Determination of Tetracycline Antibiotics in Beef and Pork Tissues Using Ion-Paired Liquid Chromatography

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A simplified procedure was developed for determination of tetracycline antibiotics in tissues which improved stability of these compounds in sample extracts and eliminated the need for troublesome cleanup procedures. Tissues were homogenized in water. Acetonitrile (16 mL) and then 1 mL of 0.1 M H_3PO_4 were added to 4 mL of homogenate and the clear supernatant was filtered. The filtrate was mixed with hexane and dichloromethane and the resulting water layer was collected, evaporated to 1−2 mL, and filtered into autosampler vials. Ion-pairing liquid chromatography was used to separate tetracyclines from interferences in sample extracts, eliminating the need for further cleanup. Analysis was isocratic using a Phenomonex Prodigy ODS(3) column with a mobile phase of 4 mM oxalic acid, 4 mM sodium oxalate, 10 mM sodium decanesulfonate−acetonitrile (70 + 30 for oxytetracycline and tetracycline; 66 + 34 for chlortetracycline). Recoveries were generally in the 90−100% range with limits of quantitatation of 0.05−0.1 ppm. The procedure was evaluated with beef and pork muscle, liver, and kidney.

Keywords: *Tetracycline; oxytetracycline; chlortetracycline; beef; pork; tissues; determination; liquid chromatography*

INTRODUCTION

The tetracycline group of antibiotics is widely used for therapeutic treatment of farm animals and also as a feed additive. These uses have the potential to result in the presence of residues in tissues. The tetracyclines are not considered to be very toxic and maximum residue limits (MRLs) in the United States were recently increased to 2 ppm in muscle, 6 ppm in liver, and 12 ppm in kidney (USCFR, 1997). However, regulations are more stringent in other countries (Botsoglou and Fletouris, 1996) and this can create problems for firms exporting meat. Our laboratory has received reports that shipments of pork to Japan were rejected because of the presence of tetracycline residues. There is therefore a continuing need for rapid and sensitive analytical procedures for residues of tetracyclines in tissues.

Procedures described for determination of tetracycline antibiotic residues in animal tissues including fish have been reviewed in several recent publications (Moats and Harik-Khan, 1996; Botsoglou and Fletouris, 1996; MacNeil et al., 1996). Most of these use some type of cleanup of sample extracts using solid-phase extraction on some type of disposable cartridge or on chelating sepharose. These procedures can be quite laborious. A more serious problem has been that recoveries were poor and inconsistent. Carignan et al. (1993) and studies in our own laboratory (Ibrahim and Moats, 1993; White et al., 1993a; Moats and Harik-Khan, 1996) have shown that tetracyclines can be separated from interferences in sample extracts by adding alkylsulfonates as ion-pairing agents.

In previous studies (Moats, 1986; White et al., 1993b), we observed that a polymeric column gave better peak shapes and separations of tetracyclines than the silica-based LC columns which were then available. Since these earlier studies, a new generation of silica-based columns has been introduced which work well with the tetracyclines. These are less expensive and more stable than the polymer-based column.

Methods used previously in our laboratory for determination of oxytetracyline in tissues used extraction with a combination of 1 N HCl and acetonitrile (Moats, 1986; Ibrahim and Moats, 1993; Moats and Harik-Khan, 1996). However, tetracycline was markedly unstable in tissue extracts prepared by this procedure. The objectives of the present study were (1) to develop extraction/deproteinization methods which did not degrade tetracycline antibiotics and which were compatible with the HPLC analysis systems, (2) to explore the use of newer silica-based columns for HPLC analysis of tetracyclines, and (3) to develop rapid determinative procedures based on use of ion-pairing liquid chromatography, which can be done without further cleanup of sample extracts.

