Veterinary Drugs *and* Growth-Promoting Agent Analyses

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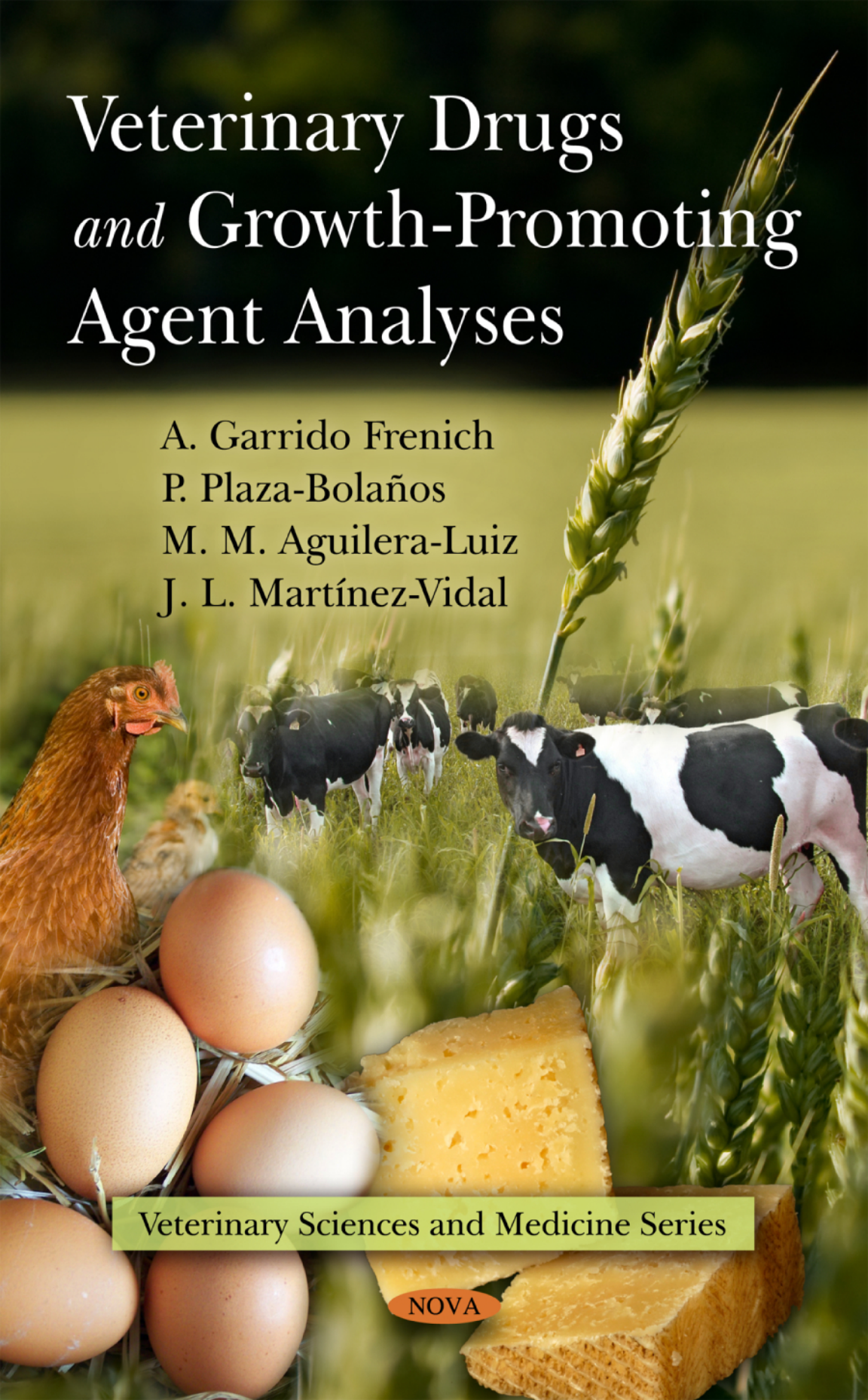

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PREFACE

This book describes the most relevant information related to the chromatographic determination of veterinary drug residues in food, environmental and biological samples, providing the main applications performed in this type of matrices, as well as the main advances related to the chromatographic techniques.

ABSTRACT

Antibiotics have been mainly used in veterinary practices and they are frequently found in food, environmental and biological matrices. Microbiological methods have been traditionally applied because they are easy to perform and are inexpensive. However they do not distinguish among several classes of veterinary drugs and they only provide semi-quantitative analysis, and sometimes give rise to false positives. They are still used due to their simplicity although they have been replaced with chromatographic and electrophoretic techniques, which allow simultaneous determination of several classes of veterinary drugs. In this sense, liquid chromatography (LC) coupled to several detectors is currently widely used and it is a reference technique for the determination of these type of compounds, even replacing gas chromatography (GC), due to they are rather polar, non-volatile and thermolabile compounds.

Although LC has been coupled to conventional detectors such as fluorescence, UV-visible or diode array (DAD), in the last few years mass spectrometry (MS) has been widely used, due to this type of detection provides more reliable identification and confirmation of these analytes than conventional detectors. Basically, triple quadrupole (QqQ) and time-offlight (TOF) analyzers have been mainly used for the determination of veterinary drugs, because confirmation and quantification are included in the same step, although in the last few years an hybrid analyzer, the quadrupole-time-of-flight (Q-TOF) has been an emerging analyzer to be coupled to LC for accurate mass measurement and unequivocal identification of veterinary drug residues and their metabolites.

However, one of the main problems associated with LC methods is the time consumed during the chromatographic analysis. The use of ultra performance liquid chromatography (UPLC) has become very popular in the last few years. This approach is based on the reduction of the particle

size of the stationary phase (< 2 μm), and it allows a decrease in the analysis time and an increase in sensitivity.

This survey describes the most relevant information related to the chromatographic determination of veterinary drug residues in food, environmental and biological samples, providing the main applications performed in this type of matrices, as well as the main advances related to the chromatographic techniques.

Chapter 1

1. INTRODUCTION

1.1. GENERAL OVERVIEW

The use of veterinary drugs (VDs) and growth-promoting agents (GPAs) is widely extended in farming practice. The estimated annual consumption of antimicrobials in the European Union (EU) and in the United States (US) is around 10,000 metric tons in each. About half the total antibiotics in the EU are used for livestock production [1]

These compounds are administered via feed additives and/or drinking water to cattle, ship, pigs, poultry, horses, and in aquaculture (also known as aquafarming). The main purposes of the application of VDs are the prevention of the outbreak of diseases, and in case of disease, for dehydration purposes and to avoid losses during transportation. Moreover, some VDs can be added to the final product in order to increase its freshness, and with respect to the application of these compounds, there is a risk for detecting them if the specified withdrawal times are not respected [2]. On the other hand, GPAs are applied to stimulate the growth in the animals by a variety of mechanisms [3,4].

In consequence, VDs and GPAs can appear in the final food product as residues and they can be included in the food chain. In this sense, the consumption of animal products (e.g. meat, milk, eggs, etc.) containing residues of these compounds for long periods is a matter of concern because of the possible effects on human health. Although it has been demonstrated that certain chemotherapeutics can show carcinogenic properties, the main concern is related to the possible development of resistant bacteria in humans [5,6] by the uncontrolled consumption of antibiotic residues. Moreover, relatively high

amounts of these compounds can provoke allergic reactions in some hypersensitive individuals [2].

A significant problem is due to the available information about the real magnitude of their adverse effects is still scarce. As aforementioned, food could be therefore a significant way to develop resistant bacteria [5], and so that analysis of VDs and GPAs in food is a key point in ensuring food safety. On the other hand, the widespread application of these substances, especially in farming areas, can provoke their transfer and occurrence in the environment, including soils and water. The occurrence of antibiotics in water, even at low concentrations, is of concern. As an example, the increase in bacterial resistance through continuous exposure has been reported in waste effluents from hospital and pharmaceuticals plants [7]. In the last years, the monitoring of pharmaceutically active compounds in the environment has been described as one of the most important problems not only for environmental reasons, but also for food safety concerns since these substances can reach the food chain through environmental paths [8].

Currently, the application of VDs and GPAs is under strict control in the EU or the US. The use of many of these products is prohibited, for instance, the utilization of antibiotics used in human medicine from being added to feed is not allowed in the EU since 1998 [3]. Furthermore, there is an increasing concern related to the occurrence of steroids estrogens (a subclass of GPAs) in the environment since they have been identified as the main contributors to estrogenic activity in sewage effluent and river systems. The presence of compounds showing estrogenic activity in the environment adversely affects the reproductive functions of aquatic organisms. At present there is an increasing interest in the analysis of two of these compounds, boldenone and stanozolol, and a non-steroid anabolic compound, zeranol (structure related to the structure of a mycotoxin, zearalenone), because of non-compliant results found in recent years [4,9-11].

In summary, the analysis of VDs and GPAs in these commodities (foodstuffs and biological tissues and environment) is of relevance in terms of food safety, public health and environmental quality.

1.2. CLASSIFICATION OF VETERINARY DRUGS AND GROWTH-PROMOTING AGENTS

In general, the term "antibacterial agent", also categorized as anti-infectives, anti-microbials or chemotherapeutics, includes natural and synthetic compounds. Natural compounds are well-known as antibiotics (e.g. aminoglycosides, β-lactams, macrolides and tetracyclines (TCs)). These substances show low molecular weight and they are produced by fungi and bacteria, inhibiting the growth of other microorganisms at low concentrations. However, the term "antibiotic" is often utilized as a synonymous with "antibacterial" as well. For this reason, synthetic compounds such as sulphonamides, quinolones, coccidiotats and high-molecular weight natural substances (e.g. polyether antibiotics) can be included within the antibiotic group [5,6]. Apart from antibiotics, there are other compounds that can be applied to livestock, such as anthelmintics and tranquilizers; these two groups can also be considered as VDs. On the other hand, GPAs can be divided into β-agonists and hormones; this last group comprises anabolic steroids (ASs), costicosteroids and thyreostats.

In the present Chapter, a more detailed classification is shown in Figure 1.

1.2.1. Anthelmintics

Anthelmintics are drugs used primarily against intestinal worms, although many of them are also active against lungworms and liver fluke. These VDs can be separated in avermectins and benzimidazoles. Avermectins are complex macrocyclic containing a 16-membered ring. They show different polarity characteristics, for instance, moxidectin is more lipophilic than ivermectin and accumulates in adipose tissue but ivermectin can stay longer half-live in fatty species (pig and sheep). In contrast, eprinomectin is a polar avermectin, with a lower association with lipids [12]. Some of the anthelmintics most frequently applied are levamisole, several compounds belonging to the imidazole group or benzimidazol (albendazole, cambendazole, fenbendazole and thiabendazole) and macrocyclic lactones, such as ivermectin and abamectin (in general known as avermectins) [4,12] (Figure 2).

In particular, benzimidazoles are widely used for prevention and treatment of parasitic infections (e.g. nematodes) in agriculture and aquaculture, although some of them have been used as pre- or post-harvest fungicides.

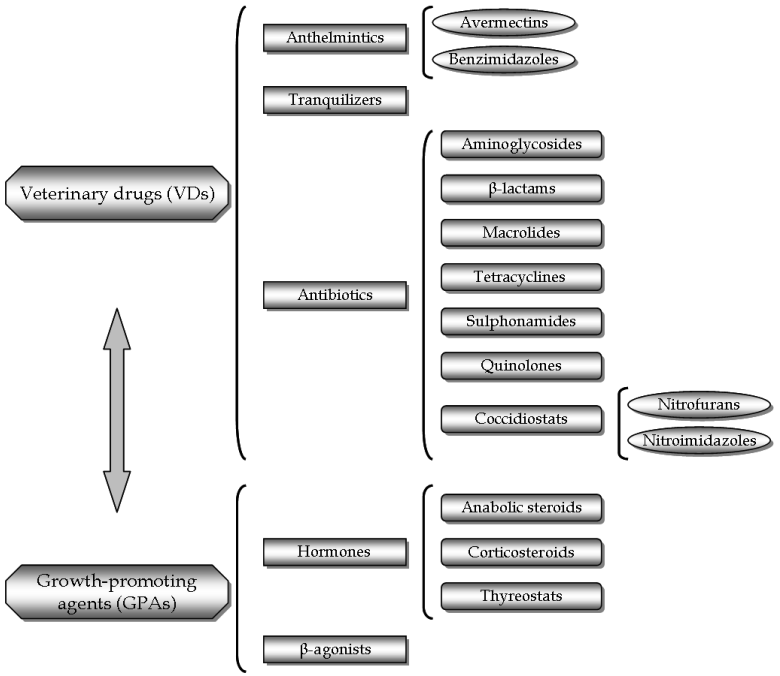

Figure 1. Classification of the VDs and GPAs discussed in the text.

Teratogenic effects and congenital malformations have been described in this sub-class of anthelmintics. It is important to notice that some benzimidazole metabolites or transformation products (TPs) show higher toxicity than the parent compounds (e.g. hydroxymebendazole has been found to be more embryotoxic than mebenzadole in rat) [13].

Anthelmintic residues can be mainly found in milk for which the corresponding withdrawal times have not been properly respected or in liver, which is the target organ for metabolism; muscle, fat and kidney are other relevant samples for these compounds [4].

1.2.2. Tranquilizers

Tranquilizers are administered to animals (mainly pigs) to reduce their stress during transport to the slaughterhouse. This stress can provoke a loss of meat quality and sometimes, premature death. Acepromazine, azaperone, chlorpromazine, propionylpromazine, xylazine and the beta-blocker carazolol are tranquilizers frequently applied for this purpose (Figure 2). Although most

tranquillizers are rapidly metabolized, the short period of time between treatment and slaughtering can result in considerable residue concentrations in meat and possible health hazards for consumers. These residues are concentrated mainly in liver and kidney. It is important to notice that the use of the majority of tranquillizers is not allowed in the EU [4,14,15].

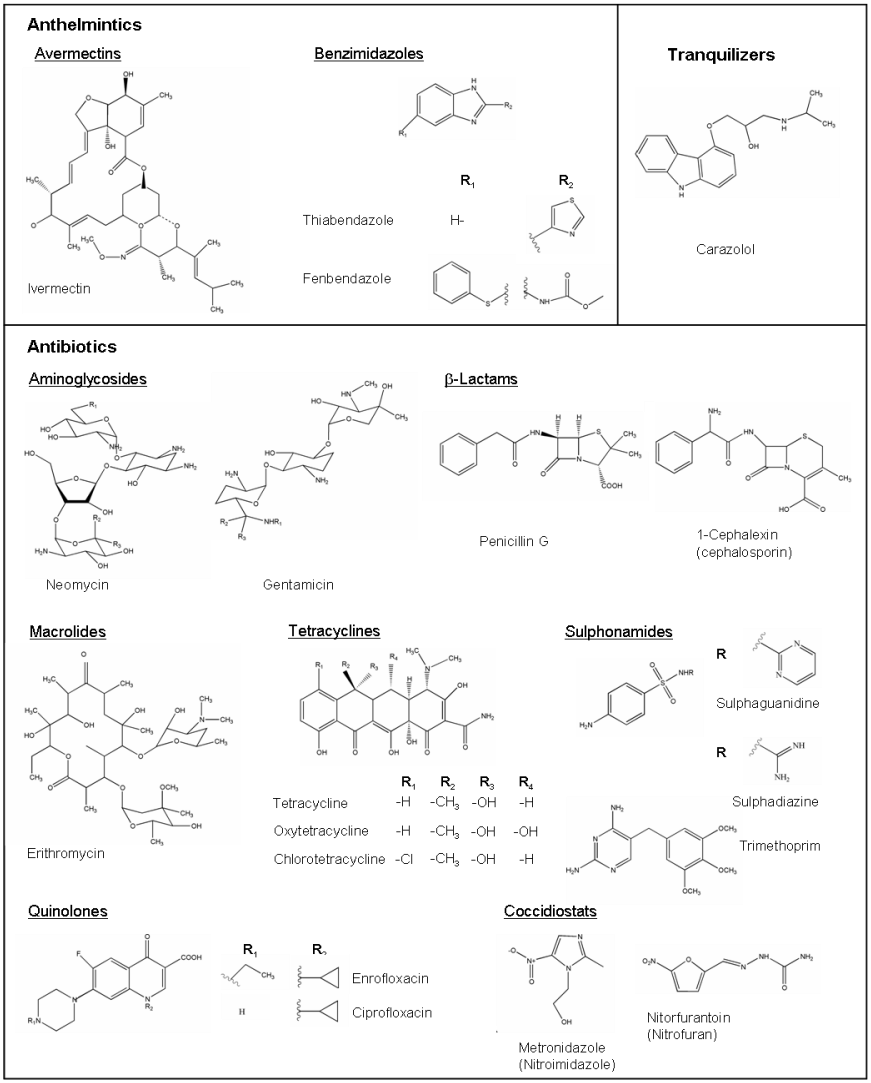

Figure 2. Examples of structures of anthelmintics, tranquilizers and antibiotics discussed.

1.2.3. Antibiotics

1) Aminoglycosides

The chemical structure of the aminoglycosides comprises two (the majority) or more aminosugars linked by glycosidic bonds to an aminocyclitol component, hence they are also known as aminocyclitols. They show basic nature due to the amino groups, whereas the hydroxyl groups are responsible for their high hydrophilic character and poor lipid solubility. These compounds are broad-spectrum antibiotics showing bactericidal activity against some Gram-positive and many Gram-negative organisms. Some well-known compounds are gentamicin, lincomycin, neomycin and streptomycin (Figure 2). Certain aminoglycosides are composed of several members with closely similar structures, such as gentamicin that is a mixture of gentamicins (C1, C2+C2a and C1a) or neomycin (mixture of neomycin B, C and fradiomycin or neomycin sulfate) [4,6,16].

Aminoglycosides are not metabolized: they can be bonded to plasma proteins to a small extent and an important amount of the original compound is excreted via urine or feces [17]. A further description of aminoglycosides characteristics can be found elsewhere [16].

Their use in humans has been limited because of side nephrotoxic and ototoxic effects, but they have been added to feed for prophylaxis purposes and as GPAs. At present, the use of aminoglycosides as GPAs is not allowed in the EU [18].

2) β-Lactams

β-Lactams are probably the most widely used class of antibiotics in veterinary practice. They are used for the treatment of bacterial infections of animals in livestock and bovine milk production [2]. The structure of these compounds shows a characteristic β-lactam ring, existing three classes: penicillins (subdivided in other subgroups), cephalosporins and monobactams (Figure 2). The β-lactam antibiotics show a limited stability: they are thermolabile, unstable in alcohols and isomerize in an acidic environment. For these reasons, precautions concerning pH and temperature have to be taken in their analysis to avoid degradation. Liver and kidney are the target organs for penicillins [4,6].

3) Macrolides

Macrolide antibiotics are macrocyclic lactones whose structure is composed of 12-, 14- or 16-membered lactone ring, to which several amino

groups and/or neutral sugars are bound. They are applied in veterinary medicine to treat respiratory diseases, enteric infections and to promote growth as feed additives. Erythromycin, lincomycin, spiramycin and tylosin are typical examples of macrolides (Figure 2). These compounds distribute extensively to tissues, especially lungs, liver and kidney. In this sense, in certain animals, toxic effects involving the cardiovascular system have been described for tilmicosin. It is important to point out that the commercial products contain small quantities of impurities and TPs (e.g. erythromycin A plus B, C, D, E and F, and several TPs) [2,4,6].

4) Tetracyclines (TCs)

The basic structure of TCs is composed by a hydro-naphtacene framework containing four fused rings and different substituents at the C5, C6 and C7 position on the backbone (Figure 2). These antibiotics show broad-spectrum activity against Gram-positive and Gram-negative bacteria, and they are also applied as additives in feed to promote growth. In this sense, the widespread utilization of these antibiotics makes necessary their monitoring in a variety of commodities since they can lead to an increasing resistance factor. The most utilized TCs in animals are chlortetracycline (CTC), oxytetracycline (OTC), tetracyline (TC) and doxytetracycline (DOX). TCs can immediately chelate to metal ions because of the presence of two ketone groups in the C1 and C11 positions; moreover, they can specifically interact with silanol groups. The isomerization of CTC and DOX to give 4-epi-TCAs in aqueous solutions at pH 2–6 has been reported [2]; in addition, keto tautomers are readily formed in aqueous solutions. As TCs are biosynthetically produced, there are some impurities in the commercial product, such as epiTCs and anhydroTCs [2,4,6,19,20]. These compounds must be taken into account when developing analytical methods for TCs. Anderson et al. [19] thoroughly described the different impurities and other interesting characteristics of TCs.

5) Sulphonamides

Sulphonamides are derivatives of sulfanilamide and show amphoteric characteristics. They comprise a large number of synthetic bacteriostatic compounds which are widely used for prophylactic and therapeutic purposes, and as growth promoters because of their low cost and broad spectrum of activity. Trimethoprim is often administered together with sulphonamides because it acts as a potentiator. Sulphanilamide, which can be considered as the basic structure for the rest of sulphonamides; sulphaguanidine, sulphacetamide and sulphadiazine are some examples of this class of

anbitiotics (Figure 2). The analysis of sulphonamides in foodstuffs is of particular concern since they show potential carcinogenic properties. Furthermore, sulphonamides show sufficient hydrophilic character as to be transferred through the aquatic environment and, therefore the monitoring of these antibiotics in water is also important [2,4,6,21].

6) Quinolones

Quinolones are synthetic antimicrobial agents showing a broad-spectrum activity against both Gram-positive and Gram-negative organisms, as well as anaerobes. They are applied in the treatment of livestock and in aquaculture. Although most quinolones are excreted unaltered by urine, others are metabolized (e.g. enrofloxacin is almost completely metabolized to another quinolone, ciprofloxacin) (Figure 2). The quinolone structure is characterized by a bicyclic structure (in some cases tricyclic). These antibiotics can show acidic or basic character [22], depending on the different substituents. They can show very different physical properties because of the variety of substituents, although most of them show native fluorescence (also named fluoroquinolones) [2,4,8].

7) Coccidiostats

Coccidiostats are widely applied in the prevention and treatment of coccidiosis. In the EU, the use of several coccidiostats as chicken feed additive in certain conditions is permitted in most countries. Feed and egg are the most common matrices in coccidiostat analysis [4].

7.1) Nitroimidazoles

Nitroimidazoles are coccidiostats utilized to prevent and treat certain bacterial and protozoal diseases in poultry and for swine dysentery. These compounds are active against most Gram-negative and many Gram-positive anaerobic bacteria. However, their activity against aerobic bacteria is limited. In relation to the structure, there is a 5-nitroimidazole nucleous which shows substituents on N1 and/or C2 positions. Nitroimidazoles have mutagenic, carcinogenic and toxic properties, and they are rapidly metabolized forming TPs with similar toxic potential as the parent compound. Thus, the EU banned their use in food-producing species [2,4,6]. Plasma and retina have been recommended as target matrices for the residue control of nitroimizadoles since these compounds are stable during sample storage and they can be detected for a long period after withdrawal time. On the contrary, for other matrices such as turkey muscle, non-homogeneous distribution of analytes and

rapid decline in analytes at storage has been observed [23]. As a solution, immediate freezing of the muscle must be performed to avoid the degradation of the nitroimidazoles and their hydroxy-metabolites; moreover, lyophilization has been recommended to achieve higher homogeneous muscle samples [24]. The most popular nitroimidazoles used as additives are metronidazole, dimetridazole, ipronidazole and ronidazole (Figure 2) [25].

7.2) Nitrofurans

Nitrofurans are synthetic chemotherapeutic agents which have been applied as food additives for the treatment of gastrointestinal infections in cattle, pigs and poultry. Nowadays, their use in food-producing animals is not allowed in the EU because of the mutagenic and cytotoxic activity observed in certain organisms. Nitrofurans are rapidly metabolized producing protein-bound TPs that are highly persistent in edible animal tissues. Only the TPs can be found in muscle, kidney, liver or egg as tissue-bond residues [26]. Thus, the formed TPs are more suitable as marker of use for the parent compounds. It is important to notice that the TPs of furazolidone, furaltadone, nitrofurantoine, and nitrofurazone still possess certain chains of the parent compound which can result in a potential toxic entity in case of release from protein-binding under acidic conditions in the stomach (Figure 2) [2,4,6].

1.2.4. Hormones (GPAs)

In general, hormones are used in livestock to increase the rate of growth of the animals and to help to protect against stress. The application is commonly performed by an implant in the ear or via feed. Currently, the use of hormones to improve animal growth is prohibited in the EU [4]. In the monitoring of these substances, urine and manure can be used in vivo, whereas liver, kidney, hair, fat or meat can be utilized after slaughtering [4,27].

