1 µg/kg	Policy of "zero tolerance": detection capability required: 1 µg/kg	Policy of "zero tolerance": detection capability required: 1 µg/kg	Policy of "zero tolerance" for any dye
Canada (Health Canada, CFIA, Env Can, DFO)	RM	Canadian Shellfish Sanitation Program (CSSP)	CSSP		None
	RA	Health Canada	Health Canada		
	RVC	Policy of "zero tolerance": 1 µg/kg	Policy of "zero tolerance": 1 µg/kg		
Japan (MHLW)	RM	Food Sanitation Act – Article 11 MHLW Notification No. 645, 2006	Food Sanitation Act – Article 11 MHLW Notification No. 645, 2006		None
	RA	MLHW	MLHW		
	RVC	Policy of "zero tolerance": 2 µg/kg	Policy of "zero tolerance": 2 µg/kg		
Australia–New Zealand (FSANZ)	RM	FSANZ Standards Code	FSANZ Standards Code – Update July 2014		None
	RA	FSANZ 2005	FSANZ 1994		

(*continued*)

Table 9.1 (Continued)

Specific regulations for dyes in food products worldwide								
Country/institution		**Malachite green**	**Leucomalachite green**	**Crystal (gentian) violet**	**Leucocrystal (gentian) violet**	**Brilliant green**	**Leucobrilliant green**	**Other dyes potentially regulated: methylene blue, Victoria blue, etc.**
	RVC	Policy of "zero tolerance": 0 µg/kg		Policy of "zero tolerance": 0 µg/kg				
Russian Federation (Rospotrebnadzor; Rosselkhoznadzor)	RM	SanPiN 2.3.2.1078-01		SanPiN 2.3.2.1078-0				None
	RA	Rospotrebnadzor		Rospotrebnadzor				
	RVC	No value announced		No value announced				
China Mainland (MoA/AQSIQ)	RM	China Food Additive Regulation – Banned since 2002 under Export Oriented Scheme according to exported countries' legislations						
	RA	Refer to JECFA						
	RVC	Policy of "zero tolerance": 0 µg/kg						
Hong Kong (CFS)	RM	Part V of the Public Health and Municipal Services Ordinance (Cap. 132) – Harmful Substances in Food Regulations – Regulation 3A						None

	RA	Refer to JECFA		
	RVC	Policy of "zero tolerance": 0 µg/kg		
India (MPEDA/EIC)	RM	According to exported countries' legislations such as EU Directive 96/23/EC and Decision 2004/25/EC		None
	RA	Refer to JECFA		
	RVC	Policy of "zero tolerance": 2 µg/kg		
Bangladesh (MOFL/DOF)	RM	According to EU Directive 96/23/EC		None
	RA	Refer to JECFA		
	RVC			
Brazil	RM	Normative Instruction SDA No. 13, (July, 15) 2015	Normative Instruction SDA No. 13, (July, 15) 2015	None
	RA	Refer to JECFA	Refer to JECFA	
	RVC	Policy of "zero tolerance": 2 µg/kg	Policy of "zero tolerance": 2 µg/kg	

(*continued*)

Table 9.1 (Continued)

Specific regulations for dyes in food products worldwide								
Country/institution		**Malachite green**	**Leucomalachite green**	**Crystal (gentian) violet**	**Leucocrystal (gentian) violet**	**Brilliant green**	**Leucobrilliant green**	**Other dyes potentially regulated: methylene blue, Victoria blue, etc.**
Chile (Sernapesca)	RM	SENASA instructions		SENASA instructions				
	RA	Refer to JECFA		Refer to JECFA				
	RVC	Policy of "zero tolerance" since 1997		Policy of "zero tolerance"				
Argentina (SENASA)	RM	SENASA instructions						None
	RA	Refer to JECFA						
	RVC							
Costa Rica (SENASA)	RM	La Gaceta n[c)]160 - 2008						None
	RA	Refer to JECFA						
	RVC	Policy of "zero tolerance": 2 µg/kg						

a) RM: risk management.
b) RA: risk assessment.
c) RVC: recommended value for control purposes.
d) RM and value recommended in line with Decision 2004/25/EC.
e) RPA: reference point for action.
f) A risk assessment (RA) pending by EFSA.

9.5.1 Procedures to Extract and Analyze Triphenylmethane Dye Residues in Fish and Shellfish Muscle

In 1983, Poe and Wilson[26] reported that frozen muscle from fish previously treated in an MG bath would develop a green surface color on the muscle tissue. Prior to this, it was believed that MG was not absorbed by fish muscle. These authors performed the first muscle extraction using methanol and chloroform with separation of the green color from lipids on a silica column. The green extract was analyzed by infrared and absorbance spectroscopy and matched the spectra of MG standards.[26] This was the beginning of many studies to understand tissue uptake, metabolism, and elimination of dye residues from fish muscle. Many analysis methods for fish were developed in the late 1980s and 1990s for separation of residues by HPLC and visible absorbance detection of the intensely colored dyes. The green-blue MG and BG absorb strongly at 618 and 627 nm, respectively, while purple CV absorbs at 588 nm; all wavelengths are far from many interfering compounds. Early extraction methods were based on solvent extraction under acidic conditions to ensure that the dye–carbinol equilibrium would be shifted to the dye form[121–124]. Later methods incorporated procedures to detect the residue contribution of the primary leuco metabolites.

Bauer et al.[125] introduced a procedure in 1988 to oxidize half of a trout extract with lead oxide, sequentially analyze both portions by HPLC-VIS, and then determine the contribution of LMG by difference. Addition of lead oxide to acetonitrile–perchloric acid extracts was also used by Dafflon et al.[126] Roybal and Munns[127] developed a chromatographic analysis for simultaneous determination of CV, LCV, demethylated metabolites, and MB with electrochemical detection rather than by absorbance measurement. This technique was applied to analyze chicken muscle with acetate buffer (pH 4.5) and acetonitrile extraction, liquid partitioning into dichloromethane, and subsequent solid-phase clean-up using alumina and carboxylic acid (CBA) weak cation exchange extraction cartridges.[128] Allen and Meinertz[129] demonstrated the feasibility of introducing a post-separation reaction column based on lead oxide oxidation to permit simultaneous HPLC-VIS analysis of MG, LMG, CV, and LCV. The PbO_2 post-column oxidation column formed the basis of dye and leuco analysis by HPLC-VIS for the next 15 years, with a variety of procedures for dye and leucobase extraction with acid or acidic buffer and organic solvent. Fink and Auch[130] demonstrated the success of the PbO_2 column to analyze MG, CV, BG, and leuco compounds in trout extracts. Allen et al.[131] mixed ground trout muscle, fry, and eggs with anhydrous sodium sulfate, prepared a matrix solid-phase desorption column, and extracted MG and LMG from the column with 1% acetic acid and methanol. The extract was cleaned up by partitioning into chloroform. Hajee and Haagsma[132] extracted LMG and MG from eel plasma with methanol, citrate buffer (pH 3), and ascorbic acid followed by SPE with sulfonic acid cartridges.

In 1995, Roybal et al.[133] developed a method for LMG and MG in catfish similar to the researcher's earlier electrochemical method for CV residues with a few

additions. Hydroxylamine hydrochloride (HAH) was introduced to the acetate buffer–acetonitrile extraction solution to prevent conversion of MG to LMG in the presence of fish enzymes. *para*-Toluenesulfonic acid (*p*-TSA) was included to serve as a counterion for the cationic MG, and alumina was dispersed into the extraction mixture to adsorb fat from the extract. Residue isolation was achieved with liquid-phase partitioning into dichloromethane and SPE with alumina and propylsulfonic acid cartridges. This procedure was used for pharmacokinetic and metabolism studies of LMG and MG in catfish,[24, 134] for CV and LCV residue determination in catfish[135], and also for a combined determination of MG, LMG, CV, and LCV in catfish and trout,[136] forming the basis of many later methods. For example, confirmatory analyses of dye and leuco compounds in fish were developed using particle beam LC-MS,[137] GC-MS,[138] and isotope dilution LC-MS[25, 139] to permit selected ion monitoring of molecular and fragment ions.

In another analytical approach to distinguish dye and leuco contributions, extracts were separated by HPLC with column effluent flowing through an electrochemical cell, diode array detection cell, and fluorescence cell.[140] In this procedure, MG and CV were detected by visible absorbance at 588 nm ($\lambda_{max} = 618$ and 588 nm, respectively), while LMG and LCV were detected by fluorescence emission at 360 nm with excitation at 265 nm. To confirm the identity of the residues, two injections of each extract were made – one with the electrochemical cell off to yield the expected absorbance and fluorescence signals and the subsequent injection with the electrochemical cell on to oxidize the leuco compounds to dyes. In the latter case, the fluorescence signal at the leuco retention time would drop to baseline, and the absorbance signal at the leuco retention time would increase. Similar analysis procedures were used by Mitrowska et al.[141] for simultaneous determination of MG and LMG by HPLC-VIS/FL without lead oxide oxidation and by Halme et al.[142, 143] for LC-MS/MS analysis with and without post-column oxidation.

In 2005, the Roybal extraction was simplified, and an *in situ* oxidation procedure was incorporated into the extraction procedure to convert leucobase to dye with the addition of 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (DDQ).[144] This permitted sensitive analysis of the sum of MG and LMG in a variety of seafood products with HPLC-VIS and quantification and confirmation of residue identity by LC-MS^n with no-discharge atmospheric pressure ionization at and below concentrations of 1 µg/kg for complete regulatory monitoring.[145, 146] The method was later extended to include CV, LCV, and BG residues[30] and adapted for other analytical procedures including LC-MS/MS analysis.[147]

Though extract clean-up procedures for triphenylmethane compounds often include similar procedures based on acid/organic solvent extraction with partitioning into dichloromethane and cation exchange SPE cartridge clean-up, many variations exist. Tarbin et al.[148] developed procedures to extract trout with citrate buffer (pH 4), sodium chloride, and acetonitrile. Analysis was by HPLC-VIS and electrospray ionization LC-MS, both following post-column oxidation with lead oxide. Bergwerff et al.[149] extracted trout with

McIlvaine buffer (pH 3, citric acid/disodium hydrogen phosphate buffer), *p*-TSA, *N*,*N*,*N*′,*N*′-tetramethyl-1,4-phenylenediamine dihydrochloride (TMPD), and acetonitrile for LC-MS/MS analysis with electrospray ionization and PbO_2 post-column oxidation. TMPD was used in place of HAH in this procedure to stabilize the dye compounds and prevent demethylation. Though post-column oxidation is not required for analysis by mass spectrometry, these authors noted improved sensitivity and reproducibility by converting leuco residues to the cationic dye compounds. Similar extraction procedures were applied to residue analysis methods by LC-MS/MS without post-column oxidation[150] and for HPLC-VIS/FL analysis.[151]

Several methods have been described for triphenylmethane compound analysis with a simpler extraction procedure using only McIlvaine buffer (pH 3) and acetonitrile extraction followed by cation exchange SPE clean-up for direct LC-MS/MS of dye and leuco compounds.[152, 153] These procedures did not include stabilizing compounds (i.e., HAH, TMPD, *p*-TSA) and eliminated the dichloromethane partitioning as well. Storey et al.[154] developed a procedure to extract fish with McIlvaine buffer (pH 4.5), EDTA, *p*-TSA, and TMPD for an LC-MS/MS residue screening method without additional liquid- or solid-phase clean-up. Van de Riet et al.[155] developed an extraction procedure to permit sensitive LC-MS/MS determination based on a simple tissue extraction using acetonitrile and perchloric acid, with dichloromethane and SPE clean-up.