METHODS AND MATERIALS

Chemicals. Acetonitrile, hexane, and dichloromethane were of HPLC grade (EM Omnisolv or equivalent, EM Industries, Gibbstown, NJ). Sodium decanesulfonate (99%) was purchased from the Aldrich Chemical Co., Milwaukee, WI. Oxytetracycline (OTC), tetracycline (TET), and chlortetracycline (CTC) were purchased from Sigma Chemical Co., St. Louis, MO. The tetracycline standards were prepared, with corrections for purity, by dissolving 1 mg/mL in 0.01 M H_3PO_4. Dilutions of 100, 10, and 1 μg/mL were prepared in 0.01 M H_3PO_4. Standards were stable for 1 month when refrigerated.

Equipment. A blender with 100−300 mL blender jars, depending on the sample-size, was used. Another type of homogenizer might also be used, especially with small samples. A Buchler (Ft. Lee, NJ) vortex evaporator was used to concentrate samples under reduced pressure in conical centrifuge tubes. Other glassware and equipment required include 50-mL graduated cylinders, 15-mL conical graduated centri-

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fuge tubes (calibrated to 1 mL), 125-mL separatory funnels with Teflon stopcocks, 125-mL conical flasks, and 50-mm narrow short-stem funnels. All glassware was cleaned in special detergent (MICRO, International Products, Trenton, NJ) and rinsed in ca. 0.01 M HCL or H_2SO_4 and deionized water.

Procedures. Extraction/Deproteinization. Tissue was cut into small pieces, 1 cm or less on a side. About 15 g (or larger or smaller as desired) was weighed accurately into a 300-mL blender jar. Deionized water (3 mL/g) was added and the mixture was blended at one-half full power or lower, using a variable resistance transformer, for 2 min or until no visible pieces of tissue remained. For spiked samples for recovery experiments, an appropriate volume of standard solution was added directly to the tissue in the homogenizer before adding water and blending. A 4-mL aliquot of homogenate was transferred to a 125-mL conical flask and 16 mL of acetonitrile was added and mixed thoroughly. Then, 1.0 mL of 0.1 M H_3PO_4 was added with thorough mixing and the mixture was allowed to stand for 15 min. The supernatant was decanted through a small plug of glass wool in the stem of a funnel and 12 mL (equivalent to 0.6 g of tissue) of filtrate was collected.

Evaporative Concentration. The filtrate was transferred to a 125-mL separatory funnel and 5 mL of hexane and 5 mL of dichloromethane were added, the mixture was shaken, and the lower water layer was collected in a 15-mL graduated conical centrifuge tube calibrated to 1 and 2 mL. The organic layer in the separatory funnel was washed with 0.5 mL of water, which was combined with the other water layer. The organic layer was discarded. The tubes were placed in the vortex evaporator and warmed to 40 °C with shaking but without vacuum for about 15 min. Then vacuum (water pump) was applied cautiously, making sure that the contents did not boil out of the tube. Addition of ca. 1 mL of *tert*-butyl alcohol or 2-propanol was helpful if tubes foamed excessively. Use of a centrifugal vacuum evaporator might be advantageous to suppress foaming. After evaporation was proceeding smoothly, the contents were evaporated to <1 or 2 mL (but not to dryness). The volume was adjusted to 1 or 2 mL with water and filtered through a 25 mm, 0.45 μM PVDF filter cartridge into a 2-mL autosampler vial.

HPLC Analysis. A Phenomonex (Torrance, CA) Prodigy ODS (3) column (4.6 × 150 mm, 5 μM particle-size) was used with an injection volume of 200 μL, a flow rate of 1 mL/min, and UV detection at 370 nm.

HPLC Mobile Phase. The mobile phase was a mixture of 4 mM oxalic acid, 4 mM sodium oxalate, and 4 mM sodium decane sulfonate in water−acetonitrile, 70:30 for oxytetracycline and tetracycline; 66:34 for chlortetracycline.

