DRUGS, COSMETICS, FORENSIC SCIENCES

Determination of Tetracycline Antibiotics in Salmon Muscle by Liquid Chromatography Using Post-Column Derivatization with Fluorescence Detection

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A simple and accurate cleanup procedure using polymeric sorbent was developed for the determination of oxytetracycline (OTC) and tetracycline (TC) residues in salmon muscle. It was applied to the analysis of 20 salmon samples during a month period. The OTC and TC residues were extracted with ethylenediaminetetracetic acid (EDTA)–McIlvaine buffer acidified at pH 4.0 and cleaned up by solid-phase extraction with a polymeric sorbent. The advantages of the polymeric sorbent over the silica-based sorbent in the cleanup of salmon muscle samples are described. A liquid chromatographic method with post-column derivatization and fluorescence detection is proposed because of its sensitivity and specificity. The average recoveries of OTC and TC from muscle salmon tissue fortified at 50, 100, and 200 μg/kg levels, ranged from 83.9 to 93.4% with a coefficient of variation between 4.09 and 5.80%. The limit of quantitation for OTC and TC in salmon muscle was 50 μg/kg.

Aquaculture production of salmon has increased worldwide in the last decade. To maximize production, aquafarming uses a wide variety of drugs for prevention and treatment of diseases. Tetracycline (TC) antibiotics are widely used for prevention and treatment of diseases in farmed salmon (1). The use of these antibiotics may lead to problems with residues in medicated fish. The concentrations found in food have no direct toxic effect, but can cause allergic hypersensitivity and bacterial resistance (2, 3). To prevent any health problems with consumers, the European Union (EU) established its maximum residue limit (MRL) as 100 μg/kg for muscle tissue (4), including salmonidae and other finfish. Thus, adequate methods for analysis of these antibiotics in salmon are needed.

Extraction and cleanup techniques for TC analysis in foods of animal origin were recently reviewed by Oka and Patterson (5). The use of pH 4.0 ethylenediaminetetracetic acid (EDTA)–McIlvaine buffer combined with solid-phase extraction (SPE) using alkyl-bonded silica cartridges for cleanup, established by Oka et al. in 1985 (6), appears to be the current standard method for the extraction of tetracyclines from tissue matrixes. However, the reversed-phase silica-based sorbents have some limitations in the analysis of TCs. TCs readily chelate to metal ions and bind with the silanol groups present in the silica-sorbents, and their recoveries vary drastically, depending on the supplier of the SPE cartridges and even from batch to batch from the same supplier (7–9).

In order to overcome these undesirable properties, pretreatment of the C18 cartridges with EDTA (10) or a silylation reagent (11), the addition of oxalic acid to the eluent of the cartridge (12), and matrix solid-phase dispersion using a C18 adsorbent with EDTA and oxalic acid (13, 14) have been tried. Metal-chelating affinity chromatography (MCAC) has also been developed (15–17). Carson (16) describes the use of ultrafiltration as further deproteinization step, while Degroodt et al. (17) use C18 SPE to concentrate the MCAC eluate, but both procedures are time-consuming. Moreover, the MCAC eluates develop a precipitate that can clog and significantly shorten the lifetime of the chromatographic column (16).

Cheng et al. (18) present a simpler method for TC analysis in porcine serum using a recently developed cartridge containing a macroporous polymer, poly(divinylbenzene-co-*N*-vinylpirrolidone). These sorbent advantages are: no impact of sorbent drying, no silanol interaction, and better recovery for polar and nonpolar compounds in complex matrixes than reversed silica-based sorbents. Zhu et al. (19) also selected this sorbent for routine analysis of TCs in groundwater and lagoon water samples because of the simplicity and ruggedness of the method relative to the C18 sorbent. This cleanup procedure was also described for swine tissues (20), shrimp (21, 22), and trout (23).

