DRUGS, COSMETICS, FORENSIC SCIENCES

Confirmation of Fluoroquinolones in Catfish Muscle by Electrospray Liquid Chromatography/Mass Spectrometry

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A multiresidue liquid chromatography/mass spectrometry (LC/MS) confirmation method for fluoroquinolones in catfish muscle was developed by using an electrospray interface. Residues of ciprofloxacin, enrofloxacin, sarafloxacin, and difloxacin were positively identified in catfish muscle fortified at 10–80 ppb as well as in incurred tissue. The extraction procedure is based on an LC method with fluorescence detection for determination of these compounds in catfish. Residues were extracted from catfish muscle with an acidic ethanol solution, and the extracts were cleaned up on a propyl sulfonic acid solid-phase extraction column. Chromatographic conditions were optimized to be compatible with the electrospray interface. Internal electrospray voltages were optimized so that 3 fragment ions, in addition to the protonated molecular ion, could be monitored for each residue. To obtain maximum sensitivity, separate MS acquisition programs were developed for ciprofloxacin/enrofloxacin and sarafloxacin/difloxacin pairs.

Fluoroquinolones (FQs) are antibacterial agents that are used in both human and veterinary medicine. Sarafloxacin (SAR) and enrofloxacin (ENR) are approved for use in poultry in the United States (1; U.S. Food and Drug Administration [FDA] Green Book), and ENR, approved in the United States for nonfood animals, is believed to be used extensively in cattle in Europe (2). Residues of FQs in tissues of food animals are of concern because of reports that humans have developed antibacterial resistance to these drugs; the FDA has recently banned the extralabel use of these drugs in food-producing animals (3, 4). Because of these concerns, several methods have been developed to determine the amount of FQ residues in food matrixes (5–11). Most quantitative methods use liquid chromatography (LC) with fluorescence detection and can measure residue levels as low as 5–20 ppb. Two methods that use a simple extraction procedure combined with LC with fluorescence detection were recently developed in our laboratory for the determination of ciprofloxacin (CIP), ENR, SAR, and difloxacin (DIF; Figure 1) in milk (10) and catfish tissue (11).

For regulatory purposes, unambiguous identification of the suspect residues found in the sample by the determinative methods is critical. Because of its inherent specificity and sensitivity, mass spectral analysis is the preferred technique for confirmation of suspect residues. Several reports of mass spectral characterization and use of mass spectrometry (MS) to confirm FQ or quinolone residues have been described. In early methods, quinolones were decarboxylated to form stable products for gas chromatography/MS analysis (12–14). More recent reports used electrospray and atmospheric pressure chemical ionization (APCI) LC/MS to characterize and confirm quinolones and FQ antibacterial drugs (15–19). For example, Schneider et al. (15) reported the confirmation of danofloxacin in cattle and chicken liver using this technique. Previous work has also confirmed FQ residues in catfish tissue. In a method developed in part for regulatory purposes, Schilling et al. (16) used electrospray LC/MS for the confirmation of SAR in catfish tissue. The method used collisionally induced dissociation (CID) in a triple quadrupole instrument to obtain appropriate fragment ions for confirmation. This confirmation method was developed for relatively high levels of residue in tissue (0.7–50 ppm), although detection limits of picogram amounts of FQs injected into the mass spectrometer were reported. Recently, a report was published that describes the detection and confirmation of 15 quinolones, including several FQs, with electrospray LC/MS/MS in several matrixes (19). The goal of this work was to design multiresidue confirmation procedures complementary to the determinative methods developed in our

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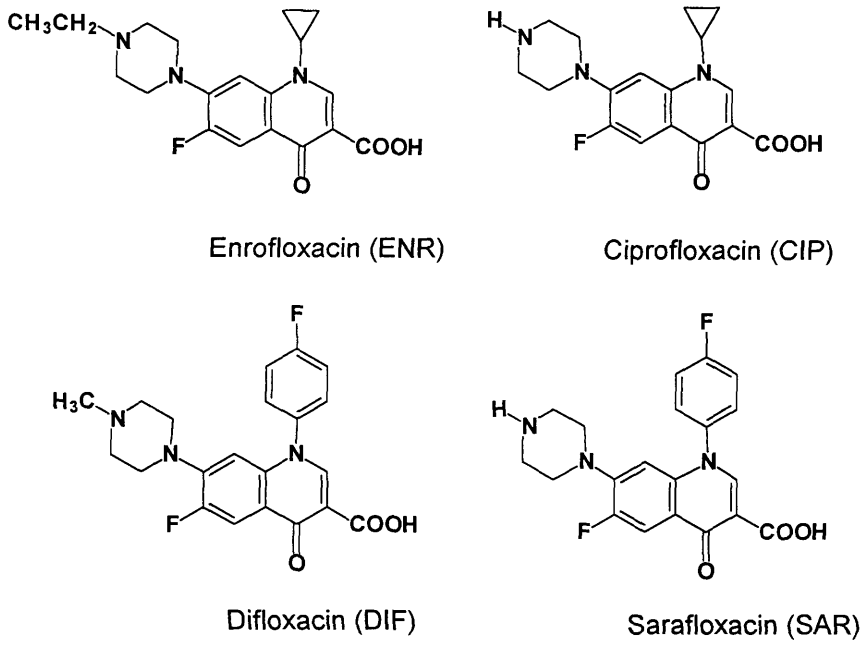

