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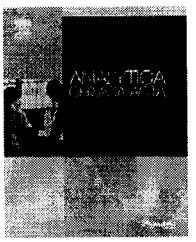

Review

A review of analytical methods for the determination of aminoglycoside and macrolide residues in food matrices

Tara A. McGlinchey[a], *Paul A. Rafter*[a], *Fiona Regan*[b], *Gillian P. McMahon*[b,*]

[a] *Department of Agriculture, Fisheries & Food, Central Meat Control, Backweston Laboratory Complex, Youngs Cross, Celbridge, Co., Kildare, Ireland*

[b] *National Centre for Sensor Research, School of Chemical Sciences, Dublin City University, Glasnevin, Dublin 9, Ireland*

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ABSTRACT

The development of antibiotic resistance in bacteria has been attributed to the overuse of antimicrobials in human medicine. Another route by which humans are exposed to antibiotics is through the animal foods we eat. In modern agricultural practice, veterinary drugs are being used on a large scale, administered for treating infection or prophylactically to prevent infection. Hence, there is pressure on analytical scientists to detect and confirm the presence of antimicrobials in foods of animal origin.

The aminoglycosides and macrolides are two families of antibiotics, each with important applications in veterinary medicine. These antibiotics are widely used in the treatment of bacterial disease, e.g., aminoglycosides for mastitis and macrolides for enteric infections. They have also been used as feed additives for growth promotion. As a result, legislation has been laid down by the European commission in which member states must meet strict criteria for monitoring residues (including antimicrobials). Testing for low levels of aminoglycosides and macrolides in foods is a priority and hence the development of fast, reliable, sensitive methods for their extraction and subsequent analysis is of great interest.

This paper reviews analytical methods for both extracting and determining these classes of antibiotics in various food matrices focusing in particular on the last 10 years. Extraction and clean-up methods such as deproteinisation, and solid-phase extraction are described. Various screening methods are also covered including thin layer chromatography (TLC), enzyme immunoassay, capillary electrophoresis (CE) and microbiological assays. Finally, liquid chromatography (LC) methods are discussed which are combined with mass spectrometry (MS) when sensitivity requirements are stringent.

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* *Corresponding author*. Tel.: +353 1 7005914; fax: +353 1 7005503.
E-mail address: gillian.mcmahon@dcu.ie (G.P. McMahon).

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1. Introduction

1.1. *Background*

Aminoglycosides are a large class of antibiotics that are characterised by two or more amino sugars linked by glycosidic bonds to an aminocyclitol component (Fig. 1). Aminoglycosides are classified according to the pattern of substitution of the cyclotol. The two most important subclasses are 4,5-disubstituted deoxystreptamine, e.g., neomycin, and 4,6-disubstituted deoxystreptamine, e.g., gentamicin, kanamycin [1]. The aminoglycosides interfere with bacterial protein synthesis by binding irreversibly to ribosomes, which causes damage to the cell membranes. Aminoglycosides are widely distributed in the body after injection, little is absorbed from the gastro-intestinal tract and they are excreted unchanged in the urine [2]. Streptomycin is an example of an aminoglycoside antibiotic produced by *Streptomyces griseus* and it is active against many gram-negative bacteria. Streptomycin is used in veterinary medicine.

Macrolides are characterised by a macrocyclic lactone ring containing 14, 15 or 16 atoms with sugars linked via glycosidic bonds (Fig. 2). The macrolides with 16 atoms in the lactone ring represent the most commonly used macrolides in veterinary medicine and examples of these include tylosin and spiramycin. Erythromycin is another example of a macrolide antibiotic—it contains 14 atoms and is produced by *Streptomyces erythrues*. It is active against gram-positive and some gram-negative bacteria [3]. Like the aminoglycosides, the macrolide mode of action is protein synthesis inhibition; however, while the aminoglycosides bind to the 30S ribosomal subunit, the macrolides bind to the 50S ribosomal unit [4].

The aminoglycosides and macrolides are both used in veterinary medicine and animal husbandry particularly for treatment of bacterial infections such as mastitis, or for prophylaxis. In some instances these veterinary drugs are being used on a large scale, administered as feed additives or via drinking water in order to prevent the outbreak of diseases and also in cases of disease, for dehydration or to prevent losses during transportation [5]. The development of antibiotic resistance in bacteria has long been attributed to the overuse of antimicrobials in human medicine but the relationship between agricultural use of antimicrobials and antibacterial resistance in humans is also the subject of much concern [6,7]. Hence, accurate determination of low levels of these antibiotics in food is of huge importance.

1.2. *Legislation*

There is increasing awareness of food safety by the consumer with respect to antimicrobial resistance due to the discovery of new resistant strains of bacteria and others that are becoming increasingly resistant over time. As a result there is increasing pressure on laboratories responsible for food safety to monitor the use of these drugs and ensure the safety of food for human consumption. Legislation regarding the control of antibiotic residues in live animals and animal products is given in Council Directive 96/23/EC [8]. In the context of this directive, details for methods and their performance criteria are described in Commission Decision 2002/657/EC [9]. Residues are divided into two groups A and B for the purposes of monitoring and defining the legislation relating to them [10].

Where a residue refers to:

> "'residues of veterinary medicinal products': means all pharmacologically active substances, whether active principles, excipients or degradation products, and their metabolites which remain in foodstuffs obtained from animals to which the veterinary medicinal product in question has been administered."

Group A refers to substances having an anabolic effect and unauthorised substances while group B refers to veterinary drugs and contaminants. The aminoglycosides and macrolides are both listed under Group B1 (antibacterial substances). The various sub-divisions of these two groups are

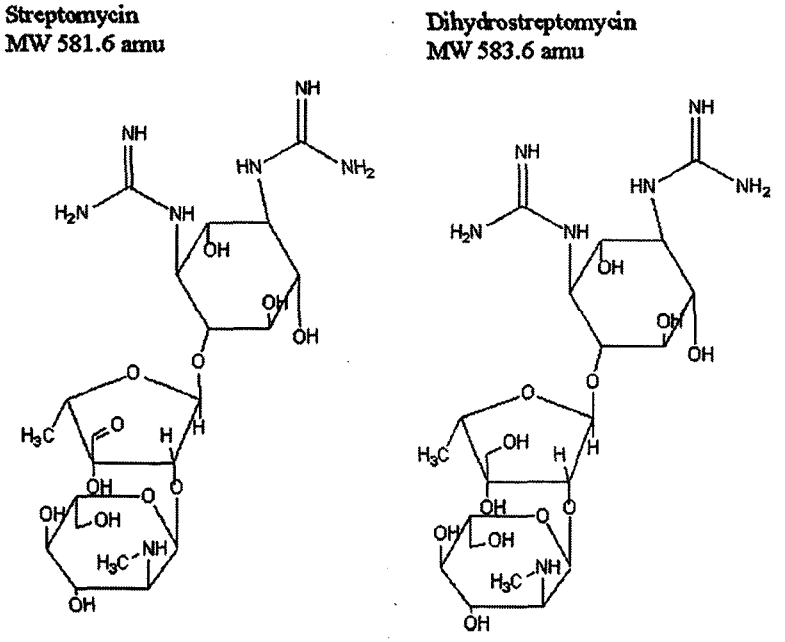

Fig. 1 – Structures of two of the main aminoglycosides.

listed in Council directive 96/23/EC [8]. The EU Council regulation 2377/90 lays down the Community procedure for the establishment of maximum residue limits (MRLs) of veterinary medicinal products in foodstuffs of animal origin [11].