The analysis of TC residues often presents difficulties largely due to their low levels in food samples and to the complexity of the sample matrixes. The method must be sensitive while providing adequate separation, isolation, and determination as rapidly as possible. Liquid chromatography (LC) with post-column derivatization and fluorescence detection has been applied for the determination of oxytetracycline (OTC) and TC in salmon muscle. The UV detection has low

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sensitivity while mass spectrometry (MS) detection in LC has made significant improvements in recent years; however, it still requires costly instruments. In general, fluorescence detection is sensitive and selective.

The derivatization technique presents 2 general goals: increase detection sensitivity by introducing suitable fluorophores, and increase selectivity by applying a specific and selective derivatization reaction, to derive only the compounds of interest and to react them selectively in complex matrixes. Fluorescence detection was performed after post-column addition of magnesium acetate in acid boric buffer at pH 9.0, according to the Haagsma procedure (24).

The objective of this work was to develop an accurate, precise, and sensitive LC method for the determination of OTC and TC in salmon muscle.

Experimental

Reagents

(**a**) *Solvents*.—Acetonitrile and methanol, all LC grade (Carlo Erba, Milan, Italy).

(**b**) *Oxalic acid, anhydrous dibasic sodium phosphate, oxalic acid, magnesium acetate, boric acid, potassium hydroxide, and sodium hydroxide*.—Analytical reagent grade chemical (Merck, Darmstadt, Germany).

(**c**) *Oasis™ HLB extraction cartridges*.—3 mL/60 mg and 6 mL/200 mg (Waters Corp., Milford, MA).

(**d**) *Standard solutions*.—OTC, TC, and demeclocycline (DMCC), used as internal standard (IS), were obtained from Sigma Chemical (Madrid, Spain).

Individual stock standard solutions of TC and OTC were prepared at 1 mg/mL in methanol, in a volumetric flask, and were stored at –20°C in brown vials for a maximum period of 1 month. The standard working solutions were a mixture of TCs prepared by dilutions of the stock solution in methanol; each solution contained 1 μg/mL DMCC as IS. These solutions were prepared daily immediately before use. Some care was taken with these solutions: they were always kept at 4°C, protected from light, and immediately after injection in the chromatographic system they were not allowed to stand in the laboratory at room temperature.

(**e**) *Mobile phase used for analysis*.—A mixture of acetonitrile and oxalic acid 0.01M (aq. pH 2.0; 20 + 80).

(**f**) *Reagent post-column and McIlvaine buffer–EDTA solution*.—Prepared as previously described (25).

All 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.

Apparatus

LC system.—Consisted of a Model 307 pump Gilson (Gilson Medical Electronics, Villiers-le-Bel, France), a Model 7125 loop, injector (Rheodyne, Cotati, CA), and a Perkin-Elmer LS-3B spectrofluorimeter (Buckinghamshire, UK) operated at excitation wavelength 385 nm and emission wavelength 500 nm. The pump for post-column reagent was a 305 Gilson model. The results were recorded on a 3390A integrator (Hewlett-Packard, Philadelphia, PA). The LC column used was a Chromspher C_8 (100 × 3 mm id, 5 μm; Chrompack, Bergen op Zoom, The Netherlands). Injection volume was 20 mL. The spectral bandwidth was 10 nm for both excitation and emission. Mobile phase flow was 0.5 mL/min. The derivatization reagent was delivered at a flow rate of 0.45 mL/min.

Sample Preparation

Salmon obtained from a local watershed were skinned and filleted. Samples were homogenized in a food blender and stored at –20°C until analysis. The entire extraction cleanup procedure and chromatographic analysis should be completed in 1 day.

Sample Fortification

The method was validated at one-half the MRL, at the MRL, and at 2 times the MRL, according to EU guidelines. The recoveries of OTC and TC were determined from blank salmon samples spiked at 50, 100, and 200 μg/kg, and allowed to stand 30 min at 4°C, protected from light. For each fortification level, 5 replicates of the same sample were used.

