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Review

Use of liquid chromatography–mass spectrometry in the analysis of residues of antibiotics in meat and milk

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Abstract

The advent of affordable LC–MS systems has led to a massive increase in the number of publications describing quantitative methods for the analysis and confirmation of veterinary drug residues. The lack of volatility and thermal instability of many antibiotics makes LC–MS the method of choice for their analysis. In this review, analytical methods for the determination of residues of each of the major classes of antibiotics are presented.

Keywords: Reviews; Antibiotics

Contents

1. Introduction

1.1. Antibiotics and antibacterials

The term ‘antibiotic’ is normally reserved for a very diverse range of compounds, both natural and semi-synthetic, that possess antibacterial activity. The antibiotics fall into five classes: penicillins, tetracyclines, macrolides, aminoglycosides and amphenicols. Strictly speaking, sulfonamides, nitroimidazoles, nitrofurans and quinolones are not antibiotics, being synthetic. Rather they are antibacterials

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but have, nonetheless, been included within the scope of this review. These compounds have been used for many years in food producing animals for the treatment of disease. Additionally, they may be used prophylactically, to prevent disease, and to promote growth.

1.2. European Union legislation

To safeguard human health, the EU — in common with other countries and trading blocks — has established safe maximum residue limits (MRLs) for residues of veterinary drugs in animal tissues entering the human food chain. MRLs have a built-in safety margin ranging between a factor of 10 and 100, to ensure that human food is entirely free from potentially harmful residues. The establishment of MRLs in the EU is governed by Council Regulation 2377/90/EEC [1]. This regulation establishes lists of compounds that have a fixed MRL (Annexe I), that need no MRL (Annexe II) or which have a provisional MRL (Annexe III). Certain compounds, including some antibiotics, are listed in Annexe IV of this Regulation. This has the effect of prohibiting their use in livestock production. With effect from 1st January 2000, the administration to food producing animals of veterinary medicinal products containing pharmacologically active substances which are not listed in Annexes I to III shall be prohibited.

Monitoring of veterinary drug residues is governed by National Surveillance Schemes, established under Council Directive 96/23/EC [2]. This establishes numbers of samples to be tested for each compound group. Criteria that define the performance expected of both screening and confirmatory methods for residues have been established in Commission Decision 93/256/EEC [3]. Amongst other criteria, this states that 'Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods'.

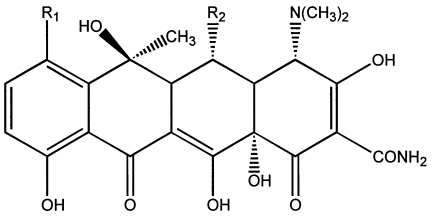
1.3. Analytical strategies for the determination of antibiotic residues

There have been a number of previous reviews that have covered the chemical analysis of antibiotics. From these, it is clear that comparatively few analytical methods capable of measuring residual concentrations of many antibiotics, at or near the MRL, exist. The low solubility of some antibiotics in organic solvents has made it difficult to develop procedures to extract and concentrate their residues from biological matrices. Other antibiotics are either insufficiently volatile or are too thermally unstable (or both) to permit their analysis using GC or GC–MS. As a consequence, many methods for measuring antibiotic residues have been developed using HPLC. However, HPLC is not regarded as being sufficiently specific for use as a confirmatory technique in the EU. The development of coupled liquid chromatography–mass spectrometry [4] has, however, increased the range of antibiotics for which assays, based on molecular spectrometry, can be developed.

2. Tetracyclines

The tetracyclines (Fig. 1), derived from *Streptomyces* spp. are active against a broad range of Gram-positive and -negative organisms. They act by inhibiting protein biosynthesis through their binding to the 30S ribosome. Tetracyclines lack the volatility required for GC–MS. LC–MS has, however, been successfully applied to the analysis of these compounds for several years.

Kijak et al. [5] developed a method for the determination of tetracycline, oxytetracycline and chlortetracycline in milk using particle beam LC–



Compound	R_1	R_2
Oxytetracycline	H	OH
Tetracycline	H	H
Chlortetracycline	Cl	H

Fig. 1. Structures of the tetracyclines.

MS. A filtrate of milk was applied to a C_{18} SPE cartridge that had previously been conditioned by washing with methanol and water. Residual tetracyclines were eluted into oxalic acid. They used an Hewlett-Packard HP59980A particle beam interface and an HP 5988A quadrupole mass spectrometer. A Nova-Pak C_{18} column was used with an isocratic mobile phase that consisted of acetonitrile–0.05 *M* oxalic acid–methanol (50:30:20, v/v/v). This gave optimum sensitivity, at the expense of incomplete chromatographic separation of the tetracyclines. They used methane as a reagent gas for negative ion chemical ionisation. Selected ion monitoring (SIM) was used to monitor four ions for each compound. Validation data for the method were presented at concentrations equivalent to 100 μg/l. Oxalic acid has been used in the mobile phase employed by many HPLC assays for the tetracyclines. It improves the recovery achieved, by preventing chelation of the tetracyclines by metal ions, and decreases degradation of these compounds. However, being non-volatile, it is not entirely compatible with LC–MS. Kijak et al. [5] reported that the particle beam skimmers needed cleaning after 10–15 analyses. The introduction of a switching valve helped to alleviate this problem.

In 1997, we reported the development of a method for tetracycline, oxytetracycline and chlortetracycline in muscle and kidney using atmospheric pressure chemical ionisation LC–MS [6]. Samples were extracted into glycine HCl. Following the addition of ammonium sulphate, the extract was centrifuged and an aliquot of the supernatant was cleaned up using Isolute cyclohexyl (CH endcapped) cartridges that had been preconditioned by washing with methanol and water. Tetracyclines were eluted with methanol, dried and residues reconstituted in the mobile phase, which consisted of 20 m*M* oxalic acid-10 μ*M* EDTA–acetonitrile (80:20, v/v). An Inertsil ODS-2 column was used. Samples were analysed using a VG Platform II quadrupole mass spectrometer. Adjustment of the voltage across the skimmer cone induced sufficient fragmentation to permit the monitoring of at least three ions. For each of the compounds, the ions monitored corresponded to $[M+H]^+$, $[M+H-NH_3]^+$ and $[M+H-H_2O]^+$. The mobile phase permitted baseline resolution of the tetracyclines. The method was validated at 50–200 μg/kg in muscle and at 300–1200 μg/g in kidney (one half to twice the MRL). Recoveries ranged from 59–72%. We found that the addition of oxalic acid was necessary to improve resolution and peak shape, while EDTA was added to improve the recovery achieved. Other workers have found that oxalic acid alone was sufficient but the addition of EDTA prevented the, occasionally complete, retention of the tetracyclines on the column. When incurred samples containing chlortetracycline were analysed using this method, chlortetracycline, 4-epi-chlortetracycline and two additional compounds, tentatively identified as keto-chlortetracycline and 4-epi-keto-chlortetracycline were observed.

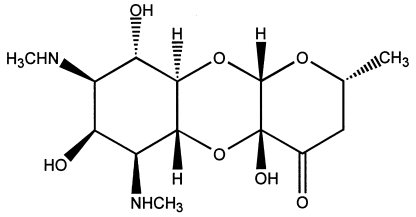
Oka and co-workers have worked on the development of chemical methods for the analysis of tetracycline residues in food animals for many years. In 1994, they reported the use of Frit fast atom bombardment (FAB) LC–MS for the analysis of tetracycline, doxycycline, oxytetracycline and chlortetracycline in honey [7]. They carried out a preliminary investigation into the potential of thermospray LC–MS in the analysis of tetracyclines [8]. In addition to the $[M+H]^+$ ion, which was detected for all three compounds tested, an additional ion corresponding to $[M+H-H_2O]^+$ for tetracycline, to $[M+H-HOCN]^+$ for oxytetracycline and to $[M+H-CONH_2]^+$ for chlortetracycline, was detected. However, the authors commented that the degree of ionisation achieved was variable, depending on the condition of the vaporiser, which declined as deposits accumulated on it. Almost simultaneously with this laboratory, Oka and co-workers also reported the development of a method for tetracycline, oxytetracycline and chlortetracycline in bovine liver, kidney and muscle, using electrospray ionisation LC–MS–MS [9] with collision-induced dissociation. Their method involved blending tissue samples with EDTA-McIlvaine buffer (pH 4.0). The mixture was centrifuged and filtered prior to solid-phase clean up using a Bond Elut C_{18} cartridge, that had been preconditioned with aqueous EDTA. Tetracycline residues were eluted using ethyl acetate, followed by methanol–ethyl acetate. This was dried and residues were dissolved in water. Separation was achieved using a TSK gel Super Octyl column, using a mobile phase that consisted of 25% acetonitrile in 0.05% aqueous trifluoroacetic acid. The mass spectrometer

was a Finnigan TSQ 7000 Triple-Stage Quadrupole. Similar fragmentation to that reported by Blanchflower et al. [6] was observed. The method was validated for all four compounds at a concentration of 100 μg/kg. Recoveries ranged from 55–79%, the poorest recoveries being obtained for doxycycline. These workers did not investigate the use of either oxalic acid or EDTA in their mobile phase, preferring not to introduce nonvolatile components into their mass spectrometer.

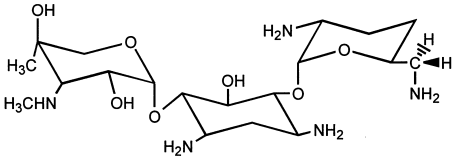
In conclusion, the use of LC–MS in the analysis of tetracycline residues has produced three well-validated methods that are capable of producing multiple ions that facilitate confirmation of these compounds at relevant concentrations. LC–MS also appears to offer an opportunity to quantify and study the structural isomers of chlortetracycline that may be important in assessing the total residue content of human foods.

3. Aminoglycosides

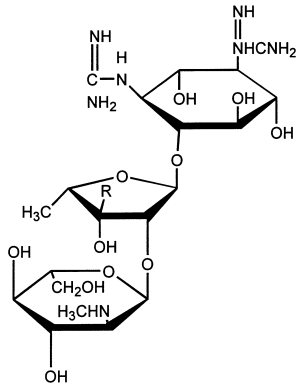
Streptomyces spp. and *Micromonospora* spp. produce aminoglycosides. Broad-spectrum antibiotics, they consist of an aminocyclitol ring (2-deoxystreptamine in most cases) connected to two or more amino sugars in a glycosidic linkage (Fig. 2). The chemical analysis of aminoglycosides has always been an exceedingly weak area for regulatory analysts. These compounds exhibit all of the physicochemical properties that impair the development of suitable confirmatory methods. They are basic and very hydrophilic, making extraction from complex biological matrices difficult; and they are thermally labile, making analysis by GC or GC–MS virtually impossible. Although LC–MS should therefore be an ideal tool to analyse these compounds, to date only two methods have been reported, both using ionspray LC–MS [10,11].



