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On-line metal chelate affinity chromatography clean-up for the high-performance liquid chromatographic determination of tetracycline antibiotics in animal tissues

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Abstract

An on-line high-performance liquid chromatographic (HPLC) method for the determination of tetracycline, oxytetracycline, chlortetracycline and demeclocycline using metal chelate affinity chromatography–reversed-phase HPLC has been developed. The drugs were extracted with succinate buffer and the extract diluted with EDTA–pentanesulphonate buffer. Diluted extract was then absorbed onto a C_8 or XAD-2 solid-phase extraction (SPE) cartridge and eluted with methanol. The eluate was then injected onto a TSKgel chelate column which had been preloaded with copper(II). The tetracyclines were eluted from this column onto the analytical column (Polymer Labs. PLRP-S) with an EDTA-containing buffer. Elution of the analytical column was via a methanol–acetonitrile gradient and detection was by UV at 350 nm. Average recoveries at the 10, 20, 50 and 300 $\mu g\,kg^{-1}$ levels were 50–80%. The limit of detection (LOD) was 10 $\mu g\,kg^{-1}$ for oxytetracycline and tetracycline and 20 $\mu g\,kg^{-1}$ for chlortetracycline and demeclocycline. The method was validated for sheep liver and cattle kidney.

Keywords: Tetracycline; Oxytetracycline; Chlortetracycline; Demeclocycline

1. Introduction

Tetracyclines (including tetracycline, oxytetracycline and chlortetracycline; Fig. 1) are widely used in animal husbandry. They are used both for the prophylaxis and treatment of disease and as feed additives to promote weight gain and increase feed conversion efficiency. They are used both in feeds, typically at dosage levels of 5–50 $mg\,kg^{-1}$ and in injectable solutions. They are licensed for use in a wide variety of food-producing animals such as cattle, pigs, sheep, poultry and fish [1]. Maximum residue limits (MRLs) have been set in a number of tissue types, including 0.6 $mg\,kg^{-1}$ in kidney, 0.3 $mg\,kg^{-1}$ in liver and 0.1 $mg\,kg^{-1}$ in muscle tissue [2].

The occurrence of residues of tetracyclines in the tissues of food-producing animals has been documented in a number of species. For example, in a study in this laboratory [3], concentrations of oxytetracycline of 14 900, 3700 and 1600 $\mu g\,kg^{-1}$ were found in kidney, liver and muscle, respectively, for a sheep slaughtered 5 h after injection at a dosage of 5 $mg\,kg^{-1}$ body weight. A second study on pigs showed concentrations of chlortetracycline of 96–136, 25–76 and 14–27 $\mu g\,kg^{-1}$ in kidney, liver and

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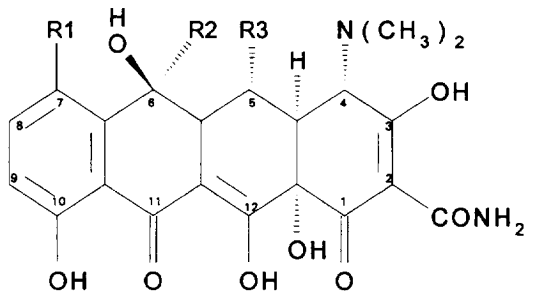

	R1	R2	R3
Tetracycline	H	CH3	H
Oxytetracycline	H	CH3	OH
Chlortetracycline	Cl	CH3	H
Demeclocycline	Cl	H	H

Fig. 1. Structure of the tetracyclines.

muscle, respectively, after slaughter 12 days after ceasing feeding at a level of 250 g per tonne. Guillot et al. [4] carried out a study on concentrations of oxytetracycline in milk and tissues of cattle treated with an injectable formulation. Residues above the limit of quantitation (LOQ) (10 $\mu g\,kg^{-1}$) were found in milk 10 milkings (5 days) after cessation of the treatment. The occurrence of residues of tetracycline in eggs [5,6] and in a number of fish species [7–9] has also been reported. Thus the occurrence of tetracycline residues in the tissues of food-producing animals is well established.

