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Determination of Tetracycline Residues in Animal Tissues by Liquid Chromatography

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A simple liquid chromatographic (LC) method was developed for the determination of tetracyclines (oxytetracycline, tetracycline and chlortetracycline) in animal tissues. Isolation of tetracyclines from biological matrices was performed with oxalic buffer followed by dechelation and deproteinization with oxalic acid – acetonitrile solution. For clean-up solid phase extraction with a SDBI (styrene-divinylbenzene) cartridge was used. LC analysis was performed on a polymeric analytical column (PLRP-S 5μm, 150 × 4.6 mm) and using an oxalic acid mobile phase (0.01 M oxalic acid – acetonitrile 75:25, v/v). The whole procedure was validated for intra- and inter-assay reproducibility determination by assaying muscle, liver and kidney samples supplemented with tetracyclines at the level of 50, 100 and 200 ng/g, respectively. The statistical evaluation demonstrates high absolute recovery (> 80%) and low coefficient of variation (< 10%) for all analysed samples. The detection limits for tetracyclines were 10–15 ng/g in muscle, and 20–25 ng/g in liver and kidney samples, depending on the analyte. © **1998 John Wiley & Sons, Ltd.**

INTRODUCTION

Like other antibiotics, tetracyclines have been used extensively to cure and prevent animal infections for many years. The most important members of the class are oxytetracycline (OTC), tetracycline (TC) and chlortetracycline (CTC). Pharmacokinetic and metabolic data indicate that the tetracyclines are concentrated in the liver and are excreted primarily through the kidney. Residues may therefore occur in food products of animal origin. The target organ for analysis is kidney; however, residues may also be determined in liver and muscles.

Liquid chromatography is most often employed for the purposes of determining residues of tetracyclines in food products. The tetracyclines have been analysed using separation on reversed-phase derivatized silica or polymer solid supports. The isolation of tetracyclines from various tissues have followed the homogenization of sample with extracting solvent. The tetracyclines are a class of antibiotics that form chelated complexes with metal ions and the method must consider the inclusion of a competing chelating agent to enhance the extraction efficiency. Additionally, another difficulty is associated with the propensity of the tetracyclines to bind with the sample matrix proteins. Consideration must also be given to providing the appropriate conditions to minimize protein binding by using a denaturing solvent. Sample clean-up is typically performed by solid-phase extraction (spe) (Oka *et al.*, 1984; Ashworth, 1985; Moats, 1986; Ikai *et al.*, 1987; Bjorklund, 1988; Rogstad *et al.*, 1988; Reimer and Young, 1990; Walsh *et al.*, 1992; White *et al.*, 1993; Horii, 1994; Moretti *et al.*, 1994).

The purpose of this study was to develop a practical, accurate and precise method for rapid extraction and quantitation of tetracycline residues in food-producing animal tissues. A simplified sample extraction and purification of sample extracts employing a styrene-divinylbenzene spe column was used for the simultaneous analysis of tetracycline residues by liquid chromatography.

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EXPERIMENTAL

Materials. Oxytetracycline, tetracycline and chlortetracycline were obtained from Sigma Chemical Co. (St Louis, MO, USA). Acetonitrile (J. T. Baker, Phillipsburg, NJ, USA) was HPLC grade. Water was freshly distilled, filtered through a 0.45 mm membrane and degassed under vacuum. Oxalic acid and sodium oxalate were from Merck (Darmstadt, Germany). Bakerbond SPE SDB1 3 mL column was from J. T. Baker.

Preparation of tetracycline standard solutions. Stock solutions of 1 mg/mL were prepared in methanol – water (1:1, v/v). The working solutions for LC were mixtures, prepared by dilution of 1 mL of each stock-solution to serial 10-fold dilutions in mobile phases of 100, 10 and 1 μg/mL. All solutions were stored in the dark at 4°C.