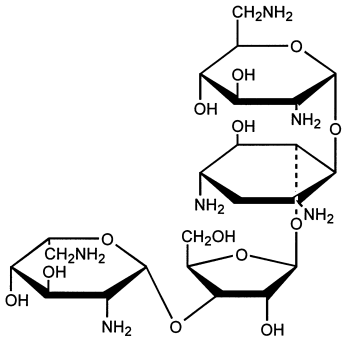
spectinomycin mol. wt. 332



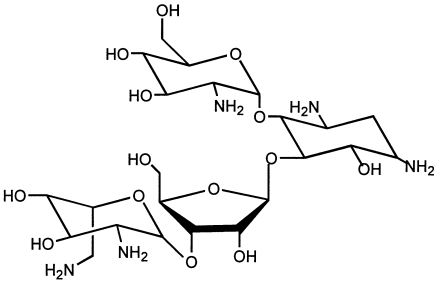
gentamicin C1a mol. wt. 449



streptomycin R= CHO mol. wt. 581
dihydrostreptomycin R = CH_2OH mol. wt. 583



neomycin B mol. wt. 614



aminosidine mol. wt. 616

Fig. 2. Structures of aminoglycosides.

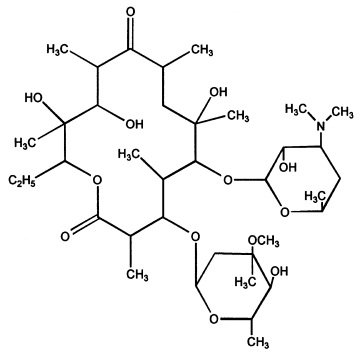
McLaughlin and Henion [10] developed a method capable of detecting spectinomycin, hygromycin B, streptomycin and dihydrostreptomycin in bovine kidney. The poor solubility of these compounds in organic solvents was overcome by the use of matrix solid-phase dispersion. Homogenised kidney was blended with Bondesil cyanopropyl 40 packing material. Following packing into an 8-ml extraction reservoir, the solid phase was washed sequentially with hexane, ethyl acetate, methanol and 50% aqueous methanol under reduced pressure (2 in.Hg; 1 in. Hg=3386.38 Pa). The analytes were eluted with water followed by 0.05 *M* sulfuric acid under reduced pressure (1 in.Hg). An aliquot of the eluate was concentrated by a factor of four and neutralised using ammonia prior to analysis using ionspray LC–MS. A Spherisorb ODS-2 column was used, with a mobile phase consisting of 8% acetonitrile containing either 10 or 20 m*M* pentafluoropropionic acid as an ion-pairing reagent. Using a split ratio of 5:1, column effluent was introduced into a Sciex Taga 6000E triple quadrupole instrument with an APCI source. Using SIM at m/z 351 for spectinomycin ($[M+H_2O+H]^+$), m/z 265 for hygromycin B ($[M+2H]^{2+}$), m/z 301 for streptomycin $[M+H_2O+2H]^{2+}$) and m/z 293 for dihydrostreptomycin ($[M+2H]^{2+}$), these antibiotics could be detected in bovine kidney fortified at a concentration of 20 000 μg/kg. These concentrations are, however, well in excess of the MRLs set by the EU, (which range from 1000 to 5000 μg/kg in bovine kidney). McLauglin et al. [11] subsequently reported that a similar extraction procedure, coupled to LC–MS–MS was capable of confirming the above mentioned compounds along with neomycin B and four components of the gentamicin C complex. Recoveries ranged from 25% (spectinomycin) to 87% (hygromycin B). The method was, however, susceptible to interference from matrix components. They experienced problems with peak shape, retention time and with ion suppression from matrix components. Consequently, they used fortified control tissue samples as standards. Using collision-induced dissociation (CID), they were able to produce three daughter ions for streptomycin, dihydrostreptomycin, neomycin B and the gentamicin C complex. Spectinomycin proved much harder to fragment. CID of the $[M+H_2O+H]^+$ precursor ion at m/z 351 resulted in the production of very weak ions at m/z 333 and 207 that had relative abundances of 4 and 2%, respectively. Hygromycin B required a higher orifice voltage to achieve adequate fragmentation. They found that it was not possible to adjust the orifice voltage as a function of m/z, nor was it possible to effect sufficient chromatographic separation of hygromycin B from other components to permit adjustment of the orifice voltage as a function of time. For these reasons, it was not possible to detect either spectinomycin or hygromycin B at the level of concern.

In conclusion, the analysis of aminoglycoside residues using mass spectrometry is still beyond the reach of most regulatory laboratories. However, the combination of LC–MS–MS and matrix solid-phase dispersion does offer the possibility of confirming all of these compounds at the MRL.

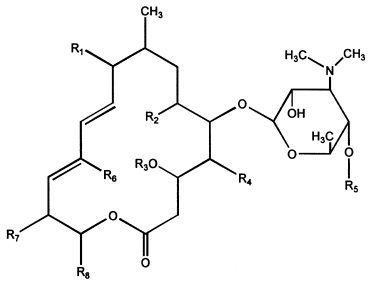
4. Macrolides

The macrolides are a group of compounds that contain a 12-, 14, or 16-membered macrocyclic lactone ring to which several amino and/or neutral sugars are attached (Fig. 3). One single-residue LC–MS test has been developed for each member of this group [12–15] with the exception of josamycin; and one multi-residue test capable of detecting all five compounds has been reported [16].

Pleasance et al. [12] reported an ionspray LC–MS method for the confirmation of erythromycin A in salmon tissues. Minced salmon muscle was homogenised twice in acetonitrile prior to centrifugation and filtration of the supernatant. The combined filtrates were extracted once with hexane to remove fats. The acetonitrile was basified with sodium hydroxide. The solution was mixed gently with dichloromethane. Following the addition of aqueous Na_2HPO_4, the mixture was shaken vigorously. Solid sodium chloride was added to facilitate separation of the aqueous and organic layers. The dichloromethane phase was washed with aqueous sodium chloride, dried and residues reconstituted in methanol. HPLC was performed on a Zorbax Rx-C_8 column, using a gradient elution system with aqueous acetonitrile containing 0.2% formic acid (5–65% acetonitrile). The mass spectrometer used was a Sciex API III triple quadrupole instrument. Using LC–MS and



erythromycin



16-member ringed macrolides

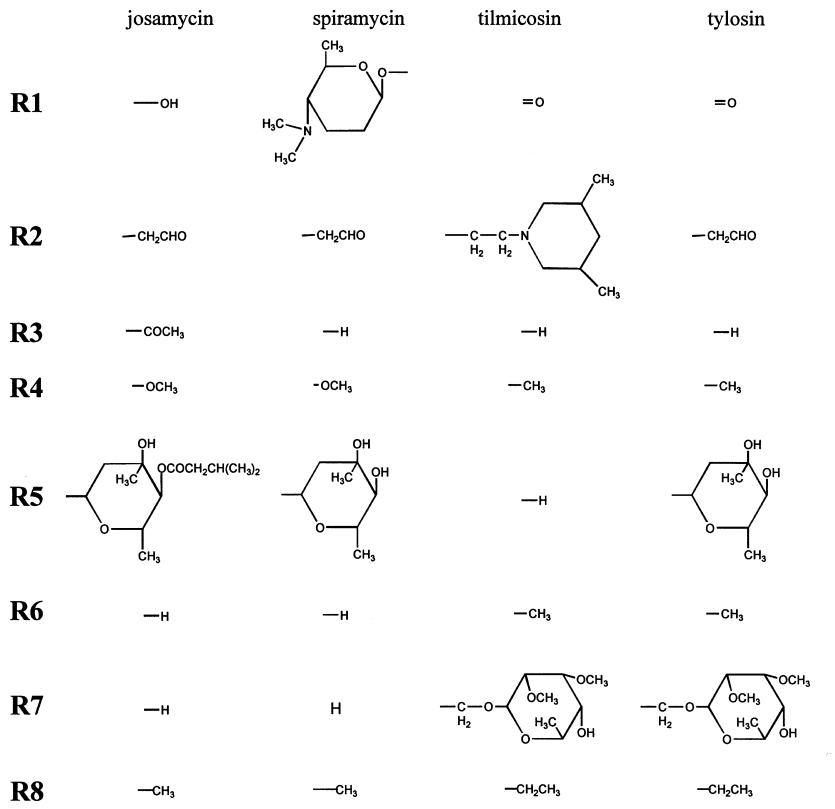


Fig. 3. Structures of the macrolides.

LC–MS–MS with CID, erythromycin could be detected at <0.01 and 0.05 μg/kg, respectively. In addition, a number of metabolites and degradation products could be detected. However, they commented that an improved clean-up procedure was required, since the analysis of only a few samples required the replacement of the guard column.

In 1994, Sanders and Delépine, the most active group in the MS analysis of macrolide residues, described a particle beam LC–MS method for the

analysis of spiramycin in bovine muscle [13]. Spiramycin was extracted from spiked muscle samples into chloroform. The extracts were applied to Bond Elut DIOL cartridges that had previously been conditioned by washing with chloroform. The cartridges were washed with chloroform and water. Spiramycin residues were eluted with 2% formic acid in 0.05 *M* ammonium acetate (50%), methanol (35%) and acetonitrile (15%). This solvent was used isocratically in the HPLC separation of spiramycin from matrix components on a LiChrospher RP_{18} endcapped column. A Hewlett-Packard 5989A particle beam mass spectrometer, using methane as the reagent gas was used to produce a range of fragment ions. The molecular ion was not present because of the lability of spiramycin, the highest molecular mass observed corresponding to the loss of water $[M-H-H_2O]^-$. In addition to the base peak at m/z 683 and its ^{13}C isotopic peak at m/z 684, four further fragment ions were detected. One of these, at m/z 348 proved to be too noisy for confirmatory purposes. However, using ions at m/z 684, 683, 475, 330 and 304 these workers were able to validate the assay in muscle at concentrations equal to half, once and twice the EU MRL of 50 μg/kg muscle.

