Improvement of chemical analysis of antibiotics XXIII[1]. Identification of residual tetracyclines in bovine tissues by electrospray high-performance liquid chromatography–tandem mass spectrometry

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Abstract

To reliably identify the residual tetracycline antibiotics (TCs), oxytetracycline (OTC), tetracycline, chlortetracycline (CTC) and doxycycline (DC), in bovine tissues, we have established a confirmation method using electrospray ionization liquid chromatography–tandem mass spectrometry (ESI LC–MS–MS) with daughter ion scan. All TCs gave $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$ as the product ions, except for DC when $[M+H]^+$ was selected as the precursor ion. The combination of C_{18} cartridge clean-up and the present ESI LC–MS–MS method can reliably identify TCs fortified at a concentration of 0.1 ppm in bovine tissues, including liver, kidney and muscle, and has been successfully applied to the identification of residual OTC in bovine liver and residual CTC in bovine muscle samples previously found at concentrations of 0.58 ppm and 0.38 ppm by LC, respectively.

Keywords: Tetracyclines; Oxytetracycline; Tetracycline; Chlortetracycline; Doxycycline

1. Introduction

Tetracycline antibiotics (TCs, Fig. 1), represented by oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC), are commonly used all over the world as veterinary medicines and feed additives. In Japan, over 60% of all antibiotics used for animals are TCs, and residual

TCs have sometimes been found in organ and muscle tissues collected from slaughtered animals [1,2]. 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 [3,4], therefore, a method combining a simple and precise

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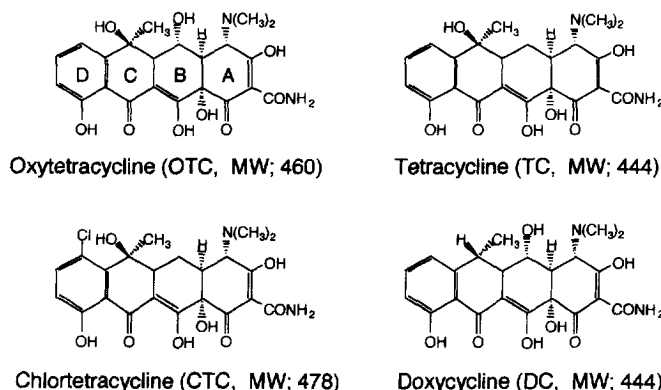*Corresponding author.
[1] Part XXII, see Ref. [10].

Fig. 1. Structures of tetracyclines.

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 [5–7]. 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 [8,9].

In our previous report [10], it was clarified that use of a well end-capped alkyl-bonded silica gel synthesized from a 99.99% pure silica gel column enables us to separate TCs without reduction of peak resolution using a volatile mobile phase that is applicable to direct interfaced LC–MS without causing clogging problems. We developed a frit fast atom bombardment (FAB) LC–MS method for the identification of residual TCs in honey using this LC system, however, we could not apply this technique to the identification of residual TCs in animal tissues due to its lack of sensitivity. These results led us to attempt to use electrospray ionization (ESI) LC–MS for the identification of TCs in animal tissues because of its high sensitivity. Although we obtained satisfactory ES mass spectra of TCs with high sensitivity when a standard solution of TCs was

injected into the ESI LC–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. This suggests that an additional sample clean-up procedure will be needed to obtain the ES mass spectra with a good S/N (signal-to-noise) ratio. Recently, tandem mass spectrometry with daughter ion scan mode in combination with ESI LC–MS (ESI LC–MS–MS) has been recognized as a rapid, sensitive and selective analytical method for the determination of complex biological samples [11–13]. The advantages gained by LC–MS–MS with daughter ion scan mode often complement those gained by extensive sample clean-up procedures. Therefore, we decided to employ ESI LC–MS–MS with daughter ion scan mode using a well end-capped column and a volatile mobile phase for the identification of TCs in animal tissues. In this paper, we report in detail the identification of residual TCs in bovine tissues using the ESI LC–MS–MS method.

2. Experimental

2.1. Materials

Methanol, acetonitrile, disodium ethylenediaminetetraacetate (Na$_2$EDTA), trifluoroacetic acid (TFA), citric acid and disodium hydrogen phosphate were of analytical reagent grade.

