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Determination of Florfenicol Amine in Channel Catfish Muscle by Liquid Chromatography

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A method for quantifying florfenicol amine (FFA) in channel catfish muscle was validated according to U.S. Food and Drug Administration guidelines. FFA is the proposed marker residue for the veterinary antibiotic florfenicol in catfish muscle for regulatory surveillance purposes. The method includes acid hydrolysis followed by sample cleanup with ethyl acetate extraction, basification, solid-phase extraction, and quantitation by liquid chromatography with UV detection. The assay was validated at 5 concentrations in the range of 0.075–35 μg/g muscle. The overall mean recovery of FFA from fish tissues fortified at these concentrations ranged from 85.7 to 92.3%, 4.8–17.2% relative standard deviation (RSD). The assay limit of detection was 0.044 μg/g muscle based on analysis of control muscle. Catfish muscle samples containing incurred florfenicol residues were analyzed in quintuplicate with RSD <5%. Acid hydrolysis has previously been demonstrated to convert florfenicol and its known metabolites to FFA and to release a significant amount of FFA from nonextractable florfenicol residues in tissues containing incurred residues in other species. By using acid hydrolysis, this method should yield a more accurate estimate of the total florfenicol-related residue level in muscle tissue from florfenicol-treated catfish than could be achieved by solvent extraction alone.

Florfenicol, a fluorinated analog of thiamphenicol, is a synthetically produced broad-spectrum antibacterial agent specifically developed for veterinary use. A 30% injectable formulation (Nuflor®) is used for treatment of respiratory disease and foot rot in cattle and respiratory disease in swine. A 50% florfenicol feed premix (Aquaflor®/Aquafen®) is used in aquaculture to control susceptible bacterial diseases. Aquaflor was registered in Japan (1990) for the control of *Pasteurella piscicida* in yellowtail, *Edwardsiella tarda* in eel, and *Vibrio anguillarum* in goldfish (1). It was also registered in Norway (1993), Chile (1995), Canada (1997; 2), and the United Kingdom (1999) for control of furunculosis in salmonid species caused by *Aeromonas salmonicida* (3).

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Enteric septicemia in channel catfish, *Ictalurus punctatus*, caused by *Edwardsiella ictaluri*, is the primary disease affecting commercial catfish production in the United States (4, 5). Initial studies have demonstrated that Aquaflor administered in feed to channel catfish at a dose rate of 10 mg/kg/day body weight for 10 consecutive days reduces mortality resulting from infection with *E. ictaluri*. As a consequence of these studies, Aquaflor is being developed for use in channel catfish for the control of mortality attributable to infection with *E. ictaluri*.

Previously published methods for quantitation of florfenicol and/or its amine metabolite (florfenicol amine, [FFA]) in the tissues of veal calves (6), rainbow trout (7, 8), broiler chickens (9), Muscovy ducks (10), and tilapia (11) have involved solvent extraction followed by sample cleanup and/or analysis. However, florfenicol metabolism studies demonstrated that nonextractable residues of florfenicol are predominant in edible tissues in poultry, swine, and cattle (12–14), although they are much less significant in salmon (15–17). Acid hydrolysis of these nonextractable residues in other species yields a significant amount of FFA (13–17), an extractable product and metabolite of florfenicol. Florfenicol and known metabolites of florfenicol are also converted to FFA by acid hydrolysis (Figure 1; 13–17). Quantitation of FFA in extracts of hydrolyzed tissues, therefore, results in a more accurate determination of total florfenicol-related residue levels than does simple solvent extraction. The purpose of the present study was to develop and validate an analytical method for quantifying FFA in catfish muscle according to U.S. Food and Drug Administration (FDA) guidelines.

Experimental

Materials

(**a**) *Hydrolysis tubes.*—Glass, 50 mL with Teflon-lined caps.

(**b**) *pH indicator strips.*—Colorphast (pH 0–14; EM Science, Gibbstown, NJ).