The same group subsequently reported the development of an assay for tylosin in bovine muscle [14] using essentially the same extraction and analytical conditions. The HPLC mobile phase however, had a higher organic content, consisting of 45% methanol, 20% acetonitrile and 35% formic acid (2%) in water. The molecular ion $[M-H]^-$ was the base peak (m/z 916) with fragment ions at m/z 898, 580, 562 and 520. Validation was attempted using these ions and the ^{13}C isotopic molecular ion at m/z 917, in bovine muscle fortified with tylosin at concentrations equal to half, once and twice the EU MRL (100 μg/kg). The variability of the abundance of ions at m/z 520 and 562 precluded their use. However, the method worked well with ions at m/z 917, 916, 898 and 580.

Kiehl and Kennington [15] reported the development of a LC–APCI-MS assay for tilmicosin in porcine liver. The authors referred to unpublished data for a description of the extraction procedure. They used an isocratic HPLC system with a mobile phase consisting of 5 m*M* ammonium acetate (pH 4.5)–acetonitrile (40:60, v/v) and a Zorbax SB-C_8 column interfaced to a Sciex API single quadrupole mass spectrometer. In addition to the $[M+H]^+$ ion at m/z 869, other prominent ions at m/z 851, 696 and 174 were detected. Using the unpublished extraction procedure and HPLC–UV analysis, a mean recovery of 86% of a 5000 μg/kg spike was achieved. While this method may be sufficiently sensitive to quantify tilmicosin at the EU MRL of 1000 μg/kg liver, it is not possible to assess the simplicity of the procedure.

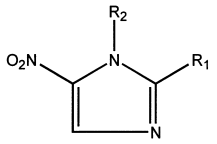
Delépine and co-workers described a method for the analysis of five macrolide residues in bovine muscle using particle beam LC–MS [16]. Having previously developed single residue assays for tylosin and spiramycin, these workers modified these methods to enable the simultaneous determination of tilmicosin, josamycin, erythromycin, spiramycin and tylosin. The extraction procedure required no modification. However, the mobile phase was modified considerably. Trifluoroacetic acid (TFA) (0.1%) was substituted for formic acid, increasing sensitivity. In addition a gradient system was introduced. This consisted of 0.1% TFA in water–methanol–acetonitrile (60:20:20, v/v) at $t=0$, to 20:55:25 (v/v) at 2 min. Using a combination of negative ion and positive ion chemical ionisation they were able to monitor four ions for each of the macrolides at 50 μg/kg.

In summary, this group of compounds is relatively well covered, with one multiresidue method capable of detecting all of the members of this group, at concentrations close to the MRL established by the EU.

5. Nitroimidazoles

The nitroimidazoles are bactericidal towards most Gram-negative and many Gram-positive anaerobic bacteria. However, they have limited activity against aerobic bacteria. MRLs have not been established for any member of this group (Fig. 4). Two compounds, dimetridazole and ronidazole, have been placed in Annexe IV of Council Regulation 2377/90 and metronidazole is not licensed for use in food-producing animals.

Few methods have been reported for the analysis of tissue or milk samples for nitroimidazoles. Matusik et al. [17] used thermospray tandem mass



Compound	R_1	R_2	Mol. wt.
Dimetridazole	CH_3	CH_3	141
Ronidazole	CH_2OOCNH_2	CH_3	200
ipronidazole	$CH(CH_3)_2$	CH_3	169
metronidazole	CH_3	C_2H_4OH	171

Fig. 4. Structures of the nitroimidazoles.

spectrometry to confirm the identity of dimetridazole (DMZ), ipronidazole (IPN) and their alcohol metabolites in turkey tissues. Separate procedures were used to extract DMZ and IPN for a determinative HPLC–UV method. These extracts were then used for confirmation. DMZ and OH-DMZ were extracted with ethyl acetate and cleaned up by acid/solvent partitioning and silica gel solid-phase extraction (SPE). The final residue was dissolved in HPLC mobile phase. IPN and OH-IPN were extracted with benzene in the presence of borax and the extract purified on a silica column. The final benzene extract was evaporated to dryness and the residue dissolved in mobile phase. HPLC was performed isocratically using a Whatman Partisil 5 C_8 RAC II 10-cm column. The mobile phase consisted of water–methanol (1:1, v/v) containing 0.1 *M* ammonium acetate, at a flow-rate of 1 ml/min. A Finnigan Model TSQ46 triple stage quadrupole MS was used with ionisation by thermospray. The protonated molecular ions of each analyte were transmitted by the first quadrupole and decomposition fragment ions were formed by collision with argon in the second quadrupole. The third quadrupole was set to scan from *m/z* 40–200. All four analytes gave ions corresponding to the loss of NO_2 and for each analyte at least three ions, including the molecular ion, were produced. The method was used to confirm the identity of each of the analytes in control tissues fortified at 2 and at 10 μg/kg.

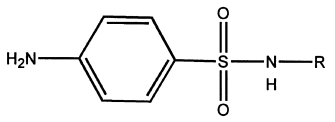
We recently reported a method for the determination of DMZ in poultry tissues and eggs using thermospray LC–MS [18]. Deuterated DMZ ($[^2H_3]$DMZ) was employed as an internal standard. Samples were extracted with dichloromethane (muscle) or toluene (liver, egg) and applied to silica cartridges. DMZ was eluted with acetone and evaporated to dryness at 40°C under nitrogen. The residue was dissolved in methanol–water and washed with hexane before LC–MS analysis. Chromatography was isocratic using a mobile phase of methanol–water (1:1, v/v) containing 0.05 *M* ammonium acetate at a flow-rate of 1 ml/min through a Whatman Partisil 5 ODS-3 column. A Hewlett-Packard 5989A MS Engine was used in positive-ion mode with ionisation by filament-assisted thermospray. SIM was employed with quantification by the ratios of the protonated molecular ions of DMZ and $[^2H_3]$DMZ at *m/z* 142 and 145, respectively. The method was validated at 5, 10 and 20 μg/kg with recoveries between 93 and 102% and R.S.D. values between 1.2 and 7.7%. The method was used to measure incurred DMZ residues in eggs and to analyse tissues from chickens fed a diet containing contamination levels of DMZ.

The two methods described above differed in that the former was a confirmatory procedure for nitroimidazoles quantified by HPLC and therefore no quantitative validation data were presented. MS–MS was employed to enhance the selectivity and definition of the method. The latter procedure was presented as a fully validated quantitative method, with specificity provided by the formation of the molecular ion for DMZ.

6. Sulphonamides

The sulphonamides (Fig. 5) are bacteriostatic, and act by competing with *p*-aminobenzoic acid in the enzymatic synthesis of dihydrofolic acid. This leads to a decreased availability of the reduced folates that are essential for purine synthesis, methionine synthesis, etc. They are inactive in necrotic tissues.

Various LC–MS techniques have been applied to the analysis of tissue or milk samples for sulphonamides over the last decade. The reported methods all employed positive-ion mode and spectra for all sulphonamides exhibited the protonated molecular ion, $[M+H]^+$, as the base peak; with the exception of sulphanilamide for which the base peak was the $[M+NH_4]^+$ adduct ion. Where CID was



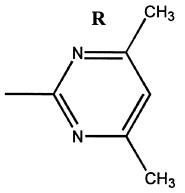
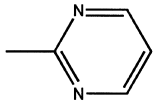
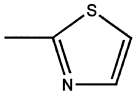
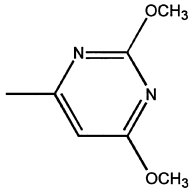
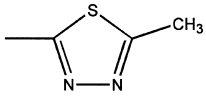
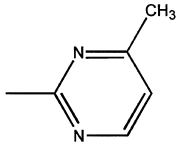
Compound	Mol. wt.	R
sulphamethazine	278	
sulphadiazine	250	
sulphathiazole	255	
sulphadimethoxine	310	
sulphamethizole	270	
sulphamerazine	264	

Fig. 5. Structures of some of the commonly used sulphonamides.

employed, either by collision with argon gas in tandem MS instruments or in APCI instruments when a high cone voltage was applied, a common fragmentation pattern was observed. Proposed fragmentation structures for sulphonamides under these conditions are shown in Fig. 6. Ions common to all sulphonamides were produced at *m/z* 156, the *p*-aminobenzenesulphonic acid moiety ($[M-RNH_2]^+$),

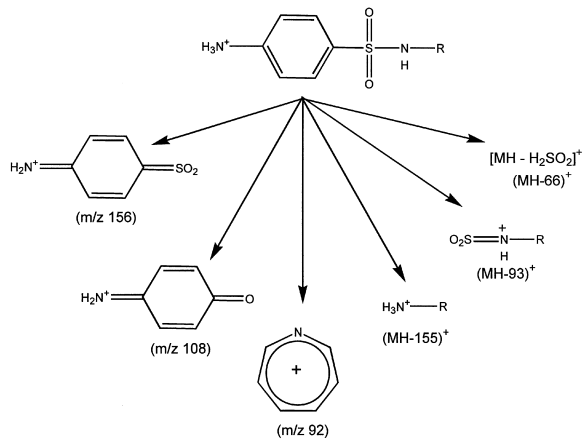


Fig. 6. Proposed fragmentation scheme for CID of protonated sulphonamides.

m/z 108 ($[M-RNH_2-SO]^+$) and *m/z* 92 ($[M-RNH_2-SO_2]^+$) and ions derived from the variable amine substituent, RNH_3 ($[MH-155]^+$). Some spectra also exhibited ions at $[MH-93]^+$ and $[MH-66]^+$ corresponding to $[O_2SNHR]^+$ and $[MH-H_2SO_2]^+$, respectively.

The EU has set an MRL of 100 μg/kg for the sum of all sulphonamides in tissues or milk. The marker residue for each sulphonamide is the parent compound.

Several authors have described thermospray methods. In 1990, Horie et al. [19] applied thermospray LC–MS to the determination of twelve sulphonamide drugs in meat. LC separation was achieved using a TSK-gel ODS-80 T_M column with a mobile phase containing 0.05 *M* ammonium acetate (pH 4.5)–acetonitrile (7:3, v/v) at a flow-rate of 0.8 ml/min. All peaks eluted between 2 and 10 min. SIM of the $[M+H]^+$ ion was employed. Chromatograms of incurred tissue at a level of 1.25 μg/g were presented.

