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A High Performance Liquid Chromatographic Method for Quantitative Determination of Tetracyclines and its Epimers in Shrimp Muscle

Shiny Thomas, Lakshmi Priya, K. P. Prasanth and P. G. Viswanathan Nair*
Sealab, Seafood Park (India) Ltd., Keltron Road, Aroor - 688 534, India

Abstract

A method for determination of tetracycline, oxytetracycline, chlortetracycline, 4-epitetracycline, 4-epioxytetracycline and 4-epichlortetracycline in shrimp muscle tissue by high performance liquid chromatographic method (HPLC) with a UV detector is described. The method involves extraction of the analytes by McIlvaine buffer, clean up by C18 SPE cartridge and analysis by HPLC and UV detection. A good separation of all the six analytes was achieved and good recovery (58 to 89%), repeatability and within laboratory reproducibility were possible with this method.

Keywords: HPLC method, tetracycline, oxytetracycline, chlortetracycline, tetracycline epimers, shrimp muscle

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* *E-mail: pgvnair@gmail.com*

Introduction

Food safety is an important global issue today and consumers are highly conscious about the quality and safety of the food products available in the market. Modern food production and processing involves use of many chemicals at different stages. A major concern in food safety is the presence of residues of such chemicals in the final product. Several antibiotics like tetracyclines are widely used as growth promoters or for treatment of disease. Tetracyclines are wide spectrum antibiotics, active against Gram-positive and Gram-negative organisms and are important as human and veterinary medicine. These substances are of low toxicity but prolonged exposure may lead to infection with resistant organisms, allergic and other undesirable reactions, vitamin B deficiency etc. (Berendsen & Van Rhijn, 2006). Studies on metabolism of tetracyclines have shown that no significant transformation occurs in the body (FAO, 1990), but formation of epimers can take place due to chemical instability under physiological conditions. The reversible formation of 4-epimers of tetracyclines is controlled by pH, presence of dissolved metals etc. (Oka et al., 2000) and is significant during sample preparation, especially under acidic conditions. Stability of tetracyclines is poor under strong acidic and alkaline conditions with reversible formation of epimers at position C4 to 4-epitetracyclines (Oka et al., 1995). Therefore it has become necessary to determine tetracyclines and their 4-epimers for evaluating the level of contamination with these antibiotics.

Tetracyclines are included in Annex I of European Council regulation (European Commission 1990) and maximum residue limits (MRLs) have been established. MRL for oxytetracycline, tetracycline and chlortetracycline are defined as the sum of tetracycline and its 4-epimer and is set at 100 $\mu g\ kg^{-1}$ in muscle for all food producing species. Therefore, the analytical methods employed should be capable of quantitative determination of both parent compounds and epimers of tetracyclines in food products of animal origin.

Several methods are available for analysis of tetracyclines (Oka et al., 2000), but detection and estimation of parent compounds and epimers together is a serious challenge (Cristofani et al., 2009). The property of these compounds to chelate with metals has been utilised for clean up process prior to liquid chromatography separation (Farringtn et al., 1991). Other clean up and concentration

methods for tissue extracts using combination of metal chelate affinity chromatography and C18 or polymeric stationary phases have also been tried (Cristofani et al., 2009). All these methods involve complex and lengthy sample preparation and clean up steps. In this paper, a simpler method of sample preparation followed by clean up using C18 SPE cartridge and separation of oxytetracycline, tetracycline, chlortetracycline and the 4-epimers of these three compounds present in shrimp muscle tissue by HPLC is described.

Materials and Methods

All reagents and chemicals used were of analytical or liquid chromatography grade. Tetracycline (TC), oxytetracycline dihydrate (OTC) and chlortetracycline hydrochloride (CTC) were obtained from Sigma Aldrich, USA and 4-epitetracycline hydrochloride, 4-epioxytetracycline and 4-epichlortetracycline hydrochloride were from Acros Organics, USA. Standard solutions of concentration of 200 μg ml^{-1} were prepared by dissolving the appropriate quantity of each of the reference standard in HPLC grade methanol. Working standards in the range of 25 ng ml^{-1} to 250 ng ml^{-1} were prepared by diluting the stock standard solution with methanol. Extraction buffer (McIlvaine buffer, pH 4.0±0.05 + EDTA) was prepared as per the AOAC procedure (AOAC, 2005).

