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Review

Liquid chromatographic–mass spectrometric methods for analyzing antibiotic and antibacterial agents in animal food products

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Abstract

Public health agencies in many countries rely on detection by mass spectrometry for unambiguous identification of residues of antibiotic and antibacterial agents in animal food products for human consumption. The introduction of relatively inexpensive and robust LC–MS systems has given a strong impulse to develop determinative and confirmatory methods for the above medicines in foodstuffs. This impulse has been also dictated by thermal instability and lack of volatility of many antibiotics and antibacterials that makes the GC–MS technique of difficult application. Analytical methods developed for analyzing components of the major classes of the medicines mentioned above are here reviewed. The discussion is focused on both sample treatment and final LC–MS analysis.

Keywords: Reviews; Food analysis; Antibiotics; Antibacterials

Contents

1. Introduction

For those chemicals able to interfere with the existence of pathogens by several action mecha-

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nisms, an old and not yet abandoned classification reserves the term 'antibiotics' to natural or semi-synthetic compounds, while synthetic ones are termed 'antibacterials'. In this review, this classification will not be adopted and the term 'antibiotic' will refer to any pharmaceutical able to act against bacteria. The use of antibiotics in veterinary medicine began in the 1950s with the use of oxytetracycline and chlortetracycline as feed additives. Today, many classes of antibiotics are widely used for preventing and treating several diseases as well as for promoting growth in food producing animals. In the 1980s, it was estimated that at least 60% of all animals used for food were exposed to antibiotics at some point in their lives. With current intense animal husbandry practices, this figure may be higher.

If recommendations for drug withdrawal times are not respected, there is a significant risk of detecting antibiotic residues in food. Moreover, some of the antibiotics can be added directly to food, mainly to milk, to prolong its freshness. Relatively high levels of antibiotic residues in foodstuff can provoke allergic reactions in some hypersensitive individuals. Of particular concern is that low-level doses of antibiotics for long periods could result in antibiotic-resistant bacteria that can transfer from food to humans [1]. In addition to immediate adverse effects, there are also long-term effects to the exposure of low levels of residue that are still unknown.

To minimize exposure of humans to antibiotics, tolerance levels of antibiotics in food have been established by both the European Union and US Food and Drug Administration (Table 1).

For detecting antibiotic residues in food, bioassay techniques are widely used in screening methods. These methods generally do not distinguish between members of a class of antibiotics, they provide only semiquantitative measurements of residues detected and sometimes give rise to false positives. Nevertheless, they continue to be used because of their simplicity and cheapness. But, before samples are condemned for containing levels of antibiotics exceeding the tolerance levels, it is well recognized these methods need to be supported by highly selective and sufficiently sensitive chemical methods. Carson et al. [2] evaluated several proposed gas chromatographic and liquid chromatographic (LC) methods using conventional detectors for the analysis of a commonly used antibiotic class, that is β-lactam antibiotics, in milk and concluded that none of the tested procedures satisfied the requirements for a suitable and reliable multiresidue confirmatory method.

Public Health Agencies in many countries relies on detection by mass spectrometry (MS) for unambiguous confirmation of antibiotics in foodstuff. The Commission Decision 93/256/EEC states that ''Methods based only on chromatographic analysis without the use of molecular spectrometric detection are not suitable for use as confirmatory methods''. Gas chromatography–mass spectrometry (GC–MS) should be the technique of choice, as it has been routinely used in the last 35 years for analyzing an enormous number of compounds in a variety of matrices. However, almost all of the antibiotics are very polar and/or thermally unstable compounds, thus complicating or precluding their analysis by GC. Research in new methodologies in MS, notably LC–MS, has greatly benefited from the international need of protecting food quality and now can serve to fulfill the goals initially sought by such a technique, that is monitoring non volatile and polar target compounds with the specificity and sensitivity similar to GC–MS. In the past 20 years, a large variety of interfaces have been developed to make the high vacuum of the mass analyzer compatible with the large amounts of liquids coming out from the LC column. LC–MS has been extensively reviewed in the past years. Several books [3–8] and review papers [9–14] devoted to illustrating principles, instrumentations and applications of LC–MS have been published. In particular, the use of LC–MS for analyzing antibiotics in food was reviewed in 1998 [15,16]. It is the general opinion that, among the various interfaces developed in the last 25 years, thermospray (TS), particle beam (PB) electrospray (ESI) and atmospheric pressure chemical ionization (APCI) are the most effective for coupling to LC instrumentation. Here, we will shortly describe typical characteristics, qualities and faults of these three devices.

1.1. General features of the LC–MS interfaces

The TS interface can accommodate reversed-phase solvents and any volatile buffer. Among the three

Table 1
Tolerance levels of antibiotics in food stipulated by the European Union (EU) and USA Food and Drug Administration (FDA)

	Maximum residue limit (MRL) (ppb)													
	Milk		Eggs		Muscle		Liver		Kidney		Fat		Fish	
	EU	FDA	EU	FDA	EU	FDA	EU	FDA	EU	FDA	EU	FDA	EU	FDA
β-Lactam antibiotics														
Amoxicillin (cattle)	4	10			50	10	50	10	50	10	50	10		
Amoxicillin (goats, poultry, swine, sheep)	4				50		50		50		50			
Ampicillin (cattle, swine)	4	10			50	10	50	10	50	10	50	10		
Ampicillin (goats, poultry, sheep)	4				50		50		50		50			
Ceftiofur[a] (cattle)	100[b]	50			200[b]	1000	2000[b]	2000	2000[b]	8000	600[b]			
Ceftiofur[a] (swine)					500[b]	NR[c]	3000[b]	NR	4000[b]	NR	600[b]	NR		
Cefazolin (sheep, goats)	50													
Cefquinome (cattle)	20													
Cephapirin (cattle)	10[b,d]	20			50[b,d]	100	50[b,d]	100	100[b,d]	100	50[b,d]	100		
Cloxacillin (cattle)	30	10			300	10	300	10	300	10	300	10		
Cloxacillin (goats, poultry, swine, sheep)	30				300		300		300		300			
Dicloxacillin	30				300		300		300		300			
Nafcillin (cattle)	30[b]				300[b]		300[b]		300[b]		300[b]			
Oxacillin	30				300		300		300		300			
Penicillin G (cattle)	4	5			50	50	50	50	50	50	50	50		
Penicillin G (chickens, swine, sheep)	4			0	50	0	50	0	50	0	50	0		
Penicillin G (turkeys)					50	10	50	10	50	10	50	10		
Tetracyclines														
Tetracycline	100[e]	80	200[e]		100[e]	2000	300[e]	6000	600[e]	12 000		12 000		
Oxytetracycline	100[e]	30	200[e]		100[e]	2000	300[e]	6000	600[e]	12 000		12 000		2000
Chlortetracycline	100[e]	30	200[e]	400	100[e]	2000	300[e]	6000	600[e]	12 000		12 000		
Doxicycline[f] (cattle)					100[e]		300[e]		600[e]					
Doxycycline[f] (Swine, poultry)					100[e]		300[e]		600[e]		300[e,g]			
Sulphonamides														
Total sulfonamide residues	100				100		100		100		100			
Sulfabromomethazine (cattle)		10				100		100		100		100		
Sulfachloropyrazine (chickens)						0		0		0		0		
Sulfachloropyridazine (calves, swine)						100		100		100		100		
Sulfadimethoxine		10				100		100		100		100		100
Sulfaethoxypyridazine (cattle)		0				100		100		100		100		
Sulfaethoxypyridazine (swine)						0		0		0		0		
Sulfamerazine (trout)														0
Sulfamethazine						100		100		100		100		
Sulfanitran (chickens)						0		0		0		0		
Sulfaquinoxaline						100		100		100		100		
Sulfathiazole (swine)						100		100		100		100		
Aminoglycosides														
Aminosidine					500[h]		1500[h]		1500[h]					
Apramycin (cattle)	NP[i]				1000[j]		10 000[j]		20 000[j]		1000[j]			
Apramycin (swine)					1000[j]		1000[j]		5000[j]	100	1000[j,k]			
Dihydrostreptomycin	200[l]	125			500[l]	500	500[l]	500	1000[l]	2000	500[l]	500		
Gentamycin (chickens, turkeys)						100		100		100		100		
Gentamycin (cattle)	100[l]				100[l]		200[l]		1000[l]		100[l]			
Gentamycin (swine)					100[l]	100	200[l]	300	1000[l]	400	100[l]	400		
Hygromycin B (swine, poultry)				0		0		0		0		0		
Neomycin (cattle, swine, sheep, goats)	500[l]	150			500[l]	1200	500[l]	3600	5000[l]	7200	500[l]	7200		
Neomycin (chickens)			500[l]		500[l]		500[l]		5000[l]		500[l]			
Neomycin (turkeys)					500[l]	1200	500[l]	3600	5000[l]		500[l]	7200[k]		

Table 1. Continued

	Maximum residue limit (MRL) (ppb)													
	Milk		Eggs		Muscle		Liver		Kidney		Fat		Fish	
	EU	FDA	EU	FDA	EU	FDA	EU	FDA	EU	FDA	EU	FDA	EU	FDA
Spectinomycin (chickens, turkeys)					300[h]	100	2000[h]	100	5000[h]	100	500[h]	100		
Spectinomycin (cattle)	200[h]				300[h]	250	2000[h]		5000[h]	4000	500[h]			
Spectinomycin (swine)					300[h]		2000[h]		5000[h]		500[h]			
Streptomycin	200[l]	125			500[l]	500	500[l]	500	1000[l]	2000	500[l]	500		
Macrolides														
Erythromycin (cattle, swine)	40[l]	0			400[l]	100	400[l]	100	400[l]	100	400[l]	100		
Erythromycin (chickens, turkeys)			200[l]	25	400[l]	125	400[l]	125	400[l]	125	400[l]	125		
Josamycin (chickens)			200[m]		200[m]		200[m]		400[m]		200[m]			
Oleandomycin (chickens, turkeys, swine)						150		150		150		150		
Spiramycin (swine)					300[n]		600[n]		300[n]		200[n]			
Tilmicosin (cattle)					50		1000	1200	1000		50			
Tilmicosin (sheep)	50				50		1000		1000		50			
Tilmicosin (swine)					50	100	1000	7500	1000		50			
Tylosin	50	50	NP[o]	200	100	200	100	200	100	200	100[k]	200		
Quinolones														
Danofloxacin (cattle)	NP[i]				200		400		400		100			
Danofloxacin (chickens)			NP[o]		200		400		400		100[k]			
Decoquinate (cattle)					500[m]		500[m]		500[m]		500[m]			
Difloxacin (chickens, turkeys)					300		1900		600		400[p]			
Enrofloxacin (chickens, turkeys)					30[q]	300	30[q]		30[q]					
Enrofloxacin (cattle)					30[q]		30[q]	100[r]	30[q]					
Enrofloxacin (swine)					30[q]		30[q]		30[q]					
Marbofloxacin (cattle)	75[h]				150[h]		150[h]		150[h]		50[h]			
Marbofloxacin (swine)					150[h]		150[h]		150[h]		50[h,k]			
Sarafloxacin (chickens, turkeys)						NR[c]	100	NR		NR	10[k]	NR		
Sarafloxacin (Salmon)													30[h,s]	
Polyether ionophores														
Lasalocid (chickens)												300[t]		
Lasalocid (cattle, rabbits)								700						
Maduramicin (chickens)						240		720				480[p]		
Monensin (cattle, goats)						50		50		50		50		
Narasin (chickens)						600		1800				1200[u]		
Semduramicin (chickens)						130		400						
Amphenicols														
Florfenicol[v] (cattle)					200	300	3000	3700	300				1000[w]	
Thiamphenicol (cattle, chickens)					40[f]		40[f]		40[f]		40[f]			
Nitroimidazoles														
Dimetridazole					10[x]		10[x]		10[x]		10[x]			
Nitrofurans														
Furazolidone (cattle, poultry, sheep)					5[y]		5[y]		5[y]		5[y]			
Furazolidone (swine)					5[y]	0	5[y]	0	5[y]	0	5[y]	0		

[a] Tolerance refers to residues desfuroylceftiofur (marker residue). [b] Provisional MRL expired 1/1/1999. [c] NR, not required. [d] Sum of cephapirin and desacetylcephapirin. [e] Sum of the parent drug and its 4-epimer. [f] Provisional MRL, expired 1/1/1998. [g] Same value also for skin. [h] Provisional MRL expired 1/7/1998. [i] NP, not permitted in lactating cattle. [j] Provisional MRL expired 1/7/1999. [k] As sum in skin and fat. [l] Provisional MRL expired 1/6/2000. [m] Provisional MRL expired 1/7/2000. [n] Provisional MRL expired 1/7/1997. [o] NP, not permitted for human egg-laying hens. [p] Fat or skin. [q] Sum of enrofloxacin and ciprofloxacin, its major metabolite. [r] As desethylene ciprofloxacin. [s] As sum of muscle and skin in natural proportion. [t] In skin with adhering fat. [u] Fat or skin with adhering fat. [v] Total residues of florfenicol measured as florfenicol amine. [w] As sum of muscle and skin in natural proportion; Provisional MRL expired 1/7/2001. [x] All residues with an intact nitroimidazole structure; Provisional MRL expired 1/1/1995. [y] All residues with an intact 5-nitro structure; Provisional MRL expired 1/7/1995.

most commonly used interfaces for LC–MS, TS is the only one that exhibits the optimum efficiency at 100% water.

The TS interface sometimes exhibits tailing for nonvolatile or thermally labile compounds due to deposition on the source and re-evaporation or decomposition. Some but not all of these unwelcome effects can be eliminated by increasing the source temperature to avoid deposition or decreasing the temperature to avoid decomposition.

TS sensitivity is analyte dependent and difficult to predict. Usually, detection limits lie in the 10–60 ng range, under full scan conditions.

Typically, ion evaporation shows $[M+H]^+$ ions for compounds having a high proton affinity, such as those basic in nature. Otherwise, $[M+NH_4]^+$ ions are formed. In negative detection, $[M-H]^-$ or [M+ buffer or solvent]$^-$ ions are observed. When using the electric discharge, gas-phase chemical ionization (CI) spectra are generated. Due to the soft ionization process, TS spectra often do not display structurally-significant fragment ions. In some cases, fragmentation can be induced by using the ''discharge-assisted thermospray'' technique or using higher repeller voltages. By this expedient the kinetic energy of the ions is increased and collision-induced decomposition (CID) processes generate fragment ions. However, high repeller voltages are not compatible with an optimal transmission efficiency, hence sensitivity is decreased. In particular cases, apparent fragmentation can be obtained by thermal degradation of the analytes while passing through the thermospray vaporizer probe. That TS spectra rarely show fragment ions is a well defined drawback of this interface. When LC–TS-MS is applied to monitoring traces of target compounds in complex matrices, the combination of retention time and molecular mass could be not sufficient for unequivocal identification of a target compound. The specificity of methods involving the TS interface can be greatly increased by making use of tandem MS. However, many regulatory laboratories cannot afford the cost of such instrumentation.

The lack of fragment ions for unequivocal determination of a certain analyte by the TS-MS arrangement encouraged several researchers to consider the use of the particle beam (PB) interface for detection of several classes of analytes, as it is able to generate classical electron-impact (EI) spectra. The ion source is the same as that used by GC–MS and EI spectra are equal to those reported in spectra libraries. This is a well-defined advantage of the LC–PB-MS technique. Another positive feature of the PB interface is that it can handle common solvents for reversed-phase (RP)-LC, including volatile buffers, at flow-rates of up to 1 ml/min.

A serious drawback of the LC–PB-MS system is that it is unsuitable to analysis of very low volatile compounds, as the interface is incapable of transferring them from the liquid to the gas phase. Moreover, effects of adsorption of polar analytes on the material composing the transmission device result in broad and tailed peaks.

Another well established drawback of the LC–PB-MS system is the relatively low sensitivity, because the transmission efficiency is not higher than 1%. Even after optimizing parameters related to the PB interface and under selected ion monitoring (SIM), Voyksner et al. were able to analyze compounds of pharmaceutical interest at level not lower than few ppm in milk and tissue extracts [17].

Another drawback of the PB-MS device is the nonlinearity of the response at low analyte concentrations. Causes related to this effect have been the object of detailed studies, but the mechanism has not yet been completely understood. To enhance the transmission efficiency at low analyte concentrations, malic acid or ammonium acetate have been added to the mobile phase. The rationale behind this approach is that a compound-specific or nonspecific 'carrier effect' can be obtained. However, this expedient is not always effective, as it depends upon the nature of the analyte.

The ESI interface is the youngest device introduced for LC–MS coupling. ESI has opened new and exciting perspectives to the LC–MS technique. It is sufficient to say that the ESI interface enables LC–MS analysis of compounds having molecular masses up to 4 000 000, as the ESI process is able to form multiply charged ions, depending on the acid/base chemistry and hydration energy of the molecules. The ability to increase charge (z) permits the analysis of large molecular masses on a conventional quadrupole limited to m/z of 2000 for singly charged

ions. The versatility of this interface has made it extremely popular among both analytical chemists and biochemists.

The ESI source apparently suffers from the limitation that it cannot accept more than 40–50 μl/min of the LC mobile-phase. These flow-rates are compatible with 1 mm I.D. LC columns. Or, the effluent from a conventional 4.6 mm I.D. LC column can be partially diverted by a split device to the ESI source. As the ESI-MS arrangement is a concentration-sensitive detector, diverting only a fraction of the LC mobile phase does not affect sensitivity. Another way of overcoming the problem of coupling LC with 4.6 mm I.D. conventional columns is that of inducing analyte ionization by gas phase ion–molecule reactions, under atmospheric pressure conditions (APCI). Reactant ion formation is achieved by the introduction of electrons from a corona discharge located in the chamber at atmospheric pressure. In this way, reversed-phase LC effluents as high as 2 ml/min can be handled.