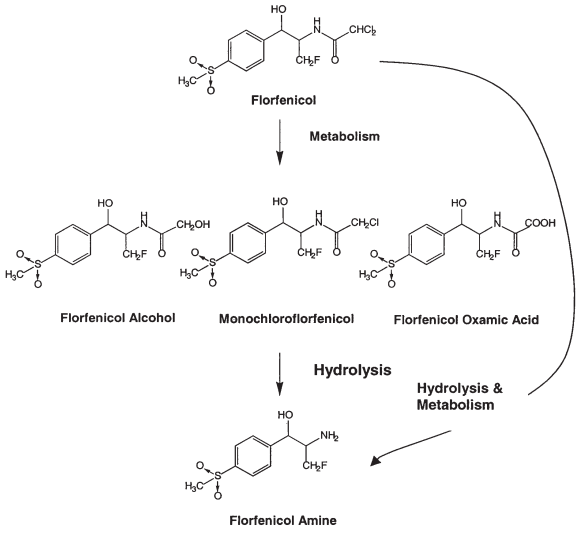

Figure 1. Metabolic and acid hydrolysis products of florfenicol.

(**c**) *Solid-phase extraction.*—Chem Elut CE1020 Sorbant Column (Analytichem, formerly Varian, Walnut Creek, CA).

(**d**) *Glass wool.*—Silane-treated (Supelco, Bellefonte, PA).

(**e**) *Collection tubes.*—Glass 60 mL (I-Chem, New Castle, DE).

(**f**) *Syringe filters.*—Acrodisc LC 25 PVDF 0.2 μm filters (Gelman Sciences, Ann Arbor, MI).

(**g**) *Mobile phase filters.*—Type HA (aqueous) and Type FA (organic), 0.45 μm filters (Millipore Corp., New Bedford, MA).

Reagents

Unless otherwise specified, all reagents were obtained from Fisher Scientific (Fair Lawn, NJ).

(**a**) *Acetonitrile, ethyl acetate, and methanol.*—Liquid chromatography (LC) or optima grade.

(**b**) *High-purity water.*—Obtained in-house from a Milli-Q system (Millipore Corp.).

(**c**) *Triethyl amine (TEA).*—LC grade.

(**d**) *Potassium phosphate monobasic (crystal).*—Laboratory grade.

(**e**) *Hydrochloric acid (HCl).*—12.1N, trace-metal grade.

(**f**) *Sodium hydroxide (NaOH) solution.*—50% (w/w).

(**g**) *Phosphoric acid, 85% (v/v).*—LC grade.

(**h**) *FFA standard.*—97.6%; Schering-Plough (Union, NJ).

(**i**) *Thiamphenicol standard.*—Schering- Plough.

(**j**) *Chloramphenicol, oxytetracycline hydrochloride, sulfadimethoxine, 3-aminobenzoic acid ethyl ester methane sulfonate salt (Tricaine).*—Sigma Chemical Co. (St. Louis, MO).

(**k**) *Glyphosate and diuron.*—Supelco.

(**l**) *Ormetroprim.*—Pfizer Animal Health (Groton, CT).

Preparation of Reagent and Standard Solutions

(**a**) *6N HCL.*—Add 500 mL 12.1N to 500 mL high-purity water, and mix carefully.

(**b**) NaOH, 30% (w/w).—Add 500 mL 50% (w/w) NaOH to 500 mL water, and mix carefully.

(**c**) *Final extract solution.*—Dissolve 2.72 ± 0.2 g potassium phosphate in 1.9 L water and add 20 mL acetonitrile. Adjust pH of solution to 4.0 ± 0.2 by adding ca 50 μL 85% (v/v) phosphoric acid. Dilute to 2000 mL with water. Store at 4°C.

(**d**) *Mobile phase A.*—Add 2.72 ± 0.2 g potassium phosphate and 150 μL TEA to 2 L water. Adjust pH of solution to 4.0 ± 0.2 by adding ca 90 μL 85% (v/v) phosphoric acid, and filter through 0.45 μm LC aqueous filter.

(**e**) *Mobile phase B.*—100% methanol.

(**f**) *Stock solution.*—FFA (100 mg, not adjusted for standard purity). Accurately weigh into a 100 mL volumetric flask. Dilute contents of flask with methanol to yield stock solution of 1000 μg/mL. Store at –20°C.

(**g**) *Fortification solutions.*—Dilute stock solution to 1.5–40 μg/mL with methanol for fortifications and store at –20°C.

(**h**) *Calibration curve standard solutions.*—Prepare serial dilutions of stock solution by using final extract solution to provide LC standards at concentrations of 0.05–2.4 μg/mL (equivalent to 0.05–2.4 μg/g muscle). Store solutions at 4°C. Remake monthly.

