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Review

Analytical strategies for residue analysis of veterinary drugs and growth-promoting agents in food-producing animals—a review

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Abstract

After a brief introduction into the field of veterinary drugs and growth-promoting agents, the most important EU regulations and directives for the inspection of food-producing animals and animal products regarding the residue control of these substances are presented and discussed. Main attention in the review is on the methods of analysis in use today for the most important classes of veterinary drugs and growth-promoting agents viz. anthelmintics, antibiotics, coccidiostats, hormones, β-agonists and tranquillizers. Emphasis is given to the potential, and limitations, of state-of-the-art analytical procedures and their performance characteristics. The most obvious conclusion is that, today (reversed-phase) liquid chromatography combined with tandem mass spectrometric detection – either triple-quadrupole or ion-trap multi-stage – is the preferred technique in a large majority of all cases. In the field of sample treatment, the combined use of liquid extraction – i.e., liquid partitioning or liquid–liquid extraction – and liquid–solid extraction – primary on- or off-line solid-phase extraction – is most popular. Finally, while the analytical tools required to meet the demands typically formulated by governments and international organizations today, generally speaking are available, several problems still do exist. To quote three examples, problems are encountered in the area of simultaneously extracting and pre-treating groups of analytes with mutually widely different polarities, with regard to identification-point – based confirmation of analyte identity, and regarding quantification errors caused by ion-suppression effects. Improving the speed of analysis is another aspect that should, and will, receive dedicated interest in the near future.

Keywords: Veterinary drugs; Growth-promoting substances; LC–MS; EU criteria

Contents

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1. Introduction

In recent years, food safety problems have become a frequently recurring phenomenon. Also as a result of media attention, expressions such as 'mad cow disease', 'dioxin chickens', 'MPA crisis' and 'chloramphenicol scandal' are familiar to the general public. In the European Union (EU), consumer protection ranks extremely high. This is expressed in the precautionary principle [1] based on the Treaty of Amsterdam [2].

To reach the required level of protection, reliable data have to be made available, to enable adequate risk evaluation and subsequent action. In other words, sophisticated and robust analytical methods have to be developed for a wide variety of, primarily organic, micro-contaminants. This review addresses one highly relevant problem, the residue analysis of veterinary drugs and growth-promoting agents in food-producing animals. In modern agricultural practice, *veterinary drugs* are being used on a large scale and administered as feed additives or via the drinking water in order to prevent the outbreak of diseases. In addition, veterinary drugs are given in the case of disease, for drying-off purposes, or to prevent losses during transportation. *Growth-promoting agents* such as hormones and certain veterinary drugs, are applied to stimulate the growth by various mechanisms.

1.1. Legislation and regulation

In the EU the use of veterinary drugs is regulated through Council Regulation 2377/90/EC [3]. This regulation describes the procedure for the establishment of maximum residue limits (MRLs) for veterinary medicinal products in foodstuffs of animal origin. Its Annexes present the following information:

- Annex I includes substances for which final MRLs have been established.
- Annex II includes substances for which it is not considered necessary for the protection of public health to establish MRL values. These substances are allowed to be used in veterinary medicinal products for food-producing species for the animal species indicated and according to the conditions established.
- Annex III includes substances with provisional MRLs. These are established, for a defined period of time, when not all requirements for the establishment of an MRL have yet been fully addressed.
- Annex IV includes substances for which no MRL could be established because residues of these substances, at whatever limit, in foodstuffs of animal origin constitute a hazard to the health of the consumer. The administration of substances listed in this Annex to food-producing species is prohibited.

The prohibition of the use of growth-promoting agents such as, e.g., hormones and β-agonists is laid down in Council Directive 96/22/EC [4]. Council Directive 96/23/EC [5] regulates the residue control (monitoring) of pharmacologically active compounds, i.e., environmental contaminants, dyes, chemical elements, etc. in products of animal origin. This Directive divides all residues into Group A compounds, which comprises prohibited substances (in conformity with [4] and Annex IV of [3]) and Group B compounds, which comprise all registered veterinary drugs in conformity with Annexes I and III of [3] and other residues as summarized in Table 1. Directive 96/23/EC includes the control of food-producing animals as well as their primary products like meat, milk, eggs and honey. This means that samples are taken from the living animal on the producing farms as well as from carcasses in the slaughterhouse. Directive 96/23/EC also establishes National Surveillance Programmes for the monitoring of residues. Control for Group A is more critical, i.e., has a higher priority, because of public-health concern: relatively large numbers of samples have to be analysed and more

Table 1
Groups A and B substances and responsible CRLs

Group	CRL[a]
Group A: substances having anabolic effects and unauthorized substances	
• Stilbenes, stilbene derivatives, and their salts and esters	NL
• Antithyroid agents	NL
• Steroids	NL
• Resorcylic acid lactones including zeranol	NL
• β-Agonists	G
• Compounds included in Annex IV to Council Regulation 2377/90/EC [3]	[b]
Group B: veterinary drugs and contaminants	
• Antibacterial substances, including sulphonamides and quinolones	F
• Other veterinary drugs	
Anthelmintics	G
Anticoccidiostats, including nitroimidazoles	G
Carbamates and pyrethroids	I
Sedatives	NL
Non-steroidal anti-inflammatory drugs (NSAIDs)	G
Other pharmacologically active substances	[b]
• Other substances and environmental contaminants	
Organochlorine compounds including PCBs	I
Organophosphorus compounds	I
Chemical elements	I
Mycotoxins	NL
Dyes	F
Others	[b]

[a] Community Reference Laboratories: NL, Rijksinstituut voor Volksgezondheid en Milieuhygiëne (RIVM, Bilthoven, The Netherlands); F, Laboratories des Médicaments Vétérinaires (CNEVA-LMV, Fougères, France); G, Bundesinstitut für Risikobewertung (BfR, Berlin, Germany); I, Instituto Superiore di Sanità (Rome, Italy).

[b] Compounds are allocated to the designated CRL, according to their pharmacological action.

stringent criteria have to be used [6] in view of the serious implications of positive results for public health. The Directive also lays down that samples collected for the National Surveillance Programme have to be analysed in accredited laboratories. Accordingly, an extensive network of analytical residue laboratories has been created for the purpose of residue inspections. This hierarchical system comprises, at the lowest level, so-called Routine and/or Field Laboratories (RFLs), next some forty National Reference Laboratories (NRLs) and, at the top, four Community Reference Laboratories (CRLs). The four CRLs, which are located in Germany, France, Italy and The Netherlands, are hierarchically equal. However, each of them is responsible for a dedicated set of compounds, as is indicated in Table 1.

Technical guidelines and performance criteria for residue control in the framework of Directive 96/23/EC are described in [6]. In contrast to other areas of food control or to what is enforced in most non-EU countries, in the EU there is no obligation to use standardised methods in the residue control of food-producing animals. Instead, a *criteria approach* applies, which lays down performance characteristics, limits and criteria that have to be met by the methods used. A significant advantage of this approach is the high degree of flexibility. It allows the ready adaptation of analytical methods to technical developments and offers the possibility to react rapidly to newly emerging problems, such as, e.g., in the case of analyte/matrix combinations which have not been considered so far. Recent examples are the presence of chloramphenicol in honey and medroxyprogesterone acetate in molasses [7].

The Decision 657/2002/EC [6] defines the performance criteria for analytical residue methods. In this revised version of earlier Commission Decisions the substance as well as the matrix spectrum has been enlarged. Briefly, hormonally and thyrostatically active substances including β-agonists, pharmacologically active substances, e.g., anthelmintics, anticoccidiostats and sedatives, growth-promoting agents such as antibiotics and also contaminants such as pesticides and heavy metals are now included. Relevant matrices now also include honey, eggs, milk and aquaculture. In addition, the decision takes recent technical developments into account, with LC–MS taking the foremost position.

Contrary to registered drugs, for which MRLs are established, no action levels exist for Group A substances. In this context, action level means the concentration level of a drug above which action has to be taken. This can, for example, be performing a second analysis for confirmation of analyte identity. Until recently, for Group A substances the so-called ‘zero tolerance’ levels had to be applied. However, this caused some ambiguity here. In order to come to harmonization in the EU, the minimum required performance limit (MRPL) has now been introduced [6] which is the minimum content of an analyte in a sample that has to be detected and confirmed. The new limit represents a minimum requirement for the detection level of a method, and has to be taken into account when establishing and validating methods for prohibited substances. The first MRPLs were published in Annex II of Commission Decision 2003/181/EC [8].

Next to the general performance requirements, e.g., detection level, selectivity and specificity, Decision 657/2002/EC defines additional requirements for confirmatory methods by introducing the concept of *identification points* (IPs) and defining criteria for ion intensities. During confirmatory analysis a specific number of IPs has to be collected. For the confirmation of the identity of Group A substances – commonly to referred to as unauthorized, illegal, banned or prohibited substances – a minimum of four IPs is required. For the confirmation of the identity of substances listed in Group B, a minimum of three IPs is required. The number of IPs earned by a specific analysis depends on the technique used. However, almost invariably (see below) these techniques have to be based on mass spectrometric detection. Table 2 shows the number of IPs that each of the basic MS techniques can earn. Table 3 shows examples for the calculation of IPs. Each MS ion may be counted only once. GC–EI-MS is regarded a different technique to GC–CI-MS. Analyte derivatives can be used to increase the number of IPs only if different reaction chemistries are used. Product ions include both MS/MS and MS^n products.

Table 2
Relationship between MS techniques and IPs earned[a]

MS technique[b]	IPs
Low-resolution (LR)MS	1.0
$LRMS^n$ precursor ion	1.0
$LRMS^n$ product ion	1.5
High-resolution (HR)MS	2.0
$HRMS^n$ precursor ion	2.0
$HRMS^n$ product ion	2.5

[a] Adapted from [134].
[b] $n \geq 2$.

Table 3
Number of IPs earned for a range of techniques and their combinations

Technique(s)	Number of ions[a]	IPs
GC–MS (EI or CI)	*N*	*n*
GC–MS (EI and CI)	2 (EI) + 2 (CI)	4
GC–MS (EI or CI); two derivatives	2 (derivative A) + 2 (derivative B)	4
LC–MS	*N*	*n*
GC–MS–MS or LC–MS–MS	One precursor and two MS^2 products	4
GC–MS–MS or LC–MS–MS	Two precursors, each with one MS^2 product	5
$LC–MS^3$	One precursor, one MS^2 product and two MS^3 products	5.5
HRMS	*N*	2*n*
GC–MS and LC–MS	2 + 2	4
GC–MS and HRMS	2 + 1	4

[a] *n* = integer.

For Group A substances, LC coupled with full-scan DAD UV or fluorescence detection (FLD) or an immunogram, and TLC coupled to UV or FLD can contribute a maximum of one IP, provided that the relevant criteria for these techniques are fulfilled.

The relative ion intensities or *ion ratios* are another important aspect for confirmatory methods. Maximum allowable variation tolerances have been laid down for the ratio of the intensity of an individual ion over that of the base ion. The permitted range of variation is greater, the smaller the relative ion intensity, as is shown in Table 4. Unfortunately, the criteria are not clear as regards the nature of the reference – a standard solution or a fortified sample.

Table 4
Maximum permitted tolerances for ion intensities[a]

Relative intensity (% of base peak)	Relative maximum tolerance (%)	
	GC–EI-MS	GC–CI-MS, $GC–MS^n$, LC–MS, $LC–MS^n$
>50	±10	±20
20–50	±15	±25
10–20	±20	±30
≤10	±50	±50

[a] Adapted from [6].

The EU concept of IPs and tolerated ion intensity ratios for the confirmation of the identity of a compound is based on consensus obtained by the members of the so-called *EU working group of experts* [9]. There is no fundamental chemometric basis for the criteria used as regards the number of IPs and the applied tolerances, but it is interesting to note that they do not differ too much from the criteria established by the Association of Official Racing Chemists (AORC), the US Food and Drug Administration (FDA), the International Olympic Committee (IOC) and the World Anti-Doping Agency (WADA) [134]. The main difference is that the EU is the only organisation that allows the use of a combination of independent techniques to confirm the identity of a substance – the other organisations only allow the use of either GC–MS or LC–MS. Tables 4 and 5 present an overview of the MS criteria used by the various regulatory bodies.

The criteria of the AORC are minimum performance criteria [10], i.e., a laboratory can decide to be more stringent. With the EU [6], FDA [11] and IOC [12], the criteria should be regarded as universal identification criteria for mass spectrometry and chromatography. In the technical document of WADA [13] the criteria are intended as "an example of acceptable criteria". Hence, each WADA-accredited laboratory can select its own criteria, if it can justify their use.

1.2. Analysis

1.2.1. Sample selection

The first selection that has to be made when setting up monitoring programme regards the type of sample material.

Table 5
Criteria for MS-based confirmation according to FDA, AORC and IOC[a]

	Full-scan MS		SIM MS		MS–MS	
	n	Tolerance	*n*	Tolerance	*n*	Tolerance
FDA	3	20% abs	3	10% abs	2	10% abs
			4	15% abs	3	20% abs
			>3[b]	10% abs		
AORC	3	10% abs or 30% rel whichever is greater	4	Tighter criteria than full scan[c]	3[d]	20% abs or 40% rel whichever is greater
IOC	3	5% abs or 20% rel whichever is greater	3	5% abs or 20% rel whichever is greater	3[d]	10% abs or 25% rel whichever is greater

[a] *n*, number of diagnostic ions [134]; abs, absolute; rel, relative.
[b] When isotopes or non-specific ions like water are included.
[c] Not specified.
[d] May include precursor ion.

For monitoring drugs having an MRL (see Annexes I and III of [3]) animal tissues such as liver, kidney, muscle, milk or fat are selected. Since the drug concentrations in the consumable parts of an animal have to be below the MRL, these matrices are therefore of most interest and, because kidney and liver are the target organs for most veterinary drugs, the drug concentrations in these organs are higher than in, e.g., heart or lungs. One disadvantage of selecting animal organs or muscle is that they can be analysed only after slaughtering.

Another group of samples frequently used to monitor Group A or B substances are animal feed and drinking water. Feed is a difficult matrix; it is not easy to extract the drugs because of the large amounts of proteins and carbohydrates. However, the drug concentrations in feed are usually much higher (1–10 mg/kg) than in animal tissues (1–100 μg/kg); consequently drugs can be more easily detected.

Manure, urine and hair are a third group of matrices. They are mostly used to monitor prohibited substances and can be taken prior to slaughtering. This has the advantage that, when 'non-compliant' results are obtained, the animals can be destroyed to prevent that they reach the market. Analysing hair has gained some popularity because it has been demonstrated that anabolic steroids can be detected in hair a long time after application of the drugs – that is, when residues cannot be detected any more in urine or manure [14,15].

Finally, there are some sample types which can be used for specific purposes. To quote two examples, the thyroid gland is used to monitor the use of thyreostatics [16] and the eye retina tissue is used for monitoring β-agonists [17].

1.2.2. Method selection

Selecting a suitable method of residue analysis will, in many instances, depend on the problem at hand as well as on the final goal. To quote two widely different situations, when large sample series have to be monitored for a group of antibiotics such as sulphonamides, sample throughput will be an important criterion since speed is of the essence. In this situation a screening method is selected because high sample throughput and speed are the characteristics of such a method. When, on the other hand, samples are suspected to contain an illegal growth-promotor such as, e.g., stanozolol, method selectivity will no doubt be the main criterion because avoiding false non-compliant results now is of overriding importance. In this situation a confirmatory method is of interest because provides full or complementary information enabling to confirm the identity of the substance.

Here we will limit the discussion on method selection to a few comments on sample preparation and detection that can be considered relevant in light of the subsequent overview of drug residue analysis.

An evaluation of the scientific literature of the past 5 years, 1998–2003 [18], shows that some 350 papers on veterinary drug residue analysis were published. As regards sample treatment, liquid extraction (LE) and liquid–solid extraction (LSE) were found to be very popular techniques which were used in, respectively, 30 and 60% of all studies. Here, LE comprises conventional liquid–liquid extraction (LLE) as well as the liquid extraction of homogenized tissues such as liver, kidney and meat, referred to as liquid-phase extraction (LPE). LSE is almost always performed in the form of solid-phase extraction (SPE) [19]; the application of matrix solid-phase dispersion [20], immunoaffinity extraction [21] and molecular imprinted polymers [22] is reported in a few papers only. In many instances LE and LSE were used in combination: after analyte isolation by means of LE, the drugs were subsequently enriched by using a suitable SPE procedure. For the rest, the introduction of various types of co-polymeric sorbents has helped to make SPE a more robust extraction technique with a wider application range than the conventional silica-based sorbents [19,23]. The most widely used polymeric sorbents are the (poly)styrene-divinylbenzene co-polymers. A polymeric sorbent frequently used for the extraction of drug residues from biological samples is Oasis-HLB. This is a hydrophilic-lipophilic balanced copolymer (HLB) of *N*-vinylpyrrolidone and divinylbenzenes. The hydrophilic *N*-vinylpyrrolidone increases the water wettability of the polymer, and the lipohilic divinylbenzene provides the reversed-phase retention necessary to retain analytes.

Other sample-preparation techniques used for the analysis of veterinary drugs are pressurized liquid extraction (PLE) and ultrafiltration (UF) [24]. PLE, and also the closely related supercritical fluid extraction (SFE), have been shown to be very effective techniques for analyte isolation from fat-containing matrices [25]. Here one should consider that, while optimisation of a PLE procedure is fairly straightforward, optimisation of SFE often is rather time-consuming because of the many parameters that are involved. The use of chemometric techniques is recommended [26]. Finally, dialysis was used in only very few studies and not a single reference was found to microwave-assisted solvent extraction (MASE) [27].

As will be amply demonstrated in the text below, more than 80% of the present-day techniques for the determination of veterinary drugs and growth-promoting agents are LC–MS based – with the LC part usually involving a gradient run on an alkyl-bound silica column. Until quite recently the most frequently used mass detection technique was single-stage quadrupole MS. Today, it is rapidly being superseded by triple-stage quadrupole (QqQ-MS) and ion-trap (IT-MS^n) MS [28]. Single-stage MS is still used for screening purposes and for the quantification of MRL substances. QqQ-MS and IT-MS^n – with their excellent selected reaction monitoring-based selectivity – are preferably used in confirmation studies. A relatively new, and extremely powerful, technique is Q-ToF-MS, where a single quadrupole is combined with a time-of-flight (ToF) instrument. The accurate mass measurement of the ToF-MS ensures a distinctly enhanced selectivity compared with the other two types of tandem MS machine.

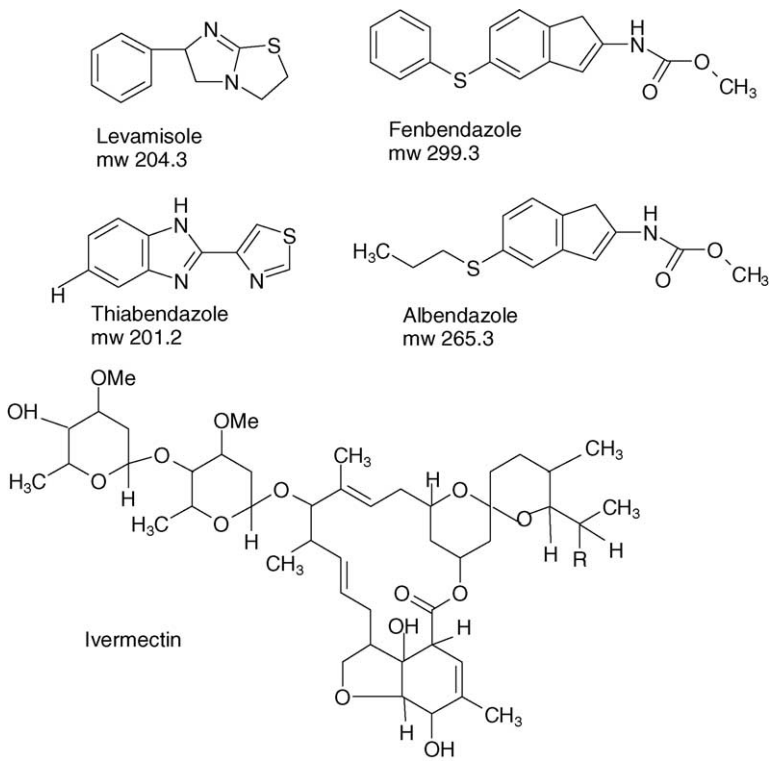


Fig. 1. Structures of representative anthelmintics.

2. Analytcial strategies

2.1. Anthelmintics

Anthelmintics are drugs acting primarily against intestinal worms, although many are active also against lungworms and liver fluke. The most frequently used anthelmintic compounds are levamisole, several compounds from the benzimidazole group (albendazole, cambendazole, fenbendazole, oxfendazole and thiabendazole) and ivermectin. Representive structures are shown in Fig. 1. Residues are most likely to be found in milk for which the withdrawal periods have not been strictly observed, or in liver tissue since this is the target organ for metabolism [29]. Muscle, fat and kidney are other samples of interest. MRLs range from 10 μg/l for oxfendazole in milk to 1000 μg/kg for albendazole in bovine liver [30].

