Review

Mass spectrometric analysis of tetracycline antibiotics in foods

Hisao Oka[a,*], Yuko Ito[a], Yoshitomo Ikai[a], Tadaaki Kagami[a], Ken-ichi Harada[b]

[a]*Aichi Prefectural Institute of Public Health, Tsuji-machi, Kita-ku, Nagoya 462, Japan*
[b]*Faculty of Pharmacy, Meijo University, Tempaku, Nagoya 468, Japan*

Abstract

We will review recent developments in mass spectrometric analysis of tetracycline antibiotics (TCs) in foods. The mass spectrometric techniques discussed are as follows: the collision-activated decomposition mass-analysed ion kinetic energy spectrometry (CAD MIKES), thin-layer chromatography (TLC)–fast atom bombardment (FAB) mass spectrometry (MS), particle beam (PB) liquid chromatography (LC)–MS, LC-frit FAB MS, thermospray (TSP) LC–MS, atmospheric chemical ionization (APCI) LC–MS and tandem electrospray (ESI) LC–MS. Their advantages and limitations are described in the confirmation of TCs in foods: CAD MIKES can confirm TCs with high sensitivity; however, its practical application is questionable because of uncommon instrumentation. TSP has a problem in reproducibility of the mass spectrum. Although TLC–FAB-MS can be applied to any kind of samples, it cannot be used for the quantitative analysis. LC–frit FAB-MS is a useful technique for the confirmation of TCs in honey, but it cannot be applied to animal tissues because of a lack of sensitivity. PB negative chemical ionization, APCI, and ESI-MS–MS can reliably confirm TCs in foods with good reproducibility. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Food analysis; Reviews; Mass-analysed ion kinetic energy spectrometry; Interfaces, LC–MS; Interfaces, TLC–MS; Mass spectrometry; Tetracyclines; Antibiotics
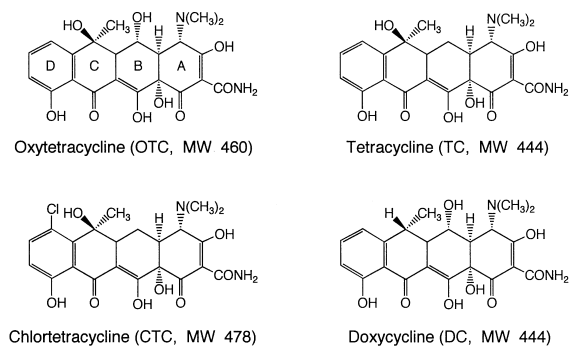
Contents

*Corresponding author.

Fig. 1. Tetracycline antibiotics. MW=molecular mass.

1. Introduction

Tetracycline antibiotics (TCs, Fig. 1) are commonly used all over the world as veterinary medicines and feed additives [1–3]. Residual TCs have sometimes been found in organ and muscle tissues collected from slaughtered animals. The determination of TC residues in edible tissues of slaughtered animals is one of the more serious analytical problems for a public health agency. Microbiological assays have been most commonly used for the detection of such residues, but they are complicated, time consuming and non-specific. Mass spectrometric techniques can confirm the residual drugs with high sensitivity and selectivity; therefore, a method combining a simple and precise chromatographic separation with an appropriate mass spectrometric determination technique would offer a significant advantage for the absolute confirmation of the residual TCs. Although high-performance liquid chromatography–mass spectrometry (LC–MS) appears best suited for this purpose, most previously reported LC conditions cannot be directly applied to existing LC–MS systems, because they require mobile phases containing such non-volatile compounds as oxalic and citric acids to improve the chromatographic resolution of TCs. However, mobile phases containing non-volatile compounds, when used in LC–MS, have been observed to cause clogging at the interface and a build-up of deposits in the ion source, so that the LC–MS cannot be operated for a prolonged period.

In order to resolve the above problem, various devices have been tried since the 1980s, and the

collision-activated decomposition mass-analysed ion kinetic energy spectrometry (CAD MIKES) [4], thin-layer chromatography (TLC)–fast atom bombardment (FAB) MS [5–7], and LC with particle beam (PB) MS [8], frit FAB MS [9], thermospray (TSP) MS [10], atmospheric chemical ionization (APCI) MS [11], and tandem electrospray ionization (ESI) MS [12] have been practically applied to the analysis of TCs in foods. In this paper, we review recent developments in the MS analysis of TCs in foods and describe the advantages and limitations of the methods.

2. Mass spectra of tetracyclines

Several reports on mass spectrometry have been published in which electron ionization [4], field desorption [13], TSP [10,14,15], PB [8,14], FAB [5–7,9,16], APCI [11], and ESI [12,17] were used. Among them, we introduce mass spectra obtained by TSP, PB, FAB, APCI, and ESI in this section, as they have been successfully applied to the analysis of residual TCs in foods.

The TSP mass spectra of TCs give abundant ions of $[M+H]^+$ (m/z 461 for OTC, 445 for TC, and 479 for CTC) with one relatively abundant fragment ion. The intensity of the fragment ion varies depending on the operation conditions. Voyksner et al. [14] reported that $[M+H–CONH_2]^+$ appeared in the mass spectra of TC (m/z 401) and CTC (m/z 435) as a fragment ion with relative strength. However, another paper reported that the relatively abundant ions, $[M+H–H_2O]^+$ for TC (m/z 427), $[M+H–HOCN]^+$ for OTC (m/z 418), and $[M+H–CONH_2]^+$ for CTC (m/z 435) were observed [10]. In TSP, vaporizer and ion source temperatures mostly affect production of ions. If the optimum temperature drifts only a few degrees, it will significantly affect the production of ions. The temperatures are significantly affected by the flow-rate of the mobile phase and the mobile phase composition; therefore, it is difficult to obtain the TSP mass spectra of TCs with good reproducibility. Furthermore, at least three ions including a molecular ion species and two structurally significant fragments are required to confirm a residual substance in the sample matrix using selected ion monitoring (SIM) [18,19]. How-

