DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Oxytetracycline, Tetracycline, and Chlortetracycline in Milk by Liquid Chromatography with Postcolumn Derivatization and Fluorescence Detection

ANGELINA L.S. PENA, CELESTE M. LINO, and IRENE N. SILVEIRA
University of Coimbra, Faculty of Pharmacy, Laboratory of Bromatology, 3000 Coimbra, Portugal

A multiresidue method for isolation and liquid chromatographic determination of oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) in milk is presented. The sensitivity of the method is adequate to meet the needs of regulatory agencies. The European Community established 100 μg/kg as the maximum residue limit (MRL) in milk for TC, CTC, and OTC. Recoveries exceeded 80% for all tetracyclines at all levels, with good precision. Correlation coefficients of standards curves for individual tetracyclines isolated from fortified samples ranged from 0.991 for CTC to 0.998 for OTC. Other antibiotics that might interfere with analysis did not interfere with elution times of OTC, TC, and CTC. The procedure is rapid, precise, and quantitative and requires minimal preparation and minimal use of organic solvents. It can be applied to routine surveillance programs. We can prepare 10 samples for analysis in about 1.45 h.

Milk has been called mother nature's perfect food. It is perceived as wholesome enough to feed to newborn babies, infants, and young children. Consumer expectation of a safe and wholesome milk supply is greater than for other food products (1).

Antibiotics are used in food-producing animals not only to treat disease but also to maintain health and promote growth. Use of unauthorized antibiotics or the failure to follow label directions for approved antibiotics could result in unsafe antibiotic residues in food products, with potential adverse effects on human health. The possibility of bacteria developing resistance to these antibiotics and the physical persistence of residues are 2 major concerns.

A range of opinions exists about the significance of antibiotic residues in milk. As with other residues, there is no way to know with certainty what the long-term, low-level effects are, if any, particularly on children. Even when residue levels are below minimum levels that produce an allergic reaction, it is not known whether toxicity may result from continuous low-level intake (2). Adverse effects associated with high levels of antibiotic residues include allergic reactions to the residues, carcinogenicity of the residues, and evolution of microorganisms that are resistant to the antibiotics (3).

Antibiotics in milk can also pose serious problems to the dairy industry. Antibiotic residues may cause partial or complete inhibition of starter cultures used in fermentation, which would interfere in the production of fermented milk products such as yogurt and cheese and in the execution of certain quality control tests (4, 5). Therefore, monitoring antibiotic residues in food forms one part of a general monitoring program to prevent unapproved uses of antibiotics.

Oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) are tetracycline antibiotics that are widely used to treat bovine mastitis. They also are used frequently in veterinary formulations to prevent and control disease, as well as in feed additives to promote weight gain and increase feed conversion efficiency (6).

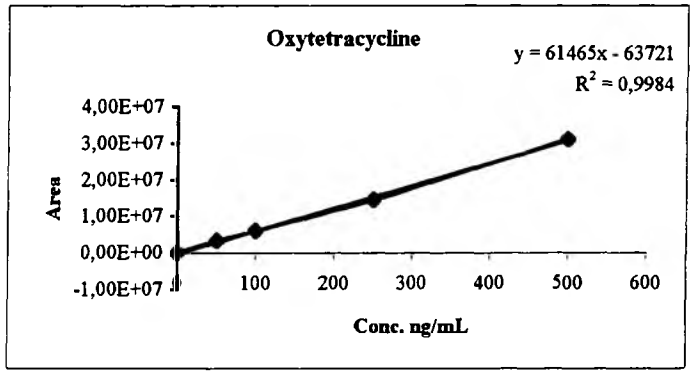
The combined total residues of OTC, TC, and CTC in bovine milk should not exceed the maximum residue level (MRL) of 100 μg/kg (7). Consequently, it is necessary to establish programs to monitor tetracycline residues in milk. These will require rapid, sensitive, and specific methods to detect and quantitate violative levels of tetracycline residues.