The definition of maximum residue limit according to this regulation is given as:

> "'maximum residue limit': means the maximum concentration of residue resulting from the use of a veterinary medicinal product (expressed in mg/kg or μg/kg on a fresh weight basis) which may be accepted by the Community to be legally permitted or recognized as acceptable in or on a food."

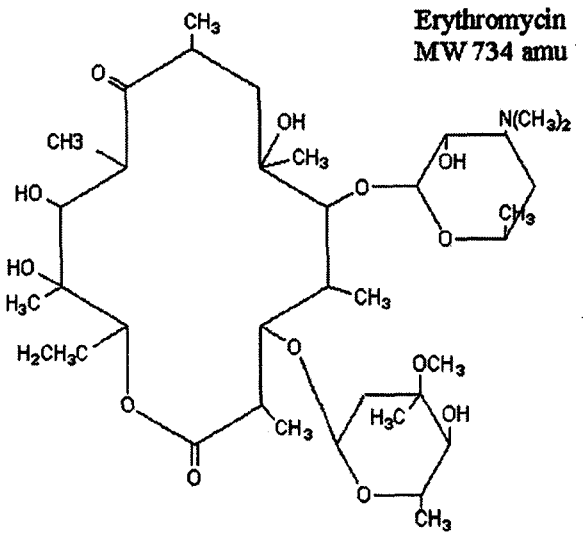

Fig. 2 – Structure of a macrolide.

This document contains four annexes I to IV which list substances with established MRL values (annex I), substances for which it is not considered necessary to establish MRL values (annex II), substances with provisional MRL values (annex III) and substances for which no MRLs could be established (annex IV). Some aminoglycosides and macrolides with established MRLs are shown in Table 1 and some aminoglycosides with provisional MRLs are shown in Table 2. EU Council regulation 2377/90 also dictates the analytical methods that can be used for confirmatory analysis and these are listed in Table 3 [11].

To ensure that aminoglycosides and macrolides are used only in approved situations and to control their use in meat-producing animals, targeted samples are taken at the slaughterhouse and screened for the presence of residues. A positive screening result means that the sample must be subjected to confirmatory analysis. This assay must adhere to Commission Decision 2002/657/EC whereby suitable confirmatory methods are based on a required number of identification points [9]. For the identity of Group B compounds such as the aminoglycosides and macrolides, a minimum of three identification points are required. As a consequence, methods that are based on chromatographic analysis followed by mass spectrometric detection are becoming the normal way of confirming identity and determining concentration.

1.3. *Analysis*

The aminoglycosides are water soluble, highly polar compounds which are not extensively bound to proteins [2]. They contain no chromophores or fluorophores and most aminoglycosides are actually composed of a mixture of closely related compounds [12]. The macrolides are more lipophilic molecules, are soluble in methanol and are unstable in acid [2]. The macrolides are weak bases with pK_a values ranging

Table 1 – Some aminoglycosides and macrolides with established MRL values (from Annex I [11])

Pharmacologically active substances	Marker residue	Animal species	MRLs ($\mu g\,kg^{-1}$)	Target tissues
Neomycin (including framycetin)	Neomycin B	All food producing species	500	Muscle
			500	Fat
			500	Liver
			5000	Kidney
			1500	Milk
			500	Eggs
Kanamycin	Kanamycin A	All food producing species except fish	100	Muscle
			100	Fat
			600	Liver
			2500	Kidney
			150	Milk
Erythromycin	Erythromycin A	All food producing species	200	Muscle
			200	Fat
			200	Liver
			200	Kidney
			40	Milk
			150	Eggs
Tylosin	Tylosin A	All food producing species	100	Muscle
			100	Fat
			100	Liver
			100	Kidney
			50	Milk
			200	Eggs

from 7.4 for tylosin A [13] to 8.9 for erythromycin and 9.2 for roxythromycin [14].

The most commonly used aminoglycoside in veterinary medicine in Europe is gentamycin [7] with neomycin, streptomycin and dihydrostreptomycin used to a lesser extent. The most commonly used macrolides are erythromycin and tylosin [7]. The use of aminoglycosides and macrolides as growth promoters has been banned in the EU, and therefore, it is impossible to rule out the use of other members of this family, e.g., spectinomycin and kanamycin. Both of these compounds have an established MRL value, which means that they must be monitored for use/abuse.

It is clear that while researchers are developing useful methods for aminoglycosides and macrolides, there is a need for more multi-analyte confirmatory methods that would include the compounds with both established and provisional MRL values in the same assay and possibly more than one class of antibiotic in the same assay. This would be of huge benefit to laboratories that carry out residue testing to have such analytical methods at their disposal. However, this is challenging and so most methods are developed for determination of antibiotics of one class or the other. In fact, much of the analytical development to date has focused on either an individual antimicrobial compound, e.g., the quantitation of tylosin in swine tissues by liquid chromatography combined with electrospray ionisation mass spectrometry [15] or a number of antimicrobial compounds from a single class, e.g., determination of 11 aminoglycosides in meat and liver by liquid chromatography with tandem mass spectrometry [16]. Two recent papers have reported detailed protocols for detecting antibiotic residues from a number of different classes, including aminoglycosides and macrolides, in foods [17,18]. The first protocol involved an initial screening microbial assay for 13 antibiotics (1 penicillin, 3 tetracyclines, 3 macrolides, 1 aminoglycoside, 1 amphenicol, 2 ionophore polyethers and 2 polypeptides). This was followed by one of seven extrac-

Table 2 – Some aminoglycosides with provisional MRL values (from Annex III [11])

Pharmacologically active substances	Marker residue	Animal species	MRLs ($\mu g\,kg^{-1}$)	Target tissues
Streptomycin and Dihydrostreptomycin	Streptomycin and Dihydrostreptomycin	Bovine Ovine Porcine Poultry	500	Muscle
			500	Fat
			500	Liver
			1000	Kidney
		Bovine Ovine	200	Milk
Gentamicin	Gentamicin	Bovine Porcine	50	Muscle
			50	Fat
			200	Liver
			750	Kidney
		Bovine	100	Milk

Table 3 – Suitable confirmatory methods for organic residues or contaminants [11]

Measuring technique	Compound group from Annex 1, 96/23/EC	Limitations
LC or GC with mass-spectrometric detection	Groups A and B	Only if following either an on-line or an off-line chromatographic separation Only if full scan techniques are used or using at least 3 (group B) or 4 (group A) identification points for techniques that do not record the full mass spectra
LC or GC with IR spectrometric detection	Groups A and B	Specific requirements for absorption in IR spectrometry have to be met
LC-full-scan DAD	Group B	Specific requirements for absorption in UV spectrometry have to be met
LC-fluorescence	Group B	Only for molecules that exhibit native fluorescence and to molecules that exhibit fluorescence after either transformation or derivatisation
2-D TLC-full-scan UV/VIS	Group B	Two-dimensional HPTLC and co-chromatography are mandatory
GC-Electron capture detection	Group B	Only if two columns of different polarity are used
LC-immunogram	Group B	Only if at least two different chromatographic systems or a second, independent detection method are used
LC–UV/VIS (single wavelength)	Group B	Only if at least two different chromatographic systems or second, independent detection method are used.

