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# IMPROVEMENT OF CHEMICAL ANALYSIS OF ANTIBIOTICS

# IX*. A SIMPLE METHOD FOR RESIDUAL TETRACYCLINES ANALYSIS IN HONEY USING A TANDEM CARTRIDGE CLEAN-UP SYSTEM

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## SUMMARY

A simple, rapid and precise analytical method for the residual tetracyclines in honey has been established using a tandem cartridge clean-up system (prepacked reversed-phase and ion-exchange cartridges) followed by high-performance liquid chromatography. The recoveries of oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC) from honey spiked at a level of 1.0 ppm are 87.1, 85.3, 98.0 and 99.0%, respectively, with coefficients of variation of 1.1–3.9%. The detection limits in honey are 0.02 ppm for OTC and TC, and 0.05 ppm for CTC and DC, respectively. The time required for the analysis of four samples is only 1 h.

## INTRODUCTION

In order to prevent foul brood of honey-bee, tetracycline antibiotics (TCs) are widely used all over the world in honey-bee culture[1,2]. Oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC) and doxycycline (DC) are added in sugared water. Such usage may result in residual TCs in honey. Therefore, for public health purposes, it is necessary to investigate the presence and the amount of TCs remaining in honey.

Several microbiological methods are available to determine TCs in foods[3–7], 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[8]. Two high-performance liquid chromatographic

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(HPLC) methods for TCs in honey have been reported[9,10]. However, Jürgens' method[9] was achieved without any clean-up operation, so many peaks of interfering substances in honey appeared on the chromatogram. Takeba's method[10] did not have enough sensitivity for routine analysis. Accordingly, it was difficult to analyze residual TCs in honey with good accuracy using published methods.

In a previous paper[11] we reported an HPLC method for the analysis of TCs in animal liver using clean up with a Baker $C_{18}$ cartridge. Although this method was simple, rapid and reliable, the clean-up operation was not sufficient for the residual analysis of TCs in honey. On the basis of various experiments, we have found that a combination of clean-up systems on prepacked reversed-phase and ion-exchange cartridges is quite effective for the residual analysis of TCs in honey.

This paper describes an useful technique for the determination of TCs in honey using a tandem cartridge clean-up system (Baker 10 $C_{18}$ and Baker 10 COOH) followed by HPLC.

## EXPERIMENTAL

### *Materials*

Methanol, acetonitrile, ethyl acetate, chloroform, benzene, acetone, oxalic acid, citric acid, disodium hydrogen phosphate and disodium ethylenediaminetetraacetate ($Na_2EDTA$) were analytical grade materials. The hydrochlorides of OTC, TC, CTC and DC were supplied by Pfizer Taito (Tokyo, Japan).

Baker 10 $C_{18}$ (100 mg, Lot No. 433089; 200 mg, Lot No. 505178; 500 mg, Lot No. 433091), 10 $C_8$ (200 mg, Lot No. 331352; 500 mg, Lot No. 433085), 10 COOH (Lot No. 427154), 10 COHCOH (Lot No. 228094) and 10 $SO_3$ (Lot No. 544102) cartridges were provided by J. T. Baker Chemical (Phillipsburgh, NJ, U.S.A.). Sep-Pak $C_{18}$ (Lot No. P5116AI) was obtained from Waters Assoc. (Milford, MA, U.S.A.).

### *Preparation of standard tetracycline solutions*

Tetracycline antibiotics (each 100 mg) were weighed accurately into 10-ml volumetric flasks and dissolved to volume in methanol and water. Dilution was sometimes necessary.

### *Pretreatment of extraction cartridges with $Na_2EDTA$*

After activation of reversed-phase cartridges with methanol and water, the cartridges were conditioned with 10 ml of saturated aqueous $Na_2EDTA$.