1) Anabolic Steroids (ASs)

Steroids comprise a large group of natural and synthetic compounds showing a similar structure made of 17 carbon atoms organized in four rings (Figure 3). Most synthetic ASs derived from the natural steroid, testosterone. These substances are responsible for regulating a variety of processes, such as those involving sexual organs (e.g. androgens and estrogens) or the development and distribution of muscle and fatty tissues. Steroid hormones can have influence on some meat tenderness parameters. Despite the use of

hormones in food-producing animals is banned in the EU, steroids are still being used. On the contrary, in other countries there are only some restrictions related to the use of these substances as GPAs.

2) Corticosteroids

Corticosteroids are anti-inflamatory substances that are not permitted in the EU as GPAs. This group comprises two sub-classes: mineralocorticoids and glucocorticoids, which are naturally produced in the adrenal cortex from cholesterol. Corticosteroids show a basic structure similar to that of the ASs, showing four rings and different substituents. Some examples of natural corticosteroids are cortisol and cortisone; dexamethasone and prednisolone are well-know synthetic corticosteroids (Figure 3). Urine, liver or meat are interesting matrices for analysis of these compounds [4,28,29].

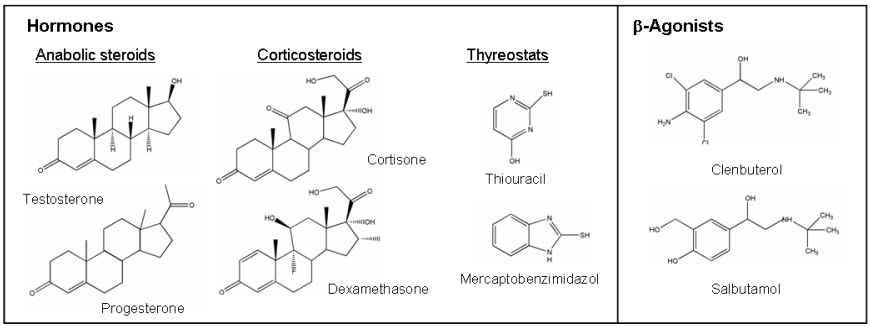

Figure 3. Examples of structures of hormones and β-agonists discussed.

3) Thyreostats

The term "thyreostats" is currently used to refer to a complex group of substances that inhibit the thyroid function. As a result of their application, there is a decrease in the production of thyroid hormones triiodothyronine and thyroxine. In the past, they were also named as "anti-hormones", although this nomenclature was not correct since anti-hormones block the action of a hormone, not its production. They can be divided into two groups: xenobiotic and natural occurring sulfur compounds, showing high polarity, low molecular weight, amphoteric characteristics, and a common N-C-S sequence. Moreover, other small inorganic molecules, such as ClO_4^- or SCN, Li^+ ions and certain VDs (e.g. sulphonamides) may present thyreostatic activity [30]. Thyreostats have been applied as GPAs because they produce high water retention in edible tissues and an increased filling of the gastro-intestinal tract, which

results in a reduction of meat quality. Besides, these compounds may be harmful to human health. Although the ban on using anabolic steroids and corticosteroids in animals is not world-wide accepted, there is an international agreement in relation to the ban on the application of thyreostats in livestock [4,31]. The most important thyreostats are 4(6)-*R*-2-thiouracil; tapazole and mercaptobenzimidazole (Figure 3) [32].

1.2.5. β-Agonists (GPAs)

β-Agonists are substances that promote lipolysis in muscle tissue; important examples of this group are clenburetol and salbutamol (Figure 3). They can be roughly classified as clenbuterol-related compounds (showing anilinic moieties) and salbutamol-related compounds (showing phenolic, catecholic or resorcinolic moieties). The use of these compounds in meat-producing animals affect growth and carcass composition, producing a high increase in muscular mass (up to 40 %) and a reduction in fat accumulation (up to 40 %). Gowik et al. [33] reported that these substances accumulate in the retina of calves, pigs and turkeys; therefore, retina is a matrix of interest for the residue control of β-agonists. In this case, while the therapeutic treatment of cattle with respiratory diseases is permitted, the use of β-agonists as GPAs in cattle is forbidden in the EU [4,29,34].

1.3. LEGISLATION: A BRIEF SUMMARY

According to the problematic previously shown, several measures have been taken by the authorities with the aim of controlling the presence of VDs and GPAs in food products and the environment.

As explained below, the EU has strictly regulated controls on the use of these products, particularly in food-animal species, by issuing several Regulations and Directives. In this sense, European Commission (EC) has established maximum residues limits (MRLs) for the different combinations VD/GPA-food for all member states. These MRLs are the levels of residues that could safely remain in the tissue or food product derived from a food-producing animal that has been treated with a VD and GPA. These residues are considered to pose no adverse health effects if ingested daily by humans over a lifetime.

The discoveries about the negative effects on human health of these veterinary drug residues brought along the development of Directives for control of supply of these compound to animals. In 1981, the first legislative documents (Directive of Council 81/851/CEE and 81/852/CEE) that established a common legislation for the different states belonging to the former European Economics Community (CEE) were developed. These documents showed the need for the establishment of a Regulation for the production and distribution of VDs. It also established protocols for the analysis and control of the production and marketing of these substances. These Regulations have been modified by other Regulations (CEE) with the objective of eliminating the handicap for the free and safety marketing of VDs among different EU members. However, all the developed modifications had to be brought together in a single document. In 2001, the European Parliament approved the Directive 2001/82/CE which established a common code about VDs. This Directive has been modify in two occasions, in 2003 with the Common position (CE) 62/2003 and in 2004, with the Directive 2004/28/CE.

The increasing interest in checking and controlling the use of VDs resulted in the set- up of the European Agency of evaluation of medicaments (EMEA) in 1995. The main responsibility of this Agency is the protection and promotion of public and animal health, through the evaluation and supervision of medicines for human and veterinary use. It contributes to the international activities of the EU through its work with the European Pharmacopoeia, the World Health Organization (WHO), and the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) and the International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Medicinal Products (VICH) trilateral (EU, Japan and USA) conferences on harmonization, among other international organizations and initiatives. Furthermore, it is important to mention the development of the "White Paper on Food Safety" in 2000 by the EC. This document was developed in order to try to guarantee a high food safety by the description of a number of actions that permit the modernization and complementation of the European legislation in terms of food consumption.

In this way of continuous improvement, another important measure was the establishment of European Food Safety Authority (EFSA) in 2002, after several food crises that emerged at the end of the 90´s. Nowadays, this institution in an essential tool for the coordination and integration of the European safety politics at European level.

In relation to the establishment of MRLs, in 1990 the first legislation document in relation to the control of veterinary drug residues was developed. The Directive of the EU Council 2377/90/CEE described the procedure for establishing these MRLs for veterinary medicinal products in foodstuffs from animal origin. From this Directive, the number of fixed MRLs has been continuously growing until the present. Numerous modifications have been developed with the purpose of controlling the new VDs and the different matrices in which they can be present. Some relevant Directives are shown below:

- Directive 92/74/CEE which established complementary regulation about homeopathic veterinary drugs.
- Directive of the Council 96/22/CE which prohibition the employed of growth promoting agents (β-agonists, hormones…).
- Regulation (CE) 324/2004 changing the Annex I of Regulation (CEE) No 2377/90.
- European Commission (EC) 17/04/2007, COM (2007) 194 final, 2007/0064 (COD) Proposal for a Regulation of the European Parliament and of the Council laying down Community procedures for the establishment of residue limits of pharmacologically active substances in foodstuffs of animal origin, and repealing Regulation (EC) 2377/90.

At this point, it is important to mention two concepts that have been developed after the first concept of MRL: the minimum required performance limits (MRPLs) and zero tolerance. On the one hand, there are VDs for which any MRL has been established in the EU. In this context, the EC has established MRPLs for these substances by the Decision 2002/657/EC. This level is the minimum concentration of residues of banned substances that an analytical method must be able to determine, with specified degrees of accuracy and precision. The first MRPLs were published in Annex II of Commission Decision 2003/181/EC, and the last modification was set in the Decision of the Commission 2004/25/CE.

On the other hand, the EU has established the principle of zero tolerance for certain residues of veterinary medical products in foodstuffs. Zero tolerance apply to all substances which are either not approved or whose use is explicitly prohibited. This last concept is applied to all substances in Annex IV and to all substances which are not listed in Annexes I-III in Regulation (EEC) 2377/90.

Finally, the EU has recently developed a new Directive (2009/9/EC) that concerns analytic norms and protocols (e.g. toxic- pharmacologic and clinic testsfor VDs), being the last modification developed since the first Regulation in 1981.

The EU is not the only institution that has established a variety of MRLs. In different countries, legislations, rules and regulations have been established regarding human health, food safety and environmental protection. In the US, MRLs or tolerances for VDs/GPAs in foodstuffs can be found in the Code of Federal Regulations, namely Title 21 (Food and Drugs, 500-600) [35]. In Canada, the Department of Health is in charge of administering a variety of pieces of legislation, and develops and enforces regulation. This Department consults with the Canadian public, industry, non-governmental organizations (NGOs) and other interested parties in the development of these rules, and it has also established MRLs for monitoring of residues of VDs in food [36].

Chapter 2

2. CHROMATOGRAPHIC TECHNIQUES

2.1. LIQUID CHROMATOGRAPHY

Liquid chromatography (LC) is one of the most powerful tools in Analytical Chemistry for the analysis of a wide range of organic compounds at trace levels. LC is greatly applied in the analysis of VDs and GPAs residues in a variety of matrices, including foodstuffs, environmental samples and biological fluids. In general, LC is suitable for the direct determination of polar compounds, such as most veterinary residues, and less polar compounds but LC-amenable after appropriate derivatization.

A variety of classical detectors can be used coupled to LC systems, such as UV and fluorescence detection (FLD). However, the use of mass spectrometry (MS) detection is currently widespread because of its advantages in the analysis of residues at trace levels:

(i) MS is greatly recommended as the most adequate detection system for the analysis of residues and organic contaminants,
(ii) MS usually provides an increase in selectivity and sensitivity in comparison to classical detectors, as well as the identification and confirmation of the compounds in a single stage, and
(iii) MS permits the increase in sample throughput because it is able to avoid a significant number of interferences, diminishing in certain cases the sample pre-treatment.

Briefly, separation of VDs and GPAs by LC is usually carried out with C18 columns (reversed-phase), and using a variety of mobile phases,

depending on the detection system employed. In MS, mixtures of water-methanol and acetonitrile (ACN) at different pH are normally used, avoiding mobile phases containing non-volatile compounds, such as phosphate buffers that can clog at the interface and produce build-up of deposits in the ion source. Acetate, formate or formic acid is also added to the mixture to improve the ionization. The selection of the most appropriate mobile phase is important in order to obtain reproducible retention times, adequate peak shapes and good sensitivity and, in MS, good ionization efficiencies [37]. A trend to use microbore LC columns (about 1 mm internal diameter, (i.d.), compared with conventional 2-4-mm i.d. columns) has been recently pointed out since it provides higher separation efficiencies and sensitivities, reducing solvent consumption and cost [37].

Nevertheless, the most outstanding approach in LC is the ultra-high pressure LC (UHPLC) or ultra performance LC (UPLC). This recent LC is based on the use of columns with a particle size lower (< 2 μm) than that of the typical columns (< 3-10 μm); working at higher pressures (6000-15000 psi) and requiring a special pump system able to work at that pressure range.

UPLC shows a variety of advantages in the analysis of VDs and GPAs and valuable characteristics for routine laboratories, such as: (i) reduction of the required time in the chromatographic separation, i.e. many UPLC methods perform the chromatographic separation in running times no longer than 10-15 min; and (ii) the decrease in peak width: UPLC peaks are comparable to GC peaks. In addition, the coupling of UPLC to MS systems can increase the possibilities of this technology and the development of fast analytical methods since complete separation of the analytes may not be always necessary thanks to the use of deconvolution software.

2.1.1. Anthelmintics

Avermectins

Avermectins residues in sample extracts are typically determined by LC techniques; separation of these compounds by reversed-phase is carried out in most methods. The utilization of C18-based columns using high percentages of organic modifier (≥ 90%) in the mobile phase is described as an easy methodology to separate avermectins. The use of buffer-based eluents is not required for most avermectins; adequate separation can be achieved using organic solvent/water mobile phase mixtures [12], which results very convenient for MS detection.

Detection: UV, FLD and MS

Avermectins can be detected by UV because of their strong chromophore characteristics; on the contrary, they cannot be detected by FLD without previous derivatization, although greater sensitivity and selectivity in comparison to UV can be obtained by FLD with pre-column derivatization. 1-methylimidazole (MI) and trifluoroacetic acid anhydride (TFAA) are typical reagents that produce fluorescence avermectin derivatives. It is important to remark that the use of FLD for the analysis of avermectins is still the most commonly applied technique, and bearing in mind limits of detection and quantification, the sensitivity provided is superior to MS detection. However, certain problems have been described when using FLD, such as the instability of fluorescent derivates (e.g. eprinomectin), slow and incomplete formation of derivatives for some compounds and the low reproducibility of the results, although this can be solved by applying on-line derivatization [12,38]. Besides, the combination of temperature and addition of acetic acid in the derivatization has been reported as an effective method to increase the stability of eprinomectin products [39].

In relation to MS detection, no derivatization is required, and thus this is one of its advantages. A number of different MS ionization sources have been applied to these residues, including electrospray ionization (ESI), atmospheric pressure chemical ionization (APCI) and atmospheric pressure photo-ionization (APPI). However, positive ESI (+) and positive/negative APCI (+/-) are most used; it is reported that no-discharge APCI (+) provides a much more intense signal than APPI (-). A more detailed description of the fragments obtained with each source can also be found in literature [40].

Benzimidazoles

The separation of benzimidazoles is mostly carried out by reversed-phase (C18 or C8) LC; alternative columns types such as silica or cation exchange have been recently used. The application of GC-based methods is very difficult due to the basic nature and low volatility of these VDs, although thiabendazole and triclabenzadole, which show higher volatility, may be determined by GC without any previous derivatization. Nevertheless, the use of LC for this kind of compounds is currently the first choice [13].

Detection: UV and MS

The determination of benzimidazoles can be carried out by UV detection since they show a strong UV chromophore. Moreover, certain compounds (namely, albendazole, cambendazole, flubenzadole, triclabenzadole,

thiabendazole and their TPs) possess naturally fluorescence, and thus FLD can also be applied. FLD can result more sensitive and selective than UV but the latter is more commonly used owning to its wider range of applicability [13].

Due to the widespread use of MS in trace analysis, this class of detection is obviously also utilized, especially in complex matrices; ESI (+) and triple quadrupole (QqQ) analyzers are mainly preferred.

2.1.2. Tranquilizers

The analysis of tranquilizers is mostly carried out by LC; the use of GC-based methods is very scarce and not recent [15,41]. Tranquilizers show strong basic properties and tendency to bind to silanol groups in silica-based materials used in reversed-phase columns and in solid-phase extraction (SPE) sorbents. For these reasons, problems with poor recoveries and unresolved peaks have been reported [14]. Several solutions can be used to deal with these drawbacks, such as the addition of trifluoroacetic acid (TFA) to the mobile phase or the use of phenyl-based columns. A significant improvement can be achieved by the use of well-deactivated reversed-phase columns (also end-capped columns) [14,42]. Furthermore, the availability of silica reversed-phase columns showing stability in alkaline conditions allows the use of mobile phases at high pH values, for instance, including 1-methyl-piperidine and ammonia at pH 10-11 [14].

Bearing in mind that tranquilizers are mainly basic drugs, the use of salts showing chaotropic effects in reversed-phase systems is described. The use of this class of salts (e.g. $NaClO_4$ and $NaPF_6$) as modifiers of the mobile phase causes an increase in the retention time, as well as a possible improvement in peak shape, and efficiency and selectivity separation [43].

Detection: UV, FLD and MS

Currently, the use of UV detection is decreasing as a consequence of the identification/confirmation features of MS detection, following the general trend. LC–UV and LC–FLD can be used for screening purposes [15] or direct quantification [42,44]. In MS, QqQ is widely used together with ESI (+); although the application of APCI (+) is also possible [45]. Due to its volatility and good ESI sensitivity, the use of ammonia-based eluents in MS is very convenient when alkaline pH in the mobile phase are required. In these conditions, the APCI source is not as adequate as ESI due to the lower analyte signals obtained [14].

2.1.3. Aminoglycosides

Aminoglycosides are very polar compounds due to the high number of amino and hydroxyl moieties. They show a low solubility in organic solvents [2] and the contact with glass should be avoided in order to prevent losses by adsorption (e.g. using other materials, such as PTFE) [1].

The separation of these compounds by reversed-phase LC is difficult because their polar nature impedes the necessary interaction with the stationary phase of alkyl-bonded silica columns, such as the typical C18-based columns [1]. Three alternatives can be used to solve this problem: ion exchange LC, ion-pair LC and pre-column derivatization.

Ion-pair LC on C18 columns is performed in the presence of an alkylsulphonate as the ion-pair reagent [4]. There is a variety of ion-pair reagents used, such as trichloroacetic acid (TCA) or sodium 1-hexane-sulfonic acid (HSA). Ion-pair LC shows several advantages compared to strong cation-exchange (SCX) columns, such as greater efficiencies, easier control over selectivity and resolution, and reversed-phase columns offer higher stability and reproducibility. Thus, ion-pair LC is normally preferred to SCX [16].

Pre-column derivatization is applied to obtain less polar compounds than the parent compound that can be adequately separated by reversed-phase LC. Ortho-phthalaldehyde (OPA, also known as 1,2-phthalic dicarboxaldehyde) and 9-fluorenylmethyl chloroformate (FMOC) have been used as derivatizing agents [4,46]. Aminoglycosides show different sites where OPA can react by introducing a chromophore group in the presence of a thiol. However, the OPA derivatives are relatively unstable when there is an excess of reagent, and thus, the experimental conditions must be carefully controlled in order to reduce the possible formation of alternative derivatives [47]. Stability problems are overcome with the use of FMOC as derivatizing agent [17]. Other suitable agents used in pre-derivatization are 1-fluoro-2,4-dinitrobenzene (DNFB) and 2,4,6-trinitrobenzene-1-sulphonic acid (TNBS) for UV, and fluoroescamine for FLD [16,48].

In spite of the extensive use of typical C18 columns for ion pair LC, the use of capillary reversed-phase C18 columns showing 1-cm length has been described. On the other hand, hydrophilic interaction chromatography (HILIC) has been proposed as an alternative to ion pair LC in order to improve sensitivity when using ESI in MS [49]. This modality is similar to normal-phase LC, but it uses polar mobile phases that are compatible with ESI. Despite a more adequate separation has been reached for gentamicin and neomycin, HILIC can show some problems related to the reproducibility of the

retention times [50]. Furthermore, this kind of LC can fail in the application to complex matrices (e.g. food), even with previous successful results in solvent [51]. Another alternative to C18 columns is the use of stationary phases based on ligands with amide groups, and the end-capping of trimethylsilyl groups to avoid the appearance of tailed peaks. This column has been used for ion-pair LC [52].

Detection: FLD, CLD and MS

Aminoglycosides cannot be detected by either UV or FLD without previous derivatization due to the lack of chromophore or fluorophore groups. Derivatizating agents, such as 1,2-naphthoquinone-4-sulphonic acid (NQS), ninhydrin and belzoyl chloride have been used [16,53], apart from the agents aforementioned. One of the most popular methods is based on the separation by ion-pair LC followed by post-column derivatization with OPA and FLD [17,54].

The utilization of chemiluminiscence detection (CLD) has also been described. The aminoglycosides show inhibitory effect on the CL reaction between luminol and hydrogen peroxide catalyzed by copper (II) [17].

The need for derivatization when analyzing amynoglicosides by UV or FLD is an important drawback for the use of these detection systems. On the contrary, these compounds can be directly analyzed by MS. In ion-pair LC, ion-pair agents compatible with MS must be employed, such as perfluorinated compounds (e.g. heptafluorobutyric acid (HFBA) or pentafluoropropionic acid (PFPA)) as they show high volatility. Moreover, the use of TFA has been recently recommended in order to reduce ion suppression [1,4].

In general, aminoglycosides are detected by ESI (+), although the use APCI has been reported [55]. These compounds produce $[M+2H]^{2+}$ pseudo ions, except spectinomycin and streptomycin, which produce $[M+H_2O+H]^{+}$ and $[M+H_2O+H]^{2+}$, respectively. Additional information about MS data related to these compounds can be found elsewhere [6,29].

2.1.4. β-Lactams

LC is the technique that best suits for the analysis of β-lactams. However, it is important to consider several questions related to the determination of these compounds. β-lactams are thermolabile and show low stability in organic solvents such as methanol (MeOH) or ACN due to the presence of an unstable four-term ring in their structures which produce their degradation by heat and

in the presence of alcohols [2,4]. Penicillins are also readily isomerized in an acidic ambient, and they can undergo transformation into their epimers in basic medium by catalization of heavy metals ions. In order to avoid these problems, it is recommended the silanization of the glassware when analyzing β-lactams [1,56].

The chromatographic separation of these compounds is normally carried out by reversed-phase LC on C18 bonded silica columns. The use of ion-pair LC is also described employing octanesulphonic acid [57], cetyltrimethyl-ammoniumchloride [58] and sodium 1-decanesulfonate [59]. Alternatively, it is possible to perform on-line separation and extraction procedures by the combination of two columns, such as a polymeric-based column (e.g. Oasis hydrophilic-lipophilic balanced (HLB) sorbent) for extraction/clean-up and a conventional column for separation/analysis [60].

Despite the aforementioned stability problems, MeOH is a very popular mobile phase solvent in LC–MS analysis of β-lactams. A slower degradation rate is observed when using MeOH/water mixtures; higher stability was found in water, ACN, and ACN/water solutions [61].

Detection: UV and MS

For the detection of β-lactams, ion-pairing LC–UV or FLD has been used in the past years. Currently, LC–MS is the selected technique as in many other VDs [4]. These antibiotics can be detected by UV without previous derivatization [57-59,62], although in some cases a previous derivatization is carried out (e.g. 1,2,4-triazole containing mercury (II) chloride [63]) to enhance UV sensitivity and specificity in the determination process.

In MS, the QqQ analyzer is mostly used, although the ion-trap (IT) analyzer is also suitable for this aim [64,65], and using ESI as ionization technique. The positive mode is more adequate for the ionization of penicillins than ESI (-), whereas the opposite is true for cephalosporins [1]. In general, ESI (-) is the most sensitive mode for the analysis of β-lactam antibiotics; however, the use of ESI (+) is preferable whenever amphoteric compounds are included in the determination [4].

2.1.5. Macrolides

Reversed-phase LC is the most common approach for the separation of macrolides. In the past, the separation of these compounds was problematic because of peak tailing problems due to the interaction with residual silanol

groups. Nowadays, the widespread use of end-capped silica based C18 columns, or in general alkyl-bonded silica columns, has fixed this problem [66]. Less common is the use of alternative columns, such as cyanopropil silica or polymer coated alumina columns [67]. An unusual modification of conventional separation of macrolides is the employ of a Biomatrix column. This column is placed before the analytical reversed-phase column; it removes the remaining macromolecules from the matrix, permits the concentration of the analytes and increases the column lifetime [29,67]. More recent is the application of sub-2-μm particle columns (e.g. UPLC technology), which has gained in popularity since it has permitted clearly to increase resolution and to reduce the running time [68-70].

In general, separation is performed under acidic conditions, although neutral media is recommended for erythromycin, due to its instability in acidic medium [4]. In certain cases, hydrophobic ion-pair reagents (e.g. HFBA, TFA or non-afluoropentanoic acid, NFPA) can be used to improve peak shape, although it is known that these compounds can increase ion suppression effects and decrease sensitivity, especially for those nitrogen containing compounds [68].

Detection: UV and MS

The detection of macrolides is possible thanks to the presence of suitable chromophore groups in the structure of certain compounds, such as spiramycin, tylosin and tilmicosin. However, many of them do not present any chromophore, and derivatization is therefore needed for UV detection. For this aim, FMOC and cyclohexa-1,3-dione (CHD) can be used as derivatizing agents [4,67,68]. Although classical detection was used in previous years [66,71-74], the more recent methodologies for the analysis of macrolides are based on MS detection due to the well-known identification/confirmation capabilities. ESI and APCI are the most common ionization techniques employed for LC–MS interfaces, although the use of ESI is considerably higher. In positive mode, macrolides normally produce $[M+H]^+$ ions; those antibiotics containing two nitrogen atoms can form $[M+H]^+$ and $[M+2H]^{2+}$ ions, and those analytes with three nitrogen atoms can also yield $[M+3H]^{3+}$. Moreover, in MS/MS experiments, *m/z* 175 and 176 are typically produced due to the loss of two characteristic sugars, desosamine and cladinose [68]. A more detailed description of different product ions is already reported [6]. In low-resolution mass spectrometry (LRMS), the QqQ analyzer is normally used; the utilization of IT analyzers is scarce [75]. Recently, the application of

high-resolution mass spectrometry (HRMS) has been described using TOF instruments [69].

