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Simultaneous determination of tetracycline, oxytetracycline, and 4-epitetracycline in milk by high-performance liquid chromatography

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Abstract

A reversed-phase high-performance liquid chromatography with photodiode-array detection (HPLC–PAD) was optimized and validated for the simultaneous determination of tetracycline (TC), 4-epitetracycline (4-epiTC) and oxytetracycline (OTC) in milk. Milk samples were extracted and cleaned-up using solid-phase extraction Discovery SPE DSC-18 tubes. The separation were accomplished in less than 8 min in a Waters Symmetry C18 column at ambient temperature with a mobile phase consisted of 0.010 M aqueous oxalic acid:acetonitrile:methanol (150:20:20 by volume). Quantitation was carried out by the peak area method, with detection limits of 2.0 μg/l of each tetracycline. Average recoveries of TC, 4-epiTC and OTC from spiked samples at the four concentrations (0.25, 0.5, 1.0 and 1.5 μg/ml) were 91.5, 71.5 and 83.1, respectively, with their standard deviations less than 4% within a day and 7% between days. This method was applied for the simultaneous determination of TC, 4-epiTC and OTC in market milk samples purchased from local supermarkets. Oxytetracycline was found being present in all samples in a concentration range of 13–106 μg/l, 4-epiTC in most samples at 18–65 μg/l, TC in one sample at 44 μg/l.

Keywords: Tetracycline; Oxytetracycline; 4-Epitetracycline; Milk; Solid-phase extraction; HPLC

1. Introduction

Tetracyclines (TCs) are a group of broad-spectrum antibiotics that have been used for more than 50 years for the treatment of bacterial infections in both humans and animals. The use of tetracyclines in the United States exceeds 5.6 million pounds annually (Mellon, Benbrook, & Benbrook, 2001; Sarmah, Meyer, & Boxall, 2006). The main applications of tetracyclines in animal husbandry are for preventative treatment of bacterial infections and to increase growth rates. The rate of metabolism of TCs in dairy cows has been estimated to be 25–75% (Elmund, Morrison, Grant, & Nevins, 1971; Rysz & Alvarez, 2004) and a significant percentage of the administrated TCs is excreted in bovine milk. If these antibiotics have been improperly administrated or if the withdrawal time for the treated cows has not been observed, TCs and their degradation products may be present in marketed milk and cause harmful effects on consumers, such as possible allergic reactions, liver damage, yellowing of teeth, and gastrointestinal disturbance due to the selective pressure of antibiotics on human gut micro flora. In addition, trace amounts of antibiotic compounds in milk favor the development of antibiotic-resistant bacteria (Robert, 1996). To protect humans from the exposure to these drug residues in milk, the World Health Organization (WHO), European Union (EU) and Chinese Ministry of Agriculture have established a maximum residue limit (MRL) of 0.1 mg/l for tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) (EC, 1990; WHO, 1990; Zhuang, 1994).

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The U.S. Food and Drug Administration has set the tolerance of 0.3 mg/l for the combined residues of TC, OTC and CTC (US Code of Federal Regulations, 2003).

Both microbiological and chromatographic methods have been described for monitoring tetracyclines in milk and animal tissues. Although the microbiological assay techniques have been recommended as official and conventional methods because of their simplicity, the bioassay methods lack specificity and provide only semi-quantitative measurements of residues detected and sometimes produce false positives (British Pharmacopoeia, 1993; United States Pharmacopocia X XIII, 1995; Kurittu, Lönnberg, Virta, & Karp, 2000). Therefore, chromatographic techniques, such as TLC, IC and HPLC, and capillary electrophoresis (CE), have been developed to replace microbiological assays, especially for the quantitative determination of tetracyclines (Andersen et al. 2005; Chen and Gu, 1995; Cinquina et al., 2003; Ding and Mou, 2000; Furasawa, 2003; Huang et al., 1997; Petkovska et al., 2006; Posyniak et al., 2005; White et al., 1993; Zhao et al., 2004). A comprehensive review of these chromatographic analyses of tetracycline antibiotics in food has been published by Oka, Ito, and Matsumoto (2000) and Anderson et al. (2005).