Sample preparation

Matrix used in this study was muscle tissue from different species of shrimp. Accurately weighed portion (about 2 g) of well homogenised tissue sample was mixed with 15 ml of extraction buffer for 10 min using a vortex mixer. The mixture was centrifuged for 5 min at 3000 rpm (at 22°C). Supernatant was filtered to another tube through Whatman No.1 filter paper. Extraction was repeated two more times using 15 ml extraction buffer each time. SPE cartridge (Waters SEP PACK) was conditioned with 12 ml methanol followed by 12 ml water and the filtered pooled sample extract was passed through the cartridge. The cartridge was allowed to drain completely. It was washed with 6 ml of water and the analytes were eluted with 12 ml of methanol. The eluate was evaporated to dryness under nitrogen at a temperature of 40°C and the residue was dissolved in 2.0 ml of mobile phase (A:B, 85:15). The solution was filtered through 0.2 μ filter and analysed by HPLC.

Chromatographic conditions were as follows: samples and standards were analysed on Agilent 1200 Series HPLC system equipped with a diode array detector using RP C18 column (150 x 3.0 mm, 3 μ particles). Chromatography was performed in gradient mode using the mobile phase: A - 0.01 M oxalic acid in water, B - acetonitrile-methanol, 80:20. 'A' was decreased from an initial value of 95 to 85% over 10 min; further decreased to 65% in the next 9 min and was finally brought to a value of 95% over the next 1 min. Flow rate used was 0.5 ml min^{-1}, detection wavelength was 356 nm and injection volume was 30 μl.

For determining the linearity and calibration curve, mixed standard solutions containing 25, 50, 100, 150 and 250 ng ml^{-1} of each of the standards were prepared and analysed following the chromatographic conditions. Blank samples were spiked at three levels viz., 0.5, 1.0 and 1.5 times the MRL. These spiked samples were extracted and analysed for determining recovery (trueness), repeatability, within laboratory reproducibility, decision limit (CCα) and detection capability (CCβ) according to the guidelines of European Commission (2002).

Results and Discussion

The chromatographic conditions gave good separation of the six compounds (Fig 1 a). Resolution and separation were often major problems with many of the methods previously described. The effect of pH and residual silanol groups of the stationary phases are known to complicate the resolution of these compounds (Oka et al., 2000). It was found that pH and the gradient composition are critical in proper separation of all the compounds. Total run time is about 20 min. Separation of the first three compounds viz., 4-epi-TC, 4-epi-OTC and OTC were the most difficult and attempts to reduce the run time with this column has resulted in merged peak. Similar good separation of the six compounds was obtained when spiked samples were analysed. Fig 1 b shows the chromatogram of a sample of shrimp muscle tissue spiked with the six compounds at the MRL (100 ng g^{-1}) level.

An extra peak was observed at about 15 min. This peak was found in all sample blanks (shrimp muscle). Replicate analysis of shrimp muscle tissue fortified at 0.5, 1.0 and 1.5 times the permitted level confirmed that the presence of this peak did not lead to false identification or influence the quantification

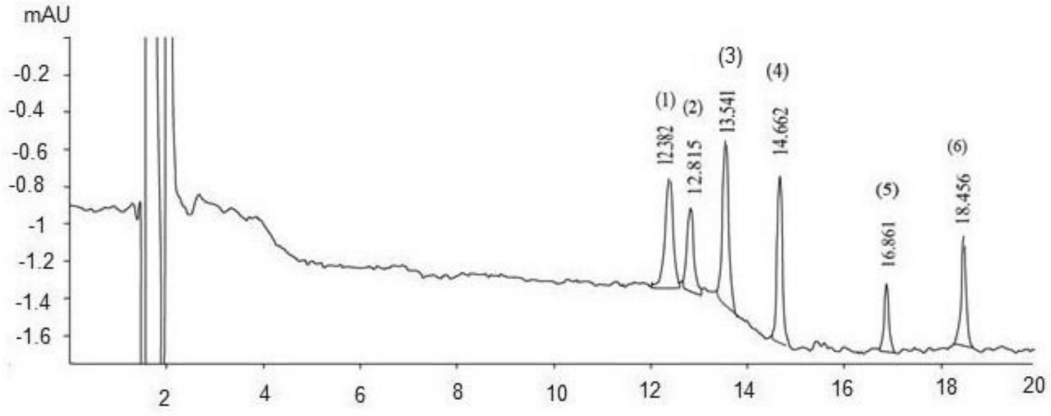

Fig. 1 a. Chromatogram of standard mixture of 100 ng ml^{-1} (1) 4-Epitetracycline, (2) 4-Epioxytetracycline, (3) Oxytetracycline, (4) Tetracycline, (5) 4-Epichlortetracycline, (6) Chlortetracycline