Key: LC: liquid chromatography; GC: gas chromatography; IR: infrared spectrometry; DAD: diode array detection; TLC: thin layer chromatography; UV/VIS: ultraviolet/visible spectrophotometry; HPTLC: high-performance thin layer chromatography.

tion procedures depending on the drug class and then HPLC analysis on a C18 reversed-phase column where experimental conditions (including detection mode) changed according to the antibiotic being determined. The second protocol examined 42 veterinary drugs (5 tetracyclines, 7 macrolides, 3 aminoglycosides, 8 β-lactams, 2 amphenicols and 17 sulfonamides) in honey by LC–MS. The extraction involved four sequential liquid–liquid extractions. Recoveries ranged from 28 to 214% for the three aminoglycosides and from 28 to 104% for the seven macrolides which demonstrates the difficulty in developing good extraction procedures for a combination of drug classes. The method worked well as a screening method for 37 of the analytes.

Due to the safety issues surrounding these compounds and the MRLs associated with them, there is huge pressure on the analytical methods to be capable of achieving extremely low limits of detection. Hence, the trend has been to see more LC–MS methods being reported. However, this is not without its challenges. For example, for Group B compounds, according to 2002/657/EC, there must be at least three mass spectral identification points which means a parent mass ion and two daughter products are necessary [9]. Overall, recent analytical methods are reporting better sensitivities. For the aminoglycosides, an LC–MS/MS method quoted a limit of quantitation (LOQ) for streptomycin of $2\,\mu g\,kg^{-1}$ in honey and $10\,\mu g\,kg^{-1}$ in milk; for dihydrostreptomycin these limits were a factor of two lower again [19]. For the macrolides a number of sensitive methods have been reported. Using LC–MS, authors reported detection limits of 5 and $2\,\mu g\,kg^{-1}$ for lincomycin and tylosin, respectively, in honey samples [20].

In summary, it is apparent that low LOD values can be achieved, but they vary widely depending on the analyte being determined, the sample preparation, the technique used and the sample matrix. Another issue is that it can be difficult to reach the required sensitivity levels for all the analytes within one run.

2. Extraction and clean-up methods

The target tissues specified by legislation that have to be monitored are so complex that extraction and clean-up methods play a very important role in the overall analysis of aminoglycosides and macrolides. Food matrices like muscle and liver contain many possible interfering substances that need to be removed selectively. The extraction and clean-up methods for macrolides and aminoglycosides that are dealt with in the literature have been applied to a variety of matrices. The array of matrices include wastewater [21–23] and river water [24,25], sewage sludge [26], liver and kidney animal tissues [15,27], human plasma [28,29] urine [30] and serum [31,32], rat plasma [33], animal feedstuffs [34], bovine and sheep milk samples [35–37], agricultural soils [38,39] and foods [40]. The usual techniques employed for extraction and cleanup of antibiotics from such matrices include protein precipitation, liquid–liquid extraction (LLE) and solid-phase extraction (SPE)

in the majority of cases and in some cases SPE preceded by pressurised liquid extraction or ultrasonic solvent extraction. While food samples are the main focus of this review, it is valuable to take into consideration the other sample matrices and their sample handling and extraction approaches. A good source of information on methodologies for extraction and clean-up of antibiotics in food matrices has been published [41].

Determination of the macrolides from some matrices has been reported to be possible with no sample clean-up. In one report, honey samples were diluted and injected directly into the LC–MS/MS system without additional steps such as solid-phase extraction or liquid–liquid extraction [20]. Normally, the issues of matrix interference and blocking of columns or injectors in systems necessitate some sample preparation prior to analysis.

2.1. *Protein precipitation*

Deproteinisation is commonly used in the extraction of antibiotics from biological matrices where removal of interferences is necessary whilst retaining good recoveries of the analytes of interest. It is a simple off-line procedure. Deproteinisation using acetonitrile for the determination of streptomycin in eggs with recovery levels of 72% has been reported [42]. This method demonstrated the effectiveness of simple precipitation of proteinaceous material using an organic solvent. Acids such as trichloroacetic acid [16] or perchloric acid [40] can also be used for protein precipitation prior to analysis of food samples. Garcia-Mayor and co-workers [35] used a protein precipitation method for the determination of macrolides in milk. Phosphate buffer and acetonitrile were used simultaneously to precipitate the proteins.

2.2. *Liquid–liquid extraction*

Liquid–liquid extraction (LLE) has been exploited as an extraction procedure for aminoglycosides and macrolides from complex matrices. In a method published on determination of the aminoglycosides streptomycin and dihydrostreptomycin, milk samples were prepared using LLE [19]. The method was validated over a linear range from 50 to 800 $\mu g\,kg^{-1}$. The recoveries were found to be slightly low at 60% due to matrix suppression. A number of papers have reported extraction with acetonitrile prior to clean-up of the extracts by LLE with hexane [43–46]. In some cases, this procedure was followed by solid-phase extraction.

Supported liquid membrane (SLM) extraction and/or enrichment is similar to liquid–liquid extraction and dialysis combined [47]. In SLM, an organic liquid is embedded in small pores of a polymer support and is held there by capillary forces. If the organic liquid is immiscible with the aqueous feed and strip streams, SLM can be used to separate the two aqueous phases. It may also contain an extractant, a diluent, which is generally an inert organic solvent to adjust viscosity, and sometimes also a modifier to avoid so-called third phase formation [48]. One of the advantages of SLM is that the relatively small volume of organic components in the membrane and simultaneous extraction and re-extraction in one technological step results in high separation factors, easy scale-up, lower energy requirements and thus lower overall running costs [48].

The use of SLM has been reported for extraction of macrolides from kidney and liver tissue [49]. The macrolides were detected following extraction at concentration levels of 0.01, 0.03 and 0.08 $\mu g\,kg^{-1}$ for tylosin tartrate, erythromycin and spiramycin, respectively. A 1-decanol/undecane (1:1) liquid membrane at pHs of 9 and 3 for donor and acceptor, respectively, was utilised.

In a case where human plasma was studied for the determination of Tamsulosin [29], a potent macrolide, sample extraction with butylmethyl ether followed by direct injection onto LC–MS resulted in successful quantitation of the drug. In a study of rat plasma analysis for macrolides and their derivatives, samples were vortexed with organic solvent, pH adjusted with Na_2CO_3 and extracted with ethyl-acetate-isopropanol prior to LC–MS for analysis [33]. Recoveries were found to be between 58 and 76%.

2.3. *Solid-phase extraction*

In many cases, solid-phase extraction (SPE) is employed to clean-up and to preconcentrate a sample [15,16,19,21–23,25–28,31,38,39,45,46,50–55]. SPE involves liquid–solid partition, where the extracting phase is a solid sorbent. This technique, and versions thereof, have been used extensively to extract and concentrate trace organic materials from samples [50]. A wide choice of sorbents is available which rely on different mechanisms for extraction/retention of analytes. While there are drawbacks associated with SPE such as the importance of packing uniformity to avoid poor efficiency, this technique can be used to extract veterinary residues from even the most challenging matrices such as shrimp [51], soil [38] or sewage sludge [26].

Bruijnsvoort et al. used a C18 SPE cartridge for the extraction of streptomycin and dihydrostreptomycin from honey and obtained recoveries of >80% [19]. Kaufmann and Maden were able to extract 11 aminoglycosides from fish, pork and liver samples using a low-pH extraction with trichloroacetic acid followed by SPE [16]. The cartridge material was a weak cation-exchanger. Babin and Fortier exploited on-line SPE for the extraction of three aminoglycosides from veal tissues [52]. This automated clean-up and analysis system enabled the analysis of 24 veal samples in half a day with recoveries of 51–76%.