Analytical methods typically used for residue analysis of anthelmintics are presented in Table 6. The animal species of interest are sheep, cattle and poultry. Sample pretreatment is mostly based on LPE or LLE, with an organic solvent mixture such as hexane/3-methylbutanol under basic conditions. For tissue analysis LPE is sometimes combined with SPE on alumina, silica or a (weak) cation exchanger (WCX). Because of the relatively high MRLs for the anthelmintics (Table 6), LC methods with UV or fluorescence (FLD) detection are most commonly used [31,32,135]. However, when a large number of anthelmintics and their metabolites has to be detected in a single run, selectivity problems can occur. These can be solved by the use of LC–MS techniques [33–36]. Combining

Table 6
Selected LC methods for anthelmintics

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL[a] (μg/kg)	Reference
Levamisole	Tissue	LPE/Si-SPE	UV	4–20	10–100	[31]
Levamisole	Milk	LLE	ESI(+)QqQ-MS	<1	–	[33]
Benzimidazoles	Milk	LLE	ESI(+)QqQ-MS	<1	10–100	[33]
Mebendazole + metabolites	Sheep muscle	LPE	ESI(+)QqQ-MS	0.5, 2–7	60–400	[34]
Albendazole + metabolites	Fish	LPE/LLE	FLD	1–25[b]	–	[135]
Benzimidazoles	Liver	SFE/Al-SPE	DAD UV	50	50–1000	[32]
Ivermectin + avermectins	Liver	LPE/C_8-SPE/Al-SPE	APCI(+)MS	25[c]	20–1500	[35]
Levamisole	Tissue	LPE/SPE	APCI(+)IT-MS^n	3–5	10–100	[36]

[a] According to [30].
[b] LOQ.
[c] LOI.

selective LPE/SPE sample treatment and LC−MS enables multi-residue analysis for analyte concentrations far below the MRL, even for complex matrices like milk and tissues. de Ruyck et al. [33] were able to detect levamisole (for which there is no MRL) and several benzimidazoles in milk down to 1 μg/l. Milk was made alkaline with 10 M sodium hydroxide and the anthelmintics were extracted with ethyl acetate. After evaporation of the organic phase, the residue was redissolved in 600 μl of eluent (0.1% aqueous formic acid–acetonitrile (50:50, v/v)) and separated on an Alltima C_{18} column. Detection was by means of ESI(+)QqQ-MS using SRM for selective detection. The overall recoveries in the concentration range 1–150 μg/l were 89–102%. de Ruyck et al. [34] used the same LC–MS procedure for the determination of mebendazole and its hydrolysed and reduced metabolites in sheep muscle. The anthelmintic compounds were extracted with ethyl acetate after the sample mixture had been made alkaline. A typical LC–ESI(+)QqQ-MS trace is shown in Fig. 2.

In summary, the most time-consuming step in the determination of anthelmintics is the selective extraction; separation

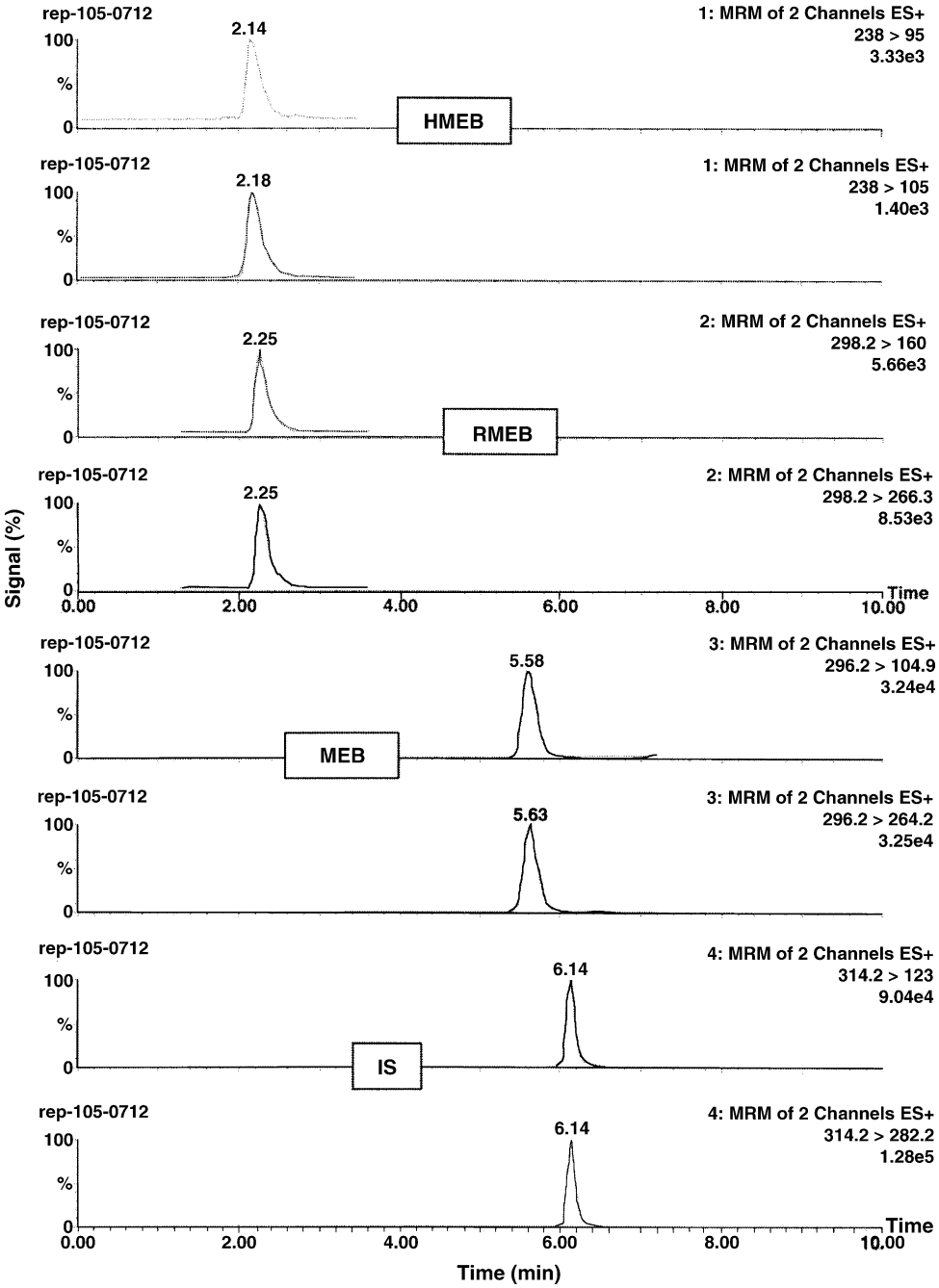


Fig. 2. LC–ESI(+)QqQ-MS of a blank sheep muscle tissue fortified with a mixture of mebendazole and its hydrolysed and reduced metabolites at 10 μg/kg and with the IS (flubendazole) at 50 μg/kg; adapted from [34].

plus detection by reversed-phase (RP)LC and MS is rapid, sensitive and selective. In most instances, the LODs are far below the MRLs, and usually approx. 10-fold better than with UV detection.

2.2. Antibiotics

Antibiotics comprise the following sub-groups:

- Aminoglycosides
- β-Lactams
- Macrolides
- Peptides
- Sulphonamides (and trimethoprim)
- Tetracyclines
- Quinolones
- Miscellaneous: Chloramphenicol, Malachite green

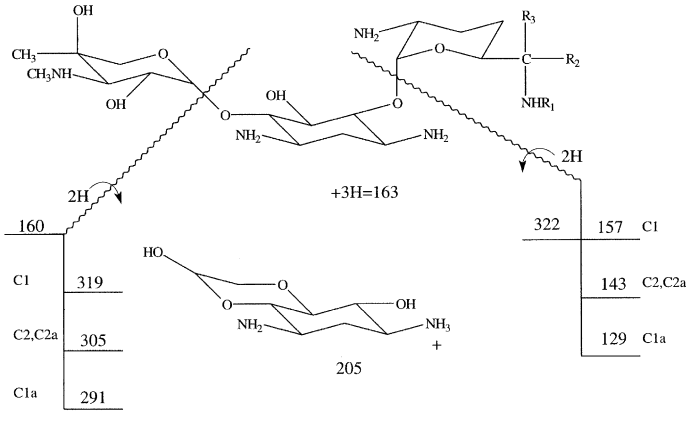
Incorrect use of antibiotics in veterinary practice may leave residues in edible tissues. These residues may have direct toxic effects on consumers, e.g., allergic reactions in hypersensitive individuals, or they may cause problems indirectly through induction of resistant strains of bacteria. The EU has set MRLs for several antibiotics in tissues, milk and eggs. In order to detect such residues in food and tissues, bioassay techniques are widely used as screening methods. These methods generally do not distinguish between members of a class of antibiotics, but provide a semi-quantitative estimate of 'total' residues detected. Nevertheless, they continue to be used because of their simplicity and low cost. However, before samples are declared to contain concentrations of antibiotics exceeding the tolerance levels, confirmation (and identification of the individual compounds) by sufficiently selective and sensitive instrumental methods such as LC–MS or GC–MS are required. One may quote Commission Decision 2002/657/EC [6] which states that for prohibited substances "methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods". Recently two interesting reviews were published, one by Corcia et al. [37] on LC–MS methods for the determination of antibiotics and antibacterial agents in food products, and one by Balizs et al. [38] on LC–tandem MS methods for residue analysis of veterinary drugs. These reviews will be frequently cited in the overview of the various groups of antibiotics presented below.

2.2.1. Aminoglycosides

Aminoglycosides (AGs) are a large and diverse class of antibiotics that characteristically contain two or more aminosugars linked by glycosidic bonds to an aminocyclitol component. Well-known AGs are gentamicin, lincomycin, neomycin and streptomycin.

Although the thirty-odd AGs may cause side effects of nephrotoxicity and ototoxicity [39], they are still occasionally used for the treatment of serious infections. The MRLs range from 50 μg/kg for gentamicin in bovine fat to 20,000 μg/kg for apramycin in bovine kidney [30]. Several factors complicate the determination of AG residues in tissues and milk. Their polar nature impedes their extraction and chromatographic separation, their volatility is low, they have no chromophores or fluorophores and, finally, most AGs consist of mixtures of compounds with closely similar structures. To quote an example, gentamicin is made up of four compounds with three molecular masses indicated in Fig. 3 as C1, C2 + C2a and C1a [40].

Microbiological assays are used for the screening of AGs in food of animal origin. A commonly used procedure is the four-plate test with which, for example, neomycin can be detected in tissues down to 0.2 μg/kg [41]. For quantification and confirmation, LC–FLD or LC–MS are used. Most reported extractions of AGs from tissue use ion exchange at high or low pH, or ion-pairing in an aqueous or methanolic solution. Milk is defatted and, then, deproteinated with trichloroacetic acid before the AGs are extracted by WCX-SPE. As in many other cases, the more or less selective extrac-



	MH^+	$MH^+ - NH_3$	R_1	R_2	R_3
C1	478	461	CH_3	H	CH_3
C2, C2a	464	447	H	(CH_3)	(CH_3)
C1a	450	433	H	H	H

Fig. 3. Structures of gentamicin components with proposed ESI(+)IT-MS^n fragmentation patterns; adapted from [40].

Table 7
Selected LC methods for aminoglycosides

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL (μg/kg)	Reference
Gentamicin	Pig tissue	LPE/C_{18}-SPE/derivative MOC-Cl	FLD	50	50–750	[42]
Neomycin	Pig tissue	LPE/C_{18}-SPE/derivative FMOC-Cl	FLD	100	500–5000	[42]
Gentamicin	Swine, calf tissue	LPE/CBA-SPE	ESI(+)IT-MS^n	0.5–2.5	100–1000	[136]
Gentamicin	Milk	Defatted/WCX-SPE	ESI(+)IT-MS^n	<15	100	[40]
Neomycin	Milk	Defatted/WCX-SPE	ESI(+)IT-MS^n	<15	1500	[40]

tion of the target analytes – especially from tissue samples – is the most time-consuming step of the analysis, notably when UV or FLD detection are used. Because of their polar character, the AGs are difficult to separate by RPLC. Options to solve this problem include (i) the use of strong cation exchange (SCX) columns, (ii) ion-pairing LC on a C_{18} column, in the presence of an alkylsulphonate as the ion-pair reagent or, in combination with MS, a volatile reagent like heptafluorobutyric or pentafluoropropionic acid (HFPA, PFPA) [40], or (iii) derivatization with, e.g., 9-fluorenylmethyl chloroformate (FMOC-Cl) to obtain less polar compounds which can be separated by RPLC [42].

A review discusses AG residue analysis in feed and food by means of LC, but also TLC and CE, procedures [43]. As an illustration we briefly quote a general approach that can be used for the determination of AG residues in, e.g., kidney, liver, muscle and fat LPE is performed with an aqueous phosphate buffer and, subsequently, clean-up is done by means of carboxypropyl (CBA)- or other WCX-SPE. Analysis is by means of PFPA-based ion-pairing LC and QqQ-MS detection in the SRM mode. This method is very selective and the LODs are far below the MRL values. Table 7 presents a selection of methods available for AG analysis based on FLD and MS detection.

2.2.2. β-*Lactam antibiotics*

β-Lactams are probably the most widely used class of antibiotics in veterinary medicine for the treatment of bacterial infections of animals used in livestock farming and bovine milk production. There are MRLs for all food-producing species ranging from 4 μg/l for ampicillin in milk to 300 μg/kg for oxacillin, cloxacillin and dicloxacillin in bovine tissues like muscle fat, liver and kidney [30]. β-Lactam antibiotics basically consist of two classes of thermally labile compounds, penicillins and cephalosporins. Both classes contain a bulky side-chain attached to 6-aminopenicillanic acid and 7-aminocephalosporanic acid nuclei, respectively, as is shown in Fig. 4 [44]. The presence of an unstable four-member ring in the β-lactam structures makes these compounds prone to degradation by heat and in the presence of alcohols. Penicillins are also readily isomerized in an acidic environment. Because of these characteristics, several precautions concerning pH and temperature have to be taken in each step of the sample-preparation procedure to avoid analyte degradation [45]. Especially at low concentrations degradation can be significant. The penicillin G concentration in milk fortified at 7.5 μg/l decreased by 18% in 16 h at room temperature. The pH of the extraction buffer is important: optimum stability was obtained at pH 6.7 for animal tissues and at pH 4.6 for milk, and degradation was less at sub-ambient temperatures.

β-Lactams are extracted from milk and animal tissues (liver and kidney are the target organs for penicillins) with salt buffers. The aqueous extract is concentrated and cleaned by C_{18}-SPE or WCX-SPE. The LC separation and detection is mostly based on ion-pairing LC with UV or, sometimes after derivatization, by FLD. The use of these conventional detection techniques is often complicated due to interfering matrix components. As has already been mentioned for aminoglycosides, the use of LC–MS can solve these selectivity problems.

Several studies [37,38] describe methods available today for the selective confirmatory analysis of β-lactam antibiotics in milk at the MRL level by LC–MS, LC–QqQ-MS or LC–IT-MS^n. Analyte extraction in combination with tandem MS detection was based on a single liquid extraction with, for example, acetonitrile followed by UF; in the case of single-stage MS detection, various LLE steps were used like addition of acetonitrile (to prevent analyte binding to proteins) followed by LLE with dichloromethane, hexane–acetonitrile, water, phosphate buffer (pH 7) and again dichloromethane [46]. The RPLC separation was performed on C_{18}-bonded silica with an acetonitrile/water gradient containing an ion-pairing reagent, for example di-*n*-butylamine acetate (DBAA) [47].

An LC–IT-MS^n method for the determination of β-lactams in kidney was reported by Fagerquist and Lightfield [44]. After extraction of the analytes with acetonitrile and water, clean-up of the extracts was by C_{18}-SPE, with subsequent RPLC on a C_{18} column; the eluent was a methanol–water gradient with 0.1% formic acid. The authors concluded that IT-MS^n is very useful for identification purposes, but they were unable to obtain reproducible quantitative results. This problem, which is probably due to ion suppression, often occurs when the final extract still contains too many matrix components. Additional clean-up has to be introduced to solve this problem. Ito et al. [48] used LC–UV_{220} to screen food for various penicillins using a specific combination of SPE columns for sample clean-up. They used a salt buffer for extraction and the extract was cleaned by C_{18}-SPE and, next, by a purification of the eluate on a QMA silica-based strong-anion-exchange cartridge. Separation was by RPLC on a C_{18} column with acetonitrile–0.02 M phosphate buffer pH 6.2 (43:57, v/v) containing 12 mM cetyltrimethylammonium chloride. Fig. 5 shows a chromatogram of bovine liver with and without the addition of penicillins. For bovine liver

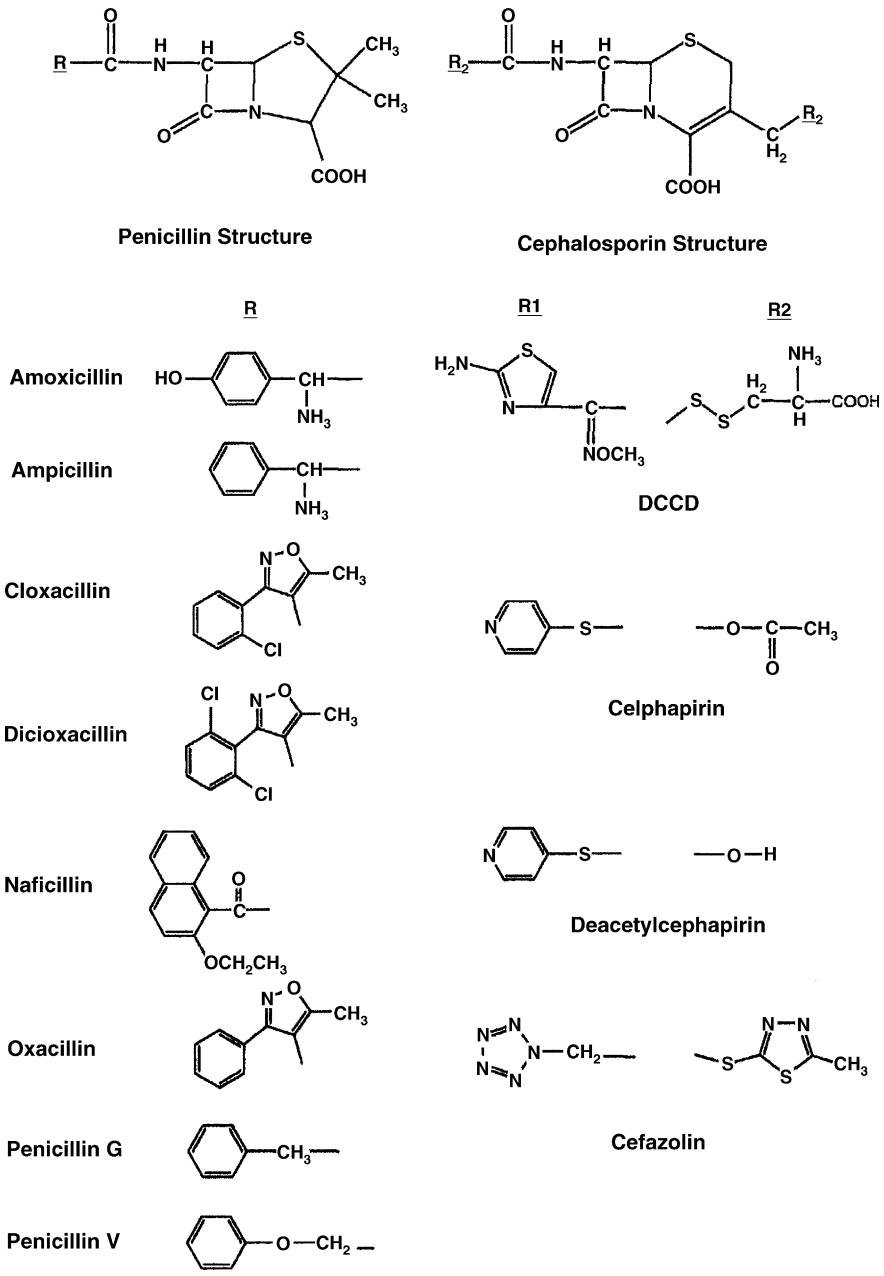


Fig. 4. Structures of eight penicillins and four cephalosporins; adapted from [44].

spiked at levels of 0.1 mg/kg the recoveries for the six penicillins were 83–96%. LODs of the penicillins in bovine liver and kidney were in the MRL range, viz. 0.02–0.05 mg/kg. The authors also reported a confirmatory LC–ESI(−)QqQ-MS method [47]. Somewhat surprisingly, although QqQ-MS is much more selective, the LODs were the same for UV and QqQ-MS detection (Table 8).

Two further studies on the determination of β-lactams, both penicillins and cephalosporins, in milk should be mentioned [45,49]. They discuss LC–ESI(+)QqQ-MS and LC–ESI(+)IT-MS^n methods which yield low LODs, and also pay attention to the selection of proper pH and temperature conditions during extraction. Two frequently selected MS–MS fragment ions of the β-lactams are *m*/*z* 160, formed due to the cleavage of the β-lactam ring and *m*/*z* 114, formed by a further loss of COOH.

Finally, amoxicillin and ampicillin are difficult to analyse due to their amphoteric nature. Generally speaking ESI(−) is the most sensitive ionisation mode for the present class of compounds, but it can only be used when the amphoteric β-lactams are not included; when these compounds are included, the ESI(+) mode is preferable.

2.2.3. Macrolides

Macrolides are an important class of antibiotics which are widely used in veterinary practice to treat respiratory dis-

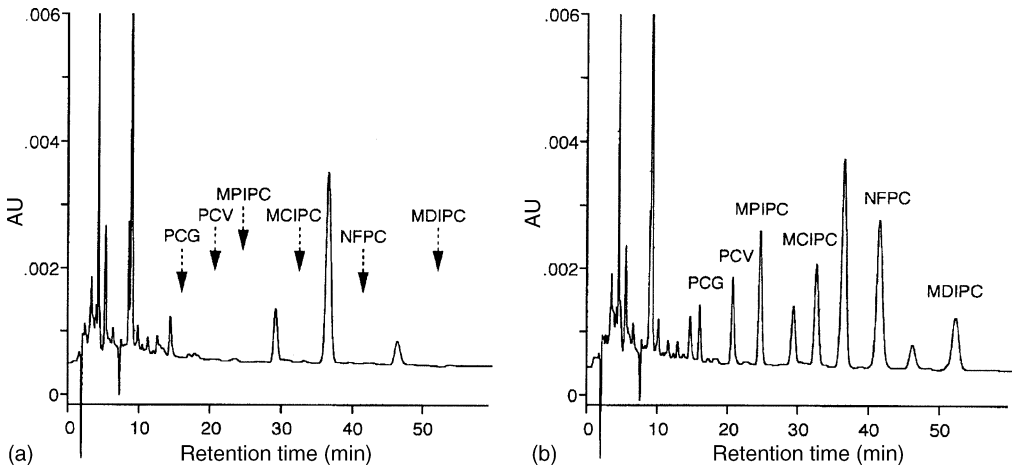


Fig. 5. LC–UV_{220} of bovine liver. (a) Bovine liver (control); (b) eight penicillins added at 0.5 mg/kg of each penicillin. PCG, benzylpenicillin; PCV, phenoxymethylpenicillin; MPIPC, oxacillin; MCIPC, cloxacillin; NFPC, nafcillin; MDIPC, dicloxacillin. Conditions: TSKgel C_{18} column; acetonitrile–phosphate buffer with 12 mM cetyltrimethylammonium chloride; flow 0.8 ml/min; adapted from [48].