2.1.6. Tetracyclines (TCs)

TCs are typically analyzed by LC; these compounds are thermolabile, their solubility in organic solvents (e.g. chloroform, ethyl acetate, and dichloromethane) is scarce, and they are insoluble in satured hydrocarbon solvents, such as *n*-hexane. Separation of TCs normally involves the use of reversed-phase columns, mainly C18 or C8-based columns. However, due to the presence of two ketone groups, several problems hinder the analysis of these antibiotics. TCs are able to chelate to metal ions, and thus they can produce complex with trace metals present in both the analytical column and the mobile phase [4,19]. The numerous double bonds and O and N substituents in TCs are possible sites of interaction; in this sense, TCs interact with silanol groups in silica packing materials, enhancing peak tailing. Therefore, end-capping reversed-phase columns are preferred owning to the minimal silanol interactions [19]. The performance of a column washing with an ethylenediaminetetraacetic acid (EDTA) solution prior to use is described to remove the peak tailing duet to the metal impurities permanently [76]. Alternative columns and stationary phases have been proposed to overcome these drawbacks, such as polymeric, phenyl or amide C16 columns. Polymeric-based phases remove silanol and trace metal interactions, whereas amide columns have no silanols available for interaction [19]. In the case of phenyl columns, they can increase retention and resolution among TCs and their impurities and/or matrix interferences [19]. Other classes of columns frequently used for the separation of other residues, such as UPLC (sub-2-µm particle columns) or monolithic columns [77] have scarcely applied in TC separation.

An additional problem is the frequently observed peak fronting in CTC and DOX, which can be reduced optimizing the column temperature; besides, quantification can be complicated owing to the formation of the TC tautomerization and epimerization products that elute before the original compounds [2,4].

An adequate selection of mobile phase may be considered as an important variable because TCs can form unexpected complexes with compounds present in the composition. Most of methods report the use of buffer and ACN-based eluents; less frequent is the utilization of MeOH and only as

organic modifier or in ACN/MeOH mixtures [19]. In order to separate OTC and its 4-epimer, the use of eluents containing tetrahydrofuran as organic modifier and high column temperature (60ºC) is described [78]. The utilization of ion-pair LC using tetrabutylammonium hydrogen sulphate, octanesulfonate sodium salt and EDTA in the eluents is also reported [79]. In relation to peak tailing problems, the use chelating agents such as oxalic acid and EDTA in the mobile phases or in any step of the sample preparation procedure is widespread [2,4]. However, the use of non-volatile compounds in the mobile phase when using MS can result in the rapid contamination of the sample cone and clogging problems. Moreover, both oxalic acid and EDTA strongly reduce the ion signal intensity, and thus, volatile buffer eluents (e.g. ammonium acetate, formic acid) are required [2,4]. Nevertheless, the use of the APCI source is reported in presence of oxalic acid by the application of high temperature that decomposes this reagent during the process [80]. A further description of mobile phases and chromatographic conditions can be found in literature [20].

Detection: UV, FLD, CLD and MS

A variety of detections systems have been employed successfully for the determination of TCs. In UV detection, these antibiotics show a strong absorbance at 360-365 nm and also at 270 nm in acidic conditions, which minimize the occurrence of mixed separation mechanisms. Different acids can be used for this purpose, including phosphate and acetate buffer, citric acid, acetic acid, *o*-phosphoric acid, perchloric acid, or TCA, but oxalic acid and formic acid are mostly used as they can also reduce silanol and metal interactions [19,20]. In relation to FLD, TCs are determined in neutral or slightly basic conditions and in presence of certain cations, such as Mg^{2+}, Ca^{2+}, Cu^{2+}, or Al^{3+}. The λ ranges commonly applied are 380-390 nm and 490-520 nm for excitation and emission, respectively. It has been reported the increase in TC fluorescence intensity with the addition of ACN in aqueous solution, and the use of ion-pairing agents [19]. TCs can also be monitored by CLD, although it is rarely applied. Chemiluminescence can be obtained as a result of the reaction between acidic $KMnO_4$ and Na_2SO_3 at λ=450-600 nm. A second strategy is based on the reaction of $KMnO_4$ and Na_2SO_3 in presence of β-cyclodextrin [81].

Currently, MS is considered as the reference technique for the analysis of TCs at trace levels in all kind of matrices. QqQ and ESI (+) are normally employed, although the use of IT [82,83] and APCI [80] are also reported. In general, all TCs show two suitable precursor ions, $[M+H-NH_3]^+$ and [M+H-

NH_3-$H_2O]^+$, except for doxycycline, in which the precursor ion is normally $[M+H]^+$ [29]. In spite of the majority of methods apply MS^2 (MS/MS), the use of MS^3 is described as an interesting alternative to distinguish isobaric ions; for instance, chlortetracycline and one of its TPs show the same $[M-H]^+$ (*m/z* 479), and also identical product ion corresponding to a neutral loss of NH_3 (*m/z* 462). However, the application of an extra MS experiment makes CTC distinguishable since it exhibits a differentiating ion corresponding to the neutral loss of H_2O (*m/z* 444) [84].

2.1.7. Sulphonamides

Frequently, because of the more marked acidic properties of sulphonamides, these compounds are normally separated by reversed-phase LC using C18 columns in ion-suppression conditions (when using MS detection) by adding formic acid, which assists the ESI process [6]. Alternatively to reversed-phase LC, restricted-access media liquid chromatography (RAM-LC) can also be applied. RAM columns use a shielded hydrophobic phase (SHP) made of a silica base covered with a polymer, which shows hydrophobic regions in a hydrophilic network. Consequently, the analytes penetrate the hydrophilic network and are retained by the hydrophobic moieties; the contrary is true for macromolecules, such as proteins. Direct injection [85] or coupling with another column [21] is described for the analysis and separation of sulphonamides. Obviously, the utilization of UPLC technology is also reported, as in other groups of compounds, following the general trend to the increasing application of sub-2-μm columns [86,87].

Detection: UV, FLD and MS

The analysis of sulphonamides using UV or FLD normally requires the performance of a previous derivatization in order to enhance sensitivity and selectivity; besides, separation efficiency can be improved using post-column derivatization, in contrast to the pre-column mode. Fluorescamine can be employed as derivatizating agent for this purpose [4,88-90].

In MS, most common MS/MS analyzers such as QqQ and IT are normally employed, specially the first one. The increasing use of hybrid instruments is also observed for the monitoring of sulphonamides, for instance as reported for the quadrupole-linear ion trap (QqLIT) hybrid analyzer [92,91]. Either ESI or APCI techniques are employed for the ionization of sulphonamides. There is a

number of common product ions for the majority of sulphonamides, including the *p*-aminobenzene sulfonic acid moiety $[M-RNH_2]^+$ (*m/z* 156), $[M-RNH_2-SO]^+$ (*m/z* 108), $[M-RNH_2-SO_2]^+$ (*m/z* 92) and other ions from the variable amine moiety RNH_3 $[MH-155]^+$ [29]. The use of APPI has been compared to both ESI and APCI, resulting in a higher sensitivity and obtaining identical fragmentation to that of the APPI source. The use of toluene as dopant agent in APPI provides the best results for sulphonamides [92]. Despite it is generally accepted that the positive mode is the most adequate for sulphonamide analysis using ESI, the utilization of ESI (-) is also reported [93]. An extensive list of CID fragments for sulphonamides is already published in literature [6,92].

2.1.8. Quinolones

Reversed-phase LC is the first choice for the separation of quinolones; GC is not applied for this purpose due to the high polarity of these VDs. Certain ampholytic compounds, such as enrofloxacine and its TP, ciprofloxacin, can show peak tailing problems because of the presence of silanol groups and metal traces that interact with them. In consequence, the use of end-capped or high purity silica columns and the optimization of pH and ion-strength conditions are strongly recommended [4]. Although C18 or C8-based columns are mostly applied, the use of other types of columns, such as styrene-divinylbenzene, phenyl and amide columns is described [6,22].

Other alkyl-based stationary phases can also be applied, for instance, C14 [94] or C5 [95]. Besides, two special columns have been coupled to the analytical column: immunoaffinity chromatography (IAC) [96] and turbulent flow columns (TFC) [97,98]. Both permit the injection of the sample without any previous pre-treatment (or a shortened pre-treatment); in the case of TFC, flow rates of 4-6 ml min^{-1} are typically used. Finally, the utilization of sub-2-μm particle columns (e.g. UPLC technology) has been recently reported as an approach in chromatographic separation, increasing the resolution and reducing the running time [99].

Detection: UV, FLD and MS

UV and FLD-based methods can be used for the determination of quinolones; earlier methods utilized UV methodologies. However, FLD provides higher selectivity and sensitivity; for this reason, FLD is preferred to UV systems. FLD is traditionally employed for the analysis of quinolones

since a high number of them show native fluorescence [4,22]. These compounds show a general absorption band at 300-350 nm, and a particular absorption band for each quinolone at 245-290 nm. The pH must be carefully controlled due to the high influence of this parameter on fluorescence intensity; the use of low pH values (2.5-4.5) is described as the most adequate values to obtain the highest fluorescence [22]. In order to increase the fluorescence properties of these analytes, terbium ions (Tb^{3+}) can be used to form complexes with quinolones that show strong emission [8].

In MS, QqQ analyzers and ESI interfaces working in positive mode are the most employed instrumentation , although the use of TOF, QqLIT and IT analyzers, and APCI sources is also reported (Figure 4) [22,98,100,101]. The pseudo-molecular ion $[M+H]^+$ is generally obtained at low voltage, whereas the fragments corresponding to $[M+H-H_2O]^+$ and $[M+H-C_2O]^+$ are produced at higher voltages.

Additional information about the observed fragments can be found in previous works [6]. The main monitored reactions correspond to neutral losses from the piperazine substituents (e.g. water, carboxylic groups, hydrofluoric acid, cyclopropyl or/and ethyl groups [22,102]. Due to the sensibility and widespread use of FLD techniques and the suitability of MS for the confirmation of analytes, FLD has been used as screening tool and MS, for quantification purposes [103].

2.1.9. Coccidiostats

Currently, the analysis of these kinds of VDs is usually performed by LC–MS, although in the past, LC–UV, TLC or GC–MS were applied [4].

Nitroimidazoles

The analysis of this subclass of coccidiostats is performed by reversed-phase LC by using common C18 columns, although recently UPLC has also been applied [104]. The chromatographic separation of these compounds is not as complicated as the separation of nitrofurans, as explained below.

Detection: UV and MS

LC is normally coupled to MS analyzers, mainly QqQ analyzers (although the QqLIT analyzer has recently been used [105]), and utilizing ESI as ionization technique, although APCI has been used for some applications [23,106]. ESI is operated in the positive mode due to its higher signal response for these compounds in comparison with ESI (-) [107]. In ESI (+), the $[M+H]^+$

is commonly obtained; the acetonitrile adduct, $[M+CH_3CN+H]^+$, has also been observed [108]. Additional fragments and fragmentation pathways have been described elsewhere [109].

Despite MS detection is preferred for the analysis of nitroimidazoles, UV detection has also been applied [110,111], for instance, as screening tool prior to LC–MS/MS with APCI or ESI techniques for confirmation purposes [29,106].

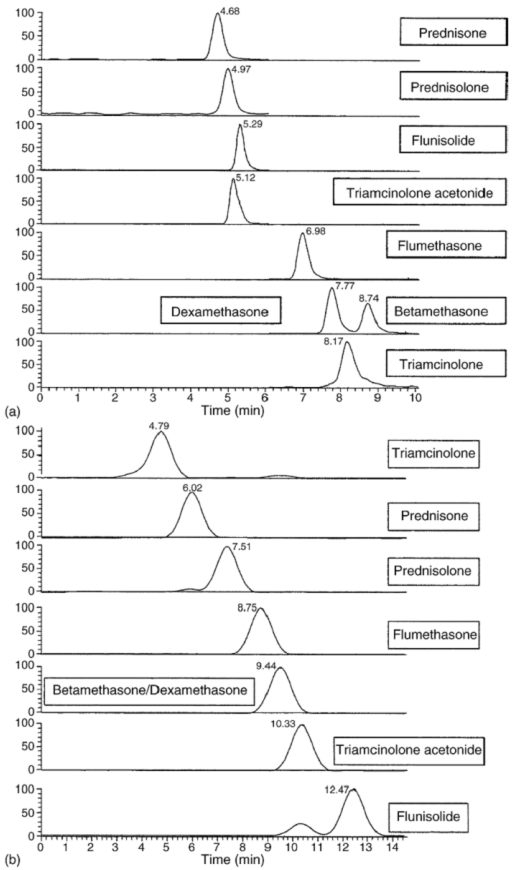

Figure 4. Reversed-phase LC–APCI(+)-MS/MS of a corticosteroid standard (50 μg kg^{-1}) showing the separation of betamethasone and dexamethasone using: (a) porous graphitic carbon column (Hypercarb, 125 mm × 4.6 mm, 5 μm, isocratic elution MeOH–CH_2Cl_2 (85:15, v/v)); and (b) C18 column (Inertsil 5 ODS-3, 250 mm × 4.6 mm, isocratic elution MeOH–water (65:35, v/v, pH 6.8, 20 mM NH_4Ac). [From [5] with permission from Elsevier B.V.].

Nitrofurans

Because of these compounds are rapidly metabolized, the analysis of nitrofuran residues is limited to the determination of their main metabolites. Due to the high polarity of the metabolites, separation on reversed-phase columns is unfavorable and the retention is poor. For this reason, derivatization is strongly recommended. 2-nitrobenzaldehyde (NBA) has been used as derivatizating agent (Note: NBA is a possible mutagen so it is important to avoid inhalation and use only in a chemical fume hood) [6,112]. For separation, the use of C18 columns is widespread, although monolithic columns have also been applied as an alternative to shorten the analysis time and solvent consumption [113].

Detection: UV, DAD and MS

For the detection of nitrofurans and metabolites, UV, DAD and MS can be used. However, the use of MS is greater and more adequate than the use the aforementioned non-selective detectors in order to obtain unequivocal identification and confirmation of these residues [112]. In general, when using classical detection, DAD is preferred to UV. As NBA is widely used as derivatizing agent, it has been reported that unreacted NBA can cause considerable interference in the LC–UV determination of some nitrofuran metabolites [114].

In MS, ESI (+) is usually applied in QqQ instruments operating in MS/MS because it normally provides higher sensitivity than APCI [29]. Nevertheless, APCI has also been utilized [115]. The use of derivatizing agents is beneficial for the detection of nitrofuran metabolites by MS since the molecular mass is increased, improving the final detection. The molecular masses of the derivatized metabolites are in the range 209-335 Da, which should reduce the influence of the MS background noise on the analyte signal [6,112]. Moreover, derivatization with NBA permits the improvement of the ionization efficiency, and avoids non-specific fragmentation processes, mainly losses of ammonia, water or carbon dioxide [116].

Recently, the simultaneous determination of nitroimidazoles and nitrofurans has been reported in meat [117]. This is the only application for the analysis of both groups in a single injection, using LC–MS. The separation is performed using a C18 column and typical eluents (water and ACN); however, the necessary hydrolysis and derivatization for nitrofurans is previously carried out.

2.1.10. Anabolic Steroids (ASs)

The analysis of ASs presents some difficulties. This class of compounds shows a low concentration in urine or edible tissue due to their short half-life time. Reversed-phase using C18 columns are the usual choice for separation; the application of UPLC is still scarce [118]. Additionally, the use of monolithic columns has also been reported [119].

The determination of ASs is mainly based on the analysis of free steroids. For this purpose, ASs have to be released from steroid glucuronide and/or sulphate conjugates in matrices such as urine or liver [4,9]. *Helix pomatia* juice (a species of snail, also known as Roman snail) is widely used to achieve this goal due to its content in β-glucuronidase and arylsulphatase. However, the presence of oxidoreductase enzyme activity can provoke the reaction of the steroid 3-ol group to a 3-oxo group [29]. LC–MS is also applied for the direct determination of steroid conjugates (e.g. sulphates, glucoronides, glycosides and glutathiones) due to the high polarity of these analytes; the combination of ESI and QqQ has been described as the optimum choice [9,120]. Moreover, LC–MS can be used for trenbolone-like steroids (thermolabile compounds), stanozolol (strong adsorption in GC) and progestagens (strong non-polarity) [32].

Detection: MS

The determination of ASs is mostly carried out by LC–MS, without any derivatization step, which is one of the main advantages of this technique [29]. The use of MS is widespread for the analysis of EAs whereas the use of classical detectors (UV, DAD or FLD) is much reduced [121]. In MS, QqQ analyzers are the preferred instruments, although some applications using IT can be found. QqLIT and linear ion-trap-orbitrap (LTQ-orbitrap) have also been applied for the analysis of stazonolol and analogues [122].

ESI, APCI and APPI ionization sources have been used for the analysis of ASs. ESI is probably the most popular ionization source for the determination of phase II metabolites (AS conjugates) whereas APCI and APPI are more suitable for the analysis of free less polar ASs [32,120]. The three ionization sources have been tested and compared, finding a MeOH-water gradient (5mM ammonium acetate and 0.01% acetic acid) as the most suitable mobile phase for the analysis of the 3 free studied EAs by ESI. Besides, the differences in sensitivity for APCI and APPI were not significant [123].

It has been reported that $[M+H]^+$ ions obtained by APCI (+) show higher intensity than those obtained by ESI or APCI (-), describing APCI (+) as the

best ionization mode for ASs [4,29]. Nevertheless, the direct ionization of many ASs to produce $[M+H]^+$ is somehow difficult due to the lack of acidic or basic moieties. The use of adducts with components of the mobile phase can result in a suitable way to ionize them by ESI. For certain ASs, the formation of adducts such as $[M+Na^+ + MeOH]^+$ or $[M+H+CH_3CN-H_2O]^+$ can be required to ionize them. Moreover, the use of adducts such as $[M+NH_4]^+$ can be more convenient in certain cases. The most appropriate ionization conditions for each AS group have been reported previously [124].

2.1.11. Corticosteroids

GC or LC can be selected for the determination of corticosteroids, although there is an increasing interest in LC–MS-based methodologies [4]. This technique is particularly adequate for the direct determination of polar metabolites [125]. Nevertheless, the separation efficiency in LC is lower than in GC, but the use of MS detection provides an increase in selectivity that allows the overcoming of this drawback. Despite LC is suited for the analysis of phase I and II metabolites, as well as their esterified forms, the separation of isomers such as dexamethasone and betamethasone with common reversed-phase columns is still problematic. The use of columns with specific stationary phases, such as graphite-based columns, has been pointed out to achieve the separation (Figure 5) [32,126,127]. In this case, it is not recommended ACN as solvent in the mobile phase owning to the observed problems affecting the ionization efficiency [127]. Another strategy to achieve the chromatographic separation of these two costicosteroids (dexamethasone and betamethasone) is the performance of a derivatization stage with ethoxyamine, obtaining two products that can be resolved by common reversed-phase columns [128]. Separation by monolithic columns [129] and polymeric reversed-phase columns [130] is also suitable, although they are rarely employed. Recently, the utilization of capillary LC (μLC) has been described for the analysis of corticosteroids coupled to MS [131]. In relation to particle size, although reversed-phase columns with 5-μm particle size are typical for the analysis of corticosteroids, the use of columns with lower particle size (1.8 μm [126]) or UPLC [132-134] is increasing.

Detection: DAD, CLD and MS

Despite the widespread use of MS for the analysis of corticosteroids, DAD [129,135] and CLD detection have also been utilized but to a lesser extent.

CLD using the luminal reaction in alkaline conditions and in the presence of hexacyanoferrate (III) as oxidant is described [136].

Currently, QqQ analyzers are preferred to monitor corticosteroids by MS, although IT analyzers were often used in the past years [127,130,137,138]. Currently, the use of TOF and Q-TOF instruments is also increasing [132,139]. ESI and APCI can be used for the monitoring of corticosteroids. In ESI (+) these compounds, which are relatively polar, show an intense $[M+H]^+$ ion (slightly acidic conditions). A number of other fragments can also be produced, mainly as a consequence of loss of water molecules and/or halogen atoms. In ESI (-), a lower fragmentation is observed, with two main ions: $[M-H]^-$ and $[M-CH_2O-H]^-$. Nevertheless, ESI (-) is often applied for the analysis of corticosteroids [126,140] and some authors have reported better sensitivity for glucocorticosteroids in negative mode [141]. On the other hand, APCI (-) has been pointed out as the most adequate ionization technique for corticosteroids since it provides better sensitivity and specificity [32,125], but the utilization of APCI (+) is also described [127,128]. In presence of ACN in the eluent, corticosteroids can form the corresponding adduct ($[M-H-CH_3CN]^+$) that can be used in screening methodologies [142].

2.1.12. Thyreostats

The analysis of thyreostats shows several difficulties: these compounds have the capacity of adopting a number of tautomeric forms (e.g. thiouracil and analogues can have up to 6 tautomeric forms), and they have low molecular weight and high polarity. Derivatization is an adequate way to lock the compound into a single tautometic form. Through this derivatization stage, sensitivity increases significantly (higher molecular weight) and separation by reversed-phase LC can be improved due to the consequent decline in polarity [32]. 7-Chloro-4-nitro-benzo-2-furazan (NBD-Cl) [31,143], and 3-iodo-benzylbromide (3-IBBr) [144] have been applied as derivatization reagents in LC. However, it is important to notice that the derivatization stage is not always applied.

Detection: DAD, CLD and MS

The determination of thyreostats is normally carried out by MS detection, although the use of DAD [145] and CLD [146] detection have been reported. LC–MS was first used in 1997, with APCI as ionization source [147]. In this

sense, 3-IBBr has been pointed out as the most efficient derivatization agent in LC–MS/MS in ESI (-) mode [32].

2.1.13. β-Agonists

As in other VDs and GPAs, GC was traditionally used; however, nowadays, the utilization of LC-based methods is increasing, especially LC–MS. Reversed-phase LC is mostly applied, although there are alternative stationary phases. Graphite-based columns [148] have been used for this aim. These phases show a strong affinity for polar analytes, and thus they allow the chromatographic interaction of charged molecules using high amounts of organic modifiers. Moreover, these columns provide stability over a wide pH-range [148]. The use of reversed-phase columns with particle size < 5 μm, including UPLC techniques is also suitable for the separation of β-agonists [149-152]. The utilization of polymeric monolith microextraction columns replacing common LC separation prior to classical detection has also been described [153].

In order to determine the conjugated metabolites of β-agonists, enzymatic hydrolysis is necessary. As in previous groups of analytes, the conjugated compounds are normally released by hydrolysis with β-glucoronidase/arylsulphatase.

Detection: UV, FLD and MS

Nowadays, MS is the first technique selected for the analysis of β-agonists; however, in the past years, classical detection systems, such as UV [154-156] or FLD [154,157-160] were often applied. For instance, salbutamol provides a fluorescence signal in medium acidic conditions and undergoes chemiluminescence reaction with potassium permanganate in the presence of sulfuric acid [153]. In the case of fenoterol, a derivatization stage prior to FLD can be carried out with N-(chloroformyl)-carbazole in alkaline conditions in order to obtain highly fluorescent derivatives [160]. Additional LC–MS analysis was sometimes applied for confirmatory purposes.

Despite the widespread use of QqQ analyzers, IT has been used in both former and recent methodologies; besides, novel hybrid instruments such as the QqLIT analyzer can also be applied [161]. The performance of MS^3 experiments with the IT analyzer has been described, although the application is not as usual as MS^2 experiments due to the problems related to the decrease in sensitivity with sequential MS stages [149,162,163]. Recently, the use of

HRMS instruments is increasing due to the advantages of full scan analysis and mass accuracy measurements with analyzers, such as Q-TOF [164]. ESI is mainly applied as ionization technique for the determination of β-agonists by LC–MS; the use of other ionization sources, such as APCI (+), is scarce [163,165,166]. Product ions commonly observed for β-agonists are $[M+H-H_2O]^+$ and $[M+H-C_4H_8]^+$ [4].

2.2. GAS CHROMATOGRAPHY

GC is less commonly used in VD and GPA analysis due to the mostly polar and water-soluble character of such substances, which require tedious derivatization steps [37]. Therefore, a number of groups of compounds are not included in the present section, since either they are not GC-amenable or the use of LC-based methods without derivatization stages are currently preferred. Anthelmintics, tranquilizers and antibiotics (except amynoglycosides) are not determined by GC; on the contrary, GPAs, including β-agonists and hormones are frequently monitored using GC-based methodologies.

2.2.1. Aminoglycosides

The number of GC methods for the analysis of aminoglycosides is scarce. These compounds are non-volatile and they show high polarity. The GC analysis requires derivatization at elevated temperatures with silylating agents. Gentamicin and kanamycin were analyzed after derivatization with a two-stages procedure by trimethylsilylation of the hydroxyl groups with 1-(trimethylsilyl)-imidazole (TMSI) and acylation of the amino groups with N-(heptafluorobutyryl)-imidazole (HFBI) [167]. Another GC-method proposed the silylation of the hydroxyl groups with TMSI and cyclisation of the guanidino groups with hexafluoroacetylacetone for the analysis of dihydrostreptomycin [168]. Despite the GC methods are time-consuming and tedious, GC is the prescribed method of the US Pharmacopoeia for quality control of spectinomycin using hexamethyldisilazane as derivatizing agent [16].

