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Simultaneous multiresidue determination of tetracyclines and fluoroquinolones in catfish muscle using high performance liquid chromatography with fluorescence detection☆

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Abstract

Efficient methods are needed for analysis of veterinary drug residues in food. A number of methods are available for single analytes. Multiresidue methods are now increasingly available. It is still rare, however, to find methods not involving mass spectrometry which allow for analysis of more than one class of drug residue. An efficient multiresidue method for the simultaneous determination of fluoroquinolones (FQs) and tetracyclines (TCs) in catfish muscle has now been developed. This method involves an extraction of the analytes with a mixture of acetonitrile and citrate buffer containing magnesium chloride. After centrifugation and evaporation of the supernatants, the residues are determined using high performance liquid chromatography with fluorescence detection. With this method, five fluoroquinolones and three tetracyclines were determined in fortified catfish muscle at levels of 20, 50, and 100 ng g^{-1}. Average recoveries for ciprofloxacin (CIP), sarafloxacin (SAR), danofloxacin (DANO), enrofloxacin (ENRO), difloxacin (DIF), oxytetracycline (OTC), tetracycline (TC), and chlortetracycline (CTC) were in the range of 60–92% with good relative standard deviations. The limits of quantitation ranged from 0.15 to 1.5 ng g^{-1}. Utilization of the method to successfully analyze catfish muscle samples incurred with enrofloxacin and with oxytetracycline is described.
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1. Introduction

Use of antibiotics in food animals has led to concerns regarding residues which might be present in food, as well as potential increases in microbial resistance. Efficient methods are required for monitoring residue levels to ensure safety of the food supply. Fluoroquinolones (FQs) and tetracyclines (TCs) are classes of antibiotics which could potentially be used in fish. Although the U.S. Food and Drug Administration does not permit the use of FQs in fish, the E.U. has set maximum residue levels (MRLs) in finfish for danofloxacin (DANO), enrofloxacin (ENRO), difloxacin (DIF), flumequine and oxolinic acid ranging from 100 to 600 ng g^{-1} (muscle and skin) [1,2]. Both the U.S. and E.U. have set tolerances or MRLs for tetracycline (TC), oxytetracycline (OTC) and chlortetracycline (CTC) in finfish of 2 μg g^{-1} (sum of the TCs, U.S.) or 100 ng g^{-1} (each of the TCs, E.U.) [1,2].

Multiresidue methods which increase the efficiency of analysis are available for determination of FQs [3–6] and TCs [7–9] in fish. Multiresidue methods which will simultaneously determine more than one class of veterinary drug in any matrix are still rare and are largely restricted to either microbiological, electrochemical or conductimetric screening assays [10–12], or liquid chromatography-mass spectrometry (LC-MS) methods [13–15]. Screening methods generally do not allow for differentiation between members of a class. LC-MS methods are capable of identifying individual antibiotics within a class but involve relatively expensive and complex instrumentation, which may not

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always be available for routine monitoring. LC-MS methods can be invaluable when confirmation is required, but are not always necessary for quantitation.

Multiresidue methods are not currently available for the simultaneous determination of individual TCs and FQs in fish. We have recently developed a LC-fluorescence method for the simultaneous determination of TCs and FQs in chicken muscle [16]. The goal of this work was to determine if this approach would be applicable to fish, a different, but also commercially important food matrix. This work is significant as it would provide a novel, alternative approach for multiresidue determination of members of more than one class of antibiotics in fish simultaneously. This efficient approach would be particularly useful in cases where multiclass LC-MS methods are not available, where an LC-MS instrument is not available for routine monitoring, or where quantitation alone is required.

2. Experimental

2.1. Reagents

Tetracycline (95%), oxytetracycline hydrochloride (OTC·HCl, 95%) and chlortetracycline hydrochloride (CTC·HCl, 83%) were obtained from Sigma (St. Louis, MO, USA), ciprofloxacin (CIP) was obtained from Bayer (Kansas City, MO, USA), danofloxacin was obtained from Pfizer (Groton, CT, USA), and sarafloxacin hydrochloride (SAR·HCl) and difloxacin hydrochloride (DIF·HCl, 89.0%) were obtained from Abbott (North Chicago, IL, USA). Enrofloxacin (99.9% and >98%) was obtained from Bayer and Sigma, respectively. Malonic acid and magnesium chloride hexahydrate (99.0%) were from Sigma, citric acid monohydrate was from Mallinckrodt (Paris, KY, USA), and ammonium hydroxide (redistilled) was from GFS chemicals (Columbus, OH, USA). Acetonitrile and methanol were obtained from J.T. Baker (Phillipsburg, NJ, USA). Deionized water prepared with a Barnstead (Dubuque, IA, USA) E-pure system was used to prepare all aqueous solutions. All solutions to be used for liquid chromatography were filtered through a nylon 0.45 μm filter before use.