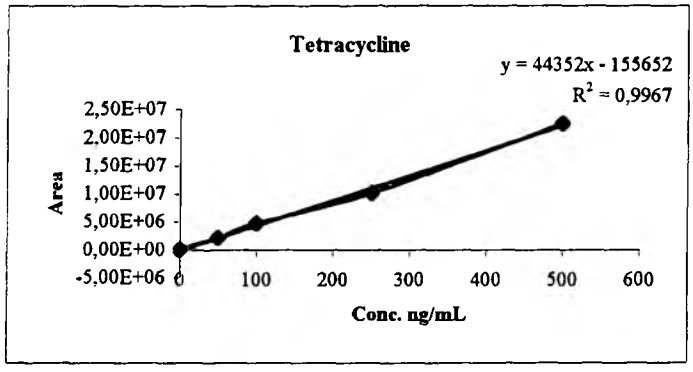
Tetracyclines in milk traditionally have been monitored through bioassays. These methods are based on inhibition of microbial growth and are useful for initial screening. But sensitivity is poor, precision appears to be variable, and specificity is questionable (8). In addition, the occurrence in milk of natural inhibitory substances such as lysozyme and lactoferrin affects the accuracy of these assays (9).

Some procedures for tetracycline residues include cleanup by solid-phase extraction (SPE) or matrix-phase dispersion. A troublesome feature of SPE for tetracyclines is poor reproducibility (10). The residues must be eluted and concentrated by evaporation, and Onji et al. (11) found that considerable losses of tetracyclines could occur during evaporation of eluates.

To avoid this problem, we developed a technique based on use of molecular weight cutoff filters (12). It consists of simple ultrafiltration of buffered milk. It was adapted from the method of Roudaut (13), which was based on the method of Thomas (14). This method avoids losses inherent in lengthy, conventional cleanup procedures (15, 16).

Received April 9, 1998. Accepted by JM August 20, 1998.





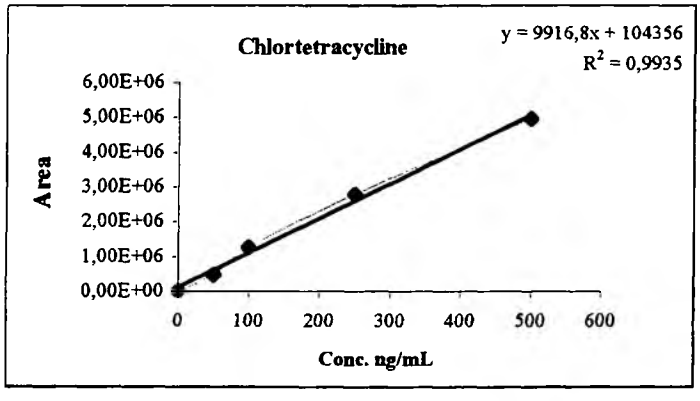


Figure 1. Calibration curves for oxytetracycline, tetracycline, and chlortetracycline.

Liquid chromatography (LC) is the method most often used to identify and quantitate tetracyclines in the greatest variety of food matrixes. Fluorescence detection is a valuable tool for tetracycline residue analysis. Interferences from food components are reduced or eliminated because of the specificity and sensitivity of this detector. Thus less extensive cleanup is required than with other detectors (17). Fluorescence detection is more specific and, in many cases, more sensitive than UV detection. Postcolumn derivatization has the advantage that a separate sample treatment step is not required and that analytes are better separated from interferences prior to derivatization. Although tetracyclines show fluorescence only under basic conditions, the fluorescence intensity of the chelate under neutral or alkaline conditions is stronger than those of free tetracyclines. This suggests the possibility of their highly sensitive detection (18).

The presented method uses the fluorescence produced when tetracyclines react with magnesium ions. Tetracyclines are separated on a reversed-phase C_8 column and made to react with magnesium acetate in boric acid buffer (pH 9.0) to produce a highly fluorescent complex (19). To resolve tetracyclines by reversed-phase LC, an acidic phase must be used, but this results in quenching of the fluorescence. The postcolumn phase at pH 9.0 adjusts the pH for optimum sensitivity.

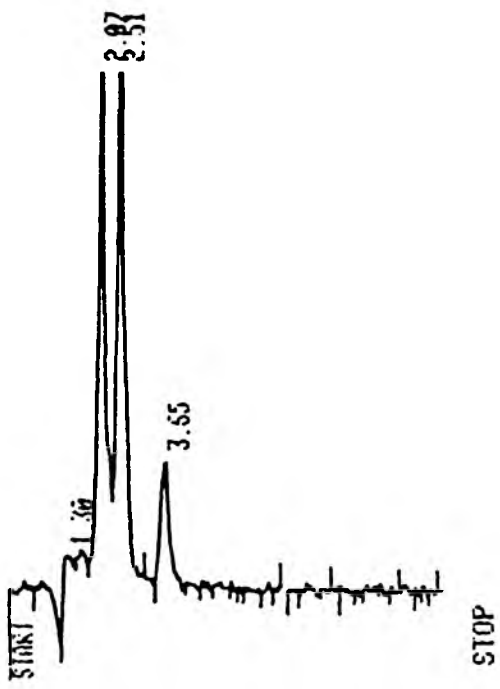


Figure 2. Chromatogram of tetracycline standards: OTC, 2.07 min; TC, 2.51 min; and CTC, 3.65 min.