Chromatographic Conditions. All analyses were performed with a Hewlett Packard 1050 liquid chromatograph (Palo Alto, CA, USA) equipped with a UV detector; the UV detector was set at $\lambda = 360$ nm. The chromatographic analyses were

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performed on PLRP-S 5 μm, 150 × 4.6 mm column (Polymer Laboratory) with a mobile phase: 0.01 M oxalic acid – acetonitrile (75:25, v/v). Separations were performed using an isocratic mode at the flow rate of 0.9 mL/min. Aliquots of 20 μL were injected onto the column. LC control, data acquisition and peak integration were performed using Hewlett-Packard HPLC ChemStation Software (DOS series).

Sample preparation. To 5 g of ground muscle tissue sample and 2 g of minced liver or kidney, 1 mL of 1 M oxalic acid and 5 mL of acetonitrile were added. The samples were stirred with a glass rod and then homogenized with 25 mL of oxalic buffer (0.01 M oxalic acid – 0.01 M sodium oxalate, 50:50, v/v). After homogenization the samples were centrifuged at 3500 rpm for 10 min (temperature 4°C). The supernatant was filtered and diluted with 70 mL of oxalic buffer.

Clean-up. The solid phase extraction was performed on a Baker-12G vacuum manifold with SDB1 column (styrene-divinylbenzene, Bakerbond, 3 mL, 200 mg). The column was conditioned with 3 mL of methanol, 3 mL of water and 3 mL of oxalic buffer – acetonitrile (95:5, v/v). The whole diluted extract was applied onto the spe SDB-1 column. After application of analysed solution, the cartridge was washed with 10 mL of oxalic buffer, 10 mL of water and dried for 3 min under vacuum. Then, *n*-hexane (5 mL) was added and the cartridge was dried for 5 min. The elution was performed with 3 mL of mobile phase: 0.01 M oxalic acid – acetonitrile (75:25, v/v), and the extract was concentrated to slightly less then 1 mL under nitrogen in 40°C. The residue was diluted with acetonitrile to 1 mL for LC analyse.

Quantitation of tetracyclines. Samples of bovine and porcine muscles, liver and kidney were spiked with tetracyclines at level 50, 100 and 200 ng/g, respectively. All spiked samples were processed according to the procedure described above. The external standard method (single-point calibration) was used for quantitation. The recovery of tetracyclines was evaluated by comparing the concentrations found in samples spiked with known amounts of the analytes to the concentrations in the standard solution. The precision of the assay was measured using the same samples.

RESULTS

Chromatography

Typical chromatograms of extracts obtained from standard solution, blank tissues and tetracyclines-fortified bovine tissue samples are shown in Figs 1–5. The chromatographic peaks of tetracyclines were well resolved, and the retention times for OTC, TC, and CTC were 3.3, 3.9 and 6.8 min, respectively. Figures 2 and 3 show representative chromatograms of process blanks (liver and kidney). The chromatograms of spiked samples (Figs 4 and 5) indicate that no endogenous compounds exist at the retention times of the three tetracyclines. The same results were found for porcine samples, the tetracycline peaks were separated from endogenous peaks.

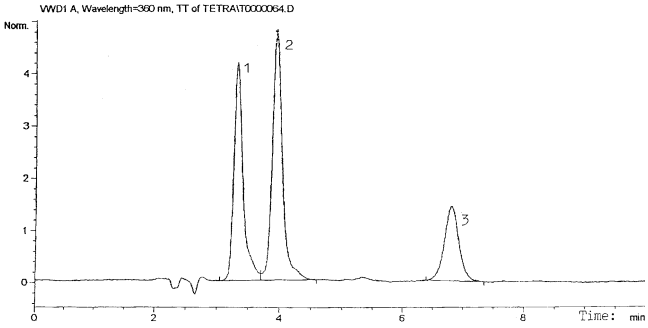

Figure 1. Separation of a standard mixture of tetracyclines (peaks: 1, oxytetracycline; 2, tetracycline; 3, chlortetracycline).