2.2.2. Coccidiostats

GC coupled to MS operating with chemical ionization in negative mode (NCI) has also been applied for the analysis of nitroimidazoles, in spite of they are mainly determined by LC–MS. In comparison to electronic ionization (EI), the fragmentation produced by CI is lower, and thus, low number of fragments is expected [169]. Additionally, due to the high polarity of these analytes, a previous derivatization stage is required. For this purpose, *N*,*O*-bis-(trimethylsilyl)acetamid (BSA) is commonly used as derivatizing agent [169,170]. For this purpose, columns with a stationary phase containing 5% phenyl-95% polydimethylsiloxane (PDMS) are selected (e.g. DB-5ms), and splitless mode is used as injection technique [169,170]. One of the main drawbacks of the derivatization step is that the compounds ronidazole and 2-hydroxymethyl-1-methyl-5-nitroimidazole produce the same derivatized product [104].

2.2.3. Anabolic Steroids (ASs)

GC–MS is widely used for the analysis of ASs mainly due to its high sensitivity in a variety of matrices. Certain ASs remain unchanged in tissues (e.g. esters of progestagens). The target residues are the modified compounds that are not GC-amenable because of probable degradation in the liner or the column [171]. Therefore, ASs require a previous derivatization, which is the main drawback of GC–MS, mainly to protect alcohol and keto functions [120]. In general, stationary phases used for the determination of ASs are non-polar (100% methylsiloxane) or slightly polar (5% phenyl or 5% cyanosiloxane) [120].

Depending on the properties of the individual ASs, silylation, acylation or oxime/silylation reactions can be used as the most popular derivatizations. Silylation with BSA, hexamethyldisilazane (HMDS) or *N*,*O*-bis(trimethylsilyl) trifluoroacetamide (BSTFA) can be used for unhindered alcohols, with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA) alone or with trimethylchlorosilane (TMCS) for hindered hydroxyl groups, and with MSTFA plus trimethyliodosilane (TMIS) or NH_4I (as catalysts) for keto function protection. Dithiothreitol (DTE) is commonly added to MSTFA as antioxidant. Acetylation is usually performed using a halogenated acid anhydride (e.g. TFAA, pentafluoropropionic acid anhydride (PFPAA) or heptaflurobutyric acid anhydride (HFBAA)) [32,120]. In specific applications

such as GC-combustion-isotope-ratio mass spectrometry (GC–C-IRMS) and solid-phase microextraction (SPME) coupled toGC–MS, MSTFA + NH_4I + DTE + CH_3CN [172] and BSTFA [173] can be used, respectively.

However, there are some difficulties in the use of derivatization agents: the lack of a universal derivatization agent, the failure of some ASs to provide a single derivatization product (e.g. trenbolone) and certain chemical rearrangement problems. These disadvantages have lead to the increase in the use of LC–MS [4,29]. Another example is stanozolol: it shows a pyrazole structure that hinders GC analysis since it forms hydrogen bonds with active sites in the chromatograph, such as injector, column head or transferline [120]. Additionally, some problems related to the use of internal standards (I.S.) in qualitative confirmatory analysis have been described due to the presence of artifacts from these I.S., which can lead to undesirable false positive results [174].

Detection: MS

As in LC–MS, QqQ is widely used for detection; IT is also utilized but to a lesser extent. Less common is the use of HRMS instruments such as magnetic sectors. In relation to the use of these analyzers, the increase in resolution cannot bring along a decrease in sample pre-treatment (e.g. sample clean-up) if the benefits of HRMS acquisition can be maintained. This is due to the fact that there is not "spectacular clean-up" in the extracted HRMS chromatograms of ASs [120].

EI is the most common ionization technique for ASs, except when using fluoroacylation due to NCI provides higher sensitivity [32]. Besides, the use of acetylation reactions in the derivatization step by using halogenated acid anhydride permits the introduction of fluorine that can reduce the exact mass of the steroid (increasing in the specificity of HRMS analysis) or increase the propensity of the steroid to trap electrons (increasing sensitivity in the analysis by NCI).

An interesting question in ASs is the unequivocal discrimination between endogenous (produced by the body) and exogenous (administered to the organism) ASs. GC–C-IRMS can be used to measure such differences, involving the determination of the $^{13}C/^{12}C$ content of steroids residues. This approach is based on differences between steroid carbon isotopic compositions of ASs which are different according to their origin [120].

2.2.4. Corticosteroids

In the past years, the use of GC-based methods, especially GC–MS methods, was widespread for the analysis of corticosteroids, even if sample preparation was tedious and derivatization and oxidation steps were needed [4]. Nowadays, the application of GC–MS is scarce due to the increasing use of LC–MS^n. Nevertheless, when single MS is performed, GC is preferred due to its higher resolution and isomer differentiation [125].

Detection: MS

EI and CI are suitable for the analysis of corticosteroids by GC–MS. Due to the moderate polarity of these compounds, derivatization is required in order to increase volatility and reduce peak tailing. Several options can be used, although depending on the applied ionization technique, better results can be obtained with a specific reaction [32,125]. Derivatives obtained by silylation with MSTFA/TMIS/DTE (typical silylation solution) normally provide large fragmentation and low intensity molecular ion. As a consequence, poor sensitivity is obtained, and this reaction is used for screening purposes in positive EI. Moreover, low sensitivity is achieved for the most polar corticosteroids (e.g. triamcinolone and prednisolone). As silylation yields numerous reaction products, oxime formation (mainly with methoxylamine (MOX)) prior to silylation can be used in order to avoid the multiple reaction products obtained. Additionally, the use of boronic esters (e.g. with methylborinic acid) prior to silylation is also suitable for the derivatization stage, providing stable derivatives and intense molecular ion for α-γ-diol compounds, although sensitivity can be insufficient in trace analysis. Finally, chemical oxidation (pyridium chlorochromate (PCC) is the most powerful reagent) and subsequent oxidation of residual –OH to –C=O is another derivatization reaction that can be applied (this reaction was frequently employed before the increasing use of LC–MS) [32,125].

In GC–MS, two combinations widely applied are the use of chemical oxidation and NCI or typical silylation and EI (+).

Eventually, GC–C-IRMS can be used to determine endogenous and exogenous costicosteroids separately [32].

2.2.5. Thyreostats

Thyreostats can also be analyzed by GC after the corresponding derivatization reactions due to the high polarity and low molecular weight of

these compounds. NBD-Cl, pentafluorobenzylbromide (PFBBr), 3-bromobenzylbromide (3-BrBBr) and MSTFA are used in these derivatization reactions. A common procedure is based on a first derivatization performed with 3-BrBBr and second one, performed with MSTFA (trimethylsilylation) [32]. Besides, the use of strong alkaline conditions has been recommended to carry out the 3-BrBBr derivatization [175].

Detection: MS

MS detection is normally applied, operating in EI [32].

2.2.6. β-Agonists

The analysis of β-agonists requires a derivatization stage prior to GC determination. As in previous groups, this drawback has lead to the increasing use of LC, which permits the direct analysis.

Detection: FID and MS

GC is widely coupled to MS for the determination of β-agonists, although the use of flame ionization detection (FID) instruments is also reported [176]. For derivatization of the analytes in order to obtain GC-amenable compounds, there is a variety of suitable reagents, which are very similar to those used in AS determinations. Trimethylsilylation (e.g. with MSTFA, MSTFA/TMSI, BSTFA/TMSI, BSTFA/TCMS), acylation (e.g. with PFPAA), combined trimethylsilylation and acylation (e.g. with MSTFA plus MBTFA, and MSTFA/TMSI plus MBTFA), and formation of cyclic derivatives (e.g. with trimethylboroxine) are the most common derivatization procedures for those compounds including *tert*-butyl, isopropyl or isopentyl chains. The use of MSTFA or BSTFA is recommended for screening purposes due to the minimal by-product formation, whereas formation of cyclic methylboronates can be useful for confirmation. More comprehensive information can found elsewhere [177,178]. Chloromethyldimethylchlorosilane (CMDCMS) as derivatizing agent has been used for the analysis of clenbuterol [179]. Further information about the stability of methyl boronic derivatives can be found elsewhere [180].

Chapter 3

3. APPLICATIONS

The present section of the chapter is focused on the description of relevant analytical methods reported in literature for the analysis of VDs and GPAs in a variety of matrices, including foodstuffs, biological fluids and environmental samples. Extraction and clean-up methods, as well as interesting conditions are highlighted; additionally, a number of tables summarizing methodologies are also included. Due to the fact that there are significant differences in the characteristics of the aforementioned compounds, the review of the applications is divided by groups in a similar way as in the classification shown in Section 1.

3.1. ANTHELMINTICS

Avermectins

The monitoring of avermectin residues is focused on food samples (mainly milk and meat), and biological samples; whereas the analysis of these VDs in environmental samples is scarcely reported (Table 1). Avermectins are typically extracted using organic solvent and clean-up by SPE or IAC. The most common solvent for extraction is ACN (or ACN/water), which also produces protein precipitation in certain samples, such as milk; the use of isooctane, MeOH and MeOH/water mixtures is also reported. C18-based sorbents are mostly employed for SPE; alternative phases, such as silica, alumina or copolymeric sorbents (e.g. Oasis HLB) can also be used. The application of FLD brings along the performance of a derivatization step prior

to chromatographic analysis (Section 2.1.1). The application of the aforementioned basic procedure can be found in milk [38,40] and meat [181,182]; biological samples, including liver [39,183-185] and plasma [186]; and water [187,188] and soil samples, where the use of pressurized-liquid extraction (PLE, also known as accelerated-solvent extraction, ASE) is preferred to common solid-liquid extraction (SLE) [188,198]. Additional applications can be found elsewhere [12].

Benzimidazoles

The number of applications related to the determination of benzimidazoles is reduced in comparison to other VDs. The developed methodologies for the extraction and clean-up of benzimidazoles are quite simple, as in the case of avermectins (Table 1). Solvent extraction with ethyl acetate (in some applications, the pH of the sample is previously made alkaline) is a generic procedure, sometimes including a subsequent SPE clean-up (e.g. C18, -CN). Ethyl acetate is widely used since it permits the extraction of complex matrices (e.g. muscle and liver tissues) avoiding the formation of emulsion interfaces and it shows good solvating power for weakly basic drugs. The aforementioned protocol has been used in food samples (e.g. meat [190-193], egg [191], milk [194] or fish [195]), and animal tissues (e.g. liver [193,196], kidney [193]). A longer procedure including some additional liquid-liquid extraction (LLE) steps using ACN and ACN saturated in *n*-hexane can be found for milk analysis [197]. Another three different extraction techniques have been used for the extraction of liquid samples, namely molecularly-imprinted SPE (MISPE) [198],SPME [199], and automated dialysis system [200] in water and plasma samples.

3.2. TRANQUILIZERS

The typical extraction procedure for the analysis of tranquilizers is based on a solvent extraction with ACN and subsequent SPE with polymeric sorbents, such as Oasis HLB (Table 1). This kind of SPE material is normally used because of the absence of silanol groups that can strongly interact with this basic analytes. Moreover, their stability at high pH values permit the use of alkaline conditions in order to suppress the protonation of the compounds, decreasing the analyte polarity and improving the recovery for the most polar

tranquilizers, such as xylazine. On the other hand, the addition of NaCl to the extracts (e.g. 10%) can increase the recovery for the most polar compounds when using C18 SPE cartridges [14].

The number of applications is reduced, but most of them apply an extraction with ACN and a subsequent SPE stage, mainly with polymeric sorbents, but also with C18 cartridges. One can find the utilization of this strategy for the analysis of tranquilizers in biological samples, such as kidney [42,45,201], liver [14] and meat [45]. The use of ACN:water mixtures and C18 SPE in liver is also reported [44].

3.3. AMINOGLYCOSIDES

In literature, there is a variety of methods for the analysis of aminoglycosides in biological fluids (mainly plasma) and food products (Table 2).

To a lesser extent, these compounds have been analyzed in environmental samples, such as water. In general, most protocols involve a solvent extraction and a sample clean-up performed by SPE. Ion pair LC and pre-derivatization are widely utilized in all kind of samples. TCA and HFBA are typical ion pair agents described. However, the procedures reported in bibliography can be classified into three general categories: (i) solvent extraction, SPE clean-up and ion pair LC coupled to MS; (ii) pre-derivatization in the extraction procedure, normally by extraction with solvent, clean-up by SPE and separation and detection by LC–FLD using a C18 analytical column; and (iii) ion-exchange LC and post-column derivatization for FLD detection.

In methodologies based on solvent extraction, TCA is commonly used. This is an ion pair agent and it is also added to release aminoglycosides bonded to proteins and for deproteinization purposes (e.g. milk samples). HFBA and 5-sulfosalycil acid dihydrate have also been employed to remove proteins from matrices such as plasma [53,202], honey and milk [203]. Aqueous buffer solutions (e.g. phosphate, citrate) at different concentrations and pH values, and acidic solutions (e.g. HCl, $HClO_4$) have been used for extraction. After extraction, SPE utilizing ionic exchange cartridges (e.g. cation exchange cartridges: CBA, CBX and Amberlite IRC-50) is carried out for sample clean-up. This kind of methodology has been applied for the analysis of aminoglycosides in animal tissues [50, 204] and plasma [53]. Alternative SPE sorbents, such as polymeric-based cartridges (e.g. Oasis) [49,51,205] have been used for the analysis of serum, animal feed, meat, liver,

Table 1. Selected applications for the analysis of anthelmintics and tranquilizers

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/ detection technique*	*LOD (units)*	*Observations*	*Ref.*
Avermectins (5)	Milk	LLE (ACN), diluted (TEA/water), SPE (C8)	LC–FLD (λ_{em}=365 nm, λ_{ex}=470 nm)	0.1 μg kg^{-1}	Waters Symmetry® C18 (150mm×3.0 mm, 5μm) Isocratic: Water/MeOH/ACN (5:47.5:47.5, v/v/v) Derivatisation: MI, TFAA, TEA, TFA	[38]
Avermectins (4)	Milk	LLE (ACN), SPE (C18+Envi-Carb)	LC–APCI(+)–IT–MS/MS	Data not provided	Zorbax SB-C18 (150 mm x 3 mm, 3.5 μm) Isocratic: Water/MeOH (10:90, v/v)	[40]
Avermectins (4)	Meat, liver	SLE (ACN), SPE (C18)	LC–FLD (λ_{em}=365, λ_{ex} = 465 nm)	0.5-1.0 ng g^{-1}	Inertsil ODS RP (250 mm x 4.6 mm , 5 μm) Isocratic: ACN/water/THF (88:4:8, v/v) Derivatization: MI/ACN (1:1, v/v), TFA/ACN (1:2, v/v)	[181]
Avermectins (4)	Meat, liver	SLE (MeOH), phosphate buffer solution, IAC	LC–ESI(+)–QqQ–MS/MS	2.5 ng g^{-1}	Atlantis C18 (150 mm x 2.1 mm, 3 μm) Isocratic: ACN/water/formic acid (95:5:0.1, v/v)	[182]
Avermectins (5)	Liver	SLE (ACN)+ SPE (C8)	LC–APCI(-)–IT–MS/MS	28-840 ng g^{-1} (a)	Luna C18 (50 mm x 4.6 mm, 3 μm) Isocratic: ACN/water/TEA (78:22:0.1, v/v)	[183]
Avermectins (2)	Liver	SLE (MeOH), IAC	LC– (APCI-)–TOF–MS	5 μg kg^{-1}	Zorbax Eclipse XDB-C8 (0.46 x 15 cm) Isocratic: MeOH/water (85:15, v/v)	[184]
Avermectins (4)	Liver	SLE (isooctane), SPE (alumina-N)	LC–FLD (λ_{em}=365 , λ_{ex}= 470 nm)	2 μg kg^{-1} (b)	Novapak C18 (150 x 3.9 mm, -) Isocratic: MeOH/ACN/water (1% TEA, 1% H_3PO_4) (61:30:9, v/v/v,) Derivatization: MI/ACN (1:1, v/v)+ TFAA/ ACN(1:2, v/v)	[185]

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/ detection technique*	*LOD (units)*	*Observations*	*Ref.*
Avermectins (1)	Plasma	SLE (ACN)	LC–(ESI+)–QqQ–MS/MS	0.2 ng ml^{-1}	Nucleosil ODS (100 mm x 3 mm,5 µm) A: Water (0.2% HAc) B: ACN (0.2% HAc)	[186]
Avermectins (2) and others	Surface water	"Dilute and shoot"	LC–ESI(+)–QqQ–MS/MS	1-7 ng l^{-1} (c)	Xterra MS C18 (200 mm × 2.1mm,3.5 µm) A: Water (0.1% formic acid) B:ACN (0.1% formic acid), C: MeOH (0.1% formic acid), D:EtAc	[187]
Avermectins (7)	Surface water (1), sediment, soil (2)	(1) SPE (2) PLE (MeOH/water (1:1, v/v)), SPE (HLB)	LC–APCI(+)–QqQ–MS/MS	(1) 2.5-14 ng l^{-1} (2) 0.5-2.5 ng g^{-1} (b)	Zorbax Eclipse XDB-C8 (150 mm × 4.6 mm, 5µm) A: Water (pH 4, 10mM NH_4OH) B: ACN	[188]
Avermectins (1)	Soils	ASE (methylene chloride), SPE (silica gel column)	LC–APCI(+)–QqQ–MS/MS	Data not provided	Synergi Hydro-RP 8A (250 mm x 4.60 mm, 4 µm) A: Water B: ACN	[189]
Benzimidazoles (3)	Milk	Homogenization (Na_2SO_4), LLE (n-hexane saturated with ACN), SPE (C18)	LC–UV (λ=295nm) LC–ESI(-)–Q–MS	0.004–0.006 µg g^{-1}	Capcell Pak C UG 120 (150 mm x 4.6 mm, 5 µm) Isocratic: Water/ACN (0.05 M NH_4Ac (50:50, v/v)	[197]
Benzimidazoles (3)	Egg (1), meat (2)	Alcalinization (NaOH), SLE (EtAc), LLE (n-hexane)	LC–APCI(+)–QqQ–MS/MS	(1) 0.19-1.14 µg kg^{-1} (2) 0.14-0.75 µg kg^{-1}	Alltima C18 RP (150 mm × 2.1 mm, 5 µm) A: Water (pH 5.2, 0.04 M NH_4Ac) B: ACN	[191]
Benzimidazoles (4)	Fish	Alcalinization (K_2CO_3), SLE (EtAc), LLE (n-hexane,EtOH/HCl)	LC–FLD (λ_{em}= 290nm, λ_{ex}= 330 nm)	0.1-6 µg kg^{-1}	Luna C18 (150 mm x 4.6 mm, 5 µm) Isocratic: ACN/MeOH/0.05 M NH_4Ac (13:15:55) and ACN/MeOH/0.05 M NH_4Ac (13:8:75)	[195]

Table 1. (Continued).

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/ detection technique*	*LOD (units)*	*Observations*	*Ref.*
Benzimidazoles (3)	Meat, fat, kidney, liver	Alcalinization (NaOH), SLE (EtAc), LLE (n-hexane)	LC–APCI(+)–QqQ–MS/MS	11-13 μg kg^{-1} (a)	Alltima C18 RP (150 mm × 2.1 mm, 5 μm) A: Water (0.1% formic acid) B: ACN	[190]
Benzimidazoles (3)	Plasma	Automated dialysis, enrichment column	LC–UV (λ= 295 nm)	1.4- 2 ng ml^{-1}	LiChroCart analytical column (125 mm x 4 mm, -) Isocratic: Water/ACN (pH 6, 0.05 M phosphate buffer)	[200]
Benzimidazoles (12)	Liver	SLE (EtAc/ Na_2SO_4/K_2CO_3), LLE (n-hexane), SPE (C18)	LC–UV (λ=298 nm)	116-1303 μg kg^{-1} (a)	Xterra TM C18 (150 mm × 3.0 mm, 3.5 μm) A: Water/MeOH/ACN (76:15:9, v/v/v, pH 6.8, $NH_4H_2PO_4$) B: Water/MeOH/ACN (52:30:18, v/v/v, pH 6.8, $NH_4H_2PO_4$) C: Water/MeOH (18:82, v/v/v, pH 6.8, $NH_4H_2PO_4$)	[196]
Benzimidazoles (7)	Water	(1) SPE (MISPE) (2) SPE (C18 discs)	LC–DAD	(1) 0.002-0.012 μg l^{-1} (2) 0.03-0.09 μg l^{-1}	(1)C18-Kromasil column (250 mm × 4.6 mm, 5μm) A: Water/HAc (96:4, v/v) B: ACN	[198]
Avermectins, benzimidazoles (9)	Milk	Alcalinization (NaOH), LLE (EtAc)	LC–ESI(+)–QqQ–MS/MS	<1 μg kg^{-1}	Alltima C18 column (150 mm x 2.1 mm, 5 μm) A: Water (0.1% formic acid) B: ACN	[194]

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/ detection technique*	*LOD (units)*	*Observations*	*Ref.*
Tranquilizers (7)	Liver	SLE (ACN), SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	< 1 μg kg^{-1}	Zorbax Extended C18 (150 mm × 2.1mm, 5μm) A: Water (30 mM, NH_4OH) B: ACN (30 mM, NH_4OH)	[14]
Tranquilizers (8)	Kidney	SLE (ACN)	LC–ESI(+)–QqQ–MS/MS	5.8-124.4 μg kg^{-1} (a)	Luna C18 (150 mm × 2 mm, 5μm) A: Water (pH 4.5, 0.05 M ammonium formate) B: ACN	[201]

(a) Decision limit (CCα); (b) Limit of quantification (LOQ); (c) Method detection limit (MDL).