Table 1
Molecular and fragment ions in particle beam negative chemical ionization mass spectra of tetracyclines

Tetracyclines	Molecular and fragment ions, m/z (relative abundance, %[a])							
	$[M]^{-\cdot}$	$[M-H_2O]^-$	$[M-CONH]^-$	$[M-CONH_2]^-$	$[M-H_2O-CONH]^-$	$[M-HCl]^-$	$[M-HCl-H_2O]^-$	$[M-H_2O-CONH_2]^-$
OTC	460 (100)	442 (48)	417 (7)	416 (33)	399 (20)	–	–	398 (78)
TC	444 (100)	426 (83)	401 (11)	400 (44)	383 (19)	–	–	382 (17)
CTC	478 (52)	460 (30)	–	434 (6)	416 (4)	442 (100)	424 (22)	–

[a] Relative abundance calculated from Ref. [14].

ever, TSP gives only two ions. Therefore, we consider that it is difficult to practically apply TSP to the analysis of TCs in foods.

Voyksner et al. [8] and Kijak et al. [14] reported PB negative chemical ionization (NCI) mass spectra of TCs using methane gas as a reagent gas. Molecular and fragment ions are summarized in Table 1. The most abundant ions are $[M]^{-\cdot}$ for OTC and TC and $[M-HCl]^-$ for CTC. In the mass spectra of OTC and TC, $[M-H_2O]^-$, $[M-CONH_2]^-$, $[M-H_2O-CONH]^-$, and $[M-H_2O-CONH_2]^-$ appeared with considerable abundance, together with the weak ion of $[M-CONH]^-$. For CTC, abundant ions of $[M]^{-\cdot}$, $[M-H_2O]^-$, and $[M-H_2O-HCl]^-$ are observed with weak ions of $[M-CONH_2]^-$ and $[M-H_2O-CONH]^-$. More than three relatively abundant ions are monitored in each mass spectrum of the TCs, so it is considered that PB-NCI is a suitable technique for the confirmation of TCs in foods.

For the measurement of the FAB mass spectra of TCs, only thioglycerol is suitable as a matrix, because with other matrices, the matrix-derived ions overlapped the molecular ion species of the TCs [5,16]. The ion species for the TCs are summarized in Table 2. OTC, TC, and CTC gave the most abundant ion, $[M+H]^+$, and two fragment ions which have been identified as $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$, however, in the mass spec-

trum of DC, only $[M+H]^+$ and $[M+H-NH_3]^+$ were observed. Because DC does not give $[M+H-NH_3-H_2O]^+$, the formation of the ionic species depends on the presence of a hydroxy group in the C ring (Fig. 1) [16], which is available for discrimination between TC and DC. The composition of the $[M+H-NH_3]^+$ of TC was established by high-resolution MS and the $[M+H]^+$ of TC gave only three ionic species, $[M+H-NH_3]^+$, $[M+H-H_2O]^+$, and $[M+H-NH_3-H_2O]^+$ in the product ion spectrum by B/E linked scanning. With *N*-pyrrolidinylmethyl tetracycline, the loss of the *N*-pyrrolidinylmethyl moiety occurred analogously. These results clearly indicate that the loss of ammonia occurs from the carboxyamide moiety in the A ring. Three ions including the molecular ion species and two fragments except for DC appear in the FAB mass spectra, so that it can be used for the confirmation of TCs in foods in a full-scan mode.

Blanchflower et al. [11] reported APCI mass spectra of TCs. Table 3 shows protonated molecules and characteristic fragment ions. The ratio of the ions varies depending on the cone voltage, and the ion ratio of TCs in Table 3 is obtained at a cone voltage of 25 V. In the spectra of OTC and TC, $[M+H-H_2O]^+$ appeared as the most abundant ion, accompanied by prominent ions, $[M+H]^+$, $[M+H-NH_3]^+$, $[M+H-NH_3-H_2O]^+$, and $[M+H-2H_2O]^+$.

Table 2
Protonated molecules and characteristic fragment ions in fast atom bombardment mass spectra of tetracyclines

Tetracyclines	Protonated molecule and fragment ion, m/z (relative abundance, %)		
	$[M+H]^+$	$[M+H-NH_3]^+$	$[M+H-NH_3-H_2O]^+$
OTC	461 (100)	444 (18)	426 (45)
TC	445 (100)	428 (20)	410 (64)
CTC	479 (100)	462 (40)	444 (44)
DC	445 (79)	428 (100)	–

Table 3
Protonated molecules and characteristic fragment ions in atmospheric pressure chemical ionization mass spectra of tetracyclines

Tetracyclines	Protonated molecule and fragment ion, m/z (Relative abundance, %[a])				
	$[M+H]^+$	$[M+H-NH_3]^+$	$[M+H-H_2O]^+$	$[M+H-NH_3H_2O]^+$	$[M+H-2H_2O]^+$
OTC	461 (77)	444 (76)	443 (100)	426 (59)	425 (50)
TC	445 (45)	428 (40)	427 (100)	410 (48)	409 (61)
CTC	479 (100)	462 (95)	461 (82)	–	443 (50)

[a] Relative abundance calculated from Ref. [11].

On the other hand, CTC gave the most abundant ion, $[M+H]^+$, and the prominent ions, $[M+H-NH_3]^+$, $[M+H-H_2O]^+$, and $[M+H-2H_2O]^+$. However, $[M+H-NH_3-H_2O]^+$, which was observed in the mass spectra of OTC and TC, did not appear in the mass spectrum of CTC. Thus, APCI-MS is considered to be a suitable technique for the confirmation of TCs in foods.

