ELSEVIER

Journal of Chromatography A, 812 (1998) 321–326

JOURNAL OF CHROMATOGRAPHY A

Short communication

Improved method for the on-line metal chelate affinity chromatography–high-performance liquid chromatographic determination of tetracycline antibiotics in animal products

A.D. Cooper*, G.W.F. Stubbings, M. Kelly, J.A. Tarbin, W.H.H. Farrington, G. Shearer

Ministry of Agriculture, Fisheries and Food, CSL Food Science Laboratory, Norwich Research Park, Colney, Norwich NR4 7UQ, UK

Abstract

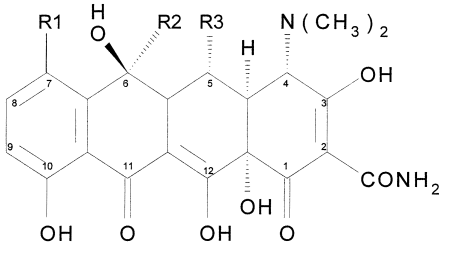
An improved on-line metal chelate affinity chromatography–high-performance liquid chromatography (MCAC–HPLC) method for the determination of tetracycline antibiotics in animal tissues and egg has been developed. Extraction was carried out with ethyl acetate. The extract was then evaporated to dryness and reconstituted in methanol prior to on-line MCAC clean-up and HPLC–UV determination. Recoveries of tetracycline, oxytetracycline, demeclocycline and chlortetracycline in the range 42% to 101% were obtained from egg, poultry, fish and venison tissues spiked at 25 μg kg^{-1}. Limits of detection less than 10 μg kg^{-1} were estimated for all four analytes. This method has higher throughput, higher recovery and lower limits of detection than a previously reported on-line MCAC–HPLC method which involved aqueous extraction and solid-phase extraction clean-up.

Keywords: Sample preparation; Food analysis; Antibiotics; Tetracyclines

1. Introduction

Tetracyclines (Fig. 1) are widely used in animal husbandry. They are used both for the prevention and treatment of disease and as feed additives to promote growth. They are licensed for use in a variety of food-producing animals including cattle, pigs, sheep, poultry and fish [1]. Maximum residue limits (MRLs) have been set for tetracycline, chlortetracycline and oxytetracycline in a number of tissue types, including 0.3 mg kg^{-1} in liver, 0.2 mg kg^{-1} in eggs and 0.1 mg kg^{-1} in muscle tissues (expressed as the sum of parent drug and 4-epimer in each case) [2].

A number of analytical methods for the high-performance liquid chromatographic (HPLC) deter-



	R1	R2	R3
Tetracycline (TC)	H	CH_3	H
Oxytetracycline (OTC)	H	CH_3	OH
Chlortetracycline (CTC)	Cl	CH_3	H
Demeclocycline (DMC)	Cl	H	H

Fig. 1. Structures of the tetracyclines.

*Corresponding author.

0021-9673/98/$19.00
PII: S0021-9673(97)01290-9

mination of tetracyclines in animal tissues at residue levels have been published. While several methods using solid-phase extraction (SPE) on silica-based packings have been described [3–7], this laboratory introduced the use of metal chelate affinity chromatography (MCAC) as a selective clean-up technique prior to HPLC determination of tetracyclines [8]. This approach (which has been adopted by other groups of workers [9,10]) utilises the tendency of tetracyclines to bind to metal ions, a feature of their chemistry which has proved to be a drawback in the more conventional SPE methods.

More recently, a variation on the MCAC approach in which the main clean-up step was carried out on-line prior to HPLC was developed in this laboratory [11]. While the use of on-line clean-up significantly improved throughput, it was still necessary to employ a preliminary clean-up of an aqueous extract by SPE on Bond-Elut C_8 or (preferably) XAD-2 in order to achieve acceptable recoveries and detection limits. Even so, the detection limits achieved by this on-line procedure were higher than those in the earlier off-line procedure [8].

This paper reports improvement of the on-line MCAC–HPLC approach by using organic solvent extraction, resulting in further increases in throughput and detection limits similar to those achievable by off-line MCAC clean-up.

