Improvement of Chemical Analysis of Antibiotics. 22.† Identification of Residual Tetracyclines in Honey by Frit FAB/LC/MS Using a Volatile Mobile Phase

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A reliable identification method for residual tetracycline antibiotics (TCs), oxytetracycline, tetracycline, chlortetracycline, and doxycycline in honey, has been established using frit FAB/LC/MS with a volatile mobile phase. The TCs were recovered from a C_{18} solid phase extraction cartridge and separated on an end-capped phenyl-bonded silica gel column synthesized from 99.99% pure silica gel (5 μm, 250 × 2.1 mm i.d.) using a mobile phase consisting of methanol–acetonitrile–0.005 M trifluoroacetic acid (2:2:11) containing 1.0% thioglycerol. The column effluent was introduced into the frit FAB ion source after splitting at a ratio of 1:20. TCs in honey were reliably identified by their FAB/MS at 0.2 ppm. Detection limits of protonated molecules are 0.1 ppm in honey. This method is one of the first applications of LC/MS technology to the identification of residual TCs in foods using a volatile mobile phase without reduction of peak resolution and clogging problems at the interface.

Keywords: *Tetracycline antibiotics; oxytetracycline; tetracycline; chlortetracycline; doxycycline; honey; LC/MS, frit FAB/MS*

INTRODUCTION

To prevent European (*Streptococcus pluton*) and American (*Bacillus larvae*) foulbrood diseases of honey bees, oxytetracycline (OTC) is mainly used worldwide in their culture (Wilson *et al.*, 1971; Lehnert and Shimanuki, 1980; Nakazawa *et al.*, 1992), and tetracycline (TC), chlortetracycline (CTC), and doxycycline (DC) are also administered in sugared water. In Japan, zero tolerance levels are provided for residues of tetracycline antibiotics (TCs, Figure 1) in honey and the government has historically set detection limits of 0.1 ppm on the basis of microbiological assay. However, TCs above 0.1 ppm have been sometimes found in honey on the market (Nakazawa *et al.*, 1992). Therefore, it is necessary for public health agencies to monitor residues of TCs in honey.

Microbiological assays have been most commonly used for the detection of residual TCs, but their precision appears to be variable and the specificity is questionable. Moreover, these methods are not suitable for the analysis of TCs in honey, because honey itself has bacteriostatic action. Since mass spectrometric techniques can confirm the residual drugs with high sensitivity and selectivity (Van Der Greef *et al.*, 1993; Harada *et al.*, 1994), a method combining a simple and precise chromatographic separation with an appropriate mass spectrometric determination technique would offer a significant advantage in the provision of absolute confirmation of the residual TCs. High-performance liquid chromatography/mass spectrometry (LC/MS) appears to be best suited for this purpose.

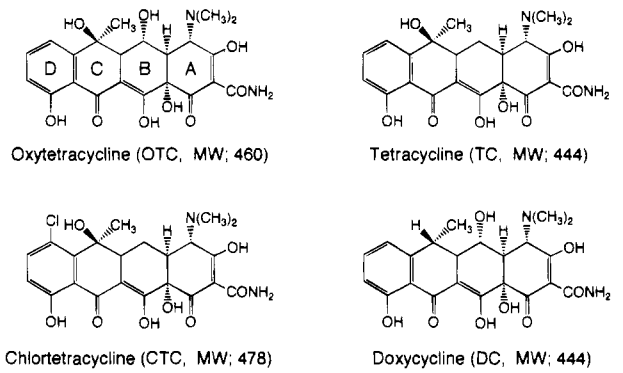

Figure 1. Structures of tetracycline antibiotics.