Extraction and Cleanup

To 5.0 g tissue in a 125 mL polypropylene centrifuge tube, IS (1 μg/mL) was added and left in contact for 30 min at 4°C, protected from light. A 20 mL volume of pH 4.0 Na_2EDTA–McIlvaine buffer solution, and 5 mL *n*-hexane were added and mixed on a Vortex mixer for 1 min. They were shaken for 10 min on a flatbed shaker at high speed, sonicated 15 min in an ultrasonication bath, and placed on ice. The tubes were centrifuged 10 min at 2500 × *g*, the upper hexane layer was discarded, and the supernates were decanted carefully into another clean centrifuge tube. The tissue plug was resuspended twice with 20 and 10 mL pH 4.0 Na_2EDTA–McIlvaine buffer solution, and all steps were repeated, until the supernates from all 3 extractions were collected.

The combined supernates were mixed with 2 mL 20% trichloroacetic acid (TCA) and mixed on a Vortex mixer for 1 min. After centrifugation at 2500 × *g* for 15 min, they were filtered through 90 mm Whatman No. 541 filter paper with a plug of glass wool. An Oasis HLB (60 and 200 mg) cartridge was conditioned with methanol (3 mL) and water (2 mL). After extract sample application, the cartridge was washed with 2 mL water and the solute was eluted with 2 mL 1% trifluoracetic acid (TFA) in methanol. The eluate was evaporated to 0.5–1.0 mL under nitrogen stream in a water bath at 30°C. Each eluate contained 1 μg/mL DMCC as IS and was kept at 4°C to avoid degradation. All the aforementioned steps were conducted in subdued light. A blank and fortification assay (at MRL level) were included in each analytical run to check for interferences (e.g., coeluting substances) and to control accuracy. In the present work, the eluates were taken from the refrigerator just before injection in the chromatographic system.

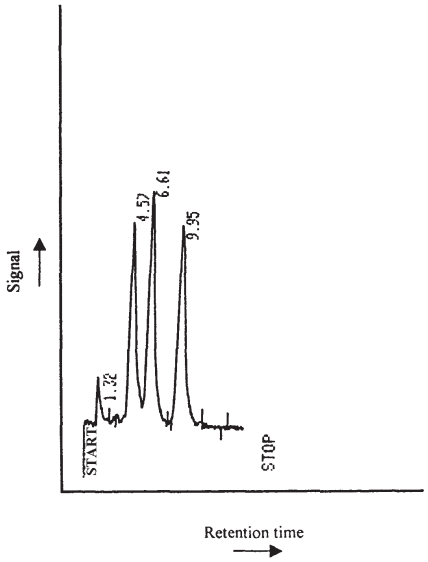

Figure 1. Liquid chromatogram of standard solution of OTC, TC (5 ng of amount injected), and IS (20 ng of amount injected).

Results and Discussion

Isocratic LC analysis on a Chromspher C_8 (100 × 3 mm id, 5 μm) column, using as mobile phase 0.01M acetonitrile–oxalic acid (pH 2.0, 20 + 80), allows complete separation of OTC, TC, and DMCC as IS. The baseline was completely flat in the area where TCs eluted (Figure 1).

The oxalate chelating buffer in the mobile phase avoids the effects caused by the presence of either residual free silanol groups or metal ion contamination in the base silica of the reversed phase, increasing the resolution of TC peaks (26). The mean retention time for OTC and TC was 4.5 and 6.7 min, respectively; the same parameter for IS was 10.0 min. On the basis of 5 parallel determinations, the precision relative standard deviation (RSD) of the retention time was 0.23% for OTC, 0.18% for TC, and 0.28% for IS, whereas the precision of peak area values was 1.15, 1.69, and 1.71% for OTC, TC, and IS, respectively.

Because many reversed-phase materials are unstable at pH 2.0, it was necessary to flush the column with a neutral solvent (e.g., water–acetonitrile, 50 + 50) for 1 h at the end of each working day. This practice contributed markedly to the prolonging of the column life (27), and the peak response was consistently high.

The spectrofluorimetric conditions followed in this study are the same as those optimized by Pena et al. (25). The spectrofluorimetric detector affords a high degree of selectivity and sensitivity to the procedure. Fluorescence detection is generally more specific than UV detection and is less prone to interferences from other compounds present in the sample matrix (28). Post-column derivatization also has the advantages that a separate sample treatment step is not required and the analytes are better separated from interferences before derivatization.