Simple QuEChERS extractions have also been developed for triphenylmethane dye determinations as well. For example, regulatory methods were developed for MG and LMG residues in salmon and shrimp using acetic acid-modified acetonitrile for extraction and LC-TOF-MS for analysis. In the first case,[156] sodium chloride assisted the extraction from salmon and the extract was cleaned up with dispersive Bondesil-NH_2 sorbent. In the second,[157] anhydrous magnesium sulfate and sodium chloride were added to the shrimp extract, and the acetonitrile supernatant was cleaned up with dispersive PSA sorbent and additional magnesium sulfate. In another procedure,[158] fish was extracted with water, acetonitrile, and formic acid, while phase separation was assisted with anhydrous sodium sulfate and sodium acetate. A portion of the supernatant was collected and filtered for analysis by UHPLC-MS/MS. Extraction methods combined with LC-MS analysis have been recently reviewed in detail.[159]

Several of the early extraction methods[123] included overnight procedures, noting higher dye extraction yields from incurred tissues when overnight extraction was used. Hall et al.[160] studied the equilibrium for extraction of LMG and MG from incurred salmon muscle using acetonitrile and acetate buffer (pH 4.5). While LMG was quantitatively extracted by the first time point (1 hour), MG required approximately 16 hours reaching an equilibrium concentration in the extraction solvent. This group also studied the interconversion of MG and LMG during the extraction process. Very little LMG converted to MG, but up to 15% of MG converted to LMG. These results combined with metabolism studies have important consequences for regulatory analysis of triphenylmethane dyes in fish. Namely,

effective methods should include analytical procedures to detect the leuco compounds. Moreover, improved quantitative results will be achieved by preventing interconversion with compounds like HAH and TMPD. It was noted that incorporating matrix-matched calibration standards into the method along with isotopically labeled internal standards for each of the dye and leuco compounds will better model the complex extraction processes.

Hurtaud-Pessel et al.[31] developed a quantitative and confirmatory method in 2011 for MG, LMG, CV, LCV, and BG residue determination in trout by LC-MS/MS. The method was validated according to EU Decision No. (EC) 2002/657[161] with retention time matching and two selected reaction monitoring product ion transitions collected for each dye or leuco compound. In this simple procedure, fish tissue was extracted with HAH, acetonitrile, and magnesium sulfate without additional liquid- or solid-phase extraction step. Residue quantification required the use of four isotopically labeled internal standards for MG, LMG, CV, and LCV, and calibration was based on using extracted matrix-matched calibrants. As predicted by Hall,[160] the use of individual internal standards and matrix-matched calibrants provided excellent normalization of the complexity of dye residue analysis in fish. For regulatory analysis, the method performance for MG, LMG, CV, LCV, and BG was characterized by decision limit and detectability (CCα and CCβ) at and below 0.5 μg/kg, trueness ranging from 100% to 110% recovery, and precision of 10% RSD. Alternative instrument parameters were additionally described for the identification of the LBG analyte in incurred trout by UHPLC-LTQ-Orbitrap™-MS.[31] The method was included in several proficiency testing studies conducted by the EU Reference Laboratory for EU Member States[162] and was the method suggested in a recent Food Emergency Response Network proficiency test conducted by the USFDA for state and federal laboratories in the USA. In 2012, the method was established as AOAC First Action Method 2012.25 for future consideration as an AOAC Official Methods of Analysis.[163] The method was independently studied and validated for salmon, catfish, shrimp, and tilapia with the method performance evaluated according to both USFDA and EU criteria for mass spectrometric confirmation of identity and method detection limit.[28] In 2015, the method was recommended by an Expert Panel Review for Final Action after review of the results of an AOAC Collaborative Study with participation from 14 regulatory, private, and academic laboratories from the USA, Canada, and France.[32] The AOAC Official Methods Board approved 2012.25 for Final Action Official Method status in February 2016.

9.5.2 Analytical Methods for Other Dyes in Seafood

Compared to the triphenylmethane dyes, there are few class-specific dye residue analysis methods for regulatory seafood monitoring. Some multi-class dye methods have been introduced in recent years, and these are described in the following section.

9.5.2.1 Phenothiazines

Like the triphenylmethane dyes, detection of MB by visible absorbance at 663 nm provides a sensitive and fairly selective analytical approach for dye residue determination. Nakagawa et al.[36] studied the uptake of MB by eels and found residues to be undetectable using a spectrophotometric analysis method. In this method, MB was extracted in *n*-butanol with zinc sulfate and analyzed spectrophotometrically. Kasuga et al.[164] developed a method to extract MB and MG residues from trout muscle with pH 3 McIlvaine buffer and acetonitrile with HPLC analysis.

In 1997, Turnipseed et al.[37] modified the earlier MG/LMG method by Roybal et al.[133] for the extraction of MB from catfish muscle. The procedure was based on initial tissue mixing with sodium acetate buffer (pH 4.5), *p*-TSA, and HAH to stabilize MB and limit demethylation to the azure metabolites. Acetonitrile was added as the extraction solvent and dispersive alumina added to adsorb fat. MB residues were partitioned into dichloromethane and then further isolated by solid-phase clean-up with alumina and weak cation exchange using a CBA SPE cartridge. The CBA SPE procedure permitted higher recoveries than the stronger propylsulfonic acid SPE used in the MG/LMG method.[133] MB residues were analyzed in fortified and incurred catfish extracts by HPLC with visible absorbance monitoring at 660–665 nm to yield 75–90% recovery over the concentration range 10–50 µg/kg. Though LMB could not be isolated for detection, it was converted to MB during the extraction and analysis. Azure B and other demethylated metabolites were present in the chromatography.[37]

The MB procedure developed by Turnipseed et al. formed the basis for MB extraction used in more recent methods for HPLC-VIS[165] and LC-MS/MS analysis.[166] For the LC-MS/MS analysis, selected reaction monitoring was used to monitor product ion transitions from both MB and LMB precursors (*m*/*z* 284 and 286, respectively) following electrospray ionization in positive ion mode. Though the researchers observed that LMB was not stable and easily oxidized to MB during the analysis, they were able to collect product ion spectra in full scan mode with weak signal for product 2 *m*/*z* units greater than the parent MB, which was indicative of the presence of LMB. For regulatory analysis, only the MB residue was validated over the concentration range 1–10 µg/kg for eel, toasted eel, and shrimp. Recovery ranged from 74% to 99% (%RSD < 17%) and the method detection limit was 0.1 µg/kg.

9.5.2.2 Xanthenes

Analytical methods for xanthene dyes in fish matrix are described in Section 9.5.3, "Multi-class Dye Residue Analysis Methods." No class-specific methods for xanthene dye residue determination were found in the literature for fish muscle. One method described supercritical fluid extraction and solvent extraction from clay soils.[167] In this procedure, uranine, eosin Y lactone, phloxine B, Rose Bengal, and erythrosine B were separated on a C_{18} HPLC column with ammonium acetate and acetonitrile gradient elution and spectrophotometric detection at 493, 525, and 546 nm.

9.5.2.3 Acridines

A residue determination method for acriflavine and proflavine was developed by Plakas et al.[48] in 1996 for catfish. Acidic methanol was used as the extraction solvent and residues were isolated with C_{18} SPE cartridges. Quantitative analysis was performed by HPLC using a cyano column with absorbance measurement at 454 nm. The method was validated for fortified muscle over the concentration range 5–80 mg/kg. Recoveries were 86–95% with less than 6% RSD. This method was also used to extract metabolite compounds, though chromatographic separation was improved with a C_8 HPLC column.[47] Though not applied to fish muscle, a method was reported to determine acriflavine residue in waste water after isolation on Oasis® HLB SPE cartridges and analysis by LC-ESI-MS/MS in positive ion mode.[168]

Park et al.[169] recently developed an extraction and analysis procedure by LC-MS/MS for acriflavine and other veterinary drugs in pork, eggs, and milk. In this method, matrix was simply extracted with 0.1% formic acid and acetonitrile, the supernatant defatted with hexane and then evaporated, reconstituted, filtered, and analyzed by LC-MS/MS using a standard C_{18} column and formic acid–acetonitrile elution gradient. This procedure[169] yielded significantly improved recovery compared to QuEChERS sample preparation. Intra-day recovery for acriflavine in pork matrix was 71% at the 5 µg/kg fortification concentration with an RSD of 15%. Kaufmann et al.[170] recently reported on the differences in identity confirmation using mass spectrometry with triple quadrupole or high-resolution techniques. Acriflavine was one of the many veterinary residues analyzed in beef liver matrix.

9.5.2.4 Azo Dyes

Methods were recently reported for the extraction and analysis of chrysoidine in fish matrix. Wang et al.[51] reported extraction of fish with methanol, solvent drying with anhydrous sodium sulfate, and clean-up with dispersive C_{18} sorbent and magnesium sulfate. Extracts were derivatized and analyzed by GC-MS for confirmatory analysis and 81% recovery (4% RSD) of residues spiked at 10 µg/kg. Gui et al.[171] developed a method for chrysoidine in yellowfin tuna by LC-MS/MS. In this method, tuna was extracted with 1 M hydrochloric acid for an hour and neutralized to pH 7 with sodium hydroxide, and then residues were adsorbed onto Oasis HLB SPE cartridge for final elution, evaporation, and reconstitution. Tuna fortified with chrysoidine at 0.5 µg/kg yielded > 85% (<15% RSD). Reyns et al.[52] extracted chrysoidine under basic conditions by adding sodium hydroxide to pangasius fish matrix and then extracting with ethyl acetate. A portion of the ethyl acetate was removed, evaporated, and dissolved in acetonitrile with formic acid and defatted with hexane prior to analysis by UHPLC-MS/MS. The method was validated according to Council Directive 2002/657/EC[161] with a 0.25 µg/kg limit of quantification.

In another azo dye analysis method, four Sudan dyes and their two metabolites were extracted from fish muscle, skin, and other animal products with acetonitrile, sodium sulfate, and ultrasound assistance. Extracts were defatted with hexane, residues collected onto basic alumina SPE cartridges, and the eluted dyes analyzed by LC-MS/MS.[172] Yamjala et al.[173] recently reviewed analytical methods for the determination of azo compounds used as food dyes.

9.5.3 Multi-class Dye Residue Analysis Methods

Many analytical methods to determine therapeutic dye residues in seafood products are class-specific methods, but as with the trend in veterinary residue analysis, larger multi-class methods began to emerge in 2008. Tarbin et al.[14] developed a quantitative multi-class LC-MS/MS residue method for triarylmethanes, phenothiazines, and a few compounds from the xanthene and phenoxazine classes (rhodamine 6G and Nile blue A) in seafood. This method included the most common and effective therapeutic dyes used in aquaculture (MG, CV, BG, and MB) and expanded the list to include other dyes that might be substituted for these to avoid regulatory detection, including pararosaniline, ethyl violet, the trinaphthylmethyl Victoria blue dyes, azure B, and new MB. Similar to other procedures,[145] the dyes were extracted from salmon using ammonium acetate buffer at pH 4.5, acetonitrile, and alumina followed by liquid–liquid extraction with dichloromethane, oxidation with DDQ, and cation exchange SPE. Because leuco metabolites are only available for MG and CV, the inclusion of a DDQ oxidation process drives leuco metabolites of triarylmethane and phenothiazine dyes to their chromic parent dye for simplified analysis.