A comparison of thermospray LC–MS and LC–UV methods for the determination of four sulphonamides and their N^4-acetyl metabolites was reported by Balizs et al. [20]. ^{13}C-labelled sulphamethazine was used as an internal standard. Samples were extracted with chloroform–acetone, evaporated to dryness, redissolved in dichloromethane–hexane and cleaned up by Sep-Pak silica SPE. Analytes were chromatographed on a Resolve C_{18}

column using a mobile phase containing 0.1 *M* ammonium acetate (pH 4.6)–acetonitrile (87:13, v/v) at a flow-rate of 1 ml/min and detected using a Vestec Model 210A thermospray LC–MS with discharge-assisted ionisation. The run time was approximately 40 min. SIM of the $[M+H]^+$ ions for each of the analytes was employed. The limits of detection (LOD) were <25 μg/kg and limits of quantification (LOQ) about 100 μg/kg based on statistical analysis of fortified tissues. LC–MS results compared well with LC–UV results.

Thermospray LC–MS was used on-line with a UV detector by Boison et al. [21] for the determination of sulphamethazine and sulphadimethoxine residues in animal tissues. Sulphaethoxypyridazine was used as an internal standard. Tissues were extracted by homogenisation with chloroform and cleaned up by SPE on Sep-Pak C_{18} cartridges. The LC mobile phase consisted of 0.01 *M* ammonium acetate (pH 4.6)–acetonitrile (72:28, v/v). The flow-rate was 1.2 ml/min and a Spherisorb C_{18} ODS-2 column was used. The run time was about 30 min. Effluent from the LC–UV system was introduced into the source of a VG Trio 2 single quadrupole MS. The $[M+H]^+$ ions for each analyte and the internal standard were monitored. The LC–UV method was validated in the range 20–200 μg/kg and good correlation between the LC–UV method and the LC–MS method was shown for several samples fortified between about 10 and 150 μg/kg.

In 1993 Abian et al. [22] described the analysis of ten sulphonamides in milk by thermospray LC–MS and thermospray LC–MS–MS on-line with HPLC–UV. Milk was deproteinised with hydrochloric acid and washed with hexane to remove lipids. The aqueous extract was evaporated, the residue dissolved in methanol, centrifuged and the methanol phase again evaporated to dryness. The residue was dissolved in water for analysis. The milk extracts were concentrated on-line in a column-switching system and chromatographed on a Spherisorb ODS-2 column. The mobile phase contained 0.1 *M* ammonium acetate and 1% formic acid and a gradient programme was used to ramp the acetonitrile content from 0% to approximately 50% in 10 min. The flow-rate was 1 ml/min. MS and MS–MS was performed on a Finnigan TSQ 70 triple quadrupole instrument. The authors reported that a thermospray interface temperature gradient was not necessary under the conditions used. Acetonitrile was reported to be a better organic modifier than methanol for this application, giving sharper peaks and less background noise. As described above, all sulphonamides produced the $[M+H]^+$ ion as the base peak, with the exception of sulphanilamide which produced the $[M+NH_4]^+$ adduct. CID of the parent ions afforded daughter ion spectra following the pattern described above. The LOD of the method in milk was 10 μg/l using SIM. Chromatograms of the $[M+H]^+$ ions were presented of a milk sample fortified at 20 μg/l with nine sulphonamides.

Thermospray LC–MS–MS was also used by Kristiansen et al. [23] to determine five sulphonamides in crude extracts of meat and blood by a LC method and a flow injection method. Sulphapyridine was employed as internal standard. Blood or minced meat was extracted with ethyl acetate, which was evaporated to dryness. The residues were dissolved in 0.05 *M* ammonium acetate–methanol (80:20, v/v) immediately before analysis. For thermospray LC–MS–MS the mobile phase was 0.05 *M* ammonium acetate–methanol (77:23, v/v) at a flow-rate of 1.2 ml/min. The column was a ChromPak Microspher C_{18}. For flow injection/thermospray LC–MS–MS 0.05 *M* ammonium acetate–methanol (80:20, v/v) was used at a flow-rate of 1 ml/min. A Finnigan TSQ 700 MS instrument was used with discharge assisted ionisation. The fragmentation patterns observed were identical to those described above. For LC analyses the authors switched the first quadrupole between the values of the molecular ions, and the third quadrupole was set to monitor the *p*-aminobenzenesulphonic acid fragment ion at m/z 156, giving a reconstructed ion chromatogram of the five chromatographically resolved analytes. For flow injection analyses the parent ion detection mode was used, with the third quadrupole again monitoring the ion at m/z 156. LODs in spiked meat samples were between 2 and 10 μg/kg using thermospray LC–MS–MS and slightly higher for the flow injection method. Replicate fortified samples of meat in the range 7–500 μg/kg and blood in the range 3–350 μg/kg were analysed. Recoveries from meat samples were about 40% at low concentrations (<40 μg/kg) and about 70% at levels >100 μg/kg. The authors noted that

recoveries could be improved to 100% by freeze-drying the ethyl acetate extracts rather than evaporating in a stream of nitrogen. However, this was achieved at the expense of a significant increase in turn-round time for the method. The authors concluded that both the LC and the flow injection methods are suitable for use as screening methods for sulphonamides in meat. The flow injection method had the advantage that it was more rapid than the LC method, and had been used to analyse more than 1000 samples without any appreciable contamination of the system. The method also had the potential to be automated and used on site in, for example, an abattoir.

LC–APCI-MS has also been used for the analysis of sulphonamide residues. Since increasing the cone voltage in an APCI source can induce fragmentation, similar spectra to those obtained by thermospray- or electrospray LC–MS–MS can be obtained using a single quadrupole instrument. In 1993 Doerge et al. [24] reported a method for the analysis of six sulphonamides in milk. Milk was purified and the analytes concentrated by sequential SPE using Sep-Pak C_{18} cartridges followed by Cyclobond I cartridges. The sample was dissolved in mobile phase which consisted of 0.025 *M* ammonium acetate (pH 6.8)–acetonitrile (80:20, v/v). A Nova-Pak C_{18} column was used with a flow-rate of 1 ml/min. MS was performed on a VG Platform single quadrupole instrument. With low cone voltages, typically 30 V, each sulphonamide produced the $[M+H]^+$ ion. SIM of the $[M+H]^+$ ions gave detection limits between 0.25 and 2.5 μg/l in milk. Increasing the cone voltage induced fragmentation, which followed the same pattern as described above. With the cone voltage selected to produce similar intensities for four ions, the ion ratios were reported to be reproducible in milk fortified at 5 μg/l with sulphadiazine, and to be within ±10% of the ion ratios for a sulphadiazine standard. By further increasing the cone voltage, only fragment ions were produced. The authors suggested that by monitoring the ion at *m/z* 156, which is common to all sulphonamides, both parent sulphonamides and their acetyl and glucuronide metabolites might be detected and quantified, since the metabolites contain labile bonds and should yield the same fragment ions as the parent compounds. However, this strategy could be used only if the metabolites and the parent compounds were chromatographically resolved, since the MRL for sulphonamides applies only to the parent compounds. The method is therefore proposed as a screening, confirmatory and determinative method, although no quantitative validation data are presented.

LC–APCI-MS was also used in 1996 by Gehring et al. [25] as a confirmatory method for sulphadiazine in salmon tissues. Sulphadiazine was quantified using a HPLC–fluorescence determinative method. For LC–MS, samples were extracted with acetonitrile, partitioned into dichloromethane and purified by SPE on propylsulfonic acid and C_{18} cartridges. The LC mobile phase consisted of acetonitrile–2% acetic acid (20:80, v/v) at a flow-rate of 1 ml/min through an Inertsil 5-μm ODS-2 column. A VG Platform MS system was used. The authors reported that it was not possible to obtain a sufficient number of diagnostic ions and the $[M+H]^+$ ion with adequate intensities at any one cone voltage, so rapid voltage stepping was employed to switch between the optimum voltage for the production of each of five ions. The ion intensity ratios were reported to be reproducible in incurred and fortified salmon tissues containing 9–20 μg/kg sulphadiazine, and were within 10% of the ratios measured in sulphadiazine standards. The method therefore meets the criteria normally accepted for electron impact ionisation (EI) MS confirmatory methods.

Electrospray (ESI) or ionspray (IS) methods have also been developed. An ionspray LC–MS–MS method was reported in 1991 by Pleasance et al. [26]. Spectral data for twenty-one sulphonamides and the two potentiators, trimethoprim and ormethoprim, were presented and the application of the method to the determination of sulphadimethoxine in salmon tissue was demonstrated. Samples were extracted with acetone in the presence of Celite and sodium sulphate. The acetone was evaporated under vacuum and the residue dissolved in dichloromethane and partitioned into 0.1 *M* sodium hydroxide. The extract was freeze-dried overnight and the remaining water evaporated the next day. The residue was dissolved in 25% methanol. Chromatography was carried out on a Vydac 201TP 5-μm, 2.1-mm I.D., column. The mobile phase was acetoni-

trile–water (35:65, v/v) containing 0.1% formic acid and the flow-rate was 100 μl/min. For simultaneous LC–diode array detection (DAD)-MS acquisitions a flow-rate of 1 ml/min was used, with approximately one quarter of the effluent split into the MS. The MS was a Perkin-Elmer Sciex API III triple quadrupole instrument. SIM of the $[M+H]^+$ ion, in conjunction with LC–DAD, was used to confirm the identity of sulphadimethoxine in incurred salmon samples, that had screened positive by HPLC–UV at levels between 25 and 1800 μg/kg. The LOD was estimated to be 10 μg/kg and recovery of sulphadimethoxine was approximately 60%.

Porter [27] described a confirmatory method for sulphonamide residues in kidney tissue using electrospray LC–MS–MS at levels at or above the MRL. Samples were extracted with acidified ethyl acetate and purified by SPE on Bond Elut NH_2 and SCX cartridges. The LC mobile phase was methanol–acetonitrile–0.01 *M* ammonium acetate (7:8:85, v/v/v) delivered at 1 ml/min. Two columns were employed; a Nova-Pak C_{18} column was used to elute four sulphonamides (sulphadiazine, sulphamerazine, sulphamethazine and sulphaquinoxaline) within about 7 min, and a Hypersil Excel 3-μm C_{18} column, which gave longer retention times. A Finnigan TSQ700 MS with electrospray source was used. The $[M+H]^+$ ion plus three daughter ions were used to confirm the presence of sulphadiazine at a concentration of 800 μg/kg in a kidney sample screened positive by HPLC. The ion ratios agreed with those obtained from a sulphadiazine standard. Approximately 77% recovery for sulphadiazine and 71% for sulphamethazine were reported.