Although the reported HPLC techniques have provided accurate and reliable measurements of tetracycline antibiotics in milk and animal tissues, most of these previous studies have been focused on parent tetracycline agents and fewer researches have been published on the determination of degradation products of TCs in milk and animal tissues (Anderson et al., 2005; White et al., 1993). It is well documented that tetracycline can undergo epimerization in solution to 4-epitetracycline (4-epiTC), which has much lower antibiotic activity. However, under certain conditions, the 4-epitetracycline can convert back to the parent tetracycline (Bergnerlang & Mikisch, 1994; Posyniak et al., 2005). Fig. 1 shows the chemical structures of tetracycline, oxytetracycline and 4-epitetracycline studied in this project. Thus, not only the concentration of tetracycline residues but also their degradation products, such as 4-epitetracycline, in animal fluids and tissues are important in understanding the potential effects of tetracycline antibiotics on human and animal health. In this study, we report on a solid-phase extraction (SPE)-HPLC-photodiode-array detection (PAD) method for the simultaneous separation and quantitation of tetracycline, oxytetracycline and 4-epitetracycline in cow milk.

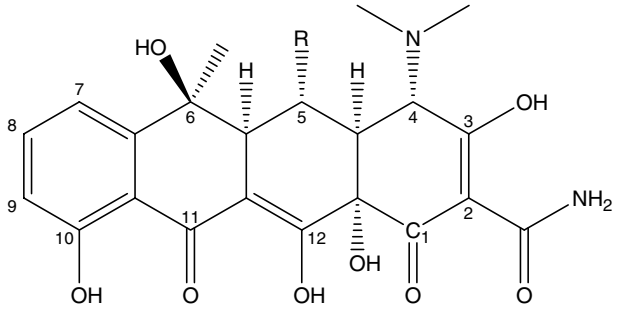

Fig. 1. Chemical structure of tetracyclines oxytetracycline R = OH; tetracycline R = H; 4-epitetracycline R = H, C4 = epimer of $N(CH_3)_2$.

2. Experimental section

2.1. Reagents and chemicals

Tetracycline and 4-epitetracycline were obtained from Spectrum Chemical Mfg. Corp. (New Brunswick, NJ). Oxytetracycline was obtained from Fluka Biochemika (Buchs, Switzerland). Methanol and acetonitrile of HPLC grade were purchased from Pharmco Chemical (Brookfield, CT). Oxalic acid, citric acid, and EDTA were from Fisher Chemical (Fairlawn, NJ). Solid-phase extraction Discovery SPE DSC-18 tubes (500 mg, 6 ml) were purchased from Aldrich Chemical (Sigma–Aldrich, St. Louis, MO). The water used was double-distilled and deionized through a Barnstead NanoPure II water purification unit (Dubuque, IA).

McIlvane/ethylendiaminetetraacetic acid (EDTA) solution used for the precipitation of protein and extraction of TCs from milk samples was prepared by adding 15.0 g of disodium hydrogen phosphate dihydrate, 3.72 g of EDTA and 13.0 g of citric acid monohydrate into 1.00 l water. The pH of this solution was adjusted to 2.90 using 1.0 M phosphoric acid or 1.0 M sodium hydroxide.

Standard stock solutions were prepared by dilution of the tetracyclines in 10 ml volumetric flasks with methanol to obtain a final concentration of 1.00×10^3 μg/ml. Standard stock solutions were stored at −10 °C and were stable for at least four weeks. These solutions were diluted to generate a series of working standard solutions for preparing calibration curves and standard addition spikes that were prepared daily.