Instrumentation

(**a**) *LC system.*—The LC system consisted of a Perkin-Elmer (Norwalk, CT) Series 200 pump, autosampler, UV-Vis detector, and column oven. Separation was accomplished on a Zorbax RX-C8 column, 250 × 4.6 mm id with a Zorbax RX-C8, 12.5 × 4.6 mm id guard column (Agilent Technologies, Palo Alto, CA). The guard column was changed after every analysis set (60–120 injections) to reduce problems with deteriorating peak shape. Chromatographic data were processed with Turbochrom Workstation software, v.6.1.1. The flow rate was 1 mL/min, detection wavelength was 220 nm, injection volume was 100 μL, and column temperature was set to 23°C. Total run time was 30 min. The column was equilibrated at 100% mobile phase A for 10 min before each injection. Upon injection, run conditions were isocratic 100% mobile phase A for 15 min followed by a linear 5 min gradient to 100% mobile phase B. These conditions were maintained for 5 min, and then a linear 5 min gradient returned to the starting conditions.

(**b**) *Homogenization.*—Skinless catfish fillets were homogenized on dry ice in a Robot Coupe Vertical Batch Processor (Robot Coupe USA, Inc., Jackson, MS).

(**c**) *Analytical balance.*—Capable of measuring to ±0.1 mg.

(**d**) *Sample balance.*—Capable of measuring to ±1 mg.

(**e**) *Shaking heated waterbath.*—Capable of maintaining 100°C water temperature.

(**f**) *Centrifuge.*—Capable of centrifuging 50 mL glass hydrolysis tubes at 2500 rpm (1303 × *g*) for 5 min.

(**g**) *pH meter.*—Capable of measurement to nearest 0.01 pH unit.

(**h**) *16-Port SPE vacuum manifold.*—Supelco.

(**i**) *Vortex mixer*

(**j**) *Ultrasonic bath*

Table 1. Recovery of florfenicol amine from catfish muscle tissue[a]

	Mean recovery, % (RSD, %)					
Fortification level, μg/g	Day 1	Day 2	Day 3	Day 4	Day 5	Overall
0.075	100.0	102.3	74.5	NA[b]	NA	92.3
	(8.6)	(11.3)	(12.0)			(17.2)
0.5	83.5	90.4	86.2	NA	NA	86.7
	(1.7)	(8.2)	(2.8)			(5.7)
1.0	83.2	97.2	78.6	102.8	88.8	90.1
	(2.2)	(2.6)	(15.1)	(2.7)	(6.3)	(11.7)
2.0	77.0	93.2	70.7	95.0	92.6	85.7
	(2.7)	(1.5)	(13.6)	(5.6)	(2.8)	(13.0)
35.0[c]	84.9	90.7	82.0	NA	NA	85.9
	(0.7)	(2.2)	(2.7)			(4.8)

[a] Results are from analysis of 3 replicate samples for each analysis day.
[b] NA = Not applicable.
[c] Final extract diluted before analysis.

(**k**) *Turbo Evaporator*.—Zymark, Turbo LV (Hopkinton, MA).

Catfish Fillet Samples

Skinless channel catfish fillets were purchased from 6 supermarkets in New Jersey and Connecticut. Skinless fillet samples were also obtained from 2 catfish that were fed florfenicol-medicated feed as part of another study. For this study, market-weight catfish were maintained in a 0.1 acre test pond under normal aquaculture conditions at a stocking density of 7000 fish/acre. Fish were fed florfenicol-medicated feed for 12 days and received an average dose of 8.1 mg/kg/day of florfenicol.

Sample Homogenization

Skinless catfish fillets were homogenized to a fine powder in the Robot Coupe processor with dry ice. The homogenized samples were transferred to storage bottles and stored at –40°C. The bottle caps were not completely tightened to allow the subliming dry ice to escape. After ca 24 h, the caps were tightened.

Sample Fortification and Workup

Aliquots (2 ± 0.2 g) of control catfish muscle homogenate were weighed into 50 mL glass tubes with Teflon-lined caps. Aliquots were fortified at 0.075–2.0 μg/g with 100 μL fortification solutions or at 35 μg/g fortification with 70 μL stock solution, and then allowed to stand at room temperature for 10–30 min. After addition of 8 mL 6N HCl, each tube was capped and mixed on a Vortex mixer for ca 1 min. The sample tubes were then placed in a shaking water bath set at 100°C, for ca 3 h. The samples were periodically (ca 30 min) removed from the water bath and mixed on a vortex mixer to ensure complete digestion. After 3 h, the tubes were removed and contents inspected to ensure that the solution was uniformly dark brown to black, with only charred black flocculent material remaining. If necessary, heating and mixing were continued until homogenates were completely digested.