Table 8
Selected LC methods for β-lactams

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL (μg/kg)	Reference
β-Lactams	Kidney	LPE/C_{18}-SPE	ESI(+)IT-MS^n	10–500	25–300	[44]
Penicillins	Kidney, liver	LPE/C_{18}-SPE/QMA-SPE	UV	20–50	25–300	[48]
Penicillins[a]	Bovine tissues	LPE/C_{18}-SPE/QMA-SPE	ESI(−)QqQ-MS	20–30	25–300	[47]
β-Lactams	Milk	LLE	ESI(+)QqQ-MS	1–5	4–100	[49]
β-Lactams	Milk	LLE/HLB-SPE	ESI(+)IT-MS^n	0.2–2	4–100	[45]

[a] Ampicillin and amoxicillin not included.

eases, or as feed additives to promote growth. Macrolide antibiotics are macrocyclic lactones with a 12–16 carbon-lactone ring, to which several amino groups and/or neutral sugars are bound (Fig. 6) [29,50]. They are easily absorbed after oral administration and distribute extensively to tissues, especially the lungs, liver and kidneys. There are MRLs for several macrolides, viz. acetylisovaleryltylosin, erythromycin, spiramycin, tilmicosin and tylosin. They range from 40 μg/kg for erythromycin in milk to 2000 μg/kg for spiramycin in porcine liver [30].

Next to commonly used organic solvents such as acetonitrile, chloroform and dichloromethane, aqueous buffer solutions can be used for the extraction of macrolides from tissues. After extraction, C_{18}-SPE or WCX-SPE purification/concentration is performed. RPLC on an alkyl-bonded silica column is the most frequently used approach for the separation of macrolides. Eluents consist of a mixture of acetonitrile and an aqueous phosphate or acetate buffer. Separation is carried out in an acidic medium, except for erythromycin, for which neutral media are preferred because of its instability under acidic conditions. Traditionally, UV absorbance is used for detection. However, macrolides like erythromycin and oleandomycin lack a suitable chromophore; consequently, instead of non-selective UV detection, MS is preferred by several authors (Table 9).

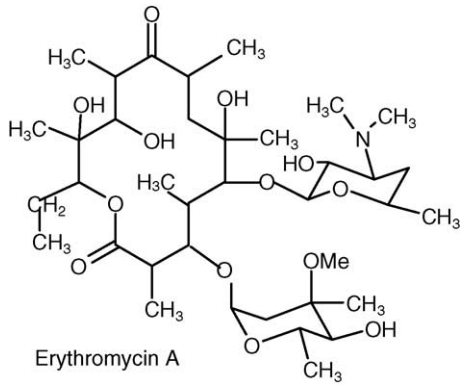


Fig. 6. Typical structure of a macrolide.

An LC–ESI(+)QqQ-MS method for the determination of five macrolides in tissue (muscle, liver, kidney), milk and eggs is described by Dubois et al. [50]. After extraction with a Tris buffer at pH 10.5 followed by protein precipitation with acetic acid and a sodium tungstate buffer in the case of milk and eggs, the extract was cleaned by hydrophilic/lipophilic balanced copolymer (HLB)-SPE. The macrolides, tylosin, tilmicosin, spiramycin, josamycin, erythromycin and roxithromycin, were separated on C_{18}-bonded silica with a gradient of aqueous 0.1 M ammonium acetate–acetronitrile. Detection was performed in the SRM mode. The method can be used for confirmation and quantification down to the 0.5 MRL level. In the (0.5–2) MRL concentration range, the recoveries ranged from 44% for erythromycin in milk to 115% for tilmicosin in muscle. Draisci et al. [51] combined a rapid electrochemical ELISA screen-

Table 9
Selected LC methods for macrolides

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL (μg/kg)	Reference
Macrolides	Tissues, milk, eggs	(LPE)LLE/Oasis HLB-SPE	ESI(+)QqQ-MS	0.01–37	40–2000	[50]
Macrolides	Manure	LLE/diol-SPE	APCI(+)QqQ-MS	0.4–20	–	[52]
Erythromycin, tylosin, tilmicosin	Muscle, liver, kidney	LPE/diol-SPE	APCI(+)QqQ-MS	20–150[a]	50–1000	[137]
Macrolides	Poultry, muscle	LPE/SCX-SPE	ESI(+)MS	<4–35	75–400	[53]
Macrolides	Muscle	LPE/SCX-SPE	DAD UV	4–20[b]	40–1000	[54]

[a] LOQ.
[b] LODs for josamicin and erythromycin, approx. 200 and 400 μg/kg, respectively.

ing with micro LC–APCI(+)QqQ-MS for the confirmation of macrolide residues in bovine meat. The LOD of the ELISA was 0.4 μg/l for erythromycin and 4.0 μg/l for tylosine isocratic separations was done on an RPLC microbore column at room temperature, with acetonitrile–methanol–1% TFA (60:20:20, v/v/v) as eluent. For both analytes the LODs were far below the MRL values of 100 μg/kg for bovine muscle. However, to achieve satisfactory results, a rather time-consuming procedure was needed, which is presented in Fig. 7.

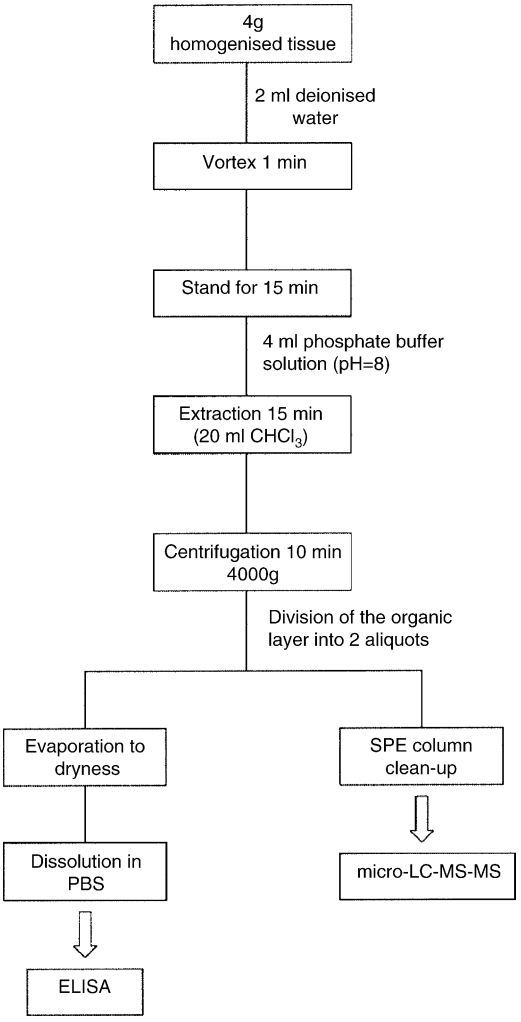


Fig. 7. Extraction procedure for erythromycin and tylosin in bovine tissue; adapted from [51].

Schlüsener et al. [52] used a combination of LPE (phosphate buffer and ethyl acetate) and diol-SPE as sample preparation technique for the determination of macrolides in liquid manure, which is a very complex matrix. In the cleaned extracts macrolides could be detected down to the 0.4 μg/kg. Such low LODs are necessary to monitor contamination of the environment with drug residues. Codony et al. [53] used extraction with aqueous methanol and SCX-SPE to isolate macrolides from poultry muscle. After separation on a C_{18} column with water–acetonitrile containing TFA as the eluent, the analytes were detected by ESI(+)-MS. Fig. 8 shows a chromatogram of spiked chicken muscle spiked with six macrolides at the MRL, which is close to the detection limit in this case. Due to the relatively high MRLs and the selectivity of SCX-SPE, DAD UV detection could be used instead of MS detection for some macrolide/matrix combinations, e.g., spiramycine in chicken muscle [54].

2.2.4. Peptides

The main peptides used as veterinary drugs are avoparcin, bacitracin (usually used as the zinc salt), efrotomycin and virginiamycin (Fig. 9). Until recently, avoparcin and virginiamycin were used as feed additives to improve feed conversion. The structural similarity of these compounds to the human glycopeptide antibiotic, vancomycin, has raised concern regarding cross-species antibiotic resistance [29]. Avoparcin and virginiamycin were banned in 1997 and 1999, respectively, as feed additives in the EU [55,56]. Before that time, analysis was based on microbiological principles. Today, there is a need for sensitive and selective instrumental methods. The number of published methods is, however, limited. Hajee et al. [56] described different LC methods for the determination of virginiamycin M1 in animal feed. After an intensive sample-preparation procedure (see Fig. 10), the separation was on an Inertsil ODS-2 column with acetonitrile–water–formic acid as the eluent. Detection was by UV_{230} while three MS detection modes (ion-source collision induced dissociation (CID), full-scan MS and MS^2) were tested as alternatives. Not surprisingly, LC–MS and LC–MS^2 were much more selective and sensitive than LC–UV. The LOQ obtained for the UV method was 2.7 mg/kg; the LOQ for MS confirmation has not been established as yet. Govaerts et al. [57] demonstrated that with LC–IT-MS^n it is possible to identify relatively complex mixtures of polypeptide antibi-

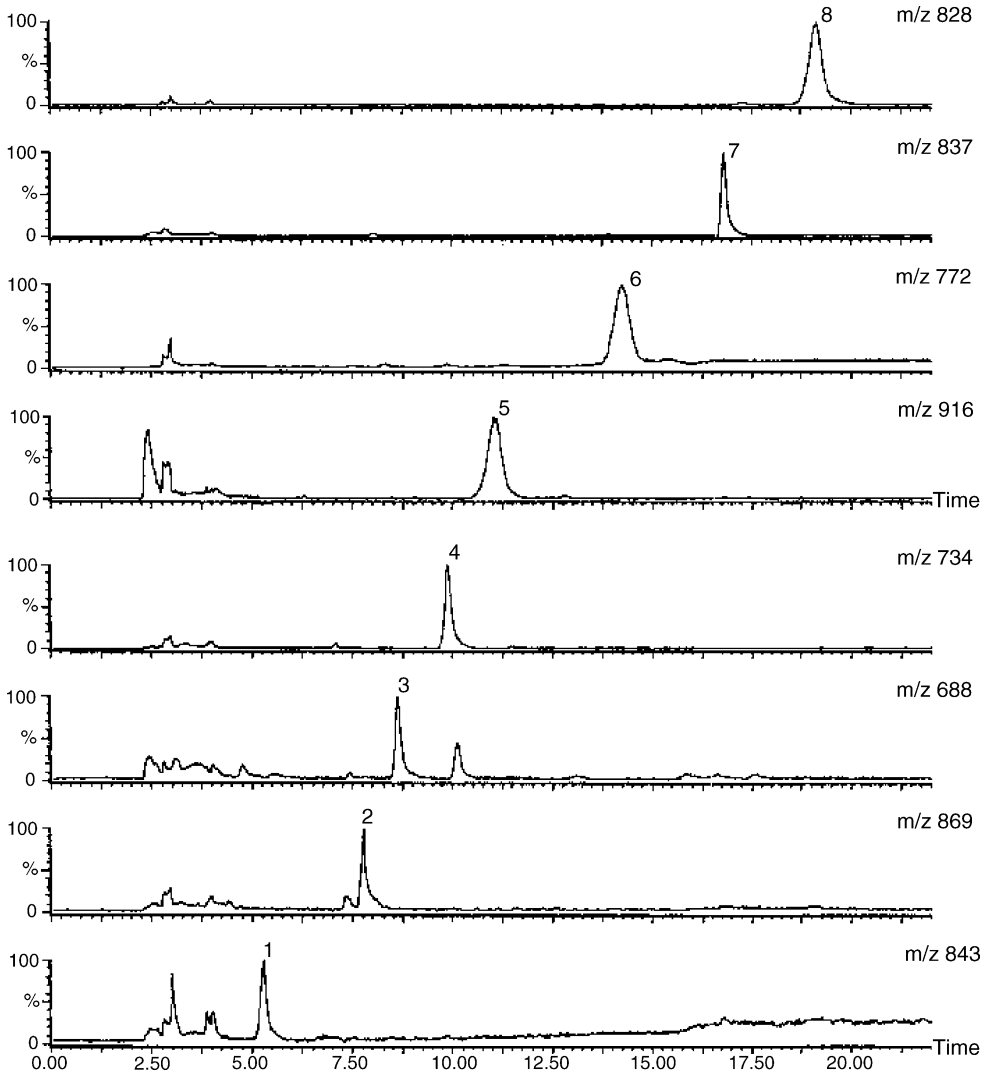


Fig. 8. LC–ESI(+)-MS of blank chicken muscle spiked at MRL level for each macrolide; 1, spiramycin; 2, tilmicosin; 3, oleandomycin; 4, erythromycin; 5, tylosin; 6, kitasamycin; 7, I.S; 8, josamycin. Conditions: Hypurity C_{18} column; gradient elution with water–acetonitrile containing TFA; adapted from [53].

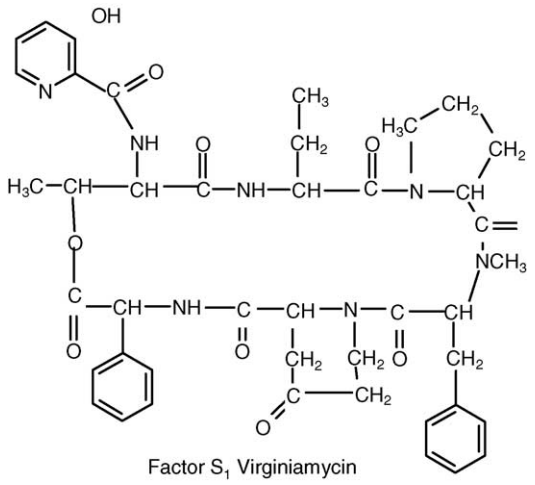


Fig. 9. Typical stucture of peptide antibiotic: virginiamycin.

otics. However, they did not apply this detection method to biological sample extracts.

The extraction of the peptides from biological matrices is a point of concern. Due to their structure they mimic naturally occurring compounds and are very difficult to be selectively extracted from feed and biological samples. Curren and King [55] tried to use PLE with hot water to extract avoparcin from kidney samples. Clean-up and separation were based on hydrophilic interaction chromatography (HILIC). In essence, this is normal-phase chromatography with a highly aqueous eluent. The stationary phase adsorbs or imbibes water and becomes hydrophilic. Polar analytes such as carbohydrates and peptides now selectively partition into the stagnant enriched aqueous layer on the surface; retention therefore increases with analyte polarity. Unfortunately, this approach dit not yield the low LOD of 1–10 μg/kg necessary for monitoring illegal use: the LOD for the PLE method was 0.5 mg/kg.

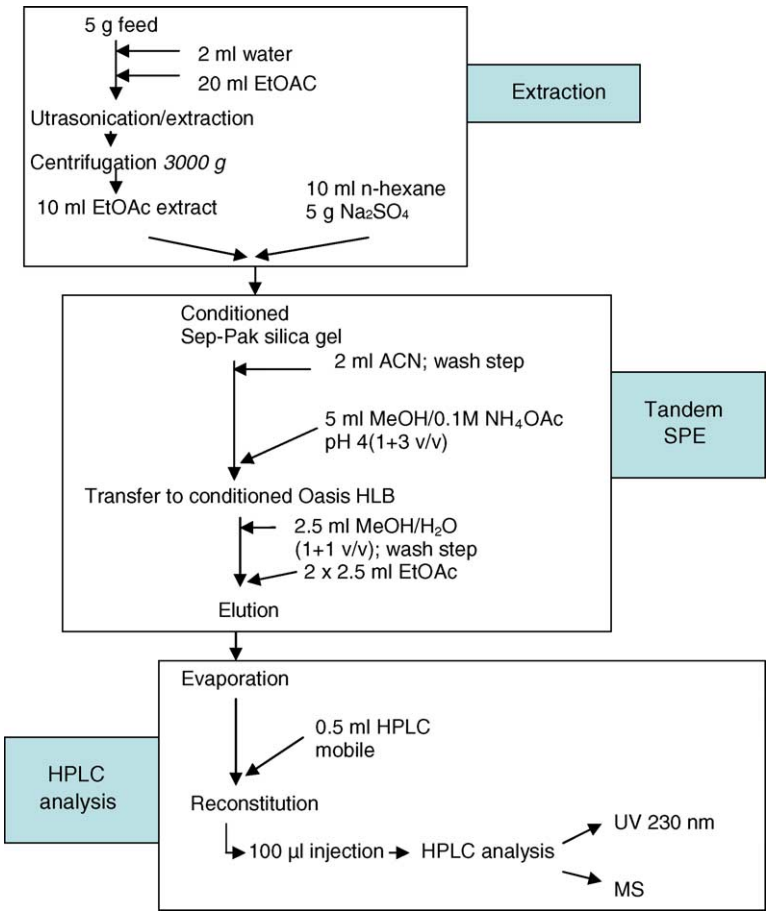


Fig. 10. Schematic of the sample preparation developed for the determination of virginiamycin M1 as marker component for virginiamycin in compound animal feed. Abbreviations: EtOAc, ethyl acetate; AC, acetonitrile; MeOH, methanol; NH_4OAc, ammonium acetate; adapted from [56].

A final example of the determination of peptide antibiotics in animal feed is given by Capitán-Vallvey et al. [58]. They developed an LC–UV method for zinc bacitracin in animal feed. Sample treatment involved extraction from the feed at pH 2, centrifugation, LPE with phosphate buffer and ethyl acetate and C_{18}-SPE. RPLC was done on a C_{18} column with a 50:50 (v/v) mixture of 0.3 M phosphate buffer, pH 3, containing 20 mM sodium dodecyl sulfate (SDS) and acetonitrile–methanol (19:1, v/v). The LOD was 5 mg/kg.

It is obvious from the literature that the trace-level determination of the present group of peptides still presents serious problems; this is illustrated by the limited information of Table 10. In other words, there is still a need to develop new selective multi-analyte procedures.

2.2.5. Sulphonamides and trimethoprim

Sulphonamides are bacteriostatics. Residues in food are of concern because of the potential carcinogenic nature of these compounds and the possibility of the development of antibiotic resistance in humans [38]. Sulphonamides are used as veterinary drugs for prophylactic and therapeutic purposes; they also act as growth-promoting substances. Trimethoprim is a potentiator often administered together with sulphonamides. EU and US regulatory bodies have set MRLs for meat and milk at 100 μg/kg. These values are for the sum of all parent sulphonamides present in meat or milk [30].

As for many antibiotics, selective extraction of sulphonamides from biological tissues is complicated due to the polar character of the analytes and matrix components.

Table 10
Selected LC methods for peptides

Polypeptides	Matrix	Sample preparation	Detection	LOD (mg/kg)	Action level (mg/kg)	Reference
Avoparcin	Kidney	PLE/HILIC-SPE[a]	(HILIC)LC-UV	0.5	Banned	[55]
Virginiamycin	Feed	LLE/SiO_2-SPE/HLB-SPE	UV or APCI(+)IT-MS^n	1	Banned[a]	[56]
Zinc bacitracin	Feed	LPE/LLE/C_{18}-SPE	UV	5	Banned	[58]

[a] Proposed Dutch MRPL, 2–4 mg/kg.

Table 11
Selected LC methods for sulphonamides

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL (μg/kg)	Reference
Sulphonamides	Shrimps	LPE/SEC-SPE	UV	10[a]	100	[62]
Sulphonamides	Liver, kidney	MSPD/PLE	ESI(+)-MS	5–14[a]	100	[59]
			ESI(+)QqQ-MS	1–8[a]		
Sulphonamides	Muscle, fish	MSPD/PLE	ESI(+)-MS	1–10	100	[60]
Sulphonamides	Milk, eggs	MSPD	ESI(+)-MS	1–6[a]	100 milk–eggs	[61]
Sulphonamides, trimethoprim	Manure	LLE	ESI(+)-MS	<100–	–	[64]
			ESI(−)-MS	1000		
Sulphonamides	Milk	LLE/UF[b]	ESI(+)QqQ-MS	5–20[a]	100	[24]
Dapson	Milk	LLE/UF	ESI(+)QqQ-MS	1–2[a]	Banned	[24]
Sulphonamides	Honey	Dissolve/SCX-SPE/HLB-SPE	FLD	2–5	–	[66]
Sulphonamides	Kidney	LPE/HLB-SPE (on-line)	ESI(+)QqQ-MS	5–14	100	[63]
Sulphonamides	Eggs	LLE/C_{18}-SPE	ESI(+)QqQ-MS	5–10[a]	–	[138]

[a] LOQ; S/N = 10.
[b] Ultrafiltration.

A recent extraction scheme involves the use of hot water as an effective extractant, sometimes in combination with matrix solid-phase dispersion (MSPD) [59–61] see (Table 11). The sample – liver, kidney, muscle or fish – is carefully mixed with sand in a porcelain mortar and the mixture extracted with hot water. Subsequent analysis is on a C_{18} column using simple water–methanol or water–acetonitrile gradients in combination with UV detection or, when more selectivity or sensitivity is necessary, MS detection. With MS–MS detection, LODs of <10 μg/kg were obtained for most of the sulphonamides in milk, muscle and kidney. With the exception of sulphaquinoxaline, recovery of the analytes at the 50 μg/kg level in both liver and kidney was 72–96% with an RSD range of 3–11% [59]. Roybal et al. [62] selectively extracted sulphonamides from shrimps by means of size-exclusion chromatography (SEC) on Sephadex LH-20 gel. After separation on a phenyl-bonded LC column, UV_{270} detection enabled analysis down to 10 μg/kg. van Eeckhout et al. [63] presented a complete on-line system based on extraction of the analytes from a primary methanol extract on an HLB column followed by LC–ESI(+)QqQ-MS. This on-line approach is very interesting because of the rapid sample preparation and, consequently, high sample throughput. This makes the method very suitable for routine analysis. Earlier, the same approach was applied to another group of antibiotics, the tetracyclines [140]. van Rhijn et al. [24] also reported a widely applicable approach for sample extraction: ultrafiltration (UF) was used for the extraction of sulphonamides and dapson – a potentiator often administered together with sulphonamides – from milk. A chromatogram is presented in Fig. 11. This UF approach was also used for the extraction of benzimidazoles. With both approaches – the on-line extraction and the off-line UF procedures – some 50 samples can be analysed per 24 h, while with combinations of LLE and SPE a sample throughput of 10 samples per 24 h is usually obtained.