### *Extraction and clean-up procedures*

A sample (5 g) was dissolved in 20 ml of 0.1 *M* $Na_2EDTA$–McIlvaine buffer (pH 4.0). After filtration of the sample solution, the filtrate was applied on a Baker 10 $C_{18}$ (100 mg) cartridge pretreated with 10 ml of saturated aqueous $Na_2EDTA$. The $C_{18}$ cartridge was washed with 20 ml of water and then air-dried under vacuum for 5 min. The $C_{18}$ cartridge containing the sample was fitted into an adapter on a Baker 10 COOH cartridge conditioned with ethyl acetate. TCs were transferred from the $C_{18}$ cartridge to the COOH cartridge with 50 ml of ethyl acetate under reduced pressure through the cartridge assembly. The $C_{18}$ cartridge and the adaptor were

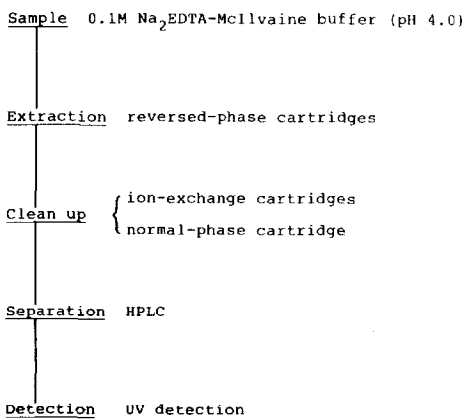
removed and the COOH cartridge was washed with 10 ml of methanol. The TCs were eluted with 10 ml of the HPLC mobile phase, methanol–acetonitrile–0.01 *M* aqueous oxalic acid (1:1.5:3), and collected in a 10-ml volumetric flask.

*High-performance liquid chromatography*

A high-performance liquid chromatograph equipped with a constant-flow pump (LC-5A; Shimadzu, Kyoto, Japan) was used with a variable-wavelength UV detector (Shimadzu SPD-2AM) operated at 350 nm. The separation was performed on Bakerbond $C_8$ (5 $\mu$m, 250 mm × 4.6 mm I.D.) with methanol–acetonitrile–0.01 *M* aqueous oxalic acid (1:1.5:3) as the mobile phase at a flow-rate of 1 ml/min at room temperature. For the determination of TCs each sample and the standard solutions (100 $\mu$l) were injected.

## RESULTS AND DISCUSSION

Although our previous method (Baker 10 $C_{18}$, HPLC) was successfully applied to the analysis of TCs in some kinds of foods including animal liver[11], it did not give satisfactory results for the residual analysis of TCs in honey. The chromatogram thus obtained showed many peaks arising from substances in honey which interfered with the precise analysis of TCs. In that analytical method only a $C_{18}$ cartridge was used for both extraction and clean-up operations, and the clean up was not always sufficient for the residual analysis of TCs in honey. So a more effective clean-up operation which can separate only TCs from impurities is essential. We investigated a tandem cartridge system for extraction and clean-up operations as shown in Scheme 1. In this procedure, the following conditions were taken into consideration: (1) a reversed-phase cartridge for extraction, and ion-exchange and normal-phase cartridges for clean up are used; (2) both cartridges are connected directly for easy operation; (3) an HPLC mobile phase is used to elute the clean-up cartridge to obtain good accuracy. Fulfilment of these conditions would lead to a simple, rapid and precise analytical method for TCs in honey. Therefore, the following conditions (ac-




Scheme 1. Strategy for establishment of analytical method for residual TCs in honey.

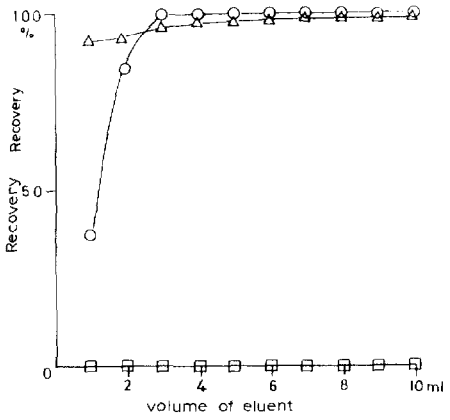



Fig. 1. Elution curves for TCs from clean-up cartridges using the mobile phase. ○ = Baker 10 COOH; △ = Baker 10 COHCOH; □ = Baker 10 $SO_3$.

cording to Scheme 1) must be optimized: (1) choice of clean-up cartridge; (2) solvents applied to the clean-up cartridge; (3) comparison of extraction cartridges; (4) elution from extraction cartridge; (5) application to residual analysis.