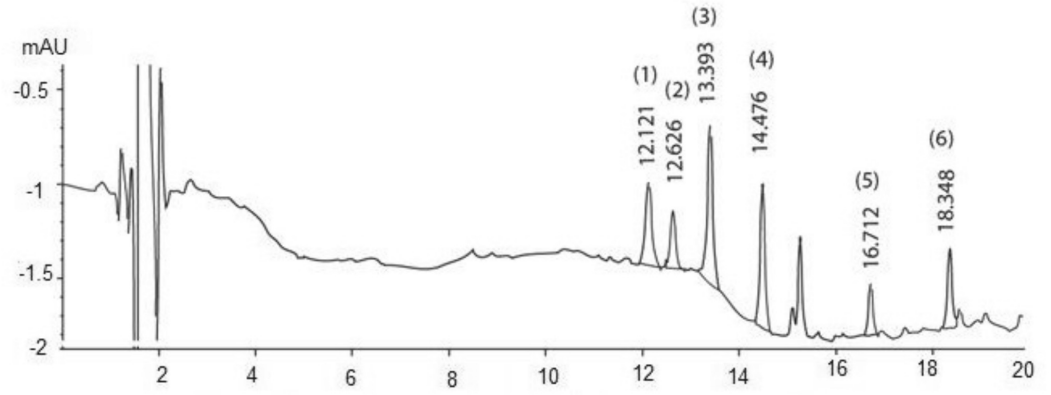

Fig. 1 b. Chromatogram of sample spiked with the six analytes at 100 ng g^{-1} concentration (1) 4-Epitetracycline, (2) 4-Epioxytetracycline, (3) Oxytetracycline, (4) Tetracycline, (5) 4-Epichlortetracycline, (6) Chlortetracycline

of the target analytes. No attempt for further clean up to eliminate this peak was made as this peak did not interfere in any way with the qualitative or quantitative results and to keep the sample preparation as simple as possible.

Linearity of response of the six analytes was tested in the range of 25 to 250 ng ml^{-1}. There was good linearity in this range as confirmed by the R^2 value, and the regression coefficients (Table 1). R^2 was above 0.999 for all compounds and the regression coefficients were highly significant in all cases.

A further confirmation of the suitability of the calibration curve was carried out by analysing standard solutions of 50, 100, 150 and 250 ng ml^{-1} and calculating the concentration using the calibration curve. It was found that the observed values agreed closely with the nominal concentrations and the deviations were less than 1% except in one case (Table 2).

Table 1. Coefficient of determination (R^2) and significance of regression coefficients

Compound	R^2	F	Significance F
Epi-TC	0.99967	3536.593	1.05E-05
Epi-OTC	0.99976	4859.789	6.5E-06
OTC	0.99975	5823.223	4.96E-06
TC	0.99968	4158.798	8.22E-06
Epi-CTC	0.99988	10923.28	1.93E-06
CTC	0.99947	2789.622	1.49E-05

Table 2. Deviations in observed concentrations calculated using calibration curve.

Compound	Nominal concentration (ppb)	Observed concentration (ppb)	Deviation (%)
TC	50, 100, 150, 250	50.14, 100.167, 149.35, 251.99	0.28, 0.16, -0.43, 0.79
OTC	50, 100, 150, 250	49.31, 100.54, 151.02, 250.71	-1.39, 0.54, 0.68, 0.28
CTC	50, 100, 150, 250	49.63, 99.72, 150.94, 249.67	-0.74, -0.27, 0.63, -0.13
Epi-TC	50, 100, 150, 250	49.68, 99.80, 149.51, 250.16	-0.64, -0.20, -0.33, 0.06
Epi-OTC	50, 100, 150, 250	50.02, 100.71, 149.56, 249.74	0.04, 0.71, -0.29, -0.10
Epi-CTC	50, 100, 150, 250	49.62, 99.189, 150.46, 251.06	-0.76, -0.81, 0.27, 0.42

Table 3. Signal to noise ratio of the six analytes spiked at 50, 100 and 150 ng g^{-1} in shrimp muscle

Compound	Signal to Noise Ratio		
	50 ng g^{-1}	100 ng g^{-1}	150 ng g^{-1}
Epi-TC	6.8	12.7	17.3
Epi-OTC	4.9	9.3	13.2
OTC	14.4	25.8	34.4
TC	12.3	23.3	32.7
Epi-CTC	4.5	8.5	11.5
CTC	7.1	13.9	18.6

The chromatographic peaks could be identified unambiguously as can be seen from the signal to noise ratio (Table 3) of the analytes.