As already observed for previously discussed antibiotics, also for the sulphonamides the use of LC–MS is very powerful. The reported procedures mostly use ESI(+)-tandem MS, were the protonated molecular ions $[M+H]^+$, undergo CID to give fragments such as shown in Fig. 12. Product ions common to the majority of sulphonamides include the *p*-aminobenzene sulphonic acid moiety, $[M-RNH_2]^+$ (*m/z* 156), $[M-RNH_2–SO]^+$ (*m/z* 108), $[M-RNH_2-SO_2]^+$ (*m/z* 92), and ions from the various amino substituents RNH_3 $[MH-155]^+$.

Manure and water are two other sample types that have to be screened for the presence of sulphonamide residues [64,65]. Sulphonamides apparently are not easily degraded and are sufficiently hydrophilic to be transferred into the aquatic environment. Lindsey et al. [65] detected sulfonamides in seven of 144 ground- and surface waters collected throughout the US. They detected sulfamethazine, sulfadimethoxine, sulfamethoxazole and sulfathiazole at concentrations of 0.07–0.15 μg/l.

A more traditional LC–FLD method for sulphonamides in honey was reported by Pang et al. [66]. Samples were dissolved in phosphoric acid solution (pH 2), filtered, and cleaned by (aromatic sulphonic acid)-SPE and HLB-SPE. After derivatization with fluorescamine, analysis was by RPLC on a C_{18} column. With FLD, LODs were 2–5 μg/kg. Spinks et al. [67] used an ELISA screening method for the detection of down to 0.1 mg/kg of sulphachlorpyridazine in eggs, milk, meat and feedstuffs.

2.2.6. Tetracyclines

Tetracycline antibiotics (TCAs) are broad-spectrum antibiotics against gram-positive as well as gram-negative bacterias. They are also used for promoting growth in cattle and poultry [38]. The basic structure of TCAs is a hydronaphthacene skeleton containing four fused rings. The various TCAs mainly differ in their substitution patterns at the C5, C6 and C7 positions (Fig. 13). Of the eight commercially available TCAs, chlortetracycline (CTC), oxytetracycline (OTC), tetracyline (TC) and doxytetracycline (DOX) are most commonly applied to food-producing animals. Their MRLs range from 100 μg/kg for muscle to 600 μg/kg for kidney [30].

Due to the presence of two ketone groups in positions 1 and 11, TCAs can readily chelate to metal ions. They can also interact with silanol groups during LC separation on a silica-based stationary phase, even if this phase is end-capped; this

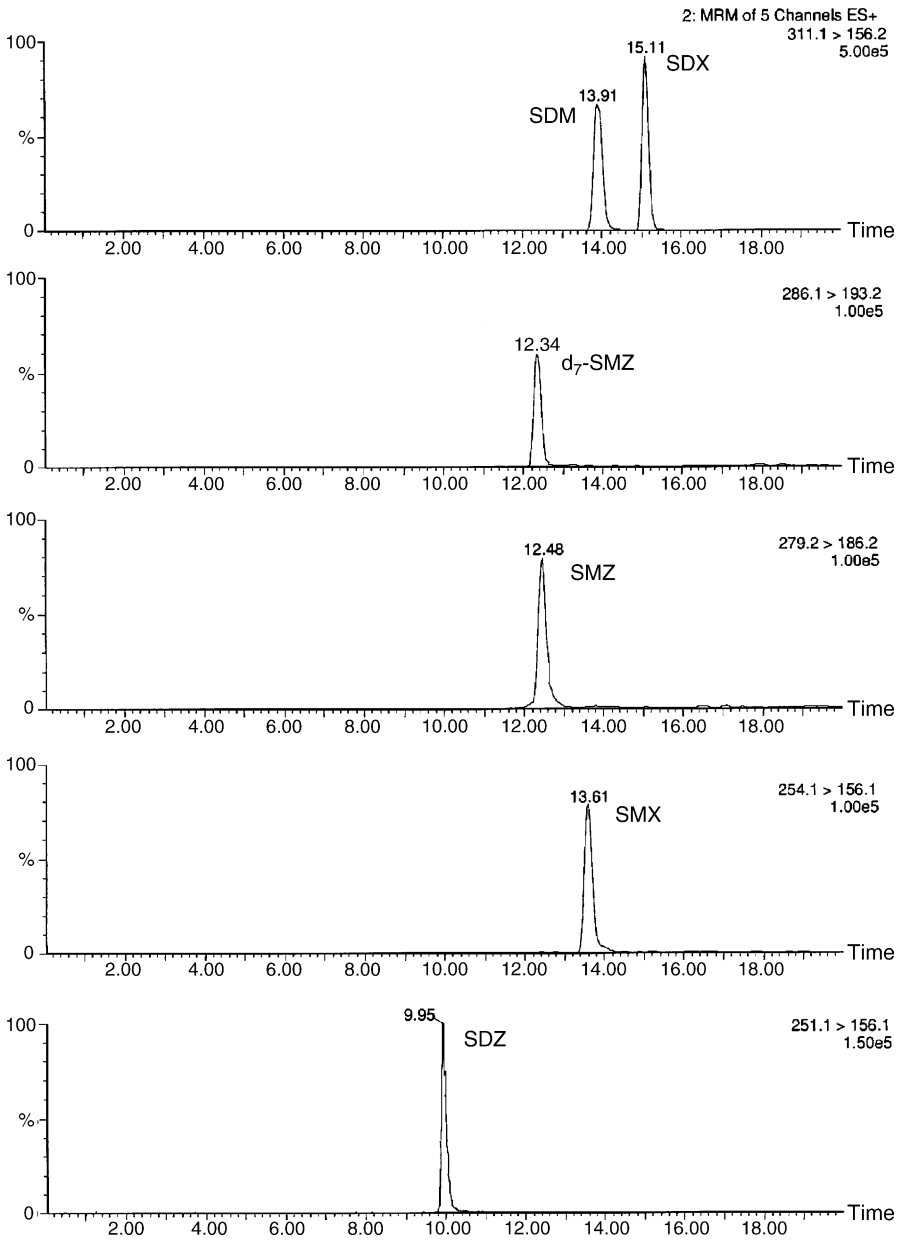


Fig. 11. LC–ESI(+)QqQ-MS chromatogram of a blank milk fortified with 100 μg/kg of sulphadoxine (SDX), sulphadimethoxine (SDM), sulphadimidine (SMZ), sulphamethoxazole (SMX) and sulphadiazine (SDZ). Concentration of d_7-sulphadimidine (I.S.), 200 μg/kg; adapted from [24].

causes severe tailing of TCA peaks. Many authors eliminate this problem by adding chelating agents, such as oxalic acid and EDTA salts, to the eluent [68]. However, the presence of non-volatile agents in the LC eluent prevents the use of ESI-MS for detection because of the rapid contamination of the sample cone orifice. Moreover, both oxalic acid and EDTA cause a drastic reduction of the ion signal intensities of TCAs. In combination with MS, volatile buffer solutions like ammonium acetate or formic acid have to be used, although this has a negative effect on the peak shape and separation.

Another problem is that CTC and DOX peaks frequently show excessive fronting. The type of column used and the LC conditions, particularly the column temperature, play a main role here. It has been reported that CTC and DOX rapidly isomerize to give 4-*epi*-TCAs in aqueous solutions at pH 2–6. In addition, keto tautomers are readily formed in aqueous solutions. The products of both tautomerization and epimerization are eluted well before the parents, OTC and DOX. This is illustrated in Fig. 14. This phenomenon, rarely mentioned in the literature, complicates quantification of CTC and DOX [68].

In a recent review on the LC analysis of TCAs in food, the authors discuss the above problems of chelate formation, silanol interactions and epimerization, and also give a very complete overview of all available LC–UV and LC–FLD techniques for TCA analysis [68]. They finally conclude that by using the chelating ability of TCAs, very selective extraction can be obtained and that the addition of EDTA or

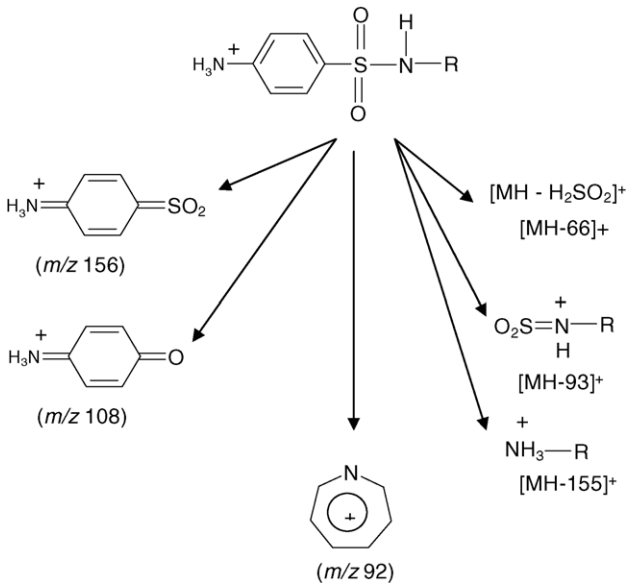


Fig. 12. Proposed fragmentation scheme for CID of protonated sulphonamides; adapted from [37].

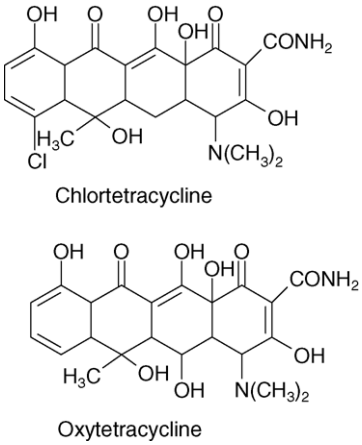


Fig. 13. Typical structures of tetracyclines.

oxalic acid during separation helps to prevent undesired secondary interactions. Most analytical method are based on the extraction of TCAs from tissues with EDTA–Mcllvaine buffer (citric acid with disodium hydrogen phosphate). For tissues and milk an additional clean-up on C_{18}-SPE or HLB-

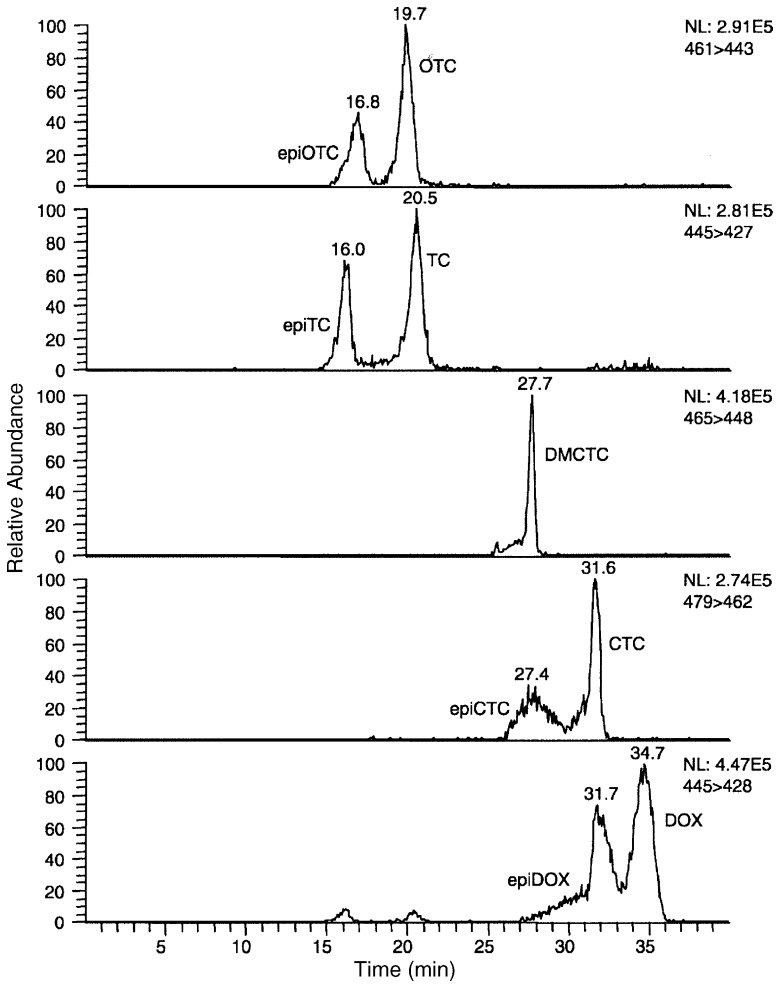


Fig. 14. LC–ESI(+)IT-MS^n of a standard solution of 4-epi-OTC, OTC, demethyl-CTC-, 4-epi-CTC, CTC, 4-epi-DOX and DOX; all compounds at approx. 500 μg/kg; adapted from [161].

Table 12
Selected LC methods for tetracyclines

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL (μg/kg)	Reference
TCAs	Bone meal	LPE	UV	0.5–1	–	[69]
Oxytetracycline + 4-epimer	Tissues	LPE/HLB-SPE	ESI(+)IT-MS^n	0.8–48	100–600	[70]
TCAs + 4-epimers	Tissues	LPE/HLB-SPE	ESI(+)IT-MS^n	0.5–4.5	100–600	[161]
TCAs	Milk, eggs	LLE/(carbograph 4)-SPE	ESI(+)-MS	2–19[a]	100–200	[139]
TCAs	Kidney	LPE/HLB-SPE (on-line)	ESI(+)QqQ-MS	18–24	600	[140]

[a] LOQ; S/N = 10.

SPE is required prior to RPLC on a C_{18} column with a water–acetonitrile or –methanol gradient. Depending on the detection technique used, EDTA–phosphate buffers (UV detection), or volatile ammonium acetate buffers or formic acid (MS detection) are used. Some analytical approaches are presented in Table 12.

An example of the more traditional LC–UV analysis of TCAs in bone meal, used for the production of feedstuff, is given by Körner et al. [69]. After LPE of the analytes from feed by means of sodium succinate buffer (pH 4), RPLC on a C_{18} column gave LODs of around 1 μg/kg, demonstrating that the method can be used for the monitoring of feed (concentrations detected were 1000–2000 μg/kg). A rapid procedure for the extraction of OTC and its 4-epimer from calf tissues involves LPE with sodium succinate buffer (pH 4) combined with clean-up by HLB-SPE and LC– IT-MS^n [70]. This simple approach gave LODs of <50 μg/kg for this specific TCA and its epimer.

As regards environmental concerns, TCAs are known to show strong sorption and are therefore expected to remain in the soil or to be transported to surface water via particulate matter after excretion. Reverté et al. [71] used LC–ESI(+)MS to determine TCAs and quinolones in waste water; Lindsey et al. [65] used the same technique for sulfonamides and tetracylines in surface and groundwater. In the latter study, the 'neutral loss' of 35 Da – i.e., the loss of ammonia plus water – was used for the selective detection of TCAs. Both groups could detect TCAs down to 10 ng/l. Since suspected concentrations in surface and groundwater are 1–500 ng/l, there is still room for more sensitive analytical methods for these antibiotics in rivers and lakes, especially in their sediments. Adequate methods and monitoring results for these compartments are scarce [72].

2.2.7. Quinolones

Quinolones are broad-spectrum synthetic antimicrobial agents used in the treatment of livestock and in aquaculture. The MRLs range from 10 μg/kg for sarafloxacin in chicken fat to 1900 μg/kg for difloxacin in poultry kidney [30]. The carboxylic group at position 3 (Fig. 15a), makes the compounds acidic. However, the 7-piperazinyl quinolones also have basic amine substituents. In aqueous solution, the 7-piperazinylquinolones therefore are cationic, zwitterionic or anionic, while the other quinolones can only be neutral or anionic (Fig. 15b). Due to the different type of substituents, quinolones have mutually rather different physical properties. As a consequence, most analytical methods have been designed for the determination of individual, or a mere two or three, quinolones. After the advent of the more widely applicable LC–MS methods, there has been a marked increase of multi-residue methods, with their distinct advantages for monitoring purposes.

Many papers have been devoted to the determination of quinolone residues. In the last 5 years over 100 articles were published [73]. Most papers discuss the analysis of fish and animal tissues such as muscle, liver, kidney, skin and fat. Milk and eggs are also often analysed. A review discussing the current methodologies for the determination of over 15 quinolones in edible animal products was recently published by Hernández-Arteseros et al. [74].

Since quinolones are polar compounds and most of them show native fluorescence, RPLC–FLD is the technique traditionally used for routine residue analysis. Ampholytic compounds such as enrofloxacin and its metabolite ciprofloxacin may give tailing peaks in RPLC due to interactions with residual silanol groups and metal impurities. Therefore, high-purity or base-deactivated columns have to be used in combination with optimized pH and ion-strength conditions. In marked contrast with the LC separation conditions, sample treatment varies greatly among the published methods, and there often is little correlation with sample type or target analyte(s). Quinolones are readily soluble in polar organic solvents and also in aqueous/organic, acid or basic, solutions. Clean-up procedures of the primary extracts are often based on SPE with C_{18}-, C_8- or C_2-bonded silicas or copolymer sorbents.

Maraschiello et al. [75] described the use of LC–UV_{295} for the determination of ofloxacin in chicken tissue. After extraction with 0.15 M HCl and clean-up by HLB-SPE, isocratic RPLC was carried out on a C_{18} column with water–acetonitrile–tri-ethylamine as the eluent. The LOQs for ofloxacin were 50 μg/kg for muscle, skin and fat, and 100 μg/kg for liver and kidney. Recoveries ranged from 80 to 100%. No MRL is defined for this specific compound. Ramos et al. [76] used LC–FLD for the determination of five quinolones, ciprofloxacin, enrofloxacin, oxolinic acid, flumequine and sarafloxacin, in pork and salmon muscle. The method includes PLE with a phosphate buffer (pH 7.4) and clean-up on C_{18}-SPE. Because of co-elution problems, two RPLC runs were required. For ciprofloxacin, enrofloxacin and sarafloxacin, acetonitrile–0.02 M phosphate buffer pH 3.0 (18:82) was used as eluent and de-

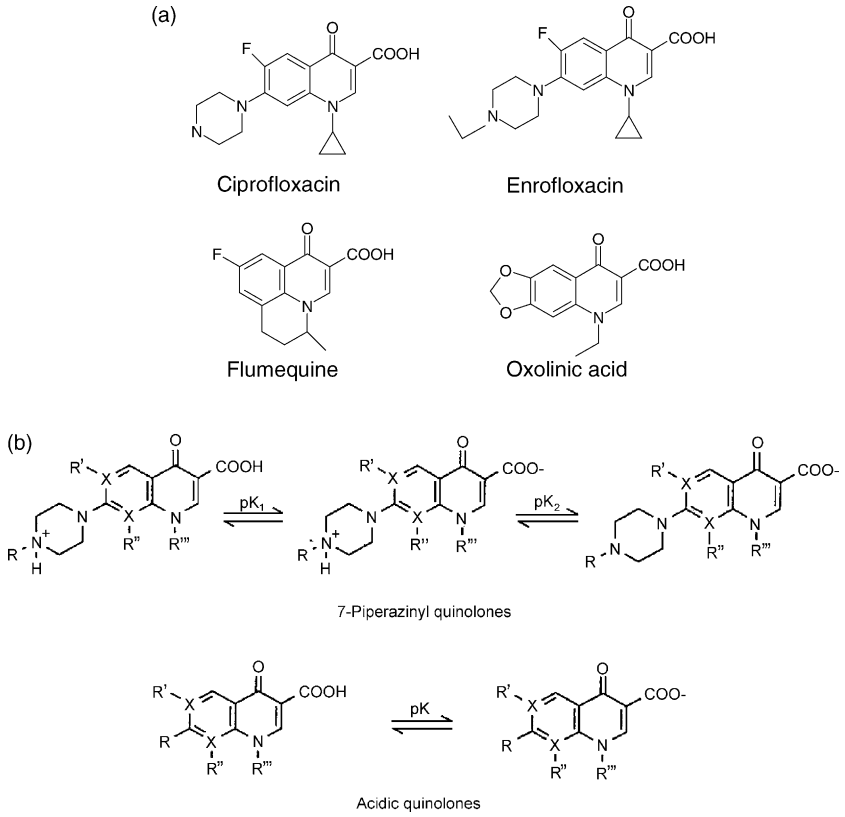


Fig. 15. (a) Structures of some well-known quinolones. (b) Acid–base equilibria for quinolones; adapted from [96].

tection was at 280/450 (exc./em.) nm. For oxolinic acid and flumequine, acetonitrile–0.02 M phosphate buffer pH 3.0 (34:66) was used at 312/366 (exc./em.) nm. LODs were as low as 5 μg/kg, except for sarafloxacin (10 μg/kg). Recoveries for the five quinolones from fortified pork muscle at the 20–300 μg/kg level were 73–86%. Only one LC run would have been necessary, had MS been used for detection.

Pecorelli et al. [77] described both LC–UV and LC–FLD for the determination of 13 quinolones in feed and successfully used PLE with an acetonitrile–metaphosphoric acid (pH 2.6) mixture as a selective multi-analyte extraction technique. Separation was by RPLC on a C_5 column with acetonitrile–tetrahydrofuran–K_2HPO_4 as the eluent. LODs ranged from 0.5 mg/kg for cinoxacin to 1.5 mg/kg for rufloxacin and recoveries from 51 to 103% for analyte concentrations of 5–25 mg/kg. These are very satisfactory results for this type of extraction. Fig. 16 presents the LC–FLD chromatogram.

A completely validated RPLC–tandem MS method for the determination of quinolones in swine kidney [78] uses acetone extraction and subsequent clean-up by mixed-mode C_8/WCX-SPE. Detection, which was by ESI(+)QqQ-MS, gives LODs far below the MRLs (Table 13). The same technique was used by Johnston et al. [79] for the determination of quinolones and fluoroquinolones – quinolones with a fluoro substituent – in fish and seafood. The authors used PLE with acetonitrile and two-stage polymeric RP and anion-exchange SPE. RPLC was done on C_{18} column with water (containing 2% formic acid)–acetonitrile as the eluent. With ESI(+)QqQ-MS in the SRM mode, LODs were 1–3 μg/kg.