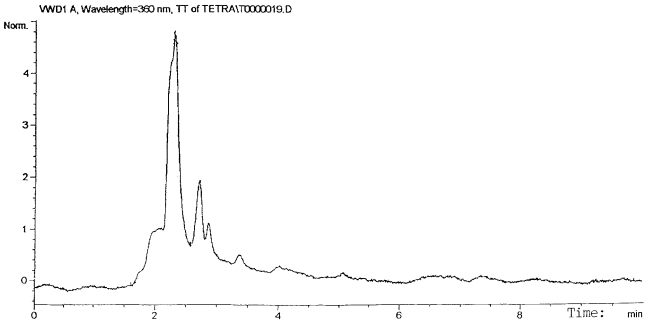

Figure 2. Separation of tissue extract from control bovine liver.

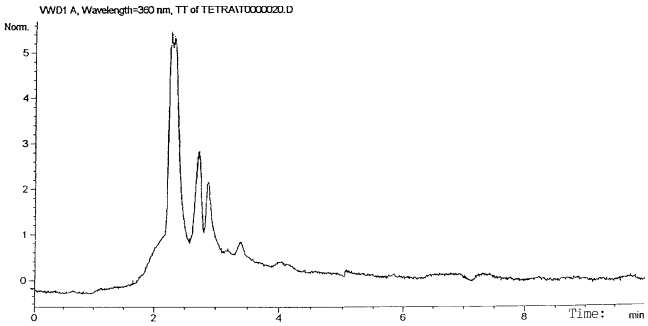

Figure 3. Separation of tissue extract from control bovine kidney.

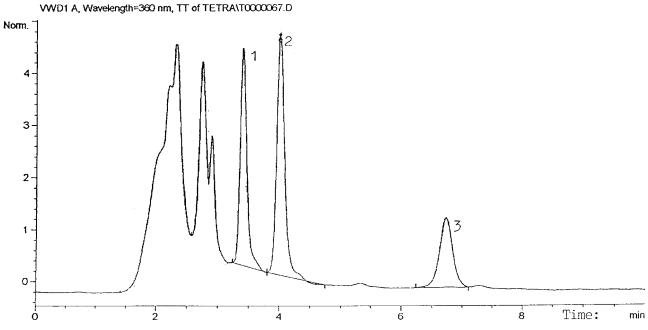

Figure 4. Separation of tissue extract from bovine liver spiked with tetracyclines (peaks: 1, oxytetracycline; 2, tetracycline; 3, chlortetracycline).

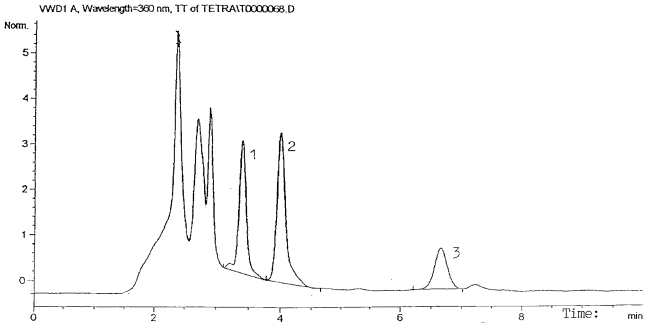

Figure 5. Separation of tissue extract from bovine kidney spiked with tetracyclines (peaks: 1, oxytetracycline; 2, tetracycline; 3, chlortetracycline).

Validation

Linearity of response. The calibration curves for the tetracycline standards and extracted samples were constructed. The ratio of peak area to analyte concentration was plotted using data derived from duplicate sample injections. The linearity was observed for tetracycline spiking tissues in the range 20–1000 ng/g. Correlation coefficients ($r = 0.9993$) were obtained for all calibration curves by using standard solutions. Standard curves for tetracycline-spiked tissues had correlation coefficients ranging from 0.9982 to 0.9991.

