DRUGS, COSMETICS, FORENSIC SCIENCES

Liquid Chromatographic Determination of Oxytetracycline in Edible Fish Fillets from Six Species of Fish

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The approved use of oxytetracycline (OTC) in U.S. aquaculture is limited to specific diseases in salmonids and channel catfish. OTC may also be effective in controlling diseases in other fish species important to public aquaculture, but before approved use of OTC can be augmented, an analytical method for determining OTC in fillet tissue from multiple species of fish will be required to support residue depletion studies. The objective of this study was to develop and validate a liquid chromatographic (LC) method that is accurate, precise, and sensitive for OTC in edible fillets from multiple species of fish. Homogenized fillet tissues from walleye, Atlantic salmon, striped bass, white sturgeon, rainbow trout, and channel catfish were fortified with OTC at nominal concentrations of 10, 20, 100, 1000, and 5000 ng/g. In tissues fortified with OTC at 100, 1000, and 5000 ng/g, mean recoveries ranged from 83 to 90%, and relative standard deviations (RSDs) ranged from 0.9 to 5.8%. In all other tissues, mean recoveries ranged from 59 to 98%, and RSDs ranged from 3.3 to 20%. Method quantitation limits ranged from 6 to 22 ng/g for the 6 species. The LC parameters produced easily integratable OTC peaks without coelution of endogenous compounds. The method is accurate, precise, and sensitive for OTC in fillet tissue from 6 species of fish from 5 phylogenetically diverse groups.

Oxytetracycline (OTC) is a drug approved by the U.S. Food and Drug Administration (FDA) to control bacterial hemorrhagic septicemia, ulcer disease, and furunculosis in salmonids and to control bacterial hemorrhagic septicemia in channel catfish (*Ictalurus punctatus;* 1). OTC also has FDA-approved uses for marking salmonids in population assessment studies and for controlling gafkemia in lobsters (1). OTC has potential as a control agent for other bacterial diseases, as well as diseases in other fish species. One of the studies required by the FDA to extend the approved uses of this antibacterial to other fish species is residue depletion.

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To conduct residue depletion studies, an analytical method that is sensitive and specific for OTC in fish fillet tissue and that can be applied to a variety of fish species would be required. Currently, no official method is listed with AOAC INTERNATIONAL for analysis of tetracyclines in fish tissue. The current method approved by the FDA for OTC in fish tissue is based on microbial inhibition and lacks the sensitivity and specificity preferred for residue depletion studies. Several sensitive and specific liquid chromatographic (LC) methods have been developed for OTC in fish fillet tissue (2–11). However, no method has shown application to fillets from multiple fish species.

The objective of this study was to develop and validate an accurate, precise, and sensitive LC method for determining OTC in edible fillets from fish species from 5 phylogenetically diverse groups. The method will be used in residue depletion studies to extend use of OTC to fish species important to public aquaculture.

METHOD

Reagents

(a) *Acetonitrile.*—LC grade (Fisher Scientific, Itasca, IL).

(b) *Water.*—Deionized to a specific resistance of >17.8 MΩ/cm with a water purification system (Barnstead E-pure, Dubuque, IA).

(c) *Ethylenediaminetetraacetic acid disodium dihydrate salt (EDTA).*—Assay purity, 99.8% (Fisher Scientific).

(d) *Citric acid monohydrate.*—Assay purity, 100.4% (Fisher Scientific).

(e) *Sodium phosphate, dibasic, heptahydrate.*—Assay purity, 98.4% (Fisher Scientific).

(f) *Trichloroacetic acid (TCA).*—6.1N, 100% w/v (Fisher Scientific).

(g) *Oxalic acid.*—Assay purity, 100.5% (Sigma Chemical, St. Louis, MO).

(h) *Octanesulfonic acid sodium salt.*—Approximately 98% pure (Sigma Chemical).

(i) *Sylon (dimethyldichlorosilane in toluene).*—Supelco, Inc., Bellefonte, PA.

(j) *OTC hydrochloride.*—Sigma Chemical.

(k) *Helium compressed gas.*—Zero grade.

(l) *Nitrogen compressed gas.*—High purity grade.