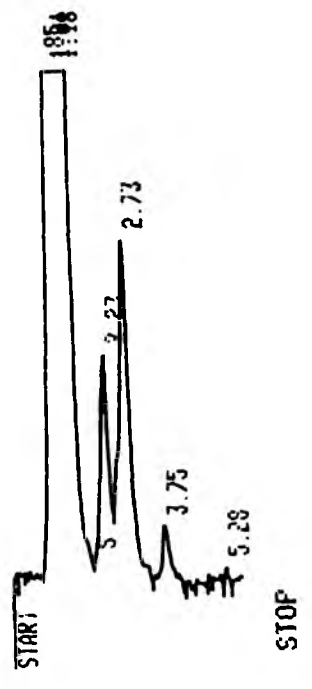


Figure 3. Chromatogram of milk spiked at 50 ng/mL.

Ten samples can be prepared in approximately 1.45 h. The procedure is rapid, precise, and quantitative and requires minimal sample preparation. It has been applied to a routine surveillance program of commercial milk in Portugal.

Experimental

Apparatus

(a) *LC system.*—Gilson Model 307 pump, a Rheodyne (Cotati, CA) Model 7125 loop injector, and a Perkin Elmer (Buckinghamshire, UK) LS-3B fluorescence detector operated at an excitation wavelength of 385 nm and an emission wavelength of 500 nm, both at a spectralband width of 10 nm. Results were recorded on a Hewlett Packard Model 3390A integrator. The LC column was Chromspher C_8, 100-3 mm (Chrompack, Bergen op Zoom, The Netherlands). Mobile phases were (*1*) 0.01M oxalate buffer–acetonitrile (60 + 40, pH 2.3) and (2) 0.01M oxalate buffer–acetonitrile–methanol (1 + 1.5 + 5, pH 2.3). They were filtered through a 0.45 μm filter under vacuum, degassed by ultrasonication, and delivered at a flow rate of 0.5 mL/min. Postcolumn reagent that had been filtered through a 0.45 μm filter under vacuum and degassed by ultrasonication was held in a brown glass bottle. This reagent was delivered at a flow rate of 0.45 mL/min and prepared daily. Isocratic elution was used throughout.

Table 1. Recovery of tetracyclines from fortified milk samples

Tetracycline	Spike level, ng/mL	Mean recovery, % ± SD (CV, %) (n = 5)
OTC	50	90.1 ± 5.6 (6.3)
	100	91.7 ± 4.8 (3.5)
	200	92.8 ± 4.0 (4.9)
TC	50	89.8 ± 5.8 (6.6)
	100	90.9 ± 6.9 (6.0)
	200	92.5 ± 7.3 (5.8)
CTC	50	84.6 ± 7.6 (8.7)
	100	86.3 ± 6.6 (7.5)
	200	89.0 ± 5.8 (6.1)

(b) *Molecular weight cutoff filters at 30 000 daltons.*—Microseparation tubes, Centricon-30 (Amicon, Beverly, MA).

(c) *Centrifuge.*—Meditronic (Selecta, Barcelona, Spain), equipped with an angular rotor.

(d) *Vortex mixer.*—Retsch (Haan, Germany).

Reagents

Chemicals were analytical reagent grade, and solvents were Lichrosolv grade.

(a) *Water.*—Purified by distillation and passage through Milli-Q System (Millipore, Bedford, MA). The water was filtered through a 0.45 μm filter under vacuum and degassed by ultrasonication.

(b) *Chemicals and solvents.*—Acetonitrile and methanol were purchased from Carlo Erba (Milan, Italy). Oxalic acid and ethylenediaminetetracetic acid (EDTA) sodium salt were reagent grade chemicals obtained from Merck (Darmstadt, Germany).