The most serious drawback of the ESI-MS system is that it cannot accommodate LC effluents containing relatively high salt concentrations. With such solutions, signal instability and plugging of the small orifice of the sample cone occur. Recently, negative effects provoked by the presence of nonvolatile additive in the LC mobile phase have been eliminated by flowing the electrosprayed solution orthogonally to the sample cone and washing the orifice continuously with a small flow of water [18].

Like the TS interface, the ESI process generates $[M+H]^+$ in the positive-ion (PI) mode or $[M-H]^-$ in negative-ion (NI) mode, even for the most thermally labile and nonvolatile compounds. In some cases, spectra from nonbasic nonionic analytes display intense signals for Na^+, K^+, NH_4^+ adduct ions, in addition to that of the protonated molecule. These cations are always present as impurities in organic solvents used as organic modifiers of the LC mobile phase. We noted that the relative abundance of cationized molecules depends mainly on the particular design of the ESI interface.

A very interesting option offered by the ESI-MS system is that, by raising the electrical field in the intermediate region of the mass analyzer, protonated molecules can be accelerated to such a point that multiple collisions with residual molecules from the drying gas generate characteristic fragment ions (Fig. 1). The rate of fragmentation is strictly dependent on the potential difference between the sample cone and the skimmer lens. Provided the target compound is not coeluted with nontarget compounds, the resulting 'in-source' collision-induced dissociation (CID) spectra closely resemble those obtained by the more costly tandem MS technique. The appearance of fragment ions in spectra from analytes is of paramount importance, considering that legal criteria for testing the presence of contaminants in real matrices usually accept, among other conditions, spectra displaying the molecular ion plus, at least, two fragment ions.

Finally, ESI-MS is a rugged technique. Since ionization occurs at atmospheric pressure, there is no worry about vacuum failure. On a daily basis, the ionization chamber and the counter electrode can be easily checked and cleaned in a matter of minutes, while vacuum is maintained in the transport and mass analyzer regions. Pump oil changes are about as frequent as with GC–MS (about 3–4 months), far less than in thermospray and particle beam LC–MS.

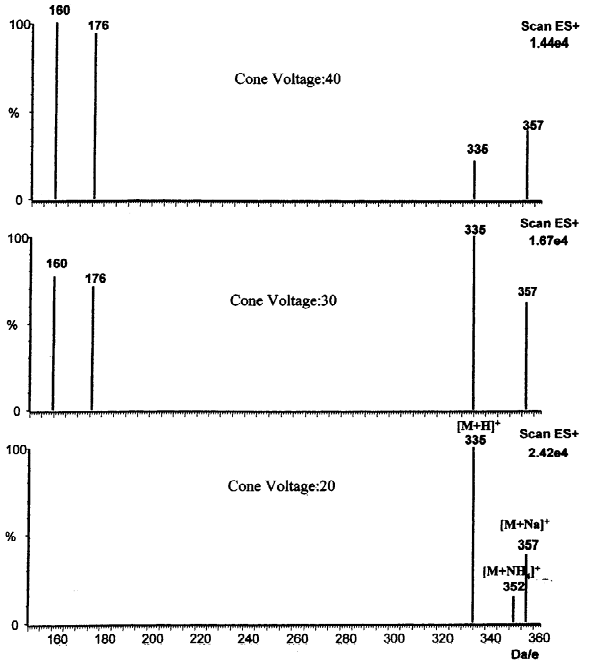


Fig. 1. Spectra of penicillin-G taken at various sample cone voltages.

APCI is another very soft ionization technique and has many similarities to ESI. Ionization takes place at atmospheric pressure and the ions are extracted into the mass detector in the same way as in ESI. Similarly, $[M+H]^+$ and $[M-H]^-$ ions are usually formed to give molecular mass information, and, when using a single quadrupole, fragmentation of the precursor ions can be induced in the source by increasing the cone voltage. Yet, the APCI process differs from the ESI one mainly in that: (i) the high voltage is applied to a corona pin, not to the probe insert capillary; (ii) the solvent evaporation and ion formation processes are separated; (iii) the APCI process does not yield multiply charged ions for high mass molecules. Using APCI, the liquid flow from the LC column is nebulized and rapidly evaporated by a coaxial nitrogen flow of nitrogen (nebulizing gas) and heating the nebulizer to high temperatures (350–500 °C). Although these temperatures may degrade the analytes, the high flow-rates of the LC mobile phase and coaxial nitrogen flow prevent breakdown of the molecules. Preformed ions can be carried into the gas phase, while ionization of analyte molecules is achieved using a corona discharge (3–6 kV) in the spray. The corona discharge produced by this high voltage causes solvent molecules entering the source to be ionized. In the atmospheric pressure region around the corona pin, a series of reactions occur that give rise to stable solvent reagent ions. Any analyte molecules, which elutes from the column and pass through this region of solvent ions, can be ionized by the transfer of a proton to form $[M+H]^+$ and $[M-H]^-$ ions. This is a form of chemical ionization, hence the name of the technique, atmospheric pressure chemical ionization. Compared to traditional chemical ionization, the APCI process is more efficient, since it occurs at a higher pressure, this resulting in a higher collision frequency.

Another major difference between APCI and ESI can be found in LC flow-rates that are used. APCI is a technique with optimal performance at high flow-rates (1 ml/min and higher). Lower flow-rates can also be used. However, when flow-rates are too low, the stability of the corona discharge may become problematic.

Although the four interfaces described above have been used in the past for developing methods aimed at detecting antibiotics in food, today ESI and APCI are become the ion sources of choice. This can be deduced from the fact that about 98% of the LC–MS works are currently performed with the above ion sources.

2. β-Lactam antibiotics

β-Lactams are probably the most widely used class of antibiotics in veterinary medicine for the treatment of bacterial infections of animals used in livestock farming and bovine milk production. β-Lactam antibiotics consist basically of two classes of thermally labile compounds: penicillins and cephalosporins. Both classes contain bulky side chain attached respectively to 6-aminopenicillanic acid or 7-amino cephalosporanic acid nuclei (Fig. 2). Manipulation of the side chain has enlarged the antibiotic spectrum to include both Gram-positive and Gram-negative bacteria.

Starting from 1989, eighteen LC–MS analytical methods have been proposed for analyzing β-lactams in food. Thirteen of these works are dealing with one or more β-lactams in milk, three works with meat, one work with fish meat and one with both milk and meat. To our best knowledge, no LC–MS method for detecting β-lactam antibiotics in eggs is quoted in the literature.

The presence of an unstable four-term ring in the β-lactam structures makes these compounds prone to degradation by heat and in the presence of alcohols. Penicillins are also readily isomerized in an acidic ambient. Because of these peculiarities, several precautions have been adopted in any step of the sample preparation procedure to avoid analyte degradation.

In 1989, Voyksner et al. [19] proposed a simple and rapid analytical procedure based on LC–TS-MS for detecting penicillin-G in bovine milk. Basically, sample pretreatment involves addition of acetonitrile to the sample to minimize analyte binding to proteins followed by ultrafiltration using a 10 000 molecular-mass cut-off filter. An aliquot of the filtrate was injected into the LC–MS apparatus. The analyte was chromatographed on a C_{18} column with a mobile phase of isopropanol–0.2 *M* ammonium acetate–acetic acid (12.5:85.5:2, v/v). The TS-MS detector

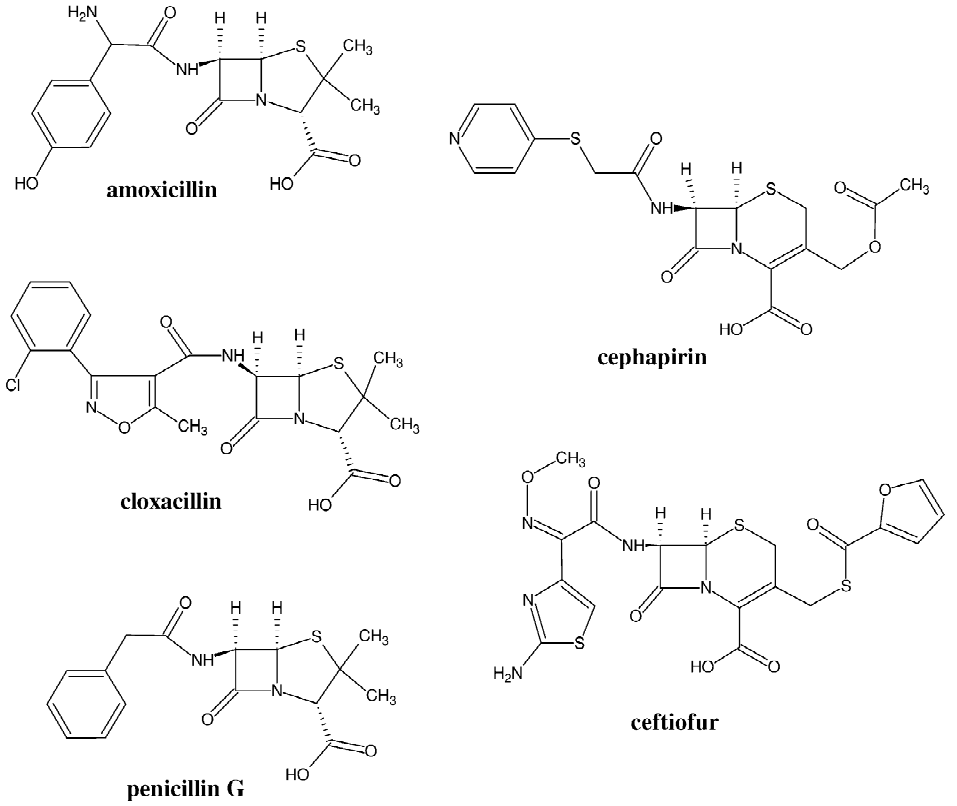


Fig. 2. Some examples of structures of β-lactam antibiotics.

was operated in the PI mode, monitoring the $[M+H]^+$ ion at m/z 335. The LOD was 100 ppb, well exceeding tolerance levels.

One year later, Voyksner et al. [17] evaluated the usefulness of adopting the PB interface for unambiguous identification of pharmaceutical interest, including β-lactams, in milk and meat samples. Sample pretreatment was the same as reported above. The authors evaluated the influence of various parameters on the response of the PB-MS system. They found that the sensitivity of the instrumentation was dependent on the nature of the organic modifier and flow-rate of the LC mobile phase, while parameters such as desolvation temperature, helium flow-rate to the nebulizer and nebulizer position resulted in minimal changes of sensitivity. Under methane CI, and with five-ions SIM, the use of LC–PB-MS permitted quantification of 14.1 ppm of cephapirin in bovine milk. The authors concluded that one way of decreasing detection limits with PB-MS is that of improving mass transmission through the interface, in particular through the momentum separators.

After experiencing the PB interface, Voyksner and co-workers [20,21] re-examined the use of the TS ion source for developing confirmatory methods for detecting cloxacillin, ampicillin, amoxicillin, cephapirin and its metabolite in bovine milk. The sample preparation procedure was almost unvaried [17]. Ammonium acetate was added to the LC mobile phase to promote formation of gas-phase ions in the TS ion source. Owing to their nature, β-lactam antibiotics can be detected both in the PI and NI modes. Preliminary studies showed that the NI mode of operation was 5–10 times less sensitive compared to the PI detection mode. The same situation is encountered when using the ESI ion source. Spectra of all of the analytes mentioned above displayed signals for protonated molecules as well as $[M+Na]^+$ adduct ions. Confirmatory ions were obtained by thermal decomposition of β-lactams. In particular,

the $[M+H-26]^+$ ion was common to all of the penicillins considered and was postulated to form from opening of the β-lactam ring followed by hydration and loss of CO_2. Unlike the previous work [17], the MS system was now set to monitor several fragment ions of the analytes, besides protonated ones. That fragment ions resulted from thermal degradation on the source walls of the penicillins was evidenced from the fact that the ions at m/z 160 and 277 maximized after several seconds after the $[M+H]^+$ ion. This anomaly could be explained assuming that penicillins are first decomposed and the formed products are then vaporized and ionized. Anyway, the two above ions could not be used as confirmatory ions due to the high background ion current in the low-mass region. This failure coupled to low enrichment factors obtained by preliminary sample treatment concurred to give high detection limits (50–100 ppb), making their method inadequate for analyzing β-lactams in food at the tolerance levels.

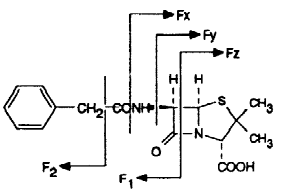
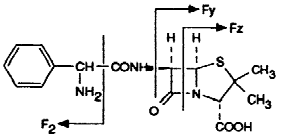
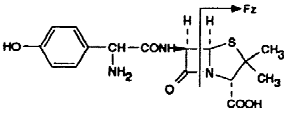
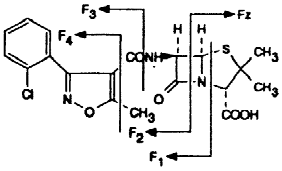
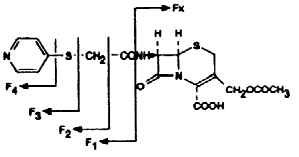
In 1993, Straub and Voyksner [22] investigated the potential of the recently introduced ESI ion source for confirming β-lactam antibiotics in bovine milk. The sample preparation procedure was the same as reported above [17]. By using classical ESI (no nebulizer gas, LC effluent delivered at the ion source at a flow-rate of 4 μl/min), the interest of the above authors was primarily focused on obtaining diagnostic fragment ions by decomposing $[M+H]^+$ or $[MH]^-$ ions in the intermediate region of the MS instrumentation (in-source CID). Compared to PI operation, the authors observed less fragmentation of the analytes considered and one-fourth decrease of the signal intensities. In the PI mode and at a relatively large potential difference between the sample cone and the skimmer lens, the spectra of all the penicillins studied, i.e. penicillin-G, ampicillin, amoxicillin and cloxacillin, displayed to a larger or lesser extent the characteristic cleavage product of the β-lactam ring at m/z 160 and a further loss of COOH at m/z 114. Obviously, cleavage of the amide moiety of penicillins formed more specific product ions. Under conditions used by the two authors, fragmentation schemes of the four penicillins are visualized in Fig. 3. Singularly, the CID spectrum of cephapirin did not display ions coming from cleavage of the β-lactam ring. Instead, loss of the carboxylic group produced the ions at m/z 220, while other fragment ions resulted from cleavage of the amide moiety (see again Fig. 3). Using flow injection ESI-MS, the effects of various mobile phase additives, such as formic, acid acetic acid, trifluoroacetic acid, ammonium acetate, ammonia and ion pair forming agents, on sensitivity were assessed. Generally, the most intense signals were achieved by adding formic acid (pH 3.1) to a water–methanol mixture.

A laborious and lengthy sample pretreatment was followed by Blanchflower et al. [23] for determining five penicillins in milk by LC–ESI-MS. Acetonitrile was used for protein precipitation and analyte extraction. Nafcillin was used as a surrogate standard. After centrifugation, the extract was purified by five liquid–liquid extraction steps using sequentially methylene chloride, hexane–acetonitrile, water, phosphate buffer (pH 7) and again methylene chloride as extractants. Following each extraction step, the extract was centrifuged. Unlike the previous work [22], the ESI process was now pneumatically assisted by a nebulizer gas and the ion source was able to accommodate mobile phase flow-rates up to 1 ml/min. In contrast to Voyksner and other researchers, the authors found that the NI mode operation gave more intense signals for penicillins than the PI mode. They had no explanation for this except that it might be due to differences in the type of instrument used. Diagnostic ions and suggested fragmentation pattern for each of the penicillin are shown in Fig. 4. Ions for operating in the SIM mode were selected after evaluating the effects of varying the electric field in the desolvation chamber on the CID spectra of the five penicillins. In spite of the fact that the LC mobile phase composed of a water–acetonitrile solution gave intense signals for the five analytes, triethylamine was added to the mobile phase to reduce shift in retention times between sample and standards due to matrix effects. In spite of a complex sample preparation procedure, the use of a surrogate standard helped to obtain relative recoveries of the analytes in the three matrices better than 70%. Owing to injection of the final extracts in the LC column equivalent to 2 ml of milk, quantification limits were below or near to stipulated EU tolerance levels.

In 1994, an active French researcher group [24]

IDENTITY OF CAD FRAGMENTS –POSITIVE ION MS OPERATION

M_r = Molecular mass of the free acid drug; CAD voltage = voltage measured at the end of the capillary (Analytica QBV 25-AL electrospray interface); m/z = mass-to-charge ratio (relative intensity of the peak in %, type of fragment); F_x, F_y, F_z, F_1, F_2, F_3, and F_4 = different collisional activated dissociation (CAD) fragments seen in the ESP spectra.