2.2. Buffer solutions

Extraction Buffer (0.1 M citrate, 100 mM magnesium chloride, pH 5.0 with NH_4OH) was prepared in the following manner: citric acid monohydrate (10.5 g) and magnesium chloride hexahydrate (10.2 g) were dissolved in <500 mL water. The pH of this solution was then adjusted to 5.0 using concentrated NH_4OH. After transfer to a 500 mL volumetric flask, water was added to a total of 500 mL, and the resultant solution mixed well.

LC Buffer (0.1 M malonate, 50 mM magnesium chloride, pH 6.5 with NH_4OH) was prepared in the following manner: malonic acid (10.4 g) and magnesium chloride hexahydrate (10.2 g) were dissolved in <1 L water. The pH of this solution was then adjusted to 6.5 using concentrated NH_4OH. After transfer to a 1 L volumetric flask, water was added to a total of 1 L, and the resultant solution mixed well.

2.3. Preparation of standards

Stock solutions (200 $\mu g\,mL^{-1}$) were prepared of OTC and CTC in methanol. A TC stock solution (200 $\mu g\,mL^{-1)}$ was prepared in acetonitrile to avoid its rapid degradation in methanol. These solutions were stored at 4 °C and prepared fresh monthly. A fortification mixture of TC, OTC and CTC (2 $\mu g\,mL^{-1}$) in LC Buffer was prepared from these stock solutions on the day of an experiment. When needed for lower fortification levels, an additional dilution (1 $\mu g\,mL^{-1}$) of the TC fortification mixture was prepared on the day of an experiment. Stock solutions (100 $\mu g\,mL^{-1}$) were each prepared of CIP, SAR, DANO, ENRO and DIF in 0.03 M NaOH. These FQ stock solutions were stored at 4 °C and were prepared fresh every 6 months. A fortification mixture of the five FQs (2 $\mu g\,mL^{-1}$) in 0.1 M phosphate buffer, pH 9 was prepared from these stock solutions, stored at 4 °C and prepared fresh monthly. When needed for lower fortification levels, an additional dilution (400 $ng\,mL^{-1}$) in LC Buffer was prepared on the day of an experiment.

2.4. Preparation of catfish muscle

2.4.1. Control fish muscle

Control catfish muscle was obtained from Carolina Classics Catfish (Ayden, NC, USA) or were supplied from experimental channel catfish stocks produced as described previously [17] at the Harry K. Dupree Stuttgart National Aquaculture Research Center (Stuttgart, AR, USA). Catfish muscle was cut up into small pieces, homogenized with a food processor (Robot Coupe, Ridgeland MS, USA), and then stored at −80 °C until use.

2.4.2. Preparation of OTC and ENRO medicated diets

Antibiotic was incorporated into a commercial production feed ration (Arkat, Dumas, AR, USA) as described [18] to provide either 100 mg OTC $kg\,fish^{-1}\,day^{-1}$ or 10 mg ENRO $kg\,fish^{-1}\,day^{-1}$ when fish are fed 3% of their body weight.

2.4.3. Preparation of incurred fish

OTC-incurred fish were produced as described [18]. ENRO-incurred fish were produced in a similar manner, except as follows: Four flow-through tanks were used, each stocked with 16 fish, 1947 ± 123 g (mean ± standard deviation). The fish were allowed to acclimate in a 13-day period, which was followed by a 10-day ENRO medication period and a 10-day withdrawal period. Water quality parameters were measured every 8 days (Hach DR/2010, HACH Chemical Co., Loveland, CO, USA) and maintained at dissolved oxygen levels of 7.4 ± 0.27 $mg\,L^{-1}$, total ammonia nitrogen of 0.04 ± 0.02 $mg\,L^{-1}$ and temperature of 21.4 ± 0.07 °C.