The occurrence of tetracycline residues in the food supply has also been documented. For example, in the UK [10], in the period 1987–1990, of 972 samples (consisting of a variety of matrices from a range of sources including home-produced and imported) analysed for residues of tetracyclines, 87 were found to contain residues of chlortetracycline, 118 were found to contain residues of oxytetracycline and 15 were found to contain residues of tetracycline. The concentrations detected ranged from 10 $\mu g\,kg^{-1}$ (the LOQ for the procedure used) to 6300 $\mu g\,kg^{-1}$.

There is a wide range of literature available describing methods of analysis for tetracycline antibiotics in animal tissues. Much of the literature focuses on the use of solid-phase extraction (SPE) [11–15] as a clean-up technique and in particular the use of silica-based cartridges. Recently the technique of metal chelate affinity chromatography (MCAC) was introduced by this laboratory [3] as a clean-up method for the determination of tetracycline residues in a large number of animal tissue and species types. Recoveries were high and LOQs low (down to at least 10 $\mu g\,kg^{-1}$). The method consists of extraction with succinate buffer followed by clean-up on a Chelating Sepharose column preloaded with copper sulphate. Elution from the chelate column is by use of an EDTA-containing buffer and the copper is removed from the eluate by chromatography on an XAD-2 resin column. The method has proved robust and has gained a measure of acceptance nationally and internationally. One criticism which has been levelled at the procedure is that it is protracted and the sample throughput is relatively low. To this end, attempts have been made to improve the procedure and the sample throughput. Carson [16] adapted this procedure by removal of the clean-up steps after the MCAC stage and changing the HPLC conditions in order to use a polymer-based column to avoid the problems of passing an extract containing a significant proportion of copper down a silica column. This procedure has published data only on the analysis of milk and includes a molecular filtration step which has, in this laboratory's experience, proved to be time-consuming. Degroodt et al. [17] modified the procedure of this laboratory by the substitution of a C_{18} SPE step in place of the XAD-2 resin. While some improvement in solvent consumption and time of analysis was claimed by the authors, overall there did not appear to be any major improvement and indeed recoveries seemed in general to be lower and coefficients of variation (C.V. values) higher, particularly as the LOQ was approached.

This paper reports studies made to improve sample throughput potential by means of off-line clean-up followed by on-line MCAC while maintaining recovery and precision for the commonly used tetracyclines.

2. Experimental

2.1. Reagents

Analytical grade citric acid, copper sulphate, sodium succinate, disodium EDTA and potassium

dihydrogen phosphate were obtained from BDH (Poole, UK). Chlortetracycline (CTC), demeclocycline (DMC), oxytetracycline (OTC), tetracycline (TC), sodium pentanesulphonate and XAD-2 resin were obtained from Sigma (Poole, UK). HPLC grade acetonitrile and methanol were obtained from Rathburn Chemicals (Walkerburn, UK). Bond-Elut cartridges were obtained from Anachem (Luton, UK) and Isolute cartridges from Jones Chromatography (Hengoed, UK).

Succinate buffer (pH 4.0) was prepared by dissolving 60 g succinic acid in 1 l water and adjusting the pH to 4.0 with 1 *M* NaOH. Succinate buffer containing 3.72% EDTA and 0.3% pentanesulphonic acid (SEPSA solution) was prepared by dissolving 37.2 g disodium EDTA and 3 g pentanesulphonic acid in 1 l succinate buffer (pH 4.0). Phosphate buffer containing pentanesulphonic acid (PPSA solution) was prepared by dissolving 3 g pentanesulphonic acid (PSA) in 1 l 0.1 *M* potassium dihydrogen phosphate.

A diluted blank sample extract for the preparation of matrix standards was prepared by taking samples of control blank tissue (2 g) through the extraction and clean-up procedure and diluting the SPE eluate (6 ml) to 12 ml with methanol.

Matrix standards to cover the concentration range 0–300 $\mu g\,kg^{-1}$ were prepared by diluting aliquots (0–200 μl) of mixed tetracycline standard solutions (1–10 $\mu g\,ml^{-1}$ in methanol) to 10 ml with diluted blank sample extract.

2.2. HPLC mobile phases

Mobile phase A was aqueous 0.1 *M* potassium dihydrogen phosphate–0.01 *M* citric acid–0.01 M EDTA, and mobile phase B [(0.1 *M* potassium dihydrogen phosphate–0.01 *M* citric acid–0.01 *M* EDTA)–methanol–acetonitrile (65:10:25, v/v)].