Recovery or trueness was determined following EU guidelines (six replicates each at 0.5, 1 and 1.5 times the MRL) and the data are presented in Table 4. Recovery was comparatively low in the case of epi-oxytetracycline, but this is comparable to several other reported results (Cristofani et al., 2009, Farrington et al., 1991) and recovery for these compounds is considered to be satisfactory. Repeatability and within laboratory reproducibility data also were (Table 4) well within the acceptable limits

Table 4. Mean recovery, repeatability (CV%) and within laboratory reproducibility (CV%)

Compound	Spiked concentration (ng g^{-1})	Recovery Mean (%)	Recovery CV %	Repeatability CV (%)	Within laboratory reproducibility CV (%)
Epi-TC	50	60.91	5.08	8.24	6.75
	100	62.69	6.48	6.51	5.27
	150	59.58	8.55	9.04	9.09
Epi-OTC	50	59.86	4.59	8.38	7.57
	100	56.94	4.36	7.78	5.30
	150	58.51	5.19	5.30	6.46
OTC	50	84.20	9.63	7.56	7.82
	100	89.35	7.22	5.41	5.11
	150	83.58	6.17	7.59	6.52
TC	50	68.26	5.29	14.65	6.25
	100	77.44	6.68	8.75	7.29
	150	77.81	8.63	6.87	8.69
Epi-CTC	50	67.65	4.28	11.26	9.27
	100	71.56	4.67	5.83	10.26
	150	71.87	5.68	7.77	9.47
CTC	50	78.58	9.01	12.62	6.06
	100	71.16	6.30	8.95	5.78
	150	72.76	6.86	8.69	5.52

specified by the EU regulations (European Commission, 2002), indicating the suitability of the method.

Decision limit (CCα) and detection capability (CCβ) were calculated from the within laboratory reproducibility values and results are presented in Table 5. CCα values were in the range of 105.58 to 117.95 ng g^{-1} and the CCβ were in the range of 116.54 to 139.8 ng g^{-1}.

Table 5. CCα and CCβ for the six compounds

Compound	CCα (ng g^{-1})	CCβ (ng g^{-1})
Epi-TC	111.08	127.47
Epi-OTC	107.33	119.37
OTC	113.61	126.76
TC	109.26	126.20
Epi-CTC	117.95	139.80
CTC	105.58	116.54

The method described involves simple sample preparation and clean up steps and gives good separation of the tetracyclines and their 4-epimers. Evaluation of the performance characteristics like linearity, recovery, repeatability and within laboratory reproducibility shows that the described method could be used for the quantitative determination of these compounds in accordance with the European Union standards.

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References

AOAC (2005) Official Methods of Analysis of AOC International, 995.09, 18th edn., AOAC International, Maryland, UA

Berendsen, B. J. A. and Van Rhijn, J. A. (2006) Residue analysis of tetracyclines in poultry muscle: Shortcomings revealed by a proficiency test. Food Additives and Contaminants, 23(11): 1141–1148

Cristofani, E., Antonini, C., Tovo, G., Fioroni, L., Piersanti, A. and Galarini, R. (2009) A confirmatory method for the determination of tetracyclines in muscle using high-performance liquid chromatography with diode-array detection. Analytica Chimica Acta. 637: 40-46

European Commission (1990) Council Regulation (ECC) 2377/90, Off. J. Eur. Commun. L. 224: 1-8

European Commission (2002) Commission Decision 2002/657/EC. Implementing Council Directive 96/23/EC concerning the performance of analytical methods and the interpretation of results. Off. J. Eur. Commun. L. 221: 8-36

Farrington, W. H., Tarbin, J., Bygrave, J. and Shearer, G. (1991) Analysis of trace residues of tetracyclines in animal tissues and fluids using metal chelate affinity chromatography/HPLC. Food Addit. Contam. 8: 55-64

FAO (1990) Residues of some veterinary drugs in animals and foods. Food and Nutrition Paper No. 41/31990. Rome, FAO

Oka, H., Ito, Y. and Matsumoto, H. (2000) Chromatographic analysis of tetracycline antibiotics in foods. J. Chromatography A. 882: 109–133

Oka, H., Nakagawa, H., Harada, K. I. and Mac Neil, J.D. (Eds) (1995) Chemical Analysis for Antibiotic used in Agriculture, pp 332–346, AOAC International