In both positive and negative ESI MS [16,17], TCs gave only the molecular ion species ($[M+H]^+$ and $[M-H]^-$) and no fragment ions at low capillary voltage. When a high capillary voltage above 150 V was used, several fragment ions appeared. Therefore, in order to confirm TCs in foods, we should measure the mass spectra at a high capillary voltage or tandem mass spectrometry should be carried out.

3. Applications

As shown in Table 4, mass spectrometric techniques have been successfully applied to the analysis of TCs in milk, animal tissues, and honey. The interface and ionization methods used are EI, PB-NCI, TLC–FAB, Frit–FAB, TSP, APCI, and ESI, and various detection modes including CAD MIKES, SIM, full-scan, and tandem mass spectrometry (MS–MS) have been examined. For the extraction and clean-up, a combination of McIlvaine buffer and a C_{18} cartridge have been mainly investigated, and successful results were obtained. Detection limits ranged from 1 ppb to 0.1 ppm. In this section, we introduce typical application studies to the analysis of TCs in foods using CAD MIKES, PB-NCI, TLC–FAB, Frit–FAB, TSP, APCI, and ESI.

3.1. Collision-activated decomposition mass-analysed ion kinetic energy spectrometry

Tandem mass spectrometric determination of OTC in bovine milk and meat was carried out in 1985 by Traldei et al. [4]. They used CAD MIKES with direct electron ionization. In the CAD MIKE spectrum, OTC gave 18 product ions and losses of H_2O (m/z 442), $CONH_2$ (m/z 416), and both H_2O and $CONH_2$ (m/z 398) mainly occurred, when $[M]^{+\cdot}$ was used as a precursor ion. To confirm and determine OTC in bovine milk and meat, a crude ethanol extract spiked with an internal standard was directly introduced into the ion source. For the confirmation, the CAD MIKE spectrum obtained from the samples was compared with that of the standard, and both spectra perfectly coincided. The determination was carried out by SIM of the most abundant ion, $[M-H_2O]^+$ (m/z 442), and the detection limit was 1 ppb. Although this method offers high sensitivity and high speed sample analysis, the practical application of this technique is limited because of uncommon instrumentation.

3.2. Thin-layer chromatography–fast atom bombardment mass spectrometry

In TLC–FAB-MS [5], the developed and air-dried TLC plate is inserted into the TLC–FAB-MS ion source, the FAB mass spectrum of the desired spot on the plate is directly measured, and the plate is removed from the ion source. In order to obtain good separation of TCs on the TLC plate, non-volatile compounds such as oxalic acid and disodium ethylenediaminetetraacetate (Na_2EDTA) are added to the

Table 4
Application of mass spectrometry to the analysis of residual tetracyclines in foods

Tetracyclines	Samples	Extraction/clean up	Stationary phase	Mobile phase	Interface/ ionization	Detection mode	Detection limit	Ref.
OTC	Bovine milk and meat	Deproteinization with ethanol	–	–	EI	CAD MIKES	1 ppb	[4]
OTC, TC, and CTC	Milk	C_{18} cartridge clean up	Novapak C_{18} column	Acetonitrile–methanol–0.01 M oxalic acid (50:20:30)	PB–NCI	SIM	0.1 ppm	[8]
OTC, TC CTC, and DC	Bovine tissues	Extraction with McIlvaine buffer (pH 4.0) containing 0.1 M EDTA; C_{18} cartridge clean up	C_8 TLC plate	Acetonitrile–methanol–0.5 M oxalic acid (pH 2.0) (1:1:4)	FAB	Full-scan	0.1 ppm	[5]
OTC, TC CTC, and DC	Honey	Extraction with McIlvaine buffer (pH 4 0) containing 0.1 M EDTA; C_{18}/COOH cartridge clean up	C_8 TLC plate	Acetonitrile–methanol–0.5 M oxalic acid (pH 2.0) (1:1:4)	FAB	Full-scan	0.1 ppm	[6]
OTC, TC CTC, and DC	Milk	Extraction with McIlvaine buffer (pH 4 0) containing 0.1 M EDTA; C_{18} cartridge clean up	C_8 TLC plate	Acetonitrile–methanol–0.5 M oxalic acid (pH 2.0) (1:1:4)	FAB	Full-scan	0.05 ppm	[7]
OTC, TC CTC, and DC	Honey	Extraction with McIlvaine buffer (pH 4 0) containing 0.1 M EDTA; C_{18} cartridge clean up	Inertsil Pheny column	Acetonitrile–methanol–0.005 M trifluoroacetic acid (2:2:11)	Frit FAB	Full-scan	0.1 ppm	[9]
OTC, TC, and CTC	Muscle and kidney	Extraction with McIlvaine buffer (pH 4.0) containing 0.1 M EDTA; C_{18} cartridge clean up	Inertsil C_8 column	Acetonitrile–0.1 M ammonium formate (pH 3), gradient (0:10 to 3:7)	TSP	SIM	0.1 ppm for OTC and TC, and 0.3 ppm for CTC	[10]
OTC, TC, CTC, and epi-CTC	Muscle and kidney	Extraction with glycine-HCl buffer; cyclohexyl cartridge clean up	Prodigy ODS 2 column	Mixture of acetonitrile containing 0.04% heptafluorobutyric acid, 0.01 M oxalic acid, 0.01 M EDTA, gradient (1:9 to 9:1)	APCI	SIM	0.01 ppm in muscle and 0.02 ppm in kidney	[11]
OTC, TC, CTC, and DC	Bovine tissues	Extraction with McIlvaine buffer (pH 4.0) containing 0.1 M EDTA; C_{18} cartridge clean up	TSK Gel Super Octyl column	Acetonitrile–0.005 M trifluoroacetic acid (1:4)	ESI	MS–MS	0.1 ppm	[12]
OTC, TC, CTC, DC DMCTC, and MINO	Bovine milk	Extraction with metal chelate affinity chromatography; polymer reversed-phase cartridge clean up	Polymer Labs. PLPRP-S column	Methanol–0.005 M oxalic acid	PB/NCI	SIM	0.03 ppm	[20]