Limits of detection and quantitation. Detection limits were estimated as the amount of analyte producing a response at five times the noise level. The detection limits in bovine and porcine muscles were 10 ng/g for OTC and TC, and 15 ng/g for CTC. The detection limits in liver and kidney were 20 ng/g for OTC and TC, and 25 ng/g for CTC. The practical limit of quantitation was estimated as the concentration equal to twice the detection limit of the method.

Interference. For muscle, liver and kidney tissue assay no interference was observed with veterinary drugs including fluoroquinolones, nitrofuranes, sulfonanides and chloramphenicol.

Recovery and Precision

The recoveries of tetracyclines were investigated from porcine and bovine muscle, liver and kidney spiked at the levels of 50, 100 and 200 ng/g. Average OTC, TC and CTC recoveries (± SD) for tetracyclines-fortified tissue samples, were generally above 80% (Tables 1 and 2).

Inter- and intra-assay reproducibility was determined by simultaneously assaying replicates of muscle and tissue samples spiked with tetracyclines. Intra-assay precision of the method was determined by replicate analyses ($n = 6$) of blank tissue samples fortified with tetracyclines. In addition, tissue samples fortified with tetracyclines at the same concentrations were analysed on four consecutive days to determine the day-to-day variation (inter-assay precision) of the method. Tables 1 and 2, respectively, show the intra-assay precision for LC determination of OTC, TC and CTC in bovine and porcine tissues. Average intra-assay variabilities (below 10%) for all types of samples and analytes were calculated.

Results of the experiments conducted to determine the day-to-day (interassay) variation of the method are shown in Tables 3 and 4. Average inter-assay variabilities were calculated at less than 10%.

Table 1. Recovery of tetracyclines from bovine tissue sample and intra-assay and accuracy of the method ($n = 6$)

Tissue	Drug	Drug added (ng/g)	Mean ± SD (ng/g)	Recovery (%)	CV (%)
Muscle	OTC	50	43.3 ± 2.3	86	5.76
		100	84.2 ± 3.6	84	4.74
		200	163.8 ± 7.8	82	4.76
Liver		50	42.8 ± 2.6	84	6.25
		100	85.4 ± 4.5	85	5.31
		200	167.7 ± 8.4	84	5.04
Kidney		50	41.8 ± 2.3	84	5.50
		100	83.8 ± 3.9	83	4.69
		200	168.8 ± 6.7	84	4.00
Muscle	TC	50	42.2 ± 1.8	84	4.26
		100	81.1 ± 3.1	81	3.78
		200	164.7 ± 5.8	82	3.52
Liver		50	40.2 ± 1.9	80	4.76
		100	84.3 ± 3.1	84	3.67
		200	162.8 ± 5.7	81	3.51
Kidney		50	41.2 ± 2.4	82	5.86
		100	83.9 ± 3.1	84	3.72
		200	164.6 ± 6.9	82	4.12
Muscle	CTC	50	40.8 ± 1.8	81	4.53
		100	83.4 ± 2.8	83	3.35
		200	162.8 ± 5.1	81	3.16
Liver		50	40.8 ± 2.4	80	5.91
		100	81.6 ± 2.9	82	3.64
		200	160.7 ± 5.5	80	3.45
Kidney		50	41.3 ± 1.8	81	4.43
		100	83.2 ± 3.2	83	3.92
		200	163.1 ± 6.3	82	3.86

Table 2. Recovery of tetracyclines from porcine tissue sample and intra-assay and accuracy of the method ($n = 6$)