Table 2. Selected applications for the analysis of aminoglycosides, β-lactams, macrolides and tetracyclines

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Aminoglycosides (2)	Egg, honey, milk, liver	Hydrolisis ($HClO_4$), SLE/LLE (0.1 M sodium pentanesulphonate)	LC–FLD (λ_{exc} = 356, λ_{em} = 439)	7.5-15 μg kg^{-1}	Discovery RP-AmideC16 (150 mm x 4.6 mm, 5 μm) Isocratic: sodium pentanesulphonate (pH 3.3)/ACN (94:6, v/v)	[52]
Aminoglycosides (7)	Milk	MSPD (hot water)	LC–ESI(+)–QqQ–MS/MS	1-6 ng ml^{-1}	HP HL C18 (250 mm x 4.6 mm, 5 μm) A: Water (1 mM HFBA) B: MeOH (1 mM HFBA)	[18]
Aminoglycosides (2)	Honey	Dilution, SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	3 μg kg^{-1} (a)	Gemini C18 (50 mm x 2 mm, 5 μm) HFBA:ACN (85:15)	[206]
Aminoglycosides (2)	Honey (1), milk (2)	(1) SLE (sodium heptasulphonic acid), SPE (C18) (2) LLE (5-sulfosalycil acid, sodium heptasulphonic acid)	LC–ESI(+)–QqQ–MS/MS	1-10 μg kg^{-1} (b)	Alltima C18 (150 mm x 2.1 mm, 5 μm) Mobile phase: data not provided	[203]
Aminoglycosides (8)	Meat, kidney	SLE (K_2PO_4, EDTA, TCA), SPE (CBX)	LC–ESI(+)–QqQ–MS/MS	22-6230 μg kg^{-1} (a)	Symmetry C18 (150 mm x 3 mm, 5 μm) A: Water (0.065% HFBA) B: MeOH (0.065% HFBA)	[50]

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Aminoglycosides (13)	Honey, meat, milk, kidney, liver	SLE/LLE (TCA, HFBA) SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	8.6–5278.8 $\mu g\ kg^{-1}$ (a)	Cap cell Pak C18 (150 mm x 2 mm, 5 µm) A: Water/ACN (95:5, v/v, 20 mM HFBA) B: Water/ACN (1:1, v/v, 20 mM HFBA)	[51]
Aminoglycosides (2)	Meat, kidney, liver	SLE (phosphate buffer/TCA), SPE (carboxypropyl, WCX)	LC–ESI(+)–IT–MS/MS	0.5-2.5 ng g^{-1}	Nucleosil C18 (100 mm x 3 mm, 5 µm) A: Water (20 mM PFPA) B: ACN/Water (50:50, v/v, 20 mM PFPA)	[204]
Aminoglycosides (5)	Serum	SPE (MCX)	LC–ESI(+)–QqQ–MS/MS	100 ng ml^{-1} (a)	ZIC-HILIC (100 mm x 2.1 mm) A: Water/ACN/formic acid (95:5:0.2, v/v/v, 2mM NH_4Ac) B: Water/ACN/ formic acid (5:95:0.2, v/v/v 2 mM NH_4Ac)	[49]
Aminoglycosides (7)	Plasma	LLE (HFBA)	Capillary LC-nanoESI(+)–Q–TOF–MS/MS	Data not provided	HP HL C18 (10 mm x 175 µm, 3 µm) Column switching B: MeOH (1 mM HFBA)	[202]
β-Lactams (10)	Milk	SPE (GBC)	LC–ESI(+/-)–Q–MS	5 ng ml^{-1} (b)	Alltima C18 (250 mm x 4.6 mm, 5 µm) A: Water (10 mM formic acid) B: MeOH (10 mM formic acid)	[213]

Table 2. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
β-Lactams (5)	Milk	LLE (phosphate buffer, *n*-hexane), SPE (C18)	LC–ESI(+)–QqQ–MS/MS	Data not provided	YMCODS-AQ (50 mm × 2 mm, 3µm) A: Water (0.1% formic acid) B: ACN/water (65:35, v/v, 0.1% formic acid)	[214]
β-Lactams (5)	Milk	LLE (phosphate buffer), SPE (C18)	LC–ESI(+)–QqQ–MS/MS	0.40-1.10 µg kg^{-1} (c)	YMC ODS-AQ (50 mm x 2 mm,3 µm) A: Water (0.1% formic acid) B: ACN/water (65:35, v/v)	[215]
β-Lactams (5)	Milk	LLE (phosphate buffer, *n*-hexane), SPE (C18)	LC–ESI(+)–QqQ–MS/MS	0.4-0.8 µg kg^{-1} (b)	YMC ODS-AQ column (50 x 2 mm 3µm) A: pure H_2O+0.1% formic acid B: 35%H_2O + 65%ACN+ 0.1% formic acid	[216]
β-Lactams (7)	Milk	LLE (ACN), SPE (HLB)	LC–ESI(+)–IT–MS/MS	0.2-2.0 ng ml^{-1} (d)	Luna C18, (250 mm x 4.6 mm, 5 µm) A: Water (1% HAc) B: MeOH (1% HAc)	[65]
β-Lactams (4)	Milk (1), kidney (2), liver (3)	SLM (*n*-undecane:di-*n*-hexyl ether)	LC–ESI(+)–IT–MS/MS	(1) 0.7-1.7 µg l^{-1} (2,3) 1-1.4 ng kg^{-1}	C18 Higgins Clipeus (150 mm x 3.0 mm, 5 µm) Isocratic: Water/MeOH (75:25, v/v, 25 mM HAc)	[64]
β-Lactams (11)	Kidney	SLE (ACN, water), SPE (C18)	LC–ESI(+)–IT–MS/MS^n	10-500 ng g^{-1} (c)	YMC ODS C18 (50 mm x 4.6 mm x 3 µm) A: 0.1% formic acid in water B: 0.1% formic acid in ACN	[211]

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
β-Lactams (10)	Kidney	SLE (ACN, water), D-SPE (C18)	LC–ESI(+)–QqQ–MS/MS	Data not provided	YMC ODS-AQ (50 mm x 4.6 mm, 3µm) A: Water (0.1% formic acid) B: MeOH/ACN (1:1, v/v, 0.1% formic acid)	[212]
β-Lactams (11)	Kidney	SLE (ACN/water), D-SPE (C18)	LC–ESI(+)–QqQ–MS/MS	Data not provided	Prodigy ODS3 (150 mm × 3 mm; 5µm) A: Water (0.1% formic acid) B: ACN (0.1% formic acid).	[61]
β-Lactams (2)	Plasma	SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	Data not provided	On-line extraction column: Oasis column (50 mm x 1 mm, 30µm) Analytical column: BDS Hypersil C18 (50 mm x 2 mm, 5 µm) Isocratic: MeOH/water (70:30, v/v, 1 mM formic acid)	[60]
β-Lactams (5)	Water and waste-water	SPE (HLB)	LC–ESI(+)–IT–MS	8-18 ng l^{-1} (d)	Xterra MS C18 column (50 mm × 2.1 mm, 2.5 µm) A :Water (0.1% formic acid) B: MeOH, C: ACN	[56]
Macrolides (9)	Meat (1)	SLE (ACN), LLE (*n*-hexane)	LC–ESI(+)–QqQ–MS/MS	0.09-0.98 ng g^{-1} (d)	Atlantis dC18 (20 mm x 3.9 mm, 3 µm) A: Water (0.1% formic acid) B: ACN	[220]
Macrolides (5)	Honey	SLE (phosphate buffer), SPE (HLB)	LC–ESI(+)–Q–MSLC–ESI(+)–QqQ–MS/MS	0.01-0.07 µg kg^{-1}	YMC ODS-AQ S-3 (50 mmm x 2 mm 120 Å) A: Water B: Water (1% formic acid) C: ACN	[219]

Table 2. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Macrolides (5)	Egg	SLE (ACN)+ LLE (n-hexane +NaCl)+ SPE(Oasis HLB)	LC–ESI(+)–QqQ–MS/MS	<1.0 μg kg^{-1}	YMC ODS-AQ S-3 (50 mmm x 2 mm, 120 Å) A: ACN B: 1% formic acid C: Water	[218]
Macrolides (7)	Milk (1), yoghurt (2)	MSPD ((1) hot water/formic acid, (2) (hot water/NH_4Ac)	LC–ESI(+)–QqQ–MS/MS	0.9-6.0 ng ml^{-1}	Alltima C_{18} (250 mm x 4.6 mm, 5 μm) A: Water (10 mM formic acid) B: MeOH (10 mM formic acid)	[221]
Macrolides (6)	Egg, honey, milk	SLE (ACN or phosphate buffer), SPE (HLB)	UPLC–ESI(+)–Q-TOF–MS (1) LC–ESI(+)–QqQ–MS/MS (2)	(1) 0.2-1.0 μg kg^{-1} (2) 0.01-0.50 μg kg^{-1}	(1) Acquity BEH C18 (100 mm x 2.1mm, 1.7 μm) A: Water (10mM NH_4Ac) B: ACN (2) YMC ODS-AQ S-3 (50 mm x 2mm, 120 Å) A: Water, B: Water (1% formic acid), C: ACN	[69]
Macrolides (7)	Kidney , liver	LLE (EDTA–McIlvaine buffer), SPE (HLB)	(1) LC–DAD (λ=200-400nm) (2) LC-ESI(+)-Q-MS	60-1005 μg kg^{-1} (a)	Kromasil 100 C18 (250 mm x 46 mm, 5μm) (1) A: Water (pH 3.5, H_3PO_4 buffer), B: ACN (2) A: Water (pH 2.8, 1% HAc), B: ACN	[71]

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Macrolides (1)	Plasma	SLE (diethyl ether)	UPLC–ESI(+)–QqQ–MS/MS	1 ng ml^{-1} (b)	Acquity BEH C18 (50 mm × 2.1 mm, 1.7 µm) A: Water (50mM NH_4Ac) B: ACN	[70]
Macrolides (2)	Feed	SLE (MeOH), LLE (MeOH/acetate buffer, *n*-hexane), SPE (cyano-propyl, alumina)	LC–UV (λ=220-350nm) LC–DAD (λ= 232 nm, 280 nm)	118-176 ng g^{-1} (a)	RP C18 (250 mm × 4 mm, -) A: Water/ACN (80:20, v/v, pH 2.5, 0.05 M K_2HPO_4) B: ACN	[72]
Macrolides (5)	River water	SPE (HLB)	LC–ESI(+)–Q–MS LC–ESI(+)–QqQ–MS/MS	0.02-1.90 µg l^{-1}	Hypurity C18 (250 mm × 2.1 mm, 5 µm) A: Water/ACN (90:10, v/v, pH 6, 10mM NH_4Ac) B: ACN	[222]
Macrolides (3)	Water, wastewater	SPE (HLB)	LC–UV (λ= 205, 215, 287nm) LC–ESI(+)–IT–MS/MS	0.03- 0.07 µg l^{-1} (d)	Xterra MS C18 (50 mm × 2.1mm, 2.5 µm) A: Water (0.1% formic acid) B: ACN (0.1% formic acid)	[75]
Tetracyclines (7)	Meat	SLE (succinic acid/MeOH), SPE (HLB)	LC–DAD (λ= 220–400 nm)	109-132 µg kg^{-1} (a)	Chromolith® Speed Rod RP-18e monolithic column (50 mm × 4.6 mm) A: Water/THF (98.5:1.5, v/v, pH 3, 0.01 M oxalic acid) B: MeOH	[77]
Tetracyclines (6)	Meat	MSPD (hot water 70°C)	LC–ESI(+)–QqQ–MS/MS	1-6 ng g^{-1}	Alltima C-18 (250 mm x 4.6 mm, 5µm) A: MeOH (10 mM formic acid) B: Water (10 mM formic acid)	[78]

Table 2. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Tetracyclines (1)	Milk	LLE (H_2SO_4/ACN, $(NH_4)_2SO_4$, H_3PO_4, CH_2Cl_2)	LC–UV ($\lambda_{detection}$=370 nm)	106.5-107.3 µg kg^{-1} (a)	Nucleosil 100-5 C18 (250 mm × 4.6 mm, 5µm) Isocratic: Water (0.01 M Na_2HPO_4)/ACN (20:80, v/v, pH 3.8, 5mM octanesulphonate sodium salt, 3 mM tetrabutylammonium hydrogen sulphate, 0.01% EDTA)	[79]
Tetracyclines (7)	Milk	LLE (Oxalate buffer (pH 4) + with 20% TCA)+ SPE (Discovery cartridges)	LC–DAD (λ=355 nm)	101.25-105.84 µg kg^{-1} (a)	Inertsil ODS-3 (250 mm x 4 mm, 5 µm) A: 0.01 M oxalic acid B: ACN	[229]
Tetracyclines (3)	Honey	SLE (citric acid buffer + phosphate buffer solution), SPE (XAD-2 resin)	LC–CLD (λ_{em}= 436, 535 nm)	0.9 -5.0 ng ml^{-1}	Zorbax Eclipse XDB-C18 (150 mm × 2.1 mm, 5 µm) Isocratic: Water/ACN (0.001 M H_3PO_4) Derivatization: $KMnO_4$/ Na_2SO_3/β-cyclodextrin	[81]
Tetracyclines (4)	Honey	SLE (oxalate buffer), SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	Data not provided	Atlantis dC18 (150 mm x 2.1 mm,3 µm) A: Water (1% formic acid) B: MeOH/ACN (50:50, v/v, 1% formic acid)	[225]

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Tetracyclines (2)	Honey	SLE (EDTA-McIlvaine buffer), SPE (HLB, CX)	LC–FLD (λ_{em}=385 nm, λ_{ex} =500 nm)	20- 21 µg kg^{-1} (a)	Nucleosil C18 column (250mm x 4 mm, 5 µm) Isocratic: Water/ACN (80:20, v/v, pH 2, 10 mM oxalate buffer) Derivatization: magnesium acetate in pH 9.0 boric acid buffer	[226]
Tetracyclines (4)	Milk (1), water (2)	(1) LLE (McIlvaine buffer) (2) SPE (HLB) (1,2) RAM: (alkyl diol silica (ADS) porous particles C8)	LC–FLD (λ_{en}= 374nm , λ_{ex} = 495 nm)	15-30 ng l^{-1}	Hypurity Elite C18 (Hypersil, 250 mm × 4.6 mm, 5µm) A: Water (pH 2.2, 0.01 M oxalic acid) B: ACN Post-column derivatisation: Mg^{2+}, pH 9	[233]
Tetracyclines (4)	Meat (1), milk (2)	SPE (McIlvaine buffer), SPE (HLB)	LC–DAD (λ=365 nm)	(1) 107.7–129.9 mg kg^{-1} (a) (2)113.2–127.2 mg kg^{-1} (a)	Hypersil C8 (250 mm x 4.6 mm, 5 µm) Isocratic: Water (0.01 M oxalic acid)/MeOH/ACN (60:25:15, v/v/v)	[94]
Tetracyclines (5)	Egg (1), milk (2)	(1) SLE (EDTA) (2) LLE (EDTA), SPE (GBC)	LC–ESI(+)–Q–MS	(1) 1-19 µg kg^{-1} (2) 0.5-7 µg l^{-1}	Hypersil C18 Hypurity Elite RP (250 mm x 4.6 mm,5 µm) A: Water (10mM formic acid) B: MeOH (10mM formic acid)	[228]

Table 2. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Tetracyclines (4)	Feed (1), meat (2), milk (3)	(1) MAE (MeOH) (2) SLE (citrate buffer/EtAc) (3) LLE (HCl/ACN), (1,2,3) SPME (MIP-coated)	LC–FLD (λ_{em}=375, λ_{ex}=535 nm)	1.0–2.3 µg l^{-1}	Luna C18 column (250 mm × 4.6 mm, 5 µm) Isocratic: Water/MeOH (70:30, v/v, pH 6.5, 0.1 M malonate, 0.05 M $MgCl_2$)	[234]
Tetracyclines (8)	Meat, fat, kidney, liver	SLE(sodium succinate), LLE (TCA), SPE (HLB)	LC–ESI(+)–IT–MS/MS	0.5- 4.5 ng g^{-1}	PLRP-S polymeric column (250 mm × 4.6 mm,8 µm) A: Water (0.001 M oxalic acid, 0.5% formic acid, 3% THF B: THF	[78]
Tetracycline (1)	Plasma	Incubation, LLE (TFA)	LC–ESI(+)–IT–MS/MS	50 ng ml^{-1} (b)	PLRP-S phase column (250 mm × 4.6 mm, 8 µm) A: Water (0.001 M oxalic acid, 0.5% formic acid) B: ACN	[82]
Tetracyclines (8)	Effluent waste-water	SPE (MAX)	LC–DAD (λ = 360 nm)	Data not provided	Aquasil C18 (150 mm × 4.6 mm, 5 µm) A: Water (10 mM oxalic acid) B: MeOH/ACN (50:50,v/v)	[232]

(a) Decision limit (CCα); (b) Limit of quantification (LOQ); (c) Limit of confirmation (LOC); (d) Method detection limit (MDL).

kidney, honey and milk; C18 cartridges have also applied for the analysis of honey and milk [203].

Additionally, ion-pair LC has been used without any SPE step but performing post-column derivatization (e.g. analysis of aminoglycosides in milk, egg, honey and kidney) with β-naphthoquinone-4-sulfonate in alkaline conditions [52]. The use of MS allows the reduction of the sample pre-treatment in all cases since the derivatization stage is not required. Matrix solid-phase dispersion (MSPD) has been applied for the analysis of seven aminoglycosides in milk samples. For the analysis of streptomycin residues in honey, direct dilution of the sample and subsequent SPE clean-up were performed. These procedures were significantly less time-consuming than methods involving typical solvent extraction due to the use of a QqQ analyzer for detection of the compounds previously separated by ion pair LC using HFBA [18,206]. An unusual derivatization agent, 7-fluoro-4-nitrobenz-2-oxa-1,3-diazole, was employed prior to LC–FLD for the analysis of plasma [207].

The use of immunoassay as screening tool has been reported for the analysis of aminoglycosides in meat, employing LC with post-column derivatization for the confirmation of the positive samples [208]. Most environmental applications are focused on the analysis of different types of water. Weakly acidic exchange SPE has been used for the analysis of these compounds in water (tap, river and wastewater), with subsequent separation and detection by SCX chromatography 0coupled to CLD using luminol and H_2O_2 (catalizer: Cu^{2+}) [17]. Ion-pair LC has also been applied for the analysis of aminoglycosides in hospital wastewater [209]. Additional applications can be found in recent literature [210].

3.4. β-LACTAMS

The analysis of β-lactams is mainly focused on the analysis of animal products and tissues, such as milk or meat. Methods reporting the analysis of environmental and biological samples are found to a lesser extent. The development of methodologies for the quantitative analysis of β-lactams has been hindered by their stability in organic solvents such as MeOH and ACN and under acidic conditions (Table 2) [29].

In general, the performance of a solvent extraction and/or SPE is the preferred procedure; moreover, subsequent LLE can also be included. ACN or ACN:water mixtures, and C18 or polymeric phases are commonly used as extraction solvent and SPE sorbent, respectively. This methodology has been

used for the analysis of β-lactams in milk [57,59,65], and biological samples, such as kidney [211]. SPE can be replaced by dispersive SPE (D-SPE) as a simplification of the extraction method; this procedure has been applied in kidney [61,212]. Either the solvent extraction or the SPE step can be omitted; only SPE can be used in liquid matrices, such as milk [213], plasma [60] and water (including wastewater) [56]. The application of LLE without further clean-up can be applied in milk, liver and kidney [62,64], but employing ACNtetraethylammonium chloride mixtures. Less common is the use of other extraction solvents such as buffer solutions (e.g. KH_2PO_4 and Na_2HPO_4) [214-216]. Additionally, supported liquid membrane (SLM) extraction can be employed as a purification and enrichment extraction (e.g. milk, liver and kidney [64]), as well as combinations of SPE cartridges (e.g. C18 plus SCX) to improve the clean-up efficiency (e.g. in liver [58]).

3.5. MACROLIDES

Most of applications are related to the monitoring of macrolides in animal products, mainly in meat (Table 2). In general, the major method employed for extraction of macrolides involves an extraction with organic solvents (e.g. ACN, MeOH, metaphosphoric acid, water/MeOH, MeOH/metaphosphoric acid) and/or buffers (e.g. metaphosphoric acid, MeOH/metaphosphoric acid, phosphate buffer, tris (tris(hydroxymethyl)-aminomethane) buffer, EDTA McIlvaine buffer (citric acid and disodium hydrogen phosphate solutions)), and sample clean up performed by SPE. In SPE, HLB and SCX cartridges, together with C18 sorbents, can be applied for this purpose; HLB sorbents can result more appropriate than SCX phases to simplify the sample-handling.

This kind of general procedure has been applied in food samples (meat [66,73,74,217], egg [69,218], honey [69,219], milk [69]), animal tissues samples (liver and kidney [71]) and feeding stuffs [72]. Occasionally, the subsequent SPE can be removed (meat samples) [220].As an alternative to the typical methodology, MSPD has been applied in milk and yogurt samples, reducing solvent consumption [221]. In this case, the erythromycin TP, anhydroerythromycin, is formed in acidic matrices, such as yogurt, and thus its monitoring is convenient. Several methods omit the solvent extraction for the analysis of macrolides in liquid samples, such as environmental waters and wastewater, performing only the SPE step [69,222]. The determination of macrolide antibiotics in biological samples such as urine or plasma is very scarce [70]. Further applications can be found elsewhere [68].

3.6. TETRACYCLINES (TCS)

The number of developed methods for the analysis of TCs in foodstuffs is considerably higher than in other kind of matrices, mainly due to the widespread use of TCs in livestock production. The applications related to the determination of these antibiotics in environmental samples is also important but to a lesser extent, and scarce in biological samples (Table 2).

There is a variety of extraction and clean-up procedures that can be used for the analysis of TCs, although the main methodology is based on the performance of a solvent extraction and/or subsequent SPE. Since these compounds can readily form chelate complexes with divalent metal ions and combine with sample matrix proteins present in matrices such as milk, egg or animal tissues, a deproteinization step with an acidic deproteinate agent as extracting solvent can be applied. In general, the use of the so-called McIlvaine buffer (citric acid and disodium hydrogen phosphate solutions) either as the extractant or the eluent solvent in SPE is frequent. The McIlvaine buffer is normally used in combination with EDTA solutions. Succinate, phosphate, citrate and oxalate buffers, and TCA have also been used. Instead of buffer solution, the extraction can be performed with an organic solvent such as ACN, ethyl acetate or acidic MeOH. The deproteinization can also be carried out via organic solvent (e.g. ACN) [19].

Different sorbents can be used for the SPE stage, although hydrophilic-lipophilic balanced cartridges are widely applied for extraction. C18, mixed-mode reversed-phase and ionic exchange (MAX), strong anion exchange (SAX), phenyl and graphitized-black carbon (GBC) sorbents have also been utilized for clean-up purposes [19,20]. In order to improve the results, the pre-treatment of the C18 cartridges for SPE with EDTA, SPE silylation and the loading of the analytes in a buffer containing EDTA is reported [19]. Additional preventing actions, such as the silanization of all glassware or the use of other materials (e.g. PTFE) are also recommended [1]. The general methodology described above can be used for the analysis of TCs in food samples (meat [78,80,223,224], honey [80,225-228], milk [80,229], egg, fish [80]) , environmental samples (manure-amended soil [230]) and certain biological samples, such as liver and kidney [78,224]. In liquid samples, the pre-treatment sample can be shortened only applying the SPE stage (e.g. environmental water [230,231], wastewater [232]), and some optimized method do not perform any further clean-up after the solvent extraction, such as in water [82], egg, soil [83] and sediment [231] samples. Additionally, the use of other pre-treatment techniques is reported, such as MSPD (meat [76])

and PLE (manure-amended soil [230]). Metal chelate affinity chromatography (MCAC) is an unusual technique that has been be employed in the determination of TCs in meat samples as clean-up step [77]. RAM is another uncommon technique that can be used in switching column LC techniques in order to perform an on-line extraction/clean-up prior to typical separation (milk, water [233]). Besides, a novel SPME fiber coated with a molecularly-imprinted polymer (MIP) has been utilized for the extraction of TCs from milk, meat and feed samples [234].

In certain samples, such as plasma, the performance of an incubation step prior to the application of the sample extraction is reported to control the keto-enol tauromerism of CTC and carry out an adequate quantification [78].

3.7. SULPHONAMIDES

The reviewed applications are focused on the analysis of sulphonamides in a variety of animal products, such as honey, egg, milk, and meat. Besides, there is a significant number of applications related to the monitoring of these antibiotics in water, including surface water or wastewater. Apart from sulphonamide residues, the monitoring of sulphonamide TPs in environmental water is currently focusing attention, as it can be observed in recent reviews [235]. On the contrary, the analysis of sulphonamide antibiotics in biological fluids is much reduced (Table 3).

There is a variety of methodologies that can be suitable for the extraction/clean-up of sulphonamides; however, SPE and SLE/LLE-based procedures are mostly employed, individually or in combination. It is important to notice that acid hydrolysis (e.g. TCA, acetic acid, phosphoric acid or HCl) is usually carried out when analyzing high-sugar content matrices, such as honey, in order to release the sugar-bonded sulphonamides. Several solvents can be used for the extraction of these analytes, including ACN, water (at high temperature), ACN/water, ACN/dichoromethane or dichoromethane/acetone. ACN is also utilized for deproteinization purposes in complex matrices (e.g. fish, egg). The most common phases employed for SPE are copolymer-based (e.g. Oasis HLB), SCX (e.g. aromatic sulfonic cation-exchange) and silica-based cartridges. SLE/LLE and subsequent SPE can be applied in food matrices, such as honey [89,90], meat [236-239], egg [240,241], milk [236], and baby food [239]. Solvent extraction can also be applied without further SPE clean-up, as described in honey [92,242], milk [243], egg [21], meat [86] and manure samples [244]. For the monitoring of

sulphonamides in water samples, SPE-based methods are widely applied, using HLB cartridges or a combination of HLB and silica sorbents [87,88,91,245,246]. Mixed hemimicelles-based SPE is an alternative to the aforementioned SPE sorbents; this phase has a metal oxid-base, such as alumina or silica, which interacts with ionic surfactants producing hemimicelles and admicelles (mixed hemimicelles), enhancing the adsorption capability of the sorbent. This sorbent shows high-breakthrough volume, easy elution and high for rate for sample loading [247].

Additionally, SPME is also applied for the analysis of sulphonamides in water. Direct immersion of fibers using polydimethysylosane/divinylbenzene (PDMS/DVB) [248] and carbowax/templated resins (CW/TPR) [249] are adequate for this aim. A number of MSPD-based procedures are also reported in food samples, such as milk, egg [250], meat, fish [251] and cheese [252]; and animal tissues, such as liver and kidney [253]. Finally, two single applications of PLE and a new microextraction technique named polymer monolith microextraction (PMME) have been described in soils [254] and egg [255], respectively.

3.8. QUINOLONES

As significant and general remarks, it is important to comment that quinolones are not a homogeneous group of compounds because of the different type of substituents that can provide rather different properties [4]. Quinolones is a group of antibiotics largely studied, and therefore the number of published methods is considerable. Most reported methods are focused on the determination of quinolones in food (i.e. muscle, milk) and environmental samples (i.e. water, soils); whereas the applications related to the determination of these VDs in biological samples is reduced (e.g. liver, kidney, plasma). Sample pre-treatment varies greatly, and the establishment of a basic procedure for the determination of these antibiotics is complicated; besides, many of the optimized procedures analyze a few quinolones (Table 3).