OTC, TC, CTC and DC, as their hydrochlorides, were supplied by Pfizer (Tokyo, Japan).

Table 1
Precursor and product ions of tetracyclines under ES MS–MS conditions

Tetracycline	Precursor		Condition 1[a]			Condition 2[b]		
	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)	

[a] Collision offset: −50 V.
[b] Collision offset: −25 V.

2.2. Extraction and clean-up procedures

A sample (5 g) was blended three times with 20, 20 and 10 ml of 0.1 M Na$_2$EDTA–McIlvaine buffer (pH 4.0) using a high-speed blender and was centrifuged (850 g for 5 min) each time. The supernatants were combined, centrifuged again (850 g for 15 min) and filtered. The filtrate was applied to a Bond Elut C$_{18}$ solid phase extraction cartridge (500 mg, 3 ml, part number 1210-2028, lot number 072944, Varian, Harbor City, CA, USA) that had been pretreated with saturated aqueous Na$_2$EDTA. The cartridge was washed with 20 ml of water and air-dried by aspiration for 5 min. 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 under reduced pressure at 30°C. The residue was dissolved in 0.1 ml of water,

and 50 μl of the solution were applied directly to ESI LC–MS–MS.

2.3. ESI LC–MS–MS conditions

The separation of TCs was performed on TSK Gel Super Octyl (2 μm, 100×4.6 mm, I.D.; Tosoh, Tokyo, Japan) using a Model 1051 pump (Hewlett-Packard, Palo Alto, CA, USA). An acetonitrile–0.05% aqueous TFA solution (1:4, v/v) was used as the mobile phase at a flow-rate of 0.5 ml/min.

The mass spectrometer and the data system used were a TSQ 7000 Triple-Stage Quadrupole (Finnigan MAT, San Jose, CA, USA) with an electrospray source and a DEC 3000 (Digital Equipment, Bedford, MA, USA), respectively. The instrument was operated at a scan rate of 400 u/s under daughter ion scan mode. A gas sheath flow of 70 p.s.i. (483 kPa)
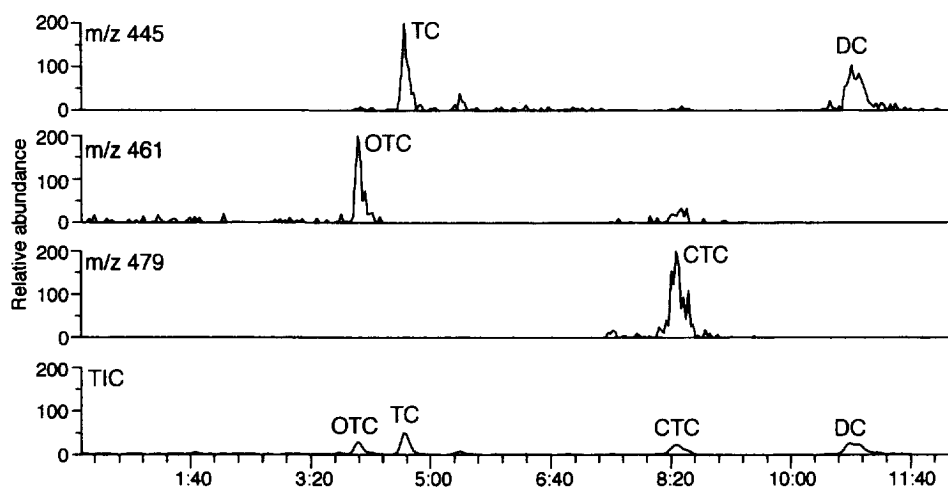

Fig. 2. Total ion and mass chromatograms of standard tetracyclines under MS–MS conditions.

nitrogen, an electrospray voltage of 4.5 kV and a capillary temperature of 175°C were used. Collision-induced dissociation was performed using argon as the collision gas at a pressure of 2.0 m Torr (0.27 Pa). The collision offset was −25 V.