2. Experimental

2.1. Reagents and apparatus

Analytical grade citric acid and sodium hydroxide were obtained from BDH (Poole, UK). Citrate buffer (1 *M*, pH 4 or 5) was prepared by dissolving citric acid (192 g) in approximately 800 ml water, adjusting the pH with 1 *M* aqueous sodium hydroxide and making up to 1 l with water. HPLC buffer (mobile phase A) was 0.1 *M* potassium dihydrogenphosphate–0.01 *M* citric acid–0.01 *M* EDTA. Mobile phase B was acetonitrile–methanol–buffer (25:10:65, v/v). A flask shaker (Stuart), centrifuge (Heraeus Varifuge 20RS), vortex mixer (Fisons Whirlimixer), filter paper (Whatman 1PS) and syringe filters (Whatman Anotop 10 mm 0.2 μm) were used. All other reagents and apparatus were used as previously described [11].

2.2. Extraction

Blended egg or thinly-sliced tissue (2 g), 1 *M* citrate buffer pH 4 (for chicken liver only) or 5 (1.2 ml) and ethyl acetate (12 ml) were homogenised for 1 min (tissue) or shaken for 15 min (egg) and then centrifuged for 10 min at 11 000 rpm. The supernatant was decanted and the residue re-extracted twice with ethyl acetate (2×12 ml). To the combined supernatants, anhydrous sodium sulphate (10 g) was added, swirled and left to stand for 5–10 min before filtration through Whatman 1PS filter paper. The filtrate was rotary evaporated to dryness (or an oily residue) under reduced pressure at 40°C and reconstituted in methanol (2 ml) by vortex-mixing. The extract was transferred to a 2-ml HPLC vial after filtration through a 0.2-μm syringe filter. HPLC analysis was carried out on 1.5 ml of the extract.

2.3. MCAC–HPLC determination

The system configuration used has been described in detail previously [11]. Briefly, an Anagel-TSK Chelate-5PW pre-column, installed in place of a sample loop on the injection valve, was pre-conditioned with aqueous copper sulphate and water. After extract loading, the pre-column was washed with water, methanol and water prior to switching the pre-column on-line for elution with mobile phase A onto the analytical column (Polymer Labs. PLRP-S 5 μm, 150×4.6 mm I.D. with 5×3 mm I.D. pre-column). After 11 min, the pre-column was switched off-line and the analytical column was eluted with a linear gradient from 100% A to 100% B in 10 min at 1 ml/min, then isocratic 100% B for 10 min, then re-equilibrated with 100% A for 10 min. During this period, the pre-column was conditioned ready for the next sample injection. The detection wavelength used was 350 nm.

2.4. Quantification

Quantification was by reference to replicate injections of matrix standard tetracyclines at a concentration equivalent to the spiking concentration.

Matrix standards to cover the concentration range 0–100 μg kg^{-1} were prepared by adding aliquots (0–200 μl) of mixed tetracycline standard solutions (1–10 μg ml^{-1} in methanol) to 2 ml of blank extract and making up to 20 ml with methanol. Retention times of the tetracyclines and their 4-epimers were determined by injection of individual matrix standards, prepared similarly.

2.5. Batch size

Samples were analysed in batches of up to 12 spikes and 2 blanks. Time of preparation for a batch of 14 samples (limited by the capacity of the centrifuge used) was 3–4 h. Two batches of this size could therefore be prepared in one working day.

3. Results and discussion

This method represents an improvement on the previously published on-line MCAC–HPLC method for tetracyclines [11], giving rise to higher throughput, higher recovery and lower limits of detection and determination. While aqueous extractants have generally been used for tetracycline determination in this and other laboratories [3–11], the use of an organic solvent extraction has in this case eliminated the need for a SPE step in the previously published method [11], the organic extract being injected into the automated MCAC–HPLC system following a solvent exchange step.

The extraction method used was based on that reported by Haagsma and Scherpenisse [12], which was used prior to off-line SPE clean-up and HPLC determination of tetracyclines in egg and tissues. The method involves extraction into ethyl acetate following addition of a small volume of a concentrated citrate buffer at pH 5.

Initially, direct loading of an ethyl acetate extract onto the MCAC column gave high recoveries and clean chromatograms, but there was a tendency for methanol-insoluble components of the extracts to precipitate in the MCAC column resulting in blockage. This problem was particularly severe with eggs, due to their high lipid content. A solvent exchange step was therefore introduced in which the ethyl acetate extract was evaporated to dryness and reconstituted in methanol before MCAC–HPLC. Methanol-insoluble components of the extract were separated by syringe filtration before injection. The MCAC column performance was then found to be robust. Approximately 150 sample extracts can be processed before the MCAC column performance starts to deteriorate, evidenced by a loss of response from tissue standards. At this point the column is repacked with fresh material.