A multiresidue method for the determination of 21 sulphonamides and trimethoprim and ormethoprim in milk using short column electrospray LC–MS–MS was reported by Volmer [28]. Milk samples were extracted by the method of Abian et al. [22] outlined above. LC was performed using a YMC 50×4 mm cartridge packed with 3-μm ODS AQ material and a mobile phase containing 0.1% formic acid with an acetonitrile–water gradient from 10:90 to 45:55, (v/v) in 7 min. The flow-rate was 1 ml/min. Chromatographic separation was achieved in less than 6 min with typical peak widths of 3–10 s. Extracts and standards were injected as 100% aqueous solutions to prevent peak broadening. ESI data were acquired using a Perkin-Elmer Sciex API 300 instrument. Prescreening and confirmation was carried out by precursor ion scan and/or multiple reaction monitoring experiments using generic product ions. Quantification of identified target compounds was achieved by time scheduled SIM of the $[M+H]^+$ ions. Further confirmation, if required, was provided by selected reaction monitoring using ions characteristic of the N-heterocyclic base moieties. Recoveries for the method were determined in milk fortified at 10 and 100 μg/l and were between 72 and 96%. LODs were between 0.1 and 1.0 μg/l based on time scheduled SIM and levels at which analytes could be confirmed using selected reaction monitoring of the precursor ion were between 0.2 and 2 μg/l.

7. Nitrofurans

The nitrofurans (Fig. 7) are synthetic chemotherapeutic agents, and have a broad spectrum of activity. They are predominantly bacteriostatic, but can be bactericidal at high doses. They act by inhibiting a number of microbial enzymes involved in carbohydrate metabolism. The nitrofurans are listed in Annexe IV of EC Council Regulation 2377/90 [1]. Therefore no MRLs have been established for these drugs and their use is prohibited in livestock production within the EU. In order to monitor breaches of the ban it is necessary to have confirmatory methods available for the detection of their residues in edible products. Nitrofurans are known to be unstable and rapidly metabolised in vivo which makes them difficult to detect. In 1995 [29] we reported a method for the detection of furazolidone

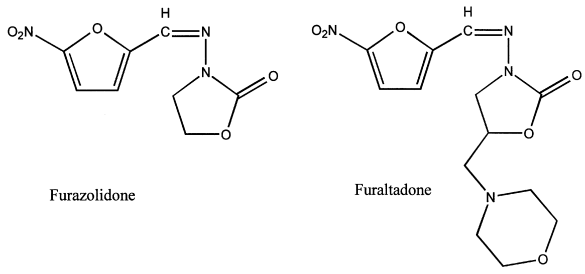


Fig. 7. Structures of nitrofurans.

residues in pig tissues, using thermospray LC–MS. Following methanol–buffer extraction of tissue, liquid partitioning and solid-phase clean up on aminopropyl cartridges, samples were analysed using a Vestec model 201A thermospray LC–MS. A Merck-LiChrocart cartridge, containing LiChrospher RP_{18}, was used with an isocratic mobile phase consisting of 0.1 *M* ammonium acetate–acetonitrile (75:25 v/v). The instrument was operated in the positive ion mode, with filament assisted ionisation, monitoring the $[M+NH_4]^+$ ion at *m/z* 243. The recoveries of furazolidone from liver and muscle, fortified at 5 μg/kg, were 66 and 70%, respectively. The LOD was 1 μg/kg. The variability of the assay was higher than normally expected but was attributed to the instability of furazolidone in tissue homogenates and the relative complexity of sample extraction and clean-up procedures. The assay was used to demonstrate the instability of furazolidone residues both in vivo and in vitro. It was necessary to snap-freeze samples in liquid nitrogen prior to analyses. This assay is the only LC–MS procedure that demonstrated the detection of intact parent drug in animal tissues.

Horne et al. [30] described a method for the determination of protein-bound metabolites of furazolidone and furaltadone in pig liver using LC–APCI-MS. Both nitrofurans contain intact side chains, 3-amino-2-oxazolidinone (AOZ) and 5-morpholinomethyl-3-amino-2-oxazolidinone (AMOZ) corresponding to furazolidone and furaltadone, respectively. These side chain moieties are capable of binding to proteins but can be released from tissue under mild acid conditions. Following extensive solvent washing of tissues, samples were simultaneously extracted and derivatised using 2-nitrobenzaldehyde in an acidic medium. The resulting NPAOZ and NPAMOZ derivatives were analysed using a VG Biotech Trio 2000 quadrupole mass spectrometer fitted with an APCI interface. NPAOZ and NPAMOZ were separated on a C_{18} column using a methanol–0.025% (v/v) acetic acid (45:55, v/v) mobile phase at a flow-rate of 1 ml/min. This flow was split, postcolumn and introduced to the mass spectrometer at 0.5 ml/min. Matrix build up on the sampling cone orifice was reduced by manual switching from mobile phase flow to methanol–water flow during the early stage of each run. The authors also employed a sample clean-up step to remove excess 2-nitrobenzaldehyde reagent from the sample extracts. The mass spectrometer was operated in single ion mode, monitoring the protonated derivatives $[NPAOZ+H]^+$ and $[NPAMOZ+H]^+$ at *m/z* 236 and 335, respectively. Daughter ions were also monitored for NPAOZ and NPAMOZ at *m/z* 206 and 291, respectively. The mean recovery from fortified samples was 70%. The limit of determination was given as 10 μg/kg in liver for both nitrofuran metabolites.

We reported the determination of protein-bound and solvent-extractable residues of the furazolidone side chain metabolite AOZ [31]. Thermospray LC–MS was used to analyse the AOZ-nitrobenzaldehyde derivative, NPAOZ. A Hewlett-Packard 5989A engine, fitted with a thermospray interface, was operated in the positive ion mode with filament assisted ionisation. Samples were passed through a C_{18} column using a mobile phase consisting of 0.1 *M* ammonium acetate–acetonitrile (65:35 v/v) at a flow-rate of 1 ml/min. The ion at *m/z* 253, corresponding to $[M+NH_4]^+$, was monitored with a dwell time of 500 ms. In this procedure there was no interference from tissue matrices or excess 2-nitrobenzaldehyde reagent thus avoiding extensive clean-up steps or column switching techniques. This thermospray method allowed the detection of AOZ in solvent extractable residues as well as the protein-bound fractions, reported by Horne et al. [30]. Recoveries from liver and muscle were 80% with a limit of determination of 10 μg/kg.

In conclusion, few LC–MS methods exist for the successful confirmation of nitrofuran residues in meat. Detection of parent compound has been shown to be difficult and of limited value [29]. However the analyses of protein-bound and solvent-extractable residues of side chain metabolites has been demonstrated using thermospray LC–MS. Since no MRLs are assigned to these compounds, the detection of these metabolites at any concentration should be sufficient to condemn carcasses.

8. Quinolones

Quinolones (Fig. 8) are broad-spectrum synthetic antimicrobial agents used in the treatment of lives-

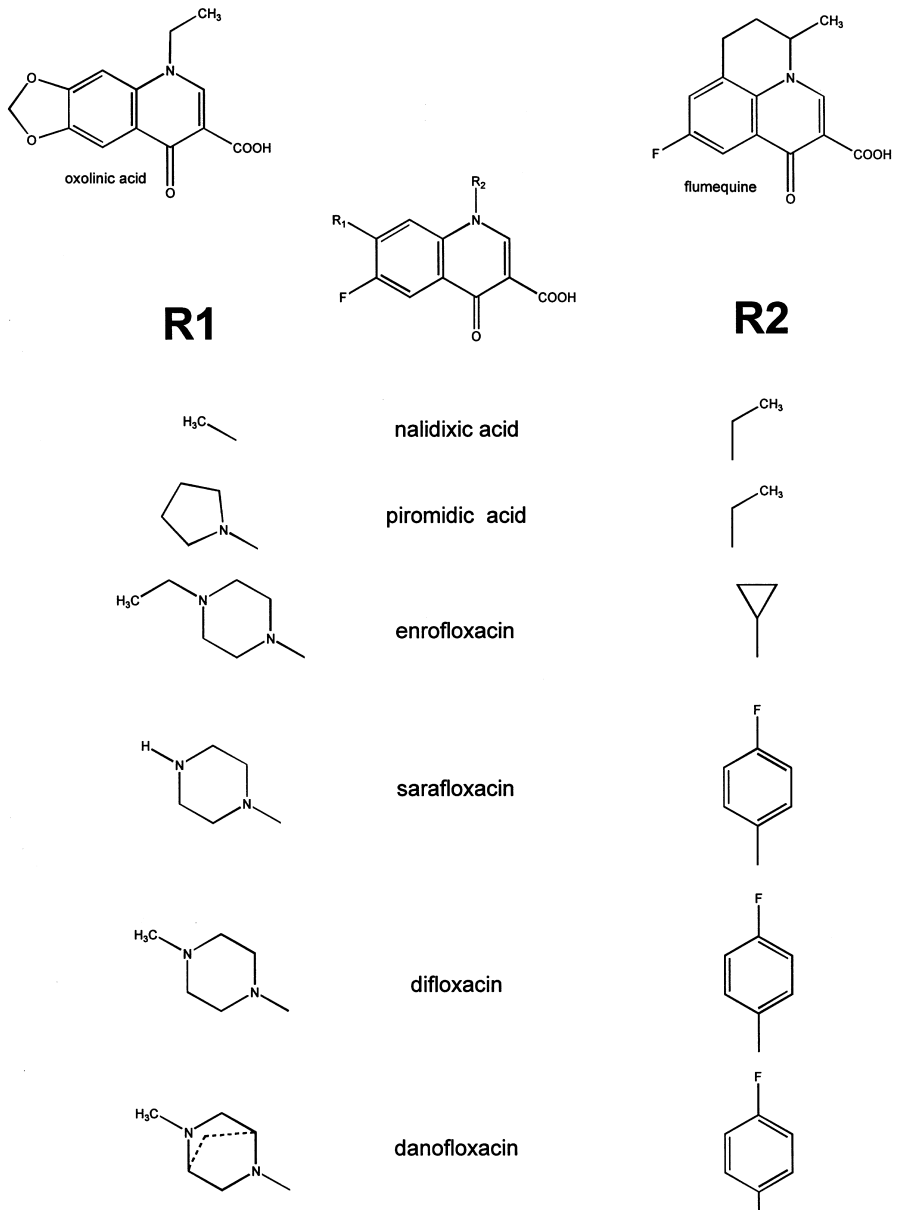


Fig. 8. Structures of some quinolones.

tock and in aquaculture. The older quinolones, e.g. nalidixic acid have limited activity against Gram-negative organisms, while the more recently introduced fluoroquinolones, e.g. enrofloxacin, have a very wide spectrum of activity. In general quinolone carboxylic acids are amphoteric with poor water solubility between pH 6 and 8.