2.2. Sample preparation

Milk samples, either slim or whole milk fortified with vitamin D from 4 different processors, were purchased from four local supermarkets in New Bedford or North Dartmouth, MA, USA, and stored in plastic containers at 4 °C in the dark until used within four days in this study. A 10.0 ml sample of milk was mixed in a 50 ml plastic centrifuge tube with 30 ml of the McIlvane/EDTA solution. The solution was agitated for 1 min using a Daigger Vortex Genie 2. The sample solutions were then centrifuged for 15 min or until the protein precipitated. The precipitate was disposed of and the solution was used for the subsequent solid-phase extraction.

The SPE cartridges were prepared by treating them with 2.0 ml of methanol at a flow rate no faster than 5.0 ml/min, followed by 2.0 ml of the McIlvane/EDTA solution. All samples were extracted by passing through the C18 SPE tube at a rate of 2.0 ml/min. The tube was then treated with 2.0 ml of 2.0% methanol in water solution to dissolve any remaining sugars. Elution was performed with 3.0 ml of

HPLC grade methanol at a rate of 5.0 ml/min. Any additional yellow characteristic color, which had not been eluted out by the initial three milliliter solvent, was eluted with extra methanol to ensure a complete recovery of TCs. Samples were dried under a stream of nitrogen to a volume of 0.2 ml and then diluted to 1.0 ml with the mobile phase. It should be noted that the fat present in the whole milk tended to clog the SPE column, resulting in long extraction time up to 15 min. However, no change in SPE recovery was observed when raw samples were spiked with standard TCs and extracted.

2.3. HPLC analysis

A Beckman System Gold 125 Solvent Module equipped with dual pumps coupled with a System Gold 168 Photodiode Array Detector (PAD) and a 508 Autosampler was employed for all analyses. A Waters Symmetry C18 column (250 × 4.6 mm, 5 μm) at ambient temperature was used for all separations. The mobile phase used was 0.010 M aqueous oxalic acid: acetonitrile: methanol (150:20:20 by volume). The mobile phase was mixed and sonicated for 5 min and then vacuum filtered through a 0.45 μm nylon filter (Whatman, Maidstone, England). The flow rate was 1.5 ml/min, with an injection volume of 50 μl, while the UV detector was set at 365 and 280 nm. All tetracycline compounds were identified by matching the retention time and their spectral characteristics examined by the PAD against those of standards (Chen, Zuo, & Deng, 2001; Zuo, Chen, & Deng, 2002). Quantitation was made based on the linear calibration curves between the concentration and peak area of standard compounds.

3. Results and discussion

3.1. HPLC separation

HPLC methods for the separation of tetracyclines in literature commonly involved the use of reversed-phase C18 or C8 columns with a mobile phase consisting of acetonitrile, methanol and aqueous oxalic acid solution (Cinquina et al., 2003; Furasawa, 2003; Posyniak et al., 2005; White et al., 1993; Oka et al., 2000). Some researchers also reported addition of ethylenediamine tetraacetic acid (EDTA) in the mobile phase. In the preliminary experiments of this investigation, several commercially available reversed-phase C18 columns with different mobile phase combinations were tested. A good baseline separation for a standard mixture of TC and OTC was achieved with an Alltech Adsorbosphere HS C18 column (4.6 × 250 mm, 7 μm) using an oxalic acid containing mobile phase (10 mM aqueous oxalic acid solution:acetonitrile:methanol, 12.5:87.5:50, v/v/v). However, this method, like the others previously reported in the literature (Cinquina et al., 2003; White et al., 1993), was found to be inadequate for our purposes because 4-epiTC cannot be resolved from the interferences in the milk sample extracts as shown in Fig. 2. A Waters Symmetry C18 column (4.6 × 250 mm, 5 μm) was finally chosen for the simultaneous determination of TC, 4-epiTC and OTC, with a mobile phase of 150:20:20 of 0.01 M oxalic acid:acetonitrile:methanol at a total flow rate of 1.5 ml/min. Fig. 3a illustrated a typical chromatogram for the separation of a mixture solution of TC, 4-epiTC and OTC. The 4-epitetracycline was eluted before OTC and TC. The mean values of retention times for three standard tetracyclines are listed in Table 1. The relative standard deviation in retention time was less than