EI: electron ionization, CAD MIKES: collisionally activated decomposition mass-analysed ion kinetic energy spectrometry, PB: particle beam, NCI: negative chemical ionization, SIM: selected ion monitoring, FAB: fast atom bombardment, TSP: thermospray, APCI: atmospheric pressure chemical ionization, ESI: electrospray, DMCTC: demeclocycline, MINO: minocycline.

solvent system, as is also the case in their analysis by LC. However, these compounds do not cause any problems in TLC–FAB-MS such as have been reported in LC–MS (clogging of the interface and deposits in the ion source) because they remain on the TLC plate and are removed with it after the measurement has been completed. Therefore, TLC–FAB-MS has been applied to the confirmation of

residual TCs in tissues from slaughtered animals using a non-volatile solvent system.

This method is based on a C_{18} cartridge clean-up, followed by separation of the TCs on a reversed-phase C_8 TLC plate. TLC–FAB-MS of the TCs are measured after condensation of the TCs spots. Among these procedures, the condensation of the spots of TCs is unique: When a matrix is deposited on a sample spot on a TLC plate, diffusion of the sample usually occurs with spreading of the matrix used, so that no satisfactory spectrum is obtained with good sensitivity, unless a large amount of sample is applied to the TLC plate. To prevent diffusion of the analyte and to obtain high sensitivity from the TLC–FAB-MS, a sample condensation technique has been developed using methanol (Fig. 2). No satisfactory spectra were obtained unless more than 5 μg/spot of TCs was applied to the TLC plate without the condensation technique. However, when the developed spot was reconcentrated on the TLC plate using the concentration technique, the $[M+H]^+$ clearly appeared at a concentration of 0.1 μg/spot. Thus, the technique can improve the detection limits of TCs by 50 times with good reproducibility in TLC–FAB-MS.

For the extraction and clean-up, a combination of McIlvaine buffer and a C_{18} cartridge has been used: A sample was extracted three times with 20, 20, and 10 ml of 0.1 M Na_2EDTA–McIlvaine buffer (pH 4.0). The supernatants were combined and filtered. The filtrate was applied to a C_{18} solid-phase ex-

traction cartridge pretreated with saturated aqueous Na_2EDTA. The cartridge was washed with 20 ml of water. TCs were eluted with 10 ml of ethyl acetate, followed by 20 ml of methanol–ethyl acetate (5:95, v/v) and the eluate was evaporated to dryness. The residue was dissolved in methanol and applied to TLC–FAB-MS. Although the recoveries (calculated by HPLC) at the 0.1 ppm fortification level were only 55–79%, the clean-up method gave good coefficients of variation of less than 6.8% at the 0.1 ppm fortification level. This means that the clean-up method provides sufficient recoveries with good reproducibility for the confirmation of residual TCs in the samples. After clean-up of TCs fortified at concentrations of 0.1 ppm in animal tissues and development of the TLC plate (TLC plate: Merck, RP-18; solvent system: methanol–acetonitrile–0.5 M oxalic acid, pH 2.0, 1:1:4), TLC–FAB-MS with the concentration technique was performed. The $[M+H]^+$ was clearly observed in the spectra for animal tissues, and some spectra also provided the fragment ions, $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$ (Fig. 3). In the mass spectra of blank tissues, no ions corresponding to $[M+H]^+$, $[M+H-NH_3]^+$, and $[M+H-NH_3-H_2O]^+$ of TCs appeared. In addition, this method was applied to the confirmation of TCs in bovine samples [5], milk [7], and honey [6], and the $[M+H]^+$ and fragment ions of the TCs were clearly visible in the spectra. This method is unique and useful for the confirmation of TCs in foods; however, it cannot be used for quantitative analysis.
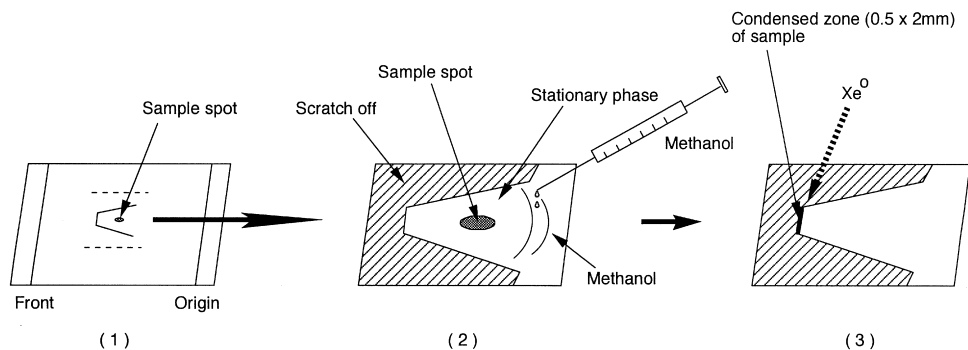