RESULTS AND DISCUSSION

With all other conditions the same, retention of tetracycline antibiotics on reversed-phase LC columns can be markedly increased by adding alkylsulfonates to the LC mobile phase (White et al., 1993a). The longer the alkyl chain, the greater the increase in retention. This was also shown by Carignan et al. (1993) who used octylsulfonate as the ion-pair. The retention of other components in extracts from biological materials is also increased by the presence of alkyl sulfonates. Retentions are also affected by the pH of the mobile phase, which can be changed by changing the buffer salt/acid ratio. Different analytes are affected differently. By systematically varying the buffer composition and the ion-pair, it was possible to select optimum conditions for separating the tetracyclines from interferences in sample extracts. There was therefore no need for additional cleanup of sample extracts.

In previous studies we found that the Polymer Labs PLRP-S column, which uses an all organic polystyrene−divinylbenzene copolymer packing, gave better separations and peak-shapes with the tetracyclines than the silica-based packings which were then available (Moats, 1986; White et al., 1993b). Nominally similar polymeric columns are available from several other manufacturers. However, in our experience, performance of these was markedly inferior to the PLRP-S columns. This may account for the poor reputation of polymeric columns with some chromatographers. The PLRP-S columns had the disadvantage that they tended to develop high back pressures and reduced efficiencies with use. Since our earlier studies, a new generation of silica-based columns has been introduced which work well with tetracyclines. These include, but are not limited to, Inertsil ODS-2, Prodigy ODS-3, and Toso-Haas 80Ts columns. These newer columns are stable and give good peak shapes and separations of the tetracyclines. They thus present an attractive alternative to the polymer based column. Not all of the newer columns work well with tetracyclines including some so-called "base-deactivated" types.

In previously published methods (Moats, 1986; Ibrahim and Moats, 1993; Moats and Harik-Khan, 1996) from our laboratory for determination of tetracyclines in tissues, tissues were first blended in 1 M HCl and an aliquot of homogenate was then mixed with acetonitrile for extraction/deproteinization. This worked well with oxytetracycline. However, tetracycline was markedly unstable in extracts prepared in this manner. When the HCl concentration was reduced in an effort to improve stability of the antibiotics, the homogenates gelled. We therefore tried blending tissues first in water, then adding acetonitrile, mixing, and adding the acid last. This worked well and the acid concentrations could be readily adjusted as required for optimum results. With the silica-based columns, high levels of acid in sample extracts caused distortion or even doubling of tetracycline peaks. This was less of a problem with the polymeric column. The concentration of acid in the sample extracts was kept as low as possible, consistent with good recovery of analytes. It was also advantageous to use phosphoric acid rather than hydrochloric in the extraction procedure.

Excessive foaming could be a problem when tissues were blended in water. Blending was done at low speed. Use of another type of homogenizer might be advantageous. After adding acetonitrile and acid, in that order, the supernatant liquid was decanted through a plug of glasswool and a measured volume was collected. Prior to analysis, it was necessary to concentrate the extract and remove acetonitrile. Two approaches have been used in our laboratory to concentrate sample extracts, (1) direct evaporation and (2) a partitioning procedure using hexane with dichloromethane as a bridge solvent which favored retention of acetonitrile in the organic layer and tetracyclines in the water layer (Moats and Harik-Khan 1995, 1996). With procedure (1), considerable water insoluble material which coextracted with the acetonitrile precipitated and was removed by filtration. Procedure (1) worked well with milk (Moats and Harik-Khan, 1995). However, with extracts from liver and kidney, the amount of precipitate was greater than could be filtered easily. With procedure (2), most of the water insoluble material remained in the organic layer. Procedure (2) had the disadvantage that additional organic solvent including dichloromethane was required. However, the volume required (5 mL of each) was small and should not create an excessive waste disposal problem. No alternative was found to the use of dichlo-