The existence of residual silanol groups and metals in LC column packing materials influences the chromatography of silanol and/or metal sensitive compounds. To avoid this influence, triethylamine and oxalic acid are added to the mobile phase. Most previously reported LC conditions for TCs are based on mobile phases containing such nonvolatile compounds as oxalic and citric acids to control peak tailing due to residual silanol groups and metal impurities in the column packing materials (Oka *et al.*, 1984; Mulders and Lagemaat, 1989; Walsh *et al.*, 1992). However, mobile phases containing nonvolatile compounds, when used

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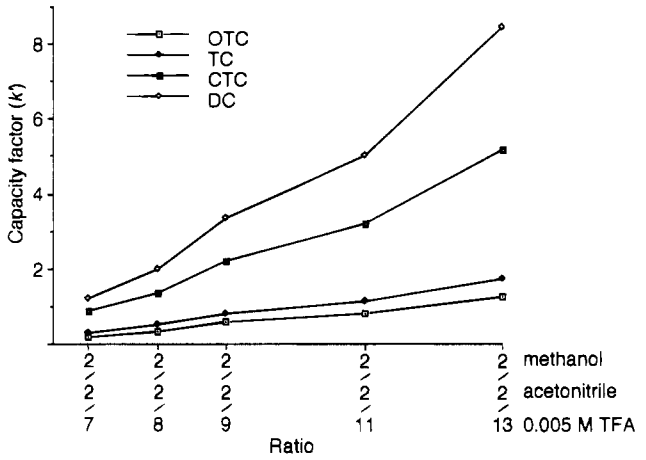

Figure 2. Effect of ratio of methanol, acetonitrile, and aqueous TFA solution on the capacity factor. Mobile phase: mixtures of methanol, acetonitrile, and 0.005 M TFA at various ratios containing 1.0% thioglycerol. For other LC conditions, see Materials and Methods.

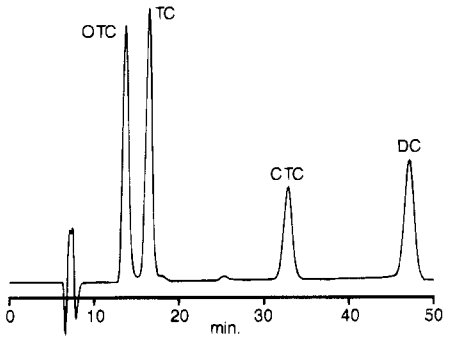

Figure 3. LC separation of TCs. For LC conditions, see Materials and Methods.

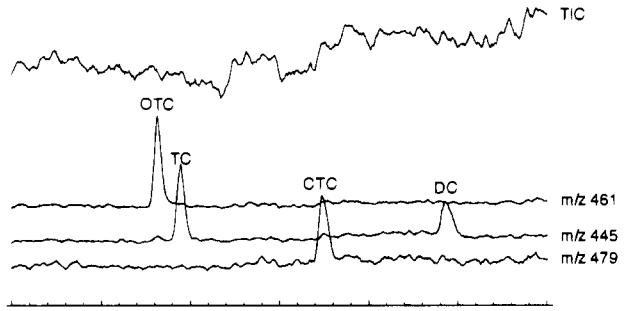

Figure 4. Total ion and mass chromatograms of standard TCs. For LC/MS conditions, see Materials and Methods.

in LC/MS, have been observed to cause clogging at the interface and a buildup of deposits in the ion source, so that the LC/MS cannot be operated for a prolonged period (Kijak *et al.*, 1991; Kenion *et al.*, 1990). Mobile phases containing such nonvolatile buffers, while necessary for the chromatography of the TCs, are therefore unsuitable for routine use in LC/MS analyses.

Recently, well end-capped alkyl-bonded silica gels have been synthesized from 99.99% pure silica gel and have been successfully applied to the separation of silanol and/or metal sensitive compounds without the above additives (Ohhira *et al.*, 1989; Horie *et al.*, 1993). Therefore, we decided to investigate this type of column packing for the identification of TCs in honey by frit fast atom bombardment (FAB)/LC/MS using a volatile mobile phase. In this paper, the application of this technique for the identification of TCs is described in detail.