Table 1. Accuracy (recovery), precision (relative standard deviation), and sensitivity (method quantitation limit) of an LC method to determine oxytetracycline (OTC) concentrations in walleye fillet tissue fortified with OTC

Tissue concentration of OTC, ng/g	*n*	Recovery, %	Relative standard deviation, %	Method detection limit, ng/g	Method quantitation limit, ng/g
6	8	80.0	12	2	6
20	9	79.7	6.0		
100	9	85.0	4.0		
1000	9	86.8	5.0		
5000	8	86.4	2.5		

Solutions

(a) *McIlvaine/EDTA extraction buffer (McI/EDTA).*—Prepare 0.10M citric acid monohydrate solution by dissolving 21 g citric acid monohydrate in 1000 mL volumetric flask with water. Adjust volume to 1000 mL with water. Prepare 0.20M dibasic sodium phosphate solution by dissolving 54 g dibasic sodium phosphate in a 1000 mL volumetric flask with water. Adjust volume to 1000 mL with water. Mix 1000 mL 0.10M citric acid monohydrate solution with 625 mL 0.20M dibasic sodium phosphate solution. Dissolve 40.6 g EDTA in the citric acid monohydrate-dibasic sodium phosphate solution. The pH of the solution is 3.9.

(b) *2.5% EDTA solution (w/v).*—Dissolve 25.0 g EDTA in a 1000 mL volumetric flask with water. Adjust the volume to 1000 mL with water.

(c) *20% Trichloroacetic acid (TCA) solution.*—Mix 20 mL 6.1N TCA with 80 mL water.

(d) *0.30M oxalic acid eluting solution.*—Dissolve 38 g oxalic acid in a 1000 mL volumetric flask with acetonitrile. Adjust the volume to 1000 mL with acetonitrile.

(e) *LC aqueous mobile phase.*—Prepare a 0.010M oxalic acid and 0.030M octanesulfonic acid sodium salt solution by dissolving 2.5 g oxalic acid and 13 g octanesulfonic acid sodium salt in 2000 mL water. Filter solution before use and sparge with helium during use.

(f) *LC organic mobile phase.*—LC grade acetonitrile. Filter before use and sparge with helium during use.

(g) *LC mobile phase.*—Mix LC aqueous mobile phase and LC organic mobile phase, 70.5 + 29.5 (v/v).

(h) *OTC hydrochloride solutions.*—Prepare a 1 mg/mL OTC stock solution by accurately weighing and dissolving 25 mg of OTC hydrochloride in a 25 mL volumetric flask with LC mobile phase. Adjust volume to 25 mL with LC mobile phase. Prepare solutions of working standards by diluting 1 mg/mL stock solution with LC mobile phase in volumetric glassware. The concentration of OTC in working standards should range from 4.8 to 500 000 ng/mL. Working standards are stable for at least 2 weeks at room temperature (ca 21°C).

Apparatus

(a) *Commercial blender.*—Waring bar blender (Dynamics Corp. of America, New Hartford, CT).

(b) *Freezer storage bags.*—Ziploc freezer bags (Dow Chemical Co., Indianapolis, IN).

(c) *Centrifuge tube.*—50 mL, polysulfone tubes (Nalgene, Rochester, NY).

(d) *Analytical balance.*—Sartorius Model 1712 MP8 balance (Brinkmann Instruments, Westbury, NY).

(e) *Centrifuge.*—Model J2-21M (Beckman, Fullerton, CA).

(f) *Centrifuge rotor.*—Model JA-17 (Beckman).

(g) *Tissue homogenizer.*—VirTishear Model Tempest I.Q.[2] homogenizer with a macro-open-blade shaft assembly and macroblades (Virtis, Gardiner, NY).

(h) *Mechanical shaker.*—Model R4600, Mistral Multi-Mixer (Lab-line Instruments, Inc., Melrose Park, IL).

(i) *Vacuum pump.*—Model DOA-102-AA pump (Gast Manufacturing Corp., Benton Harbor, MI).

(j) *Solid-phase extraction (SPE) manifold.*—Baker spe-24G manifold (J.T. Baker, Phillipsburg, NJ).