*Choice of clean-up cartridge*

Cation-exchange cartridges are expected to be effective as clean-up cartridges because TCs contain a dimethylamino group, and it is known that a normal-phase (diol) cartridge is effective for the analysis of CTC in ointments[12]. So Baker 10 COOH, 10 $SO_3$ and 10 COHCOH were examined as clean-up cartridges. First, the suitability of the mobile phase, methanol–acetonitrile–0.01 *M* aqueous oxalic acid (1:1.5:3), as the eluent for each clean-up cartridge was investigated. After retention of TCs (each 5 $\mu$g) on the clean-up cartridges using 1 ml of methanol, they were eluted with various volumes of the mobile phase and determined. The elution curves for TC from baker 10 COOH, COHCOH and $SO_3$ cartridges are shown in Fig. 1. All TCs were completely recovered from Baker 10 COOH and COHCOH cartridges with 10 ml of the mobile phase, but Baker 10 $SO_3$ was found not to be applicable (Table I). Therefore, Baker 10 COOH and COHCOH cartridges were subsequently used in the clean-up step.

TABLE I

RECOVERY OF TCs FROM CLEAN-UP CARTRIDGES USING 10 ml OF THE MOBILE PHASE

Results of three replicates.

| *Cartridge* | *Recovery (%)* | | | |
|---|---|---|---|---|
| | *OTC* | *TC* | *CTC* | *DC* |
| Baker 10 COOH | 100 | 100 | 100 | 100 |
| Baker 10 COHCOH | 100 | 97 | 90 | 83 |
| Baker 10 $SO_3$ | 0 | 0 | 0 | 0 |

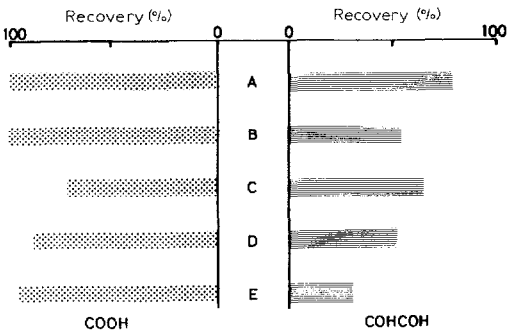



Fig. 2. Comparison of solvents applied to clean-up cartridges, showing recoveries from 50 ml of solvent containing 5 μg of TC. Results of three replicates. Solvents: A = methanol; B = ethyl acetate; C = benzene; D = chloroform; E = acetone.

*Solvent applied to clean-up cartridge*

In order to investigate whether TCs in a large amount of organic solution are effectively recovered with the clean-up cartridges (Baker 10 COOH and 10 COHCOH), TCs (each 5 μg) were applied to these cartridges with 50 ml of various organic solvents (ethyl acetate, methanol, chloroform, acetone and benzene) and then eluted with 10 ml of the mobile phase and determined. As a typical example, recoveries of TC are given in Fig. 2, which indicates that only the Baker 10 COOH cartridge gave satisfactory results when ethyl acetate, methanol and acetone were applied. The behaviour of other TCs in the clean-up cartridges was the same as in the case of TC. Therefore, we chose Baker 10 COOH as a clean-up cartridge and ethyl acetate, methanol and acetone as the solvents.