MATERIALS AND METHODS

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

Extraction and Cleanup Procedure. A sample (20 g) was dissolved in 50 mL of 0.1 M disodium ethylenediaminetetraacetate (Na_2EDTA)–McIlvaine buffer (pH 4.0). The solution was filtered through a No. 5C filter paper (Advantec Toyo, Tokyo, Japan), and the filtrate was applied to a Bond Elut C_{18} solid phase extraction cartridge (500 mg, part no. 1210–2028, lot no. 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 methanol, and the eluate was evaporated to dryness under reduced pressure at 30 °C. The residue was dissolved in 0.1 mL of water, and 90 μL of the solution was applied directly to frit FAB LC/MS.

Liquid Chromatography. A liquid chromatograph equipped with a constant-flow pump was used, with a variable-wavelength UV detector operated at 350 nm. Separations were performed on the following columns with various combinations of methanol, acetonitrile, and 0.005 M aqueous trifluoroacetic acid (TFA) solution as mobile phases at room temperature: Inertsil ODS-2 (5 μm, 250 × 4.0 mm i.d., GL Science, Tokyo), Inertsil C_8 (5 μm, 250 × 4.0 mm i.d., GL Science), Inertsil Ph (5 μm, 250 × 4.0 mm i.d., GL Science), and Inertsil Ph (5 μm, 250 × 2.1 mm i.d., GL Science).

Frit FAB/LC/MS Conditions. The separation of TCs was performed on Inertsil Ph (5 μm, 250 × 2.1 mm i.d.) using an LC-100P LC pump (Yokogawa Electric, Tokyo, Japan). Methanol–acetonitrile–0.005 M aqueous trifluoroacetic acid solution (2:2:11) containing 1% thioglycerol was used as a mobile phase at the flow rate of 0.1 mL/min.

The mass spectrometer and the data system used were a JMS-AX505W (JEOL, Tokyo, Japan) and a JMA-DA5000 (JEOL), respectively. Ion source temperature was maintained at 60 °C, and a neutral xenon beam was used as the primary beam for sample ionization by FAB. The acceleration voltages of the primary and secondary beams were adjusted to 3 and 5 kV, respectively. LC/MS data were obtained by scanning from m/z 100 to 1500 at a cycle time of 6.5 s. The TIC range was set to m/z 100–1500.

The LC and the mass spectrometer were interfaced by a laboratory-made flow splitter (Ikai *et al.*, 1991), connection tubing (fused silica 100 cm × 0.06 mm i.d.), and a frit FAB probe (JEOL). The LC effluent was split at a ratio of 1:20, and the smaller portion of the effluent was introduced into the mass spectrometer through the connection tubing at a flow rate of 5 μL/min.

Calculation of Chromatographic Parameters. Capacity factor was calculated as $k' = (t_r - t_0)/t_0$, where t_r is the retention time of the sample peak ant t_0 is the retention time for a nonretention peak.

Resolution factor was calculated as $Rs = 2(t_1 - t_2)/(w_1 + w_2)$, where t_1 and t_2 are retention times and w_1 and w_2 the corresponding peak widths at the baseline.

The asymmetry factor, *As*, is the ratio of the rear (tailing edge) to the front (leading edge) lengths of the peak along a line parallel to and 10% (of its height) distant from its base.

RESULTS AND DISCUSSION

Optimization of LC Conditions Using a Volatile Mobile Phase. Three types of well end-capped alkyl-bonded chromatographic packings synthesized from 99.99% pure silica gel are commercially available, Inertsil C_{18}, Inertsil Ph, and Inertsil C_8. In a previous study, usage of a C_8 conventional column provided better resolution between OTC and TC than did a C_{18} column (Oka *et al.*, 1984). To compare the suitability of the new C_8, Ph, and C_{18} columns (5 μm, 250 × 4.0 mm i.d.) for the analysis of TCs, we separated TCs using