2.3. HPLC columns

Pre-column: Anagel-TSK–Chelate-SPW (iminodiacetic-bonded hydrophilic polymeric support) 10 μm, 10×6 mm I.D. supplied by Anachem.

Analytical column: Polymer Labs. PLRP-S (styrene–divinylbenzene copolymer) 5 μm, 150×4.6 mm I.D. with PLRP-S 5μm, 5×3 mm I.D. precolumn supplied by Jones Chromatography.

2.4. Apparatus

Homogeniser (Ultra-Turrax, Janke & Kunkel), centrifuge (MSE Europa 24 M), filter paper (Whatman No. 1) and Vac-Elut (Varian, Jones Chromatography) were used. The HPLC system consisted of a SpectraSystem P200 binary gradient pump (Spectra-Physics), a Gilson 232 autosampler with 401 dilutur (Anachem) and a SpectraSystem UV1000 UV detector (Spectra-Physics). Data collection and analysis was by computing integrator (PE Nelson Turbochrom software).

2.5. Extraction

Thinly-sliced tissue (2 g) and 20 ml succinate buffer (pH 4.0) were homogenised for 1 min and then centrifuged for 15 min at 30 897 *g*. The supernatant was decanted and filtered through Whatman No. 1 paper. A 12-ml sample of filtrate was taken and diluted with 6 ml SEPSA solution.

2.6. Clean-up

2.6.1. Bond-Elut C_8 (500 mg/6 ml) and isolute C_8 EC (500 mg/6 ml) based clean-ups

The SPE cartridges were conditioned with methanol (6 ml), water (6 ml) and PPSA (2 ml). The diluted filtrate was passed through the cartridge and the eluate discarded. The cartridge was washed with PPSA solution (10 ml) and water (2 ml) and eluted with methanol (6 ml).

2.6.2. XAD-2 resin (3 g wet resin in a 6 ml cartridge) based clean-up

The cartridges were conditioned with methanol (10 ml), water (10 ml) and SEPSA solution (2 ml). The diluted filtrate was passed through the conditioned cartridge and the eluate discarded. The cartridge was washed with PPSA (14 ml), water (2 ml) and eluted with methanol (6 ml).

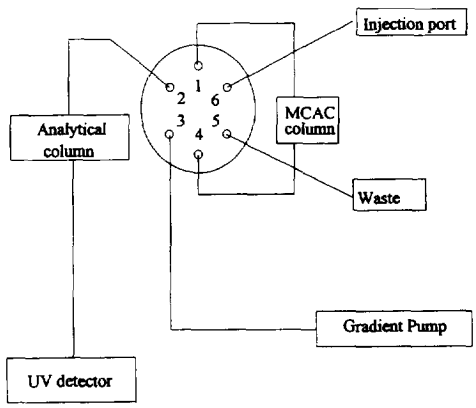

Fig. 2. System configuration.

2.7. HPLC

The system configuration used is shown in Fig. 2 and is discussed in detail later. The system was completely automated, the autosampler performing all conditioning, washing and loading operations and triggering the gradient programme and data collection. An Anagel-TSK–Chelate-5PW pre-column was installed in place of a sample loop on the injection valve. The pre-column was conditioned by the passage of copper sulphate (50 $g\,l^{-1}$) (25 μl) followed by water (0.5 ml). Samples in methanol (1.5 ml) were then loaded onto the pre-column at a rate of 0.36 ml min^{-1} and the column washed with water (0.5 ml), methanol (0.5 ml) and water (0.5 ml) prior to switching the pre-column on-line. The pre-column was eluted with mobile phase A for 11 min. The pre-column was switched off-line and the mobile phase was then programmed in a linear gradient to 100% mobile phase B in 10 min and then maintained at 100% mobile phase B for a further 10 min before re-equilibrating at 100% mobile phase A. Quantitation was by reference to replicate injections of standard tetracyclines in blank tissue extract at a level equivalent to the spiking level.

2.8. Protocol

Samples were analysed in batches of 6–10 spikes and 2 blanks. Time of preparation (unoptimised) for a batch of 12 samples was approximately 5 h including weighing out of samples.