Drug (M_r)	CAD voltage	m/z (relative intensity and tentative identification of CAD fragments)
	80	392 (54); 391 (51, $[M + K + H_2O]^+$); 367 (93, $[M + H + MeOH]^+$); 353 (41, $[M + H + H_2O]^+$); 335 (41, $[M + H]^+$); 309 (8); 279 (25); 176 (43, $[F_1 + H]^+$); 167 (33); 160 (49, $[F_z + H]^+$); 149 (14); 114 (25, $[F_z - COOH]^+$); 65 (43, $[2MeOH + H]^+$)
	160	367 (27, $[M + H + MeOH]^+$); 335 (7, $[M + H]^+$); 217 (4, $[F_x + H]^+$); 202 (3, $[F_y + H]^+$); 176 (21, $[F_1 + H]^+$); 160 (37, $[F_z + H]^+$); 149 (100); 121 (14); 114 (4, $[F_z - COOH]^+$); 93 (19); 91 (15, $[F_2]^+$); 70 (14); 57 (18)[a]; 52 (36)[a]
Penicillin G (334.4)		
	80	382 (18, $[M + H + MeOH]^+$); 350 (100, $[M + H]^+$); 65 (16, $[2MeOH + H]^+$)
	160	350 (89, $[M + H]^+$); 192 (24); 174 (20, $[F_y + H - CO]^+$); 160 (22, $[F_z + H]^+$); 114 (89, $[F_z - COOH]^+$); 108 (8); 106 (100, $[F_2]^+$)
Ampicillin (349.4)		
	80	398 (12, $[M + H + MeOH]^+$); 367 (29); 366 (100, $[M + H]^+$; 349 (19, $[M + H - NH_3]^+$); 65 (19, $[2MeOH + H]^+$)
	160	366 (62, $[M + H]^+$); 349 (85, $[M + H - NH_3]^+$); 208 (41); 160 (6, $[F_z + H]^+$); 114 (100, $[F_z - COOH]^+$); 70 (14)
Amoxicillin (365.4)		
	80	481 (28, $[M + 2Na]^+$); 437 (84, $[M + 2H]^+$); 436 (100, $[M + H]^+$); 148 (20); 74 (46); 65 (95, $[2MeOH + H]^+$)
	160	468 (56, $[M + H + MeOH]^+$); 454 (11, $[M + H_2O]^+$); 436 (36, $[M + H]^+$); 321 (<0.1, $[F_1 + H^+]^+$); 277 (64, $[F_2]^+$); 222 (19, $[F_3 + H]^+$); 220 (11); 178 (56, $[F_4 + H]^+$); 160 (100, $[F_z + H]^+$); 114 (68, $[F_z - COOH]^+$)
Cloxacillin (435.9)		
	40	446 (63, $[M + Na]^+$); 424 (25, $[M + H]^+$); 413 (100, $[M - MeOH]^+$); 234 (32); 95 (8); 59 (33)[a]; 55 (36)[a]
	160	424 (45, $[M + H]^+$); 292 (41); 226 (15, $[F_x - COOH]^+$); 193 (21); 181 (26); 152 (62, $[F_1]^+$); 141 (23); 124 (59, $[F_2]^+$); 111 (62, $[F_3 + H]^+$); 79 (33, $[F_4 + H]^+$); 52 (100[a])
Cephapirin (423.4)		

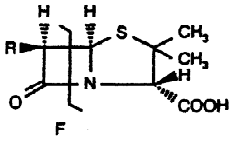
[a] The indicated mass is outside the instrument's (Finnigan MAT 4500) calibration range and is probably inaccurate.

Fig. 3. Proposed fragmentation schemes for selected β-lactam antibiotics obtained by the in-source CID process (redrawn from [22]).

showed that LC–PB-MS could be successfully used for unambiguous confirmation of three isoxazolyl penicillins, i.e. oxacillin, cloxacillin and dicloxacillin, in bovine muscle at the maximum residue limit (MRL) of 300 ppb. Negative ion chemical ionization (NICI) with methane as the reagent gas was used

Diagnostic ions (*m/z*) for each of the penicillins, obtained using LC-ES-MS in the NI mode

	M − H	M − H isotope	M − H − CO_2	F − H + H_2O
Pen V	349	—	305	208
Pen G	333	—	289	192
Ox	400	—	356	259
Clox	434	436	390	293
Diclox	468	470	424	327
Naf	413	—	369	272



	R
Pen G	C_7H_7
Pen V	C_7H_7O
Ox	$C_{10}H_9ON$
Clox	$C_{10}H_8ONCl$
Diclox	$C_{10}H_7ONCl_2$

Fig. 4. Diagnostic fragment ions for selected penicillins obtained by in-source CID of $[M-H]^-$ ions and suggested fragmentation pattern (redrawn from [23]).

because it was more sensitive than ionization by the EI mode. For identification purpose, five ions for each analyte were chosen. Among these ions, molecular ions were absent, because they were completely decomposed under the mass spectrometric conditions used. The analytes were extracted from samples by using sequentially tetrahydrofuran, 1 *M* HCl and ethyl acetate. After centrifugation and solvent removal, the residue was dissolved in the LC mobile phase. This mixture was again centrifuged prior to LC–MS analysis. Chromatography was performed with a C_{18} column and a methanol–acetonitrile–formic acid-acidified water (50:10:40, v/v) solution as mobile phase.

Tyczkowska et al. [25] developed a confirmatory method based on LC–MS with pneumatically assisted ESI for five β-lactam antibiotics in milk. The sample treatment was the same as previously reported [17]. Analytes were isocratically chromatographed on a 3-μm particle size C_{18} column with 40% acetonitrile and 1% acetic acid in water. SIM was used to monitor the $[M+H]^+$ ions and the two most intense compound-specific fragment ions for each analyte. LOD of the method did not allow analyte confirmation at the safe levels.

The performance of perfusive particle (derivatized styrene–divinylbenzene copolymer, 7–8 μm particles, 400 nm pore size) LC combined with ultrasonic assisted ESI-MS was evaluated with the aim of analyzing six β-lactam antibiotics in milk at the FDA tolerance levels [26]. The sample treatment was very similar to that previously reported [17]. Looking at the mass chromatograms, the above LC packing does not appears to give excellent results in terms of peak shape and analyte separation. Comparative data showed that the ESI process assisted by ultrasonic nebulization offered advantages over classical and pneumatically assisted ESI, in that the response of the ESI-MS detector does not depend on the nature of mobile phase additives and higher mobile phase flow-rate operation.

However, ultrasonic assisted ESI should have some drawbacks if 4 years later one of the authors re-used pneumatically assisted ESI for determining residues of ceftiofur in bovine milk [27] at tolerance level set by FDA. The sample treatment was the same as previously reported [26]. The authors failed to achieve sufficiently abundant fragment ions without sensitivity loss by the in-source CID process. So, ceftiofur was quantified by monitoring only the protonated molecule at *m/z* 524.

Heller and Ngoh [28] evaluated the feasibility of using ESI combined with ion trap MS–MS for confirming the presence of six currently approved β-lactam antibiotics and one not approved for use (cefalozin) in bovine milk. Ion trap MS provides full-scan MS–MS performance at full instrument sensitivity, in contrast to tandem MS–MS, which requires selection of only a few ions to maximize sensitivity. The CID process of protonated molecules of β-lactam antibiotics produces usually highly diagnostic ions for penicillins, and ion trap enables multiple, sequential stages of mass analysis (MS^n), which can further increases specificity and spectra detail. The sample preparation procedure involved same critical steps. Isolation of target compounds from the matrix was performed by double extracting with acetonitrile. Acetonitrile was then removed leaving 1–2 ml of water. Residue of acetonitrile still present in water could provoke successive loss of amoxicillin, when passing the extract through a C_{18}

SPE cartridge. Analytes were eluted from the cartridge with acetonitrile, which was then diluted with water. Acetonitrile was again removed leaving analytes in some water. This solution was filtered and an aliquot injected into the LC–MS system. Recovery of the analytes were not specified. By observing ion signal intensities, we deduced that amoxicillin and ampicillin were poorly recovered. The method sensitivity allowed detection of the analytes at safe levels.

An LC–ESI-MS method for simultaneously determining four β-lactam antibiotics in animal tissues was proposed by Hornazábal and Yndestad [29]. Analytes were extracted by liver, kidney, meat samples with an acetone–trichloroacetic acid solution. The water–acetone extract was partially evaporated to remove the organic solvent and the extract was purified by applying it to a C_{18} SPE cartridge. The eluate was diluted with water and filtered. The final extract was submitted to the remaining part of the analysis. Isocratic separation was performed on a C_{18} column with a methanol–ammonium acetate (50:50, v/v) solution. The ESI-MS detector was operated in the NI mode and set to collect ion at m/z 333 (penicillin-G), 349 (penicillin-V), 434 (cloxacillin) and 468 (dicloxacillin) in the one-ion SIM mode.

An LC–ESI-MS–MS method was developed for confirming the presence of amoxicillin in catfish muscle [30]. Amoxicillin in the sample was derivatized by heating the sample suspended in an acetate buffer. Amoxicillin derivatives were then extracted five times with ethyl ether. The organic solvent was removed and the dry extract dissolved in the LC mobile phase. After filtering, the final extract was ready for the final part of the analysis. The mobile phase was 18% acetonitrile in water adjusted to pH 4.4 with formic acid. The MS apparatus was set to monitor four product ions from the $[M+H]^+$ ion at m/z 203. Confirmation of amoxicillin was demonstrated in incurred fish samples containing the penicillin at 50–300 ppb levels.

Riediker and Stadler [31] developed a multiresidue method based on LC–ESI-MS–MS for the detection of amoxicillin, ampicillin, cloxacillin, oxacillin and penicillin-G in milk. d_7-pen-G was used as surrogate standard. After milk defatting by centrifugation, a phosphate buffer and hexane were added to the sample. An aliquot of the aqueous phase was then applied to a C_{18} SPE cartridge and analytes were re-extracted with a phosphate buffer–acetonitrile (1:1, v/v) solution. The eluent volume was first reduced by heating for eliminating acetonitrile and then it was diluted with other water. Before LC–MS analysis, the final extract was filtered with a cutoff filter device (nominal molecular mass limit 10 000). The analytes were separated in the gradient elution mode using a 5-cm length, 3-μm particle size C_{18} column and a mobile phase consisting of water–acetonitrile acidified with formic acid. Selected reaction monitoring (SRM) of two or more fragmentation transitions were selected to provide a high degree of sensitivity and specificity. The employment of postcolumn infusion of a standard compound (azlocillin) during chromatography of a milk extract showed significant ion suppression only in the very first part of the mass chromatogram. Thus the matrix did not significantly affect the analyte ionization. The concentration limit for analyte confirmation ranged from 0.4 to 1.1 ppb for the analytes considered. Successively [32], this method was employed for measuring concentrations of penicillins in incurred raw milk.

We have developed a simple and rapid method for quantifying ten approved-for-use β-lactam antibiotics in bovine milk below stipulated USA and EU tolerance levels [33]. The analytes are directly extracted from intact milk by passing it through a Carbograph 4 cartridge. Penicillin-V was used as I.S. The flow-rate was adjusted in such a way that time was allowed for the analytes to be desorbed from proteins and readsorbed on the sorbent material. At 5 ppb level, recovery of the β-lactams were between 70 (nafcillin) and 108% (cefalexin), with relative standard deviations ranging between 5 (oxacillin) and 11% (amoxicillin and ceftiofur). The analytes were fractionated on a C_{18} column in the gradient elution mode. The phase A was methanol and the phase B was water. Both phases were acidified with 10 m*M* formic acid. During the first part of the chromatographic run, the ESI-MS system was operated in the PI mode, while late-eluted compounds were detected in the NI mode. This was made to circumvent matrix interferences resulting in remarkable signal weaken-

ing for some of the last-eluted analytes (penicillin-G, oxacillin and cloxacillin) when detecting them as $[M+H]^+$ ions. MS data acquisition was performed by a time-scheduled three-ion SIM program. Fig. 5 shows a typical mass chromatogram resulting from analysis of a 10-ml milk sample spiked with the analytes at 10 ppb level. Analyses of milk samples taken after intramammary application of amoxicillin showed that 1.2 ng/ml of this penicillin were still present 6 days after treatment. At this concentration level, the identification power of the method was not weakened, as signals of the three monitored ions were still well distinguishable from the background noise (Fig. 6).

A sensitive LC–ESI-iontrap-tandem MS method for the quantitation and mass spectral confirmation of five penicillins and two cefalosporins commonly or potentially used in diary industry was described by Holstege et al. [34]. Antibiotics were extracted from milk with acetonitrile, followed by reversed-phase column clean-up. Analytes were separated by a C_{18} LC column using a water–methanol gradient containing 1% acetic acid. Determination was in the PI mode. Analyte detection and quantitation was based on the most abundant product ions from fragmentation of the protonated molecules for amoxicillin, ampicillin, cephapirin and ceftiofur and from fragmentation of the sodiated molecules for penicillin-G, penicillin-V and cloxacillin. An example of PI ESI mass spectrum and MS–MS spectrum of penicillin-G is shown in Fig. 7. It may be that the MS system was uncalibrated at the time the spectra were obtained, because the sodiated fragment ion corresponding to the class-specific protonated fragment ion at m/z 160 should have an m/z at 182, instead of at m/z 181. Another consideration concerns with the anomalously abundant formation of cationized molecules, which might be traced to the particular design of the ESI-ion trap device. Finally, the CID process rarely produces abundant fragment ions from decomposition of sodiated parent ions. Anyway, low limits of quantification were achieved for all of the analytes, ranging between 0.2 (ampicillin) and 2 ppb (cloxacillin and penicillin-V).

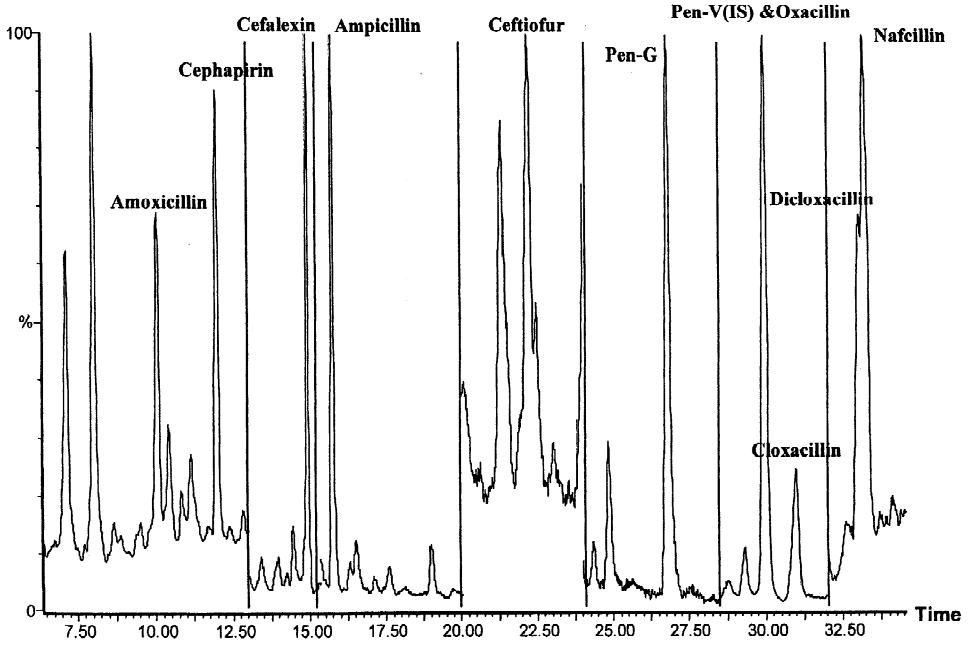


Fig. 5. Time-scheduled SIM LC–MS chromatogram resulting from analysis of 10 ml of milk spiked with β-lactams at the individual level of 10 ng/ml (from [33]).

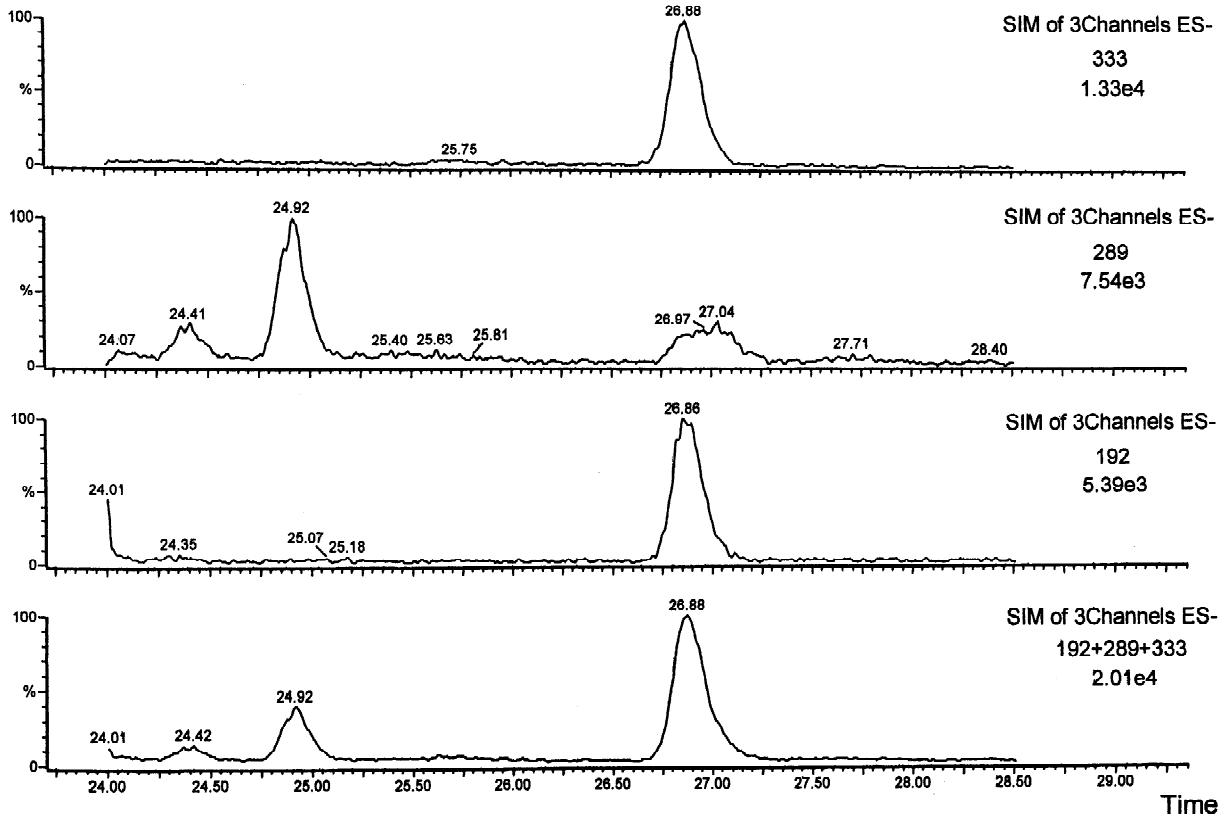


Fig. 6. Single and sum of the ion current profiles for amoxicillin resulting from analysis of a raw milk containing 1.2 ng/ml of the above antibiotic (from [33]).