3. Results and discussion

3.1. ESI LC–MS–MS of TCs

TCs give $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$ as fragment ions in the ES mass spectra, except for DC. These ions are very useful for the confirmation of TCs, as described in our previous report [14]. The loss of ammonia occurs from the carboxyamide moiety in the A ring of the TCs, and the formation of $[M+H-NH_3-H_2O]^+$ depends on the presence of an hydroxy group in the C ring, so that DC gives only $[M+H-NH_3]^+$. A combination of $[M+H]^+$, $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$ is available for confirmation of TCs. Additionally, the presence or absence of $[M+H-NH_3-H_2O]^+$ can differentiate TC from DC. Therefore, the

above three ions with adequate intensity should be observed in the tandem mass spectra obtained under daughter ion scan mode to identify the TCs. In order to obtain optimal MS–MS conditions for TCs, we measured the ES tandem mass spectra of TCs under two different collision offsets and observed carefully the intensity of these ions. As shown in Table 1, when the collision offset was set at −50 V, $[M+H-NH_3-H_2O]^+$ 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

Table 2
Recovery of TCs from bovine tissues fortified at a level of 0.1 ppm

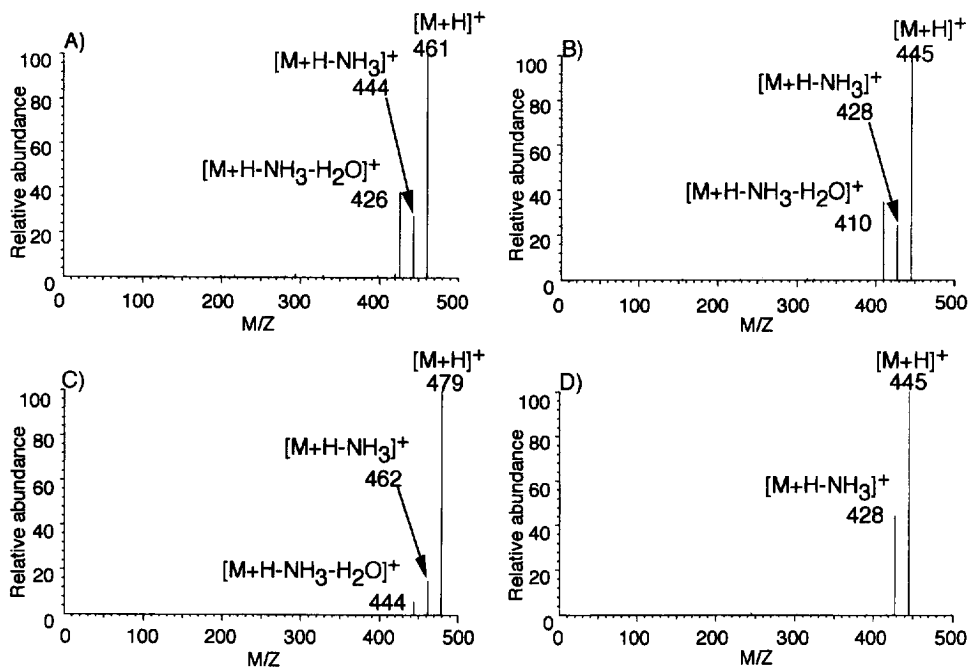| Sample	Recovery (%) and C.V. (%) ($n=5$)			
	OTC	TC	CTC	DC
Liver	73.8 (5.6)	70.3 (5.3)	61.7 (6.1)	55.5 (6.1)
Kidney	75.0 (3.1)	71.3 (5.9)	64.9 (4.8)	58.1 (6.8)
Muscle	79.0 (3.8)	79.1 (5.1)	73.0 (3.0)	69.9 (5.0)