3. Results and discussion

3.1. HPLC

The current procedure in use in this laboratory for the analysis of tetracyclines in animal tissues, at the commencement of this work, involved an extraction with succinate buffer followed by a two-stage clean-up utilising MCAC followed by reversed-phase (XAD-2 resin) SPE. Clean-up by MCAC makes use of the selective binding of tetracyclines to an iminodiacetic phase already loaded with copper ions. It is this selectivity which enables the preparation of extracts clean enough for the trace determination of tetracyclines. It was decided that the MCAC stage could be coupled to the analytical HPLC stage to shorten and automate the procedure. Initial work was carried out using an Anagel-TSK–Chelate-5PW pre-column as the MCAC column and using silica-based C_8 and C_{18} columns for the analytical HPLC. Gradient systems were employed on both the chelate side and the analytical side of the system to enable a full investigation of the parameters required to set up the automated system.

Early work demonstrated that the chelate part of the procedure could be carried out on-line. Tetracyclines loaded in a variety of aqueous- and methanol-containing buffers were retained when loaded onto the chelate column after it had been conditioned with copper sulphate. The tetracyclines could then be eluted from the chelate column using 0.01–0.1 *M* EDTA containing buffers. The elution volume from the chelate column was of the order of 10–12 ml. The tetracyclines in the chelate-column eluate could be focused by passing the eluate through a reversed-phase column, and subsequently chromatographed. It was demonstrated that in the absence of copper, the tetracyclines passed through the chelate column. Using silica-based columns for the analytical column, reducing retention times, peak heights and column efficiencies were noted on successive injections. This is presumably due to the passage of the high-ionic-strength copper-containing eluate through the silica-based column. Tetracyclines are notorious-

ly susceptible to the presence of traces of metal ions in silica packings, causing problems with irreversible binding, peak shape, etc. For this reason, further work was carried out using a polymer-based reversed-phase column as the analytical column.

3.1.1. Extraction and clean-up — simple deproteinisation procedures

Since the use of succinate buffer as an extraction solvent for tetracyclines from tissues had already been demonstrated as being effective [3], work was concentrated on determining the minimum amount of clean-up and concentration required before sample extracts could be injected onto the on-line chelate system. The tissue used was sheep liver.

Simple one-step deproteination was examined first. The following treatments of the initial succinate extracts of sheep liver spiked at 300 $\mu g\,kg^{-1}$ were examined: (1) 1:1 dilution with acetonitrile; (2) 1:1 dilution with acetonitrile followed by 10 kDa filtration; (3) 1:1 dilution with methanol; (4) 5 min at 100°C followed by 1:1 dilution with acetonitrile; (5) addition of trifluoroacetic acid (100 μl per 5 ml extract); (6) addition of Carrez reagents (0.1 *M* zinc acetate, 6% potassium ferrocyanide). Recoveries of tetracyclines were in general low and in some cases there were chromatographic interferences.

This early work also demonstrated that during the course of a batch, a reduction in response for aqueous standards occurred. For example, in a sequence of fourteen runs (standards and sample extracts), there was a reduction in peak height of 50–70% for demeclocydine and chlortetracycline. Responses from standards made up in tissue extracts did not suffer this effect. Responses from matrix standards were equivalent to the best responses from solvent standards. Therefore quantitation was performed against tissue standards in future runs.

3.1.2. Extraction and clean-up — SPE-based procedures

Due to the poor recoveries and presence of interferences in the chromatograms resulting from a simple deproteinisation procedure, the use of a variety of SPE-based procedures was investigated using methanol for elution. Initial work was carried out on sheep liver.

Low and inconsistent recoveries were obtained from spiked liver when the succinate buffer extract was loaded directly onto reversed-phase cartridges (XAD-2, Isolute C_{18}, Alltech IC-RP, Carbopack B HT) which had been washed with water or succinate buffer prior to elution. The low recoveries could be due either to break-through or to non-elution of the retained analytes. Analysis of the column loading fractions gave levels of 14–21% tetracyclines breaking through on the XAD-2 (a non-ionic polymeric resin), whilst no tetracyclines were found in the loading fraction from the Isolute C_{18} (a silica-based cartridge). This suggests that in this case, low recoveries were due to the inability to elute the analytes from the SPE cartridge with methanol.