(k) *Stopcock.*—J.T. Baker.

(l) *Inert needle insert.*—J.T. Baker.

(m) *Phenyl SPE column.*—1000 mg, 6 mL (YMC, Wilmington, NC).

(n) *Filtration column.*—20 mL, 20 mm id (YMC).

(o) *Frit.*—Polyethylene, 20 mm, 2 μm porosity (YMC).

Table 2. Accuracy (recovery), precision (relative standard deviation), and sensitivity (method quantitation limit) of an LC method to determine oxytetracycline (OTC) concentrations in Atlantic salmon fillet tissue fortified with OTC

Tissue concentration of OTC, ng/g	*n*	Recovery, %	Relative standard deviation, %	Method detection limit, ng/g	Method quantitation limit, ng/g
10	9	85.1	16	4	13
20	9	92.5	5.0		
100	9	87.1	3.1		
1000	9	85.0	1.8		
5000	9	84.6	1.5		

Table 3. Accuracy (recovery), precision (relative standard deviation), and sensitivity (method quantitation limit) of an LC method to determine oxytetracycline (OTC) concentrations in striped bass fillet tissue fortified with OTC

Tissue concentration of OTC, ng/g	*n*	Recovery, %	Relative standard deviation, %	Method detection limit, ng/g	Method quantitation limit, ng/g
10	9	92.0	17	6	22
20	9	91.3	6.2		
100	8	85.6	1.5		
1000	9	86.8	1.6		
5000	8	85.5	0.9		

(p) *Solvent filters.*—Nylaflo, 47 mm, 0.45 μm, nylon membrane, aqueous filter and FP-200, 47 mm, 0.2 μm, FP-vericel membrane, organic filter (Gelman Sciences, Ann Arbor, MI).

(q) *Sample filters.*—Acrodisc 25 mm, 0.45 μm, LC PVDF filter and Acrodisc 4 mm, 0.45 μm, CR PTFE filter (Gelman Sciences).

(r) *Syringes.*—Plastic 1 cc tuberculin syringe (Becton Dickinson and Co., Franklin, NJ).

(s) *LC sample vial.*—1.5 mL, amber, screw-cap vial with 8 mm TFE/silicon septum (Sun Brokers, Wilmington, NC).

(t) *LC system.*—Beckman Model 110A pumps (2), Model 507 autosampler (100 μL full-loop injection), and Model 406 analog interface module. Waters Model 486 tunable absorbance detector (absorbance set at 355 nm; Millipore, Bedford, MA), Alltech 330 column heater (temperature set at 45°C; Alltech, Deerfield, IL), YMC C_{18}, 250 × 4.6 mm, 5 μm analytical column and a YMC C_{18}, 20 × 4.0 mm, 5 μm guard column. Beckman version 7.11 System Gold software.

Sample Preparation

Acquire skin-off fillets from walleye (*Stizostedion vitreum*), channel catfish, striped bass (*Morone saxatilis*), and white sturgeon (*Acipenser transmontanus*) and skin-on fillets from Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*). Harden fillets in a freezer and section them into 1 cm squares. Homogenize fillet sections to a fine powder with dry ice in a commercial stainless steel blender (12). Pour fillet–dry ice powder into plastic freezer bag and store open freezer bag at ca –20°C overnight (ca 18 h) to allow dry ice to sublimate from tissue. Mix tissue periodically to promote sublimation. Seal bag and store tissue frozen until use.

Fortification of Tissue

Prepare 50 mL polysulfone centrifuge tubes by vigorously shaking capped tube with ca 2 mL 2.5% EDTA solution. Discard solution and accurately weigh 5 g homogenized tissue in rinsed tube. Spike tissue sample with an appropriate working standard to result in a nominal OTC tissue concentration of 10, 20, 100, 1000, or 5000 ng/g. The spike volume should be ≤100 μL to minimize potential solvent effects on extraction. Prepare procedure standards by spiking three 5 mL volumetric flasks with the same spike volume of working standard used to spike the tissue. Adjust volume of flasks to 5 mL with LC mobile phase and filter through a 4 mm, 0.45 μm sample filter. Procedure standards are used to verify spiking technique and amount of OTC applied to sample for recovery calculations.

