Improvement of Chemical Analysis of Antibiotics. 18.† Identification of Residual Tetracyclines in Bovine Tissues by TLC/FABMS with a Sample Condensation Technique

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To reliably identify residual tetracycline antibiotics (TCs) oxytetracycline, tetracycline, chlortetracycline, and doxycycline in bovine tissues, a confirmation method using thin-layer chromatography/fast atom bombardment mass spectrometry (TLC/FABMS) has been established. This method consists of the following four steps: (1) extraction of TCs from bovine tissues with McIlvaine buffer (pH 4.0) containing 0.1 M ethylenediaminetetraacetate (Na_2EDTA); (2) elution of the TCs from prepacked C_{18} cartridges, pretreated with Na_2EDTA, with ethyl acetate followed by methanol–ethyl acetate (5:95 v/v) as eluents; (3) separation of the TCs on a reversed-phase C_8 TLC plate with methanol–acetonitrile–0.5 M oxalic acid solution (pH 2.0) (1:1:4 v/v) as a solvent system; and (4) TLC/FABMS with a condensation technique employing thioglycerol as a matrix. The method can reliably identify TCs fortified at concentrations of 0.1 ppm in bovine tissues, including muscle, liver, and kidney, and has been successfully applied to the identification of residual TCs in bovine incurred samples.

INTRODUCTION

Tetracycline antibiotics (TCs, Figure 1) are widely used in modern agriculture, with those most frequently used being oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC). In Japan, more than 60% of all antibiotics used for veterinary treatment or growth promotion of food-producing animals are TCs, and residual TCs have been found in organ and muscle tissues collected from slaughtered animals (Oka et al., 1991). The determination of residues of TCs in edible tissues of slaughtered animals is therefore 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 nonspecific. A method combining a simple and precise chromatographic separation with an appropriate mass spectrometric determination technique to provide absolute confirmation of the residual substance would offer significant advantages. Although high-performance liquid chromatography/mass spectrometry (LC/MS) appears to be 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 nonvolatile compounds as oxalic and citric acids to improve the chromatographic resolution of TCs (Oka et al., 1984a,b; Mulders and Lagemaat, 1989, Walsh et al., 1992). When LC conditions with a mobile phase containing nonvolatile compounds are applied to an LC/MS analysis, clogging at the interface and a buildup of deposits in the ion source are observed, so that the LC/MS cannot be operated for a prolonged period (Kijak et al., 1991; Kenion et al., 1990).

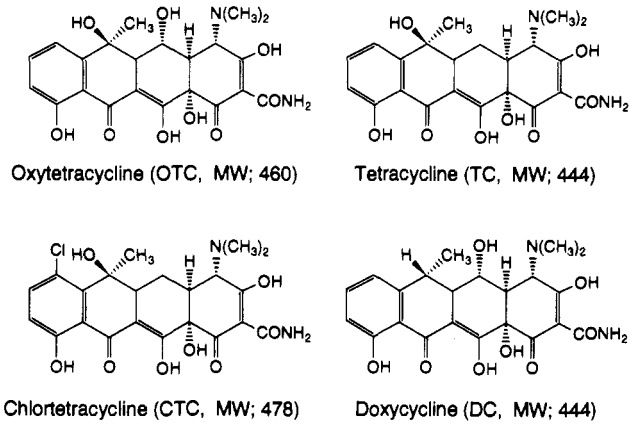

Figure 1. Tetracycline antibiotics.

Recently, we have developed a sample condensation technique for thin-layer chromatography/fast atom bombardment mass spectrometry (TLC/FABMS) that provides high sensitivity, and we have successfully applied this to the analysis of food dyes (Harada et al., 1991; Oka et al., 1992). In TLC/FABMS, the developed and air-dried TLC plate is inserted into the TLC/FABMS 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. To obtain good separation of TCs on the TLC plate, nonvolatile compounds such as oxalic acid (Oka et al., 1984a,b) and ethylenediaminetetraacetate (Na_2-EDTA) (Oka et al., 1983) are added to the solvent system, as is also the case in their analysis by LC. However, these compounds do not cause any problems in TLC/FABMS such as have been reported in LC/MS (clogging of interface and deposits in ion source) because they remain on the TLC plate and are removed with it after the measurement has been completed.