Six macrolides were extracted from eggs, honey and milk using initial clean-up with acetonitrile or phosphate buffer pH 8.0 followed by SPE [53]. Across all macrolides and all spiked concentration levels, recoveries were greater than 88%. Berrada et al. used the same Oasis HLB cartridges as employed by Wang et al. [46] for extraction of seven macrolides from liver and kidney samples [27]. Recoveries were >67% for most of the antibiotics studied at the 200 $\mu g\,kg^{-1}$ spiking level. Recoveries of 74–107% were obtained for six macrolides in animal feeds using the Oasis HLB cartridges again and an extra back extraction step [34]. Two macrolides were extracted using silica SPE cartridges but recovery was poor—estimated to be 40–55% [54].

Some valuable knowledge on extraction of aminoglycosides is gained from work by Loffler and Ternes in

their application to wastewater [23]. Hospital wastewaters are reported to contain high levels of hydrophilic aminoglycosides (gentamicin and kanamycin) and thus a weak cation-exchanger was selected for the SPE process. In two other wastewater applications [21,22], macrolide compounds were extracted by SPE using Oasis HLB cartridges. In the study by Yang and Carlson [22], samples were pH adjusted to ~5.0 prior to extraction. Of 10 target compounds measured, the SPE was capable of measuring at environmentally relevant concentrations in influent and effluent.

When liver and kidney tissue samples were analysed, samples were homogenised and centrifuged in buffer to a pellet form [15,27]. Repeated centrifuging with buffer was followed by SPE using OASIS HLB. In these studies a range of tissue samples from a variety of animals (pig, lamb, chicken, rabbit and cow) in Spain were investigated. Studied macrolide compounds were not found in any tested samples except in one case of rabbit. Strong cation-exchange was used to extract tylosin from pig tissue (muscle, skin, kidney and liver) and calibration around the MRL was achieved [15].

Carson reviewed the use of ion-pair SPE in 2000, and discussed its potential application to multiclass multi-residue analysis [55].

2.4. *Matrix solid-phase dispersion*

Matrix solid-phase dispersion (MSPD) is a sample pretreatment procedure that is increasingly used for extracting/purifying analytes from a variety of solid and semi-solid, foodstuffs. MSPD is primarily used because of the possibility of performing extraction and clean-up in one step (illustrated in Fig. 3), leading to a faster overall analysis time and lower consumption of solvents [56].

The aminoglycosides have been extracted using MSPD. Nine aminoglycosides were extracted from milk with heated water (70 °C), followed by LC–MS/MS. After acidification and filtration, 0.2 mL of the aqueous extract was injected into the LC column. Recoveries ranged between 70 and 92%. The LOQ values for this method were between 2 and 13 $\mu g L^{-1}$ [36].

An extraction method for the macrolides based on the MSPD technique with hot water as extractant proved to be robust as matrix effects, even though present, did not significantly affect the accuracy of the method. After dispersing samples of milk and yogurt on sand, target compounds were eluted from the MSPD column by passing through it 5 mL of water acidified with 30 mmol L^{-1} formic acid and heated at 70 °C. After pH adjustment and filtration, a volume of 200 μL of the aqueous extract was directly injected onto the LC column. Hot water was found to be an efficient extracting medium, since absolute recoveries of the analytes from milk and yogurt were 68–86% and 82–96%, respectively [57].

2.5. *Pressurised liquid extraction*

In the case of a number of complex sample matrices, pressurised liquid extraction (PLE) was employed [26,38,39] using an automated Dionex ASE 200 system. PLE is an accelerated liquid extraction (ASE) procedure, whereby increased temperature accelerates the extraction kinetics, and elevated pressure keeps the solvent below its boiling point. ASE is reported to use the same aqueous and organic solvents as traditional extraction methods, and the method uses the solvents more efficiently. The extracts are completely transferred for further solid-phase extraction, typically using Oasis HLB sorbent or equivalent. The advantage of using PLE is the online capability and it was found to compare well against ultrasonic solvent extraction for extraction of macrolides [26].

In summary, there are many different ways to extract aminoglycosides and macrolides from food matrices. However, sometimes compromises are required. For example, for screening methods time and cost issues are more important than the removal of matrix interferences so that a simple extraction system might be more suitable than a more complex extraction with higher recoveries. Of consideration also is the number and type of analytes the method must selectively extract.

3. Screening methods

3.1. *Chemical methods*

3.1.1. *Thin layer chromatography*

Thin layer chromatography (TLC) is one of the most popular and widely used screening methods for antibiotics due to a number of factors including simplicity, wide applicability, good sensitivity, speed and low cost. The use of TLC as

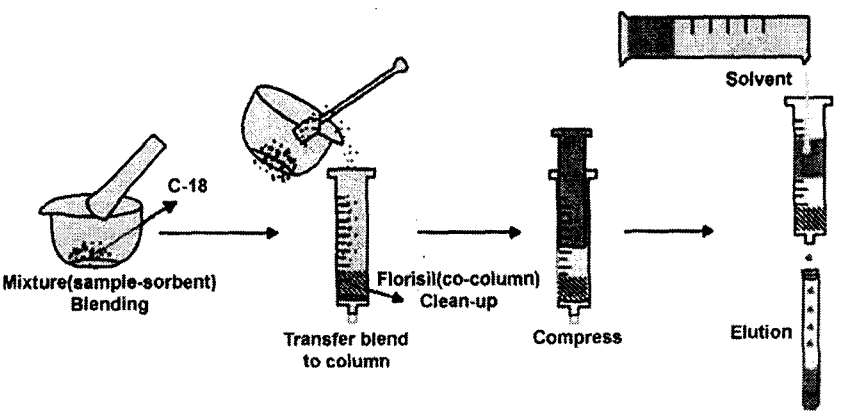

Fig. 3 – Schematic representation of a typical MSPD extraction procedure [56].

a qualitative method for the aminoglycosides has been well documented [1]. Both normal phase and reverse phase TLC can be utilised and detection limits of 0.4–0.6 μg for streptomycin, kanamycin, gentamycin and tobramycin are possible [58]. Macrolides have also been assayed by TLC [3].

3.1.2. *Capillary electrophoresis*

Capillary electrophoresis (CE) has many advantages as a separation technique. It exhibits very high efficiencies, meaning hundreds of components can be separated at the same time, only minute amounts of sample and reagents are required and it is quantitative. However, one of the drawbacks associated with the use of CE for determining trace levels of residues is that it is sometimes not sensitive enough due to the lower sample injection volumes required and short optical pathlength for on-capillary detection [59].

The aminoglycosides are difficult to detect by CE with spectrophotometric detection as these compounds lack chromophores. Hence, initial work focused on indirect UV detection of the aminoglycosides following separation by CE using imidazole as the background electrolyte under low pH and reversed polarity conditions [60]. Subsequent work showed that borate buffers could be used as electrolyte for separation of the aminoglycosides with direct UV detection at 195 nm [61]. Kowalaski et al. described the determination of streptomycin in egg samples. The analysis was performed with a buffer solution of 30 mM sodium dihydrogenphosphate, 5 mM boric acid and 5 mM sodium tetraborate and UV detection [42]. A further paper by the same research group determined a number of antibiotics (including streptomycin and dihydrostreptomycin) in poultry and porcine tissues by CE with UV detection [62]. The method was capable of identifying drug residues in tissues at levels below 20 $\mu g\,kg^{-1}$.