Reyns et al.[174] recently expanded on this method for the detection of illegal therapeutic dye use in aquaculture. The 12 dyes included were the same as in the Tarbin et al. method,[14] though the extraction procedure was modified to extract eel matrix with acetonitrile and sodium acetate and eliminate the dichloromethane extraction. The DDQ oxidation was included to convert the leuco metabolites, and an additional CBA cartridge was coupled to the strong cation exchange solid-phase extraction procedure. This method was validated over the concentration range 0.25–1.0 μg/kg using UHPLC-MS/MS for analysis.

Xu et al.[175] reported a procedure for the extraction of MG, LMG, CV, LCV, MB, and three azure dye (A, B, and C) residues from silver carp with analysis by UHPLC-MS/MS. The extraction was based on the Roybal procedure,[133] though the choice of SPE sorbent was optimized. Strong cation exchange adsorbed MB and the azure dyes too strongly; weak cation exchange did not retain LMG and MG well. A combined C_8-cation exchange cartridge (MCAX, Supelco) was found to be suitable for the clean-up of all the dye residues. Two product ion ratios were monitored for each dye to permit residue identification, and residue recovery was 75% or greater at the 0.5 μg/kg fortification concentration with RSD < 15%.[175]

Other multi-class methods have been developed with the intention of detecting dyes primarily used as food product dyes, some of which are also pharmacologically active dyes with possible aquaculture applications. Kirschbaum et al.[176] developed an HPLC-DAD method to test colored fish roe for permitted colorants from azo, xanthene, and triarylmethane dye classes. The dyes were extracted in aqueous ammonia, defatted with hexane, acidified to pH 2, and extracted onto polyamide powder for later elution and analysis. While this method was not intended to regulate therapeutic use of dyes in fish eggs, the method certainly is applicable for that purpose. Qi et al.[177] developed analyses for a similar group of permitted food dyes in fatty meat matrix with HPLC-DAD and LC-MS/MS. In this method, matrix was first extracted with hexane to remove fat and then extracted with ammoniated methanol with ultrasound assistance. Extracts were cleaned up with polymeric weak anion exchange cartridges. Sun et al.[178] reported a method for microwave-assisted extraction of 21 illegal dyes from meat and fish sausage. The 21 dyes included azo and xanthene dyes as well as triphenylmethanes and their leuco bases. Meat products were extracted in methanol/water with microwave irradiation for 5 minutes and then cooled and centrifuged. The dyes were absorbed onto C_{18} SPE cartridges and then eluted for UHPLC-DAD absorbance analysis. All 21 compounds were separated using gradient elution with a pH 5 ammonium acetate buffer and acetonitrile and absorbance measurement at 254 and 600 nm. Limits of detection were 2 μg/kg or less and recovery ranged from 61% to 105% for the fish products.

9.5.4 Bioanalytical Screening Methods

In addition to chromatographic analyses coupled with spectrophotometric or mass spectrometric detection, the sensitivity and selectivity of immunoassay techniques make them useful for quickly screening large numbers of regulatory samples. Polyclonal antibodies have been reported for MG and LMG[179] and for LMG with cross-reactivity with MG and LCV.[180] ELISA test kits are also commercially available for screening fish products for MG/LMG (Bioo Scientific, EuroProxima), CV/LCV (Bioo Scientific), and MG or LMG (GlycoNex, Beacon Kits, Abraxis, Neogen).

Oplatowska et al.[181] produced a hybridoma cell line to generate a monoclonal antibody (mAb) with cross-reactivity for MG, CV, BG, methyl violet, methyl green, and Victoria blue R. This antibody did not bind the leuco metabolites, but LMG was effectively detected at 1 μg/kg in the rapid ELISA assay when DDQ oxidation was used in the extraction procedure for fish tissues. A similar procedure was used to produce a mAb for MG, CV, and oxidized leuco metabolites against a more effective carrier protein to enhance sensitivity and selectivity of the ELISA.[182] Jiang et al.[183] developed a hybridoma procedure to develop an antibody for LMG. The antibody had 100% cross-reactivity with MG, but did not bind CV or BG. Dong et al.[184] reported a non-competitive immunoassay based on phage anti-immune complex assay (PHAIA) detection for LMG. In

this technique, a specific peptide sequence was selected from a phage library with specific binding for a mAb–LMG complex. The assay was applied to tilapia extracts reduced with potassium borohydride to convert all MG residues to the leuco base. This PHAIA technique was reported to yield a 16-fold sensitivity enhancement for LMG detection compared to a competitive ELISA method with the same mAb. ELISA immunoassays have been developed to detect dyes from other classes including chrysoidine,[185] the Sudan azo dyes,[186, 187] and rhodamine B[188] residues in food products.

In other screening techniques, Stead et al.[189] developed an oligonucleotide RNA sequence as an aptamer to bind MG and provide a simple and sensitive fluorescence assay for the MG–aptamer complex. Xu et al.[190] developed a lateral flow immunoassay based on a colloidal gold-labeled mAb against MG. The assay had sufficient cross-reactivity with CV to permit rapid and sensitive detection of both residues on a test strip.

9.5.5 Other Notable Analytical Procedures

A number of analytical procedures have been designed to add extraction selectivity to the analysis of triphenylmethane dyes or concentrate the residues in the presence of the bulk fish extract. Several researchers[191–193] have developed molecularly imprinted polymer (MIP) materials for cartridge extraction to selectively adsorb dye compounds from fish extracts. One procedure provided sensitive detection for combined LMG/MG residues based on direct electrochemiluminescence analysis of the extract, where the highly selective MIP extraction was required to reduce matrix interference prior to analysis.[192] Dispersive sorbents for dye residues have been demonstrated using magnetic nanoparticles, where the dye-bound sorbent can be easily separated from the bulk fish extract by holding a magnet to the side of the extraction tube.[194] In recent research, MIPs were generated on the surface of magnetic nanoparticles for enhanced selectivity for MG extraction.[195, 196]

In other examples of the application of new solid sorbent materials for dye extraction, graphene oxide nanosheets were used for solid cartridge extraction of MG and LMG from fish extracts.[197] Magnetic graphene oxide nanocomposite material was used as a dispersive sorbent to concentrate MG residues extracted from trout for sensitive spectrophotometric analysis.[198] A graphene oxide sorbent was developed with an MIP coating for phloxine B residue extraction.[199]

Many novel sorbent materials based on graphene oxide have been studied for their ability to remove dyes from environmental effluents. Materials designed for effective adsorption of CV, MB, rhodamine B, acriflavine, and other dyes may have applications for fish extraction procedures as well.[200–203]

Liquid micro-extraction techniques have also been applied to concentrate dye residues from fish extracts prior to analysis. Dispersive liquid–liquid micro-extraction (DLLME) techniques were developed to concentrate triphenylmethane residues from fish and shrimp matrix into small volumes of immiscible

solvent[204] and ionic liquids.[205] In this research, DLLME permitted direct spectroscopic analysis of the dye residues from an optical cell without chromatographic separation. Direct analysis of MG, CV, and MB residues in fish extracts have been studied by surface-enhanced Raman scattering (SERS) as well.[206–209] Sorbent and liquid micro-extraction techniques were described in greater detail in Chapter 2.

9.6 Recent Trading Issues with Dye Alerts

In line with the countries' food laws, seafood inspection programs have been established across the world. These programs have been in place for more than 20 years in the largest seafood importing countries such as the Member States of the EU, the USA, Canada, and Japan. Regulatory agencies/administrations of importing countries (Table 9.1) are responsible for inspection of both the domestic farmed fish production and the imported aquaculture products. The veterinary drug residue content of this production and imports is carefully monitored in order to mitigate unintentional human exposures that may pose health risks. Seafood inspections also have to include checks for proper labeling and documentation, sensory evaluations, and laboratory screening for contaminants such as heavy metals, PCBs, toxins, and microbial pathogens.

The enforcement for the non-authorized dyes in aquaculture began in the early 2000s for the control of MG/LMG and was extended to CV/LCV soon after. They are still today the main officially controlled d-ye substances.

Love et al.[210] recently acquired sets of interesting data from the official inspection programs of several large seafood importing countries: EU members, the USA, Canada, and Japan. Through the extraction of data from several governmental websites, from published literature, and also from direct queries to governmental bodies, they examined the trends in the alerts for seafood contaminant violations over the period 2000–2009.

The records for EU seafood violations from domestic and imported products were available online from the RASFF portal.[211] USA seafood inspection data were acquired through a Freedom of Information Act (FOIA) request to the USFDA and included all tests for domestic and imported seafood from 1999 to 2006. Canada's Fish, Seafood and Production Division of the Canadian Food Inspection Agency (CFIA) provided non-compliant test results for seafood products containing veterinary drugs from 2000 to 2009. Japan's Ministry of Health, Labour and Welfare provided yearly totals for seafood inspections and violations online from 2004 to 2009 and positive tests for veterinary drugs from 2007 to 2009.[212]

Love et al.[210] examined the sets of non-compliant data collected from 2000 to 2009 in the major importing countries as a function of species of aquatic animals, exporting countries, drug types, and concentrations. The triphenylmethane dyes were one of the families of drugs included in their evaluation, considering primarily MG and CV, as these started to be controlled in the mid-2000s. Results

Table 9.2 Percentage of veterinary drugs (dyes) violations collected over 2000–2009 period by seafood type and by inspection body.

	Inspecting bodies			
Seafood types	**European Union 2001–2008**	**USA 2001–2006**	**Canada 2000–2009**	**Japan 2000–2009**
Shrimp and prawns	None 100% other drugs	None 100% other drugs	3% malachite green 97% other drugs	<1% malachite green >99% other drugs
*n**	545	27	239	205
Fin-fish	31% malachite green 3% crystal violet 66% other drugs	77% malachite green 6% crystal violet 17% other drugs	68% malachite green 32% other drugs	None 100% other drugs
*n**	211	81	435	97
Molluscan shellfish		None 100% other drugs		8% malachite green 92% other drugs (75% chloramphenicol)
*n**	0	1	0	13
Crabs	None 100% other drugs (56% chloramphenicol; 42% nitrofurans)	None 100% other drugs (chloramphenicol)	None 100% other drugs (98% nitrofurans)	66% malachite green 33% other drugs (nitrofurans)
*n**	36	40	45	3

*n**: number of violations recorded over the mentioned period.
Source: David 2011 [210]. Reproduced with permission from American Chemical Society.

of their evaluation (Table 9.2) showed that fin-fish was the major species for violations with MG residues as reported by the EU ($n = 65$), the USA ($n = 62$), and Canada ($n = 296$). However, a few cases of MG violative contamination were reported as well in shrimp and prawns in Canada ($n = 7$) and Japan ($n = 2$) and also in "molluscan shellfish" ($n = 1$) and crabs ($n = 2$) in Japan. A few cases of violations with CV residues were found in imported fin-fish in the EU ($n = 6$) and the USA ($n = 5$). According to Love et al.,[210] it was not systematically reported in the data extracted whether the violation was derived from a domestic sample or from an import sample.