TCs were extracted from tissues using pH 4.0 McIlvaine buffer, added with EDTA, because they form chelates with metal ions in the tissue analyzed. The calcium content of raw salmon is 20 mg/100 g (29) and does not interfere in the analysis, as shown by the accuracy values obtained. Compared with Oka's method (10), this method reports the use of a sonicated step in addition to blending the tissue sample, in order to increase extraction efficiency in salmon muscle tissue. We obtained slightly salmon-colored extracts, which can be explained by the permitted addition of cantaxantine and astaxantine as additives in salmon production (30). In order to obtain cleaner extracts, 5 mL *n*-hexane was added to the extract to remove fats and pigments. The filtration through filter paper with a plug of glass wool was also effective in obtaining colorless filtrates.

OTC and TC interact not only with surface silanols, but also with metals in silica-based sorbents, which leads to low recoveries of TCs. In this method, we improved the accuracy by using a polymeric reversed-phase sorbent (Oasis HLB sorbent) for cleanup of salmon extracts, because of the advantages already mentioned: no impact of sorbent drying, no silanol interact, and no breakthrough of polar analyte (31). Two amounts of this sorbent, 60 and 200 mg, with the same particle size, were assayed. Better results, i.e., cleaner extracts, were obtained with 200 mg.

Like other authors (32, 33), we observed that addition of TCA to the combined supernatant as an additional protein denaturing step reduced SPE column blockage. Only 2 mL 1% TFA in methanol is necessary to elute TCs from the polymeric cartridge. We proceeded to a second elution with another 2 mL 1% TFA in methanol and did not detect any peaks in the chromatogram. It was not necessary to add oxalic acid to the eluent solvent and, consequently, the concentration of eluates at low volumes was possible.

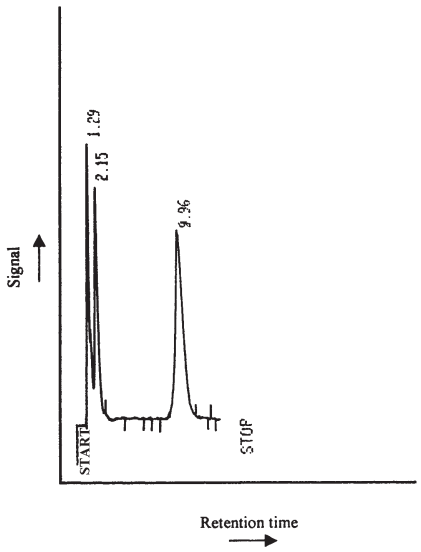

Figure 2. Liquid chromatogram of blank salmon muscle sample containing IS (20 ng of amount injected).

Table 1. Inter- and intra-assay validation results for 5 assays

Tetracycline	Fortification level, μg/kg	Recovery variation, %	Recovery mean, %	CV, % within-day	CV, % between-day
OTC	50	85.3–89.3	87.9	4.85	5.66
TC	50	83.9–87.7	85.0	5.29	7.17
OTC	100	90.8–92.0	91.7	4.23	5.00
TC	100	89.2–91.7	90.4	4.94	5.80
OTC	200	91.0–93.4	92.8	4.09	4.89
TC	200	90.5–92.7	91.6	4.85	5.38

The calibration curve was generated by plotting the ratio of peak area of the standards and the IS against their concentration ratio. Within the concentration range described, 50–1000 μg/g, linear plots were obtained for OTC and TC. The mean correlation coefficients were 0.9991 and 0.9989 for OTC and TC, respectively (n = 10, over 5 days), indicating a good correlation between TC concentrations and peak areas.

The selectivity of the method is good and no endogenous material interfered with the separation and determination of OTC and TC. Figure 2 shows a typical chromatogram of a blank assay. DMCC proved to be an appropriate IS: structurally similar, the blank chromatograms gave no additional peaks, and it has a retention time near that of the compounds studied. Although Aoyama et al. (34) observed that DMCC contains an impurity that coeluted with OTC, we did not observe its presence, in accordance with Santos et al. (35) and Cheng et al. (18). Due to the absence of any interfering peaks in sample chromatograms, concentrations as low as 50 μg/kg for OTC and TC could be determined (signal-to-noise ratio, 1:10). The sensitivity of the method is adequate to meet the needs of regulatory agencies.