Extraction

Add 8 mL McI/EDTA buffer to sample and homogenize with tissue homogenizer for 1 min at 5000 rpm. Then rinse any tissue residue off the blade and shaft into the centrifuge tube with ca 2 mL McI/EDTA buffer. Cap the tube and shake the sample on a mechanical shaker for 1 min at ca 90% of maximum speed. Centrifuge sample at a relative centrifugal force of 39 800 × *g* at ambient temperature for 10 min. Pour supernatant into a 50 mL centrifuge tube rinsed with EDTA solution. Resuspend tissue pellet in 8 mL McI/EDTA buffer with a polypropylene stirring rod. Rinse stirring rod with ca 2 mL McI/EDTA. Cap the tube, shake the contents, and centrifuge the sample and then combine supernatant with first supernatant. Resuspend pellet and repeat extraction. Add 3 mL 20% TCA to combined supernatant, shake for 1 min on mechanical shaker, and centrifuge.

Table 4. Acuracy (recovery), precision (relative standard deviation), and sensitivity (method quantitation limit) of an LC method to determine oxytetracycline (OTC) concentrations in white sturgeon fillet tissue fortified with OTC

Tissue concentration of OTC, ng/g	*n*	Recovery, %	Relative standard deviation, %	Method detection limit, ng/g	Method quantitation limit, ng/g
10	9	59.0	20	3	10
20	9	89.4	11		
100	9	90.4	2.9		
1000	9	89.9	1.9		
5000	9	87.5	1.2		

Table 5. Accuracy (recovery), precision (relative standard deviation), and sensitivity (method quantitation limit) of an LC method to determine oxytetracycline (OTC) concentrations in rainbow trout fillet tissue fortified with OTC

Tissue concentration of OTC, ng/g	*n*	Recovery, %	Relative standard deviation, %	Method detection limit, ng/g	Method quantitation limit, ng/g
12	9	98.4	8.5	4	12
20	9	89.1	3.3		
100	8	85.9	2.5		
1000	7	83.2	2.1		
5000	9	84.9	2.7		

Solid-Phase Extraction

Attach stopcocks and inert needle inserts to a vacuum-operated SPE manifold. Attach 1000 mg phenyl cartridge to each stopcock. To phenyl cartridge, connect an adapter, a stopcock, and a 20 mL filtration column fitted with one polyethylene frit. Apply 1 mL Sylon to SPE assembly and allow Sylon to saturate cartridge packing for 5 min. Aspirate Sylon through cartridge and condition SPE assembly with ca 12 mL acetonitrile followed by ca 12 mL 2.5% EDTA solution. Leave ca 1 cm 2.5% EDTA solution above phenyl cartridge packing and above filtration column frit and then close stopcocks. Pour deproteinized supernatant into 20 mL filtration column. Open stopcock above phenyl cartridge, allow supernatant to pass into phenyl cartridge, and then open stopcock below phenyl cartridge to maintain a flow rate of <5 mL/min. Leave ca 1 cm liquid above cartridge packing and above filtration column frit.

Resuspend protein pellet in 8 mL McI/EDTA buffer with polypropylene stirring rod. Rinse stirring rod with ca 2 mL McI/EDTA buffer. Cap the tube, shake the contents, and centrifuge the sample under conditions specified for tissue extraction. Pour supernatant into filtration column and aspirate supernatant through phenyl cartridge at a flow rate of <5 mL/min. When filter reservoir empties, but before phenyl cartridge dries, aspirate 5 mL McI/EDTA buffer through SPE assembly. Dry phenyl cartridge under vacuum for ca 1 min. Elute phenyl cartridge with five 100 μL applications of eluting solution into a 5 mL volumetric flask at a flow rate of <5 mL/min. Evaporate elution solvent from samples with nitrogen at room temperature. Adjust flask volume to 5 mL with LC mobile phase. Load a 1 cc syringe with sample and filter sample through an LC PVDF 25 mm, 0.45 μm sample filter into an LC sample vial. OTC in fillet extract is stable for at least 4 days under ambient laboratory conditions in LC sample vials.