A more recent survey was undertaken by the authors of this chapter through the EU RASFF portal.[213] The objective was to focus on the alerts exclusively derived from the dye residue violations in aquaculture products, that is, shrimp and prawns, fin-fish and "molluscan shellfish," and cephalopods, respectively. Table 9.3 shows there were a total of 129 alerts that confirmed the presence of dye residues in these various aquaculture product consignments. This number was obtained from a long period spanning from 2002 to 2016. The alerts for dye residues accounted for more than 50% of the 247 fin-fish alerts in the EU (imports and domestic production altogether). According to the same table, very few of the 672 alerts derived from shrimp and prawn aquaculture were triggered due to the presence of MG residues (<1%). Finally, none of the four alerts in molluscan shellfish/cephalopod seafood imports/production were derived from

Table 9.3 Percentage of veterinary drugs (dyes) violations by seafood type aquaculture. Extracted from the EU RASFF website over the period 2002–2016.[213]

Seafood types	Inspecting body: European Union RASFF period 2002–2016
Shrimp and prawns	0.3% malachite green 99.7% other drugs[a)]
*n**	*672*
Fin-fish	48.2% malachite green 3.6% crystal violet 0.4% Victoria blue 47.8% other drugs
*n**	*247*
Molluscan shellfish and cephalopod seafood	0.0% dyes (MG, CV, VB) 100% other drugs
*n**	*4*

*n**: number of violations recorded over the mentioned period.

a) "Other drugs" include violations for chloramphenicol and nitrofurans.

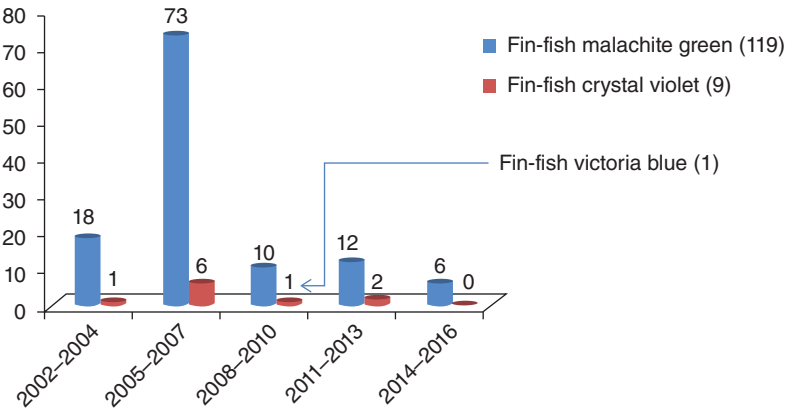


Figure 9.4 EU alerts for dye residues in fin-fish aquaculture. Extracted from the EU RASFF website over the period 2002–2016.[213]

the presence of dye residues. Overall, the major dye substance found is MG. This is perhaps not unexpected because MG was the first dye to be used for antifungal and antimicrobial treatments in fish farms. It was also the first dye to be controlled in aquaculture production, whereas the official monitoring of CV actually started after the mid-2000s. It is worth highlighting one rather unexpected alert in Table 9.3 arising from the presence of Victoria blue residues in fin-fish fillets imported from Vietnam in 2010. In fact from the 129 alerts, 119 indicated MG contamination and 9 alerts showed CV contents. Over the 2002–2016 period, after a peak of alerts in the years 2005–2007 (73 MG alerts), the data displayed in Figure 9.4 clearly demonstrate that the dyes have not disappeared yet from the fin-fish farming industry and continue to potentially enter the food chain with two or three RASFF alerts per year in the more recent years as well.

Having now a closer look at the countries of origin of the fin-fish products subjected to the 129 alerts (Figure 9.5), the top three countries accounting for more than 10 alerts each are three Asian countries with quite large volumes of fish exports to the EU. Vietnam is the source for nearly 50% of the 129 alerts followed by Indonesia (15 alerts) and China (12 alerts). There are also a significant number of countries ($n = 20$) that have been alerted (between 1 and 7 alerts each) due to the presence of MG or CV in their exported or domestic fin-fish products. Approximately half of these countries ($n = 12$) are Member States of the EU which have been facing some safety issues with regard to their domestic fish farming production (i.e., Denmark, Germany, Poland). The other roughly half of the countries ($n = 8$) are non-EU countries from Latin America and Asia (i.e., Japan, Thailand, Chile) which have been assigned a marketing authorization to export into the EU market in recognition of their implementation of an annual national residue monitoring plan demonstrating their ability to control their fish farming production in accordance with the EU regulations.[99, 214]

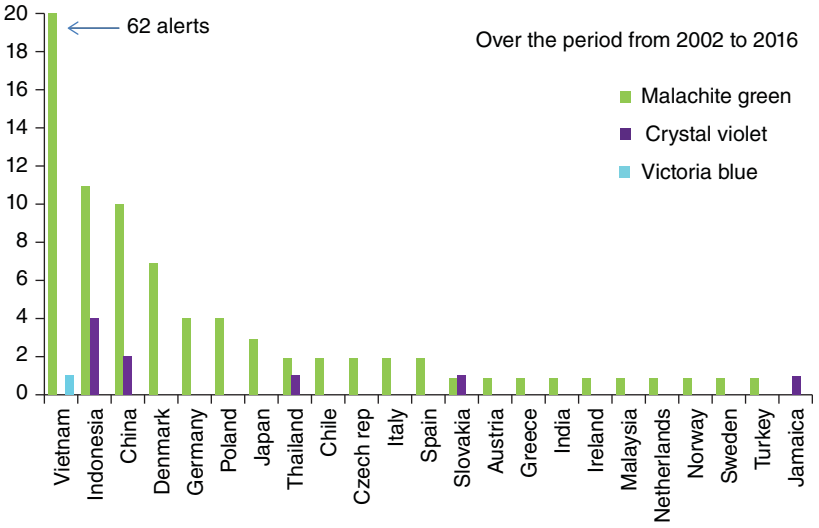


Figure 9.5 EU alerts for dye residues in fin-fish aquaculture products sorted per countries of origin. Extracted from the EU RASFF website over the 2002–2016 period.[213]

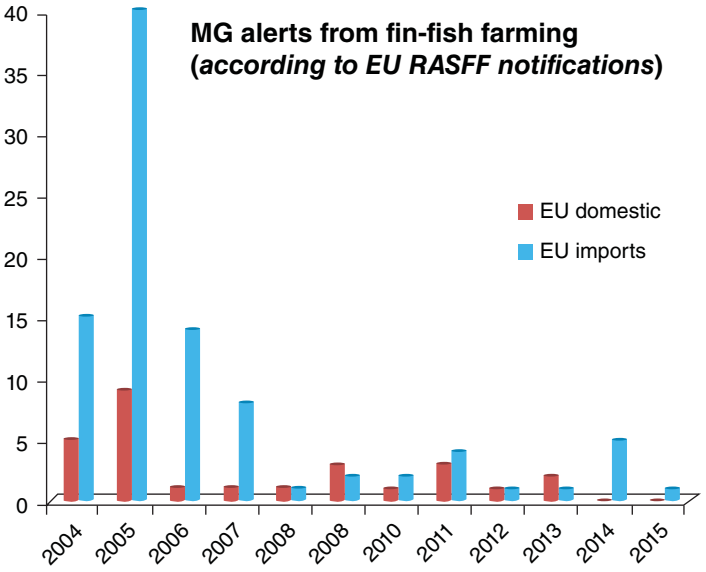


Figure 9.6 EU alerts for MG residues in fin-fish farming products sorted per year. Extracted from EU RASFF website over the period 2004–2015.[213]

It can also be clearly seen from Figure 9.6 that the larger number of alerts arises from the EU-imported products as compared to the EU domestic fin-fish production. It is clear that food safety and public health is still a big issue in aquaculture trading. Aquaculture products sold worldwide must be kept under sufficient control considering the various non-authorized chemical substances still available for fish/seafood farmers, including the dye substances and should start with serious control of MG itself.

9.7 Conclusions

The control of dye residues together with other regulatory prohibited/non-authorized or regulated chemicals in farmed fish and seafood products accounts for one of the public health concerns for this new century. The continuous rise of intensive and integrated aquaculture systems has to be seriously accompanied by appropriate controls and the various farming practices to be fully supported especially in developing countries. This area is acknowledged by the FAO to be one of the key elements to meet the urgent need worldwide to increase the efficiency and the volumes of food protein production in view of the ever faster growth of the human population.[1]

Facing this issue, most of the regulatory agencies in charge of food safety have developed programs to control these toxic chemicals in the food products derived from aquaculture. The ever-growing trading of food and in particular of fishery and farmed fish products has required governments to endorse adapted food laws in order to manage the risk of contaminated aquaculture and seafood products. Regulatory agencies of large exporting countries have been compelled to implement stricter conditions of use and even sometimes prohibition of these veterinary treatments in the intensive aquaculture practices developed in their countries over the past 20 years. As a result, there has been significant control deployed all around the world over the past 15 years. MG remains one of the key first issues to deal with for dye residue control in aquaculture around the world, together with a few other veterinary drugs of abuse such as nitrofuran and chloramphenicol, which are widely prohibited antibiotics.

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10

Method Validation and Quality Assurance/Quality Control Approaches for Multi-residue Methods

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10.1 Introduction

In modern production practices, animals are frequently treated with medicines to prevent or cure diseases or to promote growth and maximize production output. The use of veterinary pharmaceuticals in food-producing animals such as pigs, cattle, sheep, poultry, and fish may lead to residues of the substances in food products such as meat, milk, and eggs derived from the animals. To protect public health, the medicines must be used in a manner that ensures that foods do not contain residues at concentrations that may be harmful to the health of the consumer.

Avoidance of such potentially harmful residues is best achieved using a "farm to fork" approach, covering all aspects of the production, processing, and marketing of food products. With regard to veterinary medicines, to guarantee a high standard of consumer protection, the toxicity of potential residues is evaluated before a medicinal substance is authorized for use in food-producing animals. If considered necessary, maximum residue limits (MRLs) are established and in some cases the use of the relevant substance is prohibited. Laboratory analysis is an essential element of the "farm to fork" food safety system, providing monitoring and feedback on the effectiveness of the controls being implemented.

Analytical methods for veterinary drug residues in foods must be capable of reliably detecting the presence or absence of a veterinary drug of interest or concern (screening methods), determining the concentration of the analyte (quantitative methods), and providing unequivocal identification (confirmatory methods) of the drug. When an analytical method has been used to determine that the defined MRL or other regulatory limit for an approved veterinary drug has been exceeded,

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it is imperative that the test results are confirmed before regulatory action is taken. Regulatory action could include denying the product market access, destroying the product, and/or the administration of financial penalties. In cases where the detected veterinary drug is not approved or is prohibited from use in that commodity because no acceptable daily intake (ADI) and MRLs have been defined for toxicological reasons, detection of such a drug at any concentration should be confirmed, since this finding may automatically result in regulatory action. The analytical methods used to support this decision-making process must be shown to be "fit for purpose,"[1] which means that the laboratory implementing the method should have the data to show that the method is sufficiently accurate and robust to produce reliable and defensible results. Demonstration of fitness for purpose includes various criteria; the main criteria were summarized in the Codex Alimentarius Commission (CAC) Guidelines for the Assessment of the Competence of Testing Laboratories Involved in the Import and Export Control of Food in 1997[2]:

- Accreditation under a recognized system for laboratory accreditation
- Participation in appropriate proficiency testing programs
- Demonstration of an effective quality assurance (QA) system
- Use of validated methods

The initial validation of a method is usually carried out after method development and is supported during application of the method by ongoing verification using quality control (QC) procedures such as the analysis of blank and spiked control samples, analysis of certified reference materials, and participation in proficiency tests and inter-laboratory comparisons. Limits or criteria must be set for analytical performance parameters such as analytical recovery, precision, detection and/or quantification limits and instrument response and records must be maintained to show that the method performed within the quality limits when a particular analytical result was produced.