Table 3. Selected applications for the analysis of sulphonamides, quinolones and coccidiostats

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/detection technique*	*LOD (units)*	*Observations*	*Ref.*
Sulphonamides (24)	Meat	SLE (ACN), LLE (n-hexane, EtAc)	UPLC–ESI(+)–QqQ–MS/MS	0.03-0.37µg kg^{-1}	Acquity BEH C18 (100 mm × 2.1 mm, 1.7µm) A: Water (0.2% formic acid) B: MeOH (0.2% formic acid)	[86]
Sulphonamides (8)	Meat	Homogeneization (ACN/water), SPME (PDMS/DVB)	LC–APCI(+)–-IT–MS/MS	16-39 µg kg^{-1}	Supelco LC–18DB (250 mm × 4.6 mm, 5 µm) A: Water B: ACN(80% water)/Water (30:70, v/v)	[248]
Sulphonamides (10)	Egg	SLE(CH_2Cl_2/acetone/HAc), SPE (aromatic sulphonic)	LC–ESI(+)–QqQ–MS/MS	16.1-20.5 µg kg^{1} (a)	Luna ODS2 C18 (75 mm × 4.6 mm, 3 µm) A: Water (5mM NH_4Ac, 0.1% formic acid) B: MeOH	[240]
Sulphonamides (1), trimethoprim	Egg	SLE (ACN), RAM (TFA/ACN)	LC–UV (λ=240, 269 nm)	25-40 ng ml^{-1}	RAM bovine serum albumin (BSA) C18 (100 mm × 4.6 mm, 10µm) C18 analytical column (150 mm × 4.6 mm, 10 µm) Isocratic: Water/ACN (82:18, v/v, 50 mM TFA)	[21]
Sulphonamides (2)	Egg	SLE (EtOH), PMME (methacrylic acid-ethylene glycol dimethacrylate)	LC–UV (λ= 269 nm)	8.8-11.2 ng g^{-1}	Kromasil ODS (150 mm × 4.6 mm, 5 µm) Isocratic: Water/MeOH (30:70, v/v, pH 3, 0.02 M phosphate buffer)	[255]

Table 3. (Continued).

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/detection technique*	*LOD (units)*	*Observations*	*Ref.*
Sulphonamides (18)	Honey	Homogenize (TCA), LLE (Na_2HPO_4/ACN/ CH_2Cl_2)	LC–APPI(+)–QqLIT–MS/MS	0.4-4.5 µg kg^{-}	Zorbax SB C18 (210 mm × 50 mm, 1.8 µm) A: Water (1mM NFPA, 0.5% formic acid) B: MeOH /ACN (1:1, v/v, 0.5% formic acid)	[92]
Sulphonamides (5)	Milk	LLE (ACN)	LC–ESI(+)–QqQ–MS/MS	< 5 µg kg^{-1} (b) 102.8-107.1 µg kg^{-1} (a)	Symmetry C18 (150 mm x 3 mm, 5 µm) A: Water (pH 3.5, 10 mM NH_4Ac) B: ACN	[243]
Sulphonamides (1)	Meat, milk	SLE (carbonate buffer/CH_2Cl_2), SPE (silica gel)	LC–ESI(+)–QqQ–MS/MS	0.12 µg kg^{-1} (a)	Luna 5 C18 (150 mm × 2 mm, 5 µm) Isocratic: Water/ACN (80:20, v/v, 1mM ammonium formate)	[236]
Sulphonamides (13)	Meat (1), infant food (2)	PLE (hot water)	LC–ESI(+)–QqQ–MS/MS	(1) 0.6- 2.6 µg kg^{-1} (2) 0.4-1.7 µg kg^{-1}	Alltima C18 RP (250 mm x 4.6 mm, 5 µm) A: Water (1 mM formic acid) B: ACN (1 mM formic acid)	[239]
Sulphonamides (12)	Meat, fish	MSPD (hot water)	LC–ESI(+)–Q–MS	3-15 µg kg^{-1} (c)	Alltima C18 RP (250 mm x 4.6 mm, 5 µm) A: Water (5 mM formic acid) B: MeOH (5 mM formic acid)	[251]

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/detection technique*	*LOD (units)*	*Observations*	*Ref.*
Sulphonamides (12)	Kidney, liver	MSPD (hot water)	(1) LC–ESI(+)–Q–MS (2) LC–ESI(+)–QqQ–MS/MS	(1) 5-14 µg kg^{-1} (2) 1-8 µg kg^{-1}	Alltima C18 RP (250 mm x 4.6 mm, 5 µm) A: Water (5 mM formic acid) B: MeOH (5 mM formic acid)	[253]
Sulphonamides (7), trimethropim	Animal manure	SLE (EtAc)	LC–ESI(+/-)–QqQ–MS/MS	< 0.1µg kg^{-1} (c)	Nucleosil 100-5 C18 HD (125 mm x 3 mm, 5 µm) A: Water/ACN (90:10, v/v, pH 4.6, 1 mM NH_4Ac) B: ACN	[244]
Sulphonamides (16), trimethoprim	Environ-mental waters	SPE (HLB, silica)	UPLC–ESI(+)–QqQ–MS/MS	8-200 pg l^{-1} (b)	Acquity BEH C18 (100 mm × 2.1 mm, 1.7µm) A: Water (0.1% formic acid) B: MeOH (0.1% formic acid)	[87]
Sulphonamides (10)	Environ-mental waters, bottled mineral water.	SPE (HLB)	LC–ESI(+)–QqLIT–MS	0.01-461 ng l^{-1}	Atlantis C18 (150 mm × 2.1 mm, 3 µm,) A: Water (pH 2.2, 1% formic acid) B: ACN (1% formic acid)	[91]
Sulphonamides (10)	Waste-water	(1) SPME (CW/DVB) (2) SPE (MCX)	LC–ESI(+)–QqQ–MS/MS	(1) 9.04-55.3 µg l^{-1} (b) (2) 2.88-9.00 µg l^{-1} (b)	(1)Ultracarb ODS (4.6 mm × 150 mm, 5 µm) (2) XTerra MS-C18 (210 mm × 250 mm; 5 µm) A: Water (pH 3, 20mM NH_4Ac, 0.1% formic acid) B: ACN/MeOH (2:1, v/v, 20mM NH_4Ac)	[249]

Table 3. (Continued).

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/detection technique*	*LOD (units)*	*Observations*	*Ref.*
Sulphonamides (3)	Wastewater, surface water, groundwater, drinking water	SPE (SDB)	LC–ESI(+)–QqQ–MS/MS	0.01-0.02 µg l^{-1} (c)	Sunfire C18 (210 mm × 150 mm, 3.5 µm) Isocratic: Water/ACN (90:10, v/v)	[245]
Sulphonamides (6)	Water (1), soil (2)	(1) SPE (HLB) (2) MAE (ACN), SPE (HLB)	LC–FLD (λ_{ex}=405, λ_{em}= 485 nm)	(1) 1-8 ng g^{-1} (2) 1-6 ng g^{-1}	LiChrospher 100 RP-18 (250 mm × 4 mm, 5 µm) A: Water (pH 3.4, 10mM HAc) B. ACN Pre-column derivatization: fluorescamine	[88]
Sulphonamides (5)	Soil	PLE (buffered water/ACN, 85:15, v/v)	LC–ESI(+)–QqQ–MS/MS	< 15 µg kg^{-1}	Nucleodur C18 Gravity (125 mm × 2 mm, 5 µm) A: Water (pH 4, 1mM HAc) B: ACN (1mM HAc)	[254]
Quinolones (16)	Honey	TFC (styrene-divinylbenzene copolymer	LC–ESI(+)–QqQ–MS/MS	< 5 µg kg^{-1}	TFC, Zorbax SB C18 (50 mm × 2.1mm, 1.8 µm) A: Water (1mM NFPA, 0.5% formic acid) B: MeOH(0.5% formic acid) /ACN (1:1,v/v)	[88]

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/detection technique*	*LOD (units)*	*Observations*	*Ref.*
Quinolones (2)	Meat	SLE (ACN/water/formic acid)	TFC–ESI(+)–QqQ – MS/MS	25 μg kg^{-1} (c)	Chromolith Speed Rod RP18e (50 mm × 4.6 mm) A: (10mM NH_4Ac, 1.5 ml TFA) B: ACN/water (1:1, v/v, 10mM NH_4Ac, 2ml TFA)	[97]
Quinolones (10)	Meat (1), egg (2)	(1) SLE (TFA/MeOH), SPE(C18) (2) SLE (NaOH/ACN) ,SPE (C18)	LC–DAD (λ= 275, 255 nm)	(1) 5.0-12.0 μg kg^{-1}	ODS-3 (250 mm × 4 mm, 5 μm) A: Water (0.1% TFA) B: ACN, C: MeOH	[256]
Quinolones (10)	Meat, seafood	SLE (phosphate buffer), SPE (HLB)	LC–FLD (λ programm)	0.3 -1.0 ng g^{-1}	Symmetry C18 (250 mm x 4.5 mm, 5 μm) A: Water (pH 2.8, 0.02% formic acid) B: ACN	[261]
Quinolones (13)	Feed	PLE(metaphosphoric acid/ACN), SPE (HLB)	LC–DAD (λ= 278nm) LC–FLD (λ_{ex}= 278, λ_{em}= 446 nm)	0.4-1.5 mg kg^{-1}	C5 analytical column (150mm × 4.6 mm, 5 μm) A: ACN/water/THF (10:89:1, v/v/v, pH 2.6, 0.12 M KH_2PO_4) B: ACN/water/THF (50:49:1, v/v/v, pH 2.6, 0.04 M KH_2PO_4)	[95]
Quinolones (11)	Kidney	SLE (ACN), SPE (SDB-RPS)	LC–ESI(+)–QqQ–MS/MS	0.3-2.0 μg kg^{-1}	Symmetry Shield RP-8 (150 mm × 3.9 mm; 5 μm) A: Water (pH 2.5, formic acid) B: ACN/water (0.14% formic acid)	[260]

Table 3. (Continued).

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/detection technique*	*LOD (units)*	*Observations*	*Ref.*
Quinolones (9)	Pig liver	SLE (H_3PO_4/ACN), SPE (ENV+ Isolute)	(1) LC –ESI(+)–TOF–MS (2) LC–ESI(+)–Q –MS (3) LC–ESI(+)–QqQ – MS/MS	(1) 0.5-2 µg kg^{-1} (2) 0.5-2 µg kg^{-1} (3) < 0.5 µg kg^{-1}	Zorbax Eclipse XDB-C8 (150 mm × 4.6 mm) Isocratic: Water/ACN (86:14, v/v, pH 2.5, NH_4Ac)	[100]
Quinolones (2)	Sewage sludges, sludge-treated soils	ASE (H_3PO_4/ACN), SPE (MPC disk)	LC–FLD (λ_{ex}= 278 nm, λ_{em}=445 nm)	0.05-0.123 µg kg^{-1}	Discovery RP-AmideC16 (250 mm x 3 mm, 5 µm) A: Water (pH 2.4, 25 mM H_3PO_4) B: ACN	[271]
Quinolones (3)	Wastewater	SPE (WCX)	(1) LC–FLD (λ_{ex}= 278, λ_{em}= 445 nm) (2) LC–ESI(+)–Q–MS (3) LC–ESI(+)–QqQ–MS/MS	(1) 5 pg (2) 10-15 pg (3) 1-5 pg (d)	Zorbax SB-C8 (210 mm ×150 mm, 3.5 µm) Isocratic: Water/ACN/MeOH/formic acid (81.5;6:12:0.5, v/v)	[265]
Quinolones (20)	Environ-mental waters	SPE (HLB, WCX)	UPLC–ESI(+)–QqQ–MS/MS	0.6-50 ng l^{-1}	Acquity BEH C18 column (100 mm × 2.1 mm,1.7µm) A: Water (0.1% formic acid) B: MeOH	[99]
Quinolones (1)	Hospital sewage water	(1) off line-SPE (C18, SAX) (2) on-line-SPE (C18, SAX)	LC–UV (λ=275 nm, 260nm)	(1) 0.5-1.4 µg l^{-1} (2) 0.6-1.8µg l^{-1}	AtlantisTM dC18 (150 mm x 3.0 mm, 3 µm) A: Water (pH 2.5, formic acid) B: ACN	[266]

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/detection technique*	*LOD (units)*	*Observations*	*Ref.*
Nitroimidazoles (6)	Meat	SLE (EtAc), LLE (formic aqueous)	LC–ESI(+)–QqLIT–MS/MS	0.26-0.44 μg kg^{-1} (a)	Gemini C18 (150 mm x 3 mm, 5 μm) A: Water/ACN (90:10, v/v, pH 3.5 formic, 10 mM ammonium formate) B: Water/ACN (10:90, v/v, pH 3.5 formic, 10 mM ammonium formate)	[105]
Nitroimidazoles (7)	Meat	SLE (phosphate buffer, protease), LLE (n-hexane)	GC–NCI–Q–MS	0.65-2.80 μg kg^{-1} (a)	ZB-5 (30 m x 0.25 mm, 0.25 μm) Derivatization BSA	[170]
Nitroimidazoles (7)	Egg-based samples	SLE (ACN), MISPE	LC–ESI(+)–QqQ–MS/MS	< 0.34 μg kg^{-1} (a)	SymmetryShield RP18 (150 mm x 2.1 mm, 3.5 μm) A: Water (1% formic acid) B: ACN (1% formic acid)	[107]
Nitroimidazoles (7)	Egg (1), meat (2)	(1) SLE (ACN), SPE (HLB) (2) SLE (phosphate buffer, EtAc), LLE (n-hexane, formic acid aqueous)	LC–ESI(+)–QqQ – MS/MS	0.07-0.36 μg kg^{-1} (a)	SymmetryShield RP18 (150 mm x 2.1 mm, 3.5 μm) A: Water (1% formic acid) B: ACN (1% formic acid)	[109]
Nitroimidazoles (2)	Meat, kidney, liver	SLE (toluene), LLE (n-hexane), SPE (-NH_2)	GC–NCI–Q–MS	0.1-1.5 μg kg^{-1}	DB-5ms (30 m x 0.25 mm x 0.25 μm) Derivatization BSA	[169]
Nitroimidazoles (7)	Plasma	LLE (phosphate buffer/NaCl/protease, SPE (diatomaceous earth XTR)	LC–APCI(+)–QqQ–MS/MS	0.25-1.25 μg l^{-1} (a)	Genesis C18 (150 mm x 3 mm, 4 μm) A: Water (1% HAc) B: ACN	[23]

Table 3. (Continued).

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/detection technique*	*LOD (units)*	*Observations*	*Ref.*
Nitroimidazoles (7)	Kidney	SLE (EtAc), LLE (HCl, n-hexane), SPE (mixed-mode MCX)	UPLC–ESI(+)–QqQ–MS/MS	0.05-0.5 μg l^{-1}	Acquity UPLC BEH C18 (50 mm × 2.1mm, 1.7 μm) A: Water B: ACN	[104]
Nitroimidazoles (4)	Liver	LLE (EtAc), LLE (n-hexane, HCl), SPE (mixed-mode MCX)	LC–ESI(+)–QqQ–MS/MS	0.1-0.5 μg kg^{-1} (c)	SunFire C8 (100 mm x 2.1 mm, 3.5 μm) A: Water B: ACN	[273]
Nitroimidazoles (5)	Water	SPE (HLB)	LC–ESI(+)–Q–MS		C18 (150 mm x 2.1 mm, 5 μm) A: Water (pH 4.3, 5 mM NH_4Ac) B: ACN	[275]
Nitrofurans (4)	Meat	SLE (HCl), derivatization NBA, LLE (EtAc), SPE (polymeric)	LC–ESI(+)–QqQ–MS/MS	0.11-0.21 μg kg^{-1} (b)	SymmetryShield C18 (150 mm x 2.1 mm, 3.5 μm) A: Water (0.025% HAc) B: ACN	[280]
Nitrofurans (12)	Milk	Hydrolisis, LLE (n-hexane), SPE (HLB), derivatization NBA	LC–APCI(+)–QqQ–MS/MS	≤ 2 ng g^{-1}	Inertsil ODS-3 (150 mm x 2.1 mm, 5 μm) Isocratic: Water/MeOH (45:55, v/v, 20 mM NH_4Ac)	[115]
Nitrofurans (4)	Honey	SLE (HCl), derivatization NBA, LLE (EtAc), SPE (polymeric)	LC–ESI(+)–QqQ–MS/MS	0.07-0.46 μg kg^{-1} (b)	SymmetryShield C18 (150 mm x 2.1 mm, 3.5 μm) A: Water (0.025% HAc) B: ACN	[277]

Compounds (number)	*Matrix*	*Sample pre-treatment*	*Separation/detection technique*	*LOD (units)*	*Observations*	*Ref.*
Nitrofurans (4)	Honey	SLE (HCl), SPE (HLB), derivatization NBA, SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	0.2-2 µg kg^{-1}	Inertsil ODS3 (150 mm x 2 mm, 3 µm) A: Water (pH 3.4, 20 mM NH_4Ac) B: ACN	[282]
Nitrofurans (4)	Water (1), feed (2)	(1) Adjust pH (2) SLE (ACN)	LC-UV (λ = 250, 365 nm)	0.21-2.70 µg kg^{-1}	Chromolith monolithic column (100 mm x 4.6) Isocratic: Water/ACN (92:8, v/v, pH 4.5, 50 mM KH_2PO_4)	[113]
Nitrofurans (4)	Feed	SLE (alkaline EtAc), SPE (-NH_2)	(1) LC-DAD (λ = 375 nm) (2) LC-ESI (+/-)-QqQ-MS/MS	(1) 47-98 µg kg^{-1} (b) (2) 7-21 µg kg^{-1} (b)	(1) Lichorspher60 RP-select (250 mm x 4 mm, 5 µm) (2) Zorbax Eclipse XDB-C18 (150 mm x 2.1 mm, 5 µm) A: Water (pH 4.3, 14 mM NH_4Ac) B: ACN	[278]
Compounds (number)	Matrix	Sample pre-treatment	Separation/detection technique	LOD (units)	Observations	Ref.
Nitrofurans (4)	Retine	SLE (HCl), derivatization NBA, LLE (EtAc)	LC–ESI(+)–QqQ–MS/MS	Data not provided	Luna C18 (150 mm x 2 mm, 3 µm) A: Water/MeOH (80:20, v/v, 5 mM NH_4Ac) B: MeOH	[276]
Nitroimidazoles (6), nitrofurans (4)	Meat	SLE (HCl+NBA), SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	0.01-0.20µg kg^{-1}	SunFire C8 (150 mm x 2.1 mm, 5 µm) A: Water B: ACN	[117]

(a) Decision limit (CCα); (b) Method detection limit (MDL); (c) Limit of quantification (LOQ); (d) Limit of identification (LOI).

Table 4. Selected applications for the analysis of hormones and β-agonists

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
ASs (10)	Meat	Enzymatic hydrolysis, LLE (tert-butylmethyl ether, n-hexane), SPE (HLB, -NH_2)	LC–APCI(+)–QqQ–MS/MS	0.15-0.79 μg kg^{-1} (b)	Luna C18 (150 mm x 2 mm, 5 μm) A: Water/ACN (65:35, v/v) B: ACN	[302]
ASs (22)	Meat	Digestion (subtilisin), LLE (n-hexane), SPE (C18, -NH_2)	LC–ESI(+/-)–QqQ–MS/MS	< 0.5 ng g^{-1} (a)	Symmetry C18 (150 mm x 2.1 mm, 5 μm) ESI (+): Water/MeOH (65:35, v/v, 0.3% formic acid) ESI (-): Water/MeOH (65:35, v/v, 0.5% ammonia)	[299]
ASs (4)	Meat	Enzymatic hydrolysis, LLE (tert-butylmethyl ether, n-hexane), SPE (HLB)	LC–APCI(+)–IT–MS/MS	0.3 ng g^{-1}	Nucleosil 100-5 C18 (250 mm x 4.6 mm, 5 μm) A: Water B: MeOH	[300]
ASs (6)	Meat	Enzymatic hydrolysis, LLE (tert-butylmethyl ether, petroleum ether), semi-preparative LC	GC–EI–Q–MS	Data not provided	CP-SIL 5CB (25 m x 0.25 mm, 0.12 μm) Derivatization HFBA	[301]
ASs (7)	Feed	SLE (ACN), saponification, SPE (polymeric)	LC–DAD (λ = 245 nm)	27-37 μg kg^{-1} (a)	Chromolith RP-18e (100 mm x 4.6 mm) Isocratic: Water/ACN (71:29, v/v)	[119]
ASs (6)	Hair	(1) SLE (Tris buffer, tert-butylmethyl ether, SPE (C18) (2) SLE (MeOH), LLE (EtAc, H_2O, NaOH), SPE (-NH_2, Silica)	(1) UPLC–ESI(+)–QqQ–MS/MS (2) GC–EI–QqQ–MS/MS	(1) 1.3-4.6 μg kg^{-1} (a) (2) 0.7-1.7 μg kg^{-1} (a)	(1) Acquity BEH C18 (100 mm x 2.1mm, 1.7 μm) A: Water/MeOH (60:40, v/v, 0.05% ammonia) B: MeOH (0.05% ammonia) (2) OV-1 (30 m x 0.25 mm x 0.25 μm)	[118]

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
ASs (28)	Hair	Enzymatic hydrolysis, LLE (fractionation), SPE (silica and/or -NH_2)	GC–EI–QqQ–MS/MS	0.01-1.92 µg kg^{-1} (a)	OV-1 (30 m x 0.25 mm, 0.25 µm) Derivatization MSTFA, TMIS and/or DDE	[304]
ASs (4)	Urine	SPME (carbowax-divinylbenzene)	GC–EI–Q–MS	0.002-0.008 ng g^{-1}	HP-5ms (30 m x 0.25 mm, 0.25 µm)	[295]
Corticosteroids (9)	Urine	SPE (MAX) and/or hydrolisis	LC–ESI(-)–QqQ–MS/MS	≤ 3 µg l^{-1}	Zorbax Eclipse XBD (100 mm x 2.1mm, 1.8 µm) Isocratic: Water/ACN (70:30, v/v, 0.1% formic acid)	[126]
ASs (18)	Urine	Enzymatic hydrolysis, SPE (C18), LLE (tris buffer, n-pentane), SPE (HLB)	GC–EI–Q–MS	0.06-0.17 µg kg^{-1} (a)	Factor Four VF-5ms (30 m x 0.25 mm, 0.25 µm) Derivatization MSTFA	[288]
ASs (19)	Urine	SPE (C18), hydrolysis, LLE (diethy ether, Na_2CO_3, water), SPE (-NH_2)	GC–EI–IT–MS^n	0.01-1.92 µg kg^{-1} (a)	SGE BPX-5 (25 m x 0.22 mm, 0.25 µm) Derivatization MSTFA	[291]
ASs (1)	Urine	Combination of SPE (HLB), oxidation, SPE (silica), semi-preparative LC	GC–C–IRMS	Data not provided	Evaluation $^{13}C/^{12}C$ isotopic ratio	[294]
ASs (13)	Urine, water	LPME (octanol, ammonia)	LC–ESI(+)–QqQ–MS/MS	2-250 ng ml^{-1}	Purospher RP-18 (125 mm x 3 mm, 5 µm) A: Water (ph 4.2, 15 mM NH_4Ac) B: ACN/water (90:10, v/v, ph 4.2, 15 mM NH_4Ac)	[296]
ASs and others (6)	Water, blood	SPME	GC–EI–Q–MS	0.002-1.261 µg l^{-1}	HP-5ms (25 m x 0.25 mm, 0.25 µm) On-fiber derivatization BSTFA	[305]

Table 4. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Corticosteroids (1)	Milk	Deproteination (TCA), SPE (C18)	LC–APCI(+)–IT–MS/MS	41 pg ml^{-1}	PLRP-S Polymeric reversed-phase (250 mm x 4.6 mm, 8 µm) A: Water (0.1% acetic acid) B: ACN (0.1% acetic acid)	[130]
Corticosteroids (12)	Milk	LLE (MeOH/acetate buffer, n-hexane), SPE (HLB, silica, -NH_2)	UPLC–ESI(+)–QqQ–MS/MS	0.02-0.38 µg kg^{-1}	Acquity BEH C18 (100 mm x 2.1 mm, 1.7 µm) A: Water (0.1% formic acid) B: MeOH (0.1% formic acid)	[133]
Corticosteroids (12)	Meat (1), hair (2), urine (3)	(1) SLE (MeOH/buffer) and (1,2,3) hydrolysis, SPE C18, LLE (Na_2CO_3), SPE (silica)	LC–ESI(-)–QqQ–MS/MS	(1,3) 40-70 pg g^{-1} (2) 2.9-9.3 pg mg^{-1}	Nucleosil C18 (50 mm x 2 mm, 5 µm) A: Water (0.5% HAc) B: MeOH	[140]
Corticosteroids (10)	Water (1), feed (2)	(1) SPE (C18) (2)SLE/LLE (ACN), SPE (polymeric)	LC–DAD (λ = 245 nm)	(1) 26-39 µg kg^{-1} (2) 6.2-8.1 ng ml^{-1}	Chromolith RP-18e (100 mm x 4.6 mm), monolithic column Isocratic: Water/ACN (71:29, v/v)	[129]
Corticosteroids (3)	Liver	PLE (n-hexane/EtAc)	LC–APCI(-)–QqQ–MS/MS	1 µg kg^{-1} (b)	Kingsrob C18 (250 mm x 2 mm, 5 µm) Isocratic: Water/MeOH/ACN (60:35:5, v/v, 5 mM NH_4Ac)	[307]
Corticosteroids (3)	Liver	SLE (NH_4Ac)	LC–APCI(-)–IT–MS/MS	0.10-12.00 µg kg^{-1}	Hypercarb porous graphite (125 mm x 4.6 mm, 5 µm) Isocratic: MeOH/CH_2Cl_2 (85:15, v/v)	[127]