It is probably true to say that the general availability of LC–MS and the recent developments of high-purity LC columns have solved most separation and detection problems for the present group of compounds. Because of their divergent characteristics, the main remaining problem of quinolone analysis is the selective multi-analyte extraction from biological tissues, and also from less complex matrices like surface and groundwater. A rather time-consuming combination of LPE, LLE and SPE procedures has still to be used for sample preparation. Table 13 shows a selection of LC methods used for quinolone analysis.

2.2.8. Chloramphenicol

Chloramphenicol (CAP) is a broad-spectrum antibiotic active against a variety of pathogens. Although CAP was, previously, widely used in veterinary and human medicine, reports of plastic anaemia in humans arising from its use led to its ban in the US and EU in 1994. Thiamphenicol and florfenicol, which have structures similar to CAP (Fig. 17) were permitted as substitutes [37].

MRLs for thiamphenicol are 50 μg/kg for bovine and chicken tissues, and for florfenicol, 100 μg/kg for muscle to 3000 μg/kg for bovine liver. Due to the ban of CAP, very sensitive detection methods have been developed. Recently, the MRPLs of CAP for meat, eggs, milk, urine,

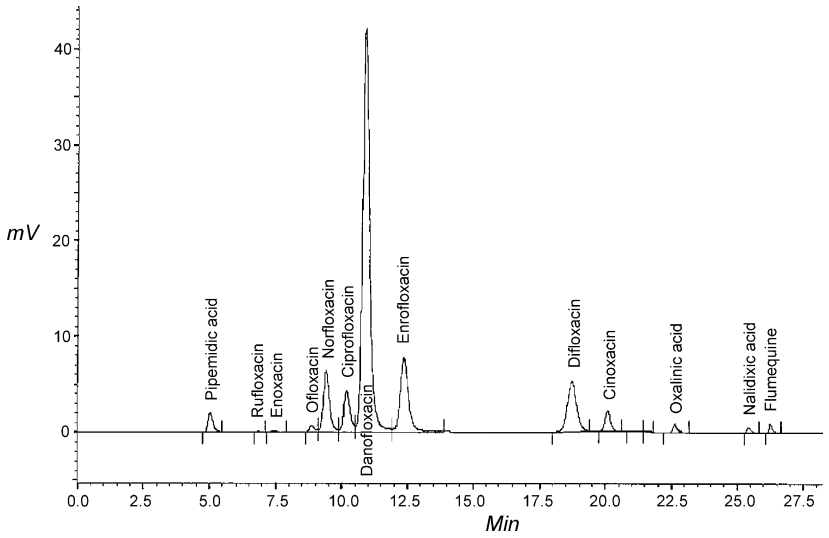


Fig. 16. RPLC–FLD of mixture of 13 quinolones (5 μg/ml); t = 0–15 min, exc./em. = 278/446 nm; t = 15–30 min exc./em. = 324/366 nm; adapted from [77].

Table 13
Selected LC methods for quinolones

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL (μg/kg)	Reference
Quinolones	Fish	LPE/dual SPE[a]	ESI(+)QqQ-MS	1–3	30–600	[79]
Ofloxacin	Chicken tissue	LPE/HLB-SPE	UV	25–60	–[c]	[75]
Quinolones	Fish, muscle	LPE/C_{18}-SPE	FLD	5–10	30–600	[76]
Quinolones	Feed	PLE/HLB-SPE	(DAD) UV	400–1500	–	[77]
Quinolones	Swine kidney	LPE/C_8 + WCX-SPE	ESI(+)QqQ-MS	0.1–19[b]	150–1500	[77]

[a] Polymeric RP and weak-anion-exchange (WAX).
[b] LOQ; S/N = 10.
[c] Some quinolones are not registered for veterinary use; no MRLs.

aquaculture products and honey were all set at 0.3 μg/kg [80].

As regards analysis, an organic solvent, predominantly ethyl acetate, or an aqueous phosphate buffer is used as extraction solvent for CAP from biological matrices. Next, the primary extract is cleaned by a variety of LLE and/or SPE steps. GC in combination with chemical ionisation (CI)-MS provides excellent analyte detectability down to 0.1 μg/kg in muscle tissues; the results for urine are less good due to matrix interferences. GC–MS in the electron impact (EI) mode is slightly less sensitive but has the distinct advantage of yielding spectra which can be searched in electronic libraries. The main drawback of using GC–MS for CAP analysis is the need for derivatization in order to improve its

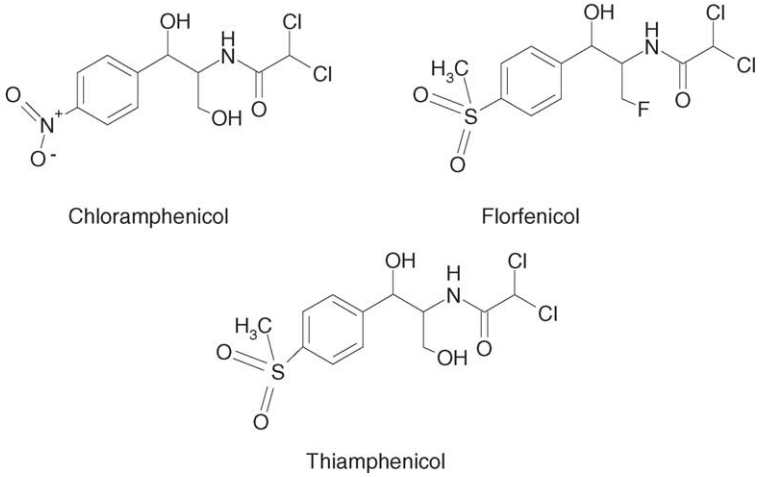


Fig. 17. Structure of amphenicols.

Table 14
Selected methods for CAP

Matrix	Sample preparation	Detection	LOD (μg/kg)	MRPL (μg/kg)	Reference
Meat, seafood	LPE/LLE/SiOH-SPE	LC–ESI(−)QqQ-MS	0.01	0.3	[83]
Shrimps	LPE/C_{18}-SPE/LLE	LC–ESI(−)-MS	0.02	0.3	[84]
Muscle, urine	LPE/C_{18}-SPE	LC–APCI(−)QqQ-MS	0.02	0.3 (muscle)	[82]
Muscle, urine	LPE/C_{18}-SPE + derivative[a]	GC–EI-MS	2	0.3 (muscle)	[82]
Muscle	MSPD/silylation	GC–ECD	2–4	0.3	[81]

[a] Derivatization with BSTFA:10% TMCS.

chromatographic properties. Gantveng et al. [82] described a GC–EI-MS method for CAP in urine. After hydrolysis, washing with ethyl acetate and clean-up by C_{18}-SPE, the analyte was derivatized with a mixture of BSTFA and 10 vol.% TMCS. A 30 m × 0.25 mm i.d., 0.25 μm film thickness HP-5MS column was used. The LOD in 'dirty' urine was 2 μg/l. One recent alternative [81] uses GC–ECD after selective extraction of CAP from muscle by means of MSPD and subsequent conversion into the trimethylsilyl derivative. Although the method is rapid and uses only a few ml of organic solvent, the LODs of 2–4 μg/kg found for cattle, pig and horse muscle tissue do not permit CAP monitoring at the MRPL level of 0.3 μg/kg.

Until recently, the interest in LC–tandem MS as a confirmatory method for CAP was limited because of the availability of GC–MS procedures. As is well-known, LC–MS does not require derivatization and, today, CAP detectability in sophisticated LC–MS procedures approaches that of GC–MS. In 2003, interest in the determination of CAP in shrimps suddenly increased due to a number of non-compliant results in the Netherlands and Germany. As a consequence, several new LC–MS procedures were developed. Gantverg et al. [82] suggested that LC–APCI(−)QqQ-MS offered sensitivity and selectivity superior to that of GC–MS. Even in urine, the LOD was 0.02 μg/kg as against 2 μg/kg for GC–MS. Mottier et al. [83] also reported an LC–tandem MS method for CAP in meat and seafood. After ethyl acetate extraction and clean-up on silica-SPE, the analysis was on a C_{18} column with a water–acetonitrile eluent. The use of ESI(−)QqQ-MS enabled highly precise quantification of CAP down to 0.05 μg/kg in fish and shrimps. The overall absolute recovery of ^{14}C-labelled CAP spiked at 2.5 μg/kg into a blank chicken meat was 60 ± 5% (n = 4). Ramos et al. [84] used LC–ESI(−)-MS for the determination of CAP in shrimps. After phosphate extraction and C_{18}-SPE clean-up, an additional LLE with ethyl acetate was performed with, next, a conventional RPLC separation; the LOQ was 0.2 μg/kg.

van de Riet et al. [85] used a LC–ESI(−)-MS to determine chloramphenicol, thiamphenicol and florfenicol in farmed aquatic species. After PLE with acetone, the extracts were partitioned with dichloromethane, the aqueous layer was removed and the organic layer evaporated to dryness. The residue was dissolved in dilute acid and defatted with hexane, and the aqueous layer prepared for LC analysis on a C_{18} column with a water–acetonitrile gradient. Recoveries were 71–107%; LODs were 0.1 μg/kg for florfenicol and chloramphenicol, and 0.3 μg/kg for thiamphenicol.

Table 14 shows that state-of-the-art LC–MS techniques are required to reach the very low MRPL of 0.3 μg/kg.

2.2.9. Malachite green

Malachite green (MG) is an antibiotic which is used to treat ectoparasites in aquaculture. Dosage rates differ depending on the species treated, and MG should be used only in closed systems such as ponds or aquaria. Among the other antibiotics, the dye MG is a very popular veterinary drug and in eel the only choice to treat and prevent fungal and parasitic infections. MG is also used world-wide with shrimps and many other cultured finfish. The use of this drug is not allowed under the current EU regulations. Because it is a potential carcinogenic, mutagenic and teratogenic compound, it is on the Annex IV list of the EU Council Regulation 2377/90/EEC [3]. MG and its primary metabolite, leuco-malachite green (LMG), are persistent and were found in muscle tissue from rainbouw trout kept at 12–14 °C at 2.4 μg/kg 10 months after a 6-day treatment at 0.2 mg/l [86].

There are only a few published procedures for the determination of MG in animal tissues. Bergwerff and Scherpenisse [87] used LC–UV_{620} for screening and LC–ESI(+)QqQ-MS for confirmation. Residues of MG were extracted from homogenized animal tissues with a mixture of McIlvaine buffer (pH 3.0) and acetonitrile, with clean-up by aromatic sulphonic acid-SPE. Ascorbic acid and *N,N,N′,N′*-tetramethyl-1,4-phenylenediamine-2HCl were added to reduce de-methylation of the dye. Responses were recorded at 620 nm (LC–UV) or by SRM (LC–MS) after on-line post-column oxidation with PbO_2. MG and LMG were determined at levels of 2.5–2000 μg/kg in various fish species with LODs of 1 μg/kg (UV) and 0.2 μg/kg (MS) for both analytes. Recoveries were acceptable for MG (44–68% at 5–100 μg/kg level) and excellent for LMG (80–105% at 25 μg/kg level). As regards stability, whereas degradation of MG was less than 20% and that of LMG less than 10% after 6 months at −20 °C, only some 60% of MG and LMG were recovered after 4 days at + 4 °C. That is, fresh fish should be analysed within a few hours after sampling. Analysis of fish bought in fish-markets and shops showed the presence of LMG in over 50% of the samples of rainbow trout (up to 15 μg/kg), eel (up to 10 μg/kg) and salmon fillets (just above LOD of 0.2–0.3 μg/kg).

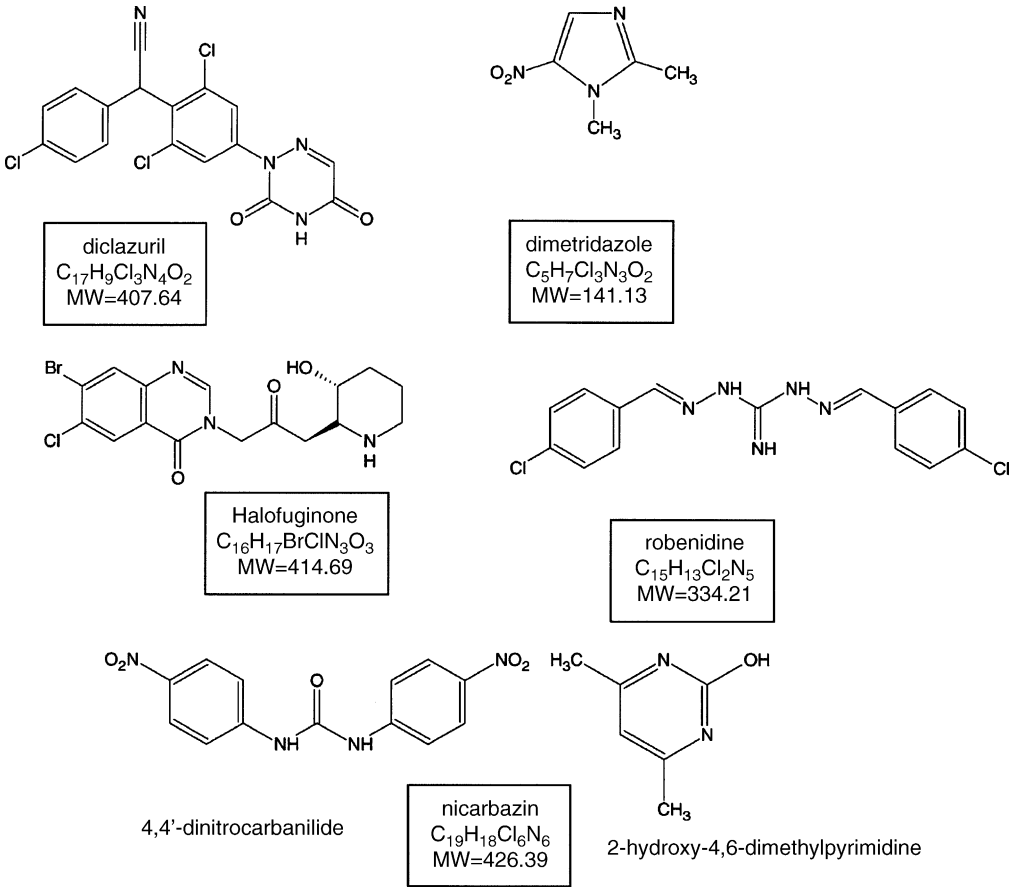


Fig. 18. Structures of five coccidiostats; adapted from [88].

2.3. Coccidiostats

Coccidostats are widely used to prevent and treat coccidiosis. In most EU countries several coccidiostats are licensed for use as feed additive in a prescribed concentration and during a certain time interval for broilers and young chickens. After administration the farmers have to wait for a specific period before the animals can be slaughtered. When this specific 'waiting period' is observed no residues will be found in the animal tissues after slaugthering. The specific waiting periods are described in [90]. Due to carry-over from previously medicated feeds, there is a risk that feed-mill production lines will be contaminated with some of the coccidiostats. Specifically, for nicarbazin – equimolar complex of 4,4′-dinitrocarbanilide and 2-hydroxy-4,6-dimethylpyrimidine, (Fig. 18) – the preparation of drug-free feed can be difficult because nicarbazin powder is strongly electrostatic. This may cause contamination of the production lines and, hence, of supposedly nicarbazin-free feeds. Although drug manufacturers have responded to the carry-over problem by introducing granular preparations of the drug that are less prone to contaminate feed-milling equipment, there are still reports of the occurrence of residues [88]. In the past it has been shown that accidental cross-contamination of feed can lead to residues of the compounds in eggs. Therefore, feed and eggs are the most frequently used matrices for coccidiostat analysis [89].

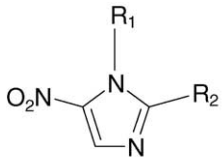
Although in the past various methods were published which used LC–UV, TLC or GC–MS [29] to analyse feed and animal tissues for coccidiostat residues, the advent of LC–MS has opened the possibility of sensitive multi-analyte strategies. Some relevant and recent LC–MS are summarized in Table 15. Two specific subgroups, the nitroimidazoles and the nitrofurans, are not included. They will be discussed separately in Sections 2.3.1 and 2.3.2. Dimetridazole, nicarbazin and robenidine are not authorised for laying hens, halofuginone is allowed until 2009 and diclazuril was allowed until December 2002. However, for neither compound may residues be found in eggs [90].

Mortier et al. [88] developed a simple and sensitive method for the determination of five coccidostats, diclazuril, dimetridazole, halofuginone, nicarbazin and robenidine in eggs. The structures are presented in Fig. 18. After LPE of the coccidiostats with acetonitrile, the organic phase is concentrated and analysed by gradient LC–ESI(+/−)QqQ-MS with water–acetonitrile on a C_{18} column. ESI(−) gave the highest sensitivity for diclazuril; all other compounds were detected by ESI(+). Table 15 presents some analytical approaches. The LODs ranged from 0.75 μg/kg for dimetridazole to 6 μg/kg for diclazuril. These results were similar (dimetridazole) or

Table 15
Selected LC methods for coccidiostats[a]

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	Reference
Coccidiostats	Eggs	LPE	ESI(+/−)QqQ-MS	0.75–6	[88]
Ionophore coccidiostats	Chicken tissue and eggs	LPE/LLE/Si-SPE	APCI(+)-MS	1–7	[91]
Ionophore coccidiostats	Feed	LPE/LLE	APCI(+)-MS	1500–2500	[92]

[a] No MRLs; specific waiting periods have to be observed; no residues may be found in the tissues.



Compound	R_1	R_2
Dimetridazole	CH_3	CH_3
Hydroxydimetridazole	CH_3	CH_2OH
Ipromidazole	CH_3	$CH(CH_3)_2$
Metronidazole	C_2H_4OH	CH_3
Ronidazole	CH_3	CH_2OOCNH_2

Fig. 19. Structure of main nitroimidazoles; adapted from [37].

better (all other compounds) than in previous procedures. Analyte recovery for the extraction from eggs spiked at the 5 μg/kg level ranged from 42% for robenidine to 113% for diclazuril.

Hormazábal and Yndestad [91] used LC–MS to determine the ionophore coccidiostats, amprolium, ethopabate, lasalocid, monensin, narasin and salinomycin in chicken tissue, plasma and eggs. After various LPE steps with methanol, water and acetone–THF, the supernatant was extracted with diethyl ether–hexane (60:40, v/v). The aqueous layer was used for the detection of amprolium and the organic layer for ethopabate and, after clean-up on Si-SPE, for the other coccidiostats. The final analysis was quite complex, with three different LC columns being used: an alkylamide column for ethopabate, a cyano-based phase for amprolium and a C_{18} column for the other four analytes. The LODs were 1–7 μg/kg for chicken tissue and 4–10 μg/kg for plasma. With feed samples, LODs down to 1.5–2.5 mg/kg were obtained [92].

2.3.1. Nitroimidazoles

Nitroimidazoles are a class of veterinary drugs used for the treatment and prevention of certain bacterial and protozoal diseases in poultry (histomoniasis in turkeys, trichomoniasis in pigeons, etc.) and for swine dysentery. Nitroimidazoles (Fig. 19) possess mutagenic, carcinogenic and toxic properties. For these reasons, the use of ronidazole, dimetridazole and metronidazole has been prohibited in the EU. The 5-nitroimidazoles are known to be rapidly metabolised. The main metabolites result from oxidation of the side-chain in the C_2 position of the imidazole ring. For dimetridazole, the major metabolite is formed by hydroxylation of the 2-methyl group to give the hydroxydimetridazole. In the same way, metronidazole gives the hydroxylated metabolite; metronidazole gives another metabolite by oxidation of the *N*-2-hydroxyethyl group to give the acetylmetronidazole. The metabolites formed sometimes have a similar mutagenic potential as the parent compound [93].

Dimetridazole, metronidazole and ronidazole are included in Annex IV of the European Union Council Regulation 2377/90 [3]. This means that any residue of these compounds found in food-producing animals or in products intended for human consumption has to be considered as a violation of the regulations.

A relatively fast, sensitive and very selective LC–MS procedure for the determination of ronidazole, metronidazole and dimetridazole in eggs uses LSE with acetonitrile. Acetonitrile was added to the whole egg sample, after mixing by vortex and ultrasonic extraction the sample was centrifuged. The supernatant was transferred into another tube and was concentrated under nitrogen followed by filtration and direct injection into the LC–ESI(+)QqQ-MS system. A C_{18} column and 0.1% formic acid and acetonitrile gradient were used. The LODs for all compounds were 0.5 μg/kg using SRM. Hurtaud-Pessel et al. [93] used a single MS mode for the determination of nitroimidazoles in poultry meat. The only sample preparation step was LSE with ethyl acetate demonstrating that these compounds are relatively easy to extract [93,94] (Table 16). With this simple LC–ESI(+)-MS method nitroimidazole residues in muscle at levels below 5 μg/kg were confirmed.

2.3.2. Nitrofurans

Furazolidone, furaltadone, nitrofurazone and nitrofurantoin (Fig. 20) are nitrofuran antibacterial agents which have been widely used as food additives for the treatment of gastronintestinal infections (bacterial enteritis caused by *Escherichia coli* and *Salmonella*) in cattle, pigs and poultry. After research had shown furazolidone to be a mutagenic and genotoxic drug, legislation was enforced to remove this and similar compounds from the market. Use of nitrofuran an-

Table 16
Selected methods for nitroimidazoles[a]

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	Reference
Dimetridazole, ronidazole, metronidazole	Chicken muscle	LPE/SCX-SPE	GC–NPD	0.2–0.5	[141]
Dimetridazole ronidazole, metronidazole	Poultry meat	LPE/LLE	LC–ESI(+)-MS	1–4	[93]
Dimetridazole ronidazole metronidazole	Porcine and chicken meat	LPE/LLE	LC–APCI(+)-MS	0.2–2.5	[94]
Dimetridazole ronidazole metronidazole	Eggs	LPE	LC–ESI(+)QqQ-MS	0.5	[93]

[a] Dimetridazole, ronidazole, metronidazole: Annex IV substances [30].