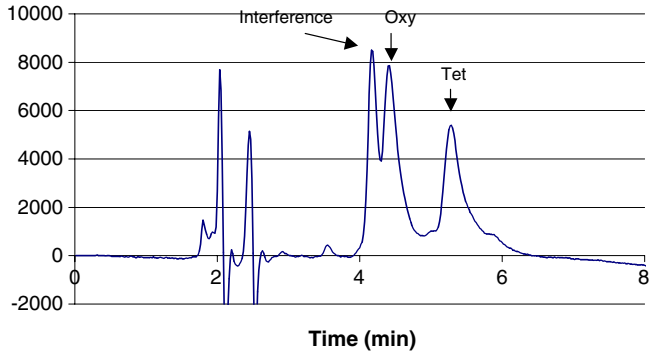

Fig. 2. Chromatogram of a milk sample spiked with tetracycline and oxytetracycline standards using a literature reported method with an Alltech Adsorbosphere HS C18 column (4.6 × 250 mm, 7 μm).

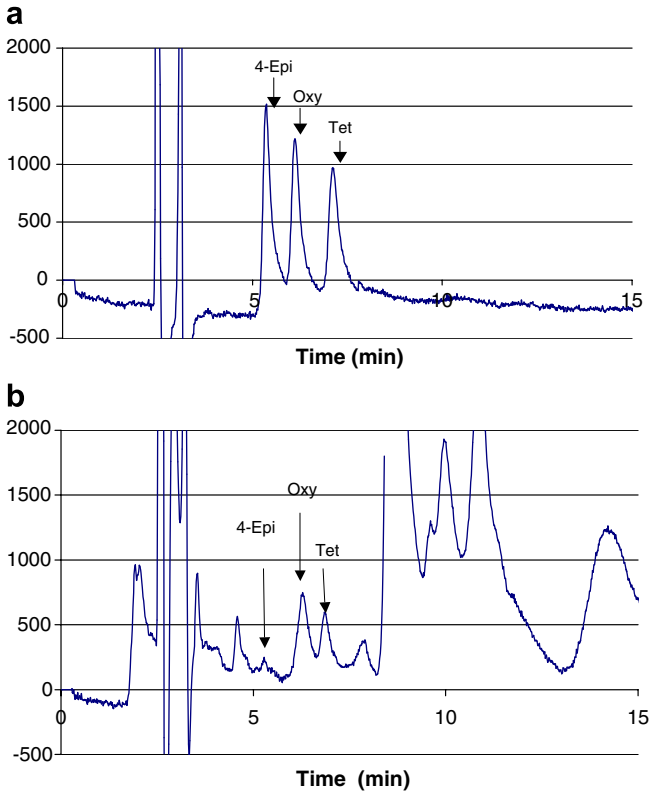

Fig. 3. (a) Chromatogram of tetracyclines (Tet), oxytetracycline (Oxy), and 4-epitetracycline (4-Epi) standard mixture (0.50 μg/ml of each TC); (b) chromatogram of milk sample 3.

Table 1
Retention times and mean recovery[a]

Tetracycline	Retention time (min)	Mean recovery (%)
TC	7.15 ± 0.02	91.5 ± 5.2
OTC	6.13 ± 0.02	83.1 ± 7.0
4-EpiTC	5.37 ± 0.02	71.5 ± 4.3

[a] All samples were introduced as 50 μl aliquots. Mean recovery is the average values of three measurements by adding standard tetracyclines into milk and water samples at concentrations of 0.5, 1.0. and 1.5 μg/l. Detection limits were measured as three times background noise.

0.04% for all TCs. Although peak tailing was apparent probably due to the adsorption on the silanol group in the reversed-phase column under the conditions used, this method produced a separation that eluted the milk interferences observed in preliminary experiments after the tetracyclines (see Figs. 3b and 4), and thus, make the simultaneous determination of TC, 4-epiTC and OTC possible.