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Corticosteroids (10)	Plasma	SPE (HLB)	µLC–ESI(+)–QqQ–MS/MS	0.2-1 pg ml^{-1}	Zorbax Eclipse C18 (150 mm x 3 mm, 3.5 µm) A: Water/ACN (90:10, v/v, pH 3.2, 2 mM NH_4Ac) B: Water/ACN (10:90, v/v, pH 3.2, 2 mM NH_4Ac)	[131]
Corticosteroids (17)	Urine	Enzymatic hydrolysis, LLE (diethyl ether)	UPLC–ESI(+)–TOF–MS	0.1-3.3 µg ml^{-1}	Acquity BEH C18 (50 mm x 2.1 mm, 1.7 µm) A: Water (0.1% formic acid) B: ACN (0.1% formic acid)	[132]
Corticosteroids (9)	Feces	LLE (diethyl ether, Na_2CO_3), SPE (silica), semi-preparative LC, derivatization (ethxyamine)	LC–APCI(+)–IT–MS/MS	Data not provided	Symmetry (150 mm x 3.9 mm, 5 µm) Isocratic: Water/MeOH (72:28, v/v)	[128]
Thyreostats (2)	Meat, thyroid	SLE (ACN), MSPD (silica gel)	GC–EI–QqQ–MS/MS	2-10 µg kg^{-1}	DB-5ms (30 m x 0.25 mm, 0.25 µm) Derivatization (PFBBr, MSTFA)	[175]
Thyreostats (6)	Meat, thyroid	SLE (MeOH, n-hexane), SPE (silica), derivatization (NBD-Cl), LLE (diethyl ether)	LC–ESI(+)–IT–MS/MS	20 µg kg^{-1} (c)	Symmetry C18 (150 mm x 2.1 mm, 5 µm) A: Water (0.73% HAc) B: MeOH	[31]
Thyreostats (6)	Milk, urine	MSPD (silica gel)	GC–EI–Q–MS	0.0016-0.004 µg g^{-1}	DB-5ms (30 m x 0.25 mm, 0.25 µm) Derivatization (PFBBr, MSTFA)	[310]
Thyreostats (5)	Thyroid, urine	SLE/LLE (EDTA/2-mercaptoethanol/EtAc), SPE (silica)	LC–APCI(-)–Q–MS	≈ 25 ng g^{-1}	Prodigy ODS3 (150 mm x 4.6 mm, 5 µm) A: Water (0.1% HFBA) B: Water/MeOH (55:45, v/v, 0.1% HFBA)	[147]

Table 4. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
Thyreostats (5)	Plasma	SLE/LLE (EDTA/2-mercaptoethanol/EtAc), SPE (silica)	LC–ESI(+)–QqQ–MS/MS	45.5-78.6 μg l^{-1} (a)	Prodigy ODS3 (150 mm x 3 mm, 5 μm) A: Water (0.1% HAc) B: MeOH	[143]
β-agonists (3)	Meat	PMME	LC–ESI (+)–Q–TOF–MS/MS	0.12-0.87 ng g^{-1}	XTerra C18 (150 mm x 2.1 mm, 3.5 μm) A: Water (0.1% formic acid) B: ACN/water (80:20 ,v/v, 0.1% formic acid)	[164]
β-agonists (12)	Meat	Digestion (protease), enzymatic hydrolysis, LLE (EtAc, n-hexane), MISPE	LC–APCI (+)–IT–MS/MS	0.13-1.00 ng g^{-1} (a)	Luna C18 (150 mm x 2 mm, 3 μm) A: Water/MeOH (95:5 ,v/v, 10 mM NH_4Ac) B: Water/MeOH (5:95 ,v/v, 10 mM NH_4Ac)	[165]
β-agonists (16)	Meat, kidney, liver	Enzymatic hydrolysis, LLE ($HClO_4$), SPE (mixed-mode, HLB, MCX)	UPLC–ESI(+)–QqQ–MS/MS	0.02-0.79 μg kg^{-1} (a)	Acquity BEH C18 (100 mm x 2.1mm, 1.7 μm) A: Water (0.1% formic acid) B: MeOH (0.1% formic acid)	[150]
β-agonists (7)	Liver, urine	SLE/LLE (acetate buffer), hydrolisis, SPE (C18), LLE (tert-butylmethyl ether)	LC–ESI(+)–IT–MS^3	0.05-0.2 μg kg^{-1} (a)	Hypersil Gold C18 (50 mm x 3 mm, 3 μm) A: Water (10 mM HAc) B: ACN	[149]
β-agonists (3)	Feed (1), urine (2)	(1) SLE (HCl) (2) Enzymatic hydrolysis, IAC	GC–EI–Q–MS		HP-5ms (15 m x 0.25 mm, 0.25 μm) Derivatization MBA	[180]

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
β-agonists (31)	Feed (1), hair (2), urine (3)	(1) SLE ($MeOH/H_3PO_4$), SPE (mixed-mode) (2) Digestion (NaOH), LLE (HCl/acetate buffer), SPE (mixed-mode) (3) Enzymatic hydrolysis, SPE (mixed-mode)	(A) LC–ESI(+)–QqQ–MS/MS (B) UPLC–ESI(+)–QqQ–MS/MS	(1,2) 5-10 μg kg^{-1} (a) (3) 0.2-0.5 μg l^{-1} (a)	(A)Alltima C18 (150 mm x 3.5 mm, 5 μm) A: Water (0.2% formic acid), B: ACN (0.2% formic acid) (B) Acquity BEH C18 (100 mm x 2.1mm, 1.7 μm) A: Water (0.1% formic acid), B: ACN (0.1% formic acid)	[151]
β-agonists (16)	Urine	Enzymatic hydrolysis, LLE (tert-butylmethyl ether)	LC–ESI(+)–QqQ–MS/MS	< 10 ng ml^{-1}	Hyper Gold C18 (50 mm x 2.1 mm, 3 μm) A: Water (15 mM NH_4Ac, 0.1% formic acid) B: ACN	[152]
β-agonists (1)	Urine	(1) SPME (2) LPME	GC–EI–IT–MS LC–UV	(1) 0.25 ng ml^{-1} (2) 3.9 ng ml^{-1}	(1) DB-5ms (30 m x 0.25 mm, 0.25 μm) Derivatization HDMS (2) Supelco 516 C18 (250 mm x 4.6 mm, 5 μm) Isocratic: Water/ACN (pH 2.5, 0.05 M Na_2HPO_4)	[155]
β-agonists (1)	Liver	MSPD, SPE (MIP+SCX)	LC-ESI (+)-IT-MS/MS	< 0.1 μg kg^{-1}	Hypercarb graphite (100 mm x 4.6 mm, 5 μm) A: Water/MeOH (60:40 ,v/v) B: ACN/ MeOH (80:20 ,v/v)	[148]
β-agonists (7)	Retina	SLE (buffer), SPE (mixed-mode C18, SCX)	GC–EI–Q–MS	70 ng g^{-1}	HP-5ms (30 m x 0.25 mm, 0.25 μm) Derivatization MSTFA	[179]

Table 4. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/ detection technique	LOD (units)	Observations	Ref.
β-agonists (15)	Sewage	SPE (MCX)	LC–UV (λ = 220 nm) LC–FLD (λ_{ex} = 230, λ_{em} = 310, 425 nm)) LC–ESI(+)–QqQ–MS/MS	6-11 ng l^{-1}	Zorbax SB-C8 (150 mm x 2.1 mm, 3.5 µm) A: Water/ACN/formic acid (94.5:5.0:0.5, v/v) B: ACN/formic acid (99.5:0.5, v/v) (3)	[265]
β-agonists (9)	Waste-water	SPE (HLB)	LC–ESI(+)–QqLIT–MS/MS	0.2-7.5 ng l^{-1}	Chirobiotic V (250 mm x 4.6 mm, 5 µm) Isocratic: Water/MeOH (10:90, v/v, 0.1% formic acid, pH 4, 20 mM NH_4Ac)	[161]

(a) Decision limit (CCα); (b) Limit of quantification (LOQ); (c) Elucidation purposes.

Many methods for the analysis of quinolones in foodstuffs perform a solvent extraction with ACN, acidified ACN (e.g. phosphoric acid, TFA, TCA) or MeOH. The majority of these methods apply a subsequent SPE with C18, copolymer, or a combination of polymer and anion exchange sorbent. This is the procedure employed for the determination of quinolones in meat [256-258], egg [256], milk [258], fish [101,259], seafood [259] animal feed [95] and biological samples, such as liver [100,258] and kidney [260]. Similarly, buffers solutions can be used for extraction purposes together with SPE, but to a lesser extent; phosphate-based buffers, and C18 and copolymer-based SPE sorbents are employed in these methodologies. Applications of the aforementioned procedure can be found in meat [261,262], sea food [261], fish [262], milk [94] and egg [103]. Recently, the use of TFC techniques for the analysis of quinolones in tissues [97] and honey [98] has also been described, simplifying the sample pre-treatment.

In water samples, including surface waters [263,264] and sewage water [99,265-267], SPE is normally used for the extraction of quinolones; several sorbents are suitable, such as HLB, C18 (cartridges and disks), weak cation exchange (WCX), mixed-phase sorbents (also named mixed-mode) and combinations of C18/SAX or polymeric and WCX. On the contrary, a variety of methodologies can be applied in soils, such as MISPE [268], ultrasonic-assisted extraction (USE) in column [269], microwave-assisted extraction (MAE) [270] and PLE (with clean-up by SPE mixed-phase cation exchange) [271].

3.9. Coccidiostats

Nitroimidazoles

The analysis of nitroimidazoles is greatly focused on food samples, although some applications in plasma and water can be found. Together with the parent compounds, hydroxy-metabolites are widely determined (Table 3).

Most of food applications are related to the analysis of nitroimidazoles and metabolites in animal products, mainly meat and egg. These compounds are relatively easy to extract directly with organic solvents; or by LLE with mixtures of organic solvent (typically ethyl acetate, ACN) and buffer solutions (phosphate-based solutions) followed by a deffating step. Normally, a subsequent clean-up step is carried out. SPE clean-up is widely extended, using polymeric, SCX, mixed-mode SCX and amino-based cartridges. Clean-up by LLE has also been applied over an aqueous extract with an organic

solvent (e.g. *n*-hexane, CCl_4) [108] or with acidic solutions [105]. As an interesting alternative, MISPE has been used for the analysis of egg-based samples [107].

These kinds of methodologies have been used for the analysis of meat [105,106,108-110,169,170], egg and egg-based products [106,107,109,272], kidney [104], liver [273], royal jelly [274] and animal feed [25].

The analysis of these VDs in environmental samples is very scarce. SPE with Oasis HLB cartridges has been used for this purpose in drinking, river and sea water samples [275]. In the case of biological fluids, the number of applications found literature is also reduced. Nitroimizadoles have been monitored in plasma [23,111] and gastric aspirate [111]. Despite different methodologies have been employed (similar to those of food samples); there are a number of common stages, such as deproteinization (e.g. using protease solutions [23,111]) and deffating stages (e.g. using LLE with *n*-hexane [111]).

Nitrofurans

Most of applications are focused on the analysis of nitrofuran metabolites in food samples since the parent compounds are easily metabolized and do not persist in edible tissue: they form protein-bound metabolites that persist longer in these tissues [4]. Retina has been pointed out as a metabolic "dead end" tissue since the accumulation of nitrofuran metabolites in this organ is significant [276].

A general procedure for the analysis of nitrofuran metabolites is based on the release of the metabolites from the proteins by mild acid hydrolysis of the homogenized sample with HCl prior to derivatization with NBA (normally overnight or 15 h). Subsequent extraction with an organic solvent (e.g. ethyl acetate, hexane, ACN) and clean-up by SPE or LLE is carried out before chromatographic analysis [4,6]. For SPE, polymeric [277] and NH_2 cartridges [278] have been used. This kind of procedure has been applied for the analysis of nitrofuran metabolites in feed [278], egg [26,279], honey [277], meat [280,281], shrimp [281], milk [115] and retina [276]. An alternative approach is the performance of an SPE extraction that is less time-consuming [112]. SPE-based procedures have been used for the analysis of meat [116]. However, the omission of the LLE stage may provide poor recoveries [277]. The performance of an additional LLE with *n*-hexane in the final extract has been used as an extra clean-up in order to remove lipidic material (e.g. meat, egg, liver) [278,279,281]. The application of sequential elution on Oasis SPE cartridges in order to obtain the parent compounds and the metabolites separately for the analysis of these compounds in honey has been described

[282]. Additionally, a dual SPE procedure using Oasis MAX and HLB cartridges has been reported for the analysis of nitrofuran compounds in liver. The purpose of applying two SPE is the removal of the excess of NBA [114]. For the analysis of nitrofuran metabolites in other food matrices where there is not any protein content, such as salt, it is necessary to perform a previous extraction of the analytes in order to facilitate the derivatization reaction [283]. Despite the widespread use of derivatization to analyze nitrofurans and metabolites, an alternative procedure that does not perform any derivatization stage has been described for the analysis of nitrofurans (parent compounds) in feed and water by using a monolithic column for separation [113]. In general, protection from light is recommended in order to avoid any photodegradation.

For quantification purposes, deuterated nitrofuran metabolites have been used preferentially as I.S. (e.g. D^3-1-aminohydantoin, D^4-3-amino-2-oxazolidinone and D^5- 3-amino-5-morpholinomethyl-2-oxazolidinone) [112]. However, certain problems relate to the utilization of underivatized isotope-labeled compounds have been reported (e.g. losses when spiking samples, overestimation of the level of bonded-metabolite). For this reason, the addition of labeled derivatized nitrofuran metabolites could be considered as an adequate approach for quantification of released analytes (metabolites) [277]. The addition of 4-NBA semicarbazone after the derivatization step as IS has also been described [116]. In this sense, the deuterated NBA metabolite derivatives have been used for quantification by isotope dilution [277]. Finally, nitrofuraxide has been applied as a suitable IS when using DAD [278].

3.10. Anabolic Steroids (ASs)

In most reviewed applications, the analysis of ASs comprises the monitoring of their residues mainly in biological samples (i.e. urine, hair), and food samples (i.e. meat), although other matrices have been evaluated, as commented below. The extraction of ASs is complicated, and the procedures are laborious and normally involve several stages, increasing total analysis time (Table 4).

The use of *H. pomatia* juice for the release of free steroids is greatly extended due to the presence of β-glucuronidase and arylsulphatase. These enzymes can also be used separately from the juice, obtaining cleaner extracts in some applications [4]. In urine samples, the application of two separate conjugation steps using β-glucoronidase (from *E. coli*) and solvolysis with sulfuric acid can also provide cleaner extracts [29].

The number of applications involving the determination of ASs in urine samples is considerable due to they are readily available. In this kind of sample, ASs normally occur as conjugates [9]. The procedure involving enzymatic hydrolysis and subsequent clean-up by dual SPE with C18 and aminopropyl sorbents, or single SPE (C18 and polymeric cartridges) is well-know [284-287]. An additional clean-up step can be performed by LLE (e.g. *n*-pentane) and/or SPE (polymeric, silica gel cartridges) [172,288,289]. Other methodologies omit the first SPE step [290] or include a fractionation stage by semi-preparative LC using normal-phase separation [172,290]. Another procedure widely utilized consists of performing an SPE extraction with C18 sorbents with subsequent hydrolysis, LLE with organic solvent (diethyl ether, diisopropyl ether) and further clean-up by LLE and/or SPE (-NH_2, silica sorbents) [174,291]. After hydrolysis, the performance of a dual SPE (C18 + aminopropyl) is also described [292,293]. The analysis of ASs by GC–C-IRMS require more complicated procedures, including SPE, LLE, semi-preparative LC and derivatization stages [120,294].

Alternatively to these common procedures, SPME and liquid-phase micro-extraction (LPME) can be applied. SPME can be used coupled to GC–MS without any "off-line" derivatization step. In this case, derivatization can be performed directly on the fiber, reducing sample-handling [295]. On the other hand, LPME can be useful for sample clean-up and analyte pre-concentration [296].

In meat, liver or kidney samples, enzymatic digestion of the proteins is normally applied in order to release the steroids. β-glucuronidase and arylsulphatase (from *H. pomatia*) [171,297,298], subtilisin A [299-301] and protease XVI [302] are enzymes used for this aim. After this procedure, extraction of ASs is carried out by extraction with organic solvents, such as MeOH [290,297,299], *tert*-butylmethyl ether [4,297,300,302] or ACN [303]. Some applications perform the solvent extraction prior to the enzymatic digestion [171]. LLE (e.g. *n*-hexane) and saponification are used as clean-up steps to remove fat content, normally including further clean-up by SPE (e.g. dual with C18 + NH_2, C18, cyanopropyl, polymeric sorbents) [4,297,299,300,302,303].

A number of applications are focused on the analysis of ASs in hair. These methodologies are very similar to those applied in meat samples. Consequently, they usually include a digestion-based treatment and a solvent extraction, and subsequent LLE and SPE clean-up stages [118,173,304].

Apart from the matrices previously commented, ASs have also been determined in biological samples, such as feces [285,286,290], serum [298]

and skin swab [286]; and feed [119,286]. Especially interesting are the analysis of ASs in environmental samples, such as water [296,305] or agricultural soils [306].

3.11. Corticosteroids

Corticosteroids are chemically related to Ass and they can be found as conjugated in a similar way. For this reason, enzymatic hydrolysis using *H. Pomatia* solutions is a widespread procedure for the analysis of the free compounds. Afterwards, SLE (for solid samples)/LLE (for liquid samples) [132,138] (e.g. diethyl ether) and/or SPE is normally carried out (Table 4).

Hydrolysis and SPE is the most common procedure for the analysis of corticosteroids in urine samples, targeted matrices for the monitoring of these compounds. C18 [140-142], polymeric-based sorbents [135,137], SCX [126] and immunoaffinity columns [135] can be used in the SPE stage. Additionally, extra LLE and SPE stages can be applied for clean-up purposes [140]. Similar methodologies involving LLE and/or SPE are applied in other kind of matrices such as hair [125,140], liver [127], plasma [131,141], feces [128], meat [140], milk [133,134], egg [134], feed [129,135] and water [129]. MeOH, MeOH/buffer mixtures or ammonium acetate can be used as extractant solvents, whereas C18 and silica sorbents are also employed in SPE stages.

Deproteinization (e.g. with TCA) and deffating (e.g. by LLE using *n*-hexane) can be used in certain complex matrices, such as plasma [141], milk [130] or liver [307], in order to improve the extraction performance. Alternative procedures using, for instance PLE, can be applied in complex solid/semi-solid samples such as liver [307].

3.12. Thyreostats

The number of applications related to the monitoring of thyreostats is not as high as in other VD and GPA groups, and they are mainly focused on the analysis of thyroid and to a lesser extent in urine (Table 4).

Due to the high polarity and low molecular weight of thyreostats, their isolation is complicated. In the past, a procedure to isolate and clean-up of these analytes based on the formation of complex on mercurated ion-exchange columns and subsequent elution was usually applied [4,308].

A well-know methodology is the performance of an extraction with MeOH (although ethyl acetate and ACN are also used) and a further clean up by SPE with silica cartridges (anion exchange, aminopropyl and alumina cartridges have also been used) [30]. This kind of protocol has been used for the determination of thyreostats in biological samples, such as thyroid tissues [31,144,145,147,309], serum, [145], plasma [143], urine [144,147], feces, hair and liver [144]: and meat [31,144] and feed samples [144]. MSPD (sorbent: silica gel) has also been used for the analysis of meat, thyroid tissues [175], urine and milk [310]; this technique can replace the SPE step [175] or can be included as an additional step prior to SPE. Gel-permeation chromatography (GPC) has also been applied for the clean-up of the raw extracts [309].

Derivatization with 3-BrBBr or PFBBr and MSTFA is the most utilized one. Additionally, certain reagents can be added to improve method recovery and clean-up efficiency, mercaptoethanol and EDTA. Mercaptoethanol is used to reduce binding to proteins (it acts as a reducing agent) and to increase recovery [145,147].

3.13. β-Agonists

The extraction of β-agonists normally involves a SPE stage; mixed-phase or mixed-mode sorbents, which combine apolar and ionic mechanisms, are widely applied (e.g. C18/C8 and SCX) (Table 4). These sorbents are able to extract and/or purify the majority of β_2-andregenic agonists and they have been selected as the most adequate phases or multi-residue extraction [4,34]. Besides, enzymatic hydrolysis with β-glucoronidase/arylsulphatase is also performed prior to the SPE stage. This methodology has been widely applied for the analysis of biological samples, mainly in urine [151,178,311] but also in hair [151,179], liver [311] and retina [312]; and in other kind of samples such as feed [151].

In environmental samples, SPE is the first choice; for sewage samples, SCX sorbents can be applied [154] whereas polymeric sorbents (e.g. Oasis HLB) can be used in wastewater [161]. In the case of analyzing clenbuterol in hair, it is important to sample black hair due to the binding to the compound to melanin. Although the hydrolysis of the conjugates is usually carried out, for clenbuterol and related substances, this step could be removed due to the lack of hydroxyl and alkylhydroxy groups responsible for sulpho or glucuronidation [34].

Extraction in acidic conditions and subsequent SPE (e.g. C18, C18 plus SCX) is another suitable strategy (less common) that has been applied for the analysis of β-agonists in biological samples (liver [149,313], retina [166,313], urine [149] and hair [179]) and food samples (meat [314]) and feed (without SPE step) [180]. After LLE, mixed-phase SPE can be applied together with polymeric SPE for the extraction of liver, kidney and meat [150].

Enzymatic hydrolysis and subsequent LLE (e.g. *tert*-butylmethyl ether) is an additional procedure that has been applied in urine [152]; in plasma, the hydrolysis step can be omitted [160]. The performance of an SPE step (C18) is also described in urine [158,315], liver [315] and meat samples [159]; in urine, mixed-mode SPE is also adequate [163]. MISPE can also be applied for the extraction of β-agonists, as a combination of MIP and SPE. MISPE has been employed for the analysis of urine [156,162,316,317], liver and feed [317]. LLE and MIP for clean-up purposes are applied in urine samples [165]. MSPD can also be used together with MIP; this procedure has been applied in solid/semi-solid samples, such as liver [148]. Applications of IAC procedures for the analysis of urine [177,180,318] and hair [177] are reported.

SPME has been used for the analysis of clenbuterol in urine samples [155] and standards solutions [176] using polyacrylate fibers with previous derivatization with HMDS by GC. Recently, PMME has been proposed for the analysis of β-agonists in meat [164] as an alternative to SPME. In this sense, the application of LPME for the analysis of urine is also reported [155].

Chapter 4

4. MULTI-RESIDUE/MULTI-CLASS METHODS

This section is devoted to the review of methodologies for the simultaneous analysis of different groups of VDs and GPAs. Currently, LC–MS-based methods are the first choice in the development of new multi-residue methods for the analysis of different classes of VDs and GPAs. There are a number or reasons that can justify the widespread use of LC–MS and the reduced application of GC-based methods for this purpose:

(i) The need for derivatization of many compounds for subsequent analysis by GC, requering a variety of derivatization agents for different groups of compounds and resulting in complicated and time-consuming methodologies in GC–MS.
(ii) There is a high number of anaytes that are not GC-amenable compounds owing to high polarity or thermolabile characteristics.
(iii) LC instruments show a higher versatility and are able to analyze compounds in a wider range of polarity, including thermolabile compounds.

One of the main difficulties in the development of multi-residue methods for the determination of a variety of groups of VDs and GPAs is due to the variety of physico-chemical properties. In other types of trace analysis, such as pesticide residue analysis; generally speaking, the variability is much lower than in VDs/GPAs, and thus the multi-residue methods can comprise the determination of a high number of pesticides (e.g. 150 in a single injection, single method). However, in the case of VDs/GPAs, the number of compounds

that can be monitored simultaneously (same extraction/clean-up method and determination) is more reduced (Table 5). Bearing in mind current literature, a few articles describe the analysis of a relatively high number of compounds (>30) [319-324]. In general, these methods are focused on the analysis of a single matrix, except the recent method developed by Mol et al. [320] that comprises the analysis of a wide range of VDs in 6 matrices using the same extraction method.