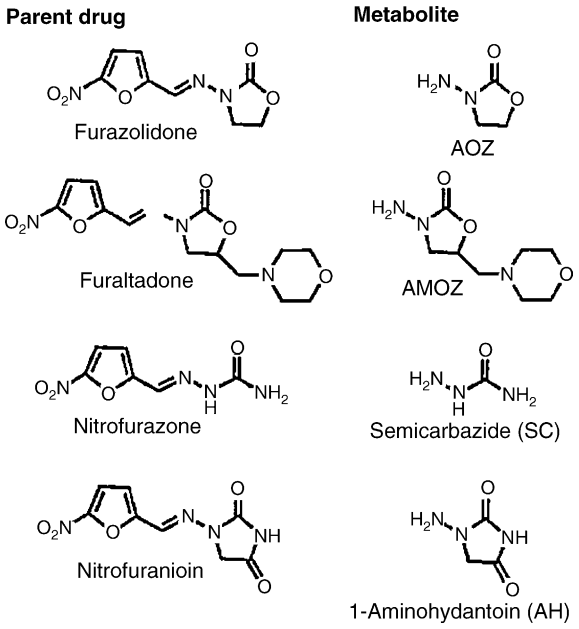


Fig. 20. Structures of the nitrofuran antibiotics and their free metabolites (AOZ, 3-amino-2 oxazolidinone; AMOZ, 3-amino-5-morpholinomethyl-2-oxazolidinone); adapted from [96].

timicrobials in food-producing animals has been prohibited within the EU since 1997.

Recently, residues of nitrofuran drugs were found in poultry and shellfish imported into the EU. Action was taken, and the MRPLs for nirofuran metabolites in poultry meat and aquaculture products were set at 1 μg/kg [80]. Methods for detecting residues of nitrofurans should not aim at measuring the concentrations of the parent drugs because these are rapidly metabolised and do not persist in edible tissues: nitrofurans form protein-bound metabolites which may persist in these tissues for considerable periods after treatment. A well-known procedure involves hydrolysis of the metabolites under acidic conditions, derivatization with 2-nitrobenzaldehyde and extraction with ethyl acetate. After further clean-up, the residues are determined by LC−UV, LC−MS or LC−tandem MS [37,38,95].

Due to the ban of nitrofurans in the EU, the identity of residues in animal tissues have to be confirmed by means of MS [6]. For this goal, ESI(+)QqQ-MS is the preferred technique (Table 17). After time-consuming sample pretreatment (Fig. 21), the separation is performed on a C_{18} column with a 1.0 mM ammonium acetate–acetonitrile gradient. The LOD was 2 μg/kg for AOZ in liver. If the same approach was used for other nitrofurans, a more extensive

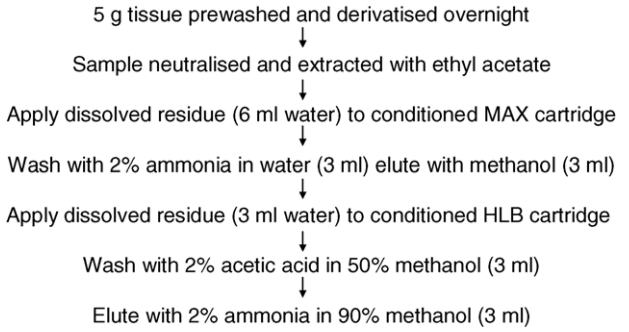


Fig. 21. SPE procedure for AOZ in liver; derivatization with NBA and extraction using MAX and HLB cartridges; adapted from [95].

SPE procedure was required [96]. In addition, methanol was used a organic modifier instead of acetonitrile, to provide a method suitable for multi-analyte analysis.

As the limited number of analytical method presented in Table 17 indicates, there is still room for improvement to obtain LODs around the MRPL of 1 μg/kg for meat. It may be good to add that contamination occurs not only from deliberate, direct misuse of the drugs but also from contaminated feed, environmental contamination (at the slaughterhouse) or transfer between animals. There are also reports on the direct migration of compounds out of the packing material which cause a non-compliant semicarbazide result [97]. This illustrates that the data obtained for residues of nitrofurans have to be evaluated very carefully.

2.4. Hormones

Hormones are administered to animals to improve the rate of growth of the animal. They can be given in the feed but are more usually implanted in the animal's ear so that the active substance can be released slowly over a long period of time into the bloodstream. In the EU, the use of hormones to enhance animal growth is prohibited. To monitor illegal use, urine and manure which are available before the animals are slaughtered and which contain the highest hormone concentrations, are mostly selected. After slaughtering, liver, kidney, hair, fat or meat can be used for monitoring. According to the EU criteria [6], chromatographic techniques combined with MS should be used to confirm the identity of hormone residues detected in the samples; four IPs have to be collected. So far, one MRPL has been set by the EU, viz. 1 μg/kg for medroxyprogesterone acetate in kidney fat [80].

Hormones comprise different sub-groups. In this section, we will discuss

Table 17
Selected LC methods for nitrofurans

Analytes	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRPL (μg/kg)	Reference
AOZ	Pig liver	Derivative LLE/MAX[b]-HLB-SPE	ESI(+)QqQ-MS	2	–	[95]
Metabolites of nitrofurans	Meat	Derivative LLE/EN[a]-SPE	ESI(+)QqQ-MS	0.25–5	1	[96]

[a] Lichrolut® EN (Merck, Darmstadt, Germany).
[b] Mixed-mode anion exchange reversed-phase sorbent.

- anabolic steroids;
- corticosteroids;
- thyreostats.

The discussion will include both the endogenous and exogenous hormones.

2.4.1. Anabolic steroids

Although the monitoring of (anabolic) steroids in cattle and pigs has revealed only a limited number of positives in the EU, the analysis of illegal preparations shows that steroids are still being used. It is known that analogues of known compounds are being synthesised. New steroids are produced legally for therapeutic use and are, then, diverted to the black market. For that reason methods used for control programmes preferably should have a multi-analyte character so that new steroids can easily be included. Fig. 22 shows some typical structures of anabolic steroids, viz. three androgens (male steroids) and an estrogen (female steroid).

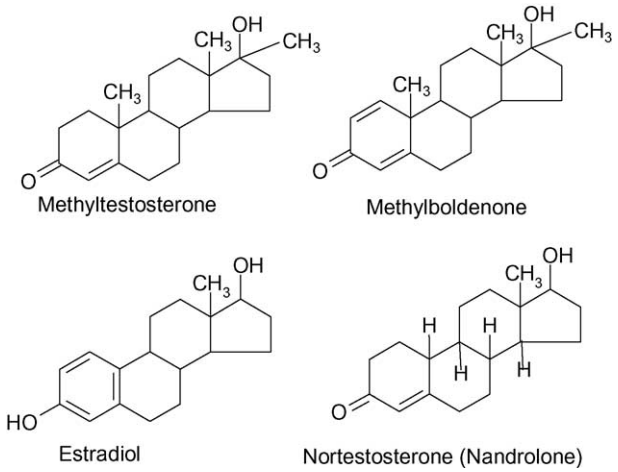


Fig. 22. Structures of three androgens (methyltestosterone, methylboldenone and nortestosterone) and an estrogen (estradiol).

Table 18
Selected methods for anabolic steroids

Anabolic steroids	Matrix	Sample preparation	Detection	LOD (μg/kg)	Action level (μg/kg)	Reference
Estrogens, gestagens, estrogens	Fat, meat	LPE/Si-SPE/NH_2-SPE/derivative	GC–IT-MS^n	0.5–5	NMRPL[a] 2–50	[162]
Anabolic steroids	Meat	Deconjugation/hydrolysed/LPE/Si-SPE/NH_2-SPE/derivative	GC–HRMS	0.005–0.1	–	[142]
Anabolic steroids	Fat	Hydrolysed/LPE/CN-SPE/derivative MSTFA++	GC–IT-MS^n	1–6	NMRPL[b] 2-50	[143]
Anabolic steroids	Urine	C_{18}-SPE/deconjugation/C_{18}-SPE/NH_2-SPE	LC–ESI(+)-QqQ-MS	<1[b]	–	[107]
Anabolic steroids	Urine	Deconjugation/C_{18}-SPE/NH_2-SPE/LC-fraction/(derivative)	LC–ESI(+/−)-QqQ-MS; (GC–HRMS)	0.2–0.4	–	[106]
Anabolic steroids	Fat	LLE/LC-fraction	LC–APCI(+)-QqQ-MS	0.1–1	MRPL MGA[b]: 1	[108]
Anabolic steroids	Muscle, urine	muscle: digest./LPE/LC-fract./derivative urine: C_{18}-SPE/deconjugation/C_{18}-SPE/NH_2-SPE/LC-fraction/derivative	GC–EI-MS	0.1–4.6	–	[100]
Anabolic steroids	Meat	digest./LPE/LC-fraction/derivative	GC–EI-MS	0.5	–	[101]
Gestagens	Fat	SFE/Alu.-SPE	LC–APCI(+)-IT-MS^n	0.5[b]	MRPL MGA[b]: 1	[103]
Gestagens	Fat	ASE/Alu.-SPE	LC–ESI(+)-QqQ-MS	0.3–0.9	MRPL MGA: 1	[104]
Boldenone	Faeces, urine	Hydrolysed/LPE/Chem-elut-SPE[e]/LC-fraction/derivative	GC–IT-MS^n	1	–	[144]
Boldenone	Urine	Deconjugation/C_{18}-SPE	LC–APCI(+)-QqQ-MS	0.2–0.5[c]	–	[145]
Boldenone	Hair	Hydrolysed/LPE/derivative (PFPA[d])	GC–EI-MS–MS	1	–	[146]
Trenbolone	Urine, serum	Deconjugation/urine: C_{18}-SPE/LLE; serum: HLB-SPE	LC–APCI(+)-QqQ-MS	0.3	–	[147]
Trenbolone, Zeranol	Muscle	LPE/HLB-SPE	LC–ESI(+/−)-MS	0.5	–	[148]
Zeranol	Liver	Hydrolysed/LPE/C_{18}-SPE	LC–ESI(–)-QqQ-MS	1[c]	–	[149]
16β-OH-stanolozol	Urine, faeces	Urine: deconjugation/Chem-elute[e]-SPE/acidic LLE faeces: LLE/acidic LLE	LC–APCI(+)-IT-MS^n	0.03–0.07	–	[150]
Stanozolol and metabolites	Urine	Deconjugation/LLE/NH_2-SPE	LC–APCI(+)-QqQ-MS	1[c]	–	[151]
Stanozolol	Meat	Digest./LPE/SFE/Alu.-SPE	LC–APCI(+)-IT-MS^n	0.2	–	[25]

[a] Belgian MRPL.
[b] melengestrol acetate.
[c] LOQ.
[d] Pentafluorpropionic anhydride.
[e] Modified diatomaceous earth.

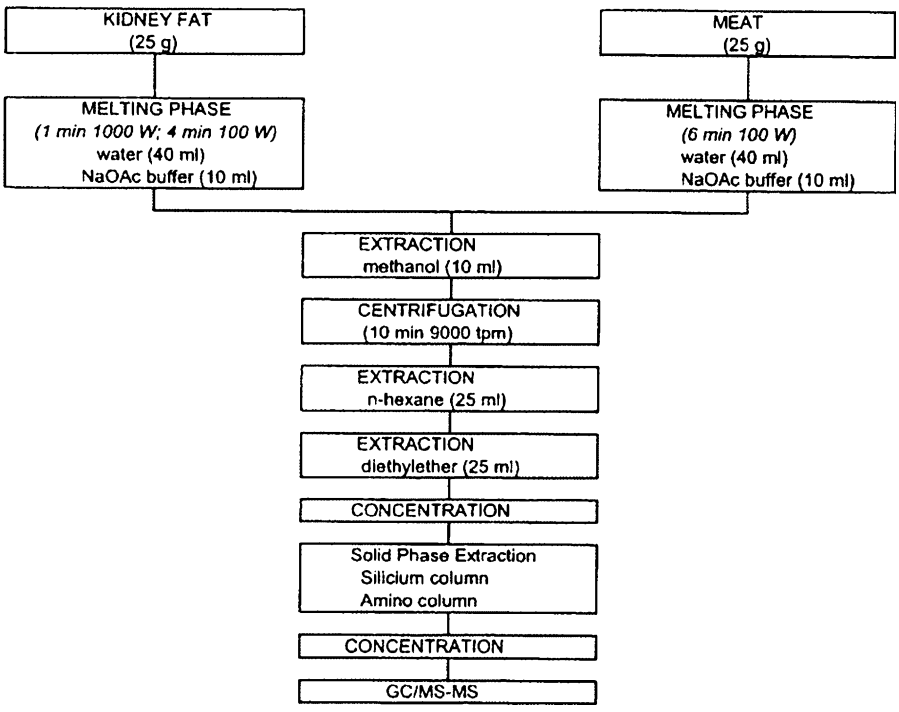


Fig. 23. Overall scheme for the extraction and clean-up procedure for anabolic compounds in kidney fat and meat; adapted from [162].

Most published methods are based on the analysis of the free steroids. This requires their release from glucuronide and/or sulphate conjugates when they have to be determined in urine of liver. *Helix pomatia* juice, whichs contains β-glucuronidase and arylsulphatase, is widely used to this end. Sometimes cleaner extracts can be obtained by using the two specific enzymes instead of the juice [98].

Kuuranne et al. [99] proposed the use of liquid-phase microextraction (LPME) combined with LC–tandem MS for the direct analysis of anabolic androgenic steroid glucuronides in urine. In LPME, a polypropylene hollow fibre membrane serves as a carrier for a thin layer of organic phase. Analytes are extracted from the sample into the organic phase. After extraction the conjugates are subjected to LC–ESI(+)QqQ-MS. The LODs typically are 2–20 μg/l.

Several authors use LLE or LPE and, next, an LC purification step [100,101] for the extraction of anabolic steroids. After the addition of Subtilisine A to digest the proteins – for example, in the case of meat – the steroids were extracted with diethyl ether. After LLE with methanol and defatting with hexane, the final residue was subjected to LC with a methanol–water gradient on a C_{18} column, and the fraction containing the steroids collected. After derivatization with heptafluorobutyric acid anhydride (HFBA), final analysis was by GC–MS. Daeseleire et al. [100] obtained analyte recoveries of 17–81% from urine and 26–65% from muscle tissue. The LODs were 0.1–3 μg/l for urine and 0.3–5 μg/kg for muscle tissue.

Some other procedures reported for steroid extraction and sample treatment are given in Table 18. A detailed scheme for the analysis of anabolic steroids in fat and meat is shown in Fig. 23 [162]. Final analysis by GC–IT-MS^n gave LODs of ca. 2 μg/kg. This is below the values reported above but, actually, not low enough in view of the MRPL of 1 μg/kg for medroxyprogesterone acetate [80].

An alternative approach for the extraction/clean-up of anabolic steroids from urine, gestagens from kidney fat and stanozolol from meat uses PLE with CO_2 [102,103,25]. Analysis was by LC–IT-MS^n or LC–QqQ-MS. The LODs for gestagens and stanozolol were 0.5 μg/kg. Hooijerink et al. [104] used PLE with hexane (defatting) and acetonitrile to extract gestagens from kidney fat. Analysis with LC–ESI(+)QqQ-MS gave LODs of 0.3–0.9 μg/kg. Recoveries were somewhat low, viz. 17–58%.

Several methods for the final separation and detection of anabolic compounds have been developed using GC–MS, since this method provides good sensitivity and is sufficiently selective for use as a confirmatory technique. However, GC–MS requires derivatization of the steroids by means of silylation, acylation or oxime/silylation, depending on the properties of the individual steroids. The lack of a universal derivatization agent, the failure of some steroids, e.g., trenbolone, to give a single reaction product, and problems with chemical rearrangement of others, strongly stimulated the development of LC–MS-based methods. In the past few years the number of LC–(tandem) MS applications has increased rapidly. Fig. 24 shows some typical MS fragment ions obtained for steroids in LC–API-MS.

A confirmatory method for anabolic steroids based on LC–IT-MS^n was developed by Schwillens et al. [105]. The EU confirmation criteria [6] were used for the determination of 16β-hydroxystanozolol, 17α/β-trenbolone, melengestrol ac-

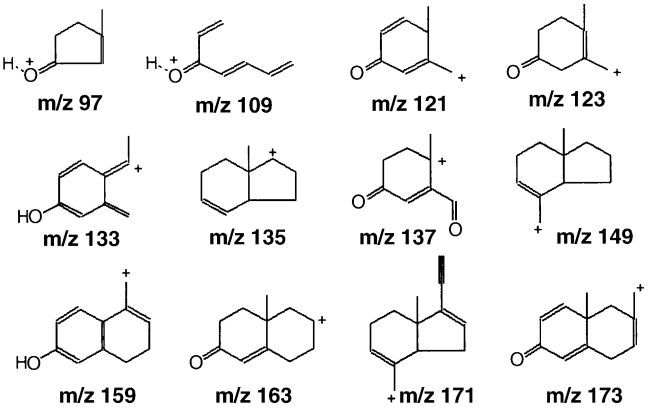


Fig. 24. Typical fragment ions of steroids; adapted from [163].

etate, methylboldenone and dexamethasone in bovine urine; at least two transitions were monitored per steroid. APCI(+) was found to be the best ionisation mode. The limits of identity confirmation were 0.5–2 μg/kg depending on the analyte/matrix combination. Some specific fragment ions were *m/z* 149, a main product ion of methylboldenone, and *m/z* 159, a main product ion of 16β-hydroxystanozolol (see Fig. 24).

Hewitt et al. [106] reported a semi-automated quantitative method for the simultaneous screening and confirming of 22 steroids in urine. Screening is based on enzymatic deconjugation followed by off-line dual-column SPE and subsequent LC–ESI(+/−)QqQ-MS. After fraction collection, the identity of suspected anabolic steroids was confirmed either by repeated ESI(+/−)QqQ-MS, using additional transitions, or by GC–HRMS after appropriate derivatization. The screening method gave LODs of 0.2–0.4 μg/l. Fig. 25 shows the schematic of this dual screening and confirmation approach.

van Poucke and van Peteghem [107] developed a sensitive and selective method for the determination of 16β-hydroxystanozolol, 17α-trenbolone, 4-chloroandrost-4-ene-3,17-dione (CLAD), α/β-boldenone and *d3*-nortestosterone in bovine urine. Since determination of most of these compounds causes problems (cf. above), analysis was done by LC–ESI(+)QqQ-MS. In order to obtain clean extracts, conjugates were isolated by C_{18}-SPE prior to deconjugation with *Helix pomatia* juice. Adding the *Helix pomatia* juice to the pre-cleaned sample reduced the hydrolysis of matrix components; consequently, less matrix components were present in the final extract. With this method, a CCβ of less than 1 μg/l was obtained. This means that reliable confirmation is possible down to at least this level. An illustrative chromatogram of the diagnostic ions of the four analytes is shown in Fig. 26.

Joos and van Ryckeghem [108] reported the determination of 36 anabolic steroids which are frequently found in kidney fat. After preparative LC, six fractions – which each contained several of the steroids – were analysed using LC–APCI(+)QqQ-MS. LODs ranged from 0.1 to 1 μg/kg; the latter is the MRPL for melengestrol acetate.

Most of the analytical methods for steroids discussed so far are multi-analyte methods. Because of a relatively large

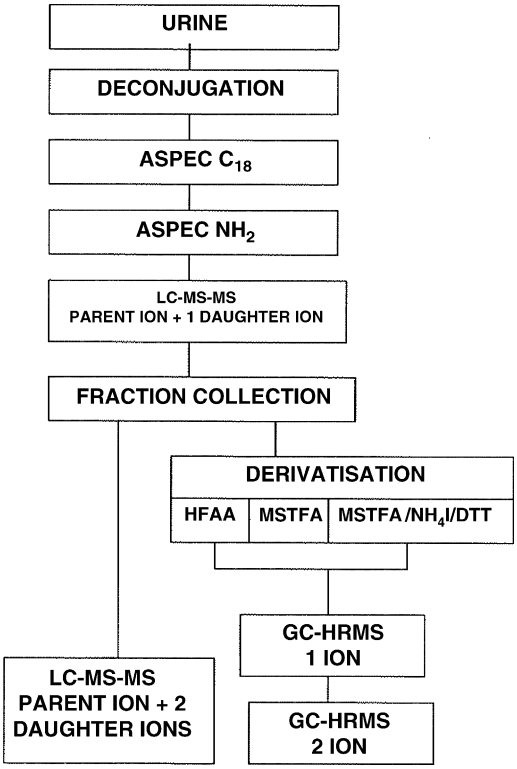


Fig. 25. Schematic of screening – confirmation approach for anabolic steroids in urine; adapted from [106]; ASPEC, automated SPE technique.

number of non-compliant results in the past 5 years, there has been created a special interest in the trace-level determination for the anabolic steroids boldenone and stanozolol and for the non-steroid anabolic agent zeranol. Their analyses will be discussed in some detail below.