Fig. 3. ESI tandem mass spectra of standard tetracyclines. (A) Oxytetracycline, (B) tetracycline, (C) chlortetracycline and (D) doxycyline.
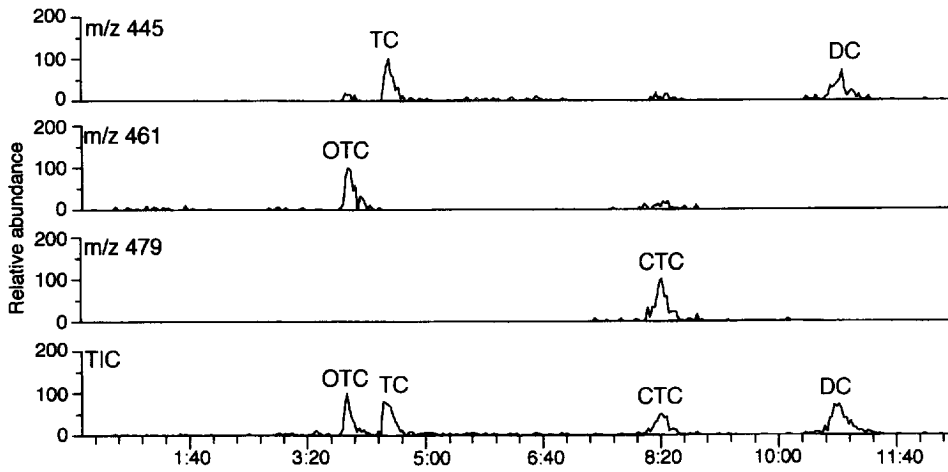
Fig. 4. Total ion and mass chromatograms of tetracyclines fortified at a concentration of 0.1 ppm in bovine liver under MS–MS conditions.

identify 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 clear product ions, [M+H−NH$_3$]$^+$ and [M+H−NH$_3$−H$_2$O]$^+$. All TCs can be identified easily under these MS–MS conditions. Therefore, we

used a collision offset of −25 V in subsequent studies.

Next, we injected 250 ng of each of the standard TCs into the LC–MS–MS system using a well end-capped octyl-bonded silica gel synthesized from 99.9% pure silica gel (TSK Gel Super Octyl) and a
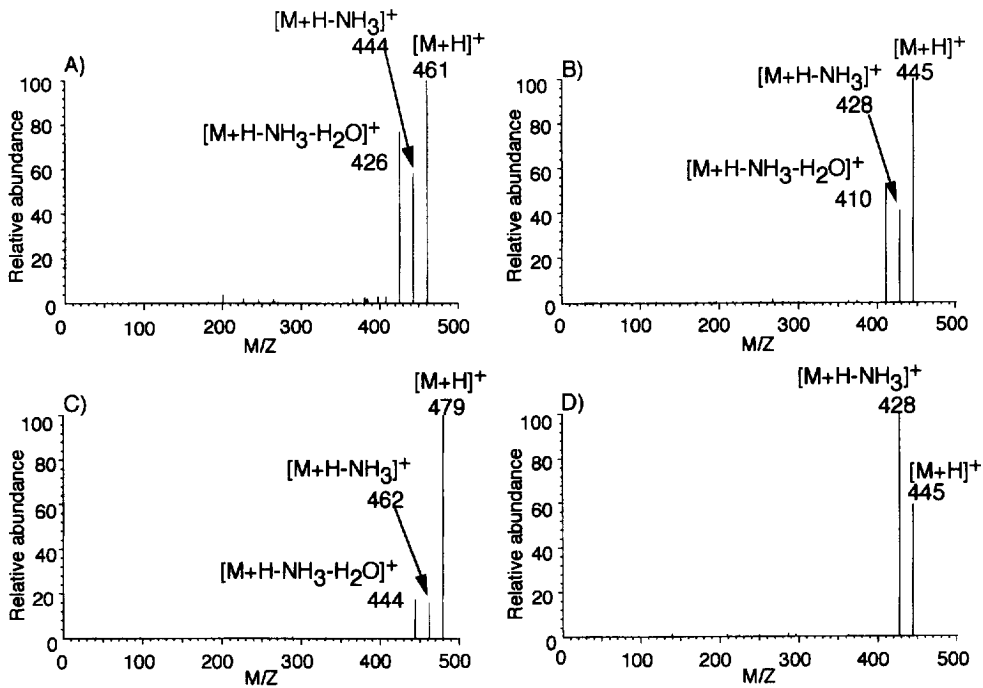

Fig. 5. ESI tandem mass spectra of tetracyclines fortified at a concentration of 0.1 ppm in bovine liver. (A) Oxytetracycline, (B) tetracycline, (C) chlortetracycline and (D) doxycyline.

volatile mobile phase (acetonitrile–0.05% aqueous
TFA solution, 1:4, v/v) as described in Section 2. As
shown in Fig. 2, the peaks of all TCs were clearly
observed at retention times of 3.9 min for OTC, 4.4
min for TC, 8.3 min for CTC and 10.8 min for DC,
on the total ion and mass chromatograms monitored
at individual protonated molecules (OTC, m/z 461;
TC, m/z 445; CTC, m/z 479 and DC, m/z 445).
Background-subtracted mass spectra of TCs taken at
the top of each peak on the mass chromatograms are
shown in Fig. 3. As summarized in Table 1, all TCs
gave $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$ as
the product ions, except for DC, when $[M+H]^+$ was
selected as the precursor ion.