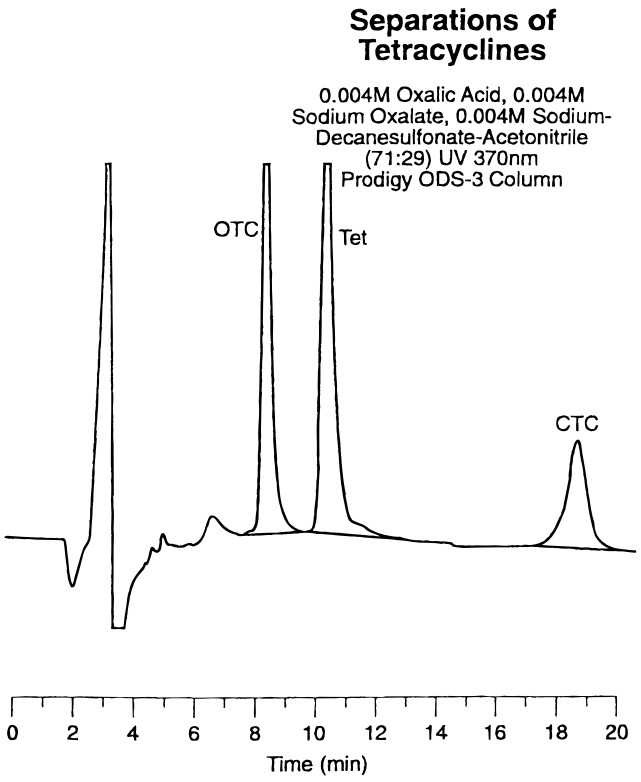

Figure 1. Isocratic elution of tetracycline standards (0.2 μg) on a Prodigy ODS-3 column.

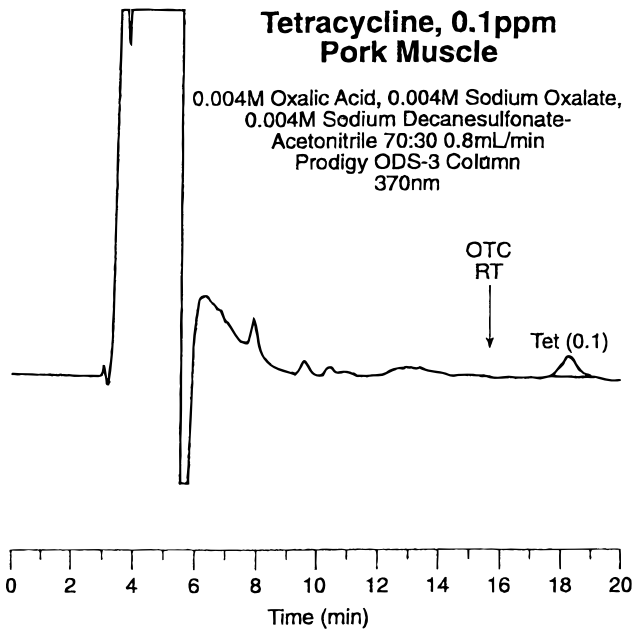

Figure 2. Tetracycline (0.1 ppm) in pork muscle.

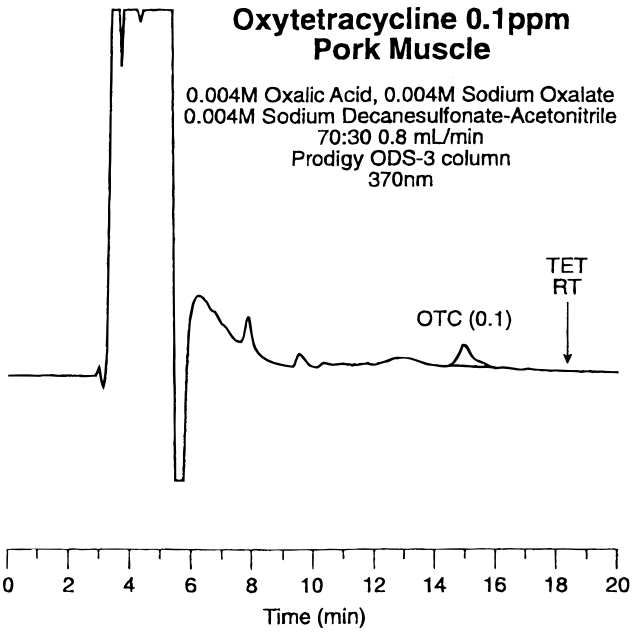

Figure 3. Oxytetracycline (0.1 ppm) in pork muscle.