LC Analysis

LC mobile phase at a flow rate of 1 mL/min is used to isolate and elute OTC from LC column. A calibration curve is developed with triplicate injections of at least 4 working standards encompassing the concentration range of OTC in sample extracts. The concentration of OTC in an extract is determined from peak area and linear regression equation of the calibration curve. The concentration of OTC in the tissue sample is calculated with the following formula:

$$\text{OTC, ng/g} = \frac{A \times B}{C}$$

where A is OTC concentration in extract as determined from linear regression equation of calibration curve (ng/mL), B is volume of extract (mL), and C is mass of tissue sample (g). Tissue concentrations of OTC were reported as ng of OTC base/g.

Method Accuracy, Precision, and Sensitivity

Accuracy was reported as percent of spiked OTC in fillet extract. Within-day precision was reported as the relative standard deviation. Method sensitivity was calculated as $10s$ (13), where s was sample standard deviation (ng/g) for the set of OTC concentrations associated with the spike level used to calculate the method detection limit. Method sensitivity was reported as method quantitation limit. Method detection limit was determined according to procedures described in the *Code of Federal Regulations* (14).

Table 6. Accuracy (recovery), precision (relative standard deviation), and sensitivity (method quantitation limit) of an LC method to determine oxytetracycline (OTC) concentrations in channel catfish fillet tissue fortified with OTC

Tissue concentration of OTC, ng/g	*n*	Recovery, %	Relative standard deviation, %	Method detection limit, ng/g	Method quantitation limit, ng/g
10	8	87.3	8.3	3	9
20	9	87.8	4.1		
100	8	89.3	1.5		
1000	8	86.0	5.8		
5000	9	89.8	1.9		