We decided to investigate the application of this technique to the identification of residual TCs in tissues from slaughtered animals. The method is based on a C_{18} cartridge cleanup, followed by separation of the TCs on

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Table I. Protonated Molecules of TCs and Cluster Ions of Matrices in the Molecular Ion Regions Using Various Matrices

matrix	protonated molecules, m/z				cluster ion of matrix in the molecular ion region of TCs, m/z
	OTC	TC	CTC	DC	
thioglycerol	461	445	479	445	ND[a]
magic bullet	461	445	479	445	461
glycerol	461	445	479	445	461
m-nitrobenzyl alcohol	461	445	479	445	460
dithioethanolamine	461	445	479	445	463
o-nitrophenyl octyl ether	ND	ND	ND	ND	ND
triethanolamine	ND	ND	ND	ND	ND
diethanolamine	ND	ND	ND	ND	ND

[a] ND, not detected.

a reversed-phase C_8 TLC plate. TLC/FAB mass spectra of the TCs are measured after concentration of the TCs spots. In the present paper, we report in detail the identification of residual TCs in bovine tissues, including muscle, liver, and kidney, by TLC/FABMS using the sample condensation technique.

MATERIALS AND METHODS

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

Extraction and Cleanup Procedure. A sample (5 g) was blended three times with 20, 20, and 10 mL of 0.1 M Na_2EDTA–McIlvaine buffer (pH 4.0) using a high-speed blender and 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 (part 1210-2028, lot 072944, Varian, Harbor City, CA) pretreated with saturated aqueous Na_2EDTA. 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 methanol or 0.5 mL of HPLC mobile-phase solution and applied directly to TLC or HPLC, respectively.

Thin-Layer Chromatography. After the application of sample and standard solutions (60 μL/spot), a C_8-modified silica gel TLC plate (RP-8, E. Merck, Darmstadt, Germany) was developed for a distance of 9 cm with methanol–acetonitrile–0.5 M oxalic acid solution (pH 2.0) (1:1:4 v/v).

Detection of TCs on TLC Plate with Spray Reagents. The TCs spots on the developed TLC plate were revealed by treatments applied in the following order: spraying with 2 M hydrochloric acid, heating at 150 °C for 1 min, and then spraying successively with 28% aqueous ammonia, 0.5% aqueous Fast Blue BB salt, and pyridine.

High-Performance Liquid Chromatography. A high-performance liquid chromatograph equipped with a constant flow pump was used, with a variable-wavelength UV detector operated at 350 nm. Separations were performed on a Bakerbond C_8 column (5 μm, 250 × 4.6 mm i.d., J. T. Baker Chemical, Phillipsburg, NJ) with methanol–acetonitrile–0.01 M aqueous oxalic acid solution (1:1.5:3) as the mobile phase at flow rate of 1.5 mL/min at room temperature. An injection volume of 10 μL was used.

Condensation Technique. The condensation technique used in the present study was described in detail in the previous paper, so only a brief summary of the procedure is presented here (Oka et al., 1992). The following condensation technique was used for TCs spots on the TLC plate: (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 30 s, the sample was condensed along the upper base of the trapezoid in a line (0.5 × 2 mm) by penetration of methanol.

Thin-Layer Chromatography/Mass Spectrometry. The location of the sample spot was estimated on the basis of that of neighboring spots of TCs standards, as located using a UV lamp (254 nm). After application of the condensation technique, the TLC plate was placed on the TLC holder, a matrix was applied on the sample spot, and the TLC/FAB mass spectra were measured using a Model JMS-AX505W mass spectrometer with a TLC/FAB ion source (JEOL, Tokyo). The FAB gun was operated with xenon gas at 5 kV, using an acceleration voltage of 3 kV for measurement of all spectra.