A review of the use of CE for aminoglycoside antibiotics to 2002 found that the choice of detector was found to have a great influence on the separations with laser-induced fluorescence (LIF) showing the best sensitivity [63]. CE-LIF was used by Serrano and Silva to determine four aminoglycosides in milk [64]. Following a derivatisation step, the separation took 20 min and the antibiotics were readily detected at 0.5–1.5 $\mu g\,kg^{-1}$ levels. A more general review of CE methods for antibiotics in a variety of matrices including foods has been reported [65].

3.1.3. *Optical biosensors*

Biosensor systems using are relatively new and provide rapid and reliable results with minimal sample preparation. A cell-based microbial biosensor for macrolides has been reported [66]. This luminescence biosensor is based on the coupling of structural luciferase genes of *Vibrio fisherii* to the regulatory control mechanism of a bacterial erythromycin resistance operon. The system was tested on its ability to isolate and characterise pikromycin from a Streptomyces species.

The detection of streptomycin and dihydrostreptomycin residues in milk, honey and meat samples using this technique has been reported [67]. This study compared a commercially available biosensor kit with a commercially available enzyme immunoassay (EIA) kit and a confirmatory HPLC method. Results demonstrated that the biosensor technology compared favourably with the immunoassay and HPLC methods. Antibody specificity for streptomycin and dihydrostreptomycin was good with <0.1% cross-reaction with other aminoglycosides or with other antimicrobials. The LOD values were 15, 30, 50 and 70 $\mu g\,kg^{-1}$ for honey, milk, kidney and muscle, respectively. Recoveries ranged from 77 to 110% using the biosensor kit. One false positive result for kidney was found but no false negatives were found (which is more important in the case of screening tests).

The Biacore 3000, an optical biosensor with four flow channels was used for the detection of five aminoglycosides in reconstituted skimmed milk, in combination with a mixture of four specific antibodies. The limits of detection were between 15 and 60 $\mu g\,kg^{-1}$, which were well below the MRLs, and the total run time between samples was 7 min [68]. Biosensors have the advantages of simple, fast, sensitive and cost-effective detection [69], thus making them suitable for use in the screening of residues in food.

3.1.4. *Resonance Rayleigh scattering*

Resonance Rayleigh scattering (RRS) is a new analytical method developed in recent years that can be used as an alternative to UV–vis or microbiological assays for screening of aminoglycosides. The technique is based on the aggregation of a conjugated structure in biological macromolecules or the ion-association complexes that are formed by the reaction between electrostatic and hydrophobic interaction in small molecules [70]. It has been reported that when Evans blue dye and some individual aminoglycosides (kanamycin, gentamycin, tobramycin and neomycin) react together, an ion-association complex is formed which enhances the individual spectra and a new RRS spectrum is observed [71]. This phenomenon has also been reported for pontamine sky blue dye with aminoglycosides [72]. While RRS of aminoglycosides has not been used for food samples, it has been used in serum [71,72], and therefore, may be applicable to food matrices.

3.2. *Biological methods*

3.2.1. *Enzyme immunoassay*

There are two main types of enzyme immunoassays—heterogeneous, where the enzyme-labelled antigen or antibody is separated from the antibody–antigen complex prior to measurement of enzyme activity, e.g., enzyme linked immunoassay (ELISA) and homogenous where the enzyme-labelled antigen or antibody is measured in the presence of the antibody–antigen complex, e.g., enzyme multiplied immunoassay technique (EMIT).

A rapid and sensitive screening ELISA for gentamicin in swine tissues has been reported. The time required for the analysis, excluding coating and blocking, was less than 45 min and there was negligible cross-reactivity with other aminoglycosides. LOD values ranged from 2.7 to 6.2 $\mu g\,kg^{-1}$ in the different tissues and recoveries were between 90 and 101% in muscle, 77 and 84% in liver and 65 and 75% in kidney [73]. In a paper by Haasnoot et al., the detection of gentamicin, neomycin, streptomycin and dihydrostreptomycin was reported using three ELISA assays for applications in milk and kidney samples [74]. The detection limits were 0.7–5.1 $\mu g\,L^{-1}$ and the recoveries were 47–78% for milk and 70–96% for kidney. Real samples were taken and analysed – kidney from

Table 4 – Selection of HPLC analytical methods for aminoglycoside and macrolide compounds

Author, year and reference	Compounds	Sample matrix	Sample clean-up	Recovery (%)	Analytical method	Range	Sensitivity
Aminoglycosides							
Vinas et al., 2007 [40]	Streptomycin	Honey, milk, eggs, liver	Acid hydrolysis and protein precipitation with perchloric acid	All matrices >90%	LC-fluorescence	–	7.5–15 $\mu g\,kg^{-1}$
	Dihydrostreptomycin						
Babin et al., 2007 [52]	Three aminoglycosides	Veal kidney, liver and muscle	On-line ion-pair SPE	51–76% (kidney)	LC–MS/MS	50–5000 $\mu g\,kg^{-1}$	0.1–0.4 $\mu g\,kg^{-1}$
Kaufmann et al., 2005 [16]	Eleven aminoglycosides	Fish, liver, pork	Acid extraction and SPE	–	LC–MS/MS	–	15–40 $\mu g\,kg^{-1}$
Bogialli et al., 2005 [36]	Nine Aminoglycosides	Bovine milk	Matrix solid-phase dispersion with hot water	70–92%	LC–MS/MS	0.2–400 $\mu g\,kg^{-1}$	LOQ 2–13 $\mu g\,L^{-1}$
Bruijnsvoort et al., 2004 [19]	Streptomycin Dihydrostreptomycin	Bovine milk Honey	SPE (honey) LLE (milk)	81–102% (honey) 60% (milk)	LC–MS/MS	50–800 $\mu g\,kg^{-1}$ (milk)	LOQ 1–10 $\mu g\,kg^{-1}$
Hornish et al., 1998 [81]	Spectinomycin	Bovine kidney, liver, muscle and fat	Acid precipitation and SPE	>80%	LC–MS/MS and LC-fluorescence	0.1–10 $mg\,kg^{-1}$	LOQ 100 $\mu g\,kg^{-1}$
Kijak et al., 1998 [82]	Gentamicin	Bovine milk	Acid precipitation and SPE	72–88%	LC-fluorescence	7.5–60 $\mu g\,L^{-1}$	15 $\mu g\,L^{-1}$
Carson et al., 1998 [83]	Spectinomycin	Bovine milk	Acid protein precipitation and ion-pair SPE	69–93%	LC–MS/MS	0.1–5 $mg\,L^{-1}$	LOQ 50–100 $\mu g\,L^{-1}$
Macrolides							
Granelli et al., 2007 [84]	Tylosin and spiramycin	Muscle and kidney for various species	Methanol extraction	80–86% from porcine muscle	LC–MS/MS	0–4 MRL	3–15 $\mu g\,kg^{-1}$
Berrada et al., 2007 [27]	Seven macrolides	Bovine liver and kidney	EDTA McIlvaines buffer and SPE	43–93% (intra-day) and 40–88% (inter-day)	LC–DAD and LC–MS	50–1000 $\mu g\,kg^{-1}$	15–50 $\mu g\,kg^{-1}$ (MS). 60–1005 $\mu g\,kg^{-1}$ (DAD)
Wang et al., 2007 [53]	Six macrolides	Eggs, milk, honey	ACN or phosphate buffer extraction and SPE	>88%	LC–MS/MS	1–80 $\mu g\,kg^{-1}$	0.01–0.5 $\mu g\,kg^{-1}$
					LC–QTOF MS		0.2–1 $\mu g\,kg^{-1}$
Zhen et al., 2007 [43]	Five macrolides	Tissue	ACN extraction and hexane clean-up	70–102%	LC–MS/MS	20–200 $\mu g\,kg^{-1}$	0.5–5.3 $\mu g\,kg^{-1}$
Garcia Mayor et al., 2006 [35]	Seven macrolides	Ovine milk	Precipitation with ACN, NaOH and ethyl acetate extraction	55–77%	LC–DAD	24–1000 $\mu g\,kg^{-1}$ for five of the macrolides	24–72 $\mu g\,kg^{-1}$
Takegami et al., 2006 [44]	Nine macrolides	Milk	ACN extraction and hexane clean-up	64–96%	LC–MS	25–1000 $\mu g\,kg^{-1}$	LOD 10 $\mu g\,kg^{-1}$
Wang et al., 2006 [45]	Five macrolides	Milk	ACN extraction, hexane clean-up and SPE	89–117% two analysts and various samples	LC–MS/MS	1–80 $\mu g\,kg^{-1}$	LOD <0.3 $\mu g\,kg^{-1}$
Wang et al., 2005 [46]	Five macrolides	Eggs	ACN extraction, hexane clean-up and SPE	95–99%	LC–MS/MS	1–50 $\mu g\,kg^{-1}$ and 50–350 $\mu g\,kg^{-1}$	LOD <1 $\mu g\,kg^{-1}$
Heller et al., 2004 [54]	Two macrolides	Eggs	Silica SPE	40–55%	LC–MS/MS	Qualitative	LOD ~1 $\mu g\,L^{-1}$
Horie et al., 2003 [85]	Eight macrolides	Meat and fish	Acid/methanol extraction and SPE	70–93%	LC–MS	10–1000 $\mu g\,kg^{-1}$	LOQ 10 $\mu g\,kg^{-1}$
Cherlet et al., 2002 [15]	Tylosin	Porcine tissue	Strong cation-exchange SPE	55–94%	LC–MS/MS	50–200 $\mu g\,kg^{-1}$	LOD 0.2–0.8 $\mu g\,kg^{-1}$ LOQ 5 $\mu g\,kg^{-1}$
Leal et al., 2001 [86]	Seven macrolides	Poultry muscle	Acid/methanol extraction and cation-exchange SPE	60–80%	LC–DAD	Varied, e.g., 50–7700 $\mu g\,kg^{-1}$ for spiramycin	6–33 $\mu g\,L^{-1}$ for five compounds, ~ 400 $\mu g\,L^{-1}$ for other two