2.4.4. Sampling of incurred fish

Immediately prior to the first feeding of medicated diet, four fish were randomly sampled as controls from each tank and the rest of the fish were weighed to determine the weight of medicated diet to be fed. This first sampling was followed by two

samplings during the dosing period (after days 3 and 10 of dosing for both OTC- and ENRO-incurred fish) and two samplings during the withdrawal period (after days 3 and 7 post medication for OTC-incurred fish, and days 1 and 10 post medication for ENRO-incurred fish). At each sampling, one fish was removed from each tank (three replicates per OTC sampling and four replicates per ENRO sampling), and weighed. All fish sampled were filleted and the fillet was frozen at −80 °C. After each tank sampling, the amount of feed administered was adjusted to account for the new body weight of the group. Frozen sample fillets were shipped to the USDA Eastern Regional Research Center on dry ice for analysis.

2.5. Fortification and extraction of FQs and TCs from fish muscle

Homogenized fish muscle samples (1.0 g) were placed in 50 mL screw-capped polypropylene centrifuge tubes. Appropriate volumes of the TC- and FQ-fortification mixtures (2 μg mL^{-1}) or a dilution (Section 2.3) were added to control fish muscle to generate the desired fortification levels. For example, addition of 50 μL of each fortification mixture (2 μg mL^{-1}) gave a 100 ng g^{-1} fortification level. Similarly, addition of 20 μL of a TC fortification mixture dilution (1 μg mL^{-1}) and 50 μL of a FQ fortification mixture dilution (400 ng mL^{-1}) gave a 20 ng g^{-1} fortification level for each analyte. In place of fortification mixture or its dilution, an equal volume of LC Buffer was added to control and incurred samples for a given experiment. Acetonitrile (1.5 mL) and Extraction Buffer (1.5 mL, pH adjusted with NH_4OH) were then added to all samples, which were then homogenized using an Ultra-Turrax T-25 homogenizer (Janke and Kunkel, Cincinnati, OH, USA, 10 mm probe, 9500 rpm). After centrifugation (5 min, 3716 × *g*), the supernatants were decanted into 18 mm × 150 mm glass culture tubes. The pellets were again homogenized with acetonitrile and Extraction Buffer as before, and the supernatants combined with those from the first extraction. The supernatants were evaporated to dryness using a TurboVap LV apparatus (Zymark, Hopkinton, MA, USA) at 40 °C under a stream of nitrogen. Acetonitrile (1 mL portions) was added, as needed to facilitate evaporation. The residues were resuspended in LC Buffer (2.0 mL), with a vortex mixer, and syringe filtered (0.2 μm, nylon) into amber autosampler vials for analysis.

2.6. HPLC-fluorescence

An Agilent (Palo Alto, CA, USA) 1100 HPLC system, consisting of a degasser, quaternary pump, autosampler, and column heater, was controlled by Chemstation software. A Jasco FP 1520 Fluorescence detector was interfaced to this system via a Hewlett Packard (Wilmington, DE, USA) 35900E A/D converter. Liquid chromatography was performed with an Agilent ZORBAX Eclipse XDB-phenyl column (3.0 mm × 150 mm, 3.5 μm) in combination with a C_{18} Security Guard column cartridge (Phenomenex, Torrance, CA, USA). A gradient elution program was used with solvent A (LC Buffer) and solvent B (methanol) as follows: 20% B (6 min), 20–40% B (4 min), 40% B (8 min), 40–80% B (3 min), 80% B (2 min), 80–20% B (3 min), 20% B (4 min). The flow rate was 0.5 mL min^{-1} and the column heater was set at 30 °C. A program on the fluorescence detector changed wavelengths between FQ conditions (λ_{ex} 275 nm, λ_{em} 425 nm) and TC conditions (λ_{ex} 375 nm, λ_{em} 535 nm) as analytes eluted from the column. Retention times for the analytes are shown in Fig. 1, as are the times for changing wavelengths. The liquid chromatography column was washed after each day's run with water/methanol (80:20 to 0:100), acetonitrile, and stored in 50/50 acetonitrile/water. The solvent A channel for the pump and degasser was flushed daily with water.