Tissue	Drug	Drug added (ng/g)	Mean ± SD (ng/g)	Recovery (%)	CV (%)
Muscle	OTC	50	41.1 ± 2.3	82	5.67
		100	82.7 ± 4.3	82	5.17
		200	168.9 ± 7.4	84	4.37
Liver		50	42.6 ± 2.3	84	5.45
		100	80.1 ± 4.5	80	5.63
		200	164.7 ± 6.8	82	4.18
Kidney		50	42.2 ± 2.0	84	4.80
		100	83.6 ± 3.7	83	4.41
		200	168.9 ± 6.7	84	3.98
Muscle	TC	50	42.2 ± 1.8	84	4.39
		100	83.4 ± 2.7	83	3.45
		200	163.4 ± 6.0	82	3.69
Liver		50	41.7 ± 2.0	83	4.69
		100	80.2 ± 3.2	80	4.03
		200	163.7 ± 5.9	82	3.63
Kidney		50	41.8 ± 1.9	82	4.58
		100	82.6 ± 3.0	83	3.62
		200	169.4 ± 5.1	85	3.01
Muscle	CTC	50	40.8 ± 2.8	81	6.88
		100	83.6 ± 4.9	84	5.87
		200	166.4 ± 6.4	83	3.84
Liver		50	41.8 ± 2.8	82	5.93
		100	81.9 ± 4.1	81	5.06
		200	163.2 ± 7.4	81	4.59
Kidney		50	40.6 ± 2.3	81	5.63
		100	83.0 ± 2.8	83	3.34
		200	160.7 ± 5.8	81	3.58

Table 3. Estimation of the interassay for tetracyclines determination in bovine tissues

			Tetracyclines found (ng/g tissue)					
Tissue	Drug	Drug Added (ng/g)	Day 1	Day 2	Day 3	Day 4	Drug found (Mean ± SD)	CV (%)
Muscle	OTC	50	43.8	40.8	39.8	46.2	42.2 ± 2.44	5.92
		100	85.3	79.9	87.7	88.2	85.21 ± 4.18	4.90
		200	171.2	159.7	163.2	162.4	164.1 ± 7.58	4.62
Liver		50	47.7	42.2	41.7	43.1	43.5 ± 2.56	5.90
		100	87.4	87.7	82.2	81.4	83.2 ± 3.31	3.98
		200	161.2	159.3	165.4	170.2	164.1 ± 7.49	4.57
Kidney		50	41.1	39.4	41.7	45.3	41.8 ± 1.89	4.54
		100	80.8	79.4	82.3	89.4	82.9 ± 3.68	4.45
		200	159.3	173.1	159.8	168.2	164.7 ± 6.27	3.81
Muscle	TC	50	44.6	41.3	42.3	39.6	41.95 ± 1.87	4.48
		100	84.4	83.6	81.7	80.3	82.5 ± 3.26	3.96
		200	164.0	159.3	160.4	161.8	168.8 ± 5.94	3.52
Liver		50	43.6	40.2	39.3	37.2	40.7 ± 2.40	5.91
		100	85.2	81.6	80.3	78.6	81.4 ± 2.45	3.01
		200	159.6	162.2	164.0	155.7	160.4 ± 6.34	3.95
Kidney		50	42.0	39.2	42.2	44.6	44.0 ± 2.04	4.65
		100	79.8	77.8	82.3	83.6	80.8 ± 2.48	3.80
		200	161.7	160.3	164.2	157.4	161.6 ± 5.68	3.52
Muscle	CTC	50	39.7	39.9	38.3	43.8	40.4 ± 2.09	5.18
		100	84.4	83.3	80.3	77.3	81.3 ± 2.79	3.44
		200	161.3	159.3	153.7	163.3	159.4 ± 5.35	3.36
Liver		50	40.2	45.6	44.2	37.3	41.8 ± 2.10	5.03
		100	83.7	80.3	79.6	80.3	82.4 ± 3.40	4.13
		200	161.2	159.6	161.2	167.8	162.4 ± 6.41	3.95
Kidney		50	41.2	37.2	39.7	45.2	40.8 ± 1.74	4.28
		100	85.4	80.6	80.5	79.4	81.4 ± 3.14	3.86
		200	161.2	165.3	159.2	160.9	161.6 ± 5.49	3.40

Table 4. Estimation of the interassay for tetracyclines determination in porcine tissues