(c) *Oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC).*—Obtained from Sigma Chemical Co. (Madrid, Spain). Individual stock standard solutions were prepared in 100 mL methanol containing 100 mg neat standard corrected to 100% purity of each tetracycline and stored at –20°C in brown glass vials for a maximum period of 1 month. A stock

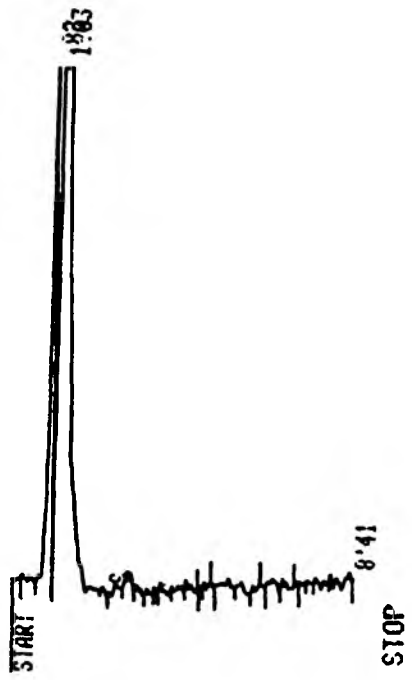


Figure 4. Chromatogram of blank sample.

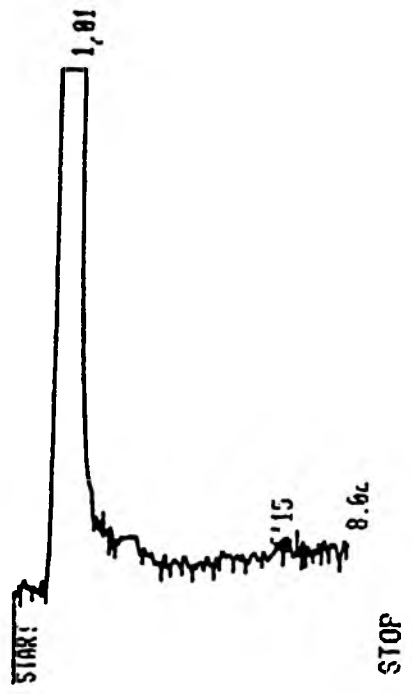


Figure 5. Chromatogram of control sample extract after addition of penicillin G, chloramphenicol, oxacillin, and neomycin.

mixed standard containing all 3 tetracyclines was prepared at 50 μg/mL in methanol. This was stable for up to 1 month when refrigerated. Working standard solutions were prepared daily from the stock mixed standard. These were stored in brown glass vials at –4°C.

(**d**) *EDTA–McIlvaine buffer (pH 4).*—Prepared by dissolving 3.72 g disodium ethylenediaminetetracetate dihydrate in McIlvaine buffer (0.2 mol/L), disodium hydrogen phosphate (7.71 mL), and 0.1 mol/L citric acid (12.29 mL).

Glassware

Glassware was cleaned with Extran MA 03 (Merck; 10%, v/v), rinsed in concentrated acid–dichromate solution, washed thoroughly with tap water, rinsed with deionized water, and dried at 80°C.

Sample Preparation

A 0.5 mL milk sample diluted with 1 mL 0.1M EDTA–McIlvaine buffer (pH 4) was stirred on a Vortex mixer, transferred to a molecular weight cutoff filter previously washed with 2 mL McIlvaine buffer to eliminate interferences, and centrifuged 60 min at 1250 × *g*. If the clear, colorless filtrates (50 μL) will not be analyzed at once, they can be stored at –20°C and protected from light for 3 days. Tetracyclines are not stable in pH 4 McIlvaine buffer (15), but no losses occur under these conditions for 3 days.

Sample Fortification

OTC, TC, and CTC solutions equivalent to 200, 100, and 50 ng/mL, respectively, were added directly to milk samples. The spiked samples were allowed to stand for 30 min before analysis.

Results and Discussion

OTC, TC, and CTC responses were linear in the range 50–500 ng/mL, with mean correlation coefficients of 0.998 for OTC, 0.996 for TC, and 0.991 for CTC (Figure 1). Because of the absence of interfering peaks, OTC, TC, and CTC concentrations as low as 50 ng/mL could be determined (signal-to-noise ratio, 1:10).

A Chromspher C_8 column consistently gave good separation and peak shape at the relatively low flow rate of 0.5 mL/min (Figure 2). Capacity factor (K') values for OTC, TC, and CTC were 1.32, 1.85, and 3.15, respectively.