The topics of method validation[3] and QA and QC[4] have been extensively covered in the previously published companion volume to this book, Chemical Analysis of Antibiotic Residues in Food.[5] The procedures for residues of non-antibiotic drugs in food are the same as those for antibiotic drug residues. The purpose of this chapter is not to repeat the information already provided in that book but to update the information where necessary, especially with regard to the increasing development and application of multi-residue methods (MRMs) for veterinary drugs, and to direct the reader to further relevant sources of information.

10.2 Sources of Guidance on Method Validation

This topic is covered in detail in Chapter 8 of the book referenced in Section 10.1,[3] so the focus in this chapter is on developments over the intervening years, particularly with respect to the validation of MRMs for veterinary drug residues.

Information and guidance on the validation of methods for the analysis of veterinary drug residues and similar chemical analyses are available from a variety of sources. These include a large body of papers published in the scientific literature, guidance issued by scientific bodies such as the International Union of Pure and Applied Chemistry (IUPAC) and Eurachem, guidance from international organizations involved in the establishment and harmonization of standards, such as the CAC, and guidance from national and regional regulatory authorities such as the US Food and Drug Administration (USFDA) and the European Commission (EC). More details on the relevant scientific bodies, international organizations, and regional regulatory authorities are provided in the companion volume to this book.[5]

Most of the validation guidelines, however, are generic and those specifically for veterinary drug residue analysis were initially developed primarily for single residue methods, or at best methods for a small number of residues of compounds of a single chemical class. With the development of broader scope MRMs for veterinary drug residues, similar to those more commonly applied for pesticide residue analysis, and even methods capable of detecting a number of different types of residues and contaminants, there is often difficulty in applying the current method validation guidelines. Although there is general agreement on the various validation parameters to be evaluated, there is considerable diversity concerning the details and process to be employed for validation and acceptance criteria.[6] Moreover, most of the guidance currently available for the validation of quantitative and confirmatory methods is appropriate for more "traditional" chromatographic methods used for veterinary drug residue analysis, such as those employing high-performance liquid chromatography with targeted detection by spectroscopic, fluorometric, or single (liquid chromatography–mass spectrometry (LC-MS)) or tandem quadrupole mass spectrometry (liquid chromatography–tandem mass spectrometry (LC-MS/MS)). A tutorial review on the validation of LC-MS methods was published in 2015 in the form of two papers.[7,8] The papers summarized the status of validation of LC-MS methods in general, clarifying the relevant terminology and providing recommendations on difficult validation-related issues in LC-MS.

The ongoing, rapid evolution in instrumentation and the development of applications for veterinary drug residue analysis using new techniques has resulted in a need for revision of many of the currently available guidance documents. For example, full-scan, with accurate mass measurement, mass spectrometric approaches offer the possibility to simultaneously analyze a virtually unlimited number of compounds, and are well suited to the analysis of multiple residues in a single analytical run. Techniques such as time-of-flight (TOF) mass spectrometry, quadrupole TOF (QTOF), and Orbitrap™ or Q-Orbitrap™ are increasingly finding applications in chemical analysis[9], as discussed in Chapter 3. The higher resolution and/or accurate mass capabilities of these techniques can greatly improve the selectivity, and consequently the sensitivity, of the analysis of compounds in complex matrices when compared with methods such as single or

triple quadrupole mass spectrometry, which typically have unit-mass resolution. For example, TOF has been applied for the multi-residue screening of veterinary drug residues in meat[10] and in animal feed.[11] Robust validation of such methods is necessary, since accurate mass determination applied without adequate or appropriate mass-resolution criteria can lead to false compliant results in both screening and confirmatory methods. Current validation guidelines do not adequately cover this area, and methods are frequently validated according to the existing criteria published in, for example, the European Commission Directive 2002/657/EC.[12] Continuous revision of these guidelines is required to keep up with advances in technology.

The following sections focus mainly on some relatively recent developments and revisions in the CAC and European Union validation guidelines.

10.2.1 CAC Guidelines

Guidelines were adopted by the CAC in 2008 for the design and implementation of national regulatory food safety programs associated with the use of veterinary drugs in food-producing animals (CAC/GL 71-2009).[13] These guidelines were designed to include general guidance on the validation of analytical methods for use with single analytes under single-laboratory validation conditions (as set out in CAC/GL 71-2009) and to be updated as necessary to permit extension to cover additional relevant areas.

The 18th session of the Codex Committee on Residues of Veterinary Drugs in Food (CCRVDF) recognized that current practice in analytical laboratories undertaking these analyses was to use MRMs wherever possible to increase the efficiency of the laboratories while keeping analytical costs to a minimum.[14] However, the same meeting also recognized that there was very limited guidance on the acceptable performance characteristics for MRMs. The CCRVDF therefore agreed to develop further guidance to address this need to be prepared as an appendix to CAC/GL 71-2009.[13] It was also recognized that developing countries may need a transition period and/or technical assistance when working toward using these guidelines.

Technical Guideline documents issued by the CAC[2, 13] to assist countries involved in the import and export control of foods in the application of requirements for trade in foodstuffs in order to protect consumers and facilitate trade recommend that laboratories engaged in regulatory analyses must be compliant with ISO/IEC 17025:2005 – "General requirements for the competence of calibration and testing laboratories."[15] Laboratories should also participate in appropriate proficiency testing schemes for food analysis that conform to the requirements laid down in "The International Harmonized Protocol for the Proficiency Testing of (Chemical) Analytical Laboratories"[16] and, whenever possible, use methods that have been validated according to the principles laid down by the CAC (see CAC/GL 27-1997).[2] In addition, the laboratories must use internal QC procedures that comply with such procedures as described in "The

Harmonized Guidelines for Internal Quality Control in Analytical Chemistry Laboratories."[17]

Validated analytical methods are methods with defined characteristic operational parameters that have been determined to be suitable for use in a regulatory control program (i.e., fit for purpose in a regulatory environment). The CAC adopted in CAC/GL 49-2003 Rev.1-2003[18] the guidelines for the single-laboratory validation of methods of analysis issued by IUPAC.[19] These have also been incorporated into the "General Criteria for the Selection of Single-Laboratory Validated Methods of Analysis" contained in the CAC Procedural Manual, 24th edition.[20] The guidance document adopted for veterinary drug residue methods detailed the attributes of MRMs used for a range of substances in the same analysis. It also set out the requirements MRMs must satisfy before they can be considered suitable for use in regulatory control programs for veterinary drug residues in foods.

10.2.1.1 Scope of the Codex Guidelines

The guidance adopted for MRMs as an appendix to CAC/GL 71-2009[13] is applicable to methods used in the analysis of veterinary drug residues, including pesticides that have an approved veterinary use. Guidance on the validation of MRMs for non-veterinary use of pesticides is contained in CAC/GL 40-1993, Rev.1-2003: Guidelines on Good Laboratory Practice in Residue Analysis.[21]

For the purposes of the CCRVDF guidelines, an MRM is considered to be a method that includes three or more analytes in the same class or more than one class of veterinary drugs in its scope. This definition, which also includes those methods sometimes referred to as multi-class multi-residue (MCMR) methods, is used throughout this chapter. MRMs are most commonly used by laboratories for screening samples for the possible presence of veterinary drugs, but they may also be used for quantitative and/or confirmatory analyses. The guidance therefore covers all three types of analyses and forms an annex to CAC/GL 71-2009.[13] It should be noted that a validated MRM may include some analytes for which performance requirements for quantitative analysis have been fully validated, while some other analytes may not meet precision and/or recovery criteria for quantitative analysis or the data requirements for confirmation of the residue. When the method has been validated as suitable to detect these analytes at a required action limit, the method may be used as a screening method for such analytes. If present, these analytes should then be quantified using a validated quantitative method or confirmed using a suitably validated confirmatory method.

As previously noted, the CAC Procedural Manual 24th edition[20] provides "General Criteria for the Selection of Single-Laboratory Validated Methods of Analysis." The "Working Instructions for the Implementation of the Criteria Approach in Codex, Table 1: Guidelines for establishing numeric values for the criteria" contained in this document are as relevant to MRMs as they are to single residue methods, as are the "Guidelines for Establishing Numeric Values for Method Criteria and/or Assessing Methods for Compliance Thereof." In

the interest of harmonization, guidance on performance criteria for analytical methods applied to veterinary drug residues should be consistent with the general guidance already approved by the CAC. In addition, a guidance document for validation and QC has been issued by the EU (Document No. SANCO/10684/2009) for pesticide residue analyses.[22] The EU document covers MRMs primarily for confirmatory analyses but also addresses multi-residue screening methods using mass spectrometry. Aspects of SANCO/10684/2009 were adopted into the CAC MRM guidance where appropriate. The SANCO document was more recently updated as SANCO/12571/2013.[23]

The principles set out in the CCRVDF guidelines for MRMs are considered practical and suitable for the determination of the performance characteristics of MRMs for use in regulatory control programs and are being considered by a number of groups in drafting and updating related guidance in other commodities, for example, marine biotoxins.[24] Indeed, a recent study sponsored by the United Kingdom[25, 26] has clearly demonstrated that the guidance developed is generic in nature and can be more widely applied to include MRMs for analytes other than veterinary drugs.

10.2.2 European Commission Decision 2002/657/EC

Commission Decision 2002/657/EC[12] establishes criteria and procedures for the validation of analytical methods to ensure the quality and comparability of analytical results generated by official laboratories. The Decision stipulates that the quality and comparability of the analytical results generated should be assured by implementing quality systems, including using methods that are validated as fit for purpose and ensuring traceability to common or agreed standards. From January 2002, official European control laboratories have been required to be accredited to ISO 17025,[15] which includes adequate analytical method validation and proof of their competence through participation in internationally recognized proficiency testing schemes. Regarding non-EU countries exporting food products to the EU, only laboratories specifically designated as official laboratories can be responsible for testing related to official control. The laboratories designated for official control by the competent authority must comply with requirements for management and technical operation, which are those of ISO 17025.[15] Only laboratories which are accredited to this standard and which apply and comply with the required analytical test methods and/or performance criteria can be designated as a laboratory responsible for official control.[27]

10.2.2.1 Performance Characteristics

The concept of routine and reference methods was superseded by a criteria approach, in which performance criteria and procedures for the validation of screening and confirmatory methods are established. A similar approach was subsequently adopted by the CAC.[13]

There is considerable similarity between Codex, Eurachem, EU, and other relevant bodies in their requirements for validation of analytical methods. All of these bodies highlight the need to assess the same range of performance characteristics, including trueness, precision, analyte stability, limits of detection and quantification, and measurement uncertainty. The major areas of difference relate to the detection and quantification limits, preferred by Codex, Eurachem, and others, and the parameters CCα (the decision limit) and CCβ (the detection capability) preferred by the EU.