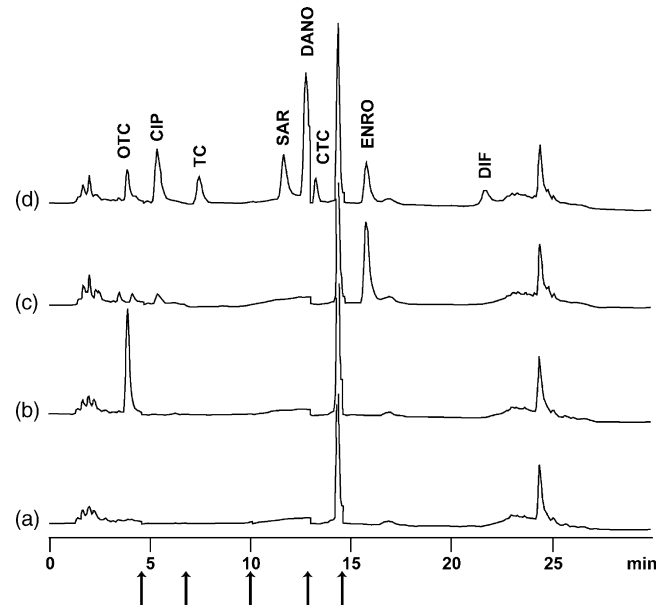

Fig. 1. Liquid chromatograms of (a) Control catfish muscle extract, (b) OTC-incurred catfish muscle extract, day 3 dosing (118 ng g^{-1}), (c) ENRO-incurred catfish muscle extract, withdrawal day 1 (1:20 dilution, 88 ng g^{-1}) and (d) Control catfish muscle extract after fortification with 50 ng g^{-1} CIP, SAR, DANO, ENRO, DIF, OTC, TC, CTC. All chromatograms are on the same *y*-axis scale. Arrows indicate times of wavelength changes.

3. Results and discussion

3.1. Method development

3.1.1. Sample extraction

Previous work had established that TCs could be successfully extracted from food matrices using a diprotic or polyprotic acid buffer at acidic pH and that EDTA was not required in these cases [19,20]. We found that FQs could be extracted along with TCs under these conditions, and that the presence of magnesium ions improved the recovery of FQs [16]. Extraction of tissue samples with aqueous buffer alone, however, led to turbid extracts, presumably due to slow and continued protein precipitation. Inclusion of an organic solvent in the extraction mixture helped to decrease or eliminate turbidity. Extraction of TCs and FQs with a 1:1 mix of acetonitrile and either 0.1 M malonate or. 0.1 M citrate, with each buffer at pH 5 and containing 50 mM magnesium ions, was examined. Citrate was chosen as the Extraction Buffer of choice as it provided better recoveries of TCs, while

Table 1
FQ and TC recoveries in fortified catfish muscle

Fortification conc.	% Recovery (%R.S.D.)							
	CIP	SAR	DANO	ENRO	DIF	OTC	TC	CTC
20 ng g^{-1} [a]	77.5 (3.7)	81.8 (4.2)	84.2 (4.2)	82.9 (7.0)	82.0 (7.0)	73.0 (5.8)	76.0 (7.0)	60.6 (9.8)
20 ng g^{-1} [a]	89.0 (2.0)	92.4 (1.3)	93.2 (1.4)	92.9 (2.5)	91.8 (1.7)	79.7 (1.9)	83.1 (1.8)	61.0 (2.5)
20 ng g^{-1} [a]	88.5 (2.2)	93.1 (2.0)	93.4 (2.0)	91.0 (3.2)	92.9 (3.3)	81.1 (1.2)	82.7 (1.8)	58.2 (4.5)
Ave [b]	85.0 (6.9)	89.1 (6.5)	90.3 (5.5)	88.9 (6.6)	88.9 (7.0)	77.9 (5.6)	80.6 (5.7)	59.9 (6.3)
50 ng g^{-1} [a]	88.5 (2.3)	91.7 (2.3)	92.0 (2.8)	91.6 (2.5)	93.7 (2.2)	81.7 (1.4)	84.3 (1.8)	70.9 (2.4)
50 ng g^{-1} [a]	86.8 (3.5)	90.2 (3.3)	90.5 (4.6)	89.5 (4.1)	90.9 (3.6)	81.3 (2.2)	83.7 (2.5)	72.2 (4.4)
50 ng g^{-1} [a]	85.9 (1.6)	89.5 (2.5)	91.8 (1.6)	91.1 (1.8)	89.9 (2.0)	80.3 (3.1)	82.0 (2.4)	71.1 (3.9)
Ave [b]	87.0 (2.7)	90.4 (2.8)	91.4 (3.1)	90.7 (2.9)	91.5 (3.1)	81.1 (2.2)	83.4 (2.4)	71.4 (3.5)
100 ng g^{-1} [a]	77.2 (4.9)	81.5 (4.4)	81.7 (4.6)	81.6 (4.7)	83.3 (4.0)	71.6 (6.0)	73.7 (5.6)	67.0 (5.6)
100 ng g^{-1} [a]	85.2 (1.0)	89.3 (1.5)	90.8 (1.3)	89.9 (1.2)	90.3 (1.5)	66.1 (2.5)	67.7 (2.4)	61.0 (2.3)
100 ng g^{-1} [a]	83.8 (1.6)	83.7 (1.7)	87.6 (1.8)	89.5 (1.6)	87.3 (2.2)	79.6 (1.5)	79.9 (1.4)	74.2 (2.4)
Ave [b]	82.1 (5.2)	84.8 (4.8)	86.7 (5.2)	87.0 (5.2)	87.0 (4.2)	72.4 (8.6)	73.8 (7.7)	67.4 (9.0)