In the procedure previously developed by this laboratory, it had been established that extracts in succinate EDTA buffer could be successfully loaded onto XAD-2 resin columns, followed by washing with water and elution with methanol. The efficacy of adding EDTA to the succinate extracts before passing them through XAD-2 or IC-RP cartridges was studied. In order to prevent problems with the on-line chelate system, it was necessary to ensure that the cartridges were washed free of EDTA prior to elution of the tetracyclines. Recoveries from the IC-RP cartridges with bed-volumes of 0.5 and 1 ml were poor. Breakthrough was identified as the problem here. Recoveries were improved by including a defatting step with hexane prior to loading the cartridge. Clean-up on XAD-2 resulted in higher recoveries, ranging from 45% for CTC to 74% for OTC. Addition of tetrabutyl ammonium phosphate to the extract prior to loading resulted in reduced recoveries for DMC, TC and OTC.

Although the addition of EDTA to the extract did result in improved recoveries, further improvement was desirable. Break-through appeared to be the problem with XAD-2 resin. In an attempt to minimise this problem, the effect of adding anionic on-pairing reagents [PSA, hexanesulphonic acid (HSA), sodium dodecyl sulphate (SDS)] was examined by spiking sheep liver extracts immediately prior to the SPE stage. Between pH 4 and 7, tetracyclines exist as dipolar ions in aqueous solution. Ion-pairing with a sulphonic acid should have the effect of reducing the polarity and increasing the retention of the tetracyclines on reversed-phase packings. The effect

of ion-pairing reagents was evaluated both on silica-based cartridges [Bond-Elut C_8, Isolute C_{18}, Isolute C_{18} (EC) and Isolute C_8 (EC)] and polymeric cartridges (XAD-2 and Alltech IC-RP). Addition of SDS to a succinate extract resulted in further deproteinisation and a filtration step was necessary before SPE. Addition of HSA resulted in a slight turbidity and addition of PSA in no turbidity with no filtration step necessary. PSA was selected as the reagent of choice as the recoveries produced were as high as with the other reagents, while also avoiding any problems with protein precipitation. In general higher recoveries were achieved in the presence of PSA, the best being obtained when PSA was added both to the loading and the washing solvents. The Alltech IC-RP column gave very much lower recoveries than the other columns. Bond-Elut C_8 gave the best recoveries at this stage and was therefore selected for the validation of the full procedure.

3.2. Validation — SPE-based procedure

The full procedure was validated at 10, 20, 50 and 300 $\mu g\,kg^{-1}$ in sheep liver (Table 1). At 10 $\mu g\,kg^{-1}$, CTC was not detected. Recoveries for the other analytes ranged from 70% for OTC to 86% for TC with C.V. values ranging from 2 to 14%. At 20 $\mu g\,kg^{-1}$, recoveries ranged from 55% for CTC to 78% for TC with C.V. values ranging from 1 to 13%. Two validation batches were performed at 50 $\mu g\,kg^{-1}$. Overall recoveries ranged from 54% for CTC to 83% for OTC with C.V. values ranging from 8 to 13%. At 300 $\mu g\,kg^{-1}$, two validation batches (1 and 2) were performed using cartridges from the same production batch as was used for the previous validation batches and two (3 and 4) from a different batch. These showed a significant batch-to-batch variation. Recoveries from the first group of cartridges remained at similar levels to those previously found with recoveries ranging from 60% for CTC to 82% for OTC with C.V. values ranging from 9 to 12%. However, the recoveries from the second group of cartridges dropped to 50–70% of the recoveries previously found. In this case, recoveries ranged from 36% for DMC to 58% for OTC.

These results point to a major problem in the use of silica-based SPE cartridges, which is one of the reasons why the literature of the trace residue analysis of tetracyclines is so extensive. The most likely explanation of these results is in lot-to-lot variation either in the purity of the base silica and/or the degree of coverage/shielding of the surface by the bonded-phase material. There are two ways around this problem. One is to use an end-capped column to reduce any silica adsorption problem or alternatively to employ polymeric-based materials. Two end-capped columns (Isolute C_{18} and C_8) were evaluated. Single validation batches were performed using Isolute C_8 (EC) on sheep liver and cattle kidney spiked at 300 $\mu g\,kg^{-1}$. Recoveries ranged from 52% for TC to 62% for OTC and DMC in sheep liver and 60% for DMC to 71% for OTC in cattle kidney. These were an improvement on the second lot of Bond-Elut cartridges. However, the tetracyclines co-chromatographed with other substances which would render quantification at the lower concentrations difficult.