Unlike gradient systems or those requiring more complex mobile phases, the simple isocratic system did not require reequilibration and eliminated the number of reagents in specific combinations required for adequate analysis.

Method accuracy was studied by spiking milk samples with each tetracycline at 3 levels (200, 100, and 50 ng/mL). Data were obtained by extracting batches of 6 samples (5 spikes and 1 reference blank) on at least 3 successive days. An external standard was used to calculate recoveries. Recoveries were generally >85% for the 3 compounds, with coefficients of variation between 8.7 and 3.5% (Table 1). Thus the method shows good accuracy and precision. A representative chromatogram of spiked sample is shown in Figure 3.

A complex formed between tetracyclines and magnesium ions is the basis for the postcolumn reaction.

The fluorescence response depends on the flow rate of postcolumn reagent, and in our study, this reached a maximum at 0.45 mL/min. The fluorescence response also depends on pH. For maximum fluorescence, a pH > 8 is essential. This pH is associated with the ionized form of the phenolic β-diketone site

of these molecules (20). The pH is adjusted with addition of postcolumn reagent, prepared at pH 9.

Excitation and emission wavelengths were optimized so that the 3 tetracyclines gave comparable peak intensities. Emission and excitation wavelengths for the 3 tetracyclines were very similar. The spectra of the products showed excitation and emission maxima at 385 and 488 nm for OTC, 380 and 499 nm for TC, and 385 and 502 nm for CTC, respectively. We have estimated the optimum excitation and emission wavelengths for the 3 tetracyclines at 385 and 500 nm, respectively.

Postcolumn derivatization does not directly affect the chromatographic properties of the tetracyclines. However, the chemical reaction must be rapid on the chromatographic time scale to preserve the chromatographic behavior. Postcolumn derivatization also has the advantages that a separate sample treatment step is not required and that analytes are better separated from interferences prior to derivatization (21).

During method development, 2 mobile phases were used—methanol–oxalic acid–acetonitrile and oxalic acid–acetonitrile. The second mobile phase gave better separation of the tetracyclines.

Because many reversed-phase materials are unstable at pH <2–3, the columns had to be flushed with a neutral solvent (e.g., water–acetonitrile, 50 + 50) for 1 h at the end of each working day. This practice significantly prolonged column life (22).

A difficulty with isolating tetracyclines is their propensity to complex with inorganic ions. Because milk contains calcium (23), which forms complexes with tetracyclines, adding EDTA to the McIlvaine buffer as a calcium chelating agent gave better recoveries.

Use of an angular rotor in the centrifuge results in a force vector that is at a constant angle to the membrane surface. As sample concentration progresses, retained material slides outward and gathers at the edge of the membrane. As a result, filtration rate remains consistently high (24).

Separations from interferences in milk extracts were good, and the selectivity of fluorescence detection puts less pressure on the need for a very efficient cleanup procedure (Figure 4). The baseline was completely flat in the area where tetracyclines eluted.

The European Community has set the MRL of OTC, TC, and CTC in milk at 100 μg/kg. The method is more than adequate to meet the needs of regulatory agencies because the detection limit for the antibiotics studied was 50 ng/mL. The method also is rapid enough to be used for direct screening of milk samples for tetracyclines.

Because other antibiotics might interfere with analysis, several compounds frequently used to treat mastitis, such as penicillin G, oxacillin, neomycin, and chloramphenicol, were also analyzed by the method. None interfered with the elution times of OTC, TC, and CTC. Chromatograms of milk containing approximately 250 ng/mL of the potentially interfering antibiotics were indistinguishable from those of control milk (Figure 5). In addition, milk samples containing 250 ng/mL of the potentially interfering drugs and 50 ng/mL of each of the 3 tetracyclines were extracted and analyzed. Recoveries of tetracyclines from these samples were similar to those from control milk fortified with tetracyclines only.

The fluorimetric detector affords a high degree of selectivity, giving the method potential to be a quantitative monitoring procedure. We have used it for this purpose for 3 months and find it to be robust and reliable.

To evaluate retention of tetracyclines in the hydrophilic membrane, we cleaned up the disposable Centricon-30 filters by soaking overnight in 0.5M $Na_2EDTA \cdot 2H_2O$ (pH 10), according to the method of Thomas (14). We chromatographically confirmed each Centricon-30 cone used after adding 2 mL McIlvaine buffer–EDTA solution and centrifuging 60 min at 1250 × *g*. No tetracycline residues were detected in any of the cleaned filters.