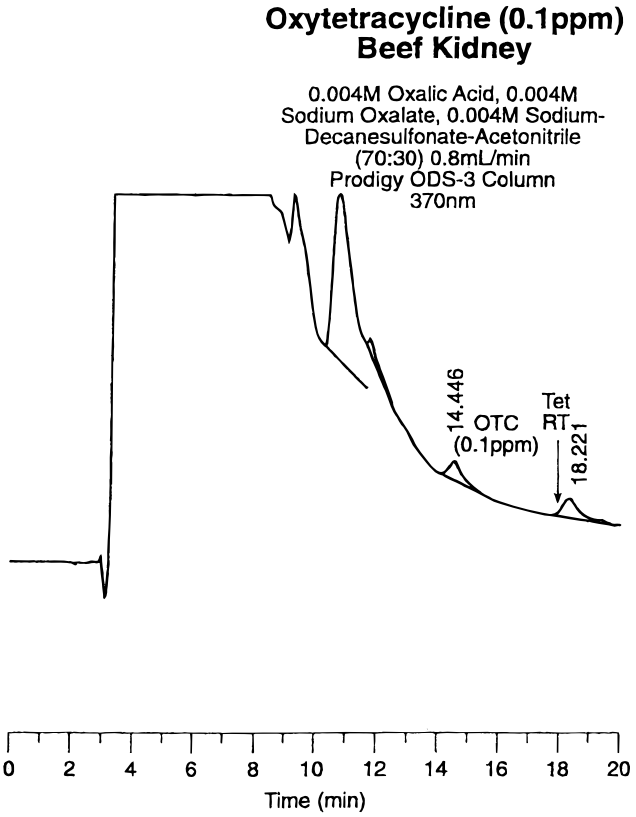

Figure 4. Oxytetracycline (0.1 ppm) in beef kidney.

romethane in this system. The tetracyclines were concentrated in the water layer which was collected. This procedure substantially reduced the volume of sample solution to be concentrated by evaporation and had the advantage that most of the water insoluble material remained in the organic layer, which was discarded. We had originally hoped that the water layer could be injected directly for HPLC analysis. However, residual organic solvent in the water layer was detrimental to column performance. The water layer was therefore concentrated to 1−2 mL by evaporation under reduced pressure. This could also be done under a stream of air or nitrogen. The concentrated sample extract was then filtered through a disposable filter cartridge into an autosampler vial for analysis. Evaporation of sample extract to dryness should be avoided since this could result in considerable losses of tetracyclines (Moats and Harik-Khan 1995, 1996).

For the present study, the Prodigy ODS(3) column was selected somewhat arbitrarily. Results with other silica-based columns were similar but not identical. In previous studies with the polymer-based column, both gradient (Moats, 1986; White et al., 1993; Ibrahim and Moats, 1993) and isocratic (Moats and Harik-Khan, 1996) were used. Gradient elution required more time

Table 1. Recovery of Tetracyclines from Tissues

tissue	amount added (ppm)	percent recovered ± sd ($N = 3$)		
		oxytetracycline	tetracycline	chlortetracycline
pork muscle	1.0	101 ± 2	100 ± 4	98 ± 2
	0.1	93 ± 5	109 ± 1	59 ± 5
pork kidney	1.0	93 ± 3	93 ± 3	110 ± 5
pork liver	1.0	99 ± 2	91 ± 1	95 ± 2
beef muscle	1.0	91 ± 1	104 ± 2	92 ± 3
beef kidney	1.0	98 ± 5	105 ± 2	92 ± 2