3.2. Quantitative analysis

Calibration curves were produced at a detection wavelength of 365 nm for the three tetracyclines in the concentration range of 0.100–2.00 μg/ml. All calibration curves were linear over the concentration range tested with correlation coefficient $R^2 > 0.996$. The relative standard deviation (RSD) values of the retention times and peak areas were generally smaller than 0.04% and 2.0%, respectively. The relative standard deviations for the intra- and inter-analysis of tetracyclines are less than 4.0% and 7.0%, indicating that the developed method was very stable and had high reproducibility. The detection limit measured as three times of the background noise was 2 ng/ml of tetracycline, oxytetracycline or 4-epitetracycline, which is a few times lower than those reported with a coulometric detector (Zhao, Zhang, & Gan, 2004).

The recoveries of TCs from milk and water samples spiked at concentrations of 0.5, 1.0. and 1.5 μg/l were determined. As presented in Table 1, the average percentage recovery was favorable. The relatively low recovery rates of 4-epitetracycline may be due to its higher solubility in aqueous solution than other TCs tested. Figs. 3b and 4 provided typical chromatograms of two milk samples and of a sample spiked with 0.25 μg/ml of each tetracycline. All tetracyclines were identified according to their retention times and the spectral characteristics of their peaks against those of standards. The standard spikes were also applied to confirm the identified analyte peaks. The concentrations of tetracyclines in milk samples are given in Table 2. The OTC was found in all samples with a concentration range from 13 to 106 μg/l and was one of the major TCs detected, 4-epitetracycline was also determined in most milk samples with a concentration range from 18 to 65 μg/l. It is not surprising that TC was only detected in one milk sample. As mentioned in Section 1, tetracycline readily undergoes epimerization to form 4-epitetracycline in animal stomach fluids.

The combined concentration of tetracyclines detected in marketed milk in this study is well below the maximum residue tolerances of 0.3 mg/l established by the US Food and Drug Administration. However, whether or not the presence of trace antibiotics in milk and animal tissues below the U.S. FDA regulated level leads to the development of antibiotic-resistant bacteria and thus, adverse effects to human is still a great scientific and public concern (Kang'ethe et al., 2005; Sarmah et al., 2006). Further study on this subject is warranted.

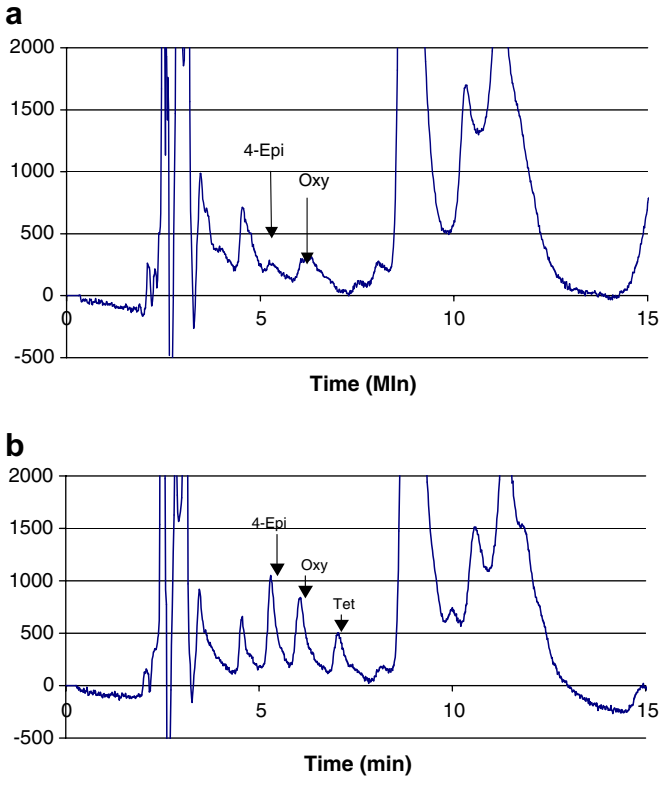

Fig. 4. Chromatogram of milk sample 6 (a) and of a sample spiked with 0.25 μg/ml of each tetracycline (b).