*Comparison of extraction cartridges*

In previous reports[11,13] we pointed out that the suitability of $C_{18}$ cartridges for the analysis of residual TCs depended markedly on the particle size, weight and supplier, and we were able to obtain satisfactory results by selecting a $C_{18}$ cartridge according to the analytical purpose. The suitability of six reversed-phase cartridges (Baker 10 $C_{18}$, 100 mg; 10 $C_{18}$, 200 mg; 10 $C_{18}$, 500 mg; 10 $C_8$, 200 mg; 10 $C_8$, 500 mg and Sep-Pak $C_{18}$) as extraction cartridges were examined. Using 20 ml of 0.1 *M* $Na_2EDTA$–McIlvaine buffer (pH 4.0), TCs (each 5 μg) were applied to extraction cartridges pretreated with saturated aqueous $Na_2EDTA$, then eluted with 10 ml of methanol and determined. Table II shows that Baker 10 $C_{18}$ (100 mg) and Sep-Pak $C_{18}$ are the most suitable for extraction. However, as the connection of Baker 10 $C_{18}$ to Baker 10 COOH is easier than in the case of Sep-Pak $C_{18}$, we selected Baker 10 $C_{18}$ (100 mg) for the extraction step.

*Elution of TCs from the extraction cartridge*

In our strategy (Scheme 1) the eluent from the extraction cartridge and the solvent applied to the clean-up cartridge must be same in order to obtain good accuracy in HPLC. The suitability of methanol, ethyl acetate and acetone was investigated as eluent from the extraction cartridge. After retention of TCs (each 5 μg) on Baker 10 $C_{18}$ (100 mg), they were transferred to Baker 10 COOH using various

TABLE II

COMPARISON OF EXTRACTION CARTRIDGES

Recovery of TCs from various reversed-phase cartridges pretreated with 10 ml of aqueous saturated $Na_2EDTA$ and eluted with 10 ml of methanol. Results of three replicates.

| *Cartridge* | *Recovery (%)* | | | |
|---|---|---|---|---|
| | *OTC* | *TC* | *CTC* | *DC* |
| Baker 10 $C_{18}$ (100 mg) | 100 | 100 | 100 | 100 |
| Baker 10 $C_{18}$ (200 mg) | 97 | 95 | 100 | 100 |
| Baker 10 $C_{18}$ (500 mg) | 84 | 71 | 78 | 75 |
| Baker 10 $C_8$ (200 mg) | 17 | 11 | 17 | 23 |
| Baker 10 $C_8$ (500 mg) | 99 | 11 | 18 | 46 |
| Sep-Pak $C_{18}$ (400 mg) | 100 | 100 | 100 | 100 |

volumes of the three solvents, eluted with the HPLC mobile phase and then determined. Elution curves for TC are shown in Fig. 3 as a typical example and good recoveries of TC were obtained by using more than 10, 50 and 50 ml of methanol, ethyl acetate and acetone, respectively. Table III shows that all TCs were recovered quantitatively from Baker 10 $C_{18}$ (100 mg) when an optimum volume of each eluent was used. Therefore, we used 10 ml of methanol, 50 ml of ethyl acetate and acetone, respectively, as eluents for Baker 10 $C_{18}$ (100 mg).

*Application to residual analysis*

The application of the extraction (Baker 10 $C_{18}$, 100 mg) and the clean-up (Baker 10 COOH) cartridges to the analysis of residual TCs in honey was investigated on the basis of the preliminary studies mentioned above. In order to examine the effect of the clean-up cartridge, chromatograms of honey (spiked at a level of 1.0

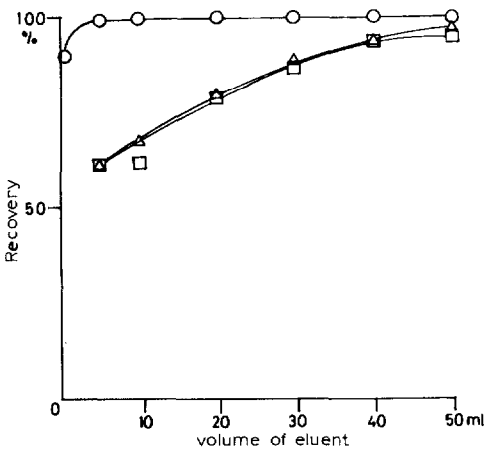



Fig. 3. Elution curves for TC from the extraction cartridge. Eluents: ○ = methanol; △ = acetone; □ = ethyl acetate.