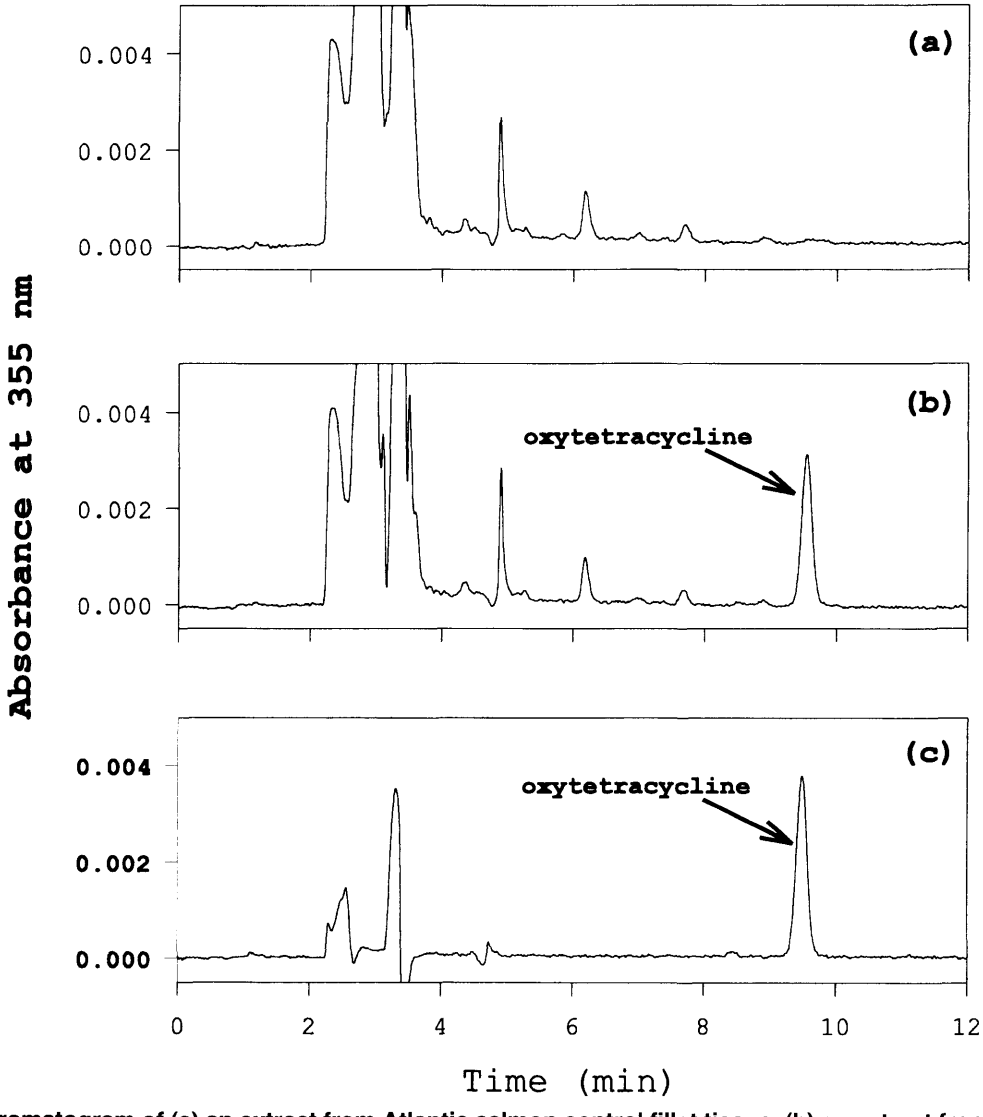

Figure 1. Chromatogram of (a) an extract from Atlantic salmon control fillet tissue, (b) an extract from Atlantic salmon fillet tissue fortified with oxytetracycline at a nominal concentration of 100 ng/g, and (c) a 100 ng/mL analytical standard of oxytetracycline. The chromatograms were developed by reversed-phase chromatography with absorbance detection at 355 nm.

Preparation of Biologically Incurred OTC

Rainbow trout weighing between 534 and 635 g were offered commercially prepared Sterling Silver Cup 5/32″ OTC-medicated fish feed (1.75 g OTC/pound feed) to obtain incurred residues of OTC. Fish were offered medicated feed once in the morning and once in the afternoon for 2 consecutive days. The mass of feed at each offering was equal to 0.55% of the total weight of fish in the culture tank. One day after the last offering of feed, fish were killed by electrocution and filleted, and the fillets were homogenized with dry ice and then analyzed for OTC.

Results

Method Accuracy, Precision, and Sensitivity

For all species, method accuracy ranged from 79.7% for walleye (Table 1) to 92.5% for Atlantic salmon (Table 2) at spike levels of 20–5000 ng/g. For all species, precision ranged from 0.9% at 5000 ng/g for striped bass (Table 3) to 11% at 20 ng/g for white sturgeon (Table 4). At OTC concentrations less than 20 ng/g, accuracy ranged from 98.4% for rainbow trout (Table 5) to 59.0% for white sturgeon (Table 4) and precision ranged from 8.3% for channel catfish (Table 6) to 20% for white sturgeon (Table 4). Method quantitation limits ranged from 6 ng/g for walleye to 22 ng/g for striped bass.

Elution profiles of fillet extracts were similar for all species. Coeluting compounds were not detected in chromatograms of extracts from any species. OTC peaks showed good symmetry and were easily integratable at all spike levels for all species. Chromatographic baselines with the greatest noise were developed from the Atlantic salmon extract (Figure 1).

Table 7. Precision (relative standard deviation) of an LC method to determine oxytetracycline (OTC) concentrations in fillet tissue from rainbow trout offered OTC medicated feed[a]

Sample name	Subsample concentration of OTC, ng/g	Mean subsample concentration of OTC, ng/g	Relative standard deviation, %
Homogenate 1A	201	212	5.5
B	199		
C	215		
D	223		
E	223		
Homogenate 2A	90	91	1.6
B	92		
C	89		
D	92		
E	92		

[a] Homogenate 1 was fillet tissue containing a biologically incurred level of OTC about one-tenth the tolerance limit for OTC in salmonids fillet tissue. Homogenate 2 was a mixture of control fillet tissue and tissue from a fish exposed to OTC (1 part control tissue and 1 part exposed tissue) resulting in a tissue OTC concentration at about one-twentieth the tolerance limit. Tissue concentrations of OTC were reported as OTC base.