Table 2
The concentrations of tetracyclines found in milk samples and relative standard deviations of analyses

Milk sample and date purchased		4-Epitetracycline		Oxytetracycline		Tetracycline	
		μg/ml	% RSD	μg/ml	% RSD	μg/ml	% RSD
1	1-Aug	–	–	0.015	–	–	–
2	7-Aug	0.020	4.08	0.047	2.68	–	–
3	10-Sep	0.019	1.10	0.106	3.68	0.044	3.76
4	6-Aug	–	–	0.013	2.7	–	–
5	14-Aug	0.018	0.31	0.043	2.04	–	–
6	18-Aug	0.023	0.81	0.044	3.24	–	–
7	30-Jun	0.033	0.84	0.033	3.15	–	–
8	3-Jul	0.031	0.39	0.025	0.68	–	–
9	21-Jul	0.031	0.36	0.030	2.25	–	–
10	21-Jul	0.029	0.72	0.015	1.05	–	–
11	15-Nov	–	–	0.019	2.11		
12	12-Dec	0.031	0.52	0.029	0.93	–	–
13	22-Dec	0.065	1.12	0.045	0.88	–	–
14	21-Jul	0.029	0.72	0.015	1.05	–	–

4. Conclusions

The SPE-HPLC-PAD method developed in this study provided a sensitive, rapid and reliable approach for the simultaneous determination of tetracycline, 4-epitetracycline and oxytetracycline in milk samples. This method was successfully applied to the measurements of tetracycline antibiotic residues in marketed milk samples. Oxytetracycline and 4-epitetracycline were found to be the most common tetracycline residues in milk with a concentration range of 13–106 and 18–65 μg/l, respectively.

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References

Anderson, W., Roybal, J., Gonzales, S., Turnipseed, S., Pfenning, A., & Kuck, L. (2005). Determination of tetracycline residues in shrimp and whole milk using liquid chromatography with ultraviolet detection and residue confirmation by mass spectrometry. *Analytica Chimica Acta, 529*, 145–150.

Anderson, C. R., Rupp, H. S., & Wu, W. (2005). Complexities in tetracycline analysis – chemistry, matrix extraction, cleanup, and liquid chromatography. *Journal of Chromatography A, 1075*, 23–32.

Bergnerlang, B., & Mikisch, E. (1994). On the importance of the 4-epimers of oxytetracycline, tetracycline and chlortetracycline residues in HPLC analyses on foodstuffs of animal origin. *Deutsche Lebensmittel-Rundschau, 90*, 39–41.

British Pharmacopoeia (1993). HMSO, London.

Chen, C., & Gu, X. (1995). Determination of tetracycline residues in bovine milk, serum and urine by capillary electrophoresis. *Journal of AOAC International, 76*, 1369–1377.

Chen, H., Zuo, Y., & Deng, Y. (2001). Separation and determination of flavonoids and other phenolic compounds in cranberry juice by high-performance liquid chromatography. *Journal of Chromatography A, 913*, 387–395.

Cinquina, A., Longo, F., & Anastasi, G. (2003). Validation of a high-performance liquid chromatography method for the determination of oxytetracycline, tetracycline, chlortetracycline and doxycycline in bovine milk and muscle. *Journal of Chromatography A, 987*, 227–233.

Ding, X., & Mou, S. (2000). Ion chromatographic analysis of tetracyclines using polymeric column and acidic eluent. *Journal of Chromatography. A, 897*, 205–214.

EC. (1990). Commission Regulation 2377/90/EC. *Off. J. Eur. Commun.* L224 (1990) 1. and Council Regulation No. 508/99, *Off. J. Eur. Commun.* L60 (1990) pp. 16–52.