since it was necessary to reequilibrate the column after each run. It had the advantage that spacing of the early and late eluting peaks was more uniform and late eluting peaks were as sharp as those eluting earlier. With the silica-based columns, gradient elution gave system peaks, unrelated to the presence of any analytes, which interfered with the analysis. Therefore, isocratic analysis was used. The HPLC mobile-phases used were evaluated with regard to the shape of the tetracycline peaks, separation of tetracyclines from interferences, and baselines. For analysis of sample extracts, the ion-pairing agent selected was sodium decanesulfonate. The longer chain dodecyl sulfate did not improve separation from interferences. When oxalic acid or phosphoric acid were used as buffers, oxytetracycline was not separated from interferences. When the corresponding buffer salts were added to raise the pH, oxytetracycline was adequately separated from interferences. With oxalate, 1:1 sodium oxalate−oxalic acid was satisfactory. With phosphate, 4:1 KH_2PO_4−H_3PO_4 was optimal. In both cases, a buffer concentration of <10 mM was most satisfactory. Concentrations of oxalate buffers >10 mM generated system peaks. Optimum results were obtained with 4 mM each of acid and salt. Oxalate buffers gave a flatter baseline. Figure 1 shows separation of standards on a Prodigy ODS(3) column with isocratic elution using the oxalate buffer. Both separations and peak shapes were good. Separations of oxytetracycline and tetracycline from interferences could be improved by reducing the acetonitrile concentration to increase retention times. However, this gave excessively long retention times for late-eluting compounds such as chlortetracycline and doxycycline. It was therefore desirable to use a different mobile-phase containing about 4% more acetonitrile for determination of these late-eluting compounds. These compounds were always well separated from interferences. The epimers of the tetracyclines, which were present as minor impurities in the standards, were not separated from the parent compound with the mobile-phase selected. They would thus presumably be determined with the parent compound although no recoveries were determined with the epimers. The epimers can be separated from the parent compounds by increasing the salt/acid ratio using either oxalate or phosphate buffers.

Figure 2 shows separation of tetracycline at a concentration of 0.1 ppm from interferences in a pork muscle extract. The acetonitrile concentration was adjusted to give retention times of 12−18 min in order to ensure separation from interferences and system peaks. The baseline was free of interferences at the retention time of oxytetracycline. It should therefore be possible to further increase sensitivity by concentrating the sample extract. Figure 3 shows results with oxytetracycline in pork muscle. There was no interference at the retention time of tetracycline. Liver and kidney were more difficult to work with than muscle. Figure 4 shows results with oxytetracycline in beef kidney. Oxytetracycline was well separated from interferences. However, tetracycline had a retention time near a small interference. With the sample containing 0.1 ppm of tetracycline (Figure 5), the tetracycline peak was fused to the interference. We later found that tetracycline could be separated from this interference simply by reducing the acetonitrile concentration by 1−2% in the mobile phase to increase the retention time. There was no interference at the retention time of oxytetracycline. Chlortetracycline (results not shown) always eluted well after any interferences. As mentioned earlier, use of a mobile phase with a higher acetonitrile concentration was advantageous for isocratic determination of late eluting tetracyclines.