Analytical methods may be of different types and suited to different uses. The performance characteristics that must be determined for screening methods, for example, are not the same as for confirmatory methods. In general, both screening and confirmatory methods may be qualitative or quantitative. According to Decision 2002/657/EC,[12] the characteristics that must be elaborated for qualitative screening methods are CCβ, the selectivity/specificity, ruggedness, and stability. For quantitative screening methods, the precision must be determined in addition to those parameters listed for qualitative screening methods. For qualitative confirmatory methods, CCα, CCβ, selectivity/specificity, ruggedness, and stability should all be determined, and for quantitative confirmatory methods, the trueness (or recovery) and the precision should be determined in addition.

10.2.2.2 Minimum Required Performance Limits

Decision 2002/657/EC[12] also establishes common criteria for the interpretation of test results and introduces a procedure to progressively establish minimum required performance limits (MRPLs) for analytical methods employed to detect substances for which no permitted limit (maximum limit) has been established. This is particularly important for substances that are not authorized for use or are specifically prohibited in the EU and is an important consideration in the validation of methods for those substances, since the MRPL provides a concentration at which the method must be able to perform satisfactorily. The MRPL is based on the technical performance of state-of-the-art analytical instrumentation rather than on toxicological or risk assessment data.[28] The original intention when this parameter was introduced was that it would not be used as a "pseudo-MRL" or an action limit for banned drugs. The MRPL was to be a target concentration for methods in all laboratories in the EU, but results from laboratories that had more sensitive methods capable of identifying and, if necessary, quantifying residues of non-permitted compounds at concentrations lower than the MRPL could lead to, for example, destruction of carcasses, instigation of follow-up actions or rejection of shipments from countries outside the EU.

The concept of the MRPL has been the subject of much controversy since its introduction. Since some laboratories in Europe could achieve decision limits lower than are required to meet the MRPL, shipments of produce were being rejected, for example, for chloramphenicol residues in shrimps from East Asia, by some EU importing countries, whereas others would accept the same shipment since their analytical methods could detect residues only at the MRPL. Exporting

countries were also unsure of how to address this situation, since they believed that if they could test products for export and show no unwanted residues at or above the MRPL, the products should be accepted in Europe.

In response to these criticisms, the European legislation was amended through Commission Decision 2005/34/EC,[29] laying down harmonized standards for the testing for certain residues in products of animal origin imported from third countries. In order to establish a harmonized and feasible approach for the control, in imported consignments, of residues of substances prohibited or not authorized in the Community, and in line with European food law and the Codex Alimentarius Working Principles for Risk Analysis,[30] it was stipulated that the isolated detection of residues of a substance below the MRPLs should be construed as not of immediate concern but that the substance should be monitored by Member States. For the purpose of the control of residues of prohibited or non-authorized substances, MRPLs were to be used as reference points for action (RPA) irrespective of the matrix tested. Furthermore, Decision 2005/34/EC states that for products that are tested and the results are below the MRPL, the products will not be prohibited from entering the food chain.[29] It was stipulated that records must be kept of any non-compliant results at concentrations below the MRPL and any repeated occurrences may lead to discussions between the EC and the competent authority of the exporting country to decide upon further action. For consistency, the application of MRPLs was changed not only for imported products but also for food of animal origin produced within the European Community.

10.2.2.3 Interpretation of Decision 2002/657/EC

Following publication of Decision 2002/657/EC,[12] there was some confusion among EU Member State laboratories about how to interpret it for analytical method validation. As a result, several guidelines were produced. The three EU Community Reference Laboratories (CRLs) for veterinary drug residues analysis developed a CRL guidance paper[31] in 2007, which aimed to improve and harmonize the performance of analytical methods used for substances for which MRLs had not been established, and which were prohibited or not approved for use in food-producing animals. The CRL guidance paper listed the substances with target matrices and recommended concentrations at which the methods should perform – the method validation should therefore demonstrate that the method applied had the performance characteristics, CCβ (detection capability) for screening methods or CCα (decision limit) for confirmatory methods, lower than the published recommended concentration. The document was intended as technical guidance for methods used in residue control and the recommended concentrations therein had no legal force. Additional guidelines for the interpretation and implementation of Decision 2002/657/EC were published in 2008[32] as a revision of a document drafted by the CRLs in 2004. In the aforementioned guidelines, there was also a lack of detail on how to validate screening methods, which was subsequently addressed by the development of a supplementary text by the CRLs in 2010.[33] None of these documents or guidelines specifically

addresses MRMs, and in practice the recommended procedures may be difficult to apply for methods covering a large number of analytes. Although no revision of Decision 2002/657/EC has been published at the time of writing, the need for revision to take into account new scientific, technological, and regulatory developments, including the capability to perform multi-residue analyses, is recognized in the Import Decision 2005/34/EC,[29] which introduces the concept of the RPA, and the Regulation of the European Parliament (EC 470/2009),[34] which further formalizes the concept for all compounds for which administration to food-providing animals is prohibited.

10.3 Practical Considerations

There are several practical considerations that make MRM validation a more complicated proposition than for single analyte methods. As mentioned previously, existing validation guidelines are mainly generic and do not address the specific problems that may be associated with MRMs. Some bodies have recognized the need for development in this area; for example, the CCRVDF developed guidance for the validation of MRMs used in the analysis of veterinary drug residues, including pesticides that have an approved veterinary use, which was adopted by the CAC as an annex to CAC/GL 71-2009,[13] as discussed in more detail in Section 10.2.1.

Some of the practical considerations to be taken into account when validating an MRM are discussed as follows.

10.3.1 Scope of the MRM

Whereas applying the guidelines to validate a method for one or two analytes can be relatively straight forward, the validation of screening, quantitative, and qualitative or confirmatory methods becomes more involved for MRMs. This is particularly true for the type of mass spectrometric methods that are becoming more common, capable of the multi-class, multi-residue analysis of veterinary drugs, pesticides, mycotoxins, environmental contaminants, and other chemicals of interest at ultratrace concentrations in diverse food and environmental sample types.[35] Even for multi-residue methods for veterinary drugs, either those that cover antimicrobial or non-antimicrobial substances (or both), the range of analytes makes validation a complex exercise. The EC has published general performance-based method validation guidelines for pesticide residues in food.[22] The authors of that document advocate the selection of diverse, representative analyte–commodity pairs for empirical method validation. They also recommend assessment of qualitative/confirmatory method performance at the same time as quantitative method validation. This approach allows the empirical evaluation of any method to determine false positive and false negative rates as well as the limit of identification.[36] Although the use of representative compounds would

greatly simplify the validation of MRMs for veterinary drug residue analysis, factors such as the complexity of meat matrices, sensitivity requirements, and the limited chemical stability of many veterinary drugs in biological matrices may make the selection of suitable representative compounds and compound–matrix pairs very difficult, if not impossible for many substances.[36]

10.3.2 Dynamic Range

The scope of an MRM for non-antibiotic drugs may encompass compounds with a wide range of MRLs, as well as prohibited compounds, and the MRLs often vary from matrix to matrix. Using existing guidelines, the performance characteristics of the method should be defined and determined for each analyte included in the scope of the MRM. For methods to be applied to demonstrate compliance with regulatory MRLs, the validation is typically performed with blank samples spiked at 0.5 × MRL, 1 × MRL, and 1.5 or 2 × MRL and at the limit of identification for prohibited substances. At least six replicates at each concentration should be analyzed for repeatability, and the validation experiment performed on three separate occasions for intra-laboratory reproducibility. For methods covering a large number of compounds, great care must be taken in preparing mixed standard solutions for spiking. The solutions must be prepared at concentrations that allow minimal volume to be used for the spiking process, in solvents suitable for all the compounds included in the scope of the method. An alternative is to perform the validation experiment with only a few of the analytes at a time, but this would require many more validation runs.[37] If this approach is taken, it may be useful to overlap the validation experiments with at least one analyte per group to give an indication of the comparability between the validation experiments.

When validating an MRM to be used for regulatory purposes to check for compliance with MRLs or other specified limits, it may be argued that it is advisable, if possible with the resources available, to validate the method at and around the MRLs, but also at the lowest concentrations that meet the performance requirements, since this will allow the method to be applied in cases where the MRL is changed on the basis of new toxicological information or reassessment. Such a change in the MRL or regulatory limit would render the method validation useless for that particular compound if the validation was initially performed only with reference to the previous MRL. This becomes especially relevant for MRMs covering a large number of compounds.

If the method is to be used for confirmation of the screening or primary test result of a prohibited compound, the lowest concentration at which the method can be validated is the concentration at which the identification criteria are met. In this situation, only false-positive results are a concern, because any analysis that meets the identification criteria for a target analyte would be reported at the determined concentration.[35]

It is often desirable for a method to be validated for a number of compounds, including both regulated and prohibited substances. In such a case the dynamic

range to be covered may be high, perhaps two (or more) orders of magnitude, typically with five or more calibration concentrations covering the range.[36] In this case, current validation guidelines may not be practical. For example, practical problems may prohibit the fortification of the validation samples at individual concentrations for each of the target analytes in an MRM, related to their MRLs or MRPLs. A unified approach was suggested by Kaufmann[10] for such cases for the validation of a TOF MRM for about 100 veterinary drugs in different meat matrices. A compromise protocol was developed using a concentrated fortification solution containing drug group-specific concentrations. Validation experiments were designed such that the concentration range encompassed the analyte-specific MRL or MRPL in each matrix. To keep the procedure practical, only two different analyte concentration groups were used, with high MRL compounds present in the mixed fortification solution at 10 times greater concentrations (spiking concentrations 10, 33.3, 100, 333, and 1000 µg/kg) than for low MRL compounds and those without an MRL (spiking concentrations 1, 3.33, 10, 33.3, and 100 µg/kg). Depending on the application and the target analytes, more than two analyte concentration groups might be required to ensure that the method is validated as fit for purpose.

Calculation or estimation of the method performance characteristics may also be difficult when the method covers a wide concentration range. The ISO approach suggests a narrow range for calculation of CCα and CCβ, for example. When a large concentration range is used, the higher concentrations typically have a greater influence on the slope of the calibration curve and therefore on the intercept with the Y axis, which is used to calculate CCα and CCβ, possibly resulting in erroneously high or low values for these parameters. Kaufmann[36] suggests that a feasible approach for the calculation of CCα and CCβ in such a situation is to use the three lowest calibration concentrations, which will produce significantly more realistic estimates for these parameters. In the same paper,[36] Kaufmann addresses several other aspects of the validation of LC-TOF MRMs, including the absence of measurable noise in some blank samples, with realistic solutions for some of the problems encountered and ideas for possible future validation strategies.

10.3.3 Internal Standards

Another consideration is the use of internal standards. Liquid chromatography–triple quadrupole mass spectrometry is currently the main mass spectrometry-based tool for veterinary drug residue analysis. For this and methods using other techniques such as QTrap® technology,[37] it is a common practice to include internal standards in quantitative analytical methods to compensate for variations introduced during sample extraction and clean-up, and in the ionization process in the mass spectrometer, thereby improving the precision and accuracy of the results. The internal standards must be substances that are distinguishable from the target analyte by the detection system, but that

behave identically, or as closely as possible, to the analytes during the extraction, clean-up, and analysis stages of the method. For mass spectrometric methods the best option is usually isotope dilution, most frequently using deuterated versions of the target compounds. However, if the analytical method covers many compounds, suitable deuterated internal standards may not be available for all compounds, or their use will increase the cost and complexity of the method beyond reasonable limits. Therefore, great care must be taken during method development to assess which internal standards may be useful to compensate for other compounds in addition to their non-deuterated analog, and the use of the various internal standards must be fully considered in the validation experiments. In some cases, suitable internal standards may not be available, and it may be necessary to quantify some of the compounds using only external standards.