[a] $n = 5$.
[b] $n = 15$.

the choice of buffer had less effect on FQs. The pH of the citrate buffer containing 50 mM magnesium ions used for extraction was varied between pH 4, 5, and 6. The pH 5 citrate/magnesium ion buffer provided the best recoveries for the TCs and FQs. Acetonitrile was found to provide significantly better recoveries for both TCs and FQs than methanol when used as a coextraction solvent. The ratio of acetonitrile:citrate/magnesium ion buffer was then varied among 1:2, 1:1 and 2:1. The optimum ratio was chosen as 1:1 based on enhanced FQ recoveries; while the ratio chosen seemed to have no consistent effect on TC recovery. The concentration of magnesium ions in the extraction mixture was varied between 50, 100, 150 and 200 mM. FQ recoveries were improved with increased magnesium ion concentration up to a plateau at 150–200 mM, while TC recoveries appeared to optimize at 100 mM. The 100 mM level was chosen as it still provided excellent recoveries of FQs, along with the best conditions for the somewhat more difficult to extract TCs. In a final experiment, the effect of a defatting cleanup by extraction of the initial extract with a 1:1 mix of ether:hexane, followed by removal of the upper organic layer was examined. The cleanup step did not result in any significant improvement in recoveries, took significantly more time, and was not judged as an overall improvement to the procedure. The above experimentation determined the most promising combination for the extraction conditions to be a 1:1 mix of acetonitrile:Extraction Buffer (0.1 M citrate, 50 mM magnesium chloride, pH 5.0).

3.1.2. HPLC-fluorescence

Optimum HPLC conditions for separation and fluorescence detection of the FQs and TCs had been previously established [16]. These conditions involved a gradient of methanol in pH 6.5 malonate buffer, with magnesium ions added to promote fluorescence of TCs. The choice of pH was made to allow FQ fluorescence (normally observed at acidic pH), while still being basic enough to allow magnesium-enhanced fluorescence of TCs. Malonate was chosen over citrate as a liquid chromatography buffer at this pH, as we had found malonate provided enhanced fluorescence of TCs over citrate [16]. Use of a new Eclipse XDB-phenyl column for this project, which had been produced from a different manufacturing lot, required a brief re-examination of the gradient conditions. Modification of the gradient, principally by starting at a higher concentration of methanol, provided a satisfactory separation of all analytes. We have noted before that FQs, in particular, appear to display sensitivity to column lot variation [21], regardless of manufacturer. Differences observed, however, can be resolved by minor modifications to the gradient.

A liquid chromatogram illustrating the separation of the analytes in a fortified fish muscle extract sample is shown in Fig. 1d. Comparison to a chromatogram of control fish muscle extract in Fig. 1a shows no significant interferences present in the control sample. In general, the control fish muscle extract sample provided less background and allowed more facile quantitation than had been encountered with chicken muscle extract [16].

3.2. Method validation

3.2.1. Linearity

Five point calibration curves were prepared for each analysis day. Quantitation utilized the fluorescence peak height for each analyte. The calibration curves were found to be linear over the 5–100 ng mL^{-1} range studied (5, 10, 20, 50 and 100 ng mL^{-1} levels were used), with correlation coefficients for each analyte >0.999.