RESULTS AND DISCUSSION

To successfully apply TLC/FABMS to residual level antibiotics in animal tissues, points which must be carefully investigated include the selection of suitable matrix, TLC conditions, cleanup procedure, and sample condensation on the TLC plate. These are discussed below.

Selection of Suitable Matrix. The following matrices were examined for the TLC/FABMS of TCs using the conventional procedure: diethanolamine (DEA), triethanolamine (TEA), *o*-nitrophenyl octyl ether (NPOE), dithioethanolamine (DTE), *m*-nitrobenzyl alcohol (NBA), glycerol, magnetic bullet (1,4-dithiothreitol–1,4-dithioerythritol, 3:1 v/v), and thioglycerol. As shown in Table I, no protonated molecules, $[M + H]^+$, appeared in the mass spectra of TCs when DEA, TEA, and NPOE were used as matrices. $[M + H]^+$ were observed using DTE, NBA, glycerol, and magic bullet at *m/z* 461 for OTC, at *m/z* 445 for TC and DC, and at *m/z* 479 for CTC, respectively. However, it was difficult to distinguish the protonated molecule of OTC from the cluster ions of the matrices in the resulting mass spectra when small amounts of OTC were deposited on the target, as the cluster ions of the matrices also appeared strongly at *m/z* 463 for DTE, at *m/z* 460 for NBA, and at *m/z* 461 for glycerol and magic bullet, respectively. Only thioglycerol gave the $[M + H]^+$ clearly, as well as prominent fragment ions, $[M + H - NH_3]^+$ and $[M + H - NH_3 - H_2O]^+$, at *m/z* 461, 444, and 426 for OTC, at *m/z* 445, 428, and 410 for TC and DC, and at *m/z* 479, 462, and 444 for CTC, respectively, without any interfering ions in their molecular ion regions, demonstrating that thioglycerol is the best matrix of those tested. Since no FABMS of TCs have previously been reported, this aspect of the investigation will be reported elsewhere (Oka et al., unpublished results).

TLC Conditions. The development of TLC conditions for the separation of TCs is complicated by their ability to chelate with metal ions which may be present in the stationary phase. We have previously reported TLC conditions for the separation of TCs using commercially available normal-phase silica gel HPTLC plates (Oka et al., 1983) and reversed C_8-modified silica gel TLC plates (Oka et al., 1984a,b). Predevelopment of plates with Na_2-EDTA and addition of oxalic acid to the solvent system can reproducibly control the chelation problem for silica gel HPTLC and C_8-modified silica gel TLC, respectively, yielding successful separations of the TCs. Because TLC/FABMS on a normal-phase plate usually provides higher sensitivity than is observed for reversed-phase plates, the detection sensitivity of TCs was first examined on silica

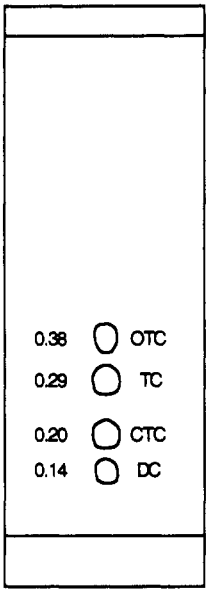

Figure 2. Separation of tetracycline antibiotics on reversed-phase C_8 TLC plate. TLC conditions: see Materials and Methods.

gel HPTLC plates under the TLC/FABMS conditions. No ions originating from TCs appeared, even if 50 μg/spot of TCs was deposited on the plate, suggesting that TCs cannot be extracted into thioglycerol from the absorbent due to binding with silanol groups and metal ions present in the silica gel. Attempts to condense TCs on silica gel HPTLC plates, using the condensation technique described in the next section, were unsuccessful. In the case of C_8-modified silica gel TLC plates, however, TLC/FAB mass spectra of TCs clearly showed the molecular ion species at concentrations of 5 μg/spot.