Further validation was carried out using XAD-2 resin cartridges prepared in-house from bulk resin. Due to the changing demands of the laboratory

Table 1
Recoveries of tetracyclines at 10, 20, 50 and 300 $\mu g\,kg^{-1}$ for sheep liver using clean-up on Bond-Elut C_8

	Recovery (blank corrected) (%)			
	OTC	TC	DMC	CTC
10 $\mu g\,kg^{-1}$ (n=4)				
Mean	70	86	85	ND[a]
S.D.$_{n-1}$	9.8	2.2	11.3	ND
C.V.$_{n-1}$	13.9	2.5	13.3	ND
20 $\mu g\,kg^{-1}$ (n=3)				
Mean	72	78	62	55
S.D.$_{n-1}$	3.9	1.0	2.7	7.0
C.V.$_{n-1}$	5.5	1.3	4.4	12.7
50 $\mu g\,kg^{-1}$ (n=12)				
Mean	83	73	61	54
S.D.$_{n-1}$	6.5	6.8	5.2	6.9
C.V.$_{n-1}$	7.8	9.4	8.7	12.7
300 $\mu g\,kg^{-1}$, cartridge batch 1 (n=11)				
Mean	82	74	60	60
S.D.$_{n-1}$	9.2	8.5	7.4	5.6
C.V.$_{n-1}$	11.2	11.6	12.4	9.4
300 $\mu g\,kg^{-1}$, cartridge batch 2 (n=12)				
Mean	58	50	36	40
S.D.$_{n-1}$	3.8	4.4	5.9	5.6
C.V.$_{n-1}$	6.5	8.8	16.2	14.0

[a] ND=not detected.

surveillance programme the target tissue was changed from sheep liver to cattle kidney. Using a modified load/wash protocol, the procedure was validated at 10, 20, 50 and 300 $\mu g\,kg^{-1}$ levels (Table 2). At 10 and 20 $\mu g\,kg^{-1}$, CTC was not detected. At 10 $\mu g\ kg^{-1}$, recoveries ranged from 69% for OTC to 89% for TC with C.V. values from 10 to 14%. At 20 $\mu g\,kg^{-1}$, recoveries ranged from 59% for DMC to 67% for OTC with C.V. values ranging from 6 to 12%. At 50 $\mu g\,kg^{-1}$, all four tetracyclines could be detected and recoveries ranged from 44% for CTC to 67% for OTC with C.V. values ranging from 12 to 19%. At 300 $\mu g\,kg^{-1}$, recoveries ranged from 58% for CTC to 64% for OTC with C.V. values ranging from 5 to 6%. Time constraints prevented an investigation of lot-to-lot variation with the XAD-2 resin. However, such variation is not recognised as a problem, unlike the silica-based materials.

A full schematic of the method is shown in Fig. 3. Typical chromatograms of blank sheep liver extract, blank sheep liver extract spiked at 50 $\mu g\,kg^{-1}$ and blank sheep liver extract spiked at 300 $\mu g\,kg^{-1}$ following clean-up by Bond Elut C_8 are shown in Fig. 4. Typical chromatograms of blank cattle kidney extract, blank cattle kidney extract at 50 $\mu g\,kg^{-1}$ and cattle kidney extract spiked at 300 $\mu g\,kg^{-1}$ following clean-up by XAD-2 are shown in Fig. 5.