An example of the use of multiple internal standards is in the validation of an MRM for 38 anthelmintic and flukicidal drugs and their metabolites in beef compounds by high performance LC-MS/MS.[38] This method was developed in the Food and Environmental Protection Laboratory of the Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture under the EU 6th Framework Integrated Project "ProSafeBeef." The method covers 38 target analytes and employs 8 internal standards, 7 of which are deuterated analogs of analytes and 1 of which is an unlabeled macrocyclic lactone (selamectin). For example, the deuterated compound fenbendazole-d_3 was found, during method development and optimization, to be suitable as internal standard for nine of the target compounds: cambendazole, fenbendazole, flubendazole, amino-flubendazole, hydroxy-flubendazole, mebendazole, amino-mebendazole, hydroxy-mebendazole, and oxibendazole, and the method was validated for these compounds accordingly. For one compound, clorsulon, it was found that none of the available deuterated compounds, or selamectin, behaved similarly in sample extraction and analysis. Clorsulon, therefore, was quantified using only an external standard curve, with correction of the result for recovery of the drug from replicate blank samples spiked at a known concentration. This method was based on a similar method developed under the same project using ultra-high performance liquid chromatography–tandem mass spectrometry (UHPLC-MS/MS).[39] In that case, the method used 11 internal standards for the 38 analytes. The purpose of the study was to assess the prevalence of residues of the target compounds in meat within Europe, not to demonstrate compliance with regulatory MRLs. The analytical method was therefore validated at low concentrations as if no MRLs had been established. An error of 1% was applied to ensure that false-positive detections were minimized rather than the 5% error normally applied for validation of a method encompassing an established permitted limit. Both of the aforementioned methods were based on a similar method for the control of anthelmintics and flukicides in milk,[40] which was validated at two concentration ranges. A low concentration range validation study was carried out for unapproved drug use, at three concentrations corresponding

to 1, 1.5, and 2 times the second lowest calibration concentration. A second validation study was performed for the MRL compounds at 0.5, 1, and 1.5 × MRL, according to Commission Decision 2002/657/EC criteria.[12] The approaches used in these examples clearly demonstrate the need to validate the analytical method according to its intended use – that is, to ensure that it is fit for purpose.

Another example of the use of selected internal standards in an MRM is in a method for the quantification and confirmation of residues of 115 veterinary drugs in milk powder, butter, fish tissue, and eggs by LC-MS/MS.[41] Although the method covered various different classes of veterinary pharmaceuticals, the use of internal standards was only feasible for some non-steroidal anti-inflammatory drugs (NSAIDs): carprofen, flunixin, ibuprofen, mefenamic acid, meloxicam, naproxen, and tolfenamic acid. This was because the only stable isotope-labeled standards available to the research group were flunixin-d_3 and meloxicam-d_3. The selection of the most suitable internal standard for each NSAID analyte was made based on the retention time of the analytes; flunixin-d_3 was used to quantify carprofen, flunixin, ibuprofen, and mefenamic acid, whereas meloxicam-d_3 was used for naproxen and tolfenamic acid.

10.4 Examples of Validation Protocols for MRMs

As stated previously, the annex to CAC/GL 71-2009[13] described in Section 10.2.1 is the only guideline focusing specifically on the validation of MRMs for veterinary drug residues. In the absence of specific, detailed, and internationally accepted guidelines for non-antibiotic and other veterinary drug residues, many published methods have been validated using the criteria outlined in Commission Decision 2002/657/EC[12] or by adapting other guidelines. Decision 2002/657/EC has been criticized due to practical aspects, including some of those mentioned in Section 10.2.2, mainly because of the large number of samples to be analyzed to estimate the characteristic performance parameters and to demonstrate ruggedness/robustness. Such analyses are cumbersome, time consuming, and financially draining, and many laboratories do not have the personnel or financial resources to fully implement the Decision. The validation protocols applied for MRMs in the literature, therefore, are often carried out with variations for the sake of practicality and may be useful to the reader in designing similar validation protocols for newly developed or adapted methods. Some of these are discussed in the following sections.

10.4.1 Validation of MRMs Using LC-MS/MS

A number of methods have been published for the simultaneous screening of a range of veterinary drug residues using LC-MS/MS.

The in-house validation and factorial effect analysis of a LC-MS/MS method for the determination of steroids in bovine muscle has been reported.[42] The method

was validated according to Commission Decision 2002/657/EC,[12] Section 3.1.3, "Alternative validation." A matrix-comprehensive in-house validation concept was developed on the basis of a variance component model, based on a fractional factorial design. Parameters calculated included CCα and CCβ, repeatability, reproducibility, recovery, calibration curves, prediction interval, and power curves. The combined measurement uncertainty and the uncertainties of individual components, such as run time, matrix, repeatability, and calibration, were estimated. Four different validation factors were selected, each of which was considered to be difficult to control in routine analysis but which could have a potential impact on the result, and each factor was systematically varied in experiments at two concentrations. The factors selected were different lot numbers of each of the two SPE cartridge types included in the method, different operators, and different storage times of extracts before analysis. The factorial effect analysis showed that the influence of the selected factors on the results of individual analyses was acceptably small.

A validation approach based on Commission Decision 2002/657/EC[12] was also adopted for the determination of 120 analytes, including veterinary drugs, feed additives, and illegal dyes, in eggs, using LC-MS/MS.[43] In this case, a validation concentration of 10 μg/kg was set for drugs without MRL or MRPL. In a quantitative screening method for 128 anti-parasitic veterinary drugs and metabolites in meat using LC-MS/MS,[44] the spiking concentrations chosen for validation were at the MRL and half of the MRL. Generally the MRL was used, but the half-MRL concentration was chosen for some compounds, especially when the MRL was established for the parent drug plus its metabolite, or for the sum of different compounds (e.g., albendazole, fenbendazole, flubendazole). A multi-class, multi-analyte LC-MS/MS method for the analysis of 84 veterinary drugs in chicken muscle[45] was again based on the EU guidelines, but since the aim of the study was the simultaneous quantification of target compounds at the lowest achievable concentration, the authors chose not to calculate CCα and CCβ.

The performance characteristics of an MRM for 29 veterinary drugs spanning three different drug groups, NSAIDS, corticosteroids and anabolic steroids, by UHPLC-MS/MS were determined.[46] The characteristic operational parameters were determined at concentrations greater than or equal to 1.0 μg/kg in muscle and kidney. In-house procedures are described for the characterization of the working range, precision, recovery, interferences, accuracy, limit of quantification, stability of the analytes, ruggedness, selectivity, and measurement uncertainty.

Confirmatory MRMs include the additional requirement of the unequivocal identification of the target analytes at the concentrations of interest. Some validation approaches for confirmatory MRMs employing LC-MS/MS have been described in the literature.

The validation of a streamlined multi-class MRM for the determination of veterinary drug residues in bovine muscle by LC-MS/MS has been reported.[37] A 3-day validation study was conducted in accordance with US Food Safety

Inspection Service (FSIS) protocols for 10 replicates, each at four concentrations (including matrix blanks) each day. Eighteen different sources of bovine muscle were used, and the analyses were carried out by three different analysts. Recoveries, within-day repeatability and between-day reproducibility, matrix effects, limits of quantification, Horowitz ratios, qualitative identification results based on ion ratios, and false-positive/false-negative rates were determined. Of the 131 veterinary drugs investigated (representing at least 13 different classes), 100 of the drugs met the quantification criteria of 70–120% recovery and Horowitz ratio < 1.0, and the remaining analytes could be screened at regulatory concentrations. This is acceptable under the guidelines in CAC/GL 71-2009,[13] which indicates that a validated MRM may include some analytes for which performance requirements for quantitative analysis have been fully validated, while some other analytes may not meet precision and/or recovery criteria for quantitative analysis or the data requirements for confirmation of identity but may be useful for screening purposes.

A MRM for the determination of 115 veterinary drug residues in milk powder, butter, fish tissue, and eggs by LC-MS/MS was described.[41] The in-house validation took into consideration the requirements outlined in Commission Decision 2002/657/EC[12] to ensure the adequate identification, confirmation, and quantification of the target compounds. Identification and confirmation was on the basis of retention times, identification points of each analyte according to EU guidelines, and the relative ion ratios of selected ion transitions. The method was intended for quantification of the target compounds at the lowest achievable concentration, so CCα and CCβ were not relevant and were not calculated, since these are parameters required for regulatory compliance testing.

10.4.2 Validation of MRMs Using Higher Resolution Mass Spectrometry

A method for the quantitative screening of more than 100 veterinary drugs, belonging to 12 different classes, in milk using ultrahigh-performance liquid chromatography–time-of-flight mass spectrometry (UHPLC-TOF-MS) has been reported.[47] The method was validated based on the procedure outlined in Commission Decision 2002/657/EC[12] for quantitative screening and with reference to the CRL Guidance[31] paper published in 2007. Repeatability, within-laboratory reproducibility, accuracy, linearity, CCβ (error probability $\beta = 5\%$), selectivity/specificity, robustness, and stability were determined. The validation concentrations were 0.5, 1.0, and 1.5 times the action limit, which was defined as the MRL or the recommended concentration. For drugs without a MRL or recommended concentration, a specific concentration of interest was defined based on the drug's characteristics or on the MRL in other matrices. The specificity as defined in Commission Decision 2002/657/EC[12] was checked by monitoring for peaks interfering with the drugs of interest in 20 blank samples. The robustness was checked by testing four samples of milk in duplicate, with slight variations in sample pre-treatment and/or extraction. UHPLC-TOF-MS

was also utilized for the screening of urine[48] and for the quantitative determination in meat[10] of residues of more than 100 analytes belonging to different families of veterinary drugs, including both antibiotics and non-antibiotics. The method for urine was designed for screening purposes and the validation focused on the capability to detect traces of the analytes. For validation of the method as applied to meat, Commission Decision 2002/657/EC[12] was used as a guideline, with some minor deviations. The target drugs were divided into two groups. One group included low MRL and banned substances, which were spiked at 1.0, 3.33, 10.0, 33.3, and 100 μg/kg, while the other group consisted of high MRL substances, which were spiked at 10.0, 33.3, 100, 333, and 1000 μg/kg. Each validation series consisted of seven blanks and four of each spiking concentration. Three validation experiments were performed for each matrix ($3 \times 3 \times 27$ samples). This approach allowed the calculation of CCα and CCβ for any MRL within the spiking range. The blank sample analyses allowed the estimation of CCα and CCβ for prohibited substances and those with no MRL. Within- and between-day reproducibilities were calculated according to ISO 5725.[49]

With increasing application of higher resolution mass spectrometry methods, such as the Q-TOF methods referenced earlier, to residue and contaminant analysis, there is a need for new criteria and guidelines on how to validate and control the performance of the methods. The main advantage of these methods is the theoretically unlimited number of compounds that can be screened simultaneously at low concentrations.[46] However, manual analysis of the huge data sets generated by these techniques is very labor intensive and time consuming. The use of libraries to filter data is, therefore, essential for the efficient management of data outputs. The quality of the results of a qualitative MRM is directly related to the performance of the mass spectrometer (sensitivity and selectivity) and the associated data processing software package. The ability of the automated software to match the mass spectrometric (accurate mass and the isotopic ratio pattern) and chromatographic (retention time) information from the sample with the information in a library is crucial.