Fig. 2. Procedure for the condensation technique: (1) an area including the desired spot on the developed TLC plate was rectangularly cut and a line was trapezoidally drawn around the sample spot; (2) the outside stationary phase of the trapezoid was scratched off along the line and a small volume of methanol was deposited on the lower base of the trapezoid; (3) after several tens of seconds, the sample had become condensed along the upper base of the trapezoid in a line (0.5×2 mm) by penetration of methanol. The TLC plate was finally set onto the TLC holder, a matrix was applied on the sample spot, and the TLC–FAB-MS was spectra was measured.
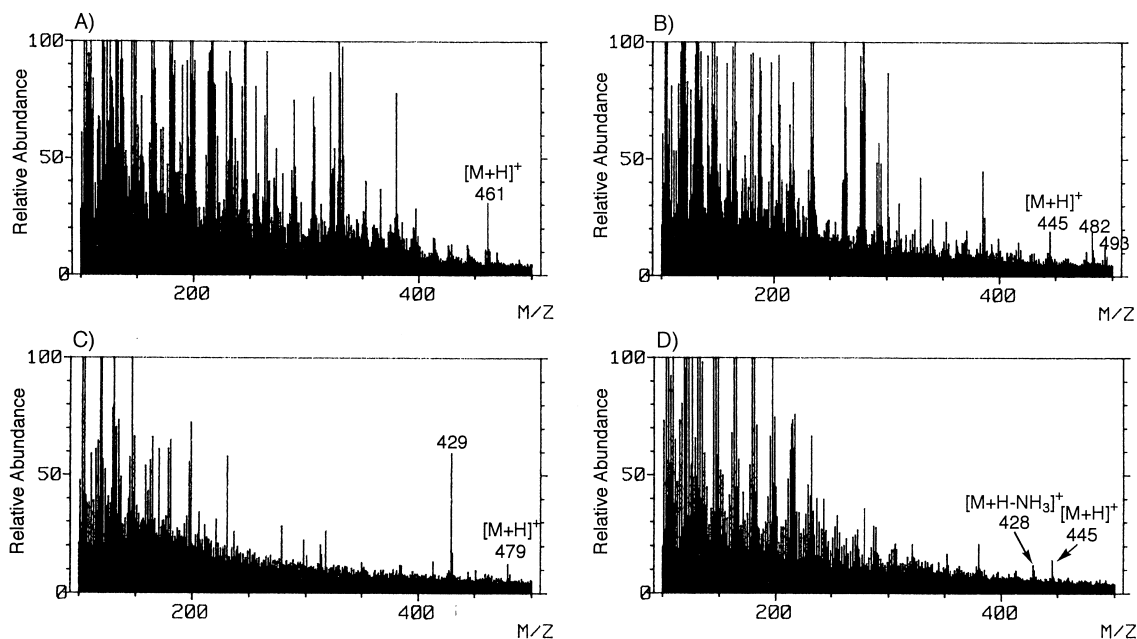
Fig. 3. TLC–FAB-MS spectra of tetracycline antibiotics fortified at concentrations of 0.1 ppm in bovine liver. (A) Oxytetracycline, (B) tetracycline, (C) chlortetracycline, (D) doxycycline.

3.3. Liquid chromatography–mass spectrometry

3.3.1. Particle beam

A confirmation method for OTC, TC, and CTC using LC–PB negative chemical ionization (NCI) MS in milk has been reported by Kijak et al. [8]. Milk was centrifuged using a molecular mass cut-off filter (25 000), and the filtrate was applied to a C_{18} cartridge. After the cartridge was washed with water, TCs retained in the cartridge were eluted with 0.1 M oxalic acid–methanol and the eluate was concentrated. The TCs were separated on a Novapak-C_{18} column with acetonitrile–methanol–0.05 M oxalic acid (5:2:3) as a mobile phase. The effluent was introduced into a PB-NCI-MS system, and the confirmation was carried out using SIM mode at m/z 460, 442, 416, and 399 for OTC, m/z 444, 426, 401, and 383 for TC, and m/z 442, 478, 460, and 434 for CTC. The detection limits of TCs in milk were 0.1 ppm. In this study, oxalic acid was added to the mobile phase and the eluent from the cartridge to improve the chromatographic resolution and recovery from the cartridge. When the effluent containing nonvolatile compounds from the column was

introduced into an LC–MS system, clogging at the interface and a build up of deposits in the ion source were observed, so that the LC–MS cannot be operated for a prolonged period. To avoid this problem, a switching valve was installed between the column and the interface. An improved method has been recently reported by Carson [20].

3.3.2. Frit fast atom bombardment

LC-frit FAB-MS has been reported for the confirmation of TCs in honey [9]. In this study, use of a well end-capped phenyl (Ph)-bonded silica gel column synthesized from 99.99% pure silica gel first enables us to separate TCs without reduction of peak resolution using a volatile mobile phase, which is applicable to direct interfaced LC-frit FAB-MS without clogging problems. Three types of well end-capped alkyl-bonded chromatographic packings synthesized from 99.99% pure silica gel were compared: Inertsil C_{18}, Inertsil Ph, and Inertsil C_8. An Inertsil Ph column was selected, because it showed the best resolution among TCs with the shortest analysis time when methanol–acetonitrile–0.005 M TFA solution
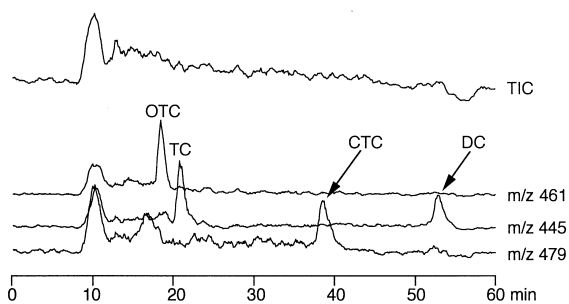
Fig. 4. Mass chromatograms of TCs fortified at concentrations of 0.2 ppm in honey.

5). In the mass spectrum of blank honey, no ions corresponding to $[M+H]^+$, $[M+H-NH_3]^+$, and $[M+H-NH_3-H_2O]^+$ of TCs appeared. Thus, TCs in honey at a 0.2 ppm can be reliably confirmed by this method, and the detection limits of the protonated molecules are 0.1 ppm in honey.