Biologically Incurred OTC

At the time this portion of the study was conducted, the FDA's tolerance limit for OTC in fish fillet tissue was 100 ng/g, and the tissue prepared for this study was at 1× and 2× the tolerance limit. Recently, however, the FDA increased the tolerance limit for OTC in fish fillet tissue to 2000 ng/g (15). Therefore, method precision was verified with fillet tissue containing biologically incurred OTC residues at one-tenth and one-twentieth of the current tolerance limit.

To verify method precision with biologically incurred OTC, 5 subsamples of a fillet from a rainbow trout fed OTC-medicated feed were analyzed (Table 7). The concentration of OTC in undiluted fillet tissue was about one-tenth the current tolerance limit. Samples of fillet tissue containing incurred OTC at about 100 ng/g were prepared by mixing control tissue with tissue from exposed fish (1 part control tissue and 1 part exposed tissue). At each concentration of incurred OTC residue, method precision was consistent with method precision found for spiked tissue.

Discussion

The described method is similar to other methods for determining OTC in animal tissue (3, 5, 6, 7, 9, 11, 16) in that OTC concentrations are determined with the classical approach of extracting tissue with a solvent, centrifuging to remove particulate material, isolating OTC by SPE, and analyzing OTC by reversed-phase LC with absorbance detection. Some of the more notable techniques or procedures gleaned from other methods to develop the described method include the addition of EDTA to the extraction solvent, the addition of TCA to the extraction procedure, and the preparation of the SPE columns with a silanizing agent.

During initial method development, use of McIlvaine buffer to extract OTC from tissue (7) was only moderately successful. The ability of tetracyclines to chelate metal ions in the tissue (17) was a potential explanation for the modest recovery. To increase method accuracy by alleviating the OTC–metal ion interaction, we replaced metal apparatus with synthetic items and added EDTA to the extraction solvent (16). Additionally, the apparatus used in the solvent tissue extraction process were rinsed with an EDTA solution.

Further improvements to the method included incorporation of TCA to the combined supernatant as an additional protein-denaturing step (11). Addition of this step alleviated SPE column blockage and reduced chromatographic baseline noise.

Unique to the described method was the use of phenyl SPE columns rather than C_{18}-type SPE columns. Initial assessments of SPE columns, including C_{18} and phenyl columns, indicated that the phenyl column had greater potential for retaining OTC during sample application and demonstrated greater elution efficiency of OTC. Column efficiency was dependent on proper preconditioning steps, including application of a silanizing agent (11). Column efficiency also increased as the molarity of oxalic acid in the eluting solution was increased to 0.3M from 0.01M (5, 7). When preconditioned phenyl columns were spiked with 5000 ng OTC and the columns were washed with 70 mL McI/EDTA buffer after spiking, the mean recovery of OTC exceeded 98%.

Development of LC parameters was based on previously reported methods (7, 8, 10, 11). Injection volumes were limited to 100 µL, because larger injection volumes produced poor peak symmetry and increased baseline noise. During development of LC parameters, chromatograms of extracts from various fish species showed interference peaks at or near the retention time of OTC. Octanesulfonic acid was added to the mobile phase (10) to modify the elution profile so that potentially interfering compounds eluted more than 15 s from OTC.

Conclusions

The intent of this study was to produce a method for determining OTC in fish fillet tissue that would be suitable for drug

residue depletion studies conducted with virtually any species of fish. The method we developed and validated was a consolidation of ideas from existing methods. In a generalized comparison, the accuracy, precision, and sensitivity of the method we described were equal to or better than those attributes reported from previously published methods.

The described method for OTC produced easily integratable OTC peaks without coelution of endogenous compounds from fillet extracts of walleye, Atlantic salmon, striped bass, white sturgeon, rainbow trout, and channel catfish. The method produces accurate and precise results at fillet concentrations ranging from about 20 ng/g (100 times less than the FDA-established tolerance level of 2000 ng/g) to 5000 ng/g. This method is suitable for conducting residue depletion studies to expand the approved uses of OTC to other fish species important to public aquaculture.

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