TABLE III

RECOVERY OF TCs FROM BAKER 10 $C_{18}$ (100 mg) USING 10 ml, 50 ml AND 50 ml METHANOL, ETHYL ACETATE AND ACETONE, RESPECTIVELY

Results of three replicates.

| *Solvent* | *Recovery (%)* | | | |
|---|---|---|---|---|
| | *OTC* | *TC* | *CTC* | *DC* |
| Acetone | 97 | 96 | 96 | 99 |
| Ethyl acetate | 93 | 95 | 97 | 96 |
| Methanol | 100 | 97 | 100 | 100 |

ppm) extracts before and after clean up were compared. Fig. 4 shows that the peaks of interfering substances in honey were eliminated by the clean-up operation and, furthermore, all TCs were subsequently recovered quantitatively. Therefore, we concluded that the Baker 10 COOH cartridge was effective for the residual analysis of TCs in honey.

Because substances adsorbed on the extraction cartridge (Baker 10 $C_{18}$, 100 mg) are not only TCs but also impurities in honey, an eluent should be used that retains impurities as much as possible and easily elutes TCs from the cartridge. As described above, ethyl acetate, methanol and acetone are suitable eluents for TCs from the $C_{18}$ cartridge. In order to clarify whether the eluents fulfil the above requirements, honey extracts were transferred from the sample-containing extraction cartridges to the clean-up cartridges using 10, 50 and 50 ml of methanol, ethyl acetate

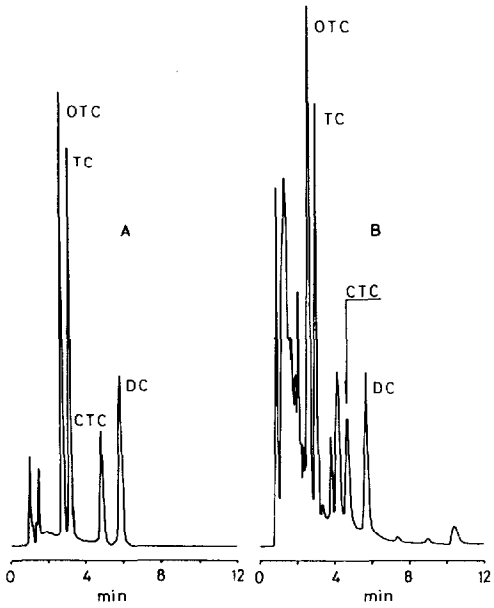



Fig. 4. Comparison of chromatograms before (B) and after (A) clean up. For chromatographic details see Experimental.

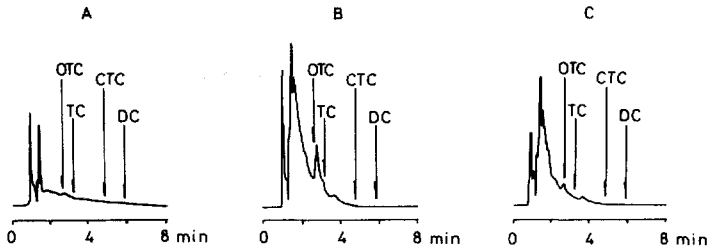



Fig. 5. Comparison of chromatograms of honey extracts using ethyl acetate (A), methanol (B) and acetone (C) as eluents for the extraction cartridge. For chromatographic details see Experimental.

and acetone, respectively, then eluted and injected for HPLC as described in Experimental. The chromatograms obtained are shown in Fig. 5 and the usage of ethyl acetate to elute the extraction cartridge gave less solvent tailing and no peaks interfering with the determination of OTC in comparison to acetone and methanol. Therefore, we chose ethyl acetate as the eluent for the extraction cartridge (Baker 10 $C_{18}$, 100 mg).