Horie et al. [32] reported a method for the determination of oxolinic, nalidixic and piromidic acids in fish muscle. Samples of tissue were extracted with 0.2% metaphosphoric acid–methanol (2:1, v/v), filtered and evaporated to about 30 ml. The concentrates were applied to C_{18} solid-phase cartridges and eluted with methanol. The eluates

were evaporated then redissolved in mobile phase. The quinolones were separated on an Inertsil ODS-2 column using a mobile phase that consisted of 0.1 *M* ammonium acetate (pH 4.5)–acetonitrile (6:4, v/v), at a flow-rate of 0.8 ml/min. A Shimadzu LC–MS QP 1000 mass spectrometer fitted with a Vestec interface was operated in the positive mode with filament assisted ionisation. The $[M+H]^+$ ions at *m/z* 262, 233 and 289 were monitored for oxolinic, nalidixic and piromidic acids, respectively. The authors recommended the use of a pure silica Inertsil column thereby avoiding the need to incorporate a nonvolatile buffer, such as oxalic acid, in the mobile phase to reduce peak tailing and to mask trace metal impurities. The concentration and pH of the ammonium acetate buffer were optimised to gain maximum intensity of the $[M+H]^+$ ions. The LOD was stated as 0.01 mg/kg and the authors demonstrated the detection of 1.9 mg/kg incurred oxolinic acid in fish tissue. However levels of recovery from tissue and other validation parameters were not presented.

The determination of residues of the fluoroquinolone, danofloxacin in cattle and chicken liver, using electrospray MS–MS, was reported by Schneider et al. [33]. Liver was homogenised and incubated with a mixture of 150 m*M* phosphoric and perchloric acids in water–methanol (1:1, v/v). The pH was adjusted to 8.5 and samples were extracted with methylene chloride. Following evaporation, samples were reconstituted in mobile phase and injected onto the LC–MS system. The mobile phase consisted of acetonitrile–0.1% trifluoroacetic acid (8:2, v/v). It was passed through a microbore Hypersil C_{18} column at a flow-rate of 50 μl/min. The column was coupled to a Sciex API III triple quadrupole mass spectrometer with an electrospray interface. The $[M+H]^+$ ions formed were sampled into the quadrupole mass filter and by optimisation of the cone, quadrupole and lens voltages, the maximum CID response of the danofloxacin daughter ions was achieved. By using selected reaction monitoring (SRM) the mass spectrometer was adjusted to selectively monitor the parent to daughter ion fragments of *m/z* 358 to 340 and *m/z* 358 to 255. Monitoring three ions and comparing ion ratios enhanced the specificity of the assay. The authors observed a four-fold increase in response of the parent ion by the addition of trifluoroacetic acid. They used this procedure to confirm the presence of danofloxacin residues in cattle and chicken liver samples down to a concentration of 50 μg/kg. The R.S.D. values for the ratios of daughter ions at *m/z* 340 and 255 were given as 13.57, for liver samples fortified at 50 μg/kg and 19.78, for incurred liver samples. Recovery levels and other validation parameters were not reported.

The multiresidue determination of four quinolones namely, flumequine, oxolinic acid, nalidixic acid and piromidic acid, in catfish tissue, was described by Doerge and Bajic [34]. After liquid–liquid extraction of samples (not described) quinolones were separated on a Dionex NSI column containing polystyrene divinylbenzene copolymer. A linear gradient using 22–38% acetonitrile against a mixture of 9% tetrahydrofuran in 25 m*M* acetic acid was run at a flow-rate of 1.0 ml/min. The LC effluent was delivered to a VG Platform single quadrupole mass spectrometer fitted with an APCI interface. Moderate cone voltages of 15–20 V yielded protonated molecular ions plus $[M+Na]^+$ and a minor ion, $[M+H-44]^+$. The loss of mass 44 was attributed to thermal decarboxylation of the quinolones in the nebuliser probe and did not derive from in-source CID reactions. The degree of in-source CID reactions was optimised so that nearly equal intensities of protonated molecules, $[M+H-18]^+$ and $[M+H-44]^+$, at a common cone voltage, were obtained. This was achieved for all compounds except nalidixic acid.

SIM of protonated molecules together with two cone voltage-induced daughter ions, resulted in the detection of the four quinolone compounds from a catfish extract fortified with 10 μg/kg of each standard. The estimated detection limits, based on the signal-to-noise ratios, were 1.7, 1.5, 1.2 and 0.8 μg/kg for piromidic acid, oxolinic acid, flumequine and nalidixic acid, respectively. The authors suggested that the determination of ratios of ion intensities for the three ions associated with each compound relative to the largest peak $[MH-18]^+$, could provide the necessary degree of selectivity for the technique to be used as a confirmatory procedure although further work was required, e.g. evaluation of reproducibility.

LC–APCI-MS–MS experiments using a VG Quattro II triple quadrupole mass spectrometer were performed in order to elucidate the fragmentation

pathways for each compound. Increased specificity and sensitivity were also achieved with this technique. A loss of H_2O $[M-18]^+$ was common for all four compounds in both in-source CID and in MS–MS scans. This constant neutral loss of mass 18 was used as a multiresidue screening procedure to detect the presence of quinolones in catfish tissue. By using multiple reaction monitoring (MRM) a ten-fold increase in sensitivity over the MS-SIM procedure was achieved.

An LC–MS–MS procedure for the confirmation of sarafloxacin residues in catfish was reported by Schilling et al. [35]. Catfish tissue was homogenised with 1 *M* sodium hydroxide followed by the addition of acetonitrile and 85% phosphoric acid to precipitate proteins and to neutralise the pH. Extracts were subjected to an SPE step using C_{18} columns. The resulting eluates, reconstituted in mobile phase, were used for LC–MS–MS analysis. Sarafloxacin was eluted from a C_{18} column using a mobile phase consisting of acetonitrile–0.04 *M* pH 2.4 citric acid (30:70, v/v) at a flow-rate of 1 ml/min. The LC system was coupled to a Sciex API III MS–MS using an ionspray interface. Before reaching the ionspray interface the mobile phase was split to produce a flow of 20 μl/min. By using CID-MS–MS, daughter ions at *m/z* 368, 342 and 299 were obtained from the protonated molecule at *m/z* 386. The method was used to confirm the presence of sarafloxacin in catfish tissue by comparing ion ratios of the three daughter ions to the parent ion. Ratios had to fall within ±10% of the ratio obtained for a standard, analysed on the same day, for successful confirmation. Also, chromatographic retention times must come within ±20 s of the standard peak. The procedure was tested in the concentration range of 0.65–1.3 μg/g. Recovery values were determined on the basis of an HPLC with UV detection procedure. For samples spiked at 0.65 μg/g and 1.3 μg/g the recoveries were 91 and 99%, respectively.

In conclusion, the residues of a number of compounds of the quinolone group have been detected using LC–MS techniques. Common to all the methods discussed was the relatively simple extraction and clean-up steps used to prepare samples for MS analysis. The use of CID-MS–MS was successful in confirming the presence of danofloxacin and sarafloxacin residues. The production of relatively stable daughter ions from the protonated molecule has allowed ion ratios to be calculated thereby enhancing the specificity of methods. Techniques such as CNL and MRM are useful for multiresidue determinations. In general MS–MS techniques added a degree of specificity and sensitivity over that achieved with SIM-MS. However, all methods discussed are lacking in detailed validation data such as recovery levels and intra/inter assay variations which are necessary to assess the potential of a confirmatory method.

9. Amphenicols

Chloramphenicol [D-(−)-threo-2-(dichloroacetamido)-1-(*p*-nitrophenyl)-1,3-propanediol] (CAP) is a broad spectrum antibiotic active against a variety of pathogens, including aerobic and anaerobic Gram-negative and Gram-positive bacteria, rickettsia, mycoplasma and chlamydia. Although widely used in veterinary and human medicine there have been reports of aplastic anaemia in humans arising from its use. This has led to its ban within the US and the EC. Thiamphenicol (TAP) and more recently florfenicol, which have chemical structures similar to CAP (Fig. 9), have been permitted as substitutes. MRLs exist for TAP and florfenicol, but CAP has been placed in Annexe IV of Council Regulation 2377/90 [1].

The range of LC–MS procedures, which have been applied to amphenicols, has been limited. Korfmacher et al. [36] produced thermospray LC–MS data in the positive mode for CAP, dehydrochloramphenicol (DHCAP), aminodehydrochloramphenicol (ADHCAP), and nitrophenylaminopropanediol (NPAPOL). Standards only were run on a Supelco diphenyl column with a mobile phase which consisted of 0.05 *M* ammonium acetate (pH 4)–methanol–isopropanol (80:10:10, v/v) at a flow-rate of 1 ml/min. The effluent was connected to a Vestec thermospray interface in conjunction with a Delsi-Nermag thermospray source and Delsi-Nermag R1010C quadrupole mass spectrometer. The $[M+H]^+$ ion was present for all four compounds. Fragmentation produced two major ions explained by the following losses $[M+H-H_2O]^+$ and $[M+H-H_2O-CH_2O]^+$. Overall sensitivity was enhanced by the application of discharge assisted ionisation.

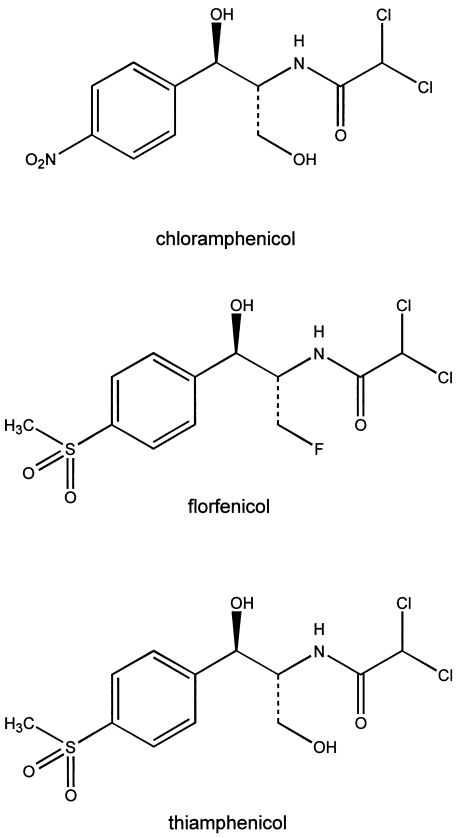


Fig. 9. Structures of the amphenicols.