3. Tetracyclines

Tetracycline antibiotics (TCAs) have a broad range of activity against gram-positive and gram-negative bacteria and are inexpensive. For these reasons, TCAs are widely used in veterinary medicine for preventing and treating several diseases as well as for promoting growth in cattle and poultry. The basic structure of TCAs consists of a hydronaphtacene framework containing four fused rings. The various TCA components differ chiefly by substitution at the C5, C6 and C7 position on the backbone (Fig. 8). Of the eight commercially available TCAs, chlortetracycline (CTC), oxytetracycline (OTC), tetracycline (TC), doxytetracycline (DC) are the most commonly applied to food-producing animals.

Because of the presence of two ketone groups in position 1 and 11 TCAs have the ability of readily chelating to metal ions. Moreover, they can specifically interact with silanol groups. To avoid analyte loss, several authors recommended the presence of chelating agents in any step of the sample preparation procedure.

On chromatographing TCAs on a silica-based stationary phase, the same unwelcome interactions can be established with unreacted silanols and metal surface impurities present in even a highly purified and well end-capped silica material. This results in TCA elution as badly tailed peaks. Many authors obviated this problem by adding some millimoles of chelating agents, such as oxalic acid and EDTA salts, to the mobile phase. However, the presence of nonvolatile agents in the LC mobile phase is not

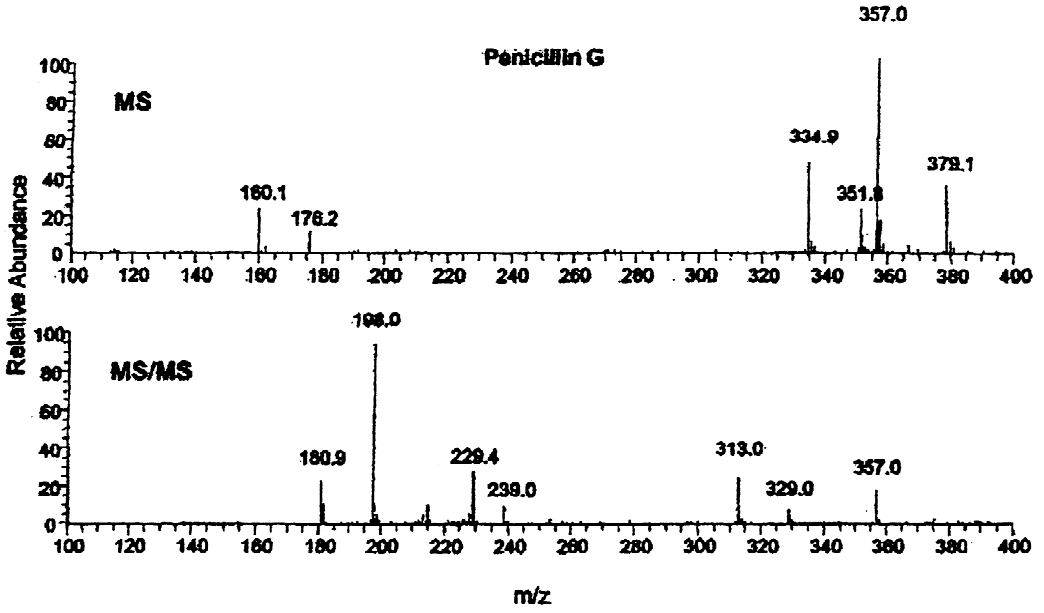
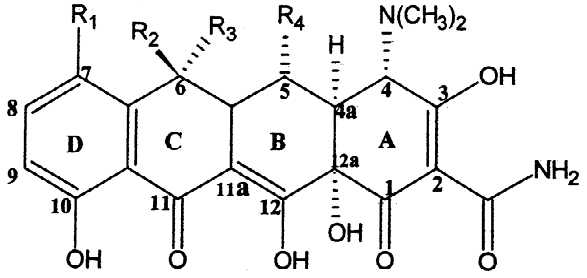


Fig. 7. Positive ion ESI mass spectra and MS–MS spectra of penicillin-G (from [34]).



	R_1	R_2	R_3	R_4
Minocycline	$N(CH_3)_2$	H	H	H
Tetracycline	H	CH_3	OH	H
Oxytetracycline	H	CH_3	OH	OH
Demeclocycline	Cl	H	OH	H
Chlortetracycline	Cl	CH_3	OH	H
Doxycycline	H	H	CH_3	OH

Fig. 8. Chemical structures of tetracyclines.

compatible with use of the ESI-MS detector, owing to rapid obstruction of the sample cone orifice. In addition, we observed that the use of both oxalic acid and EDTA resulted in drastic reduction of the ion signal intensities of TCAs.

Another problem encountered when chromatographing TCAs is that CTC and DC are eluted as peaks preceded by long and broad tailings. The extent of this unwelcome effect depends on the type of column used and chromatographic conditions selected, particularly the temperature at which the LC column operates (Fig. 9). It has been reported that CTC and DC rapidly isomerize to give 4-epiTCAs in aqueous solutions at pH 2–6 [35]. In addition, keto tautomers are readily formed in aqueous solutions [36]. It has to be pointed out that the products of both tautomerization and epimerization of CTC and DC are eluted well before the original compounds. Anomalous peak tailings for CTC and DC could then be originated by the fact the conversion processes mentioned above occur in the LC column, maybe catalyzed by residual silanol groups. This circumstance, rarely mentioned in the literature, complicates quantitation of CTC and DC.

The first report dealing with application of LC–MS to analysis of TCAs in a biological matrix

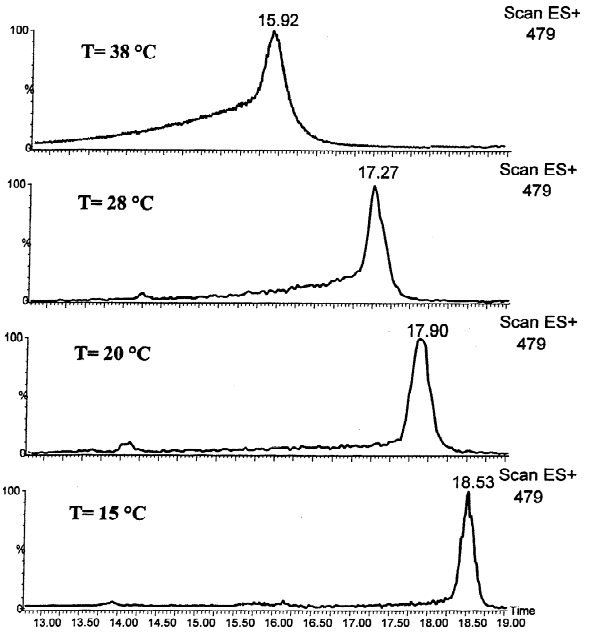


Fig. 9. Peak shape for chlortetracycline by varying the LC column temperature (from [49]).

appeared in 1991 [37]. A PB device was used to interface LC with MS. The authors devised a simple sample preparation procedure involving ultrafiltration followed by cleanup with a C_{18} SPE cartridge. They employed reversed-phase LC with a C_{18} column packing and analytes were eluted isocratically with a water–methanol–acetonitrile mixture containing 50 m*M* oxalic acid. NICI with methane as reagent and four-ion SIM mode was used to identify TCAs in milk extracts. No fragmentation scheme of the $[M-H]^-$ ions was reported. The authors observed that relative abundance of a fragment ion at m/z 442 ($[M-H_2O-H]^-$) was much greater in the OTC standard than in the OTC injected from the milk extract at the start of a series of analyses. Only after several injections of several milk extracts, relative abundance of the above ion in the analysis of standards approached that in the analysis of fortified samples. No explanation for this effect was reported. The authors concluded that the high background noise due to injection of milk extracts precluded quantification of the three TCAs at the stipulated tolerance levels.

The lack of sensitivity of the frit FAB-MS arrangement adopted in a previous study devoted to detecting TCAs in honey [38], led Oka et al. to adopt first ESI-tandem MS–MS [39] and then APCI-tandem MS–MS [40] for identifying TCAs in a variety of biological matrices. The rationale behind this change was that, unlike ESI, APCI could be operated at sufficiently high temperatures to decompose oxalic acid used as LC mobile phase additive. Oka et al. were of the opinion that only this additive can provide the best separation between TCAs and co-eluting substances. Another important modification was that the acquisition previously performed in the daughter ion scan mode [39] was replaced by the much more sensitive SRM acquisition mode [40]. After optimizing conditions of the triple quadrupole instrumentation, the mass spectra shown in Fig. 10 relative to four targeted TCAs and nontargeted DMC (internal standard) were obtained. These spectra show typical fragment ions generated by loss of NH_3 from the carboxyamide group and additional loss of water for those TCAs possessing a hydroxyl group at the C_6 position. For a variety of biological matrices, i.e. honey, milk, eggs, muscle, kidney, liver, fish, Oka et al. invariably proposed a lengthy but not complex sample preparation procedure [38–40]. Basically, it consists of triple extraction with the Na_2EDTA–McIlvaine buffer (pH 4). Each extraction is followed by centrifugation. Combined supernatants are first filtered and then passed through a C_{18} SPE cartridge. Before use, the cartridge is washed with a saturated Na_2EDTA solution to eliminate metal ions that can affect analyte recovery. TCAs are then eluted with 20 ml of methanol. This solvent was removed and the residue was reconstituted with a methanol–water solution. Chromatography was performed on a C_8 column and the mobile phase was methanol–acetontrile–5 m*M* aqueous oxalic acid.

Two years before Nakazawa et al. [40] (Oka's group), an active English research group [41] used the APCI source with a probe temperature set at 500 °C to avoid clogging of the sample cone orifice by oxalic acid added to a concentration of 10 m*M* in the mobile phase. With this LC–MS system, they developed a confirmatory assay for determining TC, OTC, CTC and its isomers in muscle and kidney. Frozen tissue samples were pulverized using a

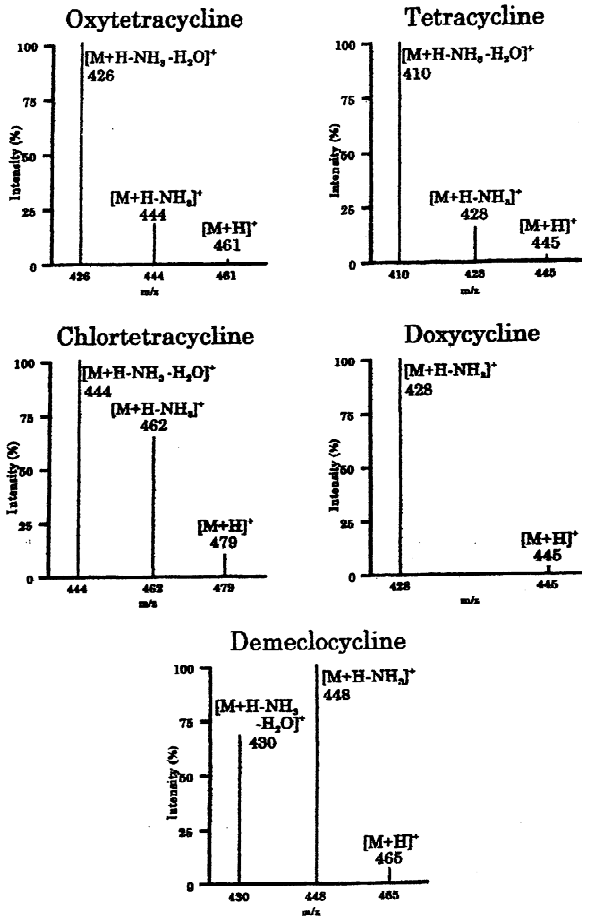
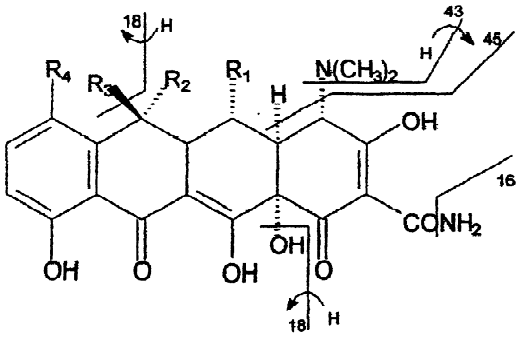


Fig. 10. CID spectra with tandem MS of tetracyclines (from [40]).

domestic food blender. Analytes were extracted from tissues by a glycine–HCl buffer. After homogenizing and centrifuging the extraction was repeated. Combined extracts were purified with a SPE cartridge filled with a cyclohexyl-bonded silica material. Analytes were eluted with methanol. This solvent was removed and replaced with 20 m*M* oxalic acid–acetonitrile (80:20, v/v). After centrifugation, an aliquot of the supernatant was injected into the LC–MS apparatus. The mobile phase composition was indeed complex, as it contained heptafluorobutyric acid (0.04%), 10 m*M* oxalic acid and 10 μ*M* Na_2EDTA, as additives. The tiny amounts of EDTA inhibited metal chelation somewhere in the system and was found not detrimental to the MS interface. The authors proposed of acquiring data for only the molecular ions of TC, OTC and CTC and, eventually, re-injecting sample extracts for collecting two additional confirmatory ions for each analyte. These diagnostic ions were obtained by in-source CID of protonated molecules. The same LC–MS instrumentation was used by the same research group to conduct important studies on the metabolism of chlortetracycline in eggs [42,43].

Based on LC–PB-MS where negative ions were generated by chemical ionization NICI using methane as reagent gas, Carson et al. [44] developed a confirmatory assay for monitoring residues of six TCAs in milk and oxytetracycline in shrimp. A rather complex but original sample preparation procedure was developed by the authors. Milk samples were defatted by centrifugation. Milk deproteinization–extraction was performed with glacial acetic acid. Shrimp tissue was homogenized with 0.1 *M* succinic acid and the homogenate was centrifuged. By exploiting the affinity of TCAs for metals, purification of both milk and tissue extracts was made with a metal chelate affinity column (Cu^{2+} ions immobilized on sepharose). The eluate was further purified by a conventional SPE cartridge. The absolute recovery for the various analytes from either milk or shrimp was near 50%. Final extracts were chromatographed on a styrene–divinylbenzene copolymer (PLRP–S) material with methanol–5 m*M* aqueous oxalic acid (58:42, v/v) as mobile phase. A fragmentation pattern of TCAs in methane NICI mode proposed by the authors is shown in Fig. 11. The problem of variations in fragment ion production occurring between standards and sample extracts was not resolved. To make the method rugged enough, acquisition in the SIM mode was then abandoned and replaced by acquisition in a narrow m/z range (m/z 378 to 483). This change increased specificity and the loss of sensitivity was compensated by large enrichment factors.

The simplest method based on LC–ESI-MS for analyzing TCAs in milk is that proposed by Lock et al. [45]. Analytes are extracted from the sample by solid-phase microextraction (SPME) with a Carbowax/templated resin. Analytes are then desorbed from the fiber by putting it in a chamber filled with



Drug	R_1	R_2	R_3	R_4	m
Chlortetracycline	H	CH_3	OH	Cl	478
Demeclocycline	H	H	OH	Cl	464
Doxycycline	OH	CH_3	H	H	444
Minocycline	H	H	H	$N(CH_3)_2$	457
Oxytetracycline	OH	CH_3	OH	H	460
Tetracycline	H	CH_3	OH	H	444

Fig. 11. Fragmentation pattern of tetracyclines in negative ion chemical ionization mode (from [44]).

acetonitrile–water (15:85, v/v) at 40 °C and set on-line with the LC–MS apparatus. After 5 min, the chamber was flushed with the LC mobile phase. By using relatively high collision energies, the authors obtained in-source CID spectra displaying class-specific product ions, namely *m/z* 154, 126 and 98, of excellent diagnostic value for identifying TCAs in complex matrices. Vartanian et al. [46] proposed possible structures for these fragments.

By exploiting the high sensitivity offered by tandem MS in the SRM mode and the relatively high EU tolerance levels for TCAs in kidney (see Table 1), a rapid sample pretreatment has been proposed by Van Eeckhout et al. [47]. TCAs are twice extracted from kidney samples with the solution used by Oka and co-workers [38–40]. After centrifugation, extract combination and filtration, a 25 μl aliquot of the about 20-ml final extract was injected into the LC column. Chromatography was performed by a short column (5 cm length, 3 μm particle size) with a mobile phase consisting of water–acetonitrile both acidified with 0.3% formic acid. All the analytes were co-eluted with a retention time of <2.5 min. For each TCA the collisional decomposition of the protonated molecule to a unique abundant fragment ion was monitored. LOQs were abundantly lower than safe level established in EU.

A semiautomated analytical method has been recently described [48] allowing simultaneous determination of TCAs and their metabolites in eggs. After sample dilution, clean-up is performed by on-line dialysis and SPE utilizing an extended ASTED (the authors did not specify what this acronym means) system followed by LC–MS–MS. For each analyte, Q_1 was set to transmit only the parent ion, while Q_3 was set to transmit only the most abundant product ion.