The method has been validated at concentrations of 25 μg kg^{-1} and above in egg, chicken muscle and liver, salmon, trout and venison muscle tissues. Validation data is detailed in Tables 1 and 2. Recoveries were generally higher than those obtained using the earlier MCAC–HPLC procedure following aqueous extraction, and ranged from 42% (tetracycline in chicken muscle) to 101% (oxytetracycline in salmon muscle). Intra- and inter-assay precision values were acceptable for a screening method, with relative standard deviation (R.S.D.) values of 20% or less obtained for oxytetracycline, tetracycline, demeclocycline and chlortetracycline in all the matrices shown.

Recoveries of tetracycline at 25, 50 and 100 μg kg^{-1} from chicken liver at pH 5 were consistently lower than from the other matrices and were below

Table 1
Recoveries of tetracyclines from egg

	Recovery (%)			
	OTC	TC	DMC	CTC
25 μg kg^{-1}				
Batch 1 (n=4)				
Mean	91	64	81	77
S.D.$_{n-1}$	19	4	16	9
R.S.D.$_{n-1}$	20	5	19	11
Batch 2 (n=4)				
Mean	94	81	105	59
S.D.$_{n-1}$	9	16	5	3
R.S.D.$_{n-1}$	9	20	4	5
Overall (n=8)				
Mean	91	71	91	69
S.D.$_{n-1}$	15	14	17	11
R.S.D.$_{n-1}$	17	20	19	16
100 μg kg^{-1} (n=6)				
Mean	104	59	66	72
S.D.$_{n-1}$	4	5	6	8
R.S.D.$_{n-1}$	4	9	8	12

Table 2
Recoveries of tetracyclines at 25 μg kg^{-1} from animal tissues

	Recovery (%)			
	OTC	TC	DMC	CTC
Chicken muscle (n=6)				
Mean	72	42	55	72
S.D.$_{n-1}$	10	2	4	6
R.S.D.$_{n-1}$	14	5	7	8
Chicken liver (n=5)[a]				
Mean	74	31	55	69
S.D.$_{n-1}$	9	8	5	10
R.S.D.$_{n-1}$	12	27	8	15
Chicken liver (n=7)[b]				
Mean	101	70	62	92
S.D.$_{n-1}$	4	7	7	4
R.S.D.$_{n-1}$	4	10	11	5
Salmon muscle (n=7)				
Mean	101	70	62	92
S.D.$_{n-1}$	4	7	7	4
R.S.D.$_{n-1}$	4	10	11	5
Trout muscle (n=7)				
Mean	58	79	50	62
S.D.$_{n-1}$	9	11	7	5
R.S.D.$_{n-1}$	15	13	13	8
Venison muscle (n=7)				
Mean	65	43	57	79
S.D.$_{n-1}$	6	2	7	11
R.S.D.$_{n-1}$	9	4	13	14

[a] Extraction at pH 4.
[b] Extraction at pH 5.

the acceptable level of 40%. A substantial increase in recovery of all four tetracyclines was observed on lowering the pH of the added buffer from 5 to 4, as shown in Table 2. This modification was therefore adopted for this tissue. The effect of this pH change on recovery from other tissues has not yet been investigated, as acceptable recoveries are obtained under the existing conditions.

Fig. 2 illustrates calibration graphs obtained for the four tetracyclines in the range 0 to 100 μg kg^{-1}. The method showed acceptable linearity in this range for the purpose of measuring recovery, with correlation coefficient (r) values of 0.9975 (oxytetracycline), 0.9955 (tetracycline), 0.9987 (demeclocycline) and 0.9967 (chlortetracycline).

Detection limits were estimated by comparing the signal in a 25 μg kg^{-1} spiked sample to the signal (''noise'') in a blank sample at the corresponding retention time and extrapolating to a signal-to-noise ratio of 3. They were thus estimated to be 3 μg kg^{-1} for oxytetracycline, 5 μg kg^{-1} for tetracycline and demeclocycline and 6 μg kg^{-1} for chlortetracycline. These represented an improvement on those obtained using the previously reported on-line procedure, particularly for chlortetracycline, which was not detectable below 50 μg kg^{-1} using the previous procedure with the preferred XAD-2 clean-up. The chromatograms also showed a generally flatter baseline than those obtained previously, facilitating interpretation. Typical chromatograms obtained from blank and spiked egg are shown in Fig. 3.