3.2. ESI LC–MS–MS of TCs in bovine tissues

Although the C_{18} cartridge clean-up method de-
scribed in Section 2 yielded recoveries in excess of
80% and good reproducibility from samples fortified
at concentration of 1.0 ppm [15], the recoveries at
the 0.1 ppm fortification level were only 55–79%,
especially for DC in bovine liver (55%) and kidney
(58%), as shown in Table 2. However, 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 identifi-
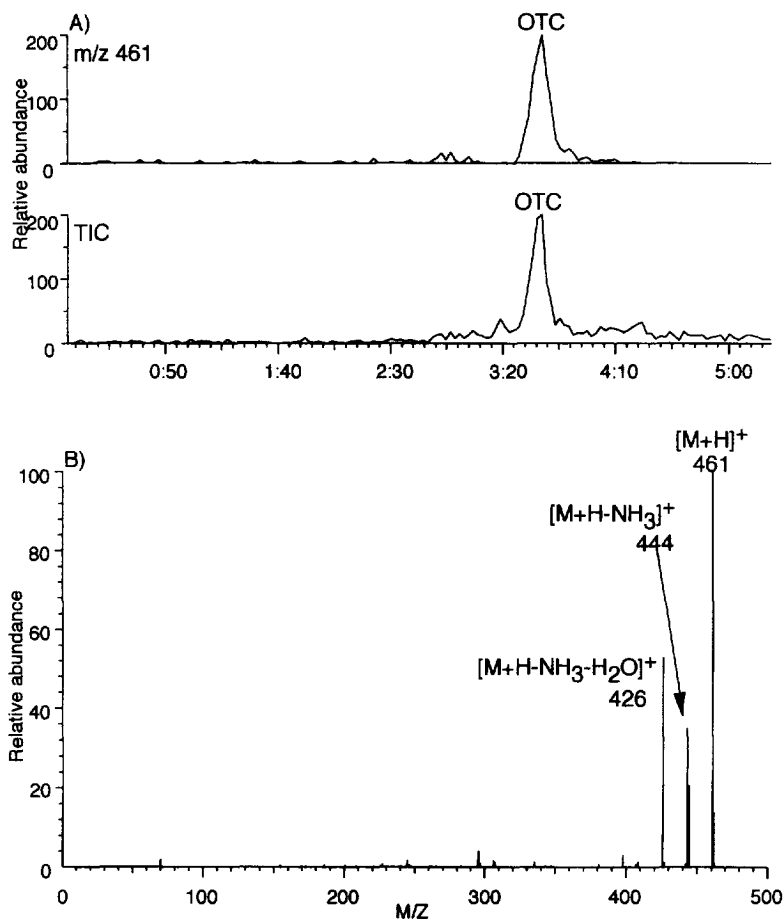cation of residual TCs in the samples, and the

Fig. 6. Total ion and mass chromatograms of residual oxytetracycline at a concentration of 0.58 ppm in bovine liver under MS–MS
conditions, and its ESI tandem mass spectrum. (A) Total ion and mass chromatograms; (B) ESI tandem mass spectrum.

method is applicable to the present ESI LC–MS–MS system. Therefore, we decided to use the C_{18} cartridge clean-up method. After clean-up of the TCs fortified at a concentration of 0.1 ppm in bovine liver, ESI LC–MS–MS was performed under the optimal conditions described in Section 2. As observed for the LC–MS–MS of the standard TCs, the peaks corresponding to the TCs appeared on the total ion and mass chromatograms monitored at individual protonated molecules as shown in Fig. 4. Fig. 5 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. We carried out the same experiments as those used for bovine liver but using bovine kidney and muscle and were able to confirm TCs at a 0.1 ppm level in bovine kidney and muscle. Thus, TCs in bovine tissues at a 0.1 ppm concentration can be reliably identified by the present ESI LC–MS–MS method.