Table IV shows the overall recoveries of OTC, TC, CTC and DC from honey

TABLE IV

RECOVERY OF TCs FROM HONEY

A 5 g amount of honey was spiked at a level of 1.0 ppm. Results of six replicates.

| | *OTC* | *TC* | *CTC* | *DC* |
|---|---|---|---|---|
| Recovery (%) | 87.1 | 85.3 | 98.0 | 99.0 |
| C.V. (%) | 2.1 | 3.9 | 1.2 | 1.1 |
| Detection limit (ppm) | 0.02 | 0.02 | 0.05 | 0.05 |

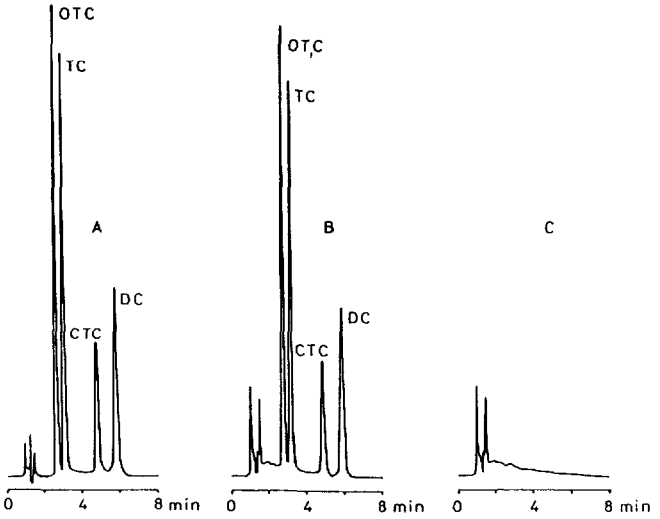



Fig. 6. Typical chromatograms of honey extracts. (A) Standard TCs (25 ng); (B) honey spiked at 1 ppm; (C) blank of honey. For chromatographic details see Experimental.

spiked at a level of 1.0 ppm, their coefficients of variation (C.V.) and the detection limits in honey. Typical chromatograms of honey extracts are shown in Fig. 6. The present method enables the analysis of TCs in honey with good recoveries (85.3–99.0%), higher accuracy (C.V. 1.1–3.9%) and better detection limits (0.02–0.05 ppm) in comparison with the reported methods[9,10]. Furthermore, it is very simple and rapid, only 1 h being required for four samples. Therefore, we recommend the tandem cartridge clean-up system (Baker 10 $C_{18}$ and Baker 10 COOH) followed by HPLC for the residual analysis of TCs in honey.

We are now analysing residual TCs in honey available on the Japanese market using our present method and the results will be reported elsewhere.

## CONCLUSIONS

A simple, rapid and precise analytical method for the residual TCs in honey has been established as shown in Scheme 2. The tandem cartridge clean-up system is effective for the extraction and the clean up of TCs in honey. The recoveries of OTC, TC, CTC and DC from honey spiked at a level of 1.0 ppm are 87.1, 85.3, 98.0 and 99.0%, respectively, with coefficients of variation of 1.1–3.9%. The detection limits are 0.02 and 0.05 ppm for OTC and TC, and for CTC and DC, respectively.

Sample (Honey, 5g)
↓
blend with 0.1M $Na_2EDTA$-McIlvaine buffer (pH 4.0, 20ml)
↓
Baker 10 $C_{18}$
↓
pretreatment with sat. $Na_2EDTA$ (aq., 10ml)
↓
wash with $H_2O$ (20ml)
↓
air-dry under vacuum for 5min.
↓
elution with $CH_3COOC_2H_5$ (50ml)
↓
Eluate
↓
Baker 10 COOH
↓
wash with $CH_3OH$ (10ml)
↓
elution with $CH_3OH$:$CH_3CN$:0.01M $(COOH)_2$ (aq.) = 1 : 1.5 : 3 (10ml)
↓
Eluate
↓
HPLC

Scheme 2. Established analytical procedure for residual TCs in honey.

Finally, we consider that the present tandem cartridge system is also effective for the analysis of other antibiotics which have ionic character, because the system contains an ion-exchange cartridge. Multi-residue analysis of antibiotics in foods using the present tandem cartridge system will be reported elsewhere.

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