Very recently, we have developed a LC–ESI-MS for analyzing five TCAs in milk and eggs at levels well below tolerance levels stipulated in EU and USA [49]. This method is similar to that proposed for quantifying β-lactam antibiotic in milk samples [33]. With milk samples, the procedure has been modified by diluting the matrix with an aqueous

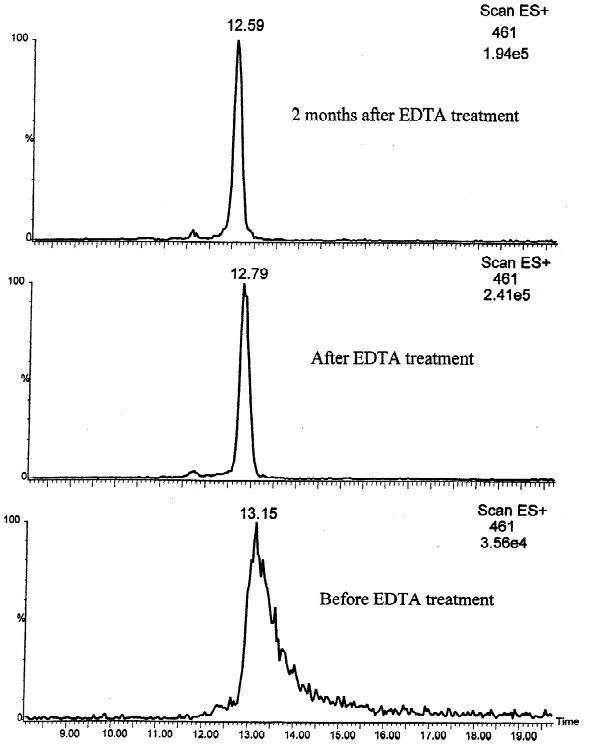
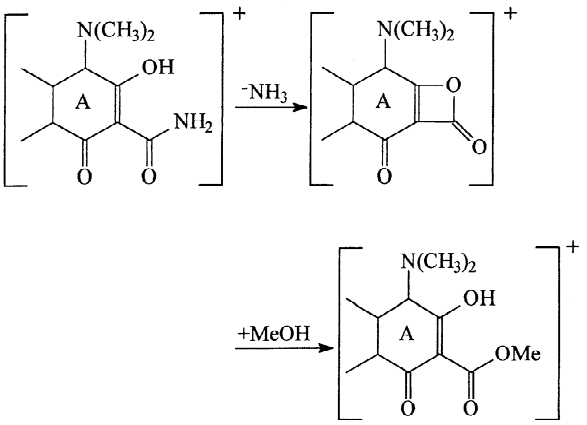


Fig. 12. Peak shape for oxytetracycline before and after treatment of the LC column with a 0.1 *M* EDTA solution (from [49]).

EDTA solution before direct application to the Carbograph 4 SPE cartridge. The presence of EDTA serves to inhibit binding of the analytes to metal ions. With egg samples, after dilution with the EDTA-containing solution and before extraction with Carbograph 4, the suspension was filtered through a paper filter to avoid clogging of the SPE cartridge. Absolute recovery of the five TCAs in the two matrices ranged between 72 and 96%. The problem of metal chelation resulting in badly tailed peaks for TCAs was once and for all solved by extensively washing the silica-based stationary phase with an aqueous Na_2EDTA solution (Fig. 12), according to a previously reported procedure [50]. By doing this, a simple and conventional mobile phase consisting of water–methanol–formic acid (10 m*M*) could be used for chromatographing TCAs in the gradient elution mode (Fig. 13). Setting the column temperature at 15 °C (see Fig. 10) minimized partial conversion of CTC and DC to their isomeric forms. By the ESI-MS device used in this work (Finnigan AQA mass spectrometer) and with methanol as mobile phase organic modifier, the in-source CID process generated reproducible nonconventional fragment ions (Fig. 14). This effect was not observed for those fragment ions produced by loss of only water, or replacing methanol with acetonitrile, or changing the ESI-MS instrumentation. We believe that the anomalous but highly diagnostic product ions are the result of a gas-phase reaction occurring in the desolvation chamber according to:



The estimated limits of quantification ($S/N=10$) of the method were 2–9 ppb TCAs in whole milk and 2–19 ppb TCAs in eggs.

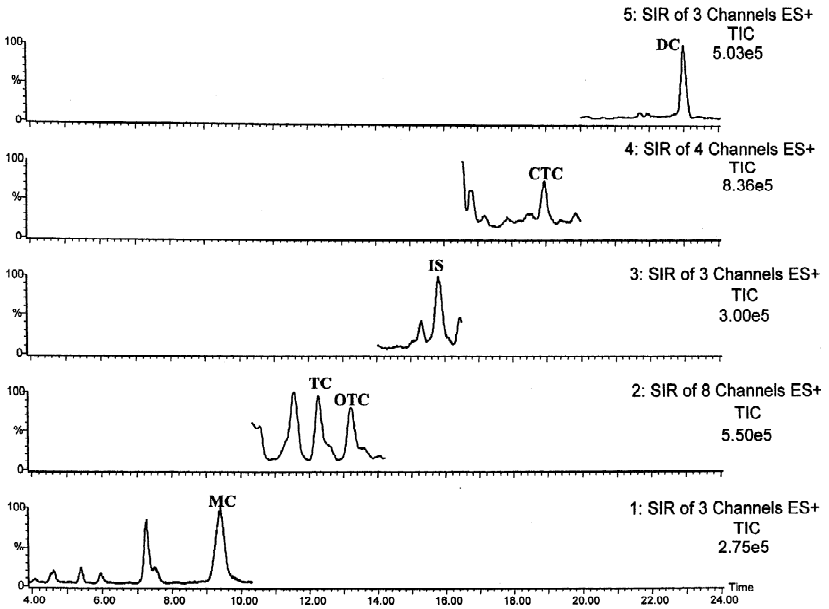


Fig. 13. LC–ES-MS multiple-ion SIM chromatogram resulting from the analysis of a raw milk sample amended with 25 ppb of tetracyclines (from [49]).

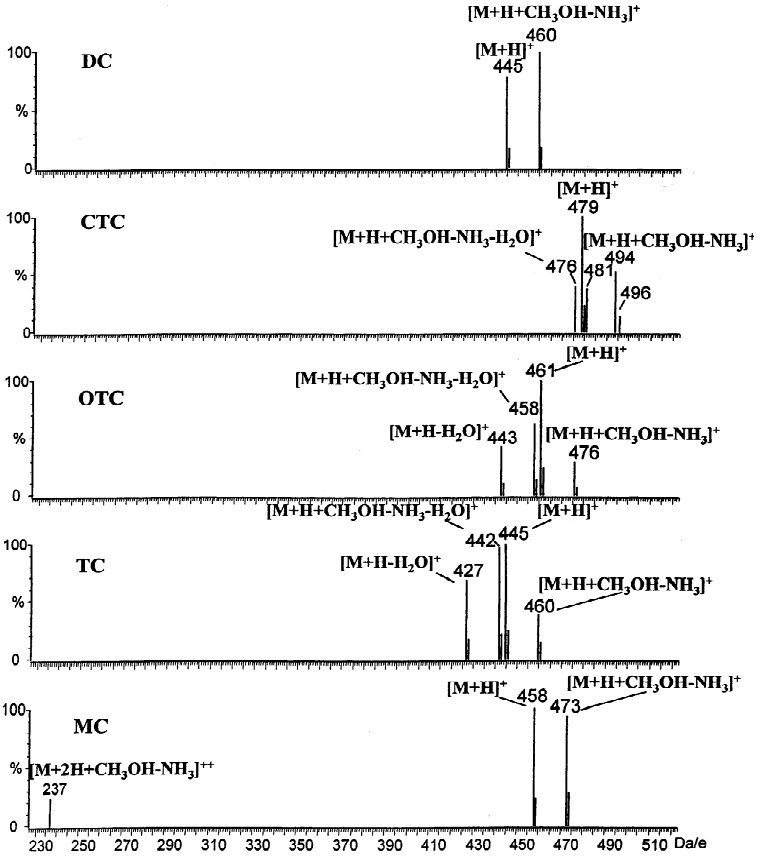


Fig. 14. In-source CID spectra of tetracyclines resulting from the use of methanol as organic modifiers of the LC mobile phase and the Finnigan AQA benchtop mass spectrometer (Thermoquest). MC, minocycline; TC, tetracycline; OTC, oxytetracycline; CTC, chlortetracycline; DC, doxycycline (from [49]).

4. Sulfonoamides

Sulphonamides (SAs) comprise a large number of synthetic bacteriostatic compounds (Fig. 15). They act by competing with *p*-aminobenzoic acid in the enzymatic synthesis of dihydropholic acid. This leads to a decreased availability of the reduced folates that are essential in the synthesis of nucleic acids. Not less than ten SAs are widely used in veterinary medicine. Analysis of SAs in foodstuff is of particular concern because of the potential carcinogenic character.

In the first half of the 1990s, some authors presented methods based on LC–TS-MS with a single quadrupole for analyzing SAs in food.

The need for obtaining diagnostic fragment ions for unambiguous identification induced Abián et al. [51] to adopt the TS-MS–MS arrangement. Proposed fragment structures of SAs are visualized in Fig. 16. For analyzing ten SAs in milk at the ppb level, the authors compared LODs obtained in the SRM mode (Q_3 focused on the group-specific fragment ion at m/z 156 and Q_1 focused alternatively on the different parent ions) and in the MS mode (one-ion SIM of the parent ions). They reported that, owing to ion scattering in the collision cell and incomplete fragmentation of the parent ions, the latter acquisition mode afforded LODs five times lower than those by the former acquisition mode. The authors observed that proteins, carbohydrates and lipids in the extracts gave a rapid decrease in the response, owing to deposition of solid material in the thermospray

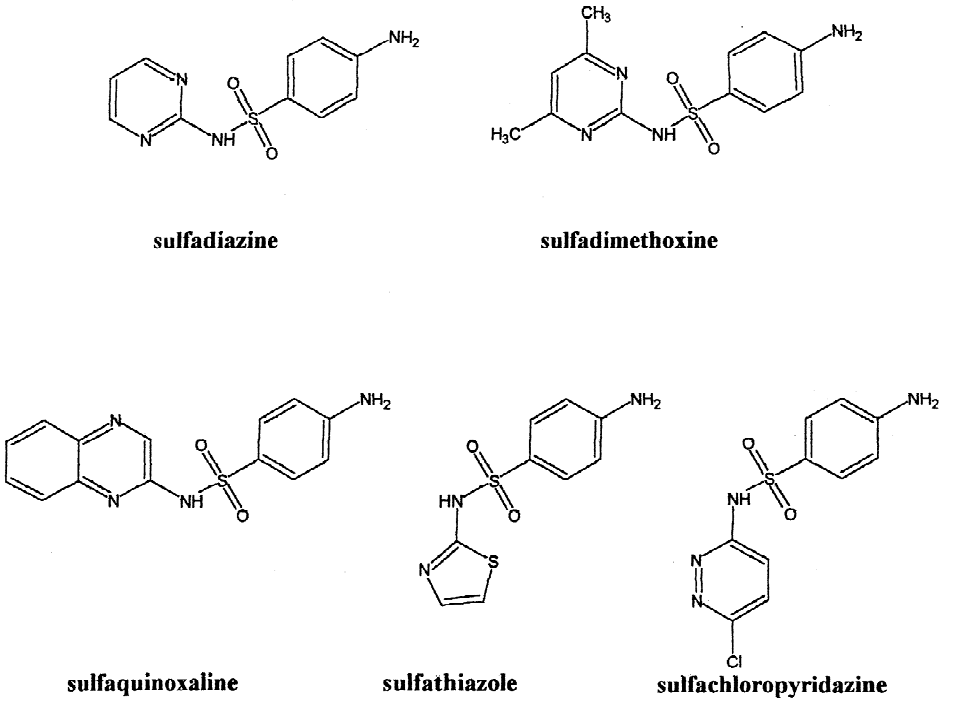


Fig. 15. Structures of some selected sulfonamides.

capillary. However, a better purification of the extract resulted in a more stable performance of the thermospray device.

Two quantitative analytical methods for measuring residues of five SAs in meat were described by Kristiansen et al. [52]. Both methods were based on TS-MS–MS detection. One method included analyte fractionation by a LC column, while the other was based simply on flow injection analysis (FIA). The sample treatment involved analyte extraction from minced meat with ethyl acetate. Sulfapyridine was used as surrogate standard. After solvent removal, the residue was redissolved with 1 ml of 80:20 0.05 *M* ammonium acetate–methanol immediately before analysis. An aliquot of 20 μl was used in the LC–MS analysis. With LC–TS-MS–MS, data acquisition was achieved by switching the mass range of Q_1 between the molecular ions of the SAs during their elution times, while Q_3 was set to pass the fragment ion at m/z 156, which is shared by all the SAs. With FIA-TS-MS–MS, the Q_3 was still set to pass the ion 156, while the Q_1 was fully scanned between 185 and 320 m/z. The authors reported that the full-scan mode gave more intense signals compared to the programmed single-reaction monitoring for each of the SAs. This reflects the time that is used to switch the potentials in the quadrupoles, which becomes significant when many ions are monitored. Both methods proved to have good and comparable sensitivity adequate to analyze SAs at concentrations well below safe levels.

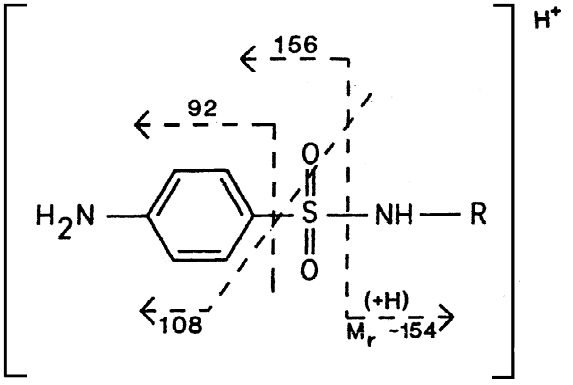


Fig. 16. Fragmentation pattern of sulfonamides (from [51]).

Pleasance et al. [53] were the first to demonstrate applicability of LC-pneumatically assisted ESI-MS to the analysis of SAs in food. Using tandem MS, spectral data were reported for twenty-one SAs. In particular, the authors described a complicated and lengthy procedure for analyzing sulfadimethoxine (SDM) in salmon fish. SDM was extracted with acetone in the presence of Celite and sodium sulfate. After acetone removal, the extract was dissolved with methylene chloride and SDM back extracted with alkalinized water. After neutralization, the extract was freeze-dried overnight and residual water was eliminated by evaporation. The residue was reconstituted with 5 ml of aqueous 25% methanol and the solution was filtered through a 0.45-μm nylon syringe filter. Detection of SDM was performed in the SIM mode of only the protonated molecule, using a triple quadrupole device as a 'single quadrupole' instrument (with transmission of ions through Q_2 and Q_3). The LOD was estimated to be 10 ppb and recovery of SDM was approximately 60%.

In 1993, Doerge et al. [54] demonstrated practicability of analyzing SAs in milk at tolerance levels by a LC–APCI-MS benchtop instrumentation. The advantage of using this instrumentation was that all of the effluent leaving the LC column at a flow-rate of 1 ml/min could be directly introduced into the atmospheric pressure corona discharge source via a heated nebulizer probe. As to sample pretreatment, they proposed extraction of six selected SAs from unadulterated whole milk sample by SPE with C_{18} material. SAs were eluted with methanol. After solvent removal and residue reconstitution with phosphate buffer, the extract was passed through a second Cyclobond I SPE cartridge. Analytes were again eluted with methanol. Following solvent removal, the extract was dissolved in 1 ml of water. Recoveries were 74–95% for milk extracted by this method, as determined by LC–UV. Although using a single quadrupole MS instrumentation, the authors demonstrated that structurally significant fragment ions could be obtained in the intermediate pressure range of the source by suitably increasing the sampling cone voltage (in-source CID spectra). Fragment ions were obtained with a minimal loss in sensitivity and the observed fragmentation reactions were consistent with those obtained using tandem MS.

The same MS instrument (VG Platform benchtop single quadrupole MS) was employed 3 years later by Doerge in collaboration with Gehring et al. [55] for developing a confirmatory method enabling residue analysis of sulfadiazine (SDZ) in salmon muscle. A rather laborious sample treatment procedure was elaborated that involved extraction of SDZ from the sample with acetonitrile followed by partitioning into methylene chloride. Taking advantage of the basic nature of SAs, the extract was purified by passing it through a strong cation exchanger (SCX) SPE cartridge. The eluate from the SCX cartridge was directly applied to a C_{18} SPE cartridge and, after washing, SDZ was eluted with methanol. The volume of the extract was concentrated down to 0.5 ml and diluted to 1 ml with water. A 20-μl volume was then injected into the LC–MS system. For confirmation of the analyte structure by SIM mode, the authors showed that it was not possible to obtain at least two fragment ions and the protonated molecule with adequate intensity at any single cone voltage. To accommodate the need for multiple ions acquisition, the remedy consisted of using the MS software to perform rapid switching of the cone voltage as a step function in concert with acquisition of the respective selected ion. The authors estimated that the presence of SDZ in salmon flesh could be confirmed at 10 ppb level.