In relation to the matrices analyzed in multi-residue/multi-class methods, the number of procedures involving the determination of VDs/GPAs in environmental samples, mainly wastewater and surface waters, is significant. Moreover, in these kinds of matrices, macrolides, TCs and sulphonamides are mostly determined; the determination of GPAs is reported only in certain cases. Besides, these methods do not comprise a high number of compounds (< 30). The development of multi methods for these matrices can result easier in comparison to other samples; the majority of these methodologies are based on an SPE stage (e.g. HLB) (Table 5) [325-336]. For other applications in solid/semi-solid samples, the use of PLE has been reported in sewage sludge [332, 337], swine manure [338] and agricultural soils [339].

In food samples, there is a variety of extraction methods that can be applied, but generally based on the use of SLE/LLE and SPE techniques, which are specified in Table 5. Meat samples focus most attention [320,321,340-342], although procedures for the determination of VDs in milk [320,343-345], egg [320,324], honey [320,346,347], fish [348,349], seafood [350], maize [320] and feed [320,351,352] are also described. In biological samples, the number of applications related to the multi-residue/multiclass analysis of VDs/GPAs is reduced in comparison to the aforementioned groups of matrices. On the contrary, applications involving the monitoring of single groups of GPAs are numerous, especially in urine samples [132,323,341,353], although methods in feces, liver and kidney can also be found. Hormones and β-agonists are typical target compounds in this class of multi-residue methods; in this sense, the monitoring of 108 analytes including VDs and GPAs in urine described by Kaufmann et al. [323] results relevant because of the unusual high number of monitored compounds and the use of HRMS instruments for this purpose.

Table 5. Selected multi-residue/multi-class methods

Compounds (number)	Matrix	Sample pre-treatment	Separation/detection technique	LOD (units)	Observations	Ref.
Environmental samples						
Sulphonamides, TCs (8)	Wastewater	SPE (Oasis HLB)	LC–ESI(+)–Q–MS	0.5-85 ng l^{-1}	Symmetry C18 column (150 mm x 2.1mm, 5 µm) A: Water (0.2% formic acid) B: MeOH C: ACN	[327]
Macrolides, sulfonamides, trimethoprim (11)	Wastewater	SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	Data not provided	Pro C18 (150 mm x 2 mm, 3 µm) A: Water (1% formic acid) B: MeOH (1% formic acid)	[334]
Macrolide, sulphonamides, trimethoprim and others (5)	Wastewater	SPE (HLB)	UPLC–ESI(+)–Q–TOF–MS/MS	10-150 ng l^{-1}	Acquity UPLC BEH C18 (50 mm × 2.1mm, 1.7 µm) A: Water (pH 4.8, 5mM NH_4Ac/HAc) B: ACN/MeOH (2:1, v/v, pH 4.8, 5mM NH_4Ac/HAc)	[330]
Macrolides, sulfonamides, trimethoprim (9)	Wastewater	SPME (carbowax-templated resin, CW/TPR, 50 µm)	LC–ESI(+)–QqQ–MS/MS	0.08-6.1 ng l^{-1}	Eclipse XDB C18 (250 mm x 4.6 mm, 5 µm) A: Water/ACN (90:10, v/v, 10 mM NH_4Ac, 0.1% formic acid) B: ACN	[328]
ASs, corticosteroids (12)	Wastewater	SPE (Strata X + silica)	LC–APCI(+)–Q–MS	0.2-4.3 ng l^{-1}	Zorbax Eclipse XDB C18 (100 mm x 2 mm, 3 µm) A: Water (pH 3.4) B: ACN	[354]

Table 5. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/detection technique	LOD (units)	Observations	Ref.
B-lactams, sulfonamides, TCs, quinolones, nitroimidazoles (12)	Water (1), sewage, sludge (2)	Water: SPE (SDVB) Solid samples: LLE (phosphate buffer, TEA/MeOH/water)	LC–ESI(+/-)–IT–MS/MS	(1) 6-160 ng l^{-1} (2) 0.1-5.3 mg kg^{-1}	Hydrosphere C18 (150 mm x 4.6 mm, 5 µm) Linear gradient of 95-50% H_2O balanced with ACN, both 0.1% formic acid	[332]
Sulphomamides, TCs, quinolones adn others (12)	Swine wastewater, lake water, groundwater	SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	0.8-104.4 ng l^{-1} (a)	Dionex Acclaim C18 reversed phase (150 mm x 2.1 mm, 4.6 µm) A: Water (0.1% formic acid) B: ACN	[325]
Macrolides, sulphonamides, TCs, quinolones (13)	Surface water and groundwater	SPE (Oasis HLB)	LC–ESI(+)–IT–MS/MS	0.03-0.19 µg l^{-1}	BetaBasic-18 C18 column (100 mm x 2.1 mm, 3 µm) A: Water (0.3% formic acid) B: ACN C: MeOH	[333]
Macrolides, sulphonamides, trimethoprim and other compounds (29)	Surface water, wastewater	SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	1-30 ng l^{-1}	Purospher Star RP-18 end-capped (125 mm × 2.0 mm, 5 µm) A: NH_4Ac 5mM/HAc B: ACN/MeOH (2:1, v/v)	[329]
β-lactams, quinolones (16)	Surface water, groundwater	SPE (on-line, C18)	LC–ESI(+)–QqQ–MS/MS	0.4-4.3 ng l^{-1}	Kromasil C18 (100 mm × 2.1 mm, 5 µm) A: Water (0.01% formic acid) B: MeOH (0.01% formic acid)	[331]

Compounds (number)	Matrix	Sample pre-treatment	Separation/detection technique	LOD (units)	Observations	Ref.
Macrolides, sulphonamides (2)	Surface water, groundwater, drinking water	SPE (Oasis MCX)	LC–ESI(+)–QqQ–MS/MSLC–ESI(+)–Q–TOF–MS/MS	10 ng l^{-}	XTerra (100 mm x 2.1 mm, 3.5 µm) A: Water (2 mM NH_4Ac) B: MeOH (2 mM NH_4Ac)	[355]
Sulphonamides, trimethoprim, quinolones, coccidiostats (17)	Surface water	SPE (HLB)	UPLC–ESI(+)–QqQ–MS/MS	10 ng l^{-1} (a)	Acquity UPLC BEH C18 (100 mm × 2.1mm, 1.7 µm) A: Water (0.1% formic acid) B: ACN (0.1% formic acid)	[326]
Suphonamides, TCs (13)	Surface water	EDTA, SPE (HLB)	LC–ESI(+)–IT–MS/MS	0.05 µg l^{-1}	XTerra C18 (50 mm x 2.1 mm, 2 .5 µm) A: Water (0.1% formic acid) B: ACN (0.1% formic acid)	[75]
Macrolides, sulfonamides, trimethoprim (11)	Sewage sludge	PLE (water/MeOH, 1:1, v/v)	LC–ESI(+)–QqQ–MS/MS	3-41 µg kg^{-1} (a)	Pro C18 (150 mm x 2 mm, 3 µm) A: Water (1% formic acid) B: MeOH (1% formic acid)	[337]
Macrolides, sulfonamides, TCs (9)	Swine manure	PLE (Citric acid/MeOH), LLE (n-hexane), SPE (SAX + HLB)	LC–ESI(+)–QqQ–MS/MS	2.7-26.9 µg kg^{-1}	XTerra MS C18 (100 mm × 2.1 mm, 3.5 µm) A: MeOH/water (95:5, v/v) B: MeOH/water (20:80, v/v, pH 3 with formic acid)	[338]
Macrolides, sulfonamides, TCs (5)	Agricultural soils	PLE (Citric acid/MeOH), SPE (SAX + HLB)	LC–ESI(+)–QqQ–MS/MS	0.6-5.6 µg kg^{-1}	XTerra MS C18 (100 mm × 2.1 mm, 3.5 µm) A: MeOH/water (5:95, 80 mM formic acid) B: MeOH/water (95:5, v/v, 80 mM formic acid)	[339]

Table 5. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/detection technique	LOD (units)	Observations	Ref.
Food samples						
β-lactams, macrolides, sulfonamides, trimethoprim, TCs, quinolones nitroimidazoles (31)	Meat	PLE	LC–ESI(+)–QqQ–MS/MS	3-15 μg kg^{-1}	XTerra MS C18 (100 mm × 2.1 mm, 3.5 μm) A: Water (10 mM formic acid) B: MeOH (10 mM formic acid)	[321]
β-lactams, macrolides, sulfonamides, TCs, quinolones (19)	Meat	LLE (MeOH:water, 70:30, v/v)	LC–ESI(+)–QqQ–MS/MS	1-30 μg kg^{-1}	Genesis C18 (50 mm × 2.1 mm, 4 μm) A: Water (0.1 mM oxalic acid, 0.2% formic acid) B: ACN	[340]
ASs, corticosteroids and others (13)	Meat	Enzymatic hydrolysis, SPE (Strata X)	UPLC–ESI(+/-)–QqLIT–MS/MS	0.09-0.19 μg kg^{-1} (b)	Eclipse Plus C18 column (50 mm x 2.1 mm, 1.8 μm) A: Water/MeOH (70:30, v/v, 0.5 mM NH_4Ac) B: Water/MeOH (5:95, v/v, 0.5 mM NH_4Ac)	[343]
Sulphonamides, quinolones, coccidiostats and others (41)	Meat	QuEChERS, SPE (SCX)	LC–ESI(+)–QqQ–MS/MS	0.27-444 μg kg^{-1} (b)	Synergi Fusion-RP (100 mm x 2 mm, 2.5 μm) A: Water (0.1% formic acid) B: MeOH (2 mM NH_4Ac)	[319]
Macrolides, quinolones (19)	Meat	SLE (ACN), SPE (on-line, C18)	LC–ESI(+)–QqQ–MS/MS	0.03-8.40 μg kg^{-1}	Luna C18(2) (50 mm × 2.1mm, 5 μm) A: Water (0.1% formic acid) B: ACN (0.1% formic acid) C: ACN, D: MeOH	[345]

Compounds (number)	Matrix	Sample pre-treatment	Separation/detection technique	LOD (units)	Observations	Ref.
Sulfonamides, trimethoprim, TCs, quinolones (5)	Fish	SLE (ACN/(citric acid/EDTA)	LC–ESI(+)–Q–MS	4-7 μg kg^{-1}	Atlantis dC18 (150 mm × 2 mm, 5 μm) A: Water (0.1% formic acid) B: MeOH	[348]
Macrolides (3) and other compounds	Fish	SLE (ACN, 0.1% HAc), D-SPE (Bondesil-NH_2)	LC–ESI(+)–TOF–MS	1-3 μg kg^{-1}	Zorbax SB-C18 (250 mm x 3 mm, 5 μm) A: Water (0.1% formic acid) B: ACN	[349]
Macrolides, sulfonamides, trimethoprim, TCs, quinolones (17)	Honey	EDTA, SPE (HLB)	UPLC–ESI(+)–QqQ–MS/MS	0.3-3.3 μg kg^{-1}	Acquity UPLC BEH C18 column (100 mm × 2.1mm, 1.7 μm) A: Water (0.05% formic acid) B: MeOH	[346]
Aminoglycosides, macrolides, sulfonamides, TCs, quinolones and others (14)	Honey	Dilution (water), SPE (Strata X)	LC–ESI(+/-)–QqQ–MS/MS	Data not provided	Phenomenex Polar-RP Synergi (50 mm x 2.0 mm, 4 μm) A: Water (0.1 % formic acid) B: ACN (0.1 % formic acid)	[347]
Anthelmintics, macrolides, sulphonamides TCs, quinolones (18)	Milk	QuEChERS	UPLC–ESI(+)–QqQ–MS/MS	1-4 μg kg^{-1}	Acquity UPLC BEH C18 column (100 mm × 2.1mm, 1.7 μm) A: Water (0.01% formic acid) B: MeOH	[344]
β-lactams, sulfonamides, TCs, quinolones (29)	Egg	SLE (sodium succinate buffer), SPE (HLB)	LC–ESI(+)–IT–MS/MS	10-50 μg kg^{-1}	Phenyl (50 mm x 4 mm, 3 μm) A: Water (0.1% formic acid) B: ACN	[324]
Sulfonamides, TCs, quinolones (18)	Shrimp	SLE (TCA 5%), SPE (HLB)	LC–APCI(+/-)–IT–MS/MS	Data not provided	Phenyl (50 mm x 4 mm, 3 μm) A: Water/ACN (95:5, v/v, 0.1 % formic acid) B: Water/ACN (15:85, v/v, 0.05% formic acid)	[350]

Table 5. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/detection technique	LOD (units)	Observations	Ref.
Anthelmintics, macrolides, TCs, sulphonamides, quinolones, tranquilizers (89)	Egg, honey, maize, meat, milk, feed	SLE (ACN/water/formic acid, 25:74:1)	UPLC–ESI(+)–QqQ–MS/MSUPLC–ESI(+)–TOF–MS	0.01-0.05 mg kg^{-1}	Acquity UPLC BEH C18 column (100 mm × 2.1mm, 1.7 µm) A: Water (1 mM ammonium formiate, 20 µl formic acid) B: MeOH/water (95:5, 1 mM ammonium formiate, 20 µl formic acid)	[320]
Macrolides, sulphonamides, trimethoprim, TCs, quinolones (25)	Chlorinated drinking water	EDTA , SPE (HLB)	LC–ESI(+)–QqQ–MS/MS	0.5-6.0 ng l^{-1}	Pursuit C18 (150 mm x 2 mm, 3 µm) A: Water (0.1% formic acid) B: ACN	[322]
ASs, corticosteroids (18)	Feed	LLE (Diethyl ether), SPE (Silica)	LC–DAD (λ = 245, 200 nm)	34-198 µg kg^{-1} (c)	Hypersil ODS (250 mm × 4.6 mm, 5 µm) Isocratic: Water/ACN (65:35, v/v)	[351]
ASs, β-agonists (-)	Feed	SLE (MeOH/phosphoric acid), SPE (mixed-mode) or IAC	LC–ESI(+)–Q–TOF–MS/MS	Data not provided	Symmetry C18 column (150 mm x 3.0mm, 5 µm) A: Water (0.1% formic acid) B: ACN (0.1% formic acid)	[352]
Biological samples						

Compounds (number)	Matrix	Sample pre-treatment	Separation/detection technique	LOD (units)	Observations	Ref.
β-agonists, ASs, corticosteroids, thyreostats (6)	Meat, fat, organ tissues, urine, faeces	SPE (C18 disk), frationation	GC–EI/NCI–IT–MS/MS LC–ESI(-)–IT–MS/MS	Data not provided	GC: Derivatization: MSTFA/ ethanediol/ammoniumdiodide, BPX-5 (25 m x 0.22 mm, 0.25 µm), BPX-35 (25 m x 0.22 mm, 0.25 µm) LC: Hypercarb (100 mm x 2.1. mm, 5 µm) A: Water (0.2% HAc) B: ACN (0.2% HAc)	[341]
TCs, quinolones (21)	Meat, liver, kidney	SLE (EDTA-McIlvaine), SPE (HLB)	UPLC–ESI(+)–QqQ–MS/MS	0.01-0.79 µg kg^{-1}	Acquity UPLC BEH C18 (100 mm × 2.1mm, 1.7 µm) A: Water (0.2% formic acid) B: ACN/MeOH (40:60, v/v, 0.2% formic acid)	[342]
Anthelmintics, aminoglycosides, β-lactams, macrolides, sulfonamides, TCs, quinolones, nitroimidazoles, tranquilizers and others (108)	Urine	"Dilute and shot"	UPLC–ESI(+)–TOF–MS	0.2-45 µg l^{-1}	Acquity UPLC BEH C18 column (50 mm × 2.1mm, 1.7 µm) A: Water/formic acid/ACN (94:3:50, v/v) B: Water/formic acid/ACN (50:3:97, v/v)	[323]
Costicosteroids, β-agonists (22)	Urine	Enzymatic hydrolysis, LLE (diethyl ether)	UPLC–ESI(+)–TOF–MS	0.1-3.3 µg l^{-1}	Acquity UPLC BEH C18 (50 mm × 2.1mm, 1.7 µm) A: Water (0.1% formic acid) B: ACN (0.1% formic acid)	[132]

Table 5. (Continued).

Compounds (number)	Matrix	Sample pre-treatment	Separation/detection technique	LOD (units)	Observations	Ref.
Corticosteroids, tranquilizers (6)	Serum	SPE (HLB)	LC–UV (1) LC–ESI(+/-)–Q–MS (2)	20-50 μg l^{-1}	Kromasil C18 (150 mm × 4 mm, 5 μm) (1) MeOH/water (70:30, v/v, 0.1% HCl) (2) MeOH/water (70:30, v/v, 0.1% HAc)	[44]

(a)Limit of quantification (LOQ); (b) Decision limit (CCα); (c) Detection capability (CCβ).

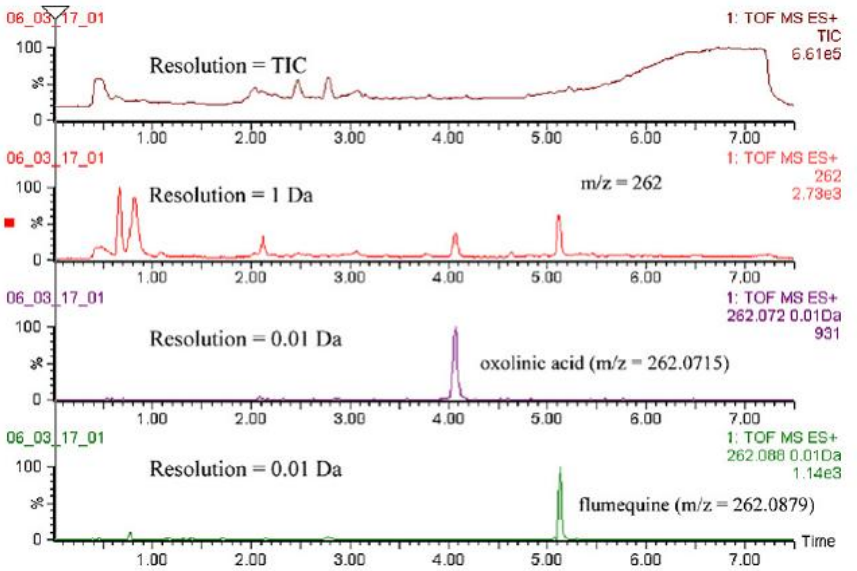

Figure 5. Urine sample spiked with two isobaric chinolones showing the influence of the mass extraction window width on selectivity; the mass exaction window width is reduced from 1000 Da (TIC) to unit resolution (1 Da) and TOF resolution (0.01 Da). The S/N ratio of the two analytes is improved by using a narrow extraction window of 0.01 Da in comparison to unit resolution, reducing significantly matrix related MS signals [From [323] with permission from Elsevier B.V.].

The use of LRMS for routine applications is widespread, namely QqQ and IT (but to a lesser extent) (Figure 6). It is well-known that these instruments offer a suitable choice for target analysis and high versatility, and therefore, they are utilized in most of methods reported in literature. However, in the recent years, a trend towards the use of HRMS instruments has been observed [132,320,330,335,348,353]. The potentiality of this kind of analyzers for screening purposes has been evaluated due to the high number of compounds that need to be monitored, such as regulated compounds (those analytes for which an MRL is established) or illegal compounds.

The main advantage of this technology is the performance of mass accuracy measurements in the full scan mode and the possibility of carrying out retrospective analysis for non-targeted compounds or compounds not included in a first data analysis. In relation to this last characteristic, the use of (Q-)TOF or orbitrap analyzers for the elucidation and identification of VD and GPA TPs is also focusing attention and it is a general trend in the trace analysis field.

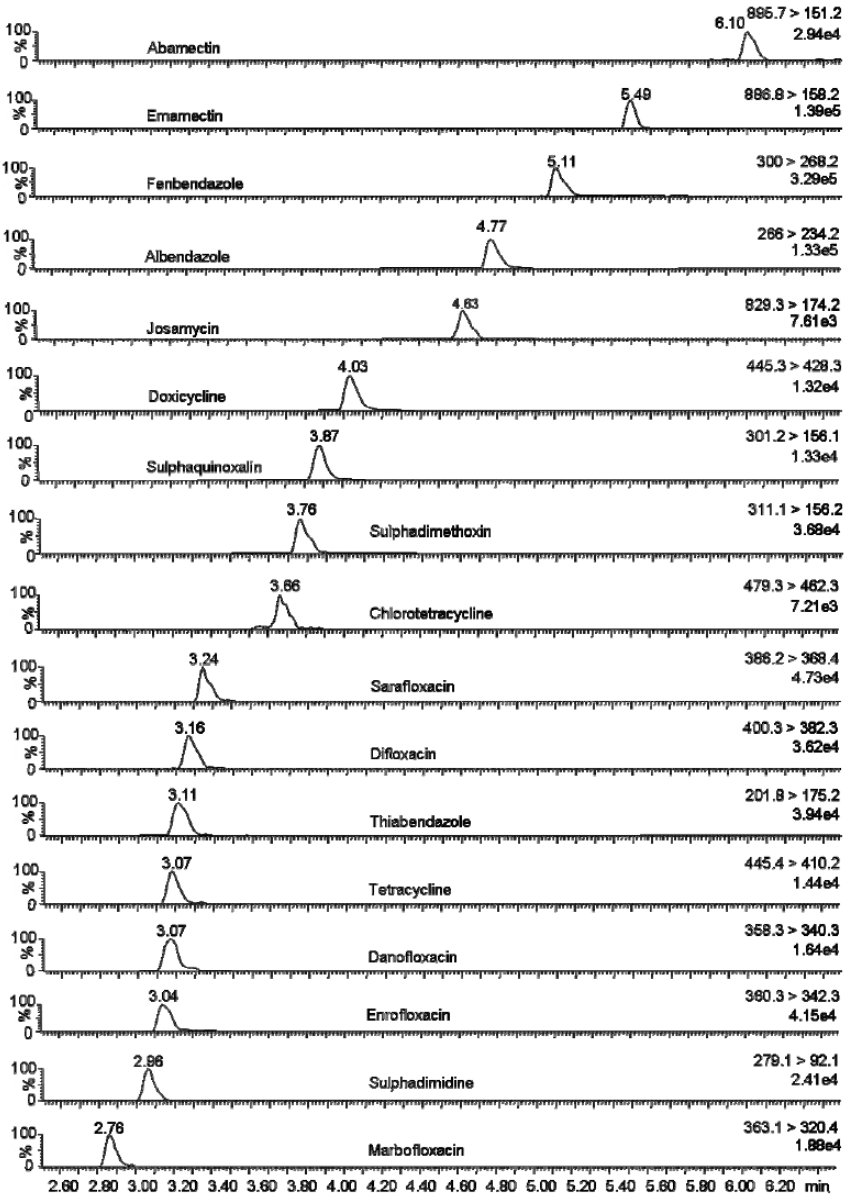

Figure 6. UPLC–ESI(+)-QqQ-MS/MS chromatogram of an egg sample (100 µg kg^{-1}) spiked with different groups of VDs.

Chapter 5

CONCLUSIONS AND FUTURE TRENDS

Bearing in mind current literature and recent applications in the determination of VDs and GPAs, there are several points to be highlighted: (i) the reduction in the use of GC-based methods, (ii) the higher utilization of sub-2-μm particle columns (e.g. UPLC technology), (iii) the increase in the use of HRMS instruments in routine applications, and (iv) the difficulty in the development of multi-residue/multi-class methods.

Nowadays, the application and development of GC-based methods is focused on the analysis of certain groups of compounds that are not LC-amenable or analytes that show worse sensitivity by LC. In general, LC is preferred to GC for the analysis of VDs and GPAs due to the fact that derivatization stages are not normally needed.

The use of columns with a particle size lower than 2 μm provides lower running times and width peak, higher resolution, and it increases sample-throughput. This column technology has dramatically reduced the former differences between GC and LC-based methods, which were normally longer. The coupling of UPLC systems to MS analyzers is an additional and important approach since the application of deconvolution tools does not require complete resolution of the analytes, increasing the capability of the system: more compounds in less time. The utilization of LRMS (QqQ, IT) is widespread in routine applications and it is the first choice when using MS. Nevertheless, HRMS (TOF) is more and more utilized, either as screening tool or as identification/confirmation/quantification instrument. The monitoring of full scan spectra brings along a number of advantages, such as the possibility of performing retrospective analysis (monitoring of non-targeted compounds) or the establishment of analyte degradation pathways. However, the high price

of this technology in comparison to typical LRMS instruments is still an important drawback.

Finally, the development of generic methods for the analysis of different groups of compounds is enormous, considering the variety of physico-chemical properties and chromatographic aspects. The use of LC for this kind of "multi" methods is widespread, as the most versatile technique. However, the sample pre-treatment seems to be the bottle-neck since it is complicated to find some generic conditions that suits for the different groups. In this sense, according to literature, there are certain groups that can be simultaneously monitored, whereas in other cases this is not possible.

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