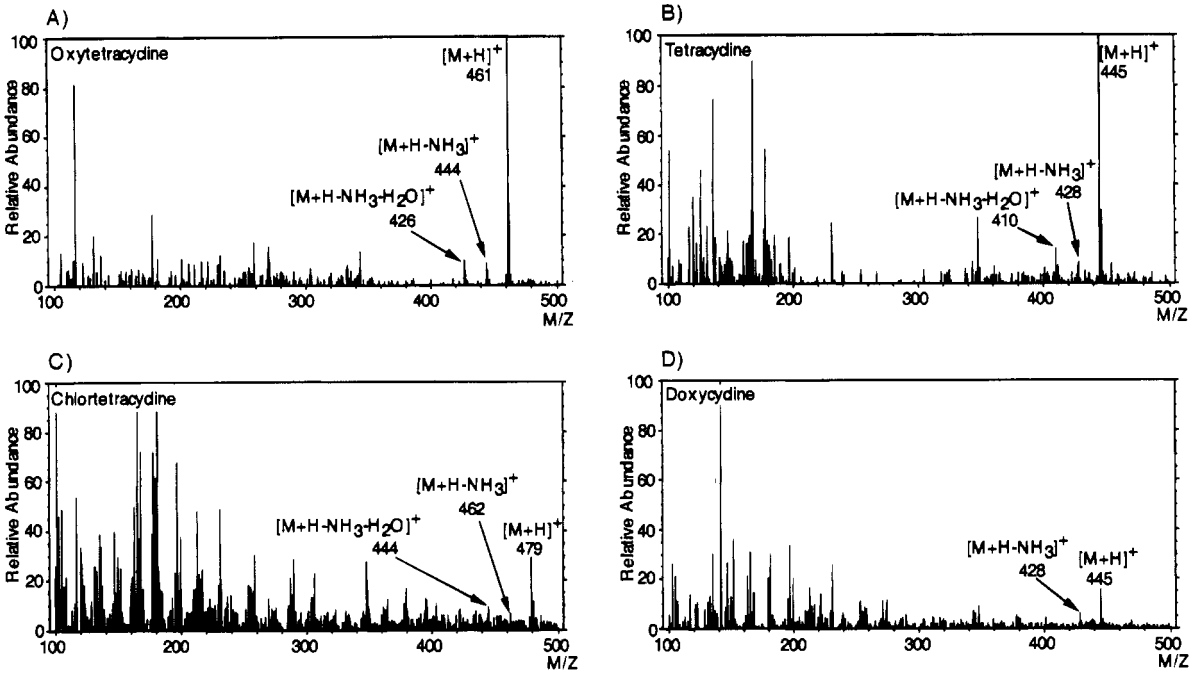

Figure 5. Background-subtracted mass spectra of standard TCs: (A) oxytetracycline; (B) tetracycline; (C) chlortetracycline; (D) doxycycline. For LC/MS conditions, see Materials and Methods.

methanol–acetonitrile–0.005 M TFA solution (1:1:5) as a mobile phase and investigated mainly peak shape and resolution as performance indicators. On all columns *As* factors of TCs were between 1.00 and 1.05, indicating no tailing and no leading peaks. For the separation of TCs, although excellent separations were obtained on all columns between TC and CTC and between CTC and DC, different chromatographic behavior was observed in the separation of OTC and TC. The *Rs* values between OTC and TC were 1.5, 1.9, and 1.0 on the C_8, Ph, and C_{18} columns, respectively. When the composition of mobile phase was adjusted to obtain the same *Rs* values between OTC and TC on the columns, the shortest retention time of DC without reduction of peak resolution was obtained on the Ph column. Therefore, we selected Inertsil Ph as the column packing material.

The optimum feed rate of the effluent from LC column for frit FAB/MS ranges between 1 and 5 μL/min. Using an LC column with internal diameter of 4.6 mm, the optimum flow rate ranges from 0.5 to 2.0 mL/min. Therefore, the effluent from a column with internal diameter of 4.6 mm must be introduced after split at a ratio of more than 1:100. We cannot obtain enough sensitivity on frit FAB/MS for residual analysis using such a high split ratio. When we use a column with internal diameter of 2.1 mm, the optimum flow rate of mobile phase ranges between 0.1 and 0.4 mL/min. The desired split ratio is then 1:20 and provides adequate sensitivity for the analysis. Therefore, we used an LC column with an internal diameter of 2.1 mm and a length of 250 mm.