Tarbin et al. [56] elaborated a screening method based on LC–API-MS for determining residues of fifteen SAs in egg. d^4-Sulphadiazine and d^4-sulphamethazine were used as internal standards. Acetonitrile was chosen as extractant. By exploiting the amphoteric nature of most of the common SAs, the clean-up procedure involved the sequential use of SCX and SAX cartridges. Following acetic acid acidification of the acetonitrile extract, this was passed through the SCX cartridge. After methanol washing, all the SAs retained on the cation exchanger were re-extracted by using ammonia in acetonitrile. This eluate was then passed through the SAX cartridge. The eluate (fraction 1) was not discharged because it contained sulfaguanidine, which being basic, was not retained at all by the anion exchanger. After methanol washing, the other 14 SAs were re-extracted with 5% acetic acid in acetonitrile (fraction 2). The method was validated at 100 and 25 ppb levels, equivalent to the MRL and one quarter of the MRL. Recoveries of the SAs

ranged from 33% for sulfaguanidine to 92% for sulfamethazine and sulfadimethoxine. For identification and quantitation, an evolved model of the above mentioned Platform APCI-MS system equipped with a single quadrupole was used. Mass data acquisition was performed in the SIM mode by monitoring the $[M+H]^+$ ions.

Application of ESI-MS–MS in the SRM mode to monitoring SA residues in food dates back to 1994. Based on the above detection system, Porter [57] described a method for analyzing five SAs in pig kidney. Compared to ESI-MS with SIM acquisition mode, the use of ESI-MS–MS in the SRM mode offered sensitivity more than 13 times higher, due to reduction in noise. For each analyte, three transitions between the parent ion and three class-specific fragment ions, i.e. m/z 92, 108, 156 were used. Sample pretreatment was carried out by extracting SAs with acidified ethyl acetate. The extract was purified by first passing it through a SPE cartridge filled with a weak cation-exchange sorbent material and then through a SCX extraction cartridge.

Casetta et al. [58] devised a LC–ESI-MS–MS method for determining sulfamethazine and sulfathiazole in honey. Mass data acquisition was performed in the SRM mode by monitoring the transitions 279→124 and 256→108 for sulfamethazine and sulfathiazole, respectively. In spite of the fact that the ion source (high flow ionspray) could accept the entire LC eluent flow (1 ml/min), the authors decided to split the column effluent by a factor of 1:25 before entering the source. In this way, any matrix component makes the ion source 25 times less dirty than with a full flow-rate with no loss of sensitivity, as the ESI-MS system is a concentration-dependent detector. Sample preparation was carried out by extracting the two analytes with methylene chloride from water-diluted and salted honey. The extract was passed through a Florisil SPE cartridge and analytes were eluted with methanol. This was removed by evaporation and SAs redissolved in 1 ml of an acidified water–acetonitrile solution (1:1).

Based on LC–ESI-MS–MS, Volmer [59] elaborated a multiresidue assay for determining 21 SAs in milk. He used a short LC column (5 cm length, 3 μm particle size) and SAs were eluted within 7 min with several peak overlappings. The sample preparation procedure was very similar to that developed by Abián et al. [51]. A three-step analytical approach was adopted to detect, confirm and quantify SAs in milk samples: (1) prescreening (and confirmation) of target compounds by precursor-ion scan and/or multiple reaction monitoring (MRM) experiments using class-specific fragment ions; (2) quantification of identified target compounds by monitoring their protonated molecules by time-scheduled SIM; (3) further confirmation, if necessary, by time-scheduled SRM using compound-specific fragment ions. The author concluded that simultaneous confirmation of all analytes present at levels between 0.2 and 10 ppb was readily possible using time-scheduled MRM.

Ito et al. [60] presented a LC–ESI-MS–MS method able to quantify ten SAs in animal liver and kidney at the tolerance levels with a high identification power achieved by CID spectra obtained by scanning the third quadrupole. The SAs were extracted with ethyl acetate. After solvent removal, SAs were dissolved with ethylacetate–hexane (50:50, v/v). For clean-up of the crude extract, this solution was passed through a WCX extraction cartridge. Analytes were re-extracted by 5 ml of 20% acetonitrile–0.05 ammonium formate and 20 μl of this solution were injected into the LC–MS–MS apparatus.

5. Aminoglycosides

Aminoglycosides (AGs) are active against a broad spectrum of Gram-negative and Gram-positive bacteria. They exert their antibacterial effect by binding to the 30S ribosome, which disrupt bacterial protein synthesis. The chemical structures of AGs are based on a aminocyclitol ring (2-deoxystreptamine, in most cases) connected to two or more amino sugars in a glycoside linkage (Fig. 17). It is believed that improper use of this antibiotic class may generate residues that are potentially harmful due to oto- and nephrotoxicity.

McLaughlin and Henion [61] evaluated the feasibility of using LC–MS for analyzing four AGs, i.e. spectinomycin, hygromycin B, streptomycin and dihydrostreptomycin in bovine tissues. The scarce solubility of AGs in organic solvents induced the authors to use the technique of matrix solid-phase dispersion (MSPD) for analyte extracting from a bovine kidney sample. A 0.5-g amount of the homogenized sample was mixed with 2 g of

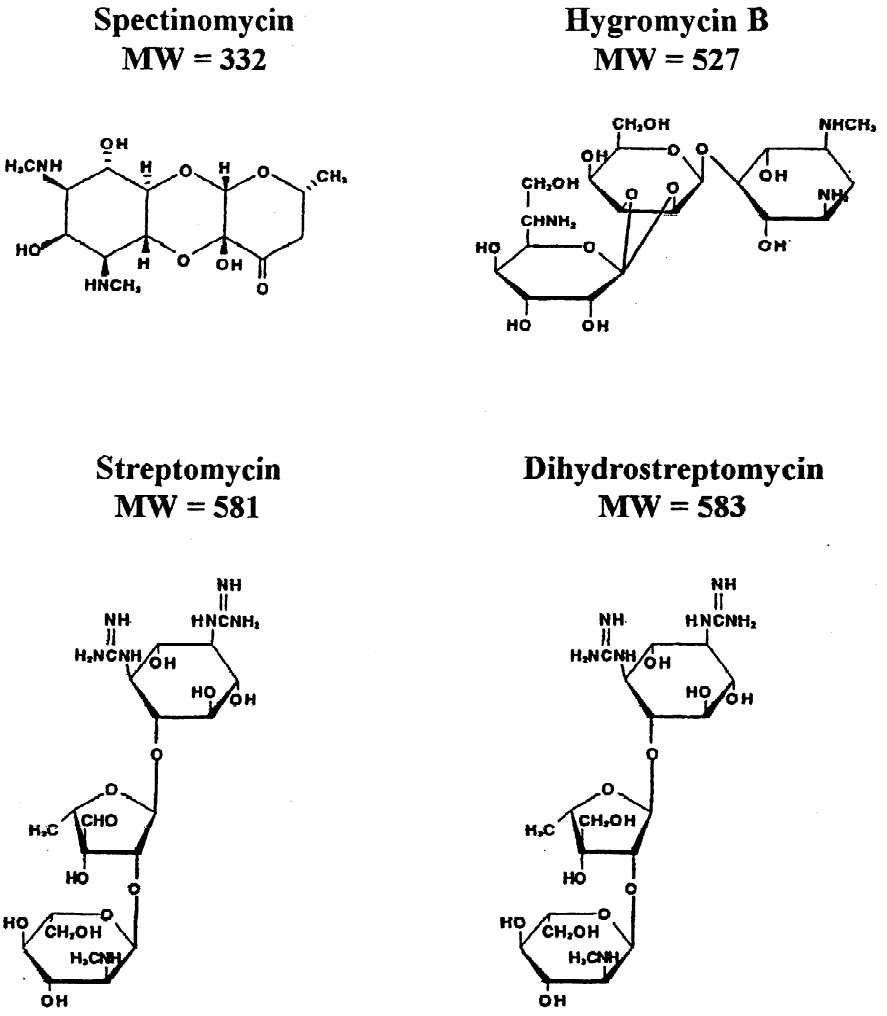


Fig. 17. Structures of the aminocyclitol and aminoglycoside antibiotics (redrawn from [61]).

cyanopropyl-bonded silica. After blending, the solid material was packed into an empty SPE cartridge. The cartridge was then washed sequentially with hexane, ethylacetate, methanol and 50% methanol in water. The analytes were eluted with 1 ml of water followed by 8 ml of 0.05 *M* sulfuric acid. The eluate volume was reduced by a factor 4, neutralized with ammonia and 10 μl of the final solution was injected into the LC–ESI-MS apparatus, which was operated in the PI mode. A C_{18} analytical column was used and the analytes were chromatographed by ion-pair LC with pentafluoropropyl acid as ion pair reagent. The authors studied the effects of the nature and concentration of pairing ions on LC separation and ionization efficiency of the analytes in the electrosprayed solutions. SIM acquisition was adopted for detecting the $[M+H_2O+H]^+$ ion at m/z 351 and 301 relative to respectively spectinomycin and streptomycin, the $[M+2H]^{2+}$ ions at m/z 265 and 293 relative to respectively hygromycin B and dihydrostreptomycin. These compounds could be detected in bovine kidney at 20 ppm level, which is well above levels of regulatory interest.

Two years later, McLaughlin et al. [62] improved

the above method in terms of sensitivity and specificity. In addition to the four AGs mentioned above, neomycin B and four components of the gentamycin C complex were considered. Improvement in the sensitivity was achieved mainly by concentrating the original eluate by a factor of 53. With tandem MS, data acquisition in the SRM mode by choosing three transitions for each analyte (where possible) provided a high level of specificity. The authors studied the fragmentation patterns of the compounds considered and reported m/z values of their product ions in their work [62]. The remedy of analyte ion suppression by matrix components was that of adopting fortified control tissue samples as standards. All compounds could be detected (while monitoring three ions by SRM) in bovine kidney at or below the regulatory level of concern, with the exception of spectinomycin. The failure in detecting spectinomycin at the regulatory level was also due to a severe loss of the analyte occurring during sample treatment.

Carson and Heller [63] developed a confirmatory method for residues of spectinomycin in bovine milk. Adding trichloroacetic acid and centrifuging eliminated proteins. An aliquot of the middle aqueous layer was first diluted with an aqueous solution containing an ion pair reagent, i.e. heptafluorobutyric acid (HFBA), and then applied to a C_{18} SPE cartridge. Spectinomycin was eluted with a methanol–water mixture (4:1, v/v) containing HFBA. The eluate was concentrated to 1 ml and filtered through 0.2-μm pore size, 13 mm diameter PDVF syringe filter (Whatman). A 20-μl aliquot of the final extract was injected into a LC–ESI-ion trap MS system. The column was a PLRP–S 15×0.21 cm, 5 μm particle size. The mobile phase was a water–methanol solution containing HFBA as ion pair reagent. In full scan MS mode, the spectrum displayed the protonated hydrated molecule (m/z 351) as the major ion, while the molecular ion (m/z 333) was present with a 30% relative abundance. In the MS–MS mode, CID of the 351 ion substantially produced only the 333 molecular ion. Since this condition was not sufficiently specific for confirmatory purpose, the authors selected the latter ion as the precursor ion for MS–MS analysis. The optimum collision energy value was rather critical and reproducible fragment ion spectra were obtained under a condition giving a poor signal for the precursor ion. The fragment ions observed were qualitatively comparable to those reported using a triple quadrupole instrument. However, a poor chromatographic performance (peak tailing, peak splitting, some significant differences of retention times between standard and extract residues) and high day-to-day variability of the ion trap quantitative response (sum of fragment ions m/z 140, 186–190 and 227) induced the authors to conclude that the method needed some improvement to meet guidelines for regulatory determination in the US.

LC–APCI-MS–MS was employed by Hornish and Wiest [64] for elaborating a confirmatory method of analysis for spectinomycin in various bovine tissues. Spectinomycin was extracted from ground tissue by adding a citric acid buffer and trichloroacetic acid to the sample. Methylene chloride was also added to eliminate non polar potentially interfering compounds. After shaking and centrifuging, the aqueous extract was purified by passing it through a C_{18} SPE cartridge and spectinomycin was eluted from the cartridge by a citric acid buffer. Recovery of spectinomycin ranged between 85 and 94%, irrespective of the type of the tissue and of the fortification level (0.1–10 ppm). The target compound was chromatographed on a C_{18} column with a mobile phase consisting of 1% acetic acid–methanol (92:8, v/v). Q_1 was set to m/z 333 (molecular ion), while Q_3 was set to scan the selected fragment ions at m/z 98, 116, 158 and 189. The limit of confirmation was estimated to be 50 ppb.

A procedure for the confirmation of neomycin and gentamicin in milk at 30 ppb level has been recently reported [65]. This method is based on weak cation-exchange extraction and analyte monitoring by ESI-ion trap-MS. Milk was first treated with acid and centrifuged. The supernate was buffered at neutral pH by sodium citrate and applied to a WCX SPE cartridge. Targeted compounds were eluted with acidic methanol. Following separation by ion-pair LC, analytes were ionized with an ESI interface. Protonated molecular ions were selectively stored in an ion trap MS, then decomposed giving CID spectra displaying several diagnostic fragment ions. To obviate matrix effect causing the absolute response to increase in extracts as compared to pure standards, quantitation was performed using fortified control tissue samples as standards.

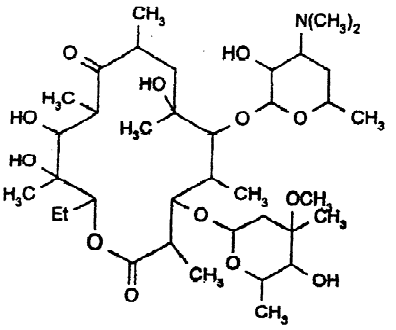
6. Macrolides

Macrolide antibiotics are macrocyclic lactones isolated first from Streptomyces spp. The chemical structures of macrolides consist of a 12-, 14- or 16-membered macrocyclic lactone to which sugar moieties, including amino and deoxy sugars, are attached (Fig. 18). Macrolides are an important class of antibiotics widely used in veterinary practice to treat respiratory diseases and enteric infections in cattle, sheep, swine and poultry. Studies with dogs, cattle, pigs and monkeys have revealed that the toxic effects associated with a member of the class of macrolide antibiotics, i.e. tilmicosin, involve the cardiovascular system.

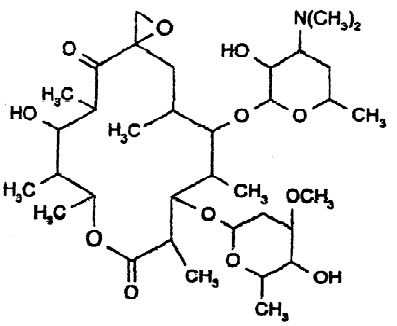
In 1992, Pleasance et al. [66] investigated the feasibility of using LC–ESI-MS–MS for determining erythromicin in salmon muscle. A minced sample was homogenized twice in acetonitrile prior to centrifugation and filtration of the supernatant. Fats were removed by hexane partitioning. Erythromicin was extracted from alkalinized and salted acetonitrile with methylene chloride. This solvent was replaced by methanol for reversed-phase LC–MS analysis. A 1/200 aliquot of the final extract was injected onto a C_8 LC column that was operated with an aqueous acetonitrile mobile phase acidified with 0.2% formic acid. Erythromicin was eluted by a linear gradient elution of 5–65% acetonitrile in 15 min. For confirmatory purpose, the authors proposed acquisition in the PI mode by monitoring two selected transitions (m/z 734→576 and 734→158). Under this condition, erythromicin could be detected under a putative tolerance level of 100 ppb. By analyzing flesh of salmon exposed to erythromicin, the authors showed that their method could enable detection of a number of metabolites and degradation product of the above antibiotic. However, the authors commented that their method needed an improved clean-up procedure, as the analysis of only a few sample required replacement of the guard column.

Kiel and Kessington [67] explored the potential for the application of LC–APCI-MS with a single quadrupole as a confirmatory method for tilmicosin in swine liver. Singularly, the authors referred to unpublished data for a description of the sample treatment. The analyte was isocratically eluted from a C_8 column using a mobile phase consisting of 5 m*M* ammonium acetate (adjusted to pH 4.5)–acetonitrile (40:60, v/v). By suitably controlling the cone voltage, in-source CID generated two diagnostic fragment ions (m/z 696, 174) from the $[M+H]^+$ ion (m/z 869). Mass spectral data were collected by using three-ion SIM. While this method may be sufficiently sensitive, nothing can be said on the simplicity and robustness of the sample treatment procedure.

It is known that a critical limitation of ESI and APCI ion sources is the susceptibility to blockage of interface orifice due to the deposition of involatile additives eventually present in the mobile phase. Bajic et al. [18] presented a method for analyzing



Erythromycin A
(mol. wt. 733)



Oleandomycin
(mol. wt. 688)

Fig. 18. Two examples of macrolide antibiotics.