This method was also successfully applied to the confirmation of residual CTC at a concentration of 0.24 ppm in a honey sample collected at the market level. However, this method cannot be applied to animal tissues, because of a lack of sensitivity.

(2:2:11) containing 1.0% thioglycerol was used as a mobile phase.

After clean-up of TCs fortified at a concentration of 0.2 ppm in honey using a C_{18} cartridge, LC-frit FAB-MS was performed. No peaks corresponding to TCs appeared on the total ion chromatogram. However, the peaks of all TCs were clearly observed on mass chromatograms monitored at individual protonated molecules (Fig. 4). Although an ion originating from co-extractive substances from honey appears at m/z 438 in the spectrum of TC, the fragment ions are clearly observed in the spectra of all TCs (Fig.

3.3.3. Thermospray

After clean-up of bovine and porcine muscle and kidney extracts with a C_{18} cartridge, the TCs were separated using a gradient elution mode with a mixture of acetonitrile and ammonium formate on an Inertsil C_8 column which was well end-capped modified silica gel synthesized from 99.99% pure silica gel [10]. The effluent from the column was then introduced into TSP MS under SIM conditions monitored at $[M+H]^+$. The detection limits in the samples are 0.1 ppm for OTC and TC and 0.3 ppm for CTC.
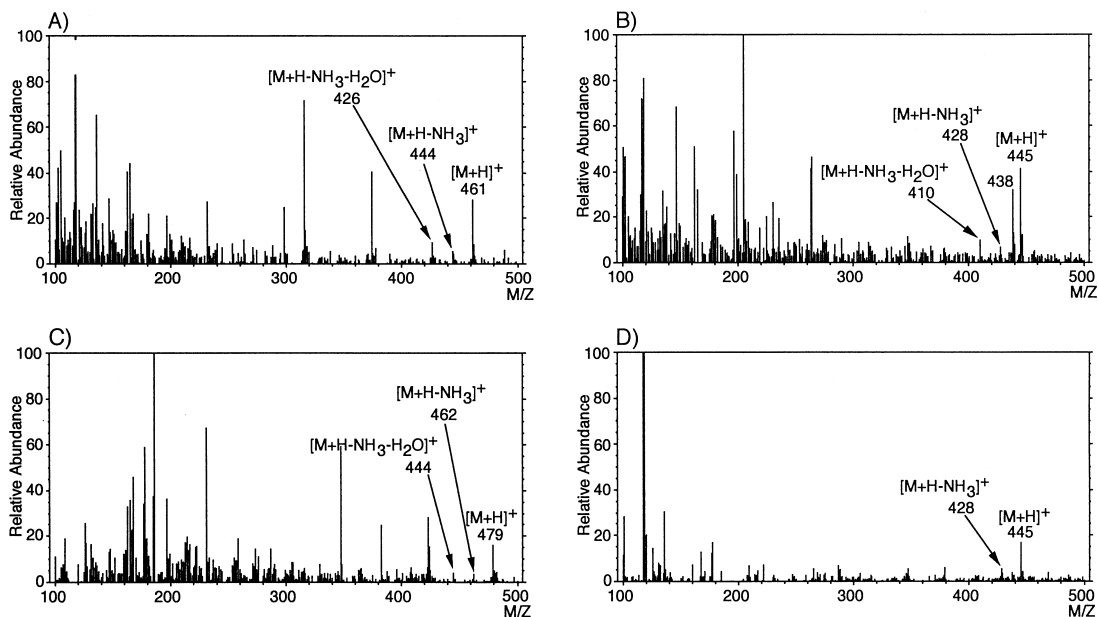

Fig. 5. Background subtracted mass spectra of TCs fortified at concentrations of 0.2 ppm in honey. (A) oxytetracycline; (B) tetracycline; (C) chlortetracycline; (D) doxycycline.

3.3.4. Atmospheric pressure chemical ionization mass spectrometry

A confirmatory method using LC–APCI-MS for OTC, TC, and CTC in muscle and kidney was reported by Blanchflower et al. [11]. The TCs were extracted with glycine–HCl buffer from the samples, and the extract was applied to an Isolute cyclohexyl cartridge (CH-end-capped). The TCs were eluted with methanol after washing the cartridge, the methanol fraction was evaporated to dryness and the residues were dissolved in 20 m*M* oxalic acid–methanol. TCs were separated on a well end-capped Prodigy ODS2 column synthesized from pure silica gel using a mixture of acetonitrile, water containing 0.04% heptafluorobutyric acid, 10 m*M* oxalic acid and 10 μ*M* EDTA under a gradient elution mode. The TCs from the column were introduced into APCI-MS. The detection limits were 0.01 ppm and 0.02 ppm in muscle and kidney, respectively.

Recovery tests have been carried out at the levels of 0.05 ppm, 0.10 ppm, and 0.2 ppm. The recoveries were 59–82% for muscle and 67–83% for kidney, and the relative standard deviations (R.S.D.s) were 12.8–22.9% for muscle and 11.7–28.1% for kidney. It is considered that this method can be used for routine analysis of TCs in foods.

3.3.5. Electrospray

Although ES mass spectra of TCs were obtained with high sensitivity when a standard solution of TCs was injected into an LC–ESI-MS system, it is hard to distinguish the ions originating from the sample matrices and the ions of TCs when TCs in animal tissues were analysed. Tandem mass spectrometry in combination with LC–ESI-MS (LC–ESI-MS–MS) has been recognized as a rapid, sensitive and selective analytical method for the determination of TCs

in complex biological samples. The advantages gained by LC–MS–MS often complement those gained by extensive sample clean-up procedures. Therefore, LC–ESI-MS–MS has been applied using a well end-capped TSK Gel Super Octyl column and a volatile mobile phase (acetonitrile–0.05% trifluoroacetic acid, 4:1) for the confirmation of TCs in animal tissues [12].