This method was successfully applied to the identification of residual OTC at a concentration of 0.58 ppm in a bovine liver and residual CTC at a concentration of 0.38 ppm in a bovine muscle sample obtained from a slaughterhouse, which were previously found to be positive by LC. $[M+H]^+$, $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$ were
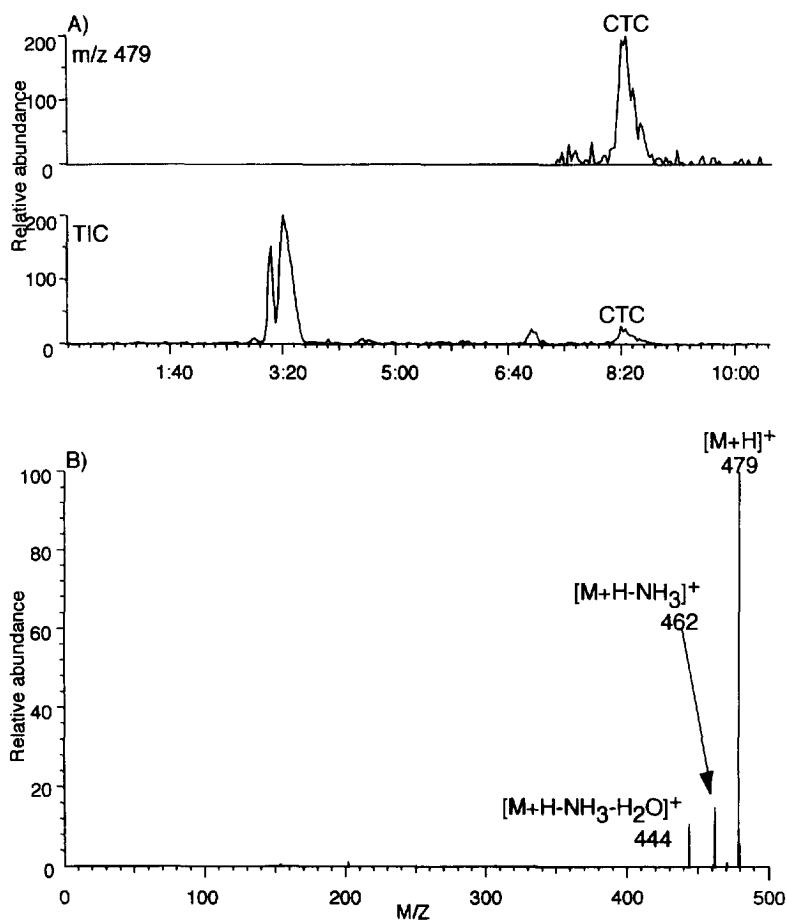

Fig. 7. Total ion and mass chromatograms of residual chlortetracycline at a concentration of 0.38 ppm in bovine muscle under MS–MS conditions, and its ESI tandem mass spectrum. (A) Total ion and mass chromatograms; (B) ESI tandem mass spectrum.

clearly observed at m/z 461, 444 and 426, and at m/z 479, 462, and 444, respectively, confirming the identity of OTC and CTC, and thereby demonstrating the effectiveness of this method for the identification of TCs in bovine tissues (Fig. 6 and 7).

4. Conclusion

Although Frit FAB LC–MS and ESI LC–MS could not be applied to the identification of residual TCs in animal tissues, using ESI LC–MS–MS with daughter ion scan mode, which is a rapid, sensitive and selective analytical method, we were able to establish a technique for the identification, with the following characteristics.

(1) All TCs gave $[M+H-NH_3]^+$ and $[M+H-NH_3-H_2O]^+$ as the product ions, except for DC, when $[M+H]^+$ was selected as the precursor ion.

(2) The clean-up procedure using a C_{18} cartridge was effective for the identification of residual TCs by ESI LC–MS–MS with daughter ion scan mode.

(3) The method can reliably identify TCs fortified at a concentration of 0.1 ppm in animal tissues and has been successfully applied to the identification of TCs in bovine samples that were previously found by LC.

Acknowledgments

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