In the separation of TCs, it is well-known that use of a mixture of methanol and acetonitrile as an organic modifier in the mobile phase is more effective than the individual use of methanol or acetonitrile (Oka *et al.*, 1984). With the ratio of methanol and acetonitrile fixed at 1:1, various ratios of the organic solvent and 0.005 M aqueous TFA solution were tested as mobile phases. In this experiment, thioglycerol was added to mobile phases as a matrix for frit FAB/MS at a concentration of 1.0%, because thioglycerol is the most suitable matrix for FAB/MS of TCs (Harada *et al.*, 1993). As shown in

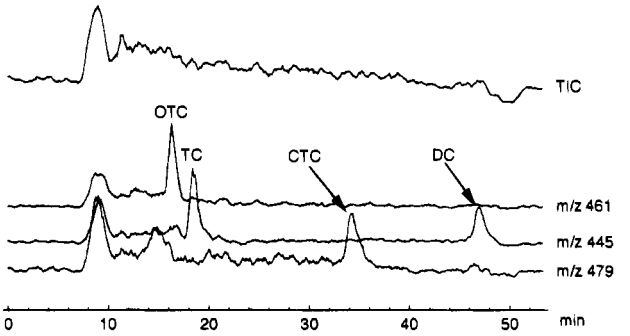

Figure 6. Total ion and mass chromatograms of TCs fortified at concentrations of 0.2 ppm. For LC/MS conditions, see Materials and Methods.

Figure 2, a poor separation between OTC and TC was obtained using methanol–acetonitrile–0.005 M TFA solution (2:2:7). The best separation was achieved when methanol–acetonitrile–0.005 M TFA solution (2:2:13) was used, but the retention time of DC was too long. Therefore, we recommend methanol–acetonitrile–0.005 M TFA solution (2:2:11) containing 1.0% thioglycerol as a mobile phase. Under the optimum conditions, a liquid chromatogram of TCs is shown in Figure 3.

Frit FAB/LC/MS of TCs. As described under Materials and Methods, the LC and the mass spectrometer were interfaced by a laboratory-made flow splitter (Ikai *et al.*, 1991). The effluent from the LC was split at the ratio of 1:20 by the splitter, and the smaller portion of the effluent was introduced into the mass spectrometer through the connection tubing at a flow rate of 5 μL/min. First, we injected 1 μg of each of the standard TCs into the LC/MS system. Although no peak corresponding to TCs appeared on the total ion chromatogram as shown in Figure 4, the peaks of all TCs were clearly observed on 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 Figure 5. The protonated molecules, $[M + H]^+$, and typical