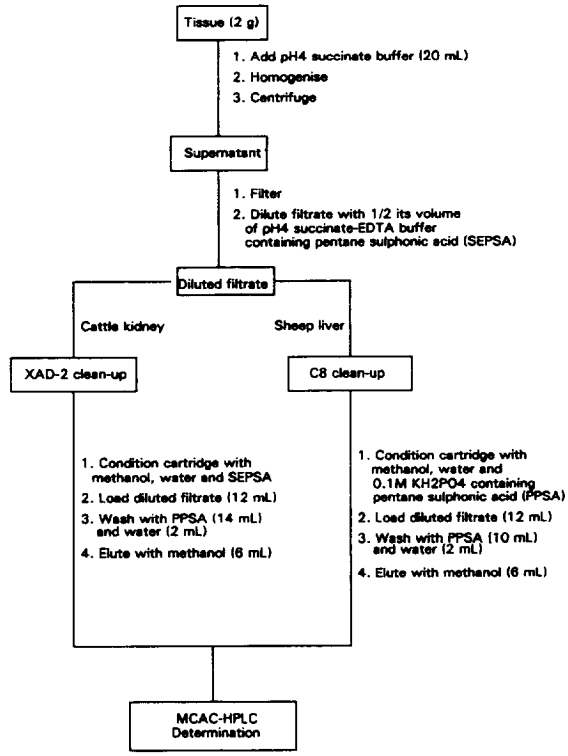

Fig. 3. Schematic of the procedure for the extraction and clean-up of tetracyclines from animal tissues using on-line MCAC clean-up.

Table 2
Recoveries of tetracyclines at 10, 20, 50 and 300 $\mu g\,kg^{-1}$ for cattle kidney using clean-up on XAD-2

	Recoveries (blank corrected) (%)			
	OTC	TC	DMC	CTC
10 $\mu g\,kg^{-1}$ (n=4)				
Mean	69	89	88	ND[a]
$S.D._{n-1}$	9.6	11.8	9.2	
$C.V._{n-1}$	13.8	13.3	10.5	
20 $\mu g\,kg^{-1}$ (n=4)				
Mean	67	65	59	ND
$S.D._{n-1}$	4.2	4.8	7.3	
$C.V._{n-1}$	6.3	7.4	12.3	
50 $\mu g\,kg^{-1}$ (n=5)				
Mean	67	61	64	44
$S.D._{n-1}$	8.3	8.5	10.1	8.3
$C.V._{n-1}$	12.5	13.8	15.9	18.7
300 $\mu g\,kg^{-1}$ (n=17)				
Mean	64	63	62	58
$S.D._{n-1}$	3.6	3.6	3.5	3.1
$C.V._{n-1}$	5.6	5.7	5.7	5.4

[a] ND=not detected.

DMC was included in this study because of its potential for use as an internal standard (I.S.). Examination of the validation data suggests that it would make an effective I.S. Table 3 summarises the difference in precision when using DMC as I.S. as against external standards. At spiking concentrations of 50 to 300 $\mu g\,kg^{-1}$ the in-batch C.V. values are generally lower when ratioed against the DMC recovery. In order to check the linearity of response, standard curves in matrix were measured for all four analytes. Matrix standard curves for all four analytes were linear over the range 0 to 300 $\mu g\,kg^{-1}$ with correlation coefficients of 0.998 to 0.999.

A significant increase in sample throughput has been achieved by transferring the MCAC clean-up from an off-line open column type of clean-up to an on-line column-switching type. Typical sample prep-

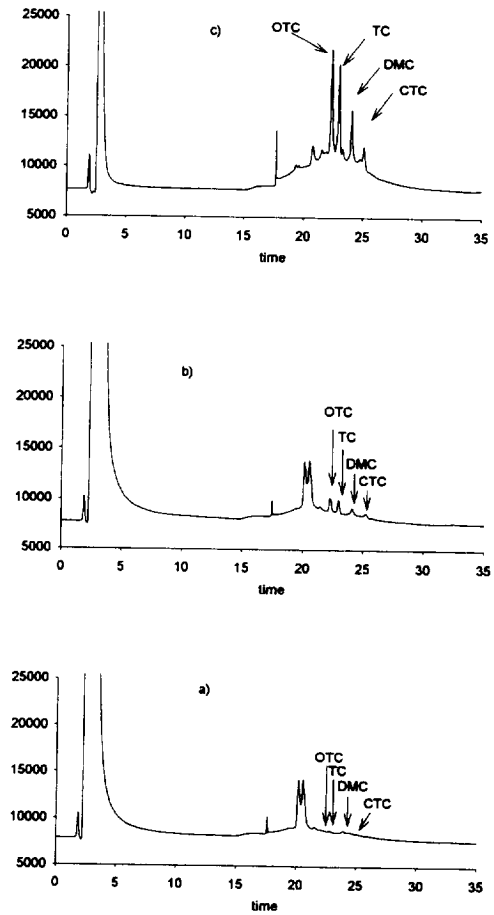

Fig. 4. Chromatograms of (a) blank sheep liver extract, (b) blank sheep liver extract spiked at 50 $\mu g\,kg^{-1}$ and (c) blank sheep liver extract spiked at 300 $\mu g\,kg^{-1}$. Clean-up by Bond-Elut C_8.