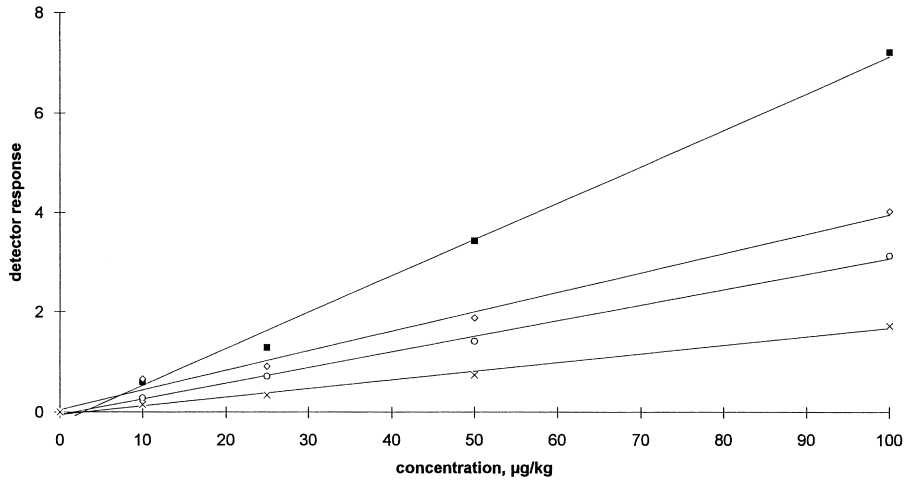


Fig. 2. Calibration graphs obtained for matrix standard oxytetracycline (■), tetracycline (◇), demeclocycline (○) and chlortetracycline (×).

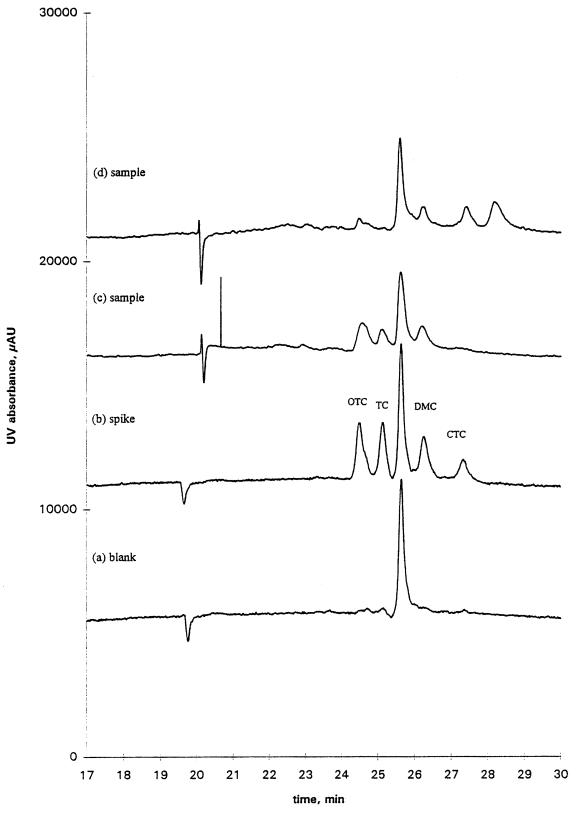


Fig. 3. Chromatograms obtained from MCAC–HPLC analysis of (a) blank egg; (b) blank egg spiked at 25 μg kg^{-1} with oxytetracycline (OTC), tetracycline (TC), demeclocycline (DMC) and chlortetracycline (CTC); (c) egg containing incurred residues of TC spiked at 25 μg kg^{-1} with DMC (internal standard); (d) egg containing incurred residues of CTC spiked at 25 μg kg^{-1} with DMC.

Within-batch variation of retention times was 0.03 min or less for all four tetracyclines. During 22 analytical batches over a 4-month period, the between-batch variation of retention times was 0.3 min or less. The mean resolution (R_s) values between each analyte and the nearest peak over the same period were 1.7 (oxytetracycline, tetracycline), 1.9 (demeclocycline) and 2.5 (chlortetracycline). The minimum R_s values observed were 1.0, 1.3, 1.6 and 1.9, respectively.