			Tetracycline found (ng/g tissue)					
Tissue	Drug	Drug Added (ng/g)	Day 1	Day 2	Day 3	Day 4	Drug found Mean ± SD	CV/ (%)
Muscle	OTC	50	43.3	45.8	39.7	41.3	42.5 ± 2.33	5.49
		100	85.2	84.7	89.2	83.6	84.4 ± 3.80	4.57
		200	171.4	161.2	163.8	163.6	164.9 ± 7.09	4.30
Liver		50	39.4	47.4	45.7	43.1	53.9 ± 3.23	6.01
		100	85.2	84.6	85.0	79.2	83.5 ± 4.19	5.02
		200	161.2	167.8	165.2	163.8	164.5 ± 7.33	4.46
Kidney		50	49.8	45.6	42.8	40.7	45.2 ± 3.07	6.81
		100	89.6	85.4	81.6	83.8	85.1 ± 3.78	4.45
		200	167.3	163.6	164.4	169.6	166.0 ± 7.33	4.42
Muscle	TC	50	38.2	42.6	43.0	45.2	42.2 ± 1.75	4.15
		100	83.6	79.3	77.8	84.9	81.4 ± 2.96	3.64
		200	162.8	169.0	159.9	155.6	163.3 ± 5.21	3.26
Liver		50	42.3	43.0	37.7	39.2	41.5 ± 2.27	5.49
		100	83.8	79.8	84.0	85.6	82.4 ± 3.37	4.94
		200	166.0	154.8	157.9	161.3	162.2 ± 7.60	4.69
Kidney		50	35.8	43.6	42.3	40.6	41.2 ± 2.23	5.42
		100	87.9	83.6	84.3	81.3	83.7 ± 3.86	4.62
		200	162.2	167.2	158.4	160.3	162.3 ± 6.58	4.06
Muscle	CTC	50	41.8	45.3	39.6	38.2	41.22 ± 2.74	6.66
		100	84.6	81.7	80.2	77.3	80.95 ± 4.28	5.29
		200	163.8	159.2	154.6	164.4	160.5 ± 7.14	4.47
Liver		50	42.0	37.2	36.7	44.6	40.42 ± 3.42	8.47
		100	85.5	80.3	81.8	82.3	82.45 ± 2.69	3.27
		200	169.0	159.7	157.3	162.0	162.0 ± 6.01	3.71
Kidney		50	41.3	43.8	41.0	39.5	41.4 ± 2.36	5.71
		100	82.4	81.7	77.3	82.6	81.25 ± 3.20	3.95
		200	162.3	159.2	156.8	163.7	160.0 ± 5.88	3.68