Elmund, G. K., Morrison, S. M., Grant, D. W., & Nevins, M. P. (1971). Role of excreted chlorotetracycline in modifying the decomposition process in feedlot waste. *Bulletin of Environmental Contamination and Toxicology, 6*, 129–132.

Furasawa, N. (2003). Isolation of tetracycline in milk using a solid-phase extraction column and water eluent. *Talanta, 59*, 155–159.

Huang, T. S., Du, W. X., Marshall, M. R., & Wei, C. I. (1997). Determination of oxytetracycline in raw and cooked channel catfish by capillary electrophoresis. *Journal of Agricultural and Food Chemistry, 45*, 2602–2605.

Kang'ethe, E. K., Aboge, G. O., Arimi, S. M., Kanja, L. W., Omore, A. O., & McDermott, J. J. (2005). Investigation of the risk of consuming marketed milk with antimicrobial residues in Kenya. *Food Control, 16*, 349–355.

Kurittu, J., Lönnberg, S., Virta, M., & Karp, M. (2000). Qualitative detection of tetracycline residues in milk with a luminescence-based microbial method: the effects of milk composition and assay performance in relation to an immunoassay and a microbial inhibition assay. *Journal of Food Protection, 63*, 953–957.

Mellon, M., Benbrook, C., & Benbrook, K. (2001). *Hogging it: Estimates of antimicrobial abuse in livestock*. Cambridge, MA, USA: Union of Concerned Scientists (UCS) Publications.

Oka, H., Ito, Y., & Matsumoto, H. (2000). Chromatographic analysis of tetracycline antibiotics in foods. *Journal of Chromatography A, 882*, 109–133.

Petkovska, E., Slaveska-Raicki, R., & Rafajlovska, V. (2006). Determination of tetracycline, oxytetracycline and chlortetracycline in milk by TLC and column chromatography using Amberlite XAD-2. *Chemia Analityczna, 51*, 275–283.

Posyniak, A., Mitrowska, K., Zmudzki, J., & Niedzielska, J. (2005). Analytical procedure for the determination of chlortetracycline and 4-epi-chlortetracycline in pig kidneys. *Journal of Chromatography A, 1088*, 169–174.

Robert, M. C. (1996). Tetracycline resistance determinants: Mechanisms of action, regulation of expression, genetic mobility, and distribution. *FEMS Microbiology Review, 19*, 1–24.

Rysz, M., & Alvarez, P. (2004). Amplification and attenuation of tetracycline resistance in soil bacteria: Aquifer column experiments. *Water Research, 38*, 3705–3712.

Sarmah, A. K., Meyer, M. T., & Boxall, A. B. A. (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere, 65*, 725–759.

US Code of Federal Regulations. (2003). Title 21, Part 556, Sections 150, 500, and 700. US Government Printing Office, Washington DC. (Chapter 1).

United States Pharmacopocia X XIII. (1995). *United States Pharmacopoeial Convention*, Rockville, MD.

White, C. R., Moats, W. A., & Kotula, K. (1993). Optimization of a liquid chromatographic method for the determination of oxytetracycline, tetracycline and chlortetracycline in milk. *Journal of AOAC International, 76*, 549–554.

WHO (1990). Technical Report Series No. 799. In: *Thirty-sixth report of the joint FAO/WHO expert committee on food additives.* WHO, Switzerland.

Zhao, F., Zhang, X., & Gan, Y. (2004). Determination of tetracyclines in ovine milk by high- performance liquid chromatography with coulometric electrode array system. *Journal of Chromatography A, 1055*, 109–114.

Zhuang, W. J. (1994). *The comprehensive compilation of maximum residue limits for pesticide, veterinary drugs in foodstuffs in the world.* Beijing: Chinese Ministry of Foreign Economics and Trade Press, p. 983.

Zuo, Y., Chen, H., & Deng, Y. (2002). Simultaneous determination of catechins, caffeine and gallic acids in green, Oolong, black and Pu-Erh teas using HPLC with a photodiode array detector. *Talanta, 57*, 307–316.