We reported [37] a method for the determination of chloramphenicol in plasma, milk and tissue. Deuterated [$^2H_5$]chloramphenicol was used as internal standard. Aliquots of milk or plasma, after the addition of water, were cleaned up on C_{18} Sep-Pak cartridges. Tissue was extracted by homogenisation with ethyl acetate in the presence of piperonyl butoxide, which inhibits in vitro metabolism of chloramphenicol. The dry extract was dissolved in dichloromethane–hexane (1:1, v/v) and cleaned up using silica gel cartridges. Subsequent elution and partition between 0.05 *M* Tris–HCl buffer (pH 10) and ether gave a residue suitable for analysis. LC was performed on a LiChrosphere C_{18} endcapped cartridge using an acetonitrile–0.1 *M* ammonium acetate (35:65, v/v) mobile phase at a flow-rate of 1 ml/min. The LC system was interfaced with a Vestec dedicated thermospray LC–MS system.

In contrast to the method described by Korfmacher et al. [36], optimum sensitivity was achieved in the negative ion mode using filament assisted ionisation. The predominant ion was the $[M]^-$ molecular ion at m/z 322 formed by electron capture, together with minor contributions from the ion $[M-HCl]^-$ at m/z 286 and the ion $[M-2HCl]^-$ at m/z 250. The method was validated at 5 and 10 μg/kg in liver with R.S.D. values of 6.5 and 2.9%, respectively. LODs for tissue and milk were 1 and 2 μg/kg, respectively, based on a signal-to-noise ratio of 3:1.

Yoshida and Kondo [38] described a method for the determination of thiamphenicol in milk by ethyl acetate solvent partition on a Kieselguhr support. After evaporation, the residue was chromatographed on a Wakosil-II 5 C_{18} HG column using an isocratic mobile phase of acetonitrile–water (1:1, v/v) at a flow-rate of 1 ml/min. The effluent was interfaced to a Hitachi M2008 mass spectrometer via a Hitachi APCI interface in the positive mode. The $[M+H]^+$ ion at m/z 357 was monitored. The LC–APCI-MS procedure acted as a confirmatory method to compliment a HPLC–UV assay. Validation data for the LC–APCI-MS technique was not presented.

A simple and rapid particle beam LC–MS procedure for the determination of CAP, DHCAP, ADHCAP and NPAPOL in calf muscle was suggested by Delépine and Sanders [39]. Tissue was vortex-extracted with ethyl acetate. On removal of the solvent the residue was partitioned between hexane–carbon tetrachloride and water. The aqueous fraction was chromatographed on a LiChrospher C_{18} endcapped column using a mobile phase of methanol–0.2% formic acid (43:57, v/v) at a flow-rate of 0.6 ml/min. The effluent was interfaced with a Hewlett-Packard Engine equipped with a particle beam interface. Mass spectral data was equivalent to that observed by ourselves except for greater abundance of the two fragment ions formed by consecutive loss of HCl. Validation was performed on tissues fortified at four levels (2, 5, 10 and 20 μg/kg) on different days giving R.S.D. values of less than 15%. The replicate analysis of incurred positive controls (n=6–8) gave R.S.D. values of less than 30% as recommended by EU guidelines [3]. The LOQ was set at 2 μg/kg. A chromatogram of a muscle from a treated animal was presented.

In conclusion, the use of LC–MS in the analysis of amphenicol residues has produced one well

validated method for CAP and its metabolites. However the interest in LC–MS as a confirmatory method is limited due to the availability of many excellent GC–MS procedures.

10. Penicillins

The penicillins (Fig. 10) are administered to food producing animals both therapeutically and prophylactically. The basic structure of penicillin (6-aminopenicillanic acid) consists of a thiozolidine ring fused to a β-lactam ring with a side chain. Manipulation of the side chain has altered the antibacterial spectrum of penicillins to include both Gram-positive and Gram-negative bacteria.

A range of LC–MS procedures has been applied to the analysis of penicillins in tissue or milk during the past decade. The reported methods employ both positive and negative-ion mode. Positive ion spectra exhibit the protonated molecular ion $[M+H]^+$ while negative ion spectra contain the ion $[M-H]^-$ produced by proton abstraction. The relative comparison in sensitivity of the two modes is dependent on the instrument and the ionisation technique.

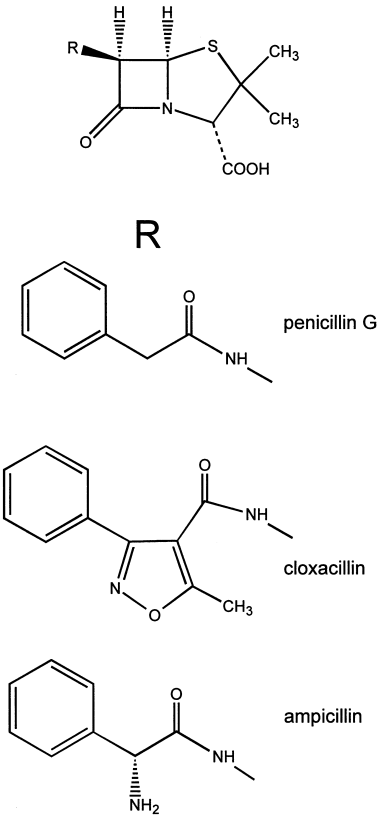


Fig. 10. Structures of some penicillins.

Thermospray investigations have been described by a number of authors [40–43] using the positive mode. The fragmentation mechanism proposed involves the opening of the β-lactam ring, followed by hydration and loss of carbon dioxide $[M+H+H_2O-CO_2]^+$. Subsequent cleavage of the penicilloic acid, produced two major components, one common to all penicillins $[C_6H_9HSO_2+H]^+$ at m/z 160 and the other specific to the particular analogue $[F+H]^+$. These fragments are interpretable as arising from the chemical degradation processes, induced by thermal energy transfer to the molecules as they pass through the thermospray vaporiser probe.

In 1989, Tyczkowska et al. [43] applied thermospray LC–MS to the confirmation of penicillin G in bovine milk. Milk was diluted with an equal volume of acetonitrile–methanol–water (40:20:40, v/v/v) to precipitate protein. After centrifugation an aliquot of the ultrafiltrate was injected into a LC system equipped with a Finnigan Mat thermospray interface and a Finnigan Mat 4800 quadrupole mass spectrometer. The LC system consisted of a Brownlee Phenyl Spheri-5 cartridge with a mobile phase containing isopropanol–0.2 *M* ammonium acetate–acetic acid (12.5:85.5:2, v/v/v) at a flow-rate of 1 ml/min at 40°C. The LOD was 100 μg/l, based on monitoring the $[M+H]^+$ ion at m/z 335 and $[M+H+H_2O-CO_2]^+$ ion at m/z 309. A comparison of the reported levels of penicillin G in milk, produced by udder infusion, was in close agreement when measured by LC–UV or thermospray LC–MS.

Voyksner et al. [44] extended this method to the confirmation of cloxacillin, ampicillin and amoxycillin in bovine milk. To optimise the sensitivity of the method for amoxycillin the $[M+H]^+$ ion at m/z 366 and the $[F+H]^+$ ion at m/z 207 were monitored due to the low abundance of the $[M+H+H_2O-CO_2]^+$ ion at 340. The LODs based on a 3:1 signal-to-noise ratio were estimated to be 100, 200 and 200 μg/l, respectively. While the authors employed thermospray LC–MS primarily for confirmation, linearity was achieved over the range 100 to 500

μg/l with typical correlation coefficients of 0.9996. A comparison of the level of cloxacillin in milk from treated animals measured by LC–UV and thermospray LC–MS gave close agreement.

Thermospray LC–MS was used in conjunction with UV detection by Boison et al. [45] to confirm penicillin G in milk. Penicillin V was used as internal standard. After protein precipitation with sodium tungstate, samples were applied to a Bond Elut C_{18} SPE cartridge, from which the penicillins were eluted with acetonitrile–0.0125 *M* ammonium acetate (60:40, v/v). LC separation was achieved using a Nova-Pak C_{18} column with a gradient mobile phase running from acetonitrile–0.15 *M* ammonium acetate (10:90, v/v) to acetonitrile–0.15 *M* ammonium acetate (37:63, v/v) at a flow-rate of 0.9 ml/min. The eluate was introduced into a VG Trio II mass spectrometer fitted with a thermospray/plasmaspray interface. SIM of the $[M+H]^+$ ion at *m/z* 352; $[M+H+H_2O-CO_2]^+$ at *m/z* 335 for penicillin G and *m/z* 160 for penicillin G/penicillin V was employed. The authors stated that the response of the penicillin G ion *m/z* 352 at concentrations of 5 μg/kg was low. Intra-assay precision ($n=4$) at six levels in the range 3–60 μg/kg and inter-assay precision at two levels, 6 and 50 μg/kg ($n=2$), for 4 days were determined for the LC–UV method. However data for the correlation between LC–UV and thermospray LC–MS was not included. A thermospray LC–MS chromatogram for milk fortified at 5 μg/l penicillin G was presented.

Voyksner and Pack [46] studied the fragmentation of standards using electrospray or ionspray techniques with CID, to produce structural information on penicillin G and cloxacillin. Thermal fragment ions $[M+H+H_2O-CO_2]^+$, often observed in thermospray, were not detected in electrospray. As the potential was increased, fragment ions arising from the cleavage of the β-lactam ring were formed. At higher potential differences, the majority of the ion current was carried by ions having values of *m/z* below 100.

Similar studies on standards were carried out by Straub and Voyksner [47] who examined the influence of CID voltage on the positive ion spectra of penicillins to penicillin G, ampicillin, amoxycillin and cloxacillin. They employed a Fusia C_{18} capillary column attached to an Analytica ESP-MS interface Model QBV 25AL, installed on a type 4500 single quadrupole Finnigan MAT mass spectrometer for flow infusion experiments. In general, all four penicillins showed higher $[M+H]^+$ intensity at +160 V. The fragmentation data followed a similar pattern for all the penicillins studied, showing a characteristic cleavage product of the β-lactam ring $[C_6H_9HSO_2+H]^+$ at *m/z* 160 and a further loss of COOH at *m/z* 114. Fragments formed by the cleavage of the amide moiety are unique for the individual penicillins.