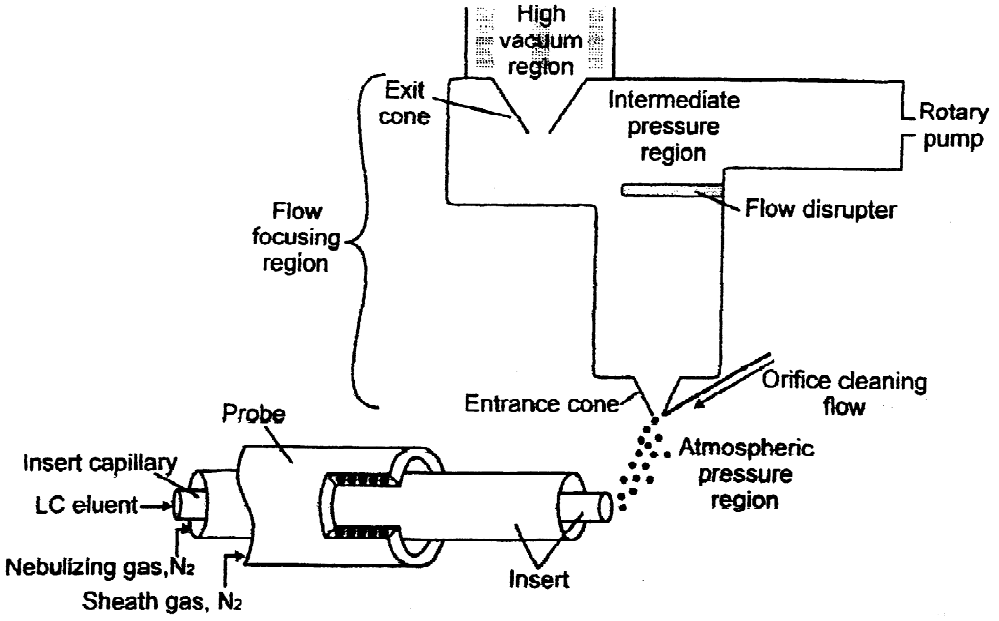


Fig. 19. Sketch of the AQA API source/probe (from [18]).

erythromycin in chicken and beef liver, and catfish muscle by making use of a novel API source (Fig. 19) able to accept a mobile phase containing 10 m*M* sodium phosphate. The enhanced robustness of the new source is derived from the use of a continuous flow of aqueous solvent at the sampling cone orifice that maintain unobstructed ion transmission. With this device, the authors showed that the ion signal intensity for the analyte did not change significantly over 13 h of continuous use of the instrument. Erythromycin and oleandomycin, the latter compounds being used as surrogate internal standard, were extracted from the above matrices by ten volumes of water containing 20 m*M* of both KH_2PO_4 and sodium octane sulfonate, and 20% acetonitrile. After centrifugation and fat elimination by hexane partitioning, the pH of the aqueous solution was adjusted to 10 and erythromycin was extracted three times with ethyl acetate. After solvent exchange with methylene chloride, the extract was passed through a WCX (aminopropyl silica) SPE cartridge. Erythromycin and oleandomycin were desorbed with 2% methanol in methylene chloride. Just before analysis, solvents were removed and the residue was dissolved with 0.5 ml of the LC mobile phase. Ten μl of this solution were injected into the LC–MS apparatus. Separations were performed by a C_{18} column using a mobile phase containing 30% by volume acetonitrile in 10 m*M* sodium phosphate. The flow-rate was 1 ml/min and the entire column effluent was introduced into the ion source. A Finnigan AQA single quadrupole mass spectrometer was used with an ESI probe temperature of 450 °C. Positive ions were acquired in SIM mode. The MS method monitored the $[M+H]^+$ ions for the analyte and the surrogate at m/z 734 and 689, respectively. No attempt was made by the authors of improving the specificity of the method by monitoring fragment ions that could be obtained by raising the cone voltage. The authors showed that quantification of erythromycin at level as low as 10 ppb in all of the matrices considered was possible.

Dubois et al. [68] described an LC–ESI-MS–MS analytical procedure enabling quantification of tylosin, tilmicosin, spiramycin, josamycin and erythromicin in various tissues below stipulated EU tolerance levels. The method uses extraction in a Tris buffer at pH 10.5 followed by protein precipitation with sodium tungstate and clean-up on an Oasis SPE cartridge. LC separation was performed on a C_{18}

column with a gradient of 0.1 *M* ammonium acetate–acetonitrile as mobile phase. Protonated molecules served as precursor ions for ESI in the PI mode and the most abundant fragment ion was chosen for each analyte for MRM acquisition. Eventually, further confirmation could be achieved by including three additional product ions for multiple MRM acquisition.

A confirmatory method for three macrolides (tylosin, tilmicosin and erythromicin) in bovine tissues by micro-LC–ESI-MS–MS was presented by Draisci and co-workers [69,70]. The molecular related ion, $[M+2H]^{2+}$, at m/z 435 for tilmicosin, the $[M+H]^{+}$ ions at m/z 734 and 916 for respectively erythromicin and tylosin were the precursor ions and two fragment ions for each macrolide were used for SRM operation. Analyte extraction and clean-up was carried out according to a previously reported procedure [71]. Depending on the nature of both matrices and analytes, limits of quantification ranged between 30 and 150 ppb, and were all below MRLs issued by EU.

7. Quinolones

Quinolones are a group of relatively new antibiotics synthesized from 3-quinolonecarboxylic acid. Some representative structures are shown in Fig. 20. Quinolones show excellent activity against both Gram-positive and Gram-negative organisms, as well anaerobes. They act to inhibit DNA gyrase a key enzyme in DNA replication.

A confirmatory method for monitoring residues of danofloxacin in chicken and bovine liver by micro-LC–ESI-MS–MS was developed by Schneider et al. [72]. To liver homogenate, 0.15 *M* of both phosphoric and perchloric acids in equal volumes of methanol–water were added. Then, the sample was again homogenized and incubated 1 h at 55 °C. After centrifugation, the supernatant pH was adjusted to 8.5 and the analytes were extracted with methylene chloride. After evaporation, the residue was dissolved with 50 μl of the LC mobile phase and 1 μl was injected onto the above apparatus. The mobile phase consisted of acetonitrile–0.1% trifluoroacetic acid, while the stationary phase was a base-deactivated C_{18} column. The MS–MS system operated in the PI mode. Identification and quantitation of danofloxacin was performed in the SRM mode by choosing two transitions, i.e. 358→340 and 358→255. The fragment ion at m/z 340 formed by loss of water, while the ion at m/z 255 was postulated to originate by losses of water+*N*-methylpyrrolidine from the piperazine moiety. Authors estimated that the method can enable confirmation of danofloxacin down to 50 ppb.

Doerge and Bajic [73] evaluated performances of both LC–MS and LC–MS–MS instrumentations for determining and quantifying nalidixic, oxolinic, piromidic acids, and flumequine, in catfish muscle. Protonated molecules were obtained by an APCI ion source. With the former equipment, fragmentation of the $[M+H]^{+}$ ions was induced by in-source CID. Under these conditions, all of the analyte spectra indicated losses of water and CO_2. SIM of the protonated molecule plus the two fragment ions for each compound allowed detection in catfish muscle at levels of 0.8–1.7 ppb. A ten times lower detection limit was obtained by the MS–MS arrangement by exploiting the MRM acquisition mode. Only one transition, i.e. $[M+H]^{+}\rightarrow[M-H_2O+H]^{+}$, was chosen for each analyte. The four analytes were eluted on a styrene–divinylbenzene copolymer LC column using a mobile phase gradient consisting of 22–38% solvent A (acetonitrile) in solvent B (9% tetrahydrofuran in 25 m*M* acetic acid) over 20 min. The sample preparation procedure was not described by the authors.

Schilling et al. [74] developed an analytical procedure for confirming trace levels of sarafloxacin in catfish tissue based on LC–ESI-MS–MS. Catfish tissue was first homogenized with 1 *M* NaOH and then protein were precipitated with an acetonitrile–85% phosphoric acid solution (2:1, v/v). After centrifugation, the extract was purified by a C_{18} SPE cartridge. After washings, sarafloxacin was desorbed by an acetonitrile–0.04 *M* citric acid (pH 2.4) solution. The eluate was evaporated to a dry residue and redissolved in the LC mobile phase. Differently from Doerge and Bajic [73], an analyte-specific fragment ion was also selected for MS–MS operation. The method allowed for the detection of sarafloxacin of 200 ppb in catfish tissue.

Volmer et al. [75] described a multiresidue method for simultaneous detection and confirmation of

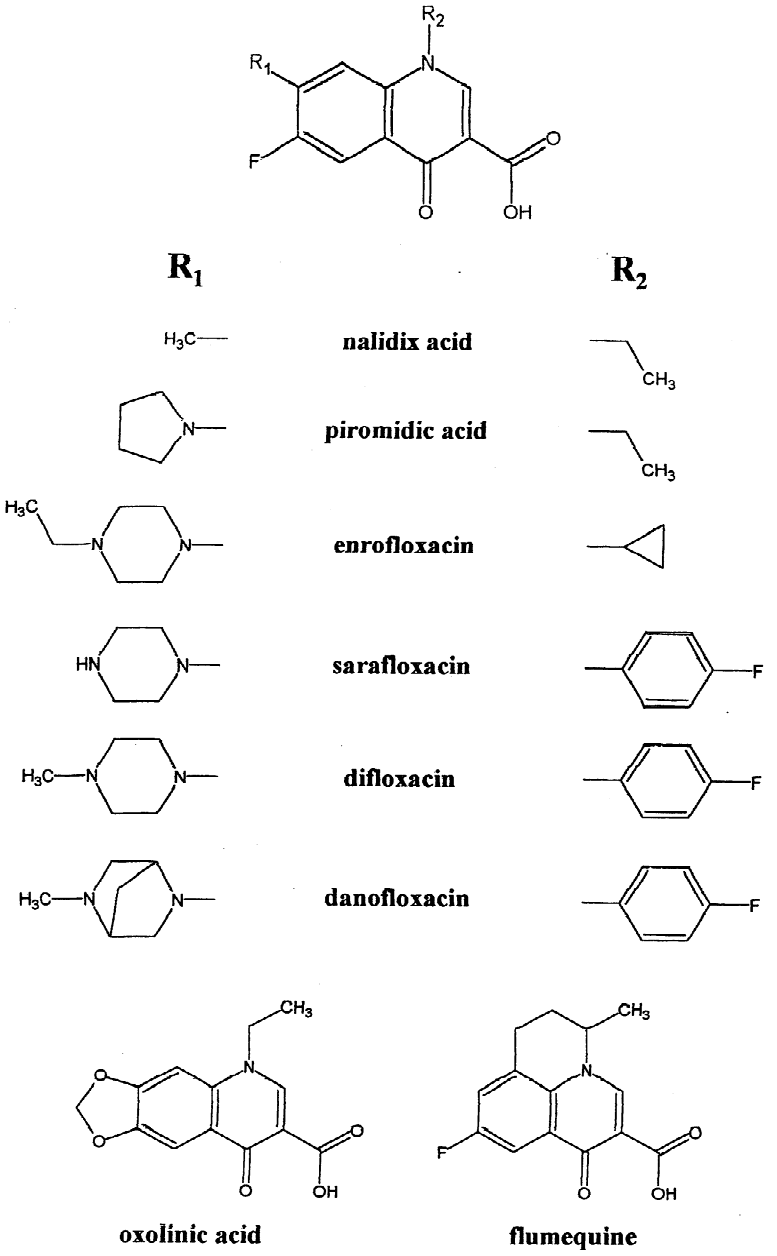


Fig. 20. Structure of selected quinolone antibiotics.

quinolones in milk and salmon tissue. With milk samples (2 ml), simultaneous protein precipitation, defattening and extraction of seven selected quinolones were obtained by adding NaOH, acetonitrile, diethyl ether and hexane. After centrifugation, the upper layer of organic solvent was discharged. The aqueous solution was acidified with phosphoric acid and methanol was added, followed by centrifugation. After solvent removal, the residue was dissolved in 0.5 ml of the LC mobile phase and filtered prior to injection. A 10-g amount of fish muscle tissue amended with four selected quinolones was

first homogenized with acetone. The watery acetone extract was defatted with hexane, and the analytes were subsequently extracted with chloroform. This solvent was evaporated to dryness and the residue was dissolved in 1 ml of water that was filtered prior to injection. Analyte recovery was 56–95%. Separations were accomplished on a short column (5 cm) packed with 3-μm phenyl-bonded silica. The mobile phase composition was acetonitrile–water (2:98, v/v) acidified with 20 m*M* formic acid programmed to 57:43 in 10 min. LC–ESI-MS–MS equipment was employed for identifying and quantifying quinolones. The strategy proposed by the authors to detect, confirm and quantify 7-piperazinyl-6-fluoroquinolones in milk samples was as follows: (a) prescreening of target and nontarget quinolone antibiotics by constant neutral loss scan experiments using the $[M+H]^+ \rightarrow [MH-87]^+$ transition; (b) quantification of identified target compounds by monitoring their protonated molecules in a time-scheduled SIM operation; (c) further confirmation, if necessary, by SRM using three characteristic transitions. The LODs in milk in SIM of protonated molecules were 0.2–2 ppb for six 7-piperazinyl-6-fluoroquinolones, and 0.5 ppb for enrofloxacin. Limits of confirmation (LOCs) by MS–MS were between 1 and 2 ppb. The SIM LODs of four acidic 4-quinolones, which are typical in aquaculture application, were about 1 ppb for all the analytes. Unlike 7-piperazinyl-6-fluoroquinolones, the four acidic 4-quinolones do not exhibit class-specific fragment ions. For confirmatory purposes, then, characteristic product ions were monitored by using three transitions for each analyte.

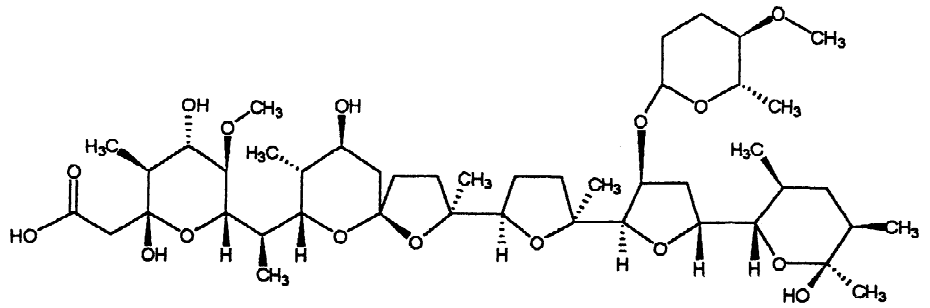
Delepine et al. [76] described an LC–APCI-MS method for the confirmation of six quinolones (enrofloxacine, ciprofloxacine, marbofloxacine, danofloxacine, sarafloxacine and difloxacine) in pig muscle. The analytes were extracted from 2 g of tissue with phosphate buffer (pH 7.4). After centrifugation, the supernatant was purified on a C_{18} SPE cartridge. After washings, the analytes were eluted from the cartridge with 1 ml of 1% trifluoroacetic acid followed by 0.5 ml of acetonitrile. After solvent removal by evaporation, the residue was redissolved in 200 μl of acetonitrile and the solution was diluted to 2 ml with water. A 100-μl aliquot of the resulting solution was injected into the LC–MS system. For fractionation, a C_{18} column was used. The mobile phase composition was acetonitrile–water containing 1% formic acid and 50 m*M* ammonium acetate (20:80, v/v) programmed to 40:60 in 5 min, then to 80:20 in 6 min. Acquisition was performed in the PI mode. Confirmatory ions were obtained by in-source CID. Four ions for each quinolone were monitored in the SIM mode. The LOCs of the method permitted the confirmation of the six quinolones down to 7.5 ppb.

A multiresidue LC–ESI-MS method was developed for identification of ciprofloxacine, enrofloxacine, sarafloxacine and difloxacine in catfish muscle at 10 ppb level [77]. Quinolones were twice extracted from 2 g of catfish muscle with ethanol–water glacial acetic acid (98:1:1, v/v). After the second centrifugation, other acetic acid was added to the combined supernatants. This solution was placed in freezer for 30 min and then centrifuged. The supernatant was cleaned up on a SCX SPE cartridge. After washing with methanol, the analytes were eluted with 30% ammonia–methanol (1:4, v/v). After drying, the residue was reconstituted in 0.5 ml mobile phase and this solution was filtered. A 50-μl aliquot was injected into the LC column. The chromatography was performed on a phenyl-bonded silica. The mobile phase consisted of 2% formic acid–acetonitrile (86:14). By adjusting the cone voltage, in-source CID provided two class-specific and one compound-specific fragment ions for each quinolone. Acquisition was performed by time-scheduled four-ion SIM.

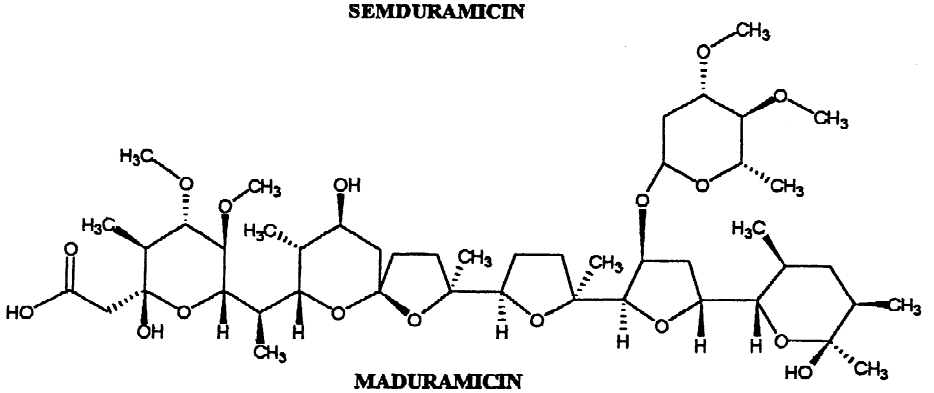
8. Polyether ionophore

Polyether ionophore antibiotics are fermentation-derived biologically active compounds characterized by the presence of a carboxylic acid group and several cyclic units (Fig. 21). The term ionophore derives from their ability to form stable complexes with alkaline cations. They are widely used as anticoccidiosis agents in broiler chickens and as feed efficiency enhancers in cattle and sheep.