TCs give $[M+H]^+$, $[M+H-NH_3]^+$, and $[M+H-NH_3-H_2O]^+$ in the ES mass spectra measured at high capillary voltage except for DC, and these ions are very useful for the confirmation of TCs. These three ions with adequate intensity should be observed in the tandem mass spectra obtained to confirm the TCs. In order to obtain optimal MS–MS conditions for TCs, the ES tandem mass spectra of TCs were measured under two different collision offsets, and the intensity of these ions were carefully observed. When the collision offset was set at -50 V (Table 5), $[M+H-NH_3-H_2O]^+$ for OTC (m/z 426), TC (m/z 410), and CTC (m/z 444) and $[M+H-NH_3]^+$ for DC (m/z 428) appeared as the most abundant ions. However, the intensity of $[M+H]^+$ and $[M+H-NH_3]^+$ was very weak, so that it is difficult to confirm TCs in the sample from these tandem mass spectra. On the other hand, using a collision offset of -25 V, $[M+H]^+$ appeared as the most abundant ion accompanied by prominent product ions, $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$. All TCs can be confirmed easily with this MS–MS condition. Therefore, a collision offset of -25 V was selected.

The clean-up method described in Section 3.2 is also applicable to this LC–MS system, because the sample solution does not contain non-volatile compounds such as oxalic acid and EDTA. Therefore, the clean-up method has been applied to this study. After clean-up of the TCs fortified at a concentration

Table 5
Precursor and product ions of tetracyclines under ES MS–MS conditions

| Tetracyclines	Precursor		Collision offset: -50 V			Collision offset: -25 V		
	Ion	m/z	Product ions			Product ions		
			m/z (relative abundance)			m/z (relative abundance)		
Oxytetracycline	$[M+H]^+$	461	461 (2)	444 (6)	426 (100)	461 (100)	444 (28)	426 (40)
Tetracycline	$[M+H]^+$	445	445 (2)	428 (6)	410 (100)	445 (100)	428 (48)	410 (81)
Chlortetracycline	$[M+H]^+$	479	479 (13)	462 (32)	444 (100)	479 (100)	462 (26)	444 (20)
Doxycycline	$[M+H]^+$	445	445 (3)	428 (100)		445 (72)	428 (100)	
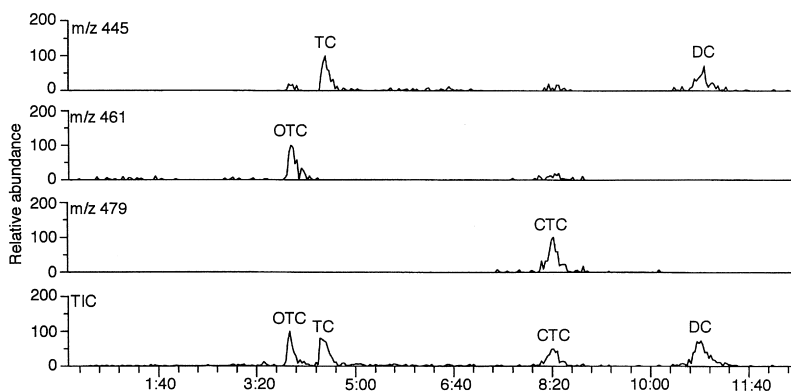
Fig. 6. Total ion and mass chromatograms of tetracyclines fortified at a concentration of 0.1 ppm in bovine liver under MS–MS conditions.

of 0.1 ppm in bovine liver using a C_{18} cartridge, LC–ESI-MS–MS was performed. The peaks corresponding to the TCs appeared on the total ion and mass chromatograms monitored at individual protonated molecules as shown in Fig. 6. Fig. 7 shows the tandem mass spectra of TCs recorded at the tops of each peak on the mass chromatograms. The product ions are clearly observed in the spectra of all TCs, but no ions corresponding to $[M+H]^+$, $[M+H-$

$NH_3]^+$, and $[M+H-NH_3-H_2O]^+$ of TCs appeared in the tandem mass spectrum of bovine liver blank. This method can also confirm TCs at a 0.1 ppm level in the bovine kidney and muscle. This method was successfully applied to the confirmation of residual OTC at a concentration of 0.58 ppm in a bovine liver sample and residual CTC at a concentration of 0.38 ppm in a bovine muscle sample obtained from a slaughterhouse. We recommend this method for the
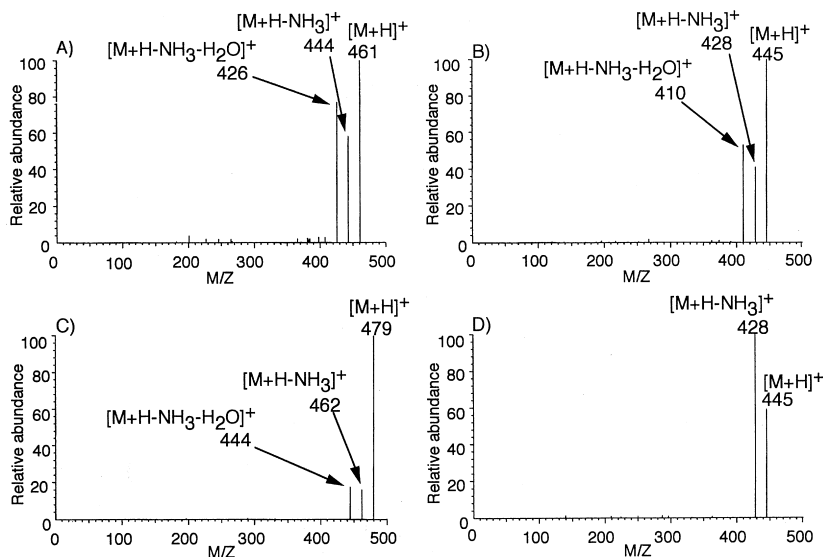

Fig. 7. ES tandem mass spectra of tetracyclines fortified at a concentration of 0.1 ppm in bovine liver. (A) oxytetracycline; (B) tetracycline; (C) chlortetracycline; (D) doxycyline.

confirmation of TCs in foods, because this method can be applied to the analysis in honey, milk, egg, and fish samples.