The 4-epimers of the tetracyclines studied (which are included in the most recent MRL regulations [2]) were all found to co-elute with oxytetracycline in the HPLC system used and give rise to similar UV responses as the parent compounds. When used for screening purposes, the method is therefore likely to detect the epimers if present. If the residues present in a positive sample were to be quantitated using this method, the epimers (if present) would have to be quantitated separately and added to the parent tetracycline concentration in order to measure correctly to the regulations.

A comparison of aqueous and ethyl acetate extraction methods in the determination of incurred oxytetracycline residues has been carried out [13]. No significant difference between the two types of method was found. The applicability of this method to the detection of incurred residues is also illustrated in Fig. 3, which shows chromatograms obtained from eggs containing trace residues of tetracycline and chlortetracycline. Note that peaks corresponding to the epimers appear at about 24.5 min in the chromatograms obtained from both of these samples.

The extension of the method to other matrices is on-going. This method has been found to be inapplicable to milk and chicken-based baby-food, due to the presence of co-eluting matrix interferences. This is also the case with the previously reported on-line MCAC method. The development of an alternative on-line cation-exchange clean-up for determination of tetracyclines in milk has recently been carried out in this laboratory and will be reported separately [14].

The method described here has been found to be robust in routine use. Over 550 samples of egg, poultry, fish and venison tissues have been analysed over a 4-month period. Demeclocycline is added to all samples during routine surveillance as an internal standard for quality assurance purposes. Internal standard recovery has been acceptable (greater than 40%) in more than 99% of the samples analysed.

4. Conclusions

The method described represents a significant improvement on a previously reported on-line MCAC–HPLC method for tetracyclines. The throughput has been increased by the use of an organic solvent extraction, eliminating the need for off-line SPE clean-up prior to the on-line MCAC clean-up. Organic solvent extraction results in recoveries which are, if anything, greater than those achieved using aqueous extraction. Fewer co-extractives are present in the extracts following the on-line

clean-up, so that the detection limits are similar to those achieved by off-line MCAC clean-up as described originally [8]. This method has been applied to the surveillance of animal products for the presence of tetracycline residues and has proved to be robust in routine use.

Acknowledgements

The development of on-line MCAC–HPLC methodology for tetracyclines was originally funded by the Veterinary Medicines Directorate and the Chief Scientist's Group of the UK Ministry of Agriculture, Fisheries and Food.

References

[1] Y. Debuf (Editor), The Veterinary Formulary, Pharmaceutical Press, London, 1988, p. 97.
[2] EC Regulation 2377/90 incorporating amending regulation 281/96.
[3] I. Nordlander, H. Johnsson, B. Oesterdahl, Food Addit. Contam. 4 (1987) 291.
[4] A. Rogstad, V. Hormazabal, M. Ynestad, J. Liq. Chromatogr. 11 (1988) 2337.
[5] H. Oka, H. Matsumoto, K. Uno, K.I. Harada, S. Kadowaki, M. Suzuki, J. Chromatogr. 325 (1985) 265.
[6] H. Oka, Y. Ikai, N. Kawamura, J. Hayakawa, J. Assoc. Off. Anal. Chem. 74 (1991) 894.
[7] J.R. Walsh, L.V. Walker, J.J. Weber, J. Chromatogr. 596 (1992) 211.
[8] W.H.H. Farrington, J.A. Tarbin, J. Bygrave, G. Shearer, Food Addit. Contam. 8 (1991) 55.
[9] M.C. Carson, J. Assoc. Off. Anal. Chem. 76 (1993) 329.
[10] J.M. Degroodt, B. Wyhowski de Bukanski, S. Srebnik, J. Liq. Chromatogr. 16 (1993) 3515.
[11] G. Stubbings, J.A. Tarbin, G. Shearer, J. Chromatogr. B 679 (1996) 137.
[12] N. Haagsma and P. Scherpenisse, in N. Haagsma, A. Ruiter and P.B. Czedik-Eysenberg (Editors), Proceedings of the EuroResidue II Conference, Veldhoven, Netherlands, 3–5 May 1993, University of Utrecht, Utrecht, 1993, p. 342.
[13] A.D. Cooper, J.A. Tarbin, W.H.H. Farrington and G. Shearer, Food Addit. Contam., submitted for publication.
[14] G.W.F. Stubbings, W.H.H. Farrington and G. Shearer, in preparation.