In order to investigate the presence of possible degradation products, the stability of stock standard solutions was studied. They were stored at –20°C and analyzed during an 8-week period. We observed degradation after 2 weeks for IS and after 4 weeks for TC. For the period of study, we did not observe any degradation of OTC, but these results must be carefully analyzed because, with our chromatographic conditions, we cannot separate OTC from its epimer, epioxytetracycline (EOTC). Under the experimental conditions mentioned above, no appreciable decomposition was observed in the working solutions for approximately 1 working day (8–12 h). Tsuji and Robertson (36) also observed that TCs are stable in methanol for at least 8 h, with no increase in epimers.

The accuracy of the method was studied by spiking salmon muscle samples at 3 fortification levels (50, 100, and 200 μg/kg) for each TC for 3 consecutive days. Within-day accuracy and precision data were determined by analyzing, on the same day, 5 replicates of spiked samples at 3 fortification levels and 1 blank (to check for interference). The between-day accuracy and precision were also determined by extracting batches of 3 fortification levels and analyzing them on 5 consecutive days. Recoveries were generally >83.9%, and the concentration dependence of recovery was negligible, showing good accuracy of the method. For the 3 fortification levels, the coefficient of variation of within- and between-day precision ranged from 4.09 to 5.29% and from 4.89 to 7.17%, respectively, showing good repeatability (Table 1). A representative chromatogram of a spiked sample is shown in Figure 3.

Other studies performed with salmon muscle tissue, using McIlvaine buffer and SPE silica C18 columns, reported lower recoveries for OTC and TC. Reimer and Young (37) reported OTC and TC recoveries of 82 and 66%, respectively, for the 200 μg/kg fortification level, but have not evaluated recovery values for lower fortification levels. Carignan et al. (38) extracted OTC from salmon tissue with 1% metaphosphoric acid and dichloromethane and omitted the SPE cleanup step. They reported accuracy values of 62.4% for OTC for the 100 μg/kg fortification level. These differences may be explained by the advantages obtained with the Oasis HLB cartridges (18, 19, 31).

The proposed method proved to be useful and reliable for OTC and TC determination in salmon muscle with regard to European regulation requirements. The cleanup procedure is

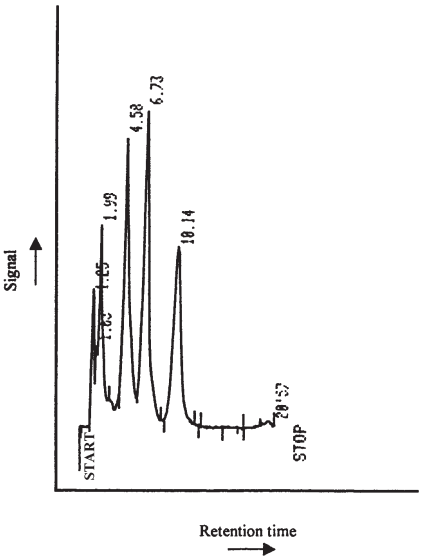

Figure 3. Liquid chromatogram of fortified assay sample containing OTC and TC (10 ng of amount injected), and IS (20 ng of amount injected).

simple and rapid and, for concentrations ranging from 50 to 200 μg/kg, this method has good accuracy, repeatibility, and sensitivity. In the 20 different farmed salmon samples analyzed, purchased in local watersheds during a 1-month period, we did not detect any residues of OTC and TC.

Conclusions

The Oasis polymer demonstrated a high efficiency as a cleanup procedure for the analysis of OTC and TC in salmon muscle. Owing to its higher sensitivity and selectivity, fluorescence detection can be regarded as a reliable substitute for UV detection. The accuracy, precision, and sensitivity of the developed method are quite appropriate for OTC and TC determination in salmon muscle with regard to the European regulation requirements.

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