To measure the TLC/FAB mass spectrum with good sensitivity and reproducibility, the location of a spot should be estimated before the measurement. However, it is difficult to visualize the TCs directly on a TLC plate at concentrations of 0.1 ppm in biological samples without a spray reagent. Since the locations of the spots of the

Table II. Recovery of TCs from Bovine Tissues Fortified at a Level of 0.1 ppm[a]

	recovery, % (CV, %)			
sample	OTC	TC	CTC	DC
liver	72.5 (5.8)	71.3 (5.9)	61.5 (6.3)	54.6 (6.5)
kidney	74.9 (3.5)	72.8 (6.5)	64.1 (4.1)	58.7 (6.0)
muscle	79.1 (4.0)	79.5 (5.0)	72.6 (3.1)	69.6 (5.7)

[a] $n = 5$.

TCs in a sample have frequently been estimated on a developed TLC plate on the basis of the R_f of neighboring standards, TLC conditions must be selected in which the positions of the TCs are not affected by coeluting substances from a sample. The reproducibility of R_f values of TCs was examined on C_8-modified silica gel plates using bovine muscle fortified at 0.1 ppm with the TCs. The samples were cleaned up as described under Materials and Methods, with the final extracts being subjected to the TLC separation. Visualization of the spots on the developed chromatograms demonstrated that the R_f values of TCs extracted from tissue matched those of the standards, so that the standards could be reliably used to estimate the location of TCs present in samples after cleanup and chromatography. The TLC conditions for TLC/FABMS for TCs were therefore standardized to use a C_8-modified silica gel plate with a solvent system containing methanol–acetonitrile–0.5 M oxalic acid solution (pH 2.0) 1:1:4 v/v), providing a separation as illustrated in Figure 2.

Condensation Technique of Spots of TCs on TLC Plate. 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. In our previous papers (Harada et al., 1991; Oka et al., 1992), to prevent diffusion of the analyte and to obtain sensitivity from the TLC/FAB mass spectrum, we developed a sample condensation technique using methanol. Although the diffusion of a sample on a reversed-phase plate is not as great as is observed using normal-phase plates when a