Table 4 – (Continued)

Author, year and reference	Compounds	Sample matrix	Sample clean-up	Recovery (%)	Analytical method	Range	Sensitivity
Dubois et al., 2001 [87]	Five macrolides	Tissue, egg, milk	Sodium tungstate precipitation and SPE	Varied with drug and matrix, e.g., tylosin >64%	LC–MS/MS	0.5–2 MRL	LOD 0.01–37 $\mu g\,kg^{-1}$
Draisci et al., 2001 [88]	Three macrolides	Bovine tissue	Chloroform extraction and SPE	>70%	LC–MS/MS	Varied, e.g., 20–200 $\mu g\,kg^{-1}$ for tilmicosin in muscle	LOQ 20–150 $\mu g\,kg^{-1}$
Aminoglycosides and Macrolides Lee et al., 2007 [17]	One aminoglycoside and three macrolides	Meats and fish	Hygromycin B: homogenisation with acid methanol and extracted from amberlite CG-50 column Macrolides: homogenisation with acidic methanol and SPE	Hygromycin B: 90% Macrolides: 89–96%	LC-fluorescence for hygromycin B and LC–UV at different λ for macrolides	50–500 $\mu g\,L^{-1}$	Hygromycin B: LOD 2 $\mu g\,L^{-1}$; LOQ 10 $\mu g\,L^{-1}$ Macrolides: LOD 6–7 $\mu g\,L^{-1}$; LOQ 15–23 $\mu g\,L^{-1}$
Hammell et al., 2008 [18]	Three aminoglycosides and seven macrolides	Honey	LLE	28–214% aminoglycosides. 28–104% macrolides	LC–MS/MS	Matrix matched 6 point calibration	LOD 20 $\mu g\,kg^{-1}$

Key: LLE: liquid–liquid extraction; SPE: solid-phase extraction, MSPD: matrix solid-phase dispersion; LC: liquid chromatography; MS: mass spectrometry; DAD: diode array detection; MRL: maximum residue limit; LOD: limit of detection; LOQ: limit of quantitation.

healthy pigs ($n = 124$) and milk ($n = 776$) – and the aminoglycoside residues found were all below the established MRLs.

An electrochemical ELISA for the detection of two macrolides (erythromycin and tylosin) in bovine muscle has been reported [75]. The detection limit of the assay was 0.4 $\mu g\,L^{-1}$ for erythromycin and 4.0 $\mu g\,L^{-1}$ for tylosin. Results were confirmed by LC–MS/MS.

3.2.2. *Microbiological assay*

Microbiological tests are inexpensive, easy to perform on a large scale and they possess a wide, non-specific spectrum in sensitivity [76]. However, a comparative study carried out on tobramycin standards and samples by ELISA, HPLC and microbiological assay found that the M-agar microbiological assay resulted in an overestimation of the actual quantity in comparison with the other procedures [77]. The aminoglycosides are commonly screened by the four-plate test in the EU. There are many drawbacks with the four-plate test such as the fact that it takes at least 6 h before the results are known [68].

4. Liquid chromatography methods

Analytical methods for the determination of these antimicrobials have been collated in a number of papers for the aminoglycosides [1,78,79] and the macrolides [3,30]. One of these papers focused on the technique of liquid chromatography (LC) [30]. In the case of the aminoglycosides and macrolides, group B compounds, where quantitative analysis at the MRL and lower is required, mass spectral detection can be employed. When a confirmatory assay for antibiotic residues in food is required, the method must provide information on the chemical structure of the analyte. A paper by Rivier describes the criteria for the identification of compounds by LC–MS and LC–MS^n in order to comply with the European Union (EU) criteria for trace level organic analysis [80]. A summary of some of the most relevant LC-based analytical methods published for the aminoglycosides and macrolides can be seen in Table 4.

4.1. *Aminoglycosides*

Many authors have overcome the problem of the absence of a UV chromophore or fluorophore for the aminoglycosides by using derivatising agents for detection by fluorescence [40,81,82,89]. Derivatisation steps, however, render the analytical process more time consuming and may even introduce impurities. Another problem associated with derivatisation is the possibility of the derivatives themselves degrading within a few hours after formation. Limits of detection using LC-fluorescence methods can be low for aminoglycosides in foods, e.g., 7.5–15 $\mu g\,kg^{-1}$ for streptomycin and dihydrostreptomycin in homey, milk, eggs and liver [40] and 15 $\mu g\,L^{-1}$ for gentamicin in milk [82]. Indirect UV or fluorescence methods have also been employed for determining the aminoglycosides, though not in foods [90,91].