The equivalent study on the negative ion spectra produced by standards using an Analytica electrospray-MS interface Model 100547-3 connected to a Hewlett-Packard 5989A Engine single quadrupole mass spectrometer. In general, the negative mode spectra produced lower signal intensities compared to the positive mode. They exhibited a $[M-H]^-$ ion, together with much less fragmentation at higher CID voltages compared to positive ion spectra. The fragmentation resulted from decarboxylation and opening of the β-lactam ring, forming ions $[M-H-CO_2]^-$ at *m/z* 289 and $[F-H-H_2O]^-$ at *m/z* 192, respectively, for penicillin G.

Tyczowska et al. [48] adjusted the composition of the mobile phase to optimise the signal response for the same four penicillins in the positive ion mode. The optimum mobile phase contained 30% (v/v) acetonitrile and 1% (v/v) acetic acid. However, the ultracentrifugal clean-up employed failed to concentrate the analytes sufficiently. The pure electrospray nature of the system required flow-rates in the region of 2–10 μl/min. Application of the necessary volume of ultrafiltrate compromised the packed reverse phase capillary chromatographic performance. The method was unsatisfactory for regulatory purposes.

Straub et al. [49] described a method for the confirmation of the above penicillins at regulatory levels in milk. The procedure involved the precipitation of protein from milk with 50% acetonitrile–water, followed by ultracentrifugation through a 10 000 molecular mass filter to provide initial clean-up. The on-column focusing of an aliquot of the ultrafiltrate to a Porous II R/H LC perfusion capillary column packed with derivatized porous polystyrene divinylbenzene was made using a mobile phase consisting of 100% water with the addition of 0.2% formic acid and 25 m*M* heptafluorobutyric acid

(HFBA). The target penicillins were eluted with a mobile phase gradient increase to 50% acetonitrile over 6 min at a flow-rate of 38 μl/min. The penicillins were detected in positive mode using an Analytica electrospray interface Model 102387 with ultrasonic nebulisation capability (ultraspray) connected to a Hewlett-Packard 5989A Engine single quadrupole mass spectrometer. The LODs for cloxacillin and penicillin G were 3–5 μg/kg. Interferences in the void volume hindered the confirmation of ampicillin and amoxycillin, resulting in detection limits of 20–30 μg/kg. Linearity over the range 10–1500 μg/kg was obtained; however inter/intra assay validation data and results from incurred samples were unavailable.

In 1993 we reported a method for the determination of penicillins in muscle, kidney and milk using ionspray LC–MS [50]. Nafcillin was employed as internal standard in the quantification of penicillin G, penicillin V, oxacillin, cloxacillin and dicloxacillin. Tissue/milk was made acidic and the ionic strength adjusted with sodium chloride before proteins were precipitated with acetonitrile. The filtrate was cleaned up using liquid–liquid extraction into dichloromethane, which was then evaporated to dryness at 400°C under nitrogen. The residue was dissolved in acetonitrile–water (1:3, v/v). Chromatography was isocratic using a mobile phase of acetonitrile–water–triethylamine (27:73:0.5, v/v/v) at a flow-rate of 0.7 ml/min through an Inertsil ODS-2 column. The LC system was coupled to the Megaflow probe of a VG Platform electrospray LC–MS instrument (ionspray) operated in the negative mode. This instrument appears to produce the highest sensitivity in the negative mode. The use of acetic acid, ammonia or ammonium acetate was found to suppress the signal. However the inclusion of triethylamine maintained the signal and buffered the chromatographic system reducing shifts in retention times between samples and standards due to matrix effects.

Increasing the voltage on the extraction cone to 15–17 V produced fragmentation. Typically 3–4 ions were monitored for each penicillin. Cloxacillin produced ions of: $[M-H]^-$ at m/z 434; $[M-H]^-$ isotope at 436 m/z; $[M-H-CO_2]^-$ at 390 m/z; $[F-H+H_2O]^-$ at m/z 293. The reproducibility of ion ratios was within the ±20% variation expected for chemical ionisation in EU criteria [3].

The method was validated at 200 and 500 μg/kg in muscle and kidney, recoveries were between 85 and 114% with R.S.D.s between 2 and 11%. The validation in milk was performed at 10 and 20 μg/l producing recoveries between 70 and 118% and R.S.D.s between 3.7 and 9.3%. The LODs for this method based on a signal-to-noise ratio of 3:1 were 25 μg/kg in meat and 2 μg/l in milk for penicillin G/oxacillin; 50 μg/kg in meat and 5 μg/l in milk for penicillin V/cloxacillin; 100 μg/kg in meat and 10 μg/l in milk for dicloxacillin.

Particle beam LC–MS also exists as an option for analysis. Hurtaud et al. [51] reported a procedure for the confirmation of oxacillin, cloxacillin and dicloxacillin in bovine muscle. Penicillins were extracted from tissues with ethyl acetate under acidic conditions. Ethyl acetate was evaporated to dryness at 40°C under nitrogen. The residue was dissolved in the mobile phase consisting of methanol–acetonitrile–2% aqueous formic acid (50:10:40, v/v/v). Chromatographic separation was achieved on a LiChrosphere endcapped C_{18} column with a flow-rate of 0.6 ml/min. The retention times of the analytes were between 5 and 9 min. The mass spectrometric system consisted of a Hewlett-Packard Engine single quadrupole MS equipped with a Hewlett-Packard 59980B particle beam interface. Negative ion chemical ionisation with methane reagent gas was used because it was more sensitive than the electron impact mode.

The molecular ion was absent for each compound, however fragmentation produced a series of 4–5 ions characteristic for each penicillin including the chlorine isotope. In the case of oxacillin, opening and cleavage of the β-lactam ring followed by proton abstraction from the amide fragment gave the ion $[M-159-H]^-$ at m/z 241. The ion at m/z 214 arises from the same fragment with the further loss of carbon monoxide $[M-159-H-CO]^-$. In addition a proposed loss of carbon dioxide from the amide by rearrangement gave the ion $[M-159-CO_2]^-$ at m/z 198, while subsequent loss of a methyl from the isoxazoyl group gave the fragment $[M-159-CO_2-CH_3]^-$ at m/z 183.

The repeatability of the assay was performed at 150, 300 and 600 μg/kg in duplicate over 3 days. For each day the difference in relative abundance of the ions was compared to the standard. The ion ratios at all three levels were in accordance with the criteria

of ±20% recommended by the EU [3]. The LOD of the method was calculated by measuring the height of peak-to-peak noise of six blank samples and equating this with three times the height of the baseline noise. For oxacillin, the LOD was 40 μg/kg and for cloxacillin and dicloxacillin it was 50 μg/kg. The authors observed that area responses were a function of concentration for the three penicillins but nonlinear responses were observed.

In summary, the use of LC–MS methods for the confirmation of LC–UV positive penicillins have lacked adequate cleanup and concentration method development and hence failed to reach the LODs required for regulatory agencies. In comparison the recent LC–MS quantitative procedures have addressed this criticism and must be expanded to include all penicillins and matrices.

11. Cephalosporins

The cephalosporins are bactericidal and inhibit the third stage of bacterial cell wall synthesis by interfering with cross-linking of linear peptidoglycan strands. They generally contain the 7-amino cephalosporonic acid nucleus and differ in basic structure from the penicillins in containing a six-membered dihydrothiazine ring instead of a five-membered thiazolidine ring fused to the β-lactam portion (Fig. 11).

The LC–MS procedures applied to the cephalosporins mirror the data already presented for the penicillins. In a number of these papers representatives of each group were isolated and monitored under similar conditions [46–48].

Tyczkowska et al. [52] reported a method for the determination of cephapirin and its desacetylcephapirin metabolite in bovine milk and serum. The isolation procedure was similar to that previously reported, using ultracentrifugation. Chromatographic separation was achieved on a Brownlee Phenyl Spheri-5 cartridge with a mobile phase containing isopropanol–30 m*M* ammonium acetate–acetic acid (6.5:93:0.5, v/v/v) at a flow-rate of 1.3 ml/min at 55°C. The thermospray LC–MS system employed was a Finnigan Mat thermospray interface and a Finnigan Mat 4800 quadrupole mass spectrometer. The thermospray mass spectrum for cephapirin exhibited an $[M+H]^+$ ion at *m/z* 424 and

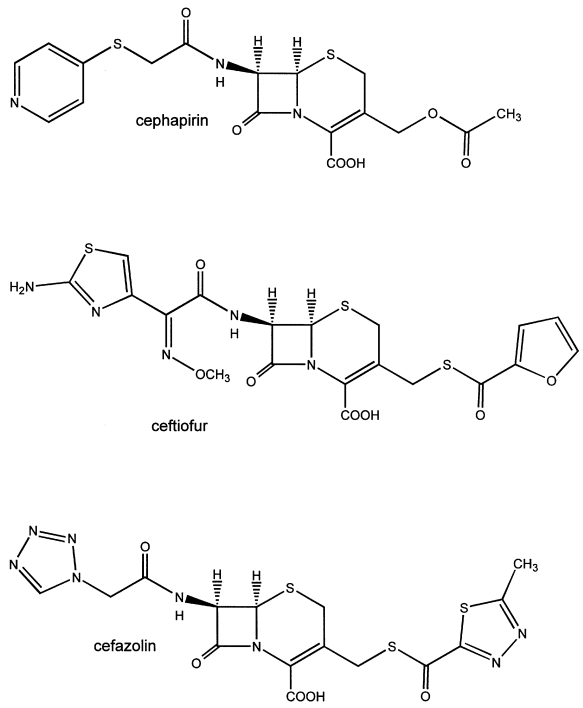


Fig. 11. Structures of some cephosporins.

several fragment ions. The peaks at *m/z* 381 and 364 are postulated to arise from the loss of $COCH_3$ and $HOCOCH_3$ respectively from the pseudomolecular ion $[M+H]^+$. The ions at *m/z* 209 and 168 are protonated fragment ions from the opening and cleavage of the cephapirin ring. The ion at 168 ($C_7H_6NO_2S$) should be indicative of all the cephalosporin antibiotics. Validation data was presented for a LC–UV method run in parallel. The thermospray LC–MS procedure remained invalidated. Sensitivity was poor and total ion current (TIC) traces of incurred milk gave poor chromatography.

The confirmation of cephalosporins by mass spectrometry is limited by the application of adequate cleanup/concentration procedures. The LC–MS technique itself provides sufficient structural information to confirm residues at the MRL of the antibiotic.

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