Immediately after hydrolysis and while still hot, the hydrolysates were extracted with 20 mL ethyl acetate, and the mixture was centrifuged for 5 min at 2500 rpm (1303 × *g*). The ethyl acetate (upper) layer was removed by aspiration and discarded. Care was taken not to disturb or transfer the black tarry residue at the interface of the 2 layers. An 8 mL amount of NaOH, 30% (w/w), was added to the extracted hydrolysate to adjust the pH to 12.5 or higher. The pH of the solution was checked with pH paper, and additional NaOH was added if necessary.

A silane-treated glass wool plug (ca 3/8 in. thick) was placed on top of Varian Chem Elut® CE1020 sorbent columns, which were then mounted on a vacuum manifold. The basified hydrolysates were poured onto the top of the columns (stopcock closed). After the solution had passed below the surface of the absorbent bed, the columns were allowed to stand for at least 45 min to allow the sample solution to fully adsorb. The columns were then eluted with three 20 mL aliquots of ethyl acetate. The SPE extraction stopcock was adjusted to allow flow of ca 1 drop/s, and care was taken not to allow columns to run dry until after the last aliquot of ethyl acetate was added.

The columns were allowed to stand after addition of the third aliquot of ethyl acetate until the eluate no longer emerged from the column tip. The SPE eluates were then evaporated to dryness under nitrogen using a Turbo Evaporator set at 45°C (ca 90 min). The dried residues were dissolved in 2 mL final extract solution using vortexing and sonication and were then filtered through a 0.2 μm pore-size Acrodisc® filter into an autosampler injection vial for LC analysis of the resulting final extracts. For samples with residue levels >2.4 μg/g (the

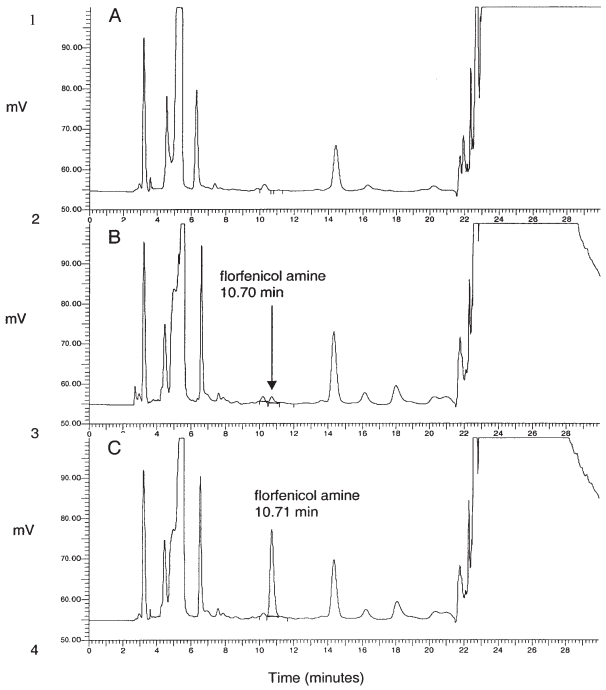

Figure 2. Representative chromatograms of extracts from (A) control catfish muscle sample; (B) catfish muscle sample fortified at 0.075 μg/g; (C) catfish muscle sample fortified at 1 μg/g.

highest calibration curve concentration), the final extract was diluted with final extract solution 20× before analysis.

Accuracy, Precision, and Method Limits of Detection and Quantitation

Method accuracy (% recovery) and precision (relative standard deviation, RSD) were determined by analyzing triplicate aliquots of homogenized muscle fortified at 0.075, 0.5, 1, 2, and 35 μg/g on 3–5 separate occasions (≥9 analyses per fortification level). Both same- and between-day accuracy and precision were calculated. Extracts from the 35 μg/g fortification level were diluted 20× before analysis.