Instead of an optical technique, evaporative light scattering detection (ELSD) can be employed. ELSD offers sensitive, universal detection of any sample less volatile than the mobile phase it is in, and both chromophores and non-

Table 5 – Time-scheduled multi-reaction-monitoring conditions for detecting aminoglycoside antibiotics and limits of quantification of the method (Adapted from [36])

Compound	MRM transition (*m/z*)	Cone voltage (V)	Collision energy (eV)	LOQ ($\mu g L^{-1}$)
Spectinomycin	351 > 315, 333	32	20	5
Dihydrostreptomycin	293 > 176, 409	20	12	3
Streptomycin	308 > 176, 263	20	15	13
Aminosidine	309 > 161, 455	15	12	–
Apramycin	271 > 163, 217	15	12	2
Gentamicin C1a	226 > 129, 322	10	6	5
Gentamicin C2, C2a	233 > 126, 143, 322	12	6	7
Gentamicin C1	240 > 139, 157, 322	15	10	6
Neomycin B	308 > 161, 455	15	10	4

chromophores can be detected [92]. A HPLC method combined with ELSD capable of analysing four aminoglycosides including amikacin, neomycin, streptomycin and tobramycin has been described [93]. In this publication, the response for all four antibiotics was much improved when detected by ELSD as opposed to UV at 220 nm. Enhancement techniques for ELSD method development are available [94]. Since the chromatographic requirements are similar, methods developed with ELSD are easily transferable to MS [95]. Rapid and simple methods for the separation and quantitation of gentamicin and neomycin by HPLC coupled with ELSD have been developed for pharmaceutical preparations [96,97].

Manyanga et al. compared a number of LC methods for the analysis of gentamicin and found, on the basis of selectivity, sensitivity and ease of use, that LC–ELSD or LC with pulsed electrochemical detection (PED) were best [98]. It was also shown that method transfer between PED and ELSD is not straightforward. LC methods combined with electrochemical detection have been reported for other aminoglycosides [99].

Mass spectrometry is the detection method of choice for the aminoglycosides due to the lack of chromophores and fluorophores in the molecule. It offers the advantages of sensitivity and confirmation of identity. However, direct mass spectral determination of the aminoglycosides can be difficult due to their thermal lability. The ionisation mode of choice for the production of the ions for residue determination is atmospheric pressure ionisation (API). This technique, coupled to high-performance liquid chromatography and tandem mass spectrometry (LC–MS/MS) has heralded a new era in qualitative and quantitative determination of veterinary drug residues [100]. API techniques include both electrospray ionisation (ESI) and atmospheric pressure chemical ionisation (APCI) and enable the determination of compounds with a range of molecular masses as well as non-volatile substances without a need to derivatise. A sensitive method for the determination of streptomycin and dihydrostreptomycin in milk and honey was developed using liquid chromatography–tandem mass spectrometry (LC–MS/MS) [19]. The method was optimised in regard to sensitivity and chromatographic efficiency, and validated by a procedure consistent with EU directive 2002/657. The mass spectrometer conditions were optimised while infusing a $0.2\,mg\,L^{-1}$ aqueous solution of the analytes, acidified with 0.1% formic acid. Streptomycin and dihydrostreptomycin generated a similar mass spectrum. The fragments *m/z* 263, 246, 221, 176 and 407 were found to be the most abundant transitions of the respective protonated molecular ions (*m/z* 582.1 for streptomycin and *m/z* 584.2 for dihydrostreptomycin) to *m/z* 263 used for screening and quantification, while the ratios with the product ion *m/z* 246 were used for confirmation of the identity. The LOQ of streptomycin was $2\,\mu g\,kg^{-1}$ in honey and $10\,\mu g\,kg^{-1}$ in milk and the values for dihydrostreptomycin were a factor of two lower again [19].

An LC–MS procedure for determining nine widely used aminoglycoside antibiotics in bovine milk were developed

Table 6 – Typical ions detected for macrolide antibiotics using LC–ESI-MS (adapted from [85])

Compound	M_w	Base peak ions	Other main ions
Erythromycin	733.9	734.5 $(M+H)^+$	716.4, 576.3
Oleandomycin	688.9	688.4 $(M+H)^+$	670.4, 544.3
Kitasamycin	771.9	772.5 $(M+H)^+$	702.5, 558.3
Josamycin	828.0	828.5 $(M+H)^+$	860.4, 786.4
Mirosamicin	727.9	728.4 $(M+H)^+$	554.3
Spiramycin	843.1	422.3 $(M+2H)^{2+}$	843.5, 699.5, 540.3
Neospiramycin	698.8	350.2 $(M+2H)^{2+}$	721.5, 699.5, 540.3
Tilmicosin	869.2	435.3 $(M+2H)^{2+}$	869.5, 695.5
Tylosin	916.1	916.5 $(M+2H)^{2+}$	742.3, 582.3

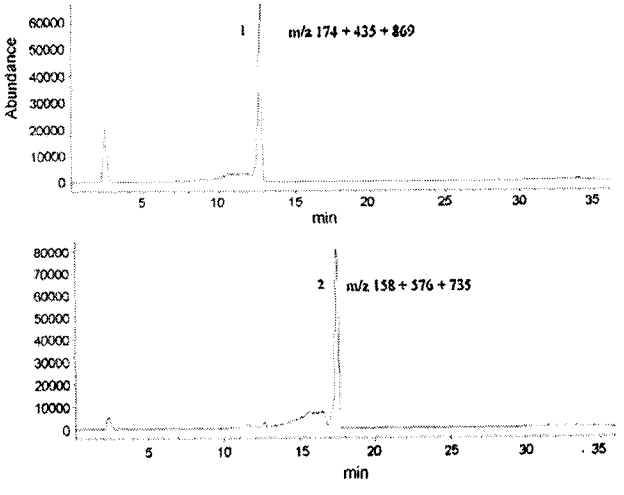

Fig. 4 – SIM chromatograms corresponding to an extract of rabbit liver sample where tilmicosin (1) was found at $250\,\mu g\,kg^{-1}$ and erythromycin (2) at $168\,\mu g\,kg^{-1}$ [27].