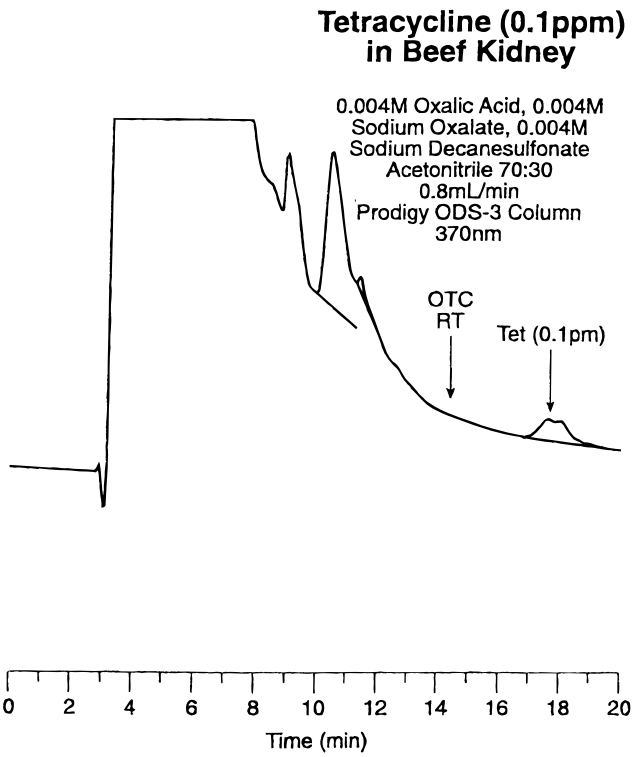

Figure 5. Tetracycline (0.1 ppm) in beef kidney.

Recoveries are summarized in Table 1. Most of the data are from samples spiked at 1 ppm and show excellent recoveries and reproducibility. Recoveries of tetracycline and oxytetracycline from muscle were also good at 0.1 ppm. Recoveries of chlortetracycline were not so good at 0.1 ppm. This was not far above the detection limit with the sample size used. Use of a larger sample size or further concentration of the extract might have improved quantitation at this level. The results shown were obtained with sample extracts concentrated to 2 mL. Sensitivity can be further improved by starting with a larger sample or concentrating the sample extracts to 1 mL or less.

In summary, the new method uses a simple extraction/deproteinization procedure, which gave nearly quan-

titative recoveries. Further cleanup of sample extracts was not required. Simple UV detection was used. The column used gave good separations and peak shapes and was used for hundreds of determinations without noticeable changes in performance. Quantitation at 0.1 ppm was readily achieved. For most purposes, UV detection was adequate. Absence of a peak of the appropriate retention time provides unequivocal evidence that an analyte is not present above the detection limits of the method. Residues, if present, can readily be quantitated by this method. With the oxalate mobile phase, LC-MS detection may also be possible if ammonium salts are used rather than sodium salts.

LITERATURE CITED

Botsoglou, N. A.; D. J. Fletouris. Analysis of Residual Antibacterials in Food of Animal Origin. In *Handbook of Food Analysis*; Nollet, L. M. L., Ed.; Marcel Dekker: New York, 1996; Vol. 2, Residues and Other Food Component Analysis, pp 1171−1264.

Carignan, G.; Carrier, K.; Sved, S. Assay of oxytetracycline residues in salmon muscle by liquid chromatography with ultraviolet detection. *J. AOAC Intl.* **1993**, 76, 325−328.

Ibrahim, A.; Moats, W. A. The effect of cooking procedures on oxytetracycline in lamb muscle. *J. Agric. Food Chem*. **1994**, *72*, 2561−2563.

MacNeil, J. D.; Martz, V. K.; Korsrud, G. O.; Salisbury, C. D. C.; Oka, H.; Epstein, R. L.; Barnes, C. J. Chlortetracycline, oxytetracycline, and tetracycline in edible animal tissues, liquid chromatographic method: collaborative study. *J. AOAC Intl.* **1996**, *79*, 405−417.

Moats, W. A. Determination of tetracycline antibiotics in tissues and blood serum of cattle and swine by HPLC. *J. Chromatogr*. **1986**, *358*, 253−259.

Moats, W. A.; Harik-Khan, R. Rapid determination of tetracycline antibiotics in milk and tissues using ion-pairing high-performance-liquid-chromatography. in *Veterinary Drug Residues: Food Safety*; Moats, W. A., Medina, M. B., Eds.; American Chemical Society: Washington, DC, 1996; pp 85−95.

United States Code of Federal Regulations. Title 21, par. 556.720, 1997.

White, C. R.; Moats, W. A.; Kotula, K. L. Optimization of a liquid chromatographic method for the determination of oxytetracycline, tetracycline, and chlortetracycline in milk. *J. AOAC Int.* **1993a**, *76*, 549−554.

White, C. R.; Moats, W. A.; Kotula, K. L. Comparative study of high performance liquid chromatographic methods for the determination of tetracycline antibiotics. *J. Liq. Chromatogr*. **1993b**, *16*, 2873−2890.

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