β-Boldenone (Fig. 27) was recently detected in an unusually high number of biological samples in various EU member states. One main question was whether the increased number of boldenone findings was due to illegal treatment of animals, or to an endogenous source [109]. Several recent studies discuss the possibility of the endogenous presence of 17α-boldenone in animals destined for human consumption, a compound which is considered to be the main metabolite of 17β-boldenone in human and horse urine. 17α-Boldenone has been detected in urine of cattle declared to be untreated at levels ranging from 0.1 to 2.7 μg/l [110]. It was also found in two out of 29 urine samples from untreated calves at concentrations of ca. 2 μg/l [111]. However, the conclusion of a Dutch/Belgian study [144] published in 1998 was that there was no evidence that either 17α-boldenone or 17β-boldenone is of endogenous origin. Recently, a collaborative study by scientists from several EU countries concluded that boldenone (metabolites) can be found in urine and faeces collected from non-treated animals; however, the excretion is not systematic [112]. Nielen et al. [113] concluded that contamination of urine samples can be excluded by analysing urine samples with and without prior enzymatic deconjugation. The authors demonstrated that non-compliant samples

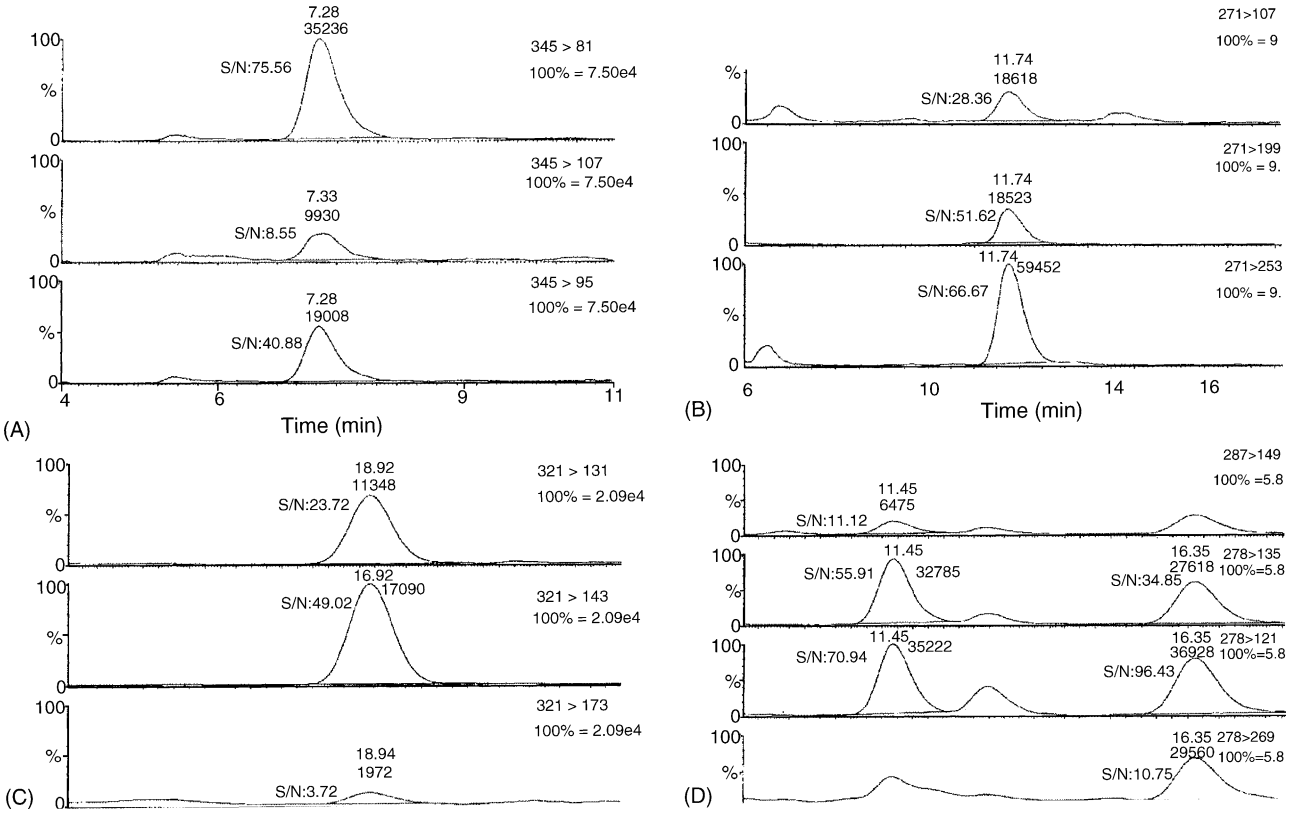


Fig. 26. LC–ESI(+)QqQ-MS chromatogram of blank urine spiked at 1 μg/l and showing the diagnostic ions of (A) 16β-hydroxystanozolol; (B) 17α-trenbolone; (C) 4-chloroandrost-4-ene 3,17-dion and (D) α- (Rt:16.35) and β-boldenone (Rt:11.45). Conditions: C_{18} column with methanol−water−formic acid; adapted from [107].

contain both free and conjugated 17β-boldenone, and false non-compliant – i.e., external contaminated – samples only the free 17β-boldenone.

A confirmatory method for 17β-boldenone, 17α-boldenone and androsta-1,4-diene3,17-dione in bovine urine by LC−APCI(+)QqQ-MS was reported by Draisici et al. [153]. After deconjugation with *Helix Pomatia* juice the extracts were cleaned by C_{18}-SPE with subsequent RPLC–ESI(+)QqQ-MS. Quantification of 17β-boldenone was possible down to 0.2 μg/l, and of 17α-boldenone and androstadienedione, down to 0.5 μg/l. The overall recovery was 92–98%. Very sensitive methods to determine boldenone in hair, urine and faeces by GC–EI-MS^2 were described by Popot et al. [146] and van Puymbroeck [144]. The latter author could detect down to 1 μg/kg of the ethoxime-trimethylsilyl derivatives of boldenone and its metabolites, after hydrolysis, clean-up on C_{18}-SPE and LC fractionation. Popot et al. extracted boldenone from horse mane samples with diethyl ether, with subsequent GC–EI-MS^2 analysis of the pentafluorpropionic anhydride (PFPA) derivatives; the LOD was around 1 μg/kg. Obviously, both GC–MS (after derivatization) and LC–MS techniques can be used successfully for steroid analysis.

Stanozolol is a relatively 'old' anabolic steroid which was first synthesised by Clinton and co-worker in 1959. Stanozolol has become an important compound in veterinary inspection: in 1999 in The Netherlands, out of 103 samples of bovine urine analysed, five were found positive for the main stanozolol metabolite, 16β-hydroxystanozolol (1–5 μg/l). Structures are shown in Fig. 28.

Various analytical methods based on deconjugation (urine, liver) followed by LPE with an organic solvent, SPE (e.g., NH_2-SPE) and LC–MS are used to control the illegal use of stanozolol (Table 19). Control is mainly targeted on

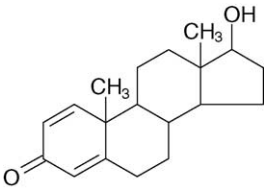


Fig. 27. Structure of 17β-boldenone.

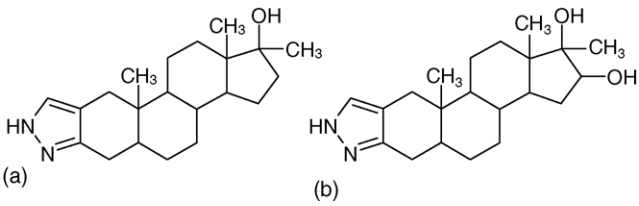


Fig. 28. Structures of (a) stanozolol and (b) 16β-hydroxystanozolol.

Table 19
Selected LC methods for corticosteroids

Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL (μg/kg)	Refence
Urine	LLE/XAD-7 resin or LLE/Extrelut	ESI(−)QqQ-MS	<1	–	[152]
Liver	PLE	APCI(−)QqQ-MS	1	–	[153]
Hair	LPE/C_{18}-SPE	ESI(−)-MS	100	–	[154]
Liver	LPE	APCI(−)-MS	0.3–12	–	[155]
Feed	LPE/NH_2-SPE/HLB-SPE or IAC-SPE	APCI(+)-MS	5	–	[21]
Urine	Deconjugation/HLB-SPE	APCI(+)IT-MS^n	2	–	[156]
Faeces	LPE/Si-SPE/C_{18}-SPE	APCI(+)IT-MS^n	1	–	[157]
Urine	LLE	ESI(−)IT-MS^n	0.5–4	–	[158]
Hair, urine, meat	Hydrolysed/C_{18}-SPE/LLE/SiOH-SPE	ESI(−)QqQ-MS	3–9	0.75–1	[159]
Milk[a]	Defatted/deprotein./C_{18}-SPE	APCI(+)IT-MS^n	0.04	0.3	[160]

[a] Dexamethasone in cow milk.

urine which contains 16β-hydroxystanozolol. For the assessment of consumer exposure and for control at the retail level, meat is the target tissue (expected concentrations, 0.2–1 μg/kg). Analysis can be performed by means of a relatively time-consuming LPE/SPE procedure and LC–MS [114], but the identification limit is rather high (2 μg/kg). Reduction of this limit to 0.5 μg/kg was achieved by means of SFE–LC–APCI(+)IT-MS^n [25]. For quantification *d3*-stanozolol was used as internal standard; for the identity confirmation multiple MS, viz. APCI(+)-MS^2 was used. The LOI was 0.2 μg/kg, a value which meats the above requirements.

Zeranol (Fig. 29) is a synthetic anabolic with estrogenic activity and is structurally related to the mycotoxin zearalenone from which it may be formed in vivo (see Fig. 29). The FAO/WHO Codex Committee recommended MRLs for trenbolone and zeranol of 2 μg/kg in muscle and 10 μg/kg in liver [115]. Within the EU these steroids are prohibited.

Fang et al. [149] developed an LC–ESI(−)QqQ-MS method for the determination of zeranol in chicken and rabbit liver. After deconjugation, zeranol was extracted using various LPE and LLE (ethyl acetate, sodium hydroxide) steps and clean-up by C_{18}-SPE. RPLC on a C_{18} column was performed with an acetonitrile–20 mM ammonium acetate gradient and ESI(−)QqQ-MS. The LOQ was 1 μg/kg. Horie and Nakazawa [148] used LC–ESI(+/−)-MS to determine trenbolone and zeranol in bovine muscle and liver. After LPE with 0.2% metaphosphoric acid–acetonitrile, the extract was cleaned by HLB-SPE, with subsequent RPLC on a C_{18} column using a 0.005% acetic acid–acetonitrile gradient. Hormone recoveries from bovine muscle fortified at 2 μg/kg were 82–85%. Somewhat surprisingly, with this single-MS technique low LODs of 0.5 μg/kg were obtained for both drugs.

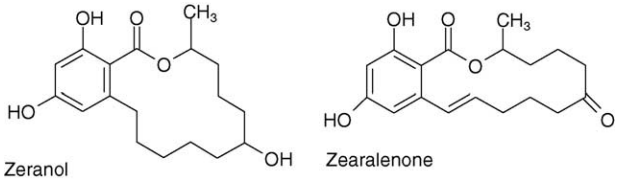


Fig. 29. Stuctures of zeranol and zearalenone.

In the framework of a current EU project [116] – which has the final aim to distinguish synthetic and naturally occurring zeranol – zeranol and its metabolites are being determined in biological tissues. For GC–MS, after hydrolysis, digestion, extraction and clean-up by C_{18}-plus NH_2-SPE, the steroids were derivatized with HFBA and determined using GC–CI(−)-MS. The LODs were as low as 0.05–0.35 μg/kg. Comparison with RPLC–ESI(−)-QqQ-MS using a methanol–water gradient showed that both methods are suitable for the detection of zeranol and its metabolites in urine, kidney, bile, meat and liver [117]. However, GC–MS is more sensitive than LC–MS method; the latter showed LODs down to 1 μg/kg.

2.4.2. Corticosteroids

Corticosteroids are anti-inflammatory drugs (Fig. 30); their use as growth-promoters is banned in the EU. MRLs have only been established for dexamethasone and betamethasone (2 μg/kg for liver, 0.75 μg/kg for muscle and 0.3 μg/l for milk), methylprednisolone (10 μg/kg for muscle,

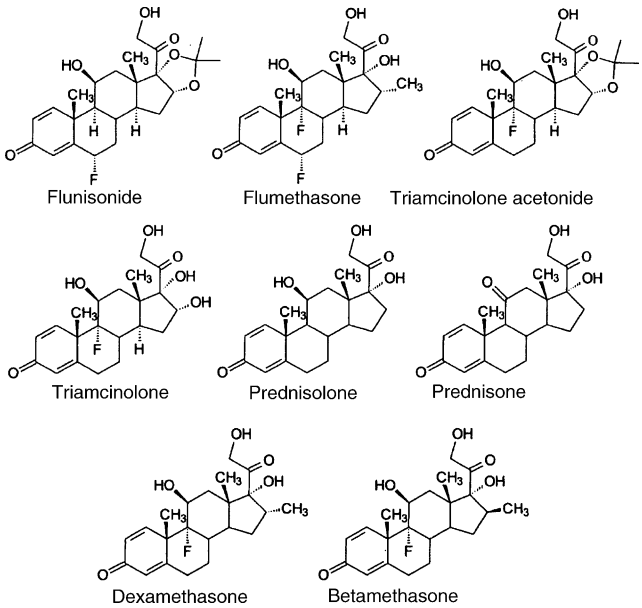


Fig. 30. Structures of some corticosteroids; adapted from [155].

fat, liver and kidney) and prednisolone (4 μg/kg for muscle and fat, 6 μg/l for milk and 10 μg/kg for liver and kidney). For a long time, methods based on GC, specifically GC–MS, were preferred for the determination of corticosteroids, despite the lengthy sample preparation and the need for derivatization or oxidation of the analytes. Courteyn et al. [118] reported a procedure for dexamethasone in urine and faeces of treated cattle by GC–CI(−)-MS after oxidation of the analyte to the 11,17-diketo derivative using pyridinium chlorochromate. For urine the LOD was 0.2 μg/l.

Today, there is an increasing interest in LC–MS-based procedures. Stolker et al. [21] discussed the potential and limitations of various LC-based procedures for the determination of corticosteroids in animal feed and bovine urine. Combination of SPE and LC–APCI(+)-MS enables the detection of down to 5 μg/kg of dexamethasone, flumethasone

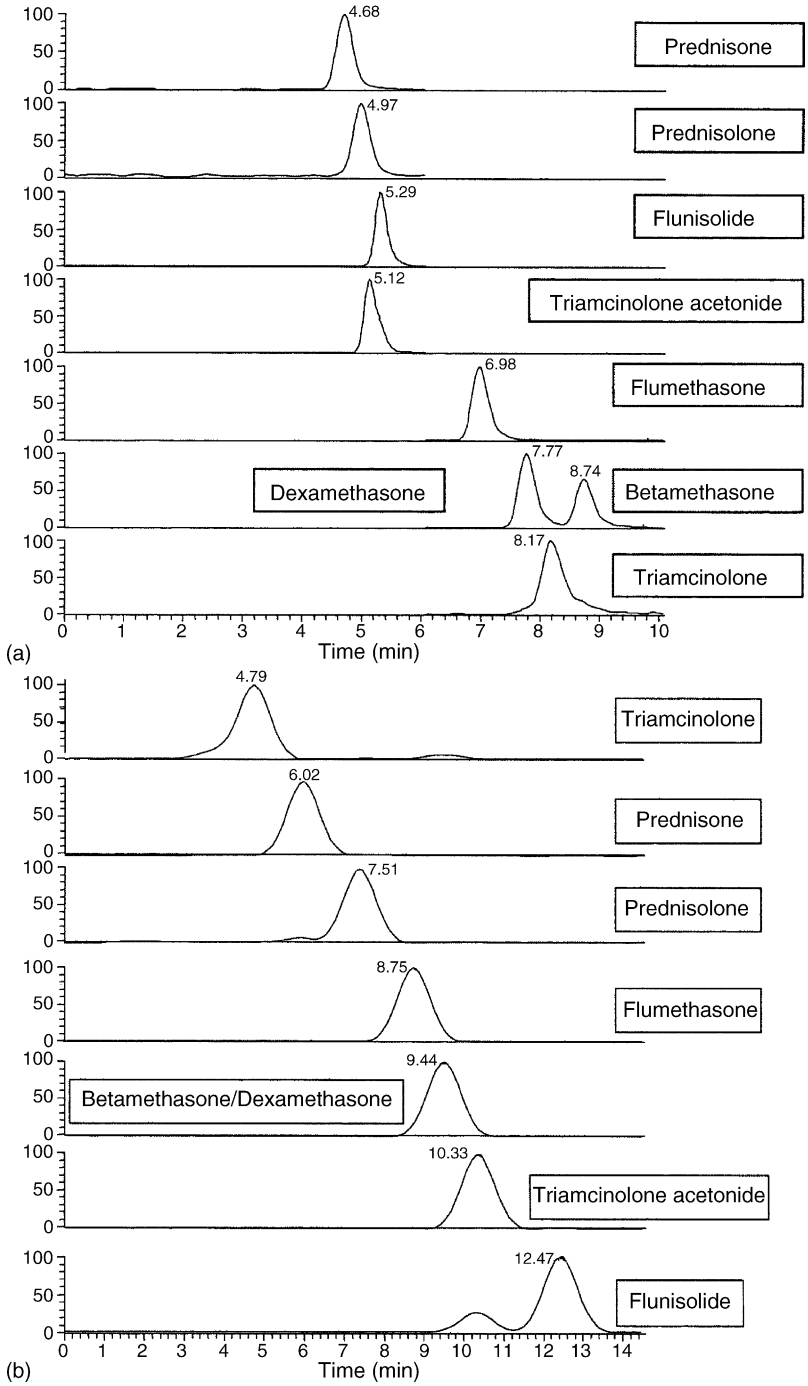


Fig. 31. RPLC–APCI(+)-MS^2 of a 50 μg/kg corticosteroid standard demonstrating the separation of betamethasone and dexamethasone. (a) Porous graphitic carbon column (Hypercarb 5 μm, 125 mm × 4.6 mm): isocratic elution methanol–dichloromethane (85:15, v/v); (b) C_{18} column (Inertsil 5 ODS-3, 250 mm × 4.6 mm): isocratic elution methanol–aqueous ammonium acetate 20 mM, pH 6.8 (65:35, v/v); adapted from [155].

and triamcinolone acetonide in feed samples. The LODs improved to 0.5 μg/l for the three corticosteroids in urine with the more sophisticated combination of IAC-SPE or HLB-SPE and LC–APCI(+)IT-MS^n. Cherlet et al. [160] presented an overview of LC methods capable of quantifying dexamethasone in biological samples.

Table 19 summarises information on some selected LC methods used for corticosteroids. The multi-analyte/multi-matrix procedure for twelve cortiocosteroids in hair, urine and meat merits attention [159]. After hydrolysis the analytes are extracted by a combination of LLE and SPE and finally determined by RPLC–ESI(−)QqQ-MS using a C_{18} column and a methanol–0.5% acetic acid gradient; LODs were 3–9 μg/kg. The recoveries from hair at a concentration level of 500 μg/kg were 32–67%. Generally, the matrix of interest for corticosteroid analysis is animal urine, liver or meat. Some research workers prefer to use hair, especially for the control of the misuse of corticosteroids in sports [154,158]. Hair is more easily obtained and the residues can be detected a long time after the illegal use of the target compounds.

Most analytical methods are based on LLE, LPE or SPE as the sample extraction/clean-up method. However, Draisci et al. [153] presented PLE for the determination of two fluorinated synthetic corticosteroids, dexamethasone and betamethasone, in bovine liver. Even after the introduction of LC–MS, differentiating between these two isomers has remained something of a problem, and several procedures focus on this separation. One option is to use a graphite LC column [155] (Fig. 31) – another one to prepare ethoxime derivatives which can be separated by conventional RPLC on a C_{18} column [157]. The disadvantage of the former solution is the increase of the LOD from about 1 to 10 μg/kg.

Recently, Antignac et al. [119] reviewed 10 years of experience with a variety of GC–MS and LC–MS techniques for the determination of corticosteroid residues in biological matrices. They concluded that LC–QqQ-MS and LC–IT-MS^n are currently the most ideal tools for monitoring corticosteroids. By applying the SRM mode, these systems achieve the best sensitivity and selectivity.

2.4.3. Thyreostats

Thyreostatic drugs (Fig. 32) are a complex group of substances which inhibit the thyroid function and, as a consequence, reduce the circulation of thyroid hormones. The weight gain obtained by treatment with thyreostats mainly consists of an increased filling of the gastro-intestinal tract and an increased water retention by the animal. Contrary to what is true for some anabolic steroids such as the natural

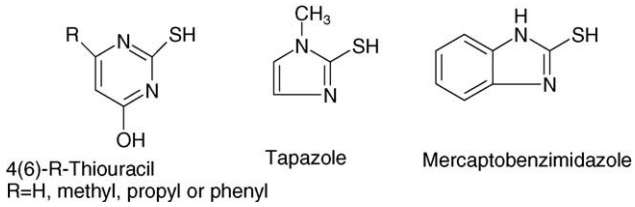


Fig. 32. Structures of thyreostatic drugs.

hormones, there is world-wide agreement on the ban of these drugs: thyreostatic drugs may be harmful to human health, the consumer is misled (being sold water for the price of meat) and the quality of the meat of animals treated with the drugs may be inferior [114]. The isolation of thyreostats from tissue is problematic because they are polar and, hence, hydrophilic. The most common isolation and clean-up procedure is to form a complex of the thyreostats on a mercurated ion-exchange column and, then, elute the thyreostats from the column. Thin-layer chromatography (TLC) with FLD and GC–MS after trimethylsilylation are among the few published analytical procedures. de Wasch et al. [120] used HPTLC for screening, with an aliquot of suspected extracts being analysed by ESI(+)IT-MS^n for confirmation. After extraction the drugs were derivatized with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole. For the confirmatory method, the LODs in thyroid glands were 25 μg/kg for methylthiouracil, thiouracil and phenylthiouracil and 100 μg/kg for tapazole (Table 16).

de Wasch et al. [16] (Table 16) also published an LC–ESI(+)IT-MS^n method for the determination of mercaptoenzimidazole, tapazole, thiouracil, methylthiouracil and phenylthiouracil in thyroid tissue and meat. After extraction with methanol – but without the use of Hg(II)-containing reagents – and clean-up by SiOH-SPE, the compounds were derivatized with 2-chloro-4-nitrobenzo-2-furazane (NBD). The NBD derivatives were separated by RPLC on a C_{18} column using a methanol–0.73% acetic acid gradient. The CCβ was 20 μg/kg for all thyreostats.

Pensabene et al. [121] (Table 20) also published an extraction method for thyreostats which does not use Hg(II) reagents. Meat was homogenised with acetonitrile–water, centrifuged, and the supernatant partitioned with petroleum ether. The acetonitrile–water extract was concentrated and cleaned by SiOH-SPE. After derivatization with MSTFA, final analysis was by GC with nitrogen-phosphorus detection. Analyte recoveries at the 100 μg/kg level were 85–94% for thiouracil, tapazole, methylthiouracil and *n*-propylthiouracil in meat. GC–IT-MS^n was used for the confirmation. The

Table 20
Selected methods for thyreostats

Matrix	Sample prep.	Detection	LOD (μg/kg)	Reference
Thyroid glands	LPE/Hg-SPE/derivative NBD-Cl	HPTLC/ESI(+)IT-MS^n	25–100[a]	[120]
Thyroid glands, meat	LPE/SiOH-SPE/derivative NBD-Cl	LC–ESI(+)IT-MS^n	1–2	[16]
Meat	LPE/SiOH-SPE/derivative MSTFA	GC–IT-MS^n	50	[121]

[a] LOI.