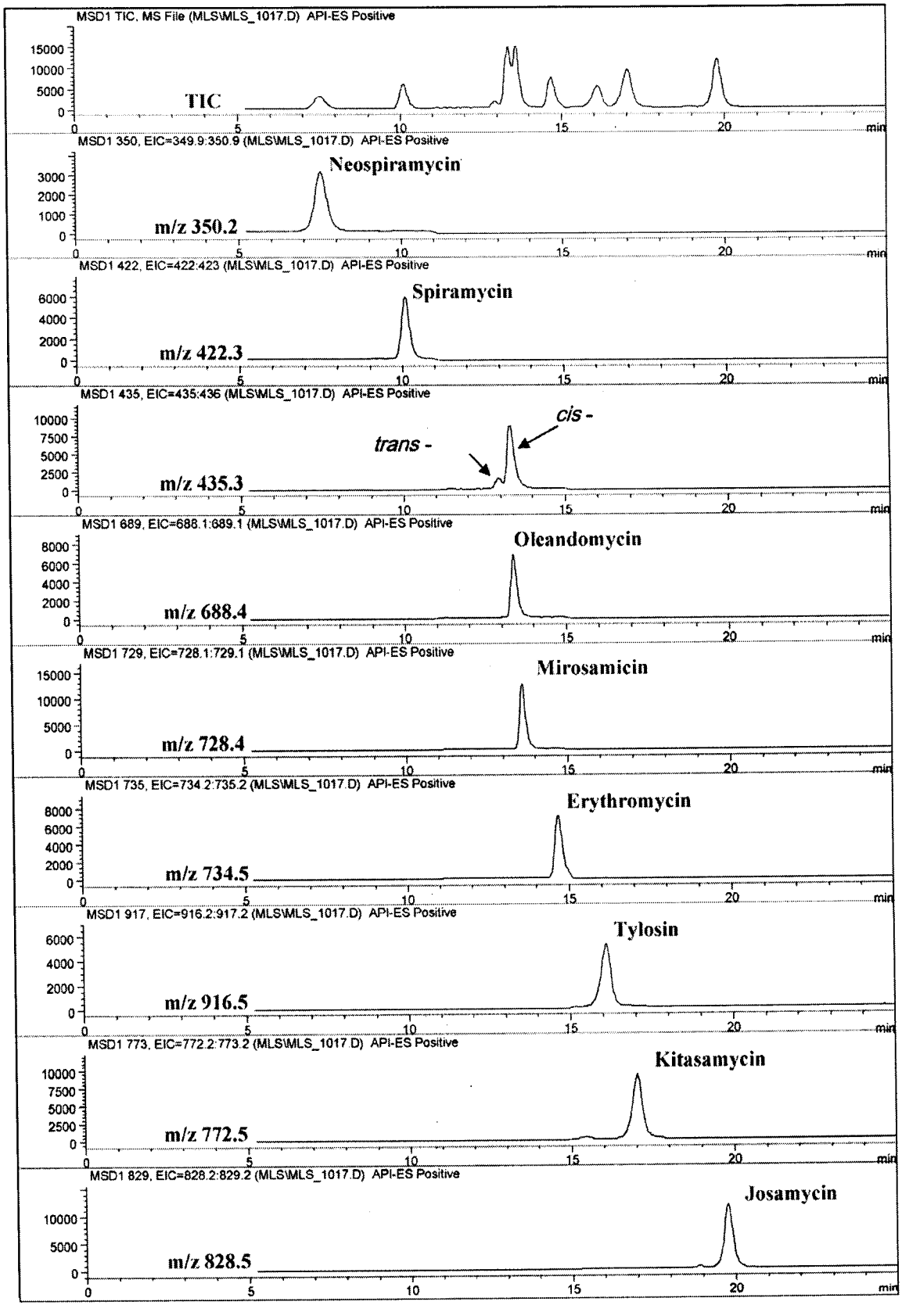

Fig. 5 – Total ion current and extracted chromatograms for a mixture of nine macrolides (eight macrolides under study and an internal standard) at 0.05 μg mL^{-1} [85].

with LOQ values between 2 $\mu g L^{-1}$ (apramycin) and 13 $\mu g L^{-1}$ (streptomycin) [36]. Extraction was carried out using matrix solid-phase dispersion (MSPD) followed by a gradient LC system using increasing methanol concentration. Heptafluorobutyric acid was included in the mobile phase as an ion-pair agent. Detection was carried out in multi-reaction-monitoring (MRM) mode and quantitation performed by selecting at least two fragmentation reactions for each analyte. Table 5 shows the mass spectral conditions and individual limits of quantitation. Babin and Fortier reported an even more sensitive LC–MS/MS method for the determination of aminoglycosides in food where the LOD values were between 0.1 and 0.4 $\mu g kg^{-1}$ in various tissue samples [52].

4.2. *Macrolides*

The macrolides do contain chromophores and hence quantitative, direct UV determination is possible. The determination of seven macrolides in sheep's milk has been described using LC–DAD [35]. Erythromycin and roxythromycin were quantified at 210 nm, josamycin and spiramycin at 231 nm, and tylosin at 287 nm. LODs ranged from 24 to 72 $\mu g kg^{-1}$. Another study using LC–DAD was shown to be capable of determining seven macrolides in animal liver and kidney samples [27]. The analytes were separated using a gradient elution system with an aqueous phosphate/phosphoric acid buffer (pH 3.5) for mobile phase A and acetonitrile for mobile phase B. Validation was carried out according to the European Commission Decision 657/2002. When the results were compared to those obtained by LC–MS detection in selected-ion monitoring (SIM) mode, the LC–DAD method was found to be robust, selective and stable. The LC–DAD method was found to be sensitive enough for detecting macrolides in liver samples with LOD values at or close to the MRLs but the LOD values were ten times lower using LC–MS (15–50 $\mu g kg^{-1}$). The method was applied to rabbit liver samples (see Fig. 4). An LC–UV method for determination of spiramycin and tylosin in feedstuffs yielded detection limits of 176 and 118 $\mu g kg^{-1}$, respectively [101].

LC–MS using electrospray ionisation has been used to successfully determine seven macrolides in chicken muscle [102]. The protonated molecular ion was used for quantitation purposes under selected ion monitoring (SIM) mode. Detection limits ranged from 1 to 20 $\mu g L^{-1}$. Another LC–MS method for determination of eight macrolides in meat and fish samples resulted in LOQ values of 10 $\mu g kg^{-1}$ (Table 6) [85]. A total ion current trace and extracted ion chromatograms for these antibiotics are shown in Fig. 5. A confirmatory method for three macrolides using micro-LC–MS/MS in bovine tissues was published in 2001 [88]. This method used an atmospheric pressure source with an ionspray interface to detect molecular ions $[M+2H]^{2+}$ at m/z 435 for tilmicosin, and $[M+H]^{+}$ ions at m/z 734 for erythromycin and 918 for tylosin. Two diagnostic daughter ions for each compound were studied to fulfill the confirmation requirements. LOQ values in kidney, liver and muscle ranged from 20 to 150 $\mu g kg^{-1}$. An LC–tandem mass spectrometric method for the determination of tylosin in honey yielded an LOD and LOQ of <3 and <5 $\mu g kg^{-1}$, respectively [103]. The assay, developed for the control of unauthorised use of antibiotics in bee-keeping, was validated according to the guidelines laid down by Commission Decision 2002/657/EC.

In recent years, sensitivity has improved using LC–MS techniques with detection limits less than 1 $\mu g kg^{-1}$ being reported for some macrolides in food matrices [15,43,45,46,53]. Building on analytical methods reported previously by the author, Wang compared two LC–MS assays for the determination of six macrolides in eggs, milk and honey [53]. The first technique was UPLC-QT of MS with an electrospray interface, which allowed unambiguous confirmation of positive findings and identification of degradation products but was not as sensitive as LC–MS/MS. The second technique was a triple quadrupole LC–MS/MS, which gave better repeatability and lower LOD concentrations of 0.01–0.5 $\mu g kg^{-1}$.

5. Conclusions

This review describes analytical methods for the determination of aminoglycosides and macrolides in food matrices focusing mainly on methods published in the past decade. Extraction of these two classes of antibiotics from a variety of matrices, focusing on food, has also been explored. This is a very important area for the monitoring of veterinary residues in agriculture, as there are so many different compounds and matrices required to be monitored under the legislation. The requirement to be able to measure even lower concentration levels is a great analytical challenge. Despite the activity in this area of research, there still exist many gaps for certain matrices and species that residue laboratories are required to monitor in their national residue plans. With this in mind, multi-residue 'catch-all' methods or even combination methods for both aminoglycosides and macrolides using definitive techniques such as LC–MS are highly appealing in terms of fulfilling the legislation requirements as well as their high throughput and sensitivity.

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