This procedure uses no organic solvents except in the mobile phase. Benefits include lower reagent costs and lower toxicity.

Compared with alternative methods, this approach minimizes sample handling and analysis time. The procedure is applicable to the routine analysis. We can prepare 10 samples for LC analysis in approximately 1.45 h. The LC analysis is also rapid; only 4 min/sample is required to elute the tetracyclines.

References

(1) Cullor, J.S., Van Eenennaam, A., Gardner, I., Perani, L., Dellinger, J., Smith, W.L., Thompson, T., Payne, M.A., Jensen, L., & Guterbock, W.M. (1994) *J. AOAC Int.* **77**, 862–870

(2) Collins-Thompson, D.L., Wood, D.S., & Thompson, I.Q. (1988) *J. Food Prot.* **51**, 632–633

(3) Charm, S.E. (1992) in *Analysis of Antibiotic/Drug Residues in Food Products of Animal Origin*, V.K. Agarwal (Ed.), Plenum Press, New York, NY, pp 31–56

(4) Brady, M.S., & Katz, S.C. (1988) *J. Food Prot.* **51**, 8–11

(5) Bishop, J.R., & White, C.H. (1984) *J. Food Prot.* **47**, 647–652

(6) Farrington, W.H.H., Tarbin, J., & Shearer, G. (1991) *Food Addit. Contam.* **8**, 55–614

(7) European Community Regulation 2377/90 (changed through No. 281/96)

(8) Boison, J.O., & MacNeil, D. (1995) in *Chemical Analysis for Antibiotics Used in Agriculture*, H. Oka, H. Nakazawa, K.-I. Harada, & J. MacNeil (Eds), AOAC INTERNATIONAL, Arlington, VA, pp 77–119

(9) Fletouris, D., Psomas, J.E., & Botsoglou, N.A. (1990) *J. Agric. Food Chem.* **38**, 1913–1917

(10) Mulders, E.J., & Van De Lagemaat, D. (1989) *J. Pharm. Biomed. Anal.* **7**, 1829–1835

(11) Onji, Y., Uno, M., & Tanigawa, K. (1984) *J. Assoc. Off. Anal. Chem.* **67**, 1135–1137

(12) Botsoglou, N.A., & Kufidis, D.C. (1992) in *Food Analysis by HPLC*, L.M.L. Nollet (Ed.), Marcel Dekker, Inc., New York, NY, pp 457–505

(13) Roudaut, B., & Garnier, M. (1993) in *Proceedings of Euroresidue II, Conference on Residues of Veterinary Drugs in Food*, Vol. 2, H. Haagsma, A. Ruiter, & P.B. Czedik-Eysenberg (Eds), Veldhoven, The Netherlands, pp 596–600

(14) Thomas, M.H. (1989) *J. Assoc. Off. Anal. Chem.* **72**, 564–567

(15) White, C.R., Moats, W.A., & Kotula, K.L. (1993) *J. AOAC Int.* **76**, 549–554

(16) Long, A.R., Hsich, L.C., Malbrough, M.S., Short, C.R., & Baker, S.A. (1990) *J. Assoc. Off. Anal. Chem.* **73**, 379–384

(17) McCracken, R.J., Blanchflower, W.J., Haggan, S.A., & Kennedy, D.G. (1995) *Analyst* **120**, 1763–1766

(18) Iwaki, K., Okumura, N., & Yamazaki, M. (1992) *J. Chromatogr.* **623**, 153–158

(19) Haagsma, N., & Sherpenisse, P. (1993) in *Proceedings of Euroresidue II, Conference on Residues of Veterinary Drugs in Food,* Vol. 2, H. Haagsma, A. Ruiter, & P.B. Czedik-Eysenberg (Eds), Veldhoven, The Netherlands, pp 342–346

(20) Bhatt, V.K., & Jee, R.D. (1985) *Anal. Chim. Acta* **167**, 233–240

(21) Bobbit, D.R., & Ng, K.W. (1992) *J. Chromatogr.* **624**, 153–170

(22) Kramer-Horaczynska, F. (1991) *J. Chromatogr. Sci.* **29**, 107–113

(23) Gonìalves Ferreira, F.A., & Silva Graìa, M.E. (1979) *Tabela de Composição dos Alimentos Portugueses*, Instituto Nacional de Saúde Ricardo Jorge, Lisbon, Portugal

(24) Centricon (1995) Operating Instructions, Publication I-259Q