Table 2a
Analysis of OTC-incurred catfish muscle

	OTC (ng g^{-1}) (%R.S.D.) [a]
Day 3 dosing	118 (1.1)
Day 10 dosing	108 (2.2)
Day 3 withdrawal	24.0 (2.1)
Day 7 withdrawal	4.4 (3.1)

[a] $n = 5$.

Table 2b
Analysis of ENRO-incurred catfish muscle

	Measured CIP ($ng\,g^{-1}$) (%R.S.D.)[a]	Measured ENRO ($ng\,g^{-1}$) (%R.S.D.)[a]	Dilution	Actual CIP ($ng\,g^{-1}$)	Actual ENRO ($ng\,g^{-1}$)
Day 3 dosing	7.16 (3.0)	106 (2.8)	1:20	143	2120
Day 10 dosing	5.95 (1.6)	84.1 (1.8)	1:40	238	3360
Day 1 withdrawal	9.07 (2.4)	88.0 (2.6)	1:20	181	1760
Day 10 withdrawal	4.21 (1.6)	29.7 (2.2)	–	4.21	29.7

[a] $n = 5$.

3.2.2. Accuracy

Accuracy of the method was tested by fortification of control fish muscle samples at three different known levels (20, 50, and 100 $ng\,g^{-1}$), extraction, analysis, and determination of the recovery for each analyte. Data for these experiments are shown in Table 1. Good results were obtained, with average recoveries ranging from 60 to 92%. It is interesting to note that, unlike with chicken muscle [16], fish muscle samples did not require matrix matched calibration curves to compensate for difficulties in measuring peak height for DIF or other analytes at low concentration, making more rapid analysis possible.

3.2.3. Precision

The method exhibited excellent precision, as shown in Table 1. Fortification/recovery experiments resulted in low intra-day relative standard deviations (R.S.D.s) for all analytes ($n = 5$, R.S.D.s < 10%). A comparison of fortification/recovery experiments conducted on three different days ($n = 15$) also displayed low inter-day R.S.D.s (<9%), confirming the excellent reproducibility of the method.

3.2.4. Limits of determination and quantitation

Fluorescence limits of detection for the analytes were determined as three times the root mean square of the noise divided by the slope of the calibration curve. Limits of quantitation (LOQs) were calculated similarly, with a factor of ten rather than three. Excellent LOQs were obtained and are as follows: 0.15 $ng\,g^{-1}$ (DANO), 0.5 $ng\,g^{-1}$ (OTC, CTC, SAR, ENRO), 1 $ng\,g^{-1}$ (TC, DIF) and 1.5 $ng\,g^{-1}$ (CIP).

3.3. Analysis of OTC- and ENRO-incurred fish

It is important, whenever possible, to test a method for detection of veterinary drug residues using actual dosed animal (incurred) samples. This was accomplished, and the results from the analyses are described below.

3.3.1. OTC-incurred fish

Results from analysis of OTC-incurred fish muscle samples are shown in Table 2a, and a sample chromatogram from day 3 of dosing is shown in Fig. 1b. Relatively high levels of OTC are present in the muscle during dosing; these levels decrease during withdrawal, as might be expected. It is interesting to note that the levels observed in this study are similar to those in which OTC-dosed catfish muscle samples were analyzed by another method [18].

3.3.2. ENRO-incurred fish

Results from analysis of ENRO-incurred fish muscle samples are shown in Table 2b, and a sample chromatogram from withdrawal day 1 (1:20 dilution) is shown in Fig. 1c. The metabolite CIP was detected in addition to ENRO in these samples. Three of the four incurred samples contained high enough levels of ENRO that required dilution of the samples with control muscle tissue prior to extraction and analysis in order for the ENRO levels to fall within range of the calibration curve for the method. The "actual" values for CIP and ENRO, after correction for the dilution, are shown in the last two columns of the table. Again, the pattern shows high levels of ENRO and CIP during dosing, and decreasing levels during withdrawal.

4. Conclusion

An efficient method for simultaneous determination of three TCs and five FQs in catfish muscle, using liquid chromatography-fluorescence, has been developed. Good recoveries (60–92%) and excellent R.S.D.s (<10%) were obtained, with low limits of quantitation, ranging from 0.15 to 1.5 $ng\,g^{-1}$. Levels of OTC and ENRO (and CIP) in OTC- or ENRO-incurred fish samples were successfully determined using the method. This method provides a novel alternative approach for the simultaneous determination of members of more than one class of antibiotics in catfish.

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