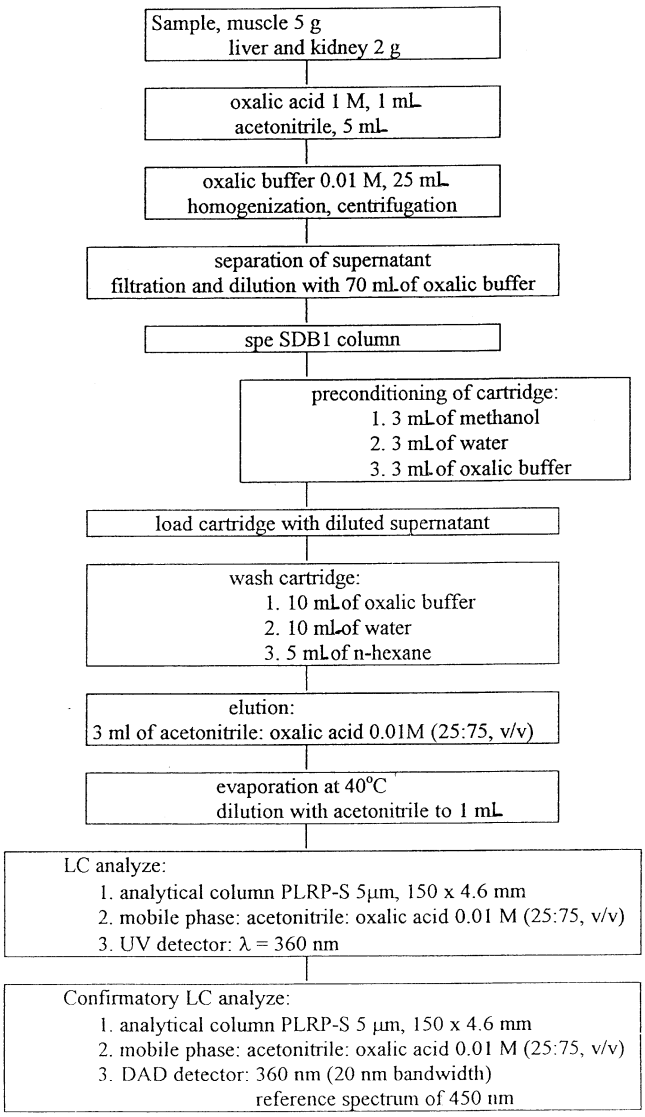

Figure 6. Flow diagram of the analytical procedure.

DISCUSSION

A critical aspect of drug residue analysis is the sample extraction step, which requires the isolation of the residues from a biological matrix. Traditionally, the isolation of tetracycline residues from tissue requires homogenization of the sample and liquid extraction solvents. The difficulty in isolating tetracyclines from tissue is associated with the propensity of those compounds to bind with sample protein and to form chelate complexes with metal ions. Usually, EDTA has been used in tetracycline isolations to facilitate their decomplexation from biological matrices (Oka *et al.*, 1984; Ashworth, 1985; Ikai *et al.*, 1987; Rogstad *et al.*, 1988; Reimer and Young, 1990; Walsh *et al.*, 1992; Moretti *et al.*, 1994; Horii, 1994).

In our method (Fig. 6) tetracyclines were extracted from tissues after dechelation and deproteinization with an oxalic acid–acetonitrile mixture. We decided that oxalic acid and acetonitrile could be useful in removing most proteins and in dechelating the tetracycline–metal complex. The introduction of an additional homogenization step with an oxalic buffer led to a higher and more reproducible recovery. The use of oxalic acid to facilitate decomplexation and extraction before spe clean-up represents the first known use of this compound for this purpose.

Several types of C_{18} reversed-phase cartridges from supplies were investigated for use in the clean-up procedures. They gave variable recoveries (Oka *et al.*, 1984; Moats, 1986; Ikai *et al.*, 1987; Bjorklund, 1988; Rogstad *et al.*, 1988; Reimer and Young, 1990; Walsh *et al.*, 1992; Moretti *et al.*, 1994; Horii, 1994;). We found, however, that using a SDB1 (styrene-divinylbenzene) catridge resulted in a higher recovery of tetracyclines. Using this cartridge we omitted interaction with tetracycline-silanol groups. Additionally, we found that tetracyclines may be applied onto the SDB1 cartridge with solution containing a small volume of acetonitrile (<7.5%).

Using the polymeric analytical column we avoided problems of the interaction of the tetracyclines with silica support. The best results were obtained using a pH 2.0 with oxalate buffer. LC mobile phase (0.01 M oxalic acid–acetonitrile, 75:25, v/v) proved to be suitable for the separation of OTC, TC and CTC on PLRP-S column. The oxalic acid appears to effectively strip or mask any metal ions that may affect tetracycline analyses. This was evidenced by OTC, TC and CTC peaks which were consistently symmetrical and well resolved from each other. Furthermore, at $\lambda = 360$ nm no interference were observed with endogenous substances of the blank tissues. Hence the wavelength of 360 nm was chosen for quantitation of the tetracyclines. This finding, in our opinion, makes the method potentially useful for simultaneous identification and quantification of tetracycline in edible tissues.

In conclusion, the results of the present study show that the proposed LC method is an efficient and reliable means of quantitating OTC, TC and CTC residue in animal edible tissues. Although it involves a multistep extraction scheme, one analyst familiar with the method can easily process eight samples in about 3 h. Therefore, the method should be useful for routine identification and confirmation of residues presumptively identified as tetracyclines by other tests.

Acknowledgement

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