The limit of detection (LOD) was determined by replicate analysis of control tissue samples from 6 different sources ($n \geq 20$). For each analysis, peak area at the retention time corresponding to FFA was determined. The LOD was calculated by adding 3 times the standard deviation of the background response to the average background response. The limit of quantitation (LOQ) was defined as the lowest level of FFA fortification in catfish muscle that yielded acceptable accuracy and precision (60–110% recovery, RSD ≤ 20%).

Method Specificity

The potential for interference with the assay by 2 florfenicol analogs (thiamphenicol and chloramphenicol) was determined by analyzing control tissue fortified with the analogs. Additionally, the potential for interference with the assay by 4 veterinary drugs used in catfish (sulfadimethoxine, ormetoprim, oxytetracycline, and tricaine), and 2 herbicides commonly used in catfish ponds (glyphosate and diuron) was determined by analyzing 1 μg/mL methanolic solutions of the compounds directly. Aliquots of the methanolic solutions containing either 2 or 4 of the compounds were added directly to 8 mL aliquots of 6N HCl and then analyzed.

Stability of FFA in Muscle and Muscle Extracts

The storage stability of FFA in frozen muscle homogenates kept at ≤ –20°C was determined by using both FFA-fortified control muscle and muscle with incurred residues. Fortification levels were 0.5 and 2 μg/g. Storage intervals were 0, 1.5, and 4.5 months.

Homogenized catfish muscle fortified at 0.5 and 2 μg/g with FFA was subjected to 3 freeze/thaw cycles (with at least 24 h separating each cycle) and then extracted and analyzed to determine residue stability. The freezer storage temperature was –20°C. Additionally, the stability of FFA was established in muscle final extracts containing residue levels of 0.5 and 2 μg/g after 24 h of controlled room temperature storage and after 2 weeks and 1 month of refrigerator storage (2–8°C).

Table 2. Stability of florfenicol amine (FFA) and incurred florfenicol residues in channel catfish fillet tissues stored at –20°C[a]

	Mean recovery, % (RSD, %)			
	Fortification level, μg/g		Incurred catfish No.	
Timepoint, months	0.25	2.0	1	2
0	87.5 (13.0)	94.6 (1.2)	100 (5.6)	100 (2.5)
1.5	97.0 (8.9)	101.1 (3.9)	109.1 (1.1)	99.3 (1.9)
4.5	114.0 (7.7)	94.2[b]	87.5 (18.6)	91.4 (2.6)

[a] Data represent mean percent FFA recovered from 3 stored tissue samples with reference to residue concentration in tissue determined at 0 months.

[b] Average of 2 analyses.

Table 3. Stability of florfenicol amine (FFA) in extracts from fortified channel catfish tissue[a]

	Fortification level, μg/g	
	Mean recovery, % (RSD, %)	
Analysis timepoint[b]	0.5	2.00
0 week	83.5 (1.7)	77.0 (2.7)
24 h RT	107.2 (7.5)	112.0 (3.6)
2 week Refr.	101.8 (2.0)	109.8 (1.8)
4 week Refr.	106.6 (7.5)	115.8 (2.6)

[a] Extracts were stored at room temperature for 24 h and at 4°C for up to 4 weeks. Data represent mean percent FFA recovered from 3 stored extracts with reference to concentration of FFA determined in extracts at 0 weeks. Results are from analyses of 3 replicate samples.

Analysis of Muscle Containing Incurred Residues

Muscle samples from 2 catfish treated with florfenicol-medicated feed and having residue levels of ca 0.4 and 1.6 μg/g FFA (ca 1/2 and 2× the proposed 1 ppm marker residue tolerance level) were analyzed in quintuplicate.

Results and Discussion

A method was developed to quantitate FFA residues in catfish muscle. The extraction procedure includes an acid hydrolysis step that converts florfenicol and its known metabolites to FFA (Figure 1) and has been shown to release a significant amount of FFA from nonextractable florfenicol residues in tissues containing incurred residues in other species (13–17). Thus, this method yields a more accurate estimate of the total florfenicol-related residue level in muscle tissue from florfenicol-treated catfish than could be achieved by solvent extraction alone.

Quantitation was by LC–UV using FFA standards in solvent (external standard method). A least-squares linear regression analysis produced the best fit for the concentration/peak area data. The overall or between-day mean recovery for fortified muscle ranged from 85.7 to 92.3%, with overall RSD ranging from 4.8 to 17.2% (Table 1). Same-day mean recovery and RSD ranged from 70.7 to 102.8% and from 0.7 to 15.1%, respectively. Figure 2(A–C) presents typical chromatograms from analysis of extracts of control and fortified (0.075 and 1 μg/g) catfish muscle.