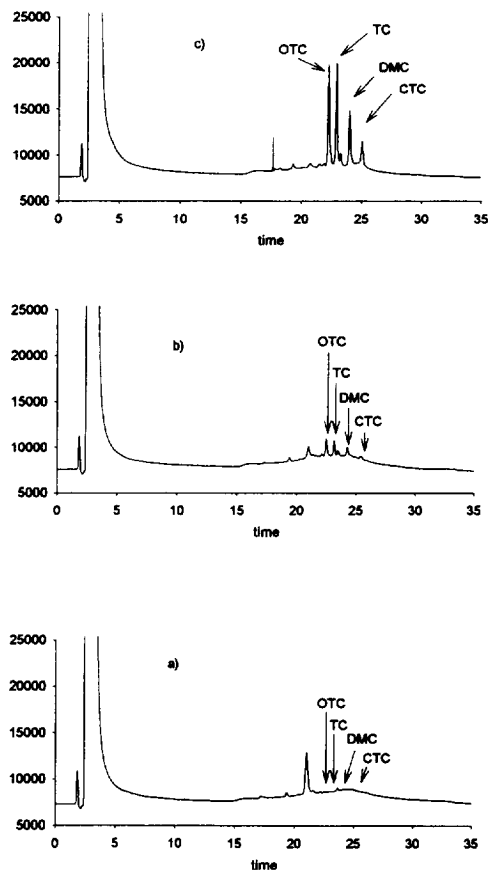

Fig. 5. Chromatograms of (a) blank cattle kidney extract, (b) blank cattle kidney extract spiked at 50 $\mu g\,kg^{-1}$ and (c) blank cattle kidney extract spiked at 300 $\mu g\,kg^{-1}$. Clean-up by XAD-2 resin.

aration times of 7–8 h for a batch of 6 samples has been reduced to approximately 4–5 h (unoptimised) for a batch of 12, with an automated HPLC clean-up/analysis which can be run overnight/weekend. Because of the nature of the sample preparation, increasing the batch size will not result in an equivalent increase in the sample preparation time. The simplified extraction and clean-up prior to HPLC analysis also has the effect of reducing the number of man-hours required for sample processing.

The applicability of the off-line MCAC clean-up procedure to a wide range of tissue and sample types has already been demonstrated [3]. Although only two tissue/species combinations have been examined during the course of this work, there is no reason why the on-line procedure should not have the same capabilities as the off-line procedure.

4. Conclusion

An HPLC procedure for the determination of tetracyclines utilising on-line MCAC clean-up and using DMC as I.S. has been developed. Simplified extraction and clean-up procedures for the determination of tetracyclines in animal tissues at trace residue concentrations, based on silica and polymeric reversed-phase SPE have been studied. Validation data for the use of both silica-based and polymeric SPE clean-ups has been obtained. The overall pro-

Table 3
Comparison of precision between quantitation using external standards and using I.S. (DMC)

Level ($\mu g\ kg^{-1}$)		C.V. (%)		
		OTC	TC	CTC
Sheep liver				
20 ($n=3$)	External	5.5	1.3	12.7
	Internal	8.9	3.1	14.0
50 ($n=12$)	External	7.8	9.4	12.7
	Internal	5.2	7.6	15.1
300, Cartridge batch 1 ($n=11$)	External	11.2	11.6	9.4
	Internal	6.7	5.6	3.8
300, Cartridge batch 2 ($n=12$)	External	6.5	8.8	14.0
	Internal	11.1	8.7	5.4
Cattle kidney				
50 ($n=5$)	External	12.5	13.8	18.7
	Internal	5.9	3.6	5.5
300 ($n=17$)	External	5.6	5.7	5.4
	Internal	2.7	1.6	2.9

cedure developed has the effect of increasing the sample throughput compared to the off-line MCAC procedure, whilst maintaining the robustness and sensitivity.

A problem area in the use of silica-based reversed-phase SPE cartridges for the analysis of tetracyclines has been highlighted and an alternative provided in this instance by the use of XAD-2 resin cartridges.

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