Selecting optimum operating parameters for data acquisition and processing is particularly critical. Parameters such as thresholds and tolerances for the mass extraction windows and mass accuracy must be carefully chosen in order to minimize the risk of false-negative or false-positive results; selection of the appropriate values is highly dependent on the actual resolution and sensitivity of the high-resolution mass spectrometry instrument, as well as the sample matrix and the target concentrations of the analytes.

The selectivity obtained during data evaluation can increase as the mass extraction window is narrowed. However, care must be exercised during the optimization of the extraction mass window because a very narrow extraction mass window width can lead to false-negative results due to insufficient or unreliable mass assignment obtained during the measurement, while a broad window can cause significant deviations in exact mass measurements, again possibly resulting in a false negative.

It has been reported that thresholds for mass accuracy (± 5 ppm) and mass extraction window (± 0.01 Da) were sufficient for screening purposes for various food contaminants using different types of analyzers.[47, 50] The values must, however, be optimized for each individual method.

10.5 Quality Assurance/Quality Control

The issues relevant to QA and QC in the veterinary drug residue laboratory have been covered in some detail in "Chemical Analysis of Antibiotic Residues in Food."[4] Quality management systems and conformity assessment are generic and apply to the management and functions of the laboratory in general. The reader is referred to the previous volume for further information. The main sections of the previous book that are most relevant for this chapter, which focuses mainly on MRMs, are the sections on analytical method requirements, analytical standards and certified reference materials, proficiency testing, and especially control of instruments and methods in the laboratory.

10.5.1 QC of Analytical Methods

Food safety laboratories must ensure the quality of their analytical results. Effective and traceable monitoring of calibration and test results provides a significant contribution to ensuring the quality of the results, as detailed in Section 5.9 of ISO/IEC 17025:2005.[15] Since standard methods are not systematically available for laboratories performing analyses for veterinary drug residues, the laboratory must develop or adapt, validate, and document methods that are fit for purpose as screening, quantitative, or confirmatory methods. One of the main factors affecting the quality of the final result is the suitability of the analytical method used; ensuring that a method is fit for purpose can be considered a basic QC criterion. Analytical method QC is one of a number of rigorous measures that can be applied to help ensure that the data produced in the laboratory are fit for their intended purpose. Some aspects of analytical method QC that are of particular importance for MRMs are discussed in the following paragraphs.

10.5.1.1 Selectivity/Specificity

One of the key QC elements for an analytical method is demonstration of its selectivity and/or its specificity. The terms selectivity and specificity are often used interchangeably in the literature, which may lead to confusion. Codex Guideline CAC/GL 71-2009[13] defines the selectivity as the ability of the method to unequivocally identify a signal response as being exclusively related to a specific compound and discourages the use of the term specificity. However, Decision 2002/657/EC[12] uses the term specificity, defined as the ability of a method to distinguish between the analyte being measured and other substances, and asserts that this characteristic is predominantly a function of the measuring technique

described but can vary according to class of compound or matrix. Eurachem[1] uses the term selectivity but accepts that specificity may be used, though with the recommendation to use "analytical specificity" to avoid confusion with "diagnostic specificity." Both terms may be encountered in the literature, applied to veterinary drug residue methods.

The ability of a method to discriminate between a target analyte and isomers, metabolites, degradation products, matrix components, and endogenous compounds, all of which may have the potential to interfere in the identification and quantification of the analyte, will define its fitness for purpose. For MRMs, which are designed to efficiently extract and analyze a number of similar compounds, there may be a greater potential for interference between compounds or their metabolites. This should have been considered during method development and validation, but should be borne in mind in the ongoing monitoring of the method's performance during routine application, since there may be variations in actual sample matrices, even though they may be of the same type as those used in the method validation.

The importance of the selectivity/specificity to the quality of the test result depends to some extent on the purpose of the test, as well as the method employed. For example, immunoassays (commonly employed as screening methods for veterinary drug residues) are more likely to be prone to interference from structurally closely related compounds and metabolites in a MRM than high-performance liquid chromatography with ultraviolet detection (HPLC-UV) and are much more susceptible to interference than tandem mass spectrometry. However, this is not necessarily a disadvantage, since the twin purposes of a screening test are the identification of potentially non-compliant samples and the avoidance of false compliant results. In contrast, it is not acceptable for a confirmatory assay to be similarly affected, particularly if the metabolite is not included in the definition of the marker residue.

10.5.1.2 Cross-Talk

Mass spectrometric methods have more recently become the methods of choice for multi-residue screening as well as for confirmatory analyses. When using LC-MS/MS for MRMs, mass spectrometric cross-talk can be a potential problem, especially in confirmatory methods. Cross-talk may occur if two (or more) mass transitions with the same product ions are acquired. If the collision cell is not completely cleared of the first fragment ion within the very short time period between the different transition settings, the product ion from the first transition can cause signal artifacts in the next transition's chromatogram. This can be particularly problematic in MRMs, where a number of closely related compounds, and/or their metabolites, may lead to identical product ions from different precursor ions. When developing an LC-MS/MS method, care must be taken to set up the instrumental conditions and parameters, such as the dwell times, inter-scan delays, and retention time windows for each transition, to avoid or minimize potential cross-talk. As a QC measure during routine application of

the method, it is important to monitor the retention time windows and ensure that chromatographic resolution is maintained within the limits set during method validation to avoid any possible cross-talk interference, which could potentially affect the measurement of ion ratios for analyte identification and confirmation.

10.5.1.3 Analytical Standards

Some areas of laboratory QC will require more effort when using MRMs rather than single residue methods. One such area is the preparation and recording of mixed standard solutions, since their preparation involves many more steps, calculations, and considerations than for single analyte solutions. Relevant issues include the choice of solvents and storage conditions to ensure that the various substances do not precipitate out of solution, the stability of the analytes in solution – the expiry date of any mixed solution must be based on the least stable substance – and system suitability checks encompassing all substances in the method.

10.5.1.4 Control Charts

The design and upkeep of control charts for a MRM also becomes a much more involved task, as does the decision mechanism if one or more analytes begins to drift out of the acceptable limits. For example, considering the time and resources required to prepare mixed standard solutions for calibration or as controls, it may be considered acceptable to make the acceptance limits for some problematic substances more flexible. Any such issues will have to be discussed and agreed between the laboratory management and the QA officer and documented accordingly.

10.5.1.5 Proficiency Testing

Both the Codex guidelines[2, 13] and Commission Decision 2002/657/EC[12] stipulate that official control laboratories must be accredited to ISO 17025. To comply with the ISO 17025 Standard, laboratories must prove their competence by regular and successful participation in internationally recognized proficiency testing schemes. Proficiency testing schemes provide a regular independent assessment of the technical performance of a laboratory and a means of assuring the validity of its analytical measurements, and can be considered as an important aspect of the ongoing verification or validation of the analytical methods applied.

A proficiency testing scheme is a system for objectively evaluating laboratory results by external means, including regular comparison of a laboratory's results with those of other laboratories.[51] Proficiency tests may be performed on a blind analysis basis for compounds within a class or group, and as such are useful for evaluating the performance of MRMs. Although it would be extremely unusual for a proficiency test round to include all compounds within the scope of an MRM, one or more of the analytes representing one or more of the classes of compounds within the scope of the method may be included, and the results can,

therefore, provide a good indication of the performance of the method overall. For example, the Progetto Trieste[52] veterinary drug residues 2016 program specifies that each test material may contain one or more substances from a group included in a published table. The proficiency test round for steroids in liver, for example, could contain a combination the analytes 17α,19-nortestosterone, 17β,19-nortestosterone, 17α-trenbolone, 17β-trenbolone, zeranol, taleranol, diethylstilbestrol (*cis-* and *trans*-DES), and hexestrol at an indicative concentration of $< 10\,\mu g/kg$ or blank. Similarly, the FAPAS[53] proficiency testing round of 06/27/16 for β-agonists in pig liver could contain one or more of the compounds bromchlorbuterol, bromobuterol, cimaterol, cimbuterol, clenpenterol, clenproperol, hydroxyclenbuterol, hydroxymethylclenbuterol, isoxsuprine, mabuterol, mapenterol, ractopamine, salbutamol, salmeterol, terbutaline, tulobuterol, and zilpaterol.

Evaluation of laboratory (and method) performance is usually made by calculating a consensus value for the test material, based on the analytical results obtained by the participants. The calculation of a robust mean, following the elimination of outliers, is the approach most commonly adopted. This approach has some drawbacks – most notably that the consensus mean may be biased when poorly controlled methods are used by a large number of participants, leading to a wide range in analytical results. Alternatively, the consensus mean can be established by reference to the results generated by a subset of the participants known to be experts in the field. Numerical assessment of an individual laboratory's performance is usually effected with a *z*-score. This is essentially a measure of the deviation of the individual result from the consensus value. Underlying the use of the *z*-score is an assumption that individual *z*-scores will approximate to a normal distribution, with a mean of zero and a standard deviation of 1. Usually, *z*-scores are interpreted as $z < 2$, satisfactory; $2 \leq z \geq 3$, questionable; and $z > 3$, unsatisfactory. The numerical values can be either positive or negative, indicating that the measurement was greater or less, respectively, than the consensus mean. The main objectives of a proficiency testing scheme are to help the participating laboratories to assess the accuracy of their test results, which helps fulfill the requirement for ongoing verification or validation of method performance, to highlight possible problems with an analytical method and to prompt the participants with unsatisfactory outcomes to undertake some further investigation of the method and to demonstrate the implementation of corrective action.

Unfortunately, there are relatively few proficiency testing schemes available to analysts working in the field of veterinary drug residues. Some schemes are organized by the EU Reference Laboratory (EURL) network and are frequently available to EU Member States only. Other schemes, however, are open to all participants. The Food Analysis Performance Assessment Scheme (FAPAS[53]; www.fapas.com) is a well-established provider of proficiency testing rounds for a wide range of analytes and matrices, including veterinary drug residues, and is widely used by a range of laboratories across the world. The Progetto Trieste proficiency testing scheme is another available scheme that organizes testing

rounds for both screening and confirmatory procedures.[52] Both of these schemes charge for participation.

10.6 Conclusion

Method validation is necessary to guarantee that analytical results produced by different laboratories are comparable and reliable. The initial validation of a method after its development should not be considered as the final proof that the method is fit for purpose but should provide a baseline for the performance of the method in routine application. Ongoing verification of the method through participation in proficiency tests and inter-laboratory comparisons, and routine QC records of method performance, can be considered as an essential part of the validation of a method and the best indication of its robustness.

The parameters to be characterized during method validation are the same for single residue methods and MRMs. However, when dealing with methods that may cover 100 or more individual analytes, the procedures become more complex, time consuming, and expensive. The existing protocols for validation may need to be amended, or at least viewed as flexible guidelines rather than prescriptive texts, in order to make them applicable in the real world. The goal is not simply to comply with protocols but to demonstrate that a method is reliable and fit for purpose.

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