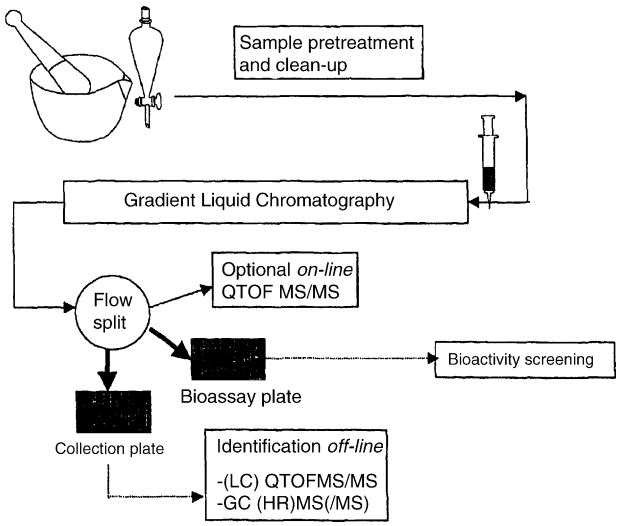


Fig. 33. Generic set-up for the fractionation and identification of unknown bioactive substances using LC–bioassay–Q-ToF-MS; adapted from [124].

estimated minimum level for a reliable measurement was 50 μg/kg in meat tissue.

2.5. β-*Agonists*

In muscle tissue, β-agonists (see Fig. 34) promote lipolysis. This may result in an up to 40% reduction of carcass fat and an increase of carcass protein up to 40%. [122]. While the therapeutic treatment of cattle with respiratory diseases is permitted, the use of β-agonists as growth promoters in cattle is forbidden in the EU [128].

Gowik et al. [17] published data on the accumulation of residues of six β-agonists – clenproperol, clenbuterol, brombuterol, cimaterol, mabuterol and propanolol – in the retinal tissue of food-producing animals. The authors concluded that all these β-agonists accumulate in the retina of calves, pigs and turkeys. Regarding clenbuterol the study showed that the concentration in the retina exceeds that in liver by at least two orders of magnitude. The retina is therefore a matrix of great interest for the residue control of these β-agonists.

In an excellent review on extraction procedures for β-agonists from many sample types, dos Ramos [123] concluded that SPE is, undoubtedly, the first choice for multi-residue β-agonist extraction, preferably with mixed-phase sorbents such as C_8 and WCX, while MSPD, which can be considered as a modified SPE approach, can be recommended for tissue samples, mainly liver.

Recently, an interesting method was developed by Nielen et al. [124] who use QToF-MS for the identification of unknown β-agonists in feed. After primary extraction with methanol–phosphate buffer and clean-up by either C_8/benzosulphonic acid or IAC-SPE, separation was done on a C_{18} column with a linear gradient of methanol–0.1% formic acid. The effluent was split and led to two identical 96-well fraction collectors, with an optional QToF-MS system for accurate mass measurement inserted in between. One 96-well plate was used for a bioassay, i.e., in order to detect the bioactivity and position of the relevant peaks in the chromatogram. The positive peak well in the second 96-well plate was used for identification by LC–QToF-MS. The approach, which is demonstrated in Fig. 33, is of interest in searches for residues of unknown growth-promoting agents in feed. The highly accurate mass measurement (0.1–2 mDa) enables to propose possible elemental compositions of all (fragment) ions monitored, i.e., to identify the 'unknown'. Frequently observed product ions for β-agonists are $[M + H - H_2O]^+$ and $[M + H - C_4H_8]^+$.

Crescenzi et al. [125] used MSPD in combination with MIP for the extraction of clenbuterol from liver. Clenbuterol was eluted from the MSPD cartridge onto the MIP-SPE cartridge with acetonitrile containing 1% acetic acid. Determination was by LC–ESI(+)IT-MS^n. The LOD was <0.1 μg/kg. MIP-SPE is very selective, but the production of a constant-quality material still causes problems. The same is true for the reproducible extraction of the analyte from a MIP cartridge, especially when biological matrices are used [126]. Recoveries from liver at the 10 μg/kg level were >90%.

Traynor et al. [127] used a surface plasmon resonance (SPR) optical biosensor for the screening of β-agonists in liver. It was possible to detect at least thirteen β-agonists in liver at concentrations ranging from <0.1 to 1.5 μg/kg. Up to sixteen liver samples can be extracted and analysed within

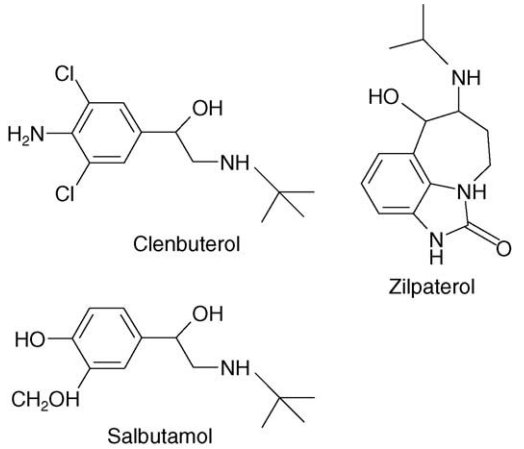


Fig. 34. Structures of some β-agonists.

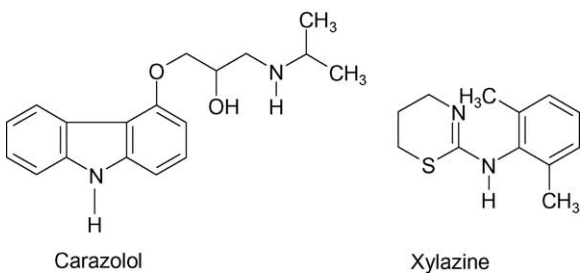


Fig. 35. Structures of tranquillizing agents.

Table 21
Selected methods for β-agonists

β-Agonists	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL (μg/kg)	Reference
Clenbuterol	Liver	MSPD/MIP-SPE	LC–ESI(+)IT-MS	<0.1	–	[125]
Zilpaterol	Feed	LPE/C_{18}-SPE/derivative	GC–EI-MS	8	–	[129]
Zilpaterol	Urine, plasma, tissues, retina	Urine + plasma + retina suspension: hydrol/LLE/mixure[a]-SPE tissues: hydrolysed/hexane defatted/Extrelut-SPE	LC–ESI(+)QqQ-MS	<0.1	–	[128]
β-Agonists	Feed	LPE/Mix[a]-SPE or IA-SPE	LC–bioassay/Q-ToF-MS	5–50	–	[124]
β-Agonists	Retina	LPE/SPE/derivative	GC–EI-MS	4–10	–	[17]
β-Agonists	Liver	Deconjugation/HLB-SPE	SPR (screening)	0.02–0.2	–	[127]

[a] C8 + benzosulphonic acid.

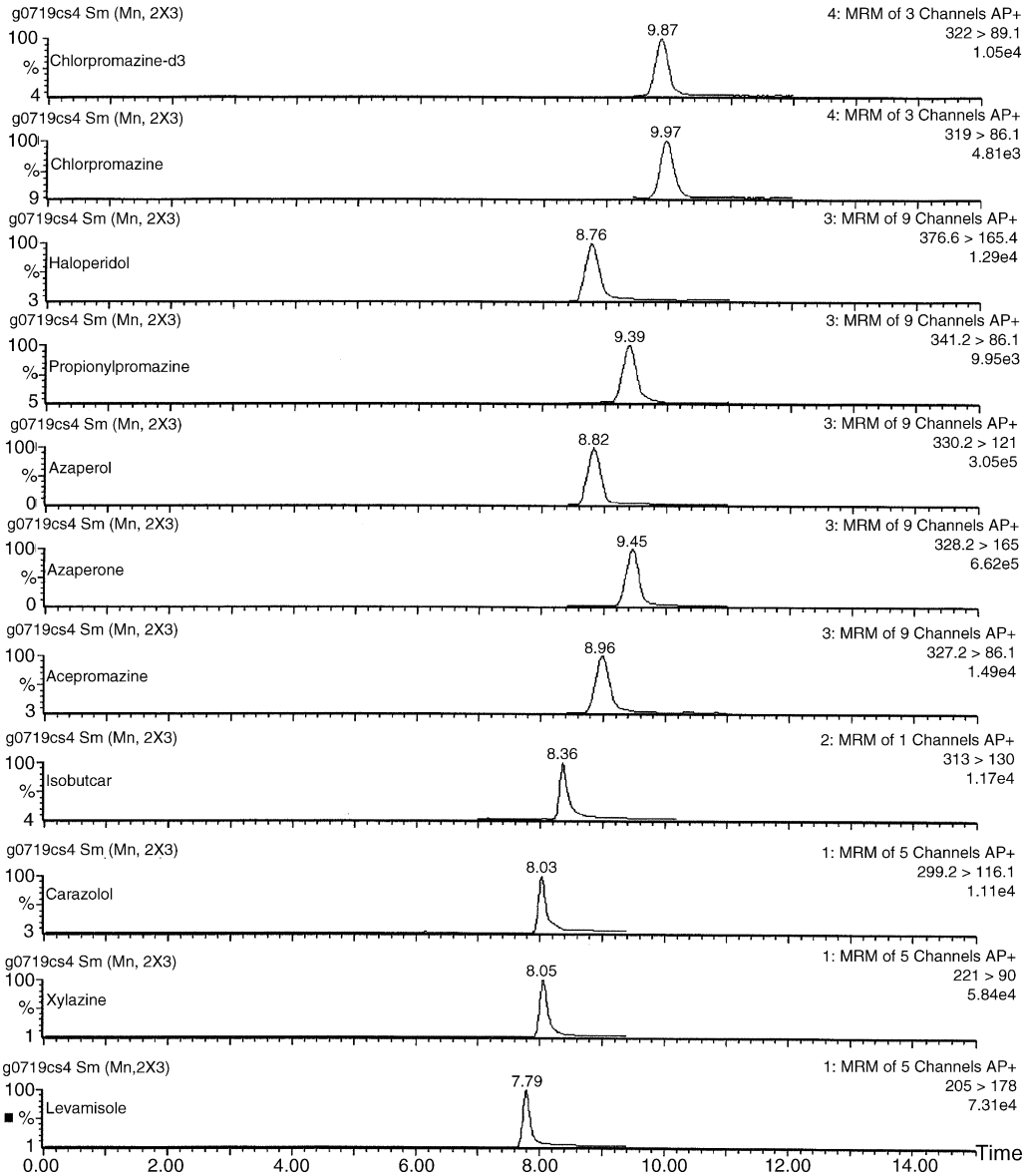


Fig. 36. LC–APCI(+)QqQ-MS of tranquillizers and internal standard in pig muscle (concentrations: MRL for carazolol, azaperone and azaperol; 5 μg/kg for all phenothiazines and xylazine); LC conditions: C_{18} column, acetonitrile–0.1 M ammonium acetate; adapted from [132].

Table 22
Selected methods for tranquillizers

Tranquillizers	Matrix	Sample preparation	Detection	LOD (μg/kg)	MRL (μg/kg)	Reference
Tranquillizers + carazolol	Muscle kidney	LPE/HLB-SPE	LC–APCI(+) QqQ-MS	1–3[a]	5–100	[132]
Tranquillizers	Urine	LLE/C_{18}-SPE	GC–MS	5–50		[130]
Tranquillizers	Liver	LPE/LLE fractionation	LC–UV	20–300	5–100	[131]

[a] Except carazolol (6–30 μg/kg) and azaperol and azaperone (approx. 60 μg/kg in pig muscle).

1.5 working days; this is a modest improvement over most LE/SPE-based procedures.

Recently, zilpaterol (Fig. 34) has become a cause for concern. Zilpaterol·HCl is a powerful β-agonist, which is more effective than ractopamine, but only about one-tenth as effective as clenbuterol. Structurally zilpaterol belongs to neither the group of anilinic (clenbuterol-like) nor phenolic (salbutamol-like) β-agonists. Zilpaterol is licensed as Zilmax™ (Hoechst Roussel Vet) in South-Africa and Mexico for use as growth promoter in cattle. Stachel et al. [128] published an LC–ESI(+)QqQ-MS method for the determination of this compound in urine, plasma, muscle, liver, kidney and the retina of cattle and pigs. LODs down to 0.1 μg/kg were obtained. Also for this β-agonist a strong accumulation was observed in retinal tissue. Bocca et al. [129] determined zilpaterol in feed after derivatization of the drug to its trimethylsilyl derivative and analysis by GC–MS. Acidic extraction followed by C_{18}-SPE (non-end-capped) for clean-up and mass characterization on ions *m/z* 308, 291, 405, 390 gave recoveries of over 75% (RSD, <3%) in feeds spiked in the range of 30–120 μg/kg. The LOD was 8 μg/kg. A summary of some selected methods for the analysis of β-agonists is presented in Table 21.

2.6. Tranquillizers

Tranquillizers (Fig. 35) are administered to animals for sedation prior to anaesthesia before transport to the market. Stress in animals is known to produce a deterioration of meat quality and pigs, in particular, easily become stressed during transport. For some tranquillizers there are MRLs but most of them are prohibited. There are MRLs for the sum of azaperone and its metabolite azaperol, viz. 100 μg/kg for pig muscle, fat, liver and kidney. The MRL for the β-blocker carazolol, which is often used as a tranquillizer, is 5 μg/kg for muscle and fat, 2 μg/l for milk and 15 μg/kg for liver and kidney of cattle, and 25 μg/kg for pig liver and kidney. Most tranquillizers are rapidly metabolized in the animal's body; any residues are concentrated in the liver and/or kidney. These organs should be discarded if tranquillizers have been administered shortly before slaughter [29].

Olmos-Carmona and Hernández-Carrasquilla [130] published a GC–MS method for tranquillizers in urine. Because of their basic nature, an alkaline environment was used for clean-up by C_{18}-SPE. Recoveries for all analytes studied (ketamine, azaperone, azoperol, haloperidol, xylazine and the phenothiazines, chloro-, aceto- and propionylpromazine) were higher than 70% at a concentration level of 50 μg/kg. The LODs were 5–50 μg/l (Table 18). This is about 10-fold better than the LODs for liver obtained by means of LC–UV (20–300 μg/kg) [131]; this is not sensitive enough to check MRL values and the procedure cannot be used at all for monitoring illegal compounds.

A rapid and straightforward method based on acetonitrile extraction, HLB-SPE clean-up and LC–APCI(+)QqQ-MS for promazine derivatives (propionylpromazine, acepromazine and chlorpromazine), xylazine, carazolol and azaperone plus azaperol in kidney and muscle is by Delahaut et al. [132] (Table 22). The LODs were 2–3 μg/kg for all prohibited compounds and up to 60 μg/kg for azaperol in pig muscle. A typical chromatogram is shown in Fig. 36.

3. Conclusions and trends

Some general conclusions regarding state-of-the-art residue analysis of veterinary drugs and growth-promoting agents in biological samples are presented below. In addition relevant trends are indicated.

In a large majority (80–90%) of all quoted studies, liquid partitioning (or, with liquid samples, LLE) is used for analyte isolation, with subsequent clean-up and analyte enrichment by means of SPE. As a rule, non-selective SPE on a conventional C_{18}-bonded silica or a hydrophilic/lipophilic-balanced co-polymer is used, with IASPE-type selectivity being applied only for well defined target-analyte procedures. The use of SFE with carbon dioxide is recommended to isolate analytes of interest such as, e.g., steroids, from fatty matrices: interferences during separation and/or detection are efficiently prevented.

For separation-and-detection, the conventional LC–(DAD) UV and LC–FLD techniques frequently used for veterinary drugs about a decade ago [133], and the GC–MS procedures then preferred for illegal drugs, are increasingly being replaced by LC–MS-based operation. With the advent of, first, robust atmospheric pressure ionisation interfaces – notably ESI which is preferred for more polar analytes as are often encountered in the present field of interest – and, next, the introduction of triple-quadrupole and ion-trap multi-stage tandem-MS instruments, almost all major classes of veterinary drugs and growth-promoting agents can be detected, identified and quantified satisfactorily. The gradual introduction of Q-ToF-MS machines with their distinctly enhanced selectivity and the possibility to calculate element composition is expected to improve performance even more in the near future. This will be

Table 23
Summary of modern analytical approaches for veterinary drugs and growth-promoting agents, and some future challenges

Compound class	Analytical approach[a]		Analytical challenges
	Conventional	Modern[b]	
Anthelmintics	LC–UV, –FLD	LC–(tandem) MS	Multi-analyte extraction from milk and liver tissues
	LODs ∼ MRLs	LODs ≪ MRLs	
Antibiotics			
Aminoglycosides	Derivative/IPLC–UV, –FLD	IPLC–(tandem) MS	Optimisation of extraction and separation, complicated by polar nature of analytes
	LODs ∼ MRLs	LODs < MRLs	
β-Lactams	IPLC–UV, –FLD	(IP)LC–(tandem) MS	Analyte instability (four-member ring) in terms of pH, temperature, alcohols
	LODs ∼ MRLs	LODs ≤ MRLs	
Macrolides	LC–UV	LC–(tandem) MS	Development of LOD<1 μg/kg methods to monitor illegal use of, e.g., tylosine and spiramycine which are prohibited compounds
	LODs > MRL; microbiological LODs ≪ MRL (for sum of antibiotics)	LODs < MRLs	
Peptide hormones	Microbiological	LC–(tandem) MS	Development of LOD < 1 μg/kg methods to monitor illegal use of prohibited compounds (avoparcine, zinkbacitracin, virginiamycin): PLE and HILIC
	LODs ≪ MRL (for sum of antibiotics)	LODs in 1–5 mg/kg (prohibited since 1997)	
Sulphonamides	LC–UV	LC–(tandem) MS	Eliminate ion-suppression
	LODs ∼ MRLs	LODs < MRLs	
Tetracyclines	LC–UV	LC–(tandem) MS	Control epimerisation and chelation: EDTA, special LC columns
	LODs ∼ MRLs	LODs < MRLs	
Quinolones	LC–FLD	LC–(tandem) MS	Multi-analyte extraction – preferably plus sulphonamides and tetracyclines
	LODs–MRLs	LODs ≤ MRLs	
Chloramphenicol[c]	Derivative/GC–MS	LC–QqQ-MS	
	LODs ∼ 1 μg/kg	LODs < 0.3 μg/kg (=MRPL)	Inclusion of other amphenicols (e.g., florfenicol)
Malachite green[c]	Pre- or post-column deriv.–LC–UV	Pre- or post-column derivative–LC–tandem MS	Control of demethylation during analysis
	LODs ∼ 1 μg/kg	LODs ∼ 0.2 μg/kg	
Coccidiostats			
Nitroimidazoles[c]	LC–UV	LC–(tandem) MS	Decrease of LODs; use as feed additive allowed; no residues may be found
	LODs ∼ 10–50 μg/kg	LODs ∼ 0.2–4 μg/kg	
Nitrofurans[c]	Deconjugation/derivative/LC–UV	Deconjugation/derivative/LC–QqQ-MS	Due to ban LOD < 1 μg/kg methods have to be developed (for metabolites)
	LODs ∼ 10–50 μg/kg	LODs ≥ MRPL (=1 μg/kg)	
Hormones[c]			
Anabolic steroids	Deconjugation/LC–fraction/derivative/GC–MS	Deconjugation/LC–tandem-MS; deconjugation/derivative/GC–(HR)-MS	Multi-analyte, multi-matrix extraction procedures for androgens, estrogens and gestagens; recognition of endogenous *vs.* exogenous origin
	LODs ∼ 1–10 μg/kg	LODs ∼ 0.5–5 μg/kg	
Corticosteroids	Derivative/GC–MS	LC–tandem-MS	Multi-analyte, multi-matrix extraction procedures
	LODs ∼ 0.3–10 μg/kg	LODs ∼ 0.5–5 μg/kg	

Table 23 (*Continued*)

Compound class	Analytical approach[a]		Analytical challenges
	Conventional	Modern[b]	
Thyreostats	Derivative/HPTLC	Derivative/LC–tandem-MS	Reduction of LODs from 10 to ≤1 μg/kg
	LODs ∼ 25–100 μg/kg	LODs ∼ 1–10 μg/kg	
β-Agonists[c]	Derivative/GC–MS	LC–tandem-MS	Development of non-target methods to detect analogues
	LODs ∼ 0.5–2 μg/kg	LODs ∼ 0.1–2 μg/kg	Efficient procedures for registered drugs *and* unauthorized compounds
Tranquillizers	LC–UV LODs ∼ MRL	LC–(tandem) MS LOD < MRL	

[a] LC: RPLC unless otherwise indicated.
[b] (Tandem) MS: tandem MS may be used – not necessary; tandem-MS: IT-, QqQ- or Q-ToF-MS should be used.
[c] Prohibited substances.

beneficial specifically in those cases where it is known that analogues of known compounds are synthesized and being used illegally – such as, e.g., β-agonist and steroid hormones (see Table 23 below). In addition, the use of Q-ToF-MS should help to improve the performance of methods using IP-based criteria for the confirmation of analyte identity: after all, a 'non-compliance' conclusion can have dramatic effects.

One main advantage of the GC-to-LC movement is that time-consuming and often not fully satisfactory derivatization is hardly required anymore. A problem that still causes concern is the adverse influence of (co-eluting) matrix constituents and, occasionally, also eluent additives on signal intensity, i.e., quantification, of the analytes of interest. Analysts are increasingly becoming aware that such ion suppression – also called matrix effect – requires careful study and that adequate sample preparation is the preferred approach to prevent such problems.

A summary of earlier, i.e., conventional, and state-of-the-art analytical procedures – and their performances – for the various (sub-)groups of analytes discussed in this review is presented in Table 23. Problems which have, today, not yet been satisfactorily solved and which, consequently, constitute tomorrow's challenges, are included. This brief summary of what can be called 'past, present and future', combined with the earlier considerations and conclusions indicates that current research should specifically address issues such as:

- Simplifying complicated and time-consuming multi-analyte sample pre-treatment, e.g., for anthelmintics, aminoglycosides and hormones.
- Improving insufficiently robust LC separations, as encountered for analyte classes of widely varying polarity.
- Developing analytical approaches for 'multi-compound class' analysis, with a typical example being tetracyclines, sulphonamides and quinolones.
- Adequately dealing with ion-suppression effects on quantification, as discussed above.
- Enhancing analyte detectability to reach LODs of typically 0.1–0.5 μg/kg for prohibited substances (which can be compared with values of 10–100 μg/kg for most registered drugs).
- Improving strategies for the confirmation of analyte identity by a careful study of IP-derived guidelines and protocols, and evaluation of the potential of Q-ToF-MS-based detection.

Finally, as in the past, so also today and in the near future, the development, optimization and implementation of such improved and/or novel techniques will require – next to the availability of state-of-the-art instrumentation – the dedicated assistance of skilled personnel.

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