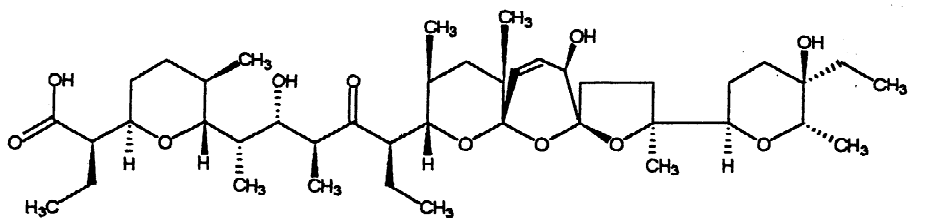
An LC–ESI-MS–MS for the residue analysis of semduramicin in chicken liver was described by Schneider et al. [78]. Semduramicin was extracted from 1.25 g of the tissue with methanol–1% aqueous



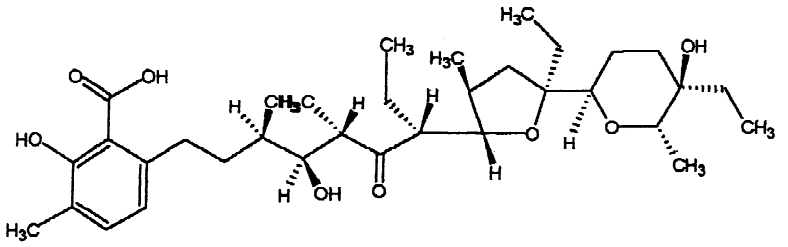
SEMDURAMICIN



MADURAMICIN



SALINOMYCIN



LASALOCID

Fig. 21. Structures of the most commonly used polyether ionofore antibiotics.

ammonia (8:2, v/v) and separated from co-extractives by using first a C_8 SPE cartridge and then a silica gel SPE cartridge. Semduramicin was eluted from the latter column with a methylene chloride–methanol (90:10, v/v) mixture. Solvents were eliminated by evaporation and the residue was redissolved

in 50 μl of LC mobile phase. One tenth of this solution was injected into the LC–MS–MS system. The analytical column was a C_{18} microbore column (250×1 mm) and the mobile phase consisted of 5 m*M* sodium acetate–acetonitrile (10:90, v/v) at a flow-rate of 40 μl/min. All of the effluent was delivered to the ESI source. Ionophores have the ability to form several adduct ions with cations present in the electrosprayed solution. This situation complicates structural analysis and dilutes the ion beam. The rationale behind the addition of sodium acetate to the mobile phase was that of shifting equilibrium toward the formation of only the sodiated adduct ion. However, CID of this ion showed very limited fragmentation, i.e. the loss of CO_2 and CO_2 + water. By monitoring both losses in the SRM mode, the authors demonstrated that semduramicin in chicken liver could be quantified at level of few teens of ppb.

Blanchflower and Kennedy [79] presented a LC–ESI-MS method for determining lasalocid in eggs. Samples were extracted under acidic conditions with acetonitrile and a simple clean-up procedure consisting of liquid–liquid partitioning using an hexane–toluene solution as extractant. Analyte identification was not highly specific as only the sodiated adduct ion at *m*/*z* 613 was monitored.

The above authors used the same instrumentation to develop a method enabling simultaneous determination of three ionophores, monensin, salinomycin and narasin in muscle, liver and eggs from domestic fowl at 1 ppb level [80]. The sample preparation scheme was the same as reported above [79]. Analytes were chromatographed on a C_{18} column with a mobile phase consisting of acetonitrile–methanol–water–trifluoroacetic acid (67:10:10:13:0.1). The ESI-MS detector was set to collect multiple single-ion data for the ions at *m*/*z* 693, 773, and 787 relative to the sodiated ions of monensin, salinomycin and narasin, respectively. Again, the confirmatory ions observed by in-source CID were not included in the acquisition program.

For determining residues of lasalocid in animal tissues and eggs, a simple and rapid method based on LC–ESI-MS–MS was developed by Matabudul et al. [81]. Following addition of anhydrous sodium sulfate to absorb water in liver, kidney and egg samples, lasalocid was extracted with acetonitrile. After centrifuging, an aliquot of the supernatant was filtered through a 0.45-μm syringe filter and 15 μl of the filtrate was injected into the LC–MS–MS system. Chromatography was performed using the same conditions reported by Blanchflower and Kennedy [80]. MS–MS parameters were suitably adjusted to give the sodiated ion at *m*/*z* 613 and two fragment ions at *m*/*z* 577 and 377. The product ions were monitored by the MRM mode. The LOQ of lasalocid in all of the matrices considered was estimated to be 1 ppb.

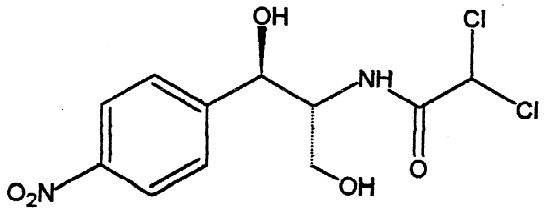
Rosén [82] developed, validated and used for routine surveillance a method based on LC–ESI-MS–MS for determining traces of narasin, monensin and salinomycin in chicken liver and eggs. Essentially, the aim of this work paper was that of demonstrating that one single method can serve very well for two different purposes, i.e. screening and confirmation. Samples were extracted with 87% methanol and extracts were centrifuged. A single-step extract purification was automatically performed by a C_{18} SPE cartridge. Analyte elution was carried out with methanol and an aliquot of it was submitted to analysis by LC–MS–MS. The stationary phase was a C_{18} material and analyte fractionation was done isocratically by acetonitrile–50 m*M* ammonium acetate (80:20, v/v). Infusion experiments for each compounds in both PI and NI modes were performed to individuate fragment ions and to find the best condition in term of sensitivity. The sodiated molecules gave the best response and the MS parameters were optimized to obtain MRM transitions for each sodiated complex. For screening purpose one transition was selected for each analyte, while four transition were monitored for narasin confirmation. By using this method, results of a 1-year monitoring campaign revealed that 50% of the Swedish eggs were contaminated by narasin at 0.2–11 ppb levels.

9. Amphenicols

Chloramphenicol is a broad spectrum antibiotic frequently used in veterinary and human medicine. After publication of studies showing anemia in humans as side effect arising from its use, chloramphenicol has been banned within the USA and the EU. Thiamphenicol and florfenicol, which have

structures similar to chloramphenicol (Fig. 22), have been permitted as substitutes.

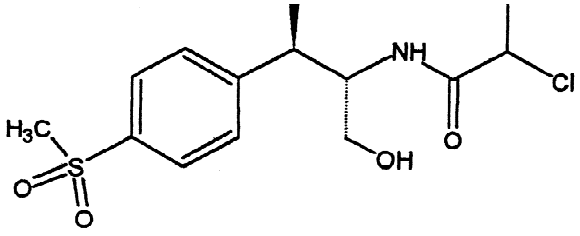
In 1992, Delépine and Sanders [83] proposed a simple and rapid method based on LC–PB-MS operated in the NICI (methane as the reagent gas) mode for detecting chloramphenicol in calf muscle. The compound was extracted from the tissue with ethyl acetate. After centrifugation and extractant removal, the dry extract was suspended in hexane–CCl_4 and extracted with 400 μl of water. One fourth of it was injected into the LC column. Chromatography was performed with a C_{18} LC column using methanol–0.2% formic acid (43:57, v/v) as mobile phase. The spectrum of chloramphenicol displayed three fragment ions formed by loss of one and two HCl from the molecular ion. The structure of the third fragment at m/z 151 was left undefined. Quantitation of chloramphenicol was performed in the six-ion SIM mode by detecting four fragment ions at m/z 151, 250, 286 (^{35}Cl) and 288 (^{37}Cl), and the two molecular ions at m/z 322 (^{35}Cl) and 324 (^{37}Cl). Validation was performed on tissues fortified at four concentration levels on different days giving RSD $<15\%$. Replicate analyses ($n=6$–8) of incurred positive controls gave RSD $<30\%$, as recommended by the EU guidelines [84].



chloramphenicol

Yoshida and Kondo [85] described a confirmatory method that employs LC–APCI-MS for the determination of thiamphenicol in milk by using ethyl acetate as extractant. After evaporation, the residue was chromatographed on a C_{18} column using an isocratic acetonitrile–water (1:1, v/v) mobile phase. The APCI ion source was operated in the PI mode and only the protonated molecule was monitored. The intent of the authors was that of promoting this method as a confirmatory method to complement a HPLC–UV assay. However, validation data for the LC–MS method were not presented.

A LC–API-MS method was recently developed by Delépine and Yndestad [86] for monitoring chloramphenicol and ketoprofen in meat and milk and chloramphenicol in egg and honey. Analytes were extracted with acetonitrile, the organic layer was separated from water with chloroform and evaporated to dryness. The residues were dissolved in methanol and the extracts were purified by a C_{18} SPE column. The analytes were recovered with methanol. After diluting with water, the final extracts was filtered and injected into the LC–MS apparatus. The MS system was set to collect multiple single-ion data in NI mode.

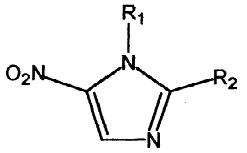


thiamphenicol

Fig. 22. Structures of selected amphenicols.

10. Nitroimidazoles

The nitroimidazoles are active against most Gram-negative and many Gram-positive anaerobic bacteria. However, they have limited activity against aerobic bacteria. Nitroimidazoles are used for the treatment and prevention of certain diseases in poultry and swine dysentery. The structures of imidazoles are visualized in (Fig. 23). Nitroimidazoles possess mutagenic, carcinogenic and toxic properties. For these reasons, the use of ronidazole, dimetridazole



Compound	R_1	R_2	Mol. wt.
Dimetridazole	CH_3	CH_3	141
Hydroxydimetridazole	CH_3	CH_2OH	157
Ipronidazole	CH_3	$CH(CH_3)_2$	169
Metronidazole	C_2H_4OH	CH_3	171
Ronidazole	CH_3	CH_2OOCNH_2	200

Fig. 23. Basic structure of nitroimidazoles.

and metronidazole has been prohibited within the EU [87–89]. However, dimetridazole may still be used as a feed additive [90]. The 5-nitroimidazoles are known to be rapidly metabolized by attack at the side-chain in the C_2 position of the imidazole ring. The metabolites formed may have a similar potential to the parent compound.

Matusik et al. [91] used LC–TS-MS–MS to confirm the identity of alcohol metabolites of dimetridazole and ipronidazole in turkey tissues. Two different solvents, i.e. ethylacetate and benzene, were used, respectively, for extracting dimetridazole and its metabolite, and ipronidazole and the related metabolite. The benzene extract was dried and the residue dissolved in the LC mobile phase, while the ethylacetate extract was purified by passing it through a C_{18} SPE cartridge. Aliquots of the final extracts were chromatographed with a C_8 column with a water–methanol (1:1, v/v) mixture containing 0.1 *M* ammonium acetate as mobile phase. Q_1 was set to pass protonated molecules, while Q_3 was set to scan from 40 to 200 *m*/*z*. All four analytes produced ions coming from the neutral loss of NO_2 and for each analyte three ions, including the parent ion, could be monitored. The identity of the four analytes could be achieved at levels down to 2 ppb in control tissues.

Sams et al. [92] developed a LC–APCI-MS method enabling detection of dimetridazole, ronidazole and their common metabolite at concentration levels of 0.1–0.5 ppb in poultry muscle and eggs. After adding sodium sulfate to samples, analytes were extracted with acetonitrile and extract purified with a SPE cartridge filled with a SCX material. Nitroimidazoles were desorbed with acetonitrile–35% ammonia (95:5, v/v). The eluate was dried and the residue was reconstituted with the LC mobile phase (13% acetonitrile–50 m*M* ammonium acetate). Confirmatory ions were obtained by in-source CID so that acquisition for each analyte was performed by the two-ion SIM mode.

Hurtaud-Pessel et al. [93] described a LC–ESI-MS assay for determining four nitroimidazoles in poultry muscle. The extraction procedure was carried out by using ethyl acetate as extractant. The oily residue obtained after solvent removal was partitioned between hexane–CCl_4 and 0.2% formic acid in water. The aqueous phase was pipetted for further analysis. The LC separation was made on a C_{18} bonded silica column with a 0.2% formic acid in water–methanol–acetonitrile (81:13:6) mobile phase. Data were acquired in the SIM mode by monitoring the $[M+H]^+$, $[M+H+1]^+$ $[M+H+CH_3CN]^+$ ions for each analyte. In spite of the fact that the selected confirmatory ions have poor diagnostic value, the authors claimed their method was specific.

A relatively fast and highly selective confirmatory assay for monitoring dimetridazole, metronidazole and ronidazole (already banned in EU at that time) in eggs using LC–tandem MS was elaborated by Daeseleire et al. [94]. After extraction with acetonitrile, extract centrifugation, partial solvent evaporation, the remaining extract was filtered and an aliquot of it was injected onto the LC–ESI-MS–MS system. The analytes were detected by SRM tuning the MS–MS system to monitor the ion current of two transitions for dimetridazole and metronidazole and one transition for ronidazole. The LOD was 0.5 ppb for all three compounds.

A LC–ESI-MS method was developed for the determination of dimetridazole, metronidazole and ronidazole, in meat [95]. Meat was extracted with acetonitrile and the organic fraction was separated from the water with NaCl and methylene chloride. An aliquot of the water residue was injected into the LC column. The LC–MS was set to collect multiple

single-ion data in the PI mode. LOQs ranged between 1 and 4 ppb.

11. Nitrofurans

Nitrofurans are synthetic antibiotics. Two examples of structures of nitrofuran antibiotics are shown in Fig. 24. They act by inhibiting a number of microbial enzymes involved in carbohydrate metabolism. Concerning the toxicological properties of nitrofurans, mutagenic activity has been observed in yeast and fungi as well as in bacterial and submammalian systems. Moreover, nitrofurans have been shown to be mutagenic in rats and mice, and cytotoxic to mammalian cells in culture. Nitrofurans are rapidly metabolized and their metabolites covalently bound to cellular protein. The potential risk to the consumer by protein-bound metabolites of furazolidone has been shown. For the above reasons, nitrofurans are not registered for use in any farm animals in the EU.

Yoshida and Kondo [96] evaluated the practicability of using LC–API-MS for determining furazolidone in avian egg. After diluting the egg sample, it was directly applied to a C_{18} SPE cartridge. The target compounds was then eluted with ethyl acetate. The solvent was eliminated and the residue dissolved with the LC mobile phase. After centrifuging, an aliquot of the final extract was injected into the LC column. The C_{18} analytical column was operated with an acetonitrile–water (40:60) mobile phase. Furazolidone was detected in the one-ion SIM mode by monitoring the protonated molecules. The authors concluded that the APCI-MS detector response was not very reproducible, thus it could be more applicable for qualitative analysis than quantitative analysis, even considering it afforded sensitivity not better than that of the UV detector for this compound.

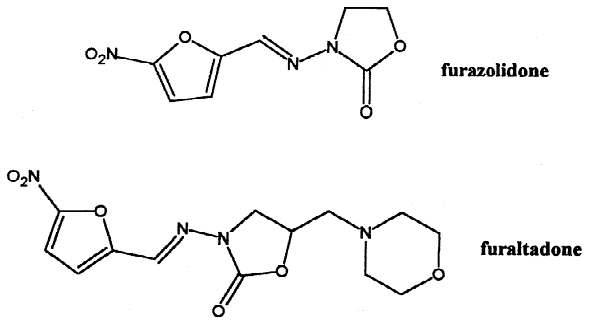


Fig. 24. Structures of two examples of nitrofuran antibiotics.

Using LC–APCI-MS, Horne et al. [97] developed a method for the quantitative and confirmatory determination of protein-bound metabolites of furazolidone and furaltadone, i.e. 3-amino-2-oxazolidinone and 5-morpholino-methyl-3-amino-2-oxazolidinone, in pig liver. After extensive solvent washing of the tissue, target compounds were simultaneously extracted and derivatized with 2-nitrobenzaldheyde under mildly acidic conditions. The two derivatives were separated on a C_{18} column by using a methanol–0.025% acetic acid (45:55, v/v) mobile phase. Analytes were detected in the two-ion SIM mode at m/z 236 and 206 for the 3-amino-2-oxazolidinone derivative, and m/z 335 and 291 for the 5-morpholino-methyl-3-amino-2-oxazolidinone derivative. Daughter ions were obtained by the in-source CID process. The LOD for both the two analytes were approximately 10 ppb in liver.

The determination of the nitrofuran residues, nitrofurazone, furazolidone and furaltadone, in eggs by LC–ESI-MS was described by Draisci et al. [98]. Nitrofurans were extracted from eggs with acetonitrile and the extracts purified by liquid–liquid partitioning. Analytes were chromatographed isocratically with a C_{18} column using a mobile phase composed of acetonitrile–water solution containing 1 m*M* ammonium acetate and 0.025% acetic acid. Based on SIM of the protonated molecules, LODs were between 1 and 3 ppb.

12. Conclusions

For methodologies devoted to residue analysis of antibiotics in food, the first and most important step is the extraction of these compounds from biomatrices and extract purification. Techniques for residue analysis have changed as different technologies have become available. Liquid–liquid extraction has been largely replaced by SPE, C_{18} being the material of choice. Matrix solid-phase dispersion (MSPD), a physical variation of SPE whereby the initial solubilization of target compounds is replaced by the

use of a solid support, is an emerging technique for extracting target compounds from animal tissues. Owing to unique chemical properties of the antibiotic classes and differences among the various components of each class, diverse strategies must be applied to extract target compounds from the particular biological matrix whereby they reside. Nowadays, LC–MS is by far the most important technique for identity confirmation of antibiotics in any type of biomatrix. Although PB and TS interfaces proved their usefulness in this area, current LC–MS confirmatory methods are invariably based on ESI or APCI as ion sources combined with single or triple quadrupole detection devices. In many cases, these methods enable detection of target compounds at levels well below maximum residue limits set by the regulatory authorities.

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