4. Conclusions

In this paper, we reviewed the application of MS to the analysis of TCs in foods. The MS techniques discussed are as follows: CAD MIKES, TLC–FAB-MS, PB-NCI, frit FAB, TSP, APCI, and ESI. Although CAD MIKES offers high sensitivity and high speed sample analysis, the practical application of this technique is limited because of uncommon instrumentation. TSP has a problem in reproducibility of the mass spectrum. TLC–FAB-MS is a unique technique and can be applied to any kinds of samples; however, it cannot be used for quantitative analysis. LC-frit FAB-MS is a useful technique for the confirmation of TCs in honey, but it cannot be applied to animal tissues because of a lack of sensitivity. PB-NCI, APCI, and ESI-MS–MS show useful fragment ions to confirm TCs and can reliably confirm TCs in foods with good reproducibility. Therefore, we recommend these techniques for routine analysis of TCs in foods at present.

References

[1] J.D. MacNeil, R.L. Ellis, in: H. Oka, H. Nakazawa, K.-I. Harada, J.D. MacNeil (Eds.), Chemical Analysis of Antibiotics Used in Agriculture, AOAC International, Arlington, VA, 1995, Ch. 1, pp. 1–29.

[2] H. Nakazawa, in: H. Oka, H. Nakazawa, K.-I. Harada, J.D. MacNeil (Eds.), Chemical Analysis of Antibiotics Used in Agriculture, AOAC International, Arlington, VA, 1995, Ch. 2, pp. 31–46.

[3] K.N. Woodward, G. Shearer, in: H. Oka, H. Nakazawa, K.-I. Harada, J.D. MacNeil (Eds.), Chemical Analysis of Antibiotics Used in Agriculture, AOAC International, Arlington, VA, 1995, Ch. 3, pp. 47–76.

[4] P. Traldi, S. Daolio, B. Pelli, R.M. Facino, M. Arini, Biol. Mass Spectrom. 12 (1985) 493.

[5] H. Oka, Y. Ikai, J. Hayakawa, K. Masuda, K.-I. Harada, M. Suzuki, V. Martz, J.D. MacNeil, J. Agric. Food Chem. 41 (1993) 410.

[6] H. Oka, Y. Ikai, J. Hayakawa, K. Masuda, K.-I. Harada, M. Suzuki, R. Himei, M. Horie, H. Nakazawa, J. Food Hyg. Soc. Japan 34 (1993) 517.

[7] H. Oka, Y. Ikai, J. Hayakawa, K. Masuda, K.-I. Harada, M. Suzuki, J. AOAC Int. 77 (1994) 891.

[8] P.J. Kijak, M.G. Leadbetter, M.H. Thomas, E.A. Thompson, Biol. Mass Spectrom. 20 (1991) 789.

[9] H. Oka, Y. Ikai, J. Hayakawa, K.-I. Harada, H. Asukabe, M. Suzuki, R. Himei, M. Horie, H. Nakazawa, J.D. MacNeil, J. Agric. Food Chem. 42 (1994) 2215.

[10] H. Oka, J. Patterson, in: H. Oka, H. Nakazawa, K.-I. Harada, J.D. MacNeil (Eds.), Chemical Analysis of Antibiotics Used in Agriculture, AOAC International, Arlington, VA, 1995, Ch. 10, pp. 333–406.

[11] W.J. Blanchflower, R.J. McCracken, A.S. Haggan, G. Kennedy, J. Chromatogr. B 692 (1997) 351.

[12] H. Oka, Y. Ikai, Y. Ito, J. Hayakawa, K.-I. Harada, M. Suzuki, H. Odani, K. Maeda, J. Chromatogr. B 693 (1997) 337.

[13] B. Crathorne, M. Fielding, C.P. Steel, D.C. Wattes, Environ. Sci. Technol. 18 (1984) 797.

[14] R.D. Voyksner, C.S. Smith, P.C. Knox, Biol. Mass Spectrom. 19 (1990) 523.

[15] G.B. Kenion, G.T. Carter, J. Ashraf, M.M. Siegel, D.B. Border, in: Liquid Chromatography Mass Spectrometry (ACS Symposium Series, No. 420), American Chemical Society, Washington, DC, 1990, Ch. 9, pp. 140–165.

[16] K.-I. Harada, K. Masuda, M. Suzuki, H. Oka, Y. Ikai, J. Hayakawa, Org. Mass Spectrom. 28 (1993) 1512.

[17] R.F. Straub, R.D. Voyksner, J. Am. Soc. Mass Spectrom. 4 (1993) 578.

[18] J.A. Sphon, J. Assoc. Off. Anal. Chem. 61 (1978) 1247.

[19] W.G. De Ruig, R.W. Stephany, G. Dijkstra, J. Assoc. Off. Anal. Chem. 72 (1989) 487.

[20] M.C. Carson, in: G. Enne, H.A. Kuiper, Valentini (Eds.), Residues of Veterinary Drugs and Mycotoxins in Animal Products, Wageningen Pree, Wageningen, Netherlands 1996, pp. 72–75.