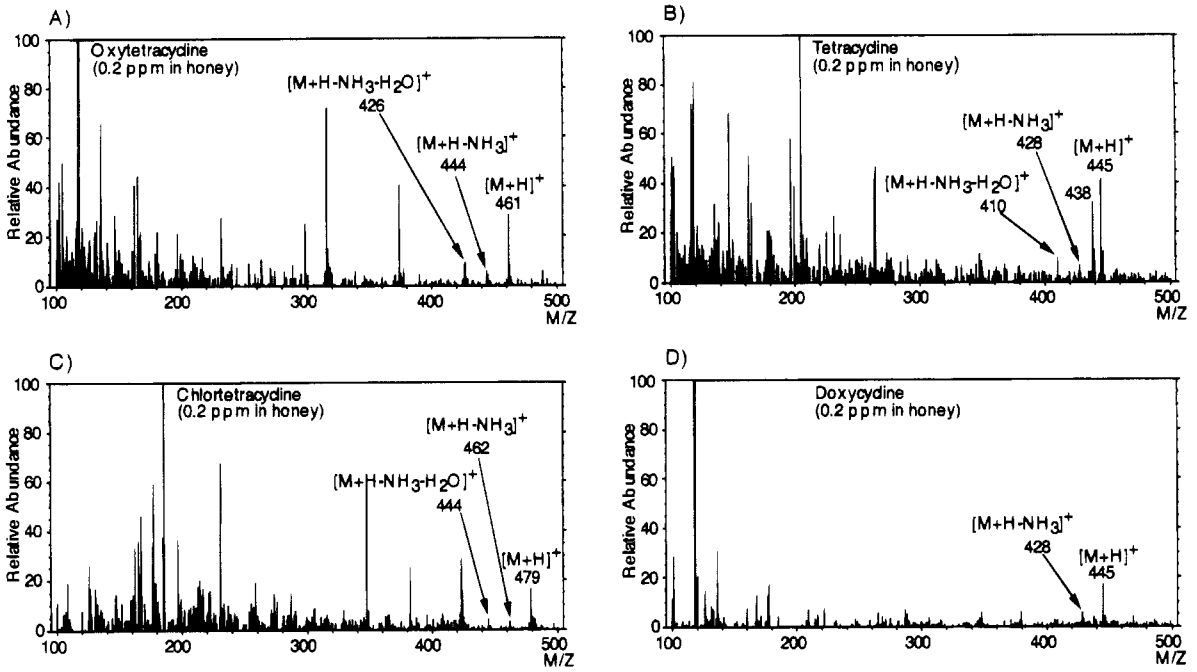

Figure 7. Background-subtracted mass spectra of TCs fortified at concentrations of 0.2 ppm: (A) oxytetracycline; (B) tetracycline; (C) chlortetracycline; (D) doxycycline. For LC/MS conditions: see Materials and Methods.

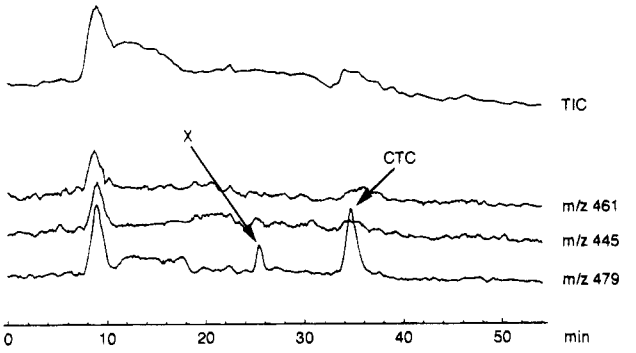

Figure 8. Total ion and mass chromatograms of residual CTC at concentration of 0.24 ppm in honey. For LC/MS conditions, see Materials and Methods.

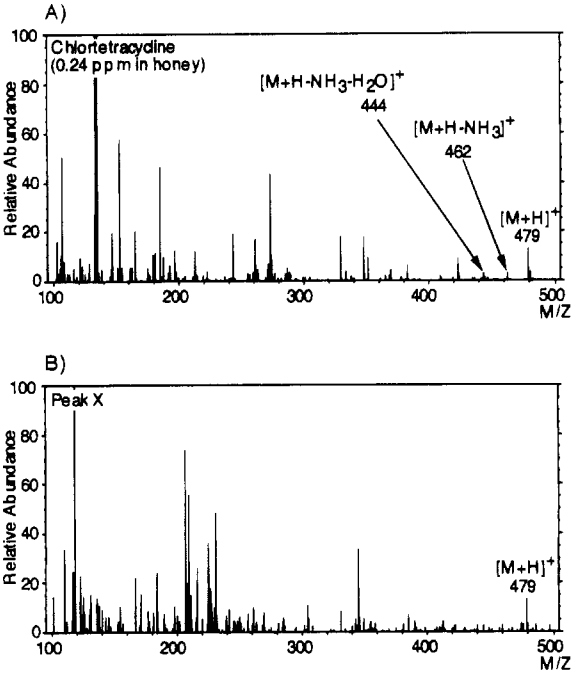

Figure 9. Background-subtracted mass spectra of residual CTC at concentration of 0.24 ppm in honey: (A) chlortetracycline; (B) compound **X**. For LC/MS conditions: see Materials and Methods.