Figure 1. Structures of fluoroquinolones.

laboratory for CIP, ENR, SAR, and DIF at low parts-per-billion (ppb) levels in catfish by using a single quadrupole instrument.

METHOD

The procedure used to extract the fluoroquinolones from catfish is essentially the same as the extraction developed for the determinative method (11) with a few modifications. An abbreviated description of the extraction procedure follows. A more detailed description of all reagents and apparatus was reported previously (10, 11).

Sample Preparation

Accurately weigh 2.0 g blended catfish muscle into 50 mL polypropylene conical tube. Fortify control catfish muscle by adding 40 µL fortification standard; wait several minutes. Add 18 mL extracting solution, absolute ethanol–water–glacial acetic acid (98 + 1 + 1), and homogenize for 20 s. Centrifuge for 5 min at 3000 rpm. Decant supernatant into 175 mL polypropylene tube. Add another 18 mL extracting solution to pellet and homogenize again. Centrifuge for 5 min at 3000 rpm, and add supernatant to first portion. Add 20 mL 1% glacial acetic acid to combined extracts. Place extracts in freezer for 30 min (to expedite flow of extracts through reservoirs and solid-phase extraction [SPE]) columns), and then centrifuge 175 mL tube at 2500 rpm for 10 min at 4°C.

Attach 75 mL reservoir (Varian, Harbor City, CA) containing single 20 µm fritted disk to propyl sulfonic acid (PRS) SPE cartridge (Bond Elut LRC PRS, 500 mg, 10 mL, Varian), using Bond Elut adapter (Varian). Place column on vacuum manifold. Condition column with ca 2 mL methanol followed by 2 mL SPE equilibrating solution (extracting solution–1% gla-

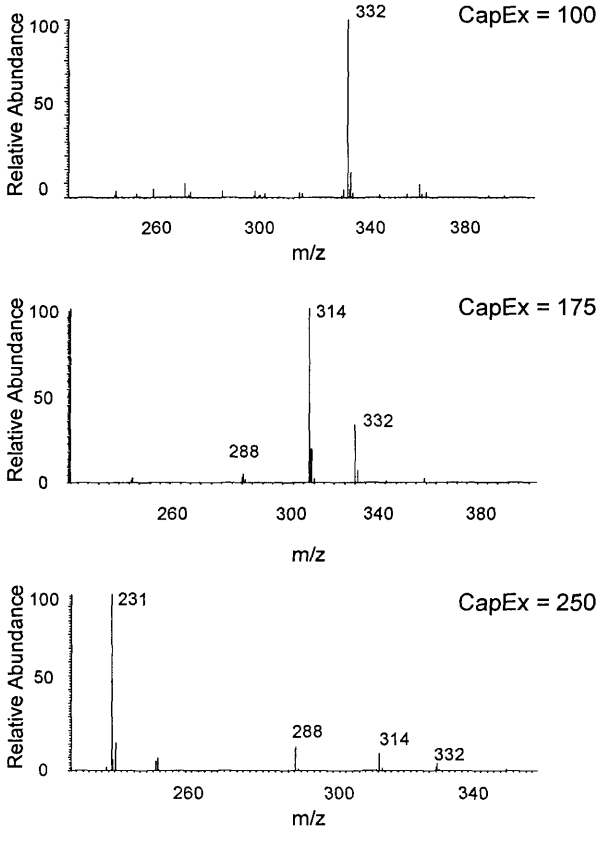

Figure 2. Electrospray mass spectra of CIP standard (100 ng injected) at 3 different CapEx voltages.