The FDA's criteria for overall recovery and RSD for a determinative method in animal tissues are 80–110% with RSD ≤10% for replicate analyses at concentrations ≥0.1 ppm, and 60–110% at concentrations <0.1 ppm (RSD ≤20%; 18). The recovery criteria were met at all levels. At the 1 and 2 ppm fortification levels, the RSD values were slightly above the 10% target level (11.7 and 13.0%, respectively) as a result of an unexplained high same-day RSD on Day 3 of the validation.

No significant interference from endogenous components was found at the retention time of FFA in the unfortified control muscle samples assayed (Figure 2A). In many cases a matrix peak, eluting approximately 1 min before FFA, was observed, but baseline or very near baseline separation between this peak and FFA was always maintained and this peak never affected quantitation. The LOD for this method was 0.044 μg/g, as determined by analysis of control tissue samples from 6 sources. The LOQ (as determined from the lowest reproducible fortification level) was 0.075 μg/g.

The selectivity of the validated LC method for FFA was confirmed by assessing the potential for interference by 2 florfenicol analogs (chloramphenicol and thiamphenicol) and 6 drugs or pesticides commonly used in catfish or catfish ponds. None of the drugs co-chromatographed or interfered with the quantitation of FFA (data not shown).

Results from stability analyses are presented in Tables 2 and 3. FFA was stable for at least 4.5 months in fortified control catfish muscle and as incurred residues in catfish muscle. Recoveries in fortified tissue were 94.2–114.0% of the levels measured in the zero-time analyses (*see* Figure 3 for a representative chromatogram). Recoveries in incurred tissue were 87.5–91.4% of the levels measured in the zero-time analyses after 4.5 months of freezer storage. FFA was stable in catfish muscle after undergoing 3 freeze/thaw cycles, with recoveries averaging 86.6–97.8% of theoretical. FFA was also stable in final extract solution stored at room temperature for

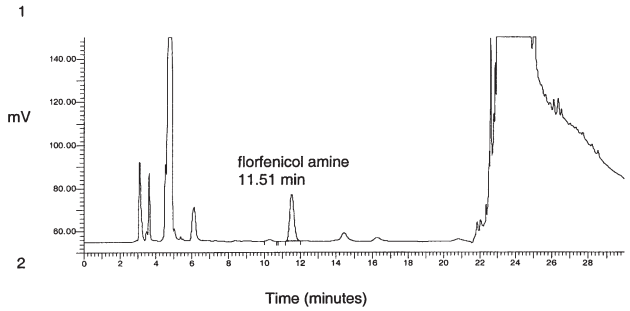

Figure 3. Representative chromatogram of an extract from catfish muscle sample containing incurred florfenicol residues (catfish 2, about 1.6 μg/g). Retention time of florfenicol amine here is about 0.8 min greater than that in Figure 2 chromatograms because of variability between mobile phase batches.

24 h and refrigerated for up to 1 month. After 24 h storage at room temperature, recoveries averaged 107.2–112.0% of the levels measured in the zero-time analyses. After 1 month of refrigerated storage, recoveries averaged 106.6–115.8% of the levels measured in the zero-time analyses.

Two samples with incurred residues from catfish treated with Aquaflor were analyzed in quintuplicate. The mean values from these analyses were 0.406 and 1.599 ppm. The RSD values for these analyses were 3.2 and 4.5%.

Conclusions

An accurate and specific method for the determination of FFA residue levels in muscle from channel catfish was developed and evaluated. The method was accurate and precise for tissue containing 0.075–35 μg/g. Overall recoveries for fortified samples ranged from 85.7–92.3% with overall RSDs of 4.8–17.2%. The LOD was 0.044 μg/g and no interference from endogenous components was noted, although a matrix peak eluted approximately 1 min before the analyte peak. FFA was stable in fortified catfish muscle and as incurred residues in catfish muscle for up to 4.5 months with freezer storage. It was also stable after 3 freeze/thaw cycles and in final extract solution after 24 h storage at room temperature and after 1 month of refrigerator storage. Because the extraction procedure includes an initial acid hydrolysis step, this method should allow for a more accurate measure of total florfenicol-related residues than do methods that rely on extraction alone.

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