fragment ions, $[M + H - NH_3]^+$ and $[M + H - NH_3 - H_2O]^+$, are clearly visible in the spectra of OTC, TC, and CTC; DC gives only $[M + H]^+$ and $[M + H - NH_3]^+$. These ions are very useful for confirmation of TCs as described in our previous paper (Harada *et al.*, 1993). The loss of ammonia occurs from the carboxyamide moiety in the A ring of TCs and the formation of $[M + H - NH_3 - H_2O]^+$ depends on the presence of a hydroxy group in the C ring. A combination of $[M + H]^+$ and $[M + H - NH_3]^+$ is available for confirmation of TCs. Additionally, the presence or absence of $[M + H - NH_3 - H_2O]^+$ can differentiate TC from DC.

Frit FAB/LC/MS of TCs in Honey. Honey samples were cleaned up for analysis using a C_{18} cartridge procedure that has previously been described (Oka *et al.*, 1983). The recoveries of the TCs from fortified honey at levels of 0.2 ppm are 86, 85, 90, and 92% for OTC, TC, CTC, and DC, respectively. Although the residue was dissolved in methanol or mobile phase after evaporation of the eluent from the cartridge in the previous study, it was dissolved in distilled water in the present study, because dissolving in methanol and mobile phase causes poor peak resolution in LC. After cleanup of TCs fortified at a concentration of 0.2 ppm in honey, frit FAB/LC/MS was performed under the optimal conditions described under Materials and Methods. As observed for the LC/MS of the standard TCs, 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 as shown in Figure 6. Figure 7 shows background-subtracted mass spectra of TCs recorded at the tops of each peak on the mass chromatograms. Although an ion originating from coextractive 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. 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 concentration can be reliably identified by the present method, and the detection limits of the protonated molecules are 0.1 ppm in honey.

The present method was applied to the identification of residual CTC at a concentration of 0.24 ppm in a honey sample collected at the market level, which was previously found positive by LC. Figure 8 shows the total ion and mass chromatograms of the sample monitored at the individual protonated molecules of TCs. CTC and an unknown peak labeled "X" appeared on the mass chromatogram monitored at m/z 479, although no peaks appear corresponding to OTC, TC, or CTC. Background-subtracted mass spectra of both peaks at the top of each peak on the mass chromatogram are shown in Figure 9. The $[M + H]^+$ ions are clearly observed in both spectra, and the spectrum of CTC also provided the fragment ions $[M + H - NH_3]^+$, and $[M + H - NH_3 - H_2O]^+$, confirming the identity of CTC and thereby demonstrating the effectiveness of the present method for the identification of TCs in honey. The molecular weight of compound **X** is 478 from the mass spectrum (Figure 9B). This compound is considered identical to 4-epichlortetracycline, which has been produced during storage of the honey, because CTC is known to reversibly epimerize to 4-epichlortetracycline in aqueous solution (Mitscher, 1978).

Conclusion. In the present study we have developed a frit FAB/LC/MS method for the identification of residual TCs in honey, with the following characteristics. Use of a well end-capped phenyl-bonded silica gel synthesized from 99.99% pure silica gel (Inertsil Ph column) enables us to separate TCs without reduction of peak resolution using a volatile mobile phase, methanol–acetonitrile–0.005 M TFA (2:2:11), which is applicable to direct interfaced LC/FAB/MS without clogging problems. The cleanup procedure using a C_{18} solid phase extraction cartridge was effective for the identification of residual TCs by LC/FAB/MS. TCs fortified at a concentration of 0.2 ppm in honey were reliably identified using the present method, and detection limits of protonated molecules were 0.1 ppm in honey. The method was successfully applied to the identification of CTC in a retail honey sample previously found positive at the concentration of 0.24 ppm by LC.

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