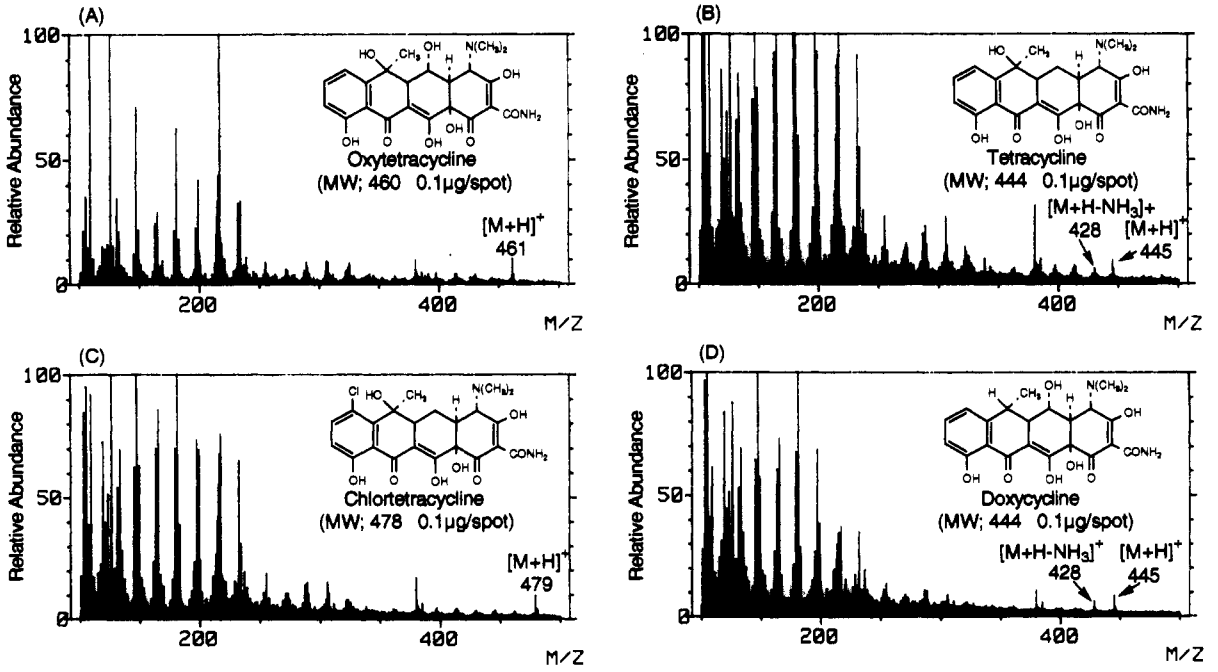

Figure 3. TLC/FAB mass spectra of tetracycline antibiotics at concentrations of 0.1 μg/spot. (A) Oxytetracycline; (B) tetracycline; (C) chlortetracycline; (D) doxycycline.

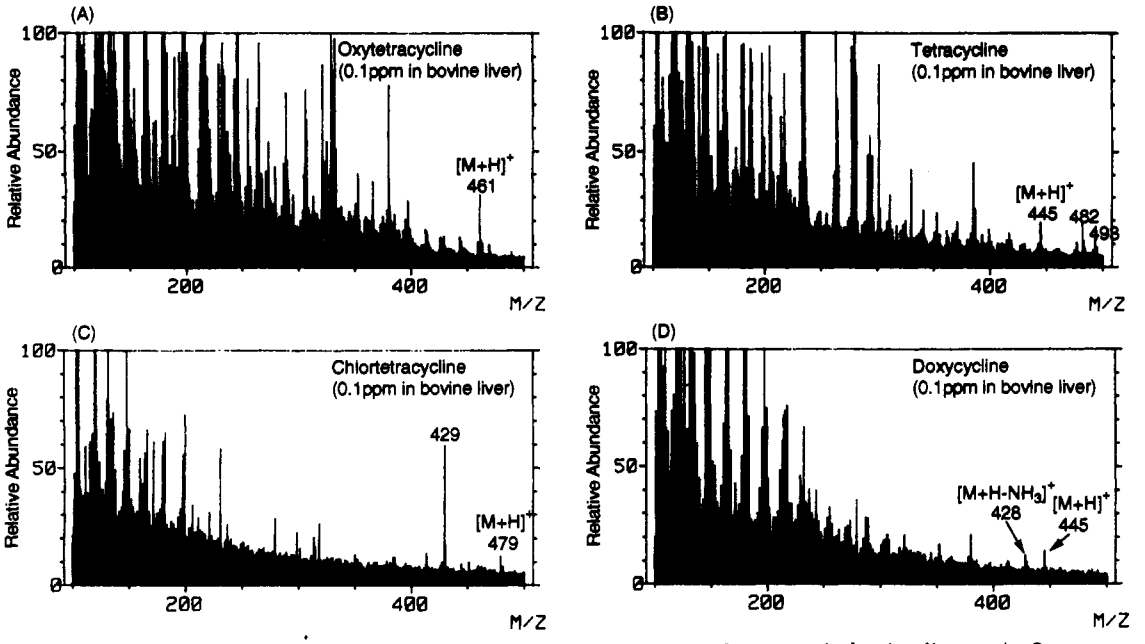

Figure 4. TLC/FAB mass spectra of tetracycline antibiotics at concentrations of 0.1 ppm in bovine liver. (A) Oxytetracycline; (B) tetracycline; (C) chlortetracycline; (D) doxycycline.

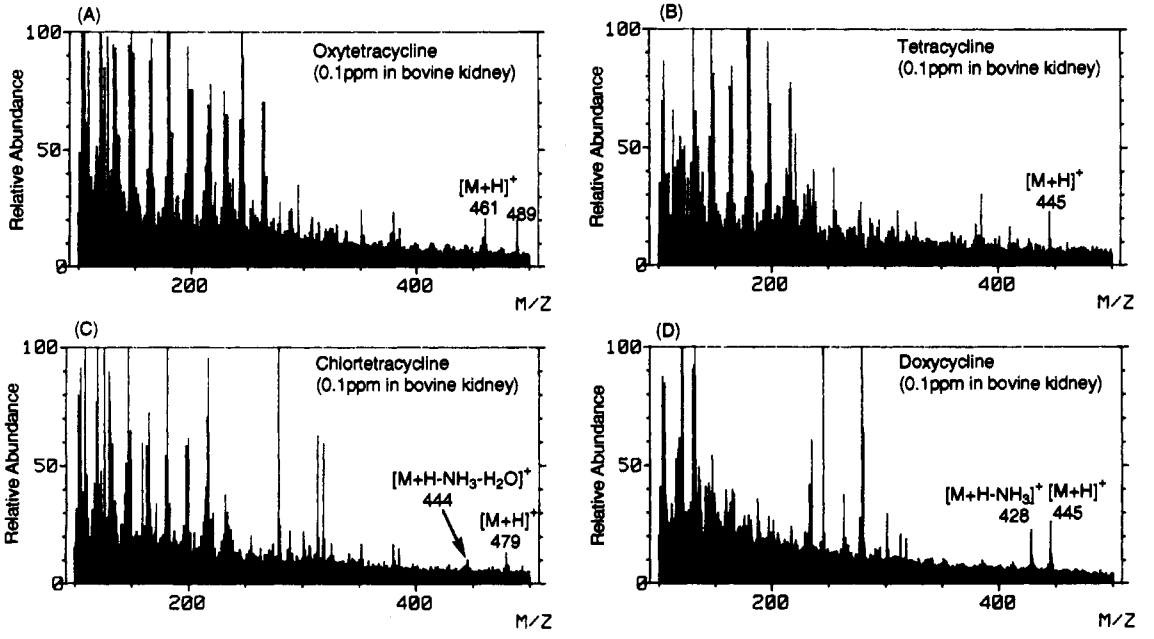

Figure 5. TLC/FAB mass spectra of tetracycline antibiotics at concentrations of 0.1 ppm in bovine kidney. (A) Oxytetracycline; (B) tetracycline; (C) chlortetracycline; (D) doxycycline.

matrix is deposited on the spot, no satisfactory spectra were obtained unless more than 5 μg/spot of TCs was applied to the TLC plate without the condensation technique. To effectively obtain high sensitivity, the developed spot was reconcentrated on the TLC plate using the concentration technique described under Materials and Methods. As shown in Figure 3, the $[M + H]^+$ appeared clearly at m/z 461 for OTC, at m/z 445 for TC and DC, and at m/z 479 for CTC, at a concentration of 0.1 μg/spot, as did the fragment ions $[M + H - NH_3]^+$ in the spectra of TC and DC. Thus, the technique can improve the detection limits of TCs by 50 times with good reproducibility in TLC/FABMS, demonstrating the suitability of the technique for identification of TCs in bovine tissues.

Cleanup Procedure. In a previous paper (Ikai et al., 1987), we reported a cleanup method for TLC analysis of TCs in bovine liver, kidney, and muscle using prepacked C_{18} solid-phase extraction cartridges. TCs can be extracted from the samples with McIlvaine buffer (pH 4.0) containing 0.1 M Na_2EDTA, the extract is applied to a C_{18} cartridge that has been pretreated with Na_2EDTA, and the TCs are eluted with ethyl acetate, followed by methanol–ethyl acetate (5:95%). Although the method yielded recoveries in excess of 80% and good reproducibility from samples fortified at concentrations of 1.0 ppm, an evaluation of the method had not been conducted for samples containing lower concentrations of TCs. The method was therefore examined for the analysis of TCs fortified at concentrations of 0.1 ppm in bovine tissues. Although the recoveries obtained were not as good as previously reported at the 1.0 ppm fortification level (Ikai et al., 1987), especially for DC in bovine liver and kidney, good coefficients of variation (CVs) were obtained, as shown in Table II. These results suggest that the cleanup method provides sufficient recoveries with good reproducibility for the identification

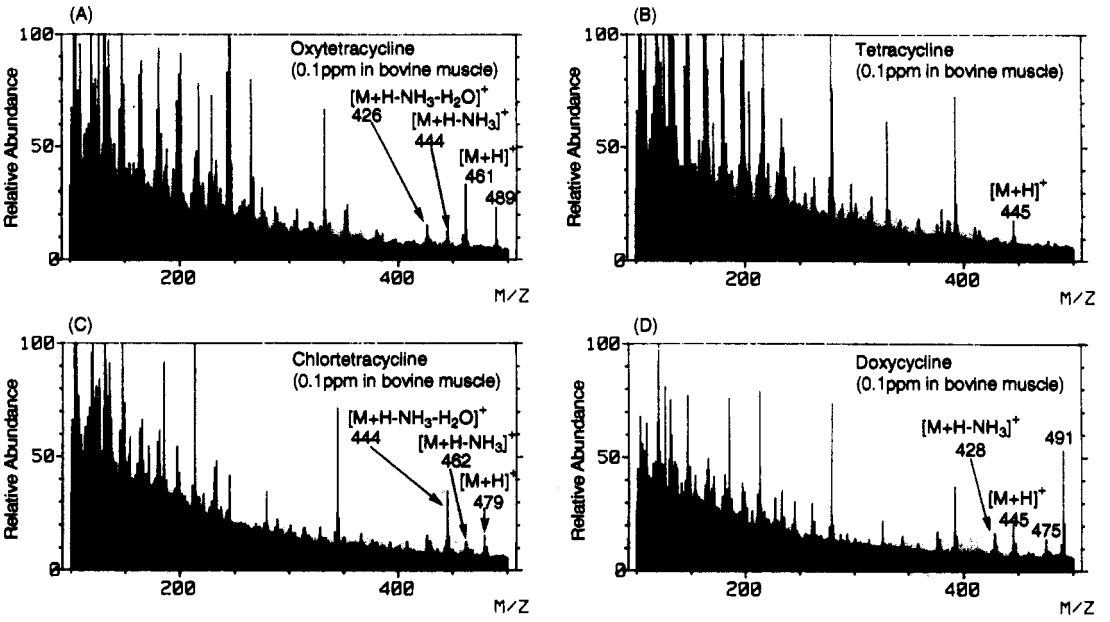

Figure 6. TLC/FAB mass spectra of tetracycline antibiotics at concentrations of 0.1 ppm in muscle. (A) Oxytetracycline; (B) tetracycline; (C) chlortetracycline; (D) doxycycline.

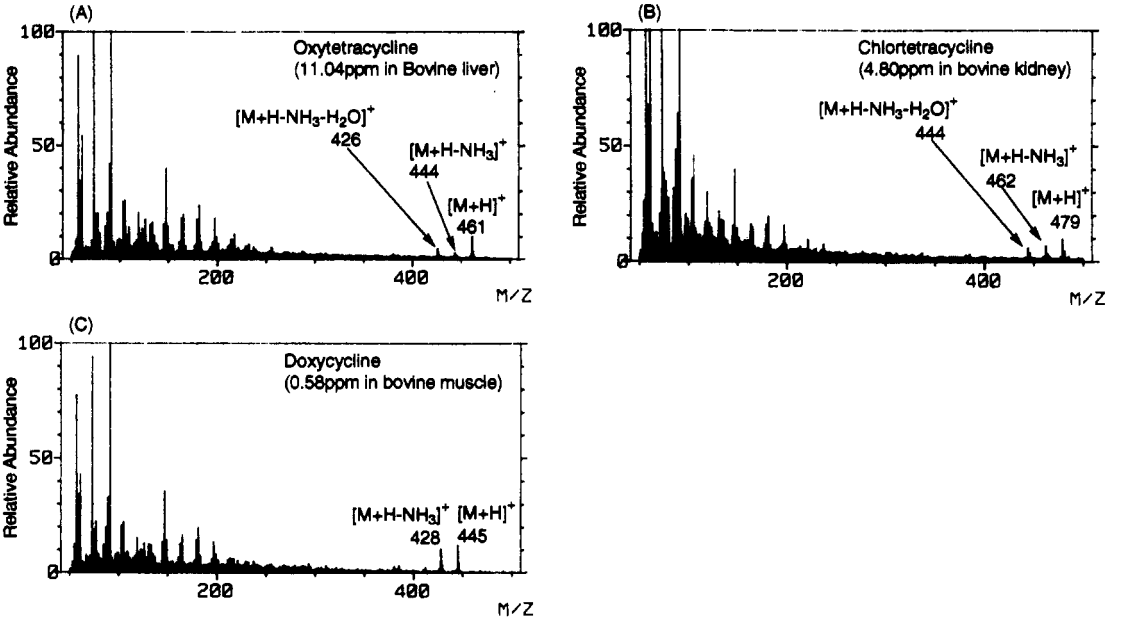

Figure 7. TLC/FAB mass spectra of residual tetracycline antibiotics in bovine incurred samples. (A) Oxytetracycline at a concentration of 11.04 ppm in bovine liver; (B) chlortetracycline at a concentration of 4.80 ppm in bovine kidney; (C) doxycycline at a concentration of 0.58 ppm in bovine muscle.

of residual TCs in the samples, and the method is applicable to the present TLC/FABMS study.

TLC/FABMS of TCs in Bovine Tissues. After cleanup of TCs fortified at concentrations of 0.1 ppm in bovine liver, kidney, and muscle, TLC/FABMS with the concentration technique was performed. Although a few ions originating from coextractives from the samples appear strongly at higher mass unit regions than the $[M + H]^+$, i.e., m/z 482 and 493 in the spectrum of TC in liver, m/z 489 in those for OTC in liver and kidney, and m/z 475 for DC in muscle (Figures 4–6), the $[M + H]^+$ are clearly observed in the spectra for all tissues and some spectra also provide the fragment ions, $[M + H - NH_3]^+$ and $[M + H - NH_3 - H_2O]^+$. 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, the present method was applied to the identification of TCs in incurred bovine samples in which TCs had already been detected by HPLC. The $[M + H]^+$ and fragment ions of TCs were clearly visible (Figure 7), confirming the identity of these substances and thereby demonstrating the effectiveness of the present method for the identification of TCs in biological samples.

Conclusions. In the present study we have developed a TLC/FABMS method incorporating an analyte condensation technique for the identification of residual TCs in bovine tissues, with the following characteristics. Use of thioglycerol as a matrix enables us to measure the FAB mass spectra of TCs at 0.1 μg/spot. Under reversed-phase C_8 TLC conditions, the location of TCs extracted from bovine tissues can be readily estimated on the basis of the location of neighboring visualized spots of standards. The cleanup procedure using C_{18} cartridges gave satisfactory recoveries and CVs for the TCs fortified at concentrations of 0.1 ppm in tissues and proved to be effective for the identification of residual TCs by TLC/FABMS. The

condensation technique improved the detection limits 50-fold with good reproducibility. TCs fortified at concentrations of 0.1 ppm in bovine tissues have been reliably identified using the present method. The method was successfully applied to the identification of TCs in incurred bovine samples previously found positive by HPLC.

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