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# Determination of tetracycline antibiotics in animal tissues of food-producing animals by high-performance liquid chromatography using solid-phase extraction ☆

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**Abstract**

A high-performance liquid chromatographic (HPLC) method was developed for the determination of oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) residues in bovine and porcine muscles. The method involved the homogenization of the sample in EDTA–McIlvaine buffer with added *n*-hexane and dichloromethane, centrifugation, precipitation of the supernatant using trichloroacetic acid and filtration. Preconcentration on Separcol SI $C_{18}$ cartridges improved the clean-up and the recovery of tetracyclines that were separated by HPLC using the optimized mobile phase of 0.01 *M* oxalic acid–acetonitrile–methanol (45:35:20) on a Spherisorb ODS 2 column (250 × 4 mm I.D.). UV detection at 360 nm was applied with a detection limit of about 50 ng/g. The diode-array spectra confirmed the applicability of this method to the study of tetracycline residues in carcasses.

## 1. Introduction

Tetracyclines are a group of broad-spectrum antibiotics that are widely used in veterinary practice (medical therapy, meat production industry). Reliable and reproducible methods are required for their monitoring. Structures of the most important tetracyclines are given in Fig. 1.

Many methods have been described for the determination of tetracyclines in various biological matrices [1–10]. However, the sensitivity and specificity of microbiological methods are poor, and chemical methods based on high-performance liquid chromatography (HPLC) are therefore widely used. Common to HPLC procedures is the need for sample pretreatment. Pretreatment procedures based on protein precipitation [1], liquid–liquid extraction [2–5] and solid-phase extraction (SPE) [6–12] have been described. The use of SPE columns in the clean-up of tissue samples for the determination of tetracycline residues appears to be efficient and time saving. In most reports, samples were spiked at levels of 1 μg/g and more.

This paper describes a rapid and sensitive method for the determination of oxytetracycline

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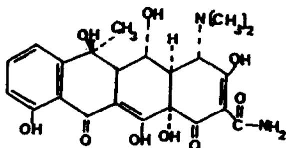
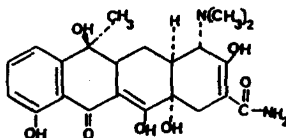
OXYTETRACYCLINE -------- $C_{22}H_{24}N_2O_9$



TETRACYCLINE ----------- $C_{22}H_{24}N_2O_8$



CHLORTETRACYCLINE ---- $C_{22}H_{23}ClN_2O_8$

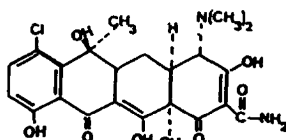


Fig. 1. Structures of oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC).

(OTC), tetracycline (TC) and chlortetracycline (CTC) in muscle tissue utilizing an off-line sample pretreatment combined with HPLC. The aim of this work was the optimization of the isolation procedure and preconcentration by SPE, improvement of the HPLC separation of tetracyclines and the application of a diode-array detector for the confirmation of tetracycline residues in animal tissues.

## 2. Experimental

### 2.1. Chemicals and solutions

Citric acid monohydrate, oxalic acid dihydrate and ethylenediaminetetraacetic acid, disodium salt dihydrate (EDTA) were supplied by Lachema (Brno, Czech Republic), perchloric acid by Ubichem (Middlesex, UK), dichloromethane by Fluka (Buchs, Switzerland), *n*-hexane by Loba (Fischamend, Austria) and disodium hydrogenphosphate dodecahydrate, HPLC-grade methanol and HPLC-grade acetonitrile by Merck (Darmstadt, Germany).

EDTA-McIlvaine buffer solution (pH 4) was prepared by dissolving 15 g of disodium hydrogenphosphate dihydrate, 13 g of citric acid monohydrate and 3.72 g of EDTA in water and diluting to 1 l. A solution of trichloroacetic acid in water (1 g/ml) was prepared.

### 2.2. Antibiotics

Oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) (Sigma, St. Louis, MO, USA) were each dissolved in methanol (1 mg/ml). A mixed working solution was prepared by diluting 1 μg/ml of OTC and TC and 2 μg/ml of CTC in the mobile phase.

### 2.3. Apparatus

The following equipment was used: LHM 20 M homogenizer (ČSAV, Brno, Czech Republic), Janetzki K 70 centrifuge (MLU, Leipzig, Germany) and Separcol SI $C_{18}$ SPE columns (500 mg) (Anapron, Bratislava, Slovak Republic).

Chromatography was performed with a Hewlett-Packard (HP) (Avondale, PA, USA) Series 1050 apparatus with a quaternary pump, autosampler, variable-wavelength detector, diode-array detector and HP 3396II integrator.

### 2.4. Method

A 5-g amount of muscle was homogenized with 20 ml of EDTA-McIlvaine buffer (4°C) and 3 ml of *n*-hexane-dichloromethane (1:3, v/v) using a homogenizer. The homogenate was centrifuged at 2400 *g* for 30 min at 4°C and the supernatant was collected. Homogenization was repeated with 10 ml of EDTA-McIlvaine buffer and centrifuged. The supernatant was collected and combined with the first sample. A volume of trichloracetic acid solution equal to 10% of the supernatant volume was slowly added to the supernatant with constant stirring. The mixture was stirred for a further 1 min, placed on a bed

of ice for 15 min and then further filtered through a paper filter into a cylinder.

The SPE cartridge was activated with 2 ml of methanol and 4 ml of EDTA–McIlvaine buffer. The outlet of the SPE cartridge was connected to a water pump and the sample was aspirated through at no more than 10 ml/min. The cartridge was flushed with 2 ml of water and disconnected. Antibiotics were eluted from the cartridge with 4 ml of 0.01 *M* methanolic oxalic acid. A 10-$\mu$l volume was injected into the liquid chromatograph.

### *2.5. High-performance liquid chromatography*

The mobile phase was methanol–acetonitrile–0.01 *M* aqueous oxalic acid (20:35:45) in an isocratic system. The column used was HP Spherisorb ODS 2 (5 $\mu$m) (250 × 4 mm I.D.) (Hewlett-Packard) with a LiChrospher 100 RP-18 (5 $\mu$m) guard column (Merck). The flow-rate was 1 ml/min and the volume injected 10 $\mu$l with detection at 360 nm.

## 3. Results and discussion

For the recovery study, porcine and bovine muscles not containing tetracycline residues were used. A blank and six replicates spiked at 1 and 0.5 $\mu$g/g were processed according to the procedure given under Experimental. As shown in Tables 1 and 2, the recoveries were comparable and there were no significant differences between spiked porcine and bovine samples. The lower recovery of CTC is known and the problem has not yet been solved. In bovine samples spiked at 0.1 $\mu$g/g, the relative standard deviations were significantly higher, being 16% for OTC, 22.4% for TC and 27.2% for CTC. The detection limit of tetracyclines in tissues was about 50 ng/g.

Table 1

Recovery of spiked oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) from porcine muscle tissue ($n$ = 6)

| Parameter | OTC | TC | CTC |
|---|---|---|---|
| Spiked level ($\mu$g/g) | 1 | 1 | 2 |
| Average recovery (%) | 86.6 | 70.0 | 63.0 |
| R.S.D. (%) | 5.4 | 12.4 | 15.2 |
| Spiked level ($\mu$g/g) | 0.5 | 0.5 | 0.5 |
| Average recovery (%) | 91.3 | 86.0 | 59.8 |
| R.S.D. (%) | 3.3 | 6.5 | 8.8 |

R.S.D. = Relative standard deviation; $n$ = number of measurements.

Table 2

Recovery of spiked oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC) from bovine muscle tissue ($n$ = 6)

| Parameter | OTC | TC | CTC |
|---|---|---|---|
| Spiked level ($\mu$g/g) | 1 | 1 | 2 |
| Average recovery (%) | 93.3 | 68.5 | 61.2 |
| R.S.D. (%) | 6.3 | 9.2 | 14.6 |
| Spiked level ($\mu$g/g) | 0.5 | 0.5 | 0.5 |
| Average recovery (%) | 93.7 | 79.8 | 72.4 |
| R.S.D. (%) | 6.8 | 5.3 | 7.4 |
| Spiked level ($\mu$g/g) | 0.1 | 0.1 | 0.1 |
| Average recovery (%) | 86.2 | 65.0 | 54.7 |
| R.S.D. (%) | 16.0 | 22.4 | 27.2 |

R.S.D. = Relative standard deviation; $n$ = number of measurements.

The chromatograms of standards, a blank, a representative spiked porcine and bovine samples are shown in Fig. 2. Fig. 3 shows the OTC, TC and CTC spectra of the standards and, spiked porcine and bovine samples.

To obtain a real sample containing OTC the following procedure was used. A calf was treated with OTC intramuscularly with a daily dosage of 6 ml (100 mg/ml) for 5 days and then slaughtered. Samples were collected and tested using the same procedure as for spiked samples. The chromatogram of OTC from a therapeutic sample is shown in Fig. 4. Spectra of OTC from a therapeutic sample and a spiked bovine sample are shown in Fig. 5.

It is difficult to extract tetracycline antibiotics from muscle tissues because they have a tendency to combine with proteins and to form chelate complexes with metal ions [13]. From muscle tissues the best recoveries were obtained by extraction in EDTA–McIlvaine buffer (pH 4)

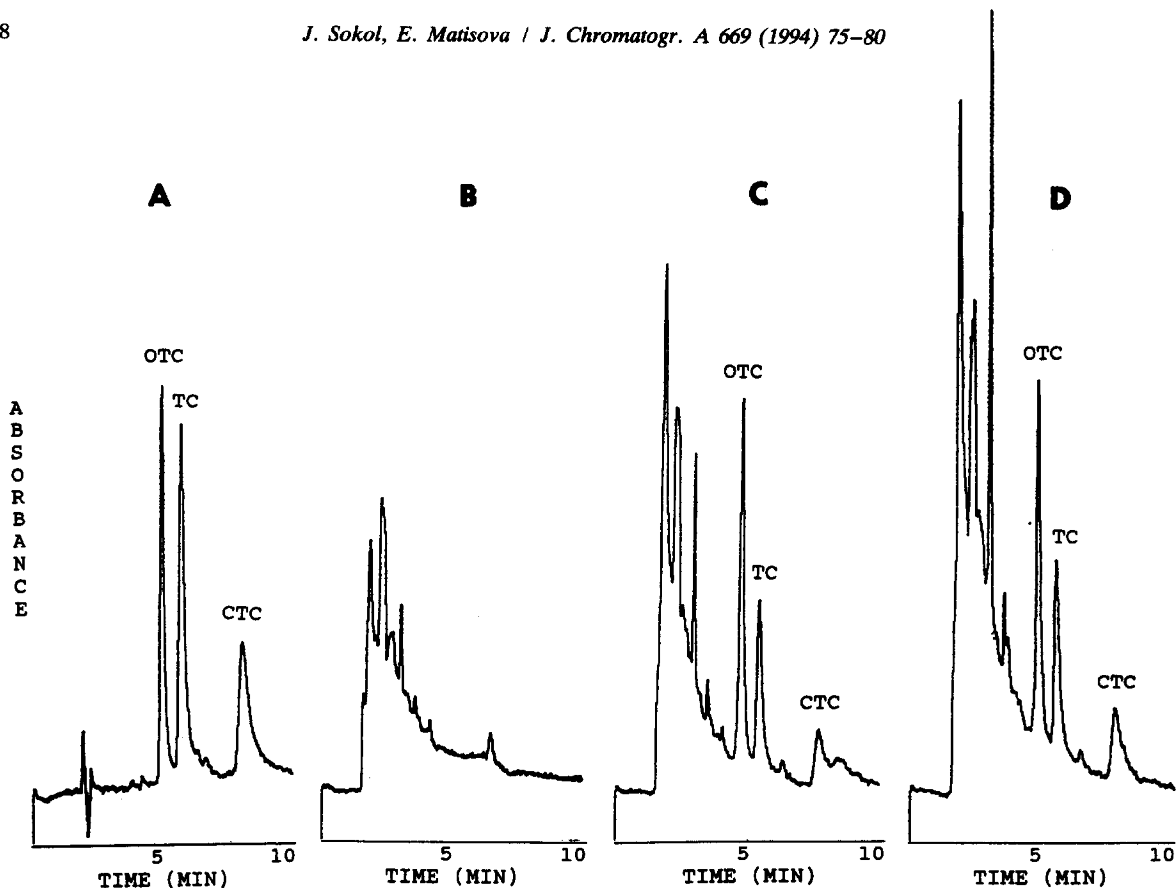



Fig. 2. (A) Chromatogram of three tetracycline standards: 10 ng of oxytetracycline (OTC) and tetracycline (TC) and 20 ng of chlortetracycline (CTC). (B) Chromatogram of blank muscle sample (10 $\mu$l). (C) Chromatogram of porcine muscle spiked with the three tetracyclines at 0.5 $\mu$g/g (10 $\mu$l). (D) Chromatogram of bovine muscle spiked with the three tetracyclines at 0.5 $\mu$g/g (10 $\mu$l). HPLC conditions: column, Spherisorb ODS 2 (250 × 4 mm I.D.); mobile phase, methanol–acetonitrile–0.01 *M* oxalic acid (20:35:45); flow-rate, 1 ml/min; detection at 360 nm.

[9,12], 0.01 *M* phosphate buffer [10,11] and 1 *M* hydrochloric acid [1]. We tested the efficiency and capacity of SPE cartridges (Separcol SI $C_{18}$). Larger volumes of samples were applied after removing the proteins with trichloroacetic acid. We did not find any significant differences between the elution properties of methanol [11] and 0.01 *M* methanolic oxalic acid [12]. Elution of the analytes from Separcol cartridges in small volumes was critical to the production of reasonable peak areas at a low concentration of tetracyclines. In this connection, high relative standard deviations of the recoveries at low spike concentrations (0.1 $\mu$g/g) indicate lower reproducibility.

Reversed-phase HPLC has often been used for determining tetracycline antibiotics in tissues. In this work a Spherisorb ODS 2 reversed-phase column was used. When using a methanol–acetonitrile–0.01 *M* oxalic acid mobile phase [6,14], the chromatograms were free from interfering peaks. We optimized the composition of the mobile phase in order to obtain sharp peaks, particularly for chlortetracycline, and to eliminate peak tailing. CTC peak tailing has been a real problem and using the present optimized mobile phase composition the peak shape of CTC was improved considerably compared with published data [2,4,8,9]. The contribution of the buffer and biological matrix to the spectrum is large (Figs. 3 and 5) and is not constant throughout the chromatogram. The retention times of

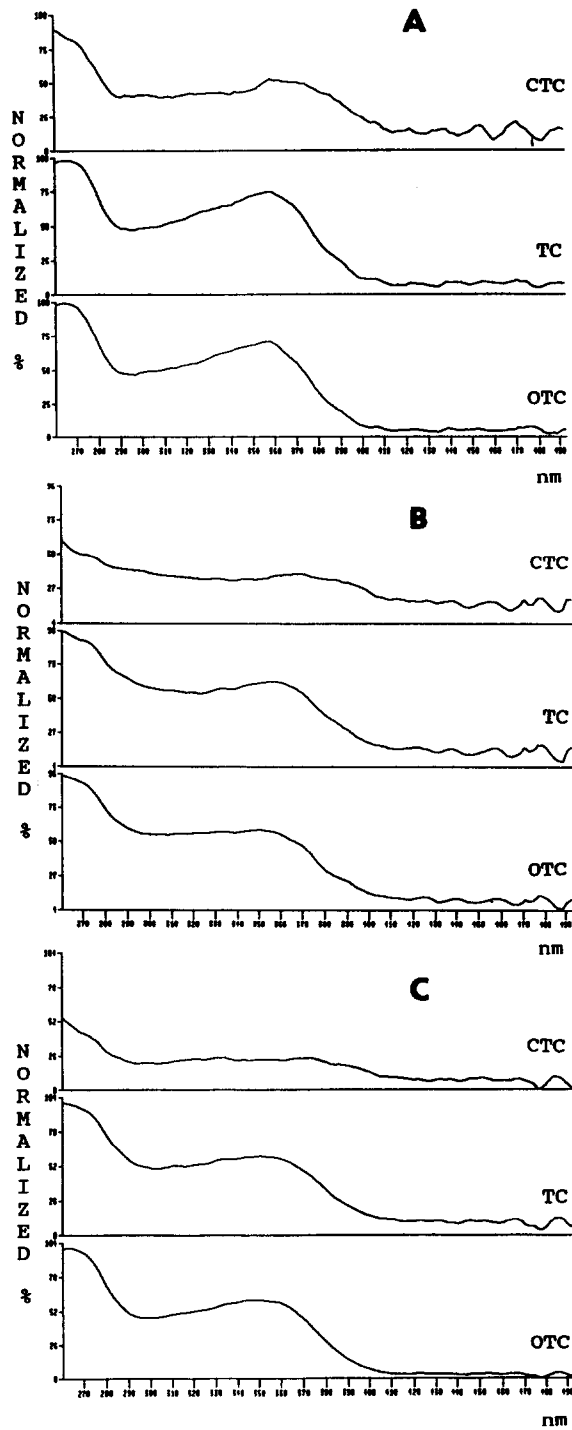



Fig. 3. Diode-array spectra of (A) standards of oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC), (B) spiked porcine muscle and (C) spiked bovine muscle.

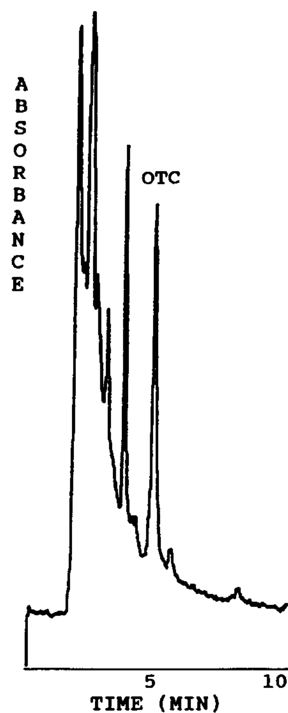



Fig. 4. Chromatogram of incurred oxytetracycline (OTC) residue sample. For experimental conditions, see Fig. 2.

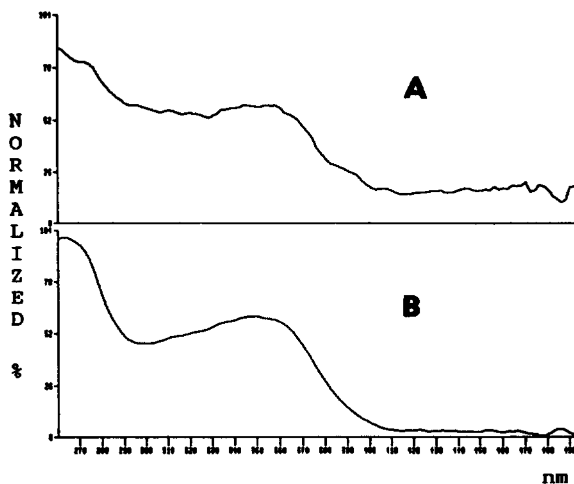



Fig. 5. Diode-array spectra of (A) incurred and (B) spiked oxytetracycline (OTC) residue from bovine muscle.

the analytes are still the primary means of identification and the spectrum is a source of confirmation.

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