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Review

Analysis of antibiotics by liquid chromatography–mass spectrometry

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Abstract

The current status of the application of LC–MS in the analysis of antibiotic and antibacterial compounds is reviewed. The main application area of LC–MS in this field is the confirmation of identity in animal food products for human consumption at maximum residue levels, set by the regulatory authorities. LC–MS is found to play an important role in the determination and confirmation of sulphonamides, β-lactam antibiotics, (fluoro)quinolone antibiotics, as well as various other groups including aminoglycosides, chloramphenicol, ionophore antibiotics, 5-nitrofuran derived compounds and macrolide antibiotics. Currently available data on these compound classes are reviewed, with special emphasis on important aspects especially relevant to LC–MS and on the mass spectral information obtained.

Keywords: Liquid chromatography–mass spectrometry; Reviews; Antibiotics; Sulfonamides; Lactams; Quinolones

Contents

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1. Introduction

In the past 10 years the on-line combination of liquid chromatography and mass spectrometry (LC–MS) has developed into a widely applied and routinely applicable detection and on-line identification approach for LC. The ease of operation and robustness of current LC–MS interface based on atmospheric-pressure ionization enable the application of LC–MS in a large variety of analytical fields. The electrospray interface and ionization technique has become the technique of choice in many areas related to the characterization of peptides, proteins and other biologically-relevant macromolecules. Furthermore, both electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) have replaced the use of thermospray in pharmaceutical application of LC–MS and have actually made LC–MS in combination with tandem mass spectrometry (MS–MS) the technique of choice in quantitative bioanalysis. In the identification of new products, profiling of impurities in synthetic products and formulations, the characterization of metabolites, as well as quantitative bioanalysis in biological samples, LC–MS has played an important role in pharmaceutical research.

The mass spectrometric analysis of antibiotics has also significantly benefitted from these developments. LC analysis of antibiotics was reviewed by Skaikh and Moats [1], while analytical strategies in screening for veterinary drug residues in edible products were discussed by Aerts et al. [2]. The analysis of antibiotic and antibacterial compounds by LC–MS and related techniques like supercritical fluid chromatography (SFC) and capillary zone electrophoresis (CZE) combined with MS is reviewed in this paper. Confirmation in residue analysis of antibiotics in food samples is the most important application of LC–MS in this field. The review is structured in terms of compound classes. Attention is paid to the LC–MS analysis of sulphonamides, β-lactam antibiotics, quinolone antibiotics as well as to other classes of antibiotic compounds, including aminoglycoside, tetracyclines, chloramphenicol, ionophore polyether, macrolide and nitrofuran antibiotics. The discussion is mainly focused on aspects related to LC–MS and MS and the information obtained, while little attention is paid to other important topics in the general analytical strategy, like sample pretreatment and separation.

2. Introduction to LC–MS

2.1. General features of LC–MS interfaces

LC–MS has been extensively studied in the past 25 years [3,4]. This research has resulted in several routinely applicable and commercially available LC–MS methods, of which the methods based on ESI and heated-nebulizer APCI currently are the most widely used ones. The progress made in LC–MS is briefly discussed here with an emphasis on the most frequently used technology in antibiotics analysis.

Three approaches can be distinguished in the development of LC–MS interfaces: (a) nebulization of the column effluent, removal of the mobile phase constituents, vaporization of the analyte and subsequent ionization, e.g., moving-belt and particle-beam interfaces, (b) direct ionization from the (miniaturized) effluent stream, e.g., continuous-flow fast atom bombardment interfaces, and (c) nebulization of the column effluent into either an atmospheric-pressure or a reduced-pressure region, desolvation of the droplets, followed by either gas-phase chemical ionization or ion evaporation, e.g., thermospray, electrospray, and heated-nebulizer APCI interfaces. In practice, the interface chosen imposes limitations to both the applicable LC methodology (flow-rate and solvent composition) and the ionization technique applicable in MS.

In terms of flow-rate, only thermospray and the

heated-nebulizer APCI are compatible with conventional LC column technology (flow-rates of 1–2 ml/min). Electrospray and particle-beam interfaces are preferentially used with flow-rates between 0.1 and 0.5 ml/min, while continuous-flow fast atom bombardment (CF-FAB) is operated at flow-rates in the 1–15 μl/min range, which is compatible with packed microcapillary columns (<0.5 mm I.D.). Postcolumn solvent splitting can be used to reduce the effective flow-rate to the LC–MS interface, but only at the expense of sensitivity as part of the analyte is split away. The latter is not the case with ESI, where the response is virtually independent of the flow-rate and the best response–sample load ratio is achieved at low flow-rates in most commercial systems.

In terms of mobile-phase composition, most interfaces are compatible with reversed-phase chromatography, using solvent systems containing of mixture of acetonitrile or methanol and water. However, the use of nonvolatile buffers, e.g., phosphates, and additives such as ion-pairing agents, e.g., alkylsulphates and -sulphonates, is prohibited in routine LC–MS applications, although the problems imposed by such additives are generally considered less in those interfaces that apply nebulization in atmospheric-pressure regions (ESI and heated-nebulizer APCI), because the ion source can easily be cleaned without disturbing the high vacuum in the mass analyser. In electrospray LC–MS, the concentration of ionic additives to the mobile phase is preferentially kept below 10 m*M*.

A crucial step in the LC–MS coupling is the ionization of the analytes. In the particle-beam interface, the analyte has to be volatilized prior to ionization. The analyte is volatilized by flash vaporization and disintegration of the particles upon collision with the ion source surface. Thus, the volatility and thermal stability of the analyte are important constraints in the applicability of this interface. A major advantage of the particle-beam interface is that it is capable of producing conventional electron ionization and chemical ionization mass spectra.

CF-FAB comprises a set of interfaces with different designs, in which FAB is used for analyte ionization. It provides soft ionization of polar analytes, resulting in protonated or deprotonated molecules with little fragmentation. The addition of a matrix solvent, usually glycerol, to the mobile phase is required. This can be performed either precolumn, where it may affect the chromatographic behaviour (pressure, retention), or postcolumn, where it will result in significant effluent dilution, as it cannot be added in a pure form. CF-FAB interfaces are relatively easy to implement on modern magnetic sector instruments equipped with a FAB gun, but considered less efficient on quadrupole instruments.

The implementation of thermospray, electrospray, and heated-nebulizer APCI interfaces require major modifications to the mass spectrometer as a special (atmospheric-pressure) ion source must be installed. Although such a modification can be performed in a few hours, it prevents the instrument from being rapidly changed from GC–MS and probe work to LC–MS and back. However, it is currently generally accepted that a dedicated mass spectrometer is required for LC–MS with these interfaces and such systems now are commercially available as benchtop LC detectors.

The working principles of thermospray, electrospray, and heated-nebulizer APCI interfaces are similar. The most important difference is in the way nebulization of the column effluent is achieved. In an electrospray interface, a high electric field at the spray needle is used to disrupt the liquid stream into small droplets. In order to allow the use of higher flow-rates in electrospray, the process is generally pneumatically assisted ('ionspray'). In a heated-nebulizer APCI interface, the liquid is disintegrated in a concentric pneumatic nebulizer by means of a high coaxial nitrogen flow and subsequent droplet evaporation in a heated quartz tube, while in thermospray the disrupting gas is the expanding vapour produced by evaporation of the liquid in a narrow-bore tube. ESI and heated-nebulizer APCI operate in an atmospheric-pressure ion source. Schematic diagrams of typical nebulizer probes for ESI and APCI are given in Fig. 1, while the general set-up of an atmospheric-pressure ionization ion-source and interface is shown in Fig. 2.

Two modes of ionization are applied in combination with these interfaces, i.e., solvent-mediated chemical ionization induced by electrons from a heated filament or a discharge electrode (applied with thermospray and heated-nebulizer APCI inter-

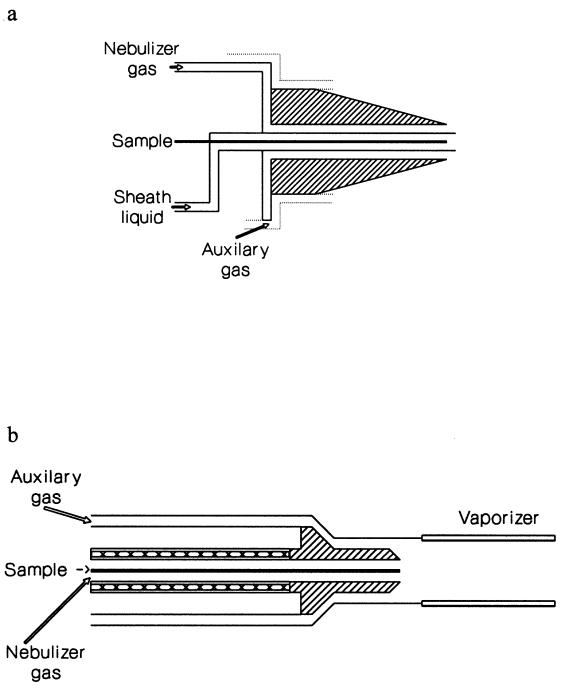


Fig. 1. Schematic diagram of (a) an electrospray probe tip, and (b) a heated-nebulizer APCI spray probe.

faces), or a field-induced ionization process, usually called ion evaporation (applicable with all three interfaces). Compared to conventional chemical ionization, applicable with a particle-beam interface, the degrees of freedom in influencing the ionization and fragmentation by selection of the reagent gas are less in the solvent-mediated chemical ionization, as the reagent gas results from the mobile phase, which in turn is determined by the chromatographic separation. Both modes of ionization provide soft ionization, resulting in cationized molecules, e.g., $[M+H]^+$, $[M+NH_4]^+$, or $[M+Na]^+$, in positive-ion mode, or anionized molecules, e.g., $[M-H]^-$, or $[M+OAc]^-$ in negative-ion mode. In general, no fragmentation is observed, unless the ions are somewhat accelerated by a potential difference in the transition area between the high-pressure ion source and the high-vacuum mass analyser (cf. Fig. 2). The ions are then collisionally activated by ion-molecule collisions and thereby prone to collision-induced dissociation (CID). This approach is termed in-source CID in this paper. Apparent fragmentation might also be due to thermal decomposition, e.g., in the thermospray vaporizer. The necessary application of heat in thermospray and heated-nebulizer APCI interfaces results in a preferential application of the electrospray interface for the analysis of highly labile molecules. Electrospray is also widely used in biochemical applications for the characterization of peptides and proteins.

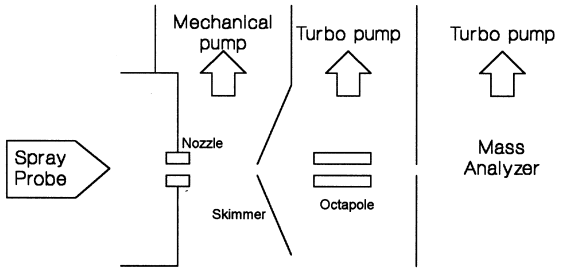


Fig. 2. Schematic diagram of an atmospheric-pressure ionization source and interface.

Although all the LC–MS interfaces discussed can, and have been, fitted on high-resolution magnetic sector instruments as well as other types of mass analysers, most of the LC–MS work is performed using (triple) quadrupole instruments. Ease of operation, cost, vacuum requirements are some of the more important reasons for this.

In choosing an appropriate LC–MS interface for a particular problem, the first principal choice is between interfaces providing electron ionization and solvent-independent chemical ionization and those providing solvent-mediated chemical ionization or ion evaporation. In the latter case, soft ionization is achieved and structural information can only be obtained by applying in-source CID or tandem mass spectrometry (MS–MS). When electron ionization is desired, the particle-beam interface is the only available choice. Otherwise, ESI and/or heated-nebulizer APCI are the techniques of choice. By estimation, 95% of the LC–MS work is currently performed with these two interfaces. The choice between ESI and heated-nebulizer APCI primarily depends on the nature of the analytes (ionic in solution, thermolability), on the sample size, and on the LC column inner diameter selected.

2.2. Important parameters of LC–MS interfaces

From the previous considerations, it may be

concluded that the various LC–MS interfaces comprise complex technology. The performance of each interface is determined by a variety of experimental parameters, which are often interrelated.

The performance of the particle-beam interface [5,6] appears to be primarily dependent on the nebulization of the column effluent, the formation of the analyte particles, the removal of the solvent vapour, and the particle disintegration prior to the ionization. With respect to the nebulization, the formation of a monodisperse aerosol is important. In some cases, improvements in the signal-to-noise ratio and linearity can be achieved by the use of various mobile-phase additives, e.g., ammonium acetate or oxalate, that may enhance particle formation and/or act as carriers [7,8]. This carrier effects is analyte dependent in a not-understood way. None of the described additives results in an improvement for all samples tested.

The influence of the various experimental parameters determining the performance of the thermospray interface [9,10] has been studied most extensively, because it was the most widely used LC–MS interface between 1987 and 1992. Beside by the mobile-phase composition, the liquid flow-rate and the nature of the analytes, the performance is determined by a number of interrelated parameters such as the vaporizer temperature, the ion source temperature, and the repeller potential, while the source contamination and the condition of the vaporizer also play a important role [9–12]. Two designs of thermospray were commercially available, which differ especially in the way the vaporizer is controlled: while with one design the stated vaporizer temperatures are in the range of 100–130°C, measured downstream of the vaporizer capillary, with the other design they are in the range of 200–250°C, measured at the vaporizer exit tip. The general trends indicated below are valid irrespective the design used.

With respect to the solvent composition, the percentage of organic modifier must be kept below 40% for thermospray buffer ionization. At higher percentages of organic modifier either filament-on or discharge-on modes must be applied. Ammonium acetate or formate are most frequently used as the electrolyte for the thermospray ionization, usually in concentrations of 50–100 m*M*.

The vaporizer temperature is primarily determined by the mobile phase composition and flow-rate. It must be adjusted to obtain an almost complete evaporation of the column effluent inside the vaporizer capillary, resulting in a supersonic jet of vapour and small droplets expanding into the ion source. Thus, with a particular solvent composition optimum vaporizer temperatures are similar for most compounds. However, with thermolabile compounds lower vaporizer temperature must be applied in order to avoid thermal decomposition of analytes. This may be an approach to achieve more structural information, although unexpected reactions may take place in thermal decomposition. Furthermore, optimization of the ion-source temperature can be useful to avoid thermal decomposition of analytes or to reduce source contamination.

In the application of CF-FAB interfaces [13,14], it is important to optimize the supply rate of glycerol as matrix solvent and to adjust the temperature of the ion source which provides the heat required for the evaporation of solvents from the FAB target. A stable equilibrium between solvent supply, evaporation and removal to the wick must be achieved, resulting in a uniform film of liquid on the target or an even distribution on the frit [13–15]. Solvents containing 1–10% of glycerol are often used. For positive-ion detection, acidic mobile phase are used, containing acetic acid or trifluoroacetic acid. Ammonium acetate can be applied as buffer in the mobile phase.

Investigation of the influence of the experimental parameters on the performance of the electrospray interface is mainly focused on its most important application area, the analysis of proteins [16–18]. In general, a needle potential of 3–5 kV is applied. The needle potential should be lower in the negative-ion mode, because of the occurrence of discharges, unless electron scavengers like SF_6 or chlorinated solvent additives are used. An important parameter in the formation of a stable electrospray is the conductivity of the liquid. For stable electrospray performance, the electrolyte concentration should be kept as low as possible, preferably below 10 m*M*. The stability of electrospray can also be enhanced by the use of solvent with low surface tension, e.g., by means of a coaxial sheath flow of 50% 2-propanol in water. In ESI, the ionization is primarily based on ion evaporation processes requiring preformed ions

in solution. Ions in solution are the result of liquid phase protonation of bases or deprotonation of acids. Therefore, the adjustment of the solvent pH is important in the analysis of neutral molecules that can be (de)protonated. The signal may be suppressed by certain mobile phase additives, e.g., by basic or acidic additives in positive-ion and negative-ion mode, respectively, although the use of trifluoroacetic acid (TFA) in positive-ion mode may also result in signal suppression due to ion-pairing in the droplets generated by electrospray, thereby reducing the ion evaporation efficiency. Although current commercial electrospray interfaces allow the introduction of flow-rates as high as 1 ml/min, a flow-rate in the range of 0.1–0.2 ml/min is considered more favourable.

While in ESI the analyte ions are generated by ion evaporation from the highly-charged droplets produced in the nebulization, in APCI electrons from a corona discharge electrode are used as a primary source of ionization, initiating a chain of ion–molecule reactions leading to (de)protonated solvent constituents which will ionize the analyte molecules by proton-transfer reactions common in chemical ionization. The heated-nebulizer APCI interface appears to be extremely robust, as no really critical experimental parameters appear to be present, although for certain analytes particular experimental conditions may be detrimental, e.g., the presence of ammonium acetate in the analysis of various pesticides [19], or the presence of acidic mobile-phase constituents in the negative-ion mode [20].

With the interfaces that rely on solvent-mediated chemical ionization and/or ion evaporation (thermospray, heated-nebulizer APCI, and ESI), the performance in the analysis of particular analytes is highly dependent on the nature of analyte. The ionization is determined by the solvent conditions. In an ammonium acetate containing mobile phase, as for instance is used in thermospray, the reagent gas in chemical ionization is determined by ammonium related ions. As a result, only those analytes are ionized and give a response that have equal or higher gas-phase proton affinities than ammonia.

2.3. Related techniques

The various LC–MS interface techniques have also been applied to achieve the on-line combination of SFC or CZE and MS. The most widely applied LC–MS interfaces in SFC–MS are direct-fluid introduction, moving-belt, thermospray and APCI interfaces [21], while in CZE–MS electrospray is the interface of choice. Because of the low solvent flow-rates in CZE, either a coaxial coupling with the use of a sheath liquid [22] or a sheathless micro- or nano-electrospray device is applied [23].

3. Sulphonamides

3.1. Introduction

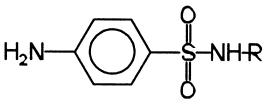
Sulphonamides, N-derivatives of 4-amino-benzenesulphonamide, comprise a large group of synthetic antibacterial compounds. The general structure and some typical examples of sulphonamides, relevant to the present discussion, are given in Table 1. They are widely used in farm animal feedstuff and fish cultures as veterinary drugs for prophylactic and therapeutic purposes. Furthermore, sulphonamides act as growth promoting substances. European Union (EU) regulations as well as US regulatory bodies have set a maximum residue limit of 100 μg/kg. The regulatory level in milk is 10 μg/kg. Sulphonamide residues in food are of concern because of their potential carcinogenic character and the possibility of development of antibiotic resistance in humans. While the sulphonamides have been used in human medicine against a wide variety of microbes, the current use is primarily in the treatment of urinary tract infections.

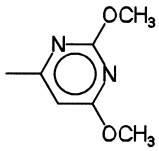
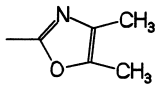
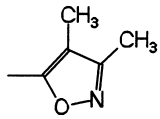
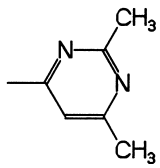
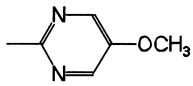
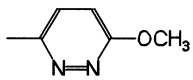
Analytical methods for the analysis of sulphonamide residues are mostly based on LC with UV detection, while GC–MS after preparative LC and/or liquid–liquid extraction and diazomethane derivatization is applied for confirmation purposes. The need for extensive sample pretreatment and analyte derivatization makes GC–MS a rather time-consuming method, thereby clearly indicating the importance of an LC–MS method for confirmation.

3.2. Residue analysis of sulphonamides

LC–MS is primarily applied in the analysis of sulphonamide residues in animal tissues and milk for

Table 1
Structures of relevant sulphonamides



Compound	Structure of R-group	M_r
Sulfadimethoxine		310
Sulfamoxole		267
Sulfisoxazole		267
Sulfisomidine		278
Sulfamethazine		278
Sulfamethoxy-pyridazine		280

human consumption. However, the first application of LC–MS in this field dates from 1982 and is concerned with the detection of residues of sulfadimethoxine in urine and plasma from racehorses [24]. The LC–MS technique applied, nebulization of the column effluent into an APCI source, was 10 years ahead of its time.

The packed-column SFC–MS analysis of ten sulphonamides via moving-belt interfacing is described by Perkins et al. [25]. The system is applied to the analysis of porcine kidney extracts containing 3.3 mg/kg sulphamethazine. With an injection volume of 8 μl, ~26 ng is injected. With the in-line UV detector the sulfamethazine peak is clearly detected, while mass chromatography at the fragment ion m/z 214 was required to detect the peak in SFC–MS. The corresponding background subtracted mass spectrum still suffers from matrix interferences.

The analysis of 20 sulphonamides by means of LC–MS with an ionspray (pneumatically assisted electrospray) interface and UV photo-diode array detection (UV-PDA) was reported by Pleasance et al. [26]. The LC separation was achieved with a 20-min linear gradient of 5–40% acetonitrile in water containing 0.1% TFA with a flow-rate of 200 μl/min on a 2.1-mm I.D. 5-μm Vydac 201TP column. The

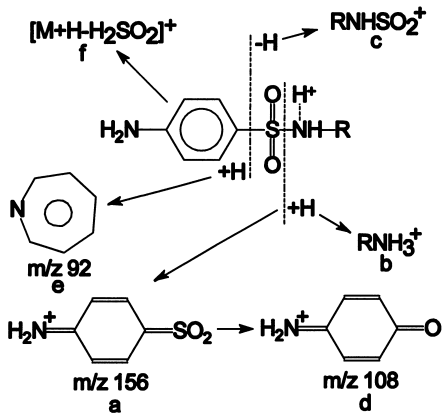


Fig. 3. Fragmentation scheme of protonated sulphonamides in collision-induced dissociation, as proposed by Pleasance et al. [26].

quantitative performance of the system is tested in column-bypass injection mode, showing acceptable linearity and an absolute detection limit of 10 pg in selected ion monitoring (SIM). Product-ion MS–MS is needed to obtain structural information. The general fragmentation scheme proposed for protonated sulphonamides in CID is shown in Fig. 3. The fragmentation yields a number of group-specific ions, i.e., at m/z 156, 108 and 92, as well as a number of compound-specific ions. The detection of the ions (a) through (f), indicated in Fig. 3, in the mass spectrum of sulfamethazine is shown in Fig. 4. From the observation of isotope peaks in this spectrum, it may be concluded that lower than unit-mass MS_1 resolution was applied. The method is applied to the determination of sulfadimethoxine in salmon flesh. The compound was detected in the salmon samples by LC–UV at levels ranging from 25 to 1800 μg/kg. In LC–MS with SIM, good linearity for

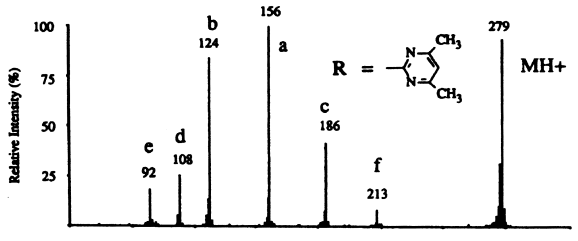


Fig. 4. Product-ion mass spectrum of sulfamethazine (from [26], ©1991, Elsevier Science).

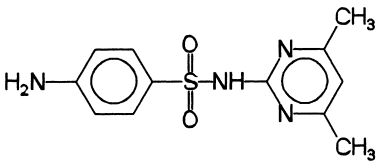
sulfadimethoxine was achieved in salmon extracts in the range of 0.08 to 80 μg/ml. A detection limit of 0.04 μg/ml was estimated. Data for the LC–MS detection of 25 μg/kg in a salmon extract are shown. From the paper, it remains unclear whether confirmation by MS–MS was actually performed for the salmon extracts.

The analysis of 20 sulphonamides by nanoscale LC–MS and CZE–MS via coaxial electrospray interfacing was reported by Perkins et al. [27]. Both methods can be used to obtain on-line full-scan mass spectra at the low picomole level. However, because the injection volume in nanoscale separations is small, the method is only applicable to residue analysis after rigorous preconcentrating sample pretreatment. In the mass spectra of the sulphonamides both protonated and sodiated molecules are detected. Sulphonamide mass spectra obtained by in-source CID were studied as well. It is demonstrated that structural informative fragments, similar to the ones shown in Fig. 3, can be induced with only a four-fold loss in base-peak intensity. The relative intensities of three compound-specific ions for sulfamethazine, i.e., m/z 213, 186 and 124, and the three class-specific ions indicated above are compared to similar data reported by others in Table 2.

The thermospray LC–MS analysis of 10 sulphonamides was reported by Abián et al. [28]. Protonated molecules are observed for most sulphonamides. Useful fragmentation is obtained by CID in MS–MS (see Table 2). Despite the higher collision energy, the yield of fragment ions is lower than, for instance, that observed by Pleasance et al. [26]. Detection limits of 20 ng in full-scan mode and 2 ng in selected reaction monitoring were reported. On-line solid-phase extraction (SPE) for sample treatment and preconcentration was applied in the analysis of milk samples. The method allows multiresidue analysis at 10 μg/kg level in milk. Data with excellent signal-to-noise ratio are shown 20 μg/kg in SIM. According to the authors, LC–MS–MS offers no advantages over LC–MS in this case, thereby ignoring the need for confirmation.

Multiresidue analysis of sulphonamides in milk by LC–MS via a heated-nebulizer APCI interface was demonstrated by Doerge et al. [29]. Using off-line SPE of spiked milk samples, six sulphonamides were analysed by LC–MS. Estimated determination limits

Table 2
CID mass spectra for sulfamethazine (M_r 279), reported by various authors [26–28,30] (cf. Fig. 3)



m/z 213 f	*m/z* 186 c	*m/z* 156 a	*m/z* 124 b	*m/z* 108 d	*m/z* 92 e	Conditions	Ref.
7	42	100	85	25	20	MS–MS, 35 eV	[26]
1	24	12	8	2	2	In-source, 25 V	[27]
8	87	79	100	45	65	In-source, 40 V	[27]
1	9	30	14	5	9	MS–MS, 50 eV	[28]
Nd	92	28	32	Nd	Nd	In-source, 51 V	[29]
6	48	100	68	37	Nd	MS–MS, 35 eV	[30]

range from 0.25 to 2.5 μg/kg in milk. Linear calibration between 1.25 and 20 μg/kg was observed. In-source CID was applied to induce fragmentation for confirmatory purposes. The relative intensities of the fragment ions showed good reproducibility. Only three fragment ions were detected for sulfamethazine, i.e., the class-specific ion at *m/z* 156 and the compound-specific ions at *m/z* 186 and 124 (see Table 2). Scanning below *m/z* 100 is not advisable in APCI, because of the presence of intense reagent gas ions.

Two multiresidue methods for the analysis of sulphonamides in spiked meat and blood samples were described by Kristiansen et al. [30], based on thermospray ionization and MS–MS. In both the LC–MS method and the column-bypass method, precursor-ion scanning on the group-characteristic ion at *m/z* 156 is applied. The CID mass spectrum obtained for sulfamethazine is compared to those of others in Table 2. The detection limits in LC–MS are slightly worse than in column-bypass injections, but still well below the maximum residue limit of 50 μg/kg in meat. While the chromatographic analysis time is 9 min, 40 to 60 samples can be analysed per hour in the column-bypass mode. Representative data for the calibration plots of sulfamethazine in meat by both methods are given in Table 3.

From the data reviewed so far, it may be concluded that the determination and confirmation of sulphonamides in animal tissue and milk samples does not pose serious problems. Obviously, robustness of the procedure is an important topic in deciding whether a method is suitable for regulatory purposes. Confirmation criteria in regulatory protocols often require within-20% reproducibility of the ion intensity ratios between diagnostic ions. From the data in Table 2, it may be concluded that this reproducibility is not achieved between instruments.

The determination of four sulphonamides and their N^4-acetyl metabolites in spiked swine muscle tissue by thermospray LC–MS in SIM mode was reported by Balisz et al. [31]. The reported limits of quantification are 100 μg/kg for most sulphonamides. The confirmation of sulphonamide residues, especially

Table 3
Results of the analysis of sulfamethazine in spiked meat samples analysed by thermospray LC–MS–MS or column-bypass MS–MS [30]

Spike level (μg/kg)	LC–MS–MS		Column-bypass MS–MS	
	Found (μg/kg)	±S.D.	Found (μg/kg)	±S.D.
7	5.6	6.9	12.8	5.7
23	17.9	6.7	30.9	5.5
115	130.5	5.7	167.3	4.8
500	510.5	11.4	492.2	9.2

sulfamethazine, sulfadiazine, sulfamerazine and sulfaquinoxaline, in kidney tissue by means of electrospray LC–MS and LC–MS–MS was reported by Porter [32]. In this study, LC–MS–MS was preferred over LC–MS with in-source CID because the latter method suffers from matrix interferences in SIM, especially at low m/z, and significant loss in response was found to occur in in-source CID. Other applications are concerned with the determination of sulfadimethoxine and sulfamethazine residues in animal tissue by thermospray LC–MS [33], the determination and confirmation of sulfadiazine residues in salmon flesh by LC–APCI-MS [34]. The determination of sulfamethazine, sulfothiazole (M_r 255), abendazole (M_r 265) and its metabolite abendazole sulfone (M_r 297) in honey and goats milk by pneumatically-assisted electrospray and selected reaction monitoring (SRM) was reported by Casetta et al. [35]. For abendazole and its metabolite, good signal-to-noise ratio were demonstrated for goats milk samples spiked with 143 μg/kg abendazole.

A multiresidue determination method for 21 sulphonamides in milk was described by Volmer [36]. Separation of all compounds was achieved in only 6 min on a 50×4.0 mm I.D. column packed with 3 μm I.D. ODS-AQ particles using a fast gradient program (10–45% acetonitrile in 0.1% aqueous formic acid in 7 min). While the column was run at a flow-rate of 1 ml/min, a postcolumn split was used to deliver 90 μl/min to the electrospray interface. A three-step approach is adopted; (a) prescreening and possibly confirmation by precursor-ion scan and SRM experiments using the group-specific ions at m/z 156, 108 and 92, (b) quantitation of identified target compounds using SIM of their protonated molecules, and (c) if necessary, further confirmation by time-scheduled SRM using compound-specific ions. It was found that the optimum collision-offset voltage for the most abundant group-specific ion (m/z 156) depended on the nature of the R-substituent (see Table 1), although the optimum was rather broad. A collision-offset of 21 V was used in the first step of the analytical procedure. The method detection limit in the SIM quantitation procedure was between 0.2 and 0.5 μg/kg for most compounds in milk. As an example, results of the prescreening and the quantitation of five sulphonamides, i.e., sulfadiazine, sulfapyridine, sulfamethazine, sulfisoxasole and sulfaquinoxaline, spiked at 1 ppb in milk, are shown in Fig. 5.

In an extension of the latter study, reported by Bateman et al. [37], CZE–MS and CZE–MS–MS are applied in the multiresidue analysis of a large number of sulphonamides in milk samples. The target compounds are detected and identified at low ppb-levels using precursor-ion scan and selective reaction monitoring, as described above. Special attention is paid to three pairs of isomeric sulphonamides, i.e., sulfanoxole and sulfisoxazole, sulfisomidine and sulfamethazine, and sulfamethoxypyridazine and sulfameter, which only differ in the position of N- and/or O-atom in the ring of the R-group (see Table 1). These pairs could be discriminated by means of a combination of in-source CID and MS–MS on the compound-specific RNH_3^+ product-ion (see Fig. 3) generated in in-source CID. The results are summarized in Table 4.

3.3. Related compounds; new leads

Electrospray MS and MS–MS was also applied in structural analysis of potentially bioactive sulphonamides of the types 1-allyl-3-arylsulphonylthioureas, 1-allyl-2-alkyl-3-aryl-sulphonylisothioureas, and 1-allyl-3-alkyl-2-aryl-sulphonylguanidines [38]. Fragmentation patterns of these compounds were elucidated by combination of MS–MS and in-source CID.

4. β-Lactam antibiotics

4.1. Introduction

The β-lactam antibiotics comprise several classes of compounds, among which the cephalosporins and the penicillins are most important. Both classes contain bulky side chain attached to the 7-aminocephalosporanic acid or 6-aminopenicillanic acid nuclei, respectively. The general structures of these two classes, together with a number of typical examples, relevant to the present discussion, are given in Table 5. The penicillins are widely used for their antimicrobial activity against both gram-positive and gram-negative organisms. Because of their use in veterinary medicine for the treatment of

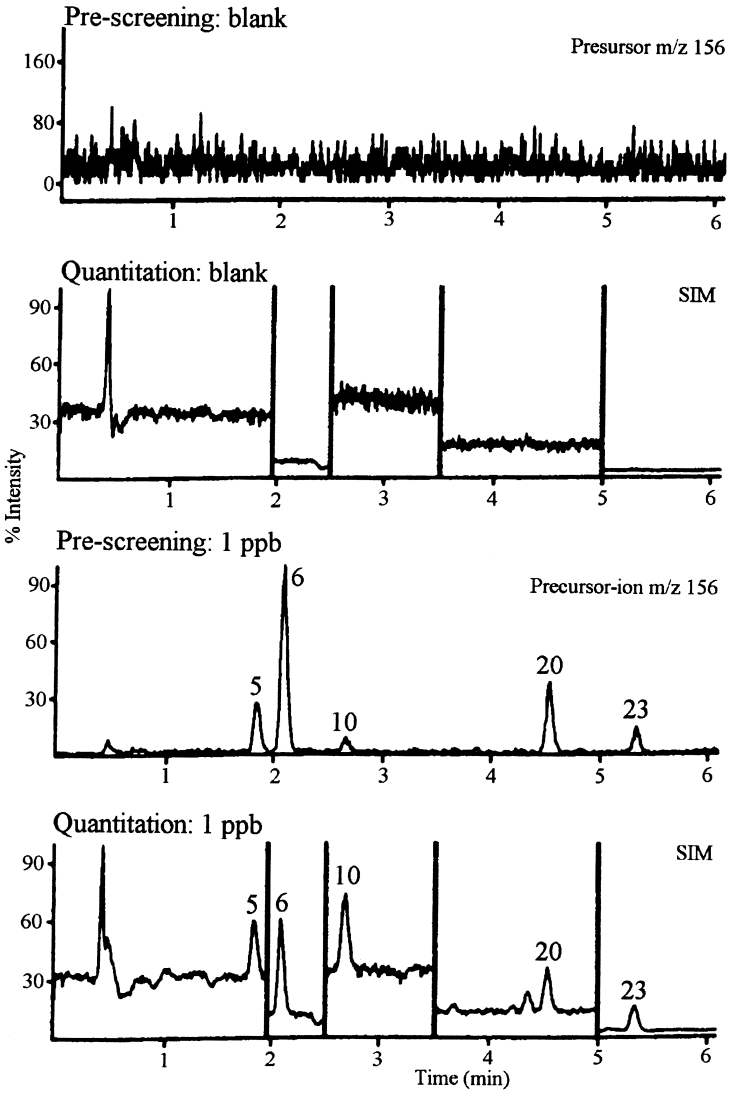
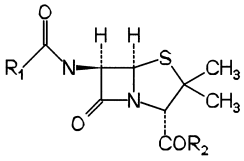
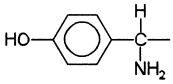
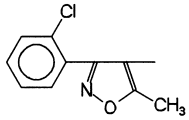
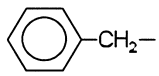
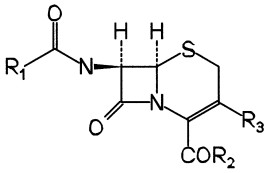
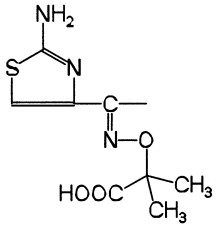
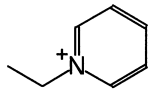


Fig. 5. Results of the prescreening and quantitation of sulphonamides at 1 μg/kg level in milk by electrospray LC–MS–MS. Peak identification: 5=sulfadiazine, 6=sulfapyridine, 10=sulfamethazine, 20=sulfisoxasole and 23=sulfaquinoxaline. (from [36], ©1996, Wiley).

Table 4
In-source CID-MS–MS procedure used in combination with the CE–MS analysis of isomeric sulphonamides [37]

Compound	$[M+H]^+$	Product from in-source CID	Product-ions in MS–MS (**base**)	Detection limit (fg)
Sulfamoxole	268	113	**70**, 78	30
Sulfisoxazole	268	113	**68**, 42, 44, 55, 71, 96	80
Sulfisomidine	279	124	**83**, 66, 107	8
Sulfamethazine	279	124	**67**, 80, 107	15
Sulfamethoxy-pyridazine	281	126	**111**, 81, 83	400
Sulfameter	281	126	**83**, 85, 111	400

Table 5
Structures of β-lactam antibiotics

Penicillin			
	R_1	R_2	
amoxicillin		—OH	
cloxacillin		—OH	
penicillin G		—OH	
Cephalosporin			
	R_1	R_2	R_3
ceftazidime		$—O^-$	

bacterial infections, residue analysis in animal food products and milk is an important topic. The β-lactams have limited stability, especially in organic solvents like methanol and acetonitrile, which may hamper accurate trace analysis. In vitro solvent degradation of cloxacillin and tentative identification of the degradation products was reported by Tyczkowska et al. [39].

Analytical methods for the β-lactams are either based on bioassays or on LC separation combined with UV, UV-PDA, or fluorescence detection. Confirmation by GC–MS is only possible after derivatization. LC–MS using a soft ionization technique can be considered as a useful alternative for confirmation purposes. Early studies comprise the use of a thermospray interface as a flow reactor for in situ thermal degradation studies on β-lactam antibiotics, i.e., cefixime, ampicillin and penicillin V, with on-line MS detection of the reaction products [40] and the identification of metabolites of ampicillin [41,42]

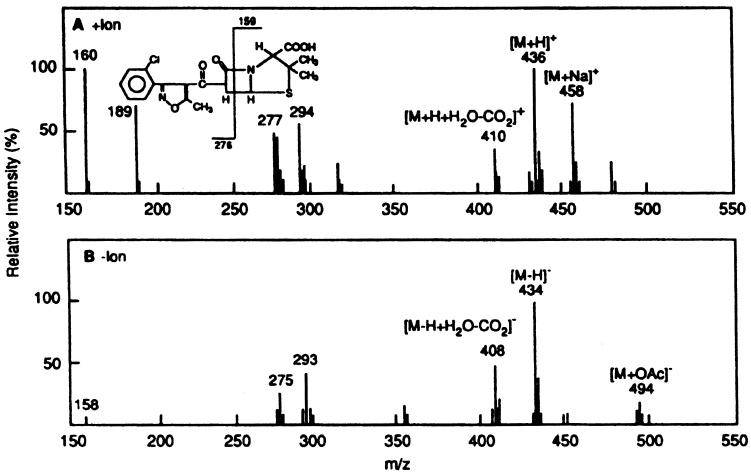


Fig. 6. Positive-ion and negative-ion thermospray mass spectra of the β-lactam antibiotic cloxacillin (from [45], ©1991, Elsevier Science).

and amoxicillin [41] by thermospray LC–MS in combination with FAB-MS–MS. In the latter case, characteristic fragment ions (see Fig. 6) in thermospray ionization of the β-lactam antibiotics are used to screen for unknown metabolites, which are subsequently identified by FAB-MS–MS. Thermal isomerization during the discharge-on thermospray analysis of benzylpencillin was investigated by Ohki et al. [43].

4.2. Residue analysis of β-lactam antibiotics

The development of an analytical method for the determination of penicillin G in bovine milk by LC with UV-PDA detection and subsequently confirmation by thermospray mass spectrometry was described by Tyczkowska et al. [44]. Penicillin G is administered in salt form with procaine, potassium or sodium as counter ion. Subsequently, a similar method was developed for cloxacillin, ampicillin and amoxicillin [45]. In the LC–UV method, a mobile phase containing alkylsulphonates, triethylamine and phosphoric acid, was applied, which is not compatible with LC–MS. Therefore, a modified phase system was applied in LC–MS, consisting of 2-propanol in 0.2 *M* aqueous ammonium acetate containing 2–5% acetic acid. Pulsed positive/negative-ion detection was performed. Typical thermospray mass spectra of cloxacillin are shown in Fig. 6. The penicillin show characteristic fragmentation under thermospray conditions, i.e., an $[M+H-26]^+$-ion attributed to thermally-induced hydrolytic opening of the β-lactam ring followed by the loss of CO_2 [40], and two characteristic protonated fragments resulting from opening and subsequent cleavage of the β-lactam ring (see Fig. 6). The same fragments are observed in negative ion mode. The m/z 160 ion in positive-ion mode can be considered as a group-specific fragment ion, because the R_2-group in the penicillins in most cases is –OH (see Table 5). Detection limits were estimated for UV-PDA detection at 10 μg/kg for penicillin G in milk [44] and 50–100 μg/kg for cloxacillin, ampicillin and amoxicillin in milk [45], and 100 μg/kg for thermospray LC–MS in SIM.

The same analytical strategy was applied to the determination of ceftazidime, a cephalosporin β-lactam antibiotic (see Table 5), in dolphin serum [46]. The main fragment ions of the cephalosporin again result from opening and cleavage of the β-lactam ring, i.e., similar to the penicillins, while no intact protonated molecule is observed in this case. Interestingly, the negative-ion mode was found to be more sensitive than the positive-ion mode for the cephalosporins, while the opposite was true for the penicillins. While in UV-PDA detection limits in the range of 50 μg/kg were achievable, the thermospray method was significantly less sensitive.

The behaviour of, amongst others, penicillin G and cloxacillin in positive-ion ESI with and without in-

source CID was studied by Voyksner and Pack [47]. At low potential, protonated penicillin molecules were observed without any fragmentation, while at high potentials structure informative fragmentation, e.g., due to opening and cleavage of the β-lactam ring, was observed. ESI of eight penicillins and nine cephalosporins was reported by Parker et al. [48] using nanoscale separation methods coupled to an electrospray source fitted onto a magnetic sector instrument. Under these conditions, the characteristic penicillin and cephalosporin fragment ions, due to opening and cleavage of the β-lactam ring, are observed next to the protonated molecule, even without deliberate in-source CID. The characteristic thermally-induced fragment at $[M+H-26]^+$, observed in thermospray, is not observed under ESI conditions. Full-scan analysis in human serum extracts spiked at 100 mg/kg is reported, which given the small injection volume corresponds to 60 pmol.

The determination of penicillin G, ampicillin, amoxicillin, cloxacillin and cephapirin by electrospray LC–MS using a 2 mm I.D. column and a 70:1 postcolumn split was described by Straub and Voyksner [49]. The influence of solvent composition was evaluated. The estimated detection limit in the simultaneous determination of these five compounds in bovine milk was 100 μg/kg. Preliminary research was also reported to replace the 2 mm I.D. column by a 320 μm I.D. packed capillary column. As a continuation of this research, a fast micro-LC method based on perfusion LC and combined with ultrasonically-assisted electrospray MS was developed for the residue analysis of six β-lactam antibiotics, i.e., penicillin G, ampicillin, amoxicillin, cephapirin, cloxacillin and ceftiofur, at 10 μg/kg level in milk [50]. Ultrafiltration through a 10 kDa cut-off filter was used as initial clean-up. On-column focusing of 75 μl sample was applied. The total chromatographic analysis time, including the on-column focusing, fast perfusion LC on a 320 μm I.D. packed capillary column operated at 50 μl/min, and reequilibration after gradient elution was only 13 min, while with a preliminary method on a conventional packed capillary column the analysis time was 40 min. All six compounds could be analysed within 6 min using this approach, as demonstrated in Fig. 7.

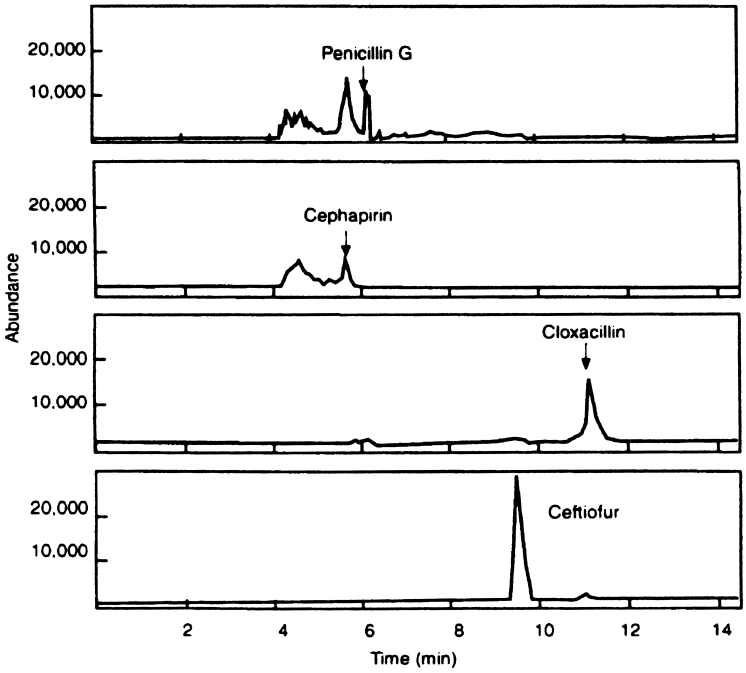


Fig. 7. Determination of β-lactam antibiotics at 10 μg/kg level in milk by perfusion LC coupled via ultrasonically-assisted electrospray to MS. (from [50], ©1994, American Chemical Society).

Linear calibration was possible in the range of 10–1500 μg/kg in milk. Control samples, spiked at 30 or 300 μg/kg, were measured to within 15% of their spiked value. These methods based on ESI were subsequently described as a simultaneous multiresidue analysis of various β-lactam antibiotics in bovine milk [51].

In another study, the development of a confirmatory assay for the simultaneous detection of five penicillins, i.e., oxacillin, cloxacillin, dicloxacillin and the penicillins V and G, in muscle, kidney and milk by electrospray LC–MS was reported [52]. Nafcillin was applied as internal standard. SIM detection on at least four ions per compound was applied for confirmation. Detection limits were 25–100 μg/kg in meat and 2–10 μg/kg in milk.

The use of particle-beam LC–MS in combination with negative-ion chemical ionization (NICI) for the confirmation of oxacillin, cloxacillin and dicloxacillin residues in bovine muscle tissue was also reported [53]. Five fragment ions per compound were monitored to achieve confirmation of these β-lactams at the 300 μg/kg level. The primary fragmentation of the β-lactams under NICI is similar to that found by other soft ionization techniques, i.e., opening and cleavage of the β-lactam ring with charge retention on either side of the molecule.

The clinical and forensic analysis of 24 cephalosporin β-lactam antibiotics via on-line SPE and a packed capillary column coupled to a frit-FAB interface was described by Kobayashi et al. [54]. Either diethanolamine or glycerol was applied as matrix. Under these conditions (de)protonated molecules as well as the common fragment ions for most cephalosporins are detected. Typical absolute detection limits are 10–50 ng injected on-column, which appeared sufficient for this type of application.

4.3. Related compounds, new leads

An important aspect of bacterial resistance to the action of β-lactam antibiotics is the hydrolysis of the lactam ring by β-lactamases. Molecular mass determination by electrospray MS on β-lactamases after treatment with for instance carbenicillin shows shifts that correspond to the formation of an acyl enzyme intermediate [55]. Subsequently, these mass shifts were investigated in more detail using 6β-bromo- and 6β-iodopenicillanic acid as enzyme inhibitors. With β-lactamases from *Entereobacter cloacae* and *Bacillus cereus*, a mass shift of 199 was observed after treatment with the inhibitor, which may be due to the production of an enzyme-bound dihydrothiazine derivative [56].

5. Quinoline antibiotics

5.1. Introduction

Quinolone and fluoroquinolone antibiotics are a group of relatively new highly-potent, synthetic antibiotics compounds, derived from 3-quinolonecarboxylic acid. Some representative structures are given in Fig. 8. The compounds were initially applied in the treatment of urinary tract infections, but now have a broad-spectrum application in the treatment of both human and veterinary diseases. Various methods for residue analysis in food products, especially fish, have been reported.

5.2. Residue analysis of (fluoro)quinolone antibiotics

A thermospray LC–MS method for the residue analysis of nalidixic acid, oxolinic acid and piromidic acid in fish tissue was reported by Horie et al. [57]. The measurement was based on SIM of the protonated molecules in positive-ion mode; no fragmentation was observed under thermospray ionization. Given the absolute detection limits in the 0.1–1 ng range, the minimum concentration detectable in fish samples after pretreatment is 10 μg/kg.

Confirmation of residues of the fluoroquinolone danofloxacin in chicken and bovine liver by means of micro-LC–MS–MS via an electrospray interface was described by Schneider et al. [58]. The com-

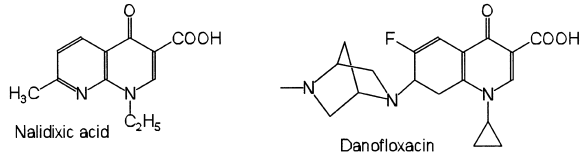


Fig. 8. Structures of the (fluoro)quinolone antibiotics nalidixic acid and danofloxacin.

pound is analysed with a mobile phase of 80% acetonitrile in 0.1% aqueous TFA on a 1-mm I.D. base-deactivated silica-based C_{18} column, operated at 50 μl/min. The product-ion spectrum of the protonated molecule at m/z 358 shows two fragments, i.e., at m/z 340 due to the loss of water and at m/z 255 due to the loss of water and the loss of N-methylpyrrolidine from the piperazine moiety. SRM is applied in the analysis of the liver samples. The peak ratio of the two fragment ions was found to be 0.16 (R.S.D. 13.57%) in liver samples spiked at 50 ppm and 0.17 (R.S.D. 19.78%) in incurred liver samples. The method allows the confirmation of danofloxacin residues down to 50 μg/kg.

The multiresidue determination of the quinolone antibiotics oxolinic acid, nalidixic acid, flumequine and piromidic acid in catfish muscle tissue by LC–MS and LC–MS–MS via a heated-nebulizer APCI interface was reported by Doerge and Bajic [59]. Two procedures were compared. First, fragmentation of the protonated molecules was induced by in source CID. All compounds show the loss of water and of CO_2 under these conditions. SIM of the protonated molecule and the two fragment ions for each compound allowed the detection in catfish muscle extracts down to 0.8–1.7 μg/kg. The intensity ratio were within 10% agreement with the ratios observed with standards. The loss of water was used in an SRM method, which yielded an estimated detection limit in catfish muscle extracts of 0.08–0.16 μg/kg.

The characterization of the fluoroquinolone antibiotics norfloxacin, enoxacin, ciprofloxacin and ofloxacin by electrospray MS was reported by D'Agostino et al. [60] with in-source CID on a magnetic sector instrument. High-resolution measurement (3000 at 10% valley definition) allowed determination of the elemental composition of the fragment ions. Typical losses observed are the loss from the protonated molecule of HF, of CO_2, of CO_2 and C_2H_5N (except for ofloxacin) and of CO_2 and C_3H_7N (except for ciprofloxacin).

The method described by Doerge and Bajic [59] was criticized by Schilling et al. [61], because in their opinion the losses of water and CO_2 are not specific enough for confirmation purpose. Therefore, an additional fragment ion, generated by CID in MS–MS, was used in the confirmation of saraflox-acin in catfish tissue, i.e., the fragment due to the loss of CO_2 and C_2H_5N, also indicated by D'Agostino et al. [60]. The confirmation is not only based on the detection of four ions, i.e., the protonated molecule and three fragment ions, but also on comparison of the percentage of relative abundance with corresponding values in reference samples. The method allowed for the detection of 200 μg/kg sarafloxacin in catfish tissue, which is well beyond the limit of 700 μg/kg aimed at in method development.

6. Other classes of antibiotics

6.1. Aminoglycoside antibiotics

The thermospray LC–MS analysis of gentamicin, a multicomponent mixture of aminoglycoside antibiotics, was described by Getek et al. [62]. Because of the small differences between the known major components of the mixture and the lack of suitable chromophores, LC–MS is one of the methods of choice. The detection of a minor impurity and the determination of the composition of various formulation was demonstrated. Detection limits were approximately 400 ng injected on-column.

The determination of the aminoglycoside antibiotics spectinomycin, hygromycin B, streptomycin and dihydrostreptomycin in bovine tissues by reversed-phase ion-pair LC coupled to pneumatically-assisted electrospray MS was reported by McLaughlin and co-workers [63,64]. Volatile ion-pairing agents (pentafluoropropionic acid and heptafluorobutyric acid) were used at a concentration of 5–20 m*M*, with the lower concentration being most favourable from the MS point-of-view. For spectinomycin the singly charged ketone hydrate at m/z 351 was the most abundant ion, while for the other three compounds doubly charged ions, corresponding to $[M+2H]^{2+}$, were detected as the most abundant ions. Absolute detection limits in SIM between 4 and 7 ng in on-column injection were reported, while data were shown for the detection of the aminoglycosides in bovine kidney extracts spiked at 20 mg/kg. The regulatory tolerance levels for these compounds are 20 μg/kg for hygromycin B and in the range of 0.1–0.5 mg/kg for the other compounds. An improved method for the compounds

mentioned above as well as neomycin B and the gentamicin C complex, implementing an improved sample pretreatment method and SRM on preferentially three product ions, was subsequently described which allows the determination of all compounds except spectinomycin in bovine kidney tissue at or below the regulatory tolerance level. Spectinomycin showed poor recovery in the sample pretreatment. Both m/z values of product-ions and their relative intensity ratios were applied in confirmation of identity.

6.2. Tetracyclines

Tetracyclines are broad-spectrum antibacterial compounds, which are applied against organisms like *Mycoplasma*, *Chlamydia* and a number of gram-positive and negative bacteria. They are widely used in veterinary medicine. Residue analysis of tetracyclines in a variety of matrices were reported: in bovine milk using particle-beam LC–MS [65], in honey using a CF-FAB interface [66], and in animal muscle and kidney using a heated-nebulizer APCI interface [67].

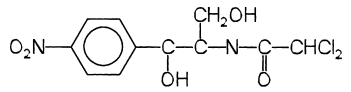
The residue analysis of tetracycline, oxytetracycline and chlortetracycline in milk by particle-beam LC–MS aims at confirmation at 100 μg/kg level [65]. The sample pretreatment comprises of ultrafiltration over a 25 kDa cut-off filter and SPE on a C_{18} cartridge. The LC separation is performed with a mobile phase of 50% acetonitrile and 20% methanol in 50 m*M* aqueous oxalic acid, which provided better sensitivity in LC–MS detection than an alternative mobile phase of 60% acetonitrile in 50 m*M* aqueous oxalic acid, which provided better separation of the tetracyclines. The confirmation was based on SIM at four ions per compound, e.g., m/z 383, 401, 426 and 444 for tetracycline, and on the requirement to have relative abundances of the three fragment ions within 15% of the values obtained by standards. The oxalic acid in the mobile phase caused skimmer clogging after 10–15 analysis. The installation of a switching valve to introduce only the relevant part of the chromatogram to the particle-beam interface cured this problem to some extent, but resulted in greater variability of the ion ratios.

The same compounds were analysed in muscle and kidney by Blanchflower et al. [67]. After extraction in a glycine–HCl buffer and SPE, gradient LC was performed with 10–90% acetonitrile in water containing 0.04% heptafluorobutyric acid, 10 m*M* oxalic acid and 10 μ*M* EDTA. The LC was coupled to an APCI interface. Under these conditions, the APCI mass spectra of tetracyclines show significant fragmentation, as demonstrated in the Fig. 9 for tetracycline. Detection limits were 10 μg/kg in muscle and 20 μg/kg in kidney. Ion ratio measurements, e.g. on m/z 410, 427 and 445 for tetracycline, are performed for confirmation. An additional problem in the residue analysis of particularly chlortetracycline is the formation of isomers, epichlortetracyclines, and the existence of ketoenol tautomerism. This results in four peaks for chlortetracycline because the isomers can be separated with the current gradient program. The sum of the isomer responses is used in quantitation, because the various isomers show different responses in APCI.

6.3. Chloramphenicol

Chloramphenicol is a broad spectrum antibiotic, which is frequently applied in veterinary medicine. Adverse reactions and side-effect in humans have been demonstrated. The maximum residue level of chloramphenicol in meat is set at 10 μg/kg by the EU.

Chloramphenicol



The applicability of thermospray LC–MS in the

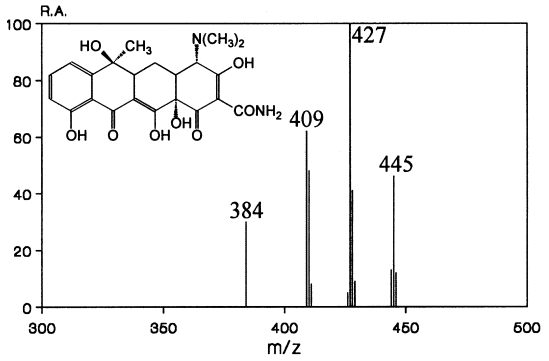


Fig. 9. APCI mass spectrum of tetracycline (redrawn from [67]).

analysis of chloramphenicol and three related compounds, which are potential metabolites of chloramphenicol, i.e., dehydrochloramphenicol, aminodehydrochloramphenicol and nitrophenylaminopropanediol, was investigated by Korfmacher et al. [68]. Under thermospray conditions, the base peak in the mass spectrum results from the loss of water from the protonated molecule, while the protonated molecule itself is detected as well.

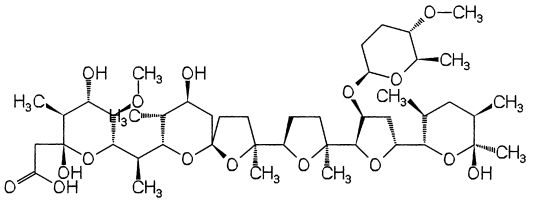
Electron-capture NICI in particle-beam LC–MS using methane as reagent gas was applied in the residue analysis of chloramphenicol and three related compounds, i.e., dehydrochloramphenicol, nitrosochloramphenicol and nitrophenylaminopropanediol, in meat by Delépine and Sanders [69]. Precision and accuracy of the method in the range of 2–20 μg/kg spiked to muscle tissue were better than 13% and ±0.8%, respectively, for chloramphenicol. Linear calibration in this range was possible using SIM of the base peak in the spectrum at m/z 322, corresponding to $M^{-\cdot}$.

An SFC–MS method for the determination of veterinary drugs, among which is chloramphenicol, was reported by Perkins et al. [70].

6.4. Polyether antibiotics

Polyether antibiotics, also called polyether ionophores, are fermentation derived biologically active compounds characterized by the presence of a carboxylic acid group and several cyclic ether units. They are able to form stable complexes with alkaline cations. They are widely used as anticoccidiosis agents in broiler chickens. A reproducible thermospray LC–MS method for confirmatory purposes was found to be difficult to develop [71].

Semduramicin:



An electrospray LC–MS for the residue analysis of the ionophore antibiotic semduramicin was described by Schneider et al. [72]. An important topic in the development of this method was the need to find a way to direct the ionization towards one cationized species instead of a variety of adducts. To this end, 5 m*M* sodium acetate (or another cation for molecular-mass confirmation) was added to the mobile phase consisting of 90% of a acetonitrile–tetrahydrofuran (THF) (85:15) mixture in water. CID of the sodium adduct shows very limited fragmentation, i.e., the loss of CO_2 and the loss of both CO_2 and water. SRM was applied in confirmation of semduramicin in poultry liver samples. By monitoring both losses semduramicin was detected with good signal-to-noise ratio in spiked liver samples down to 30 μg/kg, as demonstrated in Fig. 10.

The simultaneous determination of the ionophore antibiotics monensin, salinomycin and narasin in muscle, liver and eggs at the 1 μg/kg level by means of electrospray LC–MS was reported by Blanchflower and Kennedy [73]. The sodium adduct at $m/z = M_r + 23$, i.e., at m/z 693 for monensin, is used in SIM, while ions due to an apparent ammonium adduct, i.e., at m/z 688 for monensin, erroneously attributed to a water adduct, and the loss of 40 from the sodium adduct, i.e., at m/z 653 for monensin, possibly due to $[M+H-\text{water}]^+$, are detected as well. Because the ion ratio of the sodium and the ammonium adducts was not reproducible, only the sodium adduct is used in SIM. The mobile phase used consisted of 67% acetonitrile, 10% methanol, 10% THF in water with 0.1% TFA. No ionization

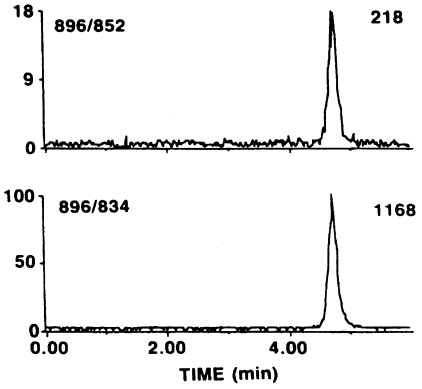


Fig. 10. Chromatograms from selective reaction monitoring of semduramicin at 30 μg/kg level in chicken liver after electrospray LC–MS–MS (from [72], ©1991, American Chemical Society).

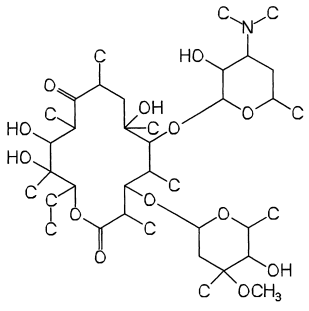
directing additive (see above) was used in this case. The ionophores can be detected in tissue and eggs down to 1 μg/kg.

6.5. Macrolide antibiotics

Macrolide antibiotics consist of a 12-, 14- or 16-membered macrocyclic lactones to which sugar moieties, including amino and deoxy sugars, are attached. They are produced by various *Streptomyces* strains. They are used in veterinary practice against gram-positive bacteria, but also in humans against various infectious diseases, where they are administered as acid-resistant esters.

The bioanalysis of erythromycin 2-ethylsuccinate in human plasma by means of a column-switching system coupled to a CF-FAB interface was described by Kokkonen et al. [74]. Column switching was used to enable the chromatography to be performed with a phosphate buffer containing mobile phase on a conventional 4.6 mm I.D. HPLC column. The relevant part of the chromatogram was heartcut to a short trapping column, where the hydrophilic compounds were washed away with water prior to the desorption of the compound of interest to the CF-FAB-MS system. Desorption was performed with a solvent readily compatible to CF-FAB, i.e., containing the glycerol matrix, and with a flow-rate of 15 μl/min, also compatible to CF-FAB. The determination limit for erythromycin 2-ethylsuccinate in plasma was 0.1 μg/ml under these conditions.

Erythromycin A:



Residue analysis of erythromycin A and its metabolites in salmon tissue using pneumatically-assisted electrospray LC–MS was described by Pleasance et al. [75]. Under ESI conditions, the mass spectrum of erythromycin A mainly contains a peak due to the protonated molecule at m/z 734, while by in-source CID some fragmentation can be induced, especially the loss of water (m/z 718) and of cladinose (m/z 576), which enables the use of a SIM method. In MS–MS the same type of fragmentation is observed. In SIM or SRM, the detection limits of erythromycin A in salmon tissue were below 10 and 50 μg/kg, respectively, while confirmatory full-scan LC–MS or LC–MS–MS was achieved at the 0.5 and 1 mg/kg level, respectively. Next to erythromycin A, a variety of metabolites were detected, e.g., anhydroerythromycin and N-desmethylerythromycin.

The nanoscale packed capillary LC–MS and CZE–MS analysis of seven 16-membered ring and three 14-membered ring macrolide antibiotics was reported by Parker et al. [76]. The ESI mass spectra of these compounds, the induction of fragmentation by in-source CID, and the separation of the compounds was investigated.

Electron-capture NICI in combination with a particle-beam interface is used in the residue analysis of ivermectin [77], which is a mixture of two macrolide antibiotic dihydroavermectin homologs. One of the homologs, H_2B_{1a}, is used as marker for ivermectin residues with a maximum residue level of 15 μg/kg in beef liver. The confirmation is based on SIM analysis of the molecular anion and four fragment ions. It was found in the analysis of milk or liver extracts, that coeluting matrix components enhanced the response and altered the abundance pattern. This is a well-known problem in particle-beam analysis. Therefore, control milk extracts were spiked with H_2B_{1a} and used in abundance matching during confirmation. The method was applicable to the confirmation of H_2B_{1a} at the 2 μg/kg level in milk and the 13 μg/kg level in liver.

Electron-capture NICI in combination of particle-beam LC–MS was applied in the residue analysis of spiramycin in bovine muscle tissue by Sanders and Delépine [78]. Mass spectra for spiramycin as well as for neospiramycin, eyrthromycin A and tylosin were reported. The latter three compounds were also used in testing the specificity of the method. The confirmation is based on the monitoring of five fragment ions of spiramycin. Variation of the ion ratios was less than 20% down to the maximum residue limit of 50 μg/kg. A similar method was subsequently developed for the confirmation of tylosin in bovine muscle [79].

6.6. Nitrofuran antibiotics

The determination of furazolidone, a 5-nitrofuran antibiotic effective in the prevention and treatment of gastrointestinal inflections caused by *Escherichia coli* and *Salmonella*, in porcine tissue by thermospray LC–MS was reported by McCracken et al. [80]. The method based on SIM of the protonated molecule at m/z 243 is applied to study the depletion of furazolidone from tissues and the stability of the compound postmortem. A detection limit of 1 μg/kg was reported. The determination of the same compound as well as two other 5-nitrofuran antibiotics, nitrofurazone and furaltedone, in eggs by LC with UV-PDA and ionspray MS detection was recently described by Draisci et al. [81]. The detection limits, based on SIM of the protonated molecule, were between 1 and 3 μg/kg, which is below the current provisional regulatory level in the EU (5 μg/kg).

6.7. Miscellaneous antibiotics

The LC–MS analysis of a number of other antibiotic substances was reported by various authors. The LC–MS analysis of the semi-synthetic antibiotic rifamycins by means of direct liquid introduction [82], thermospray or electrospray interfacing [83] was described. Thermospray LC–MS was applied in the separation and mass spectral characterization of a variety of N-acylamino dilactones and 4-butyrolactones derived from antimycin A [84].

Thermospray LC–MS was the method used by Hornish et al. [85] in the determination and confirmation of residues of the lincosamide antibiotic pirlimycin in bovine milk and liver. Quantitative determination is based on SIM of the protonated molecule (m/z 411), while for confirmation, in addition, the ^{37}Cl isotope peak at m/z 413 and fragment ions at m/z 375 and 158 were monitored. Limits of quantitation and confirmation in milk were 50 and 100 μg/kg, respectively. Method validation is described as well.

The various components in the GE2270 complex, a thiazolyl peptide antibiotic active against gram-positive bacteria, were characterized by CF-FAB-MS and LC–MS with thermospray and electrospray interfacing [86].

7. Probing noncovalent interactions between antibiotics and cell walls

The study of the noncovalent interaction of antibiotics to their target ligands, usually cell-wall substructures, represents an important mechanistic

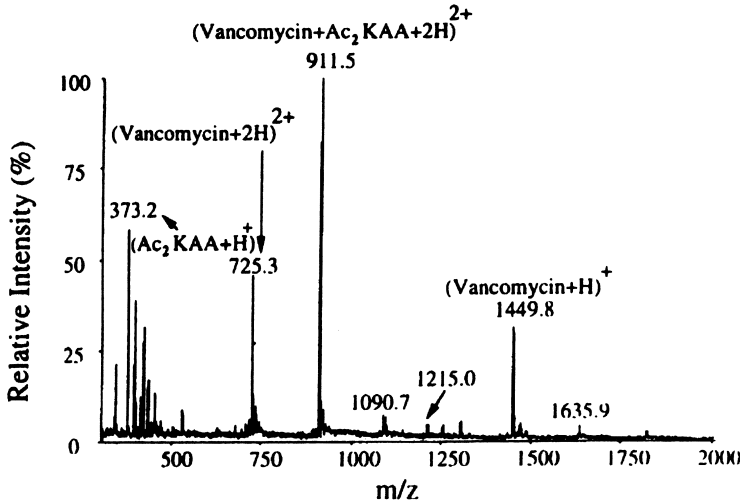


Fig. 11. Electrospray mass spectrum of an equimolar mixture of Ac_2KAA with vancomycin (25 m*M* in 50% acetonitrile in 5 m*M* aqueous ammonium acetate, pH 7) (from [87], ©1995, Wiley).

clue to the antibiotic action and/or the development of new antibiotic drugs. The interaction of vancomycin antibiotics, e.g., vancomycin and ristocetin, a family of complex glycopeptides based on a heptapeptide backbone, and bacterial cell walls appears to be related to specific interaction with X–D-Ala–D-Ala, where X is L-Lys, L-Ala or some other amino acid.

The noncovalent complexation of vancomycin and ristocetin with N,N,-diacyl–L-Lys–D-Ala–D-Ala (Ac_2KAA) was investigated using electrospray MS by two groups simultaneously [87,88]. As an example, an electrospray mass spectrum of the 1:1 complex of vancomycin and Ac_2KAA is shown in Fig. 11. Complexes with other compositions were observed as well. Furthermore, Lim et al. [87] showed that by correlation of relative ion abundances and ligand concentrations in solution the binding constants of the antibiotic–peptide complex can be measured. Similar studies for the noncovalent interaction between vancomycin, ristocetin A, and teicoplanin and Ac_2KAA by electrospray MS were reported by Hamdan et al. [88]. Both 1:1 complexes and more complex associations like $[2(\text{antibiotic})+3(\text{peptide})+3H]^{3+}$ were detected. Subsequently, CE–MS was applied to separate the complex from the peptide and the antibiotic compound, demonstrating that these complexes are in fact generated in solution prior to the mass spectrometric analysis.

8. Conclusions

Confirmation of identity during residue analysis of antibiotics in animal food products for human consumption is the most important application of LC–MS in the analysis of antibiotics. Given the polarity and, in some cases, limited thermal stability of these classes of compounds, GC–MS analysis can only be successfully applied for limited number of compound classes and after (extensive) analyte derivatization, which in general is not preferred in residue analysis for reasons of time consumption, difficulties in the derivatization of large numbers of samples for routine analysis, the problem of reproducible derivatization at trace levels, and the possible formation of artefacts. Current LC–MS technology, based on ESI or APCI interfacing and single or triple quadrupole MS, is a versatile and powerful alternative to GC–MS in this type of applications, while a number of other interfaces, especially particle-beam in combination with NICI and thermospray interfacing, also proved their usefulness in this area.

In most of the studies cited above, the real samples could be analysed at the regulatory maximum residue levels. However, clear criteria for a confirmation based on LC–MS with in-source CID or MS–MS are not yet decided upon. In most studies, criteria derived from GC–MS with chemical ionization are used, i.e., the monitoring of preferentially four ions with an additional criteria that the ion ratios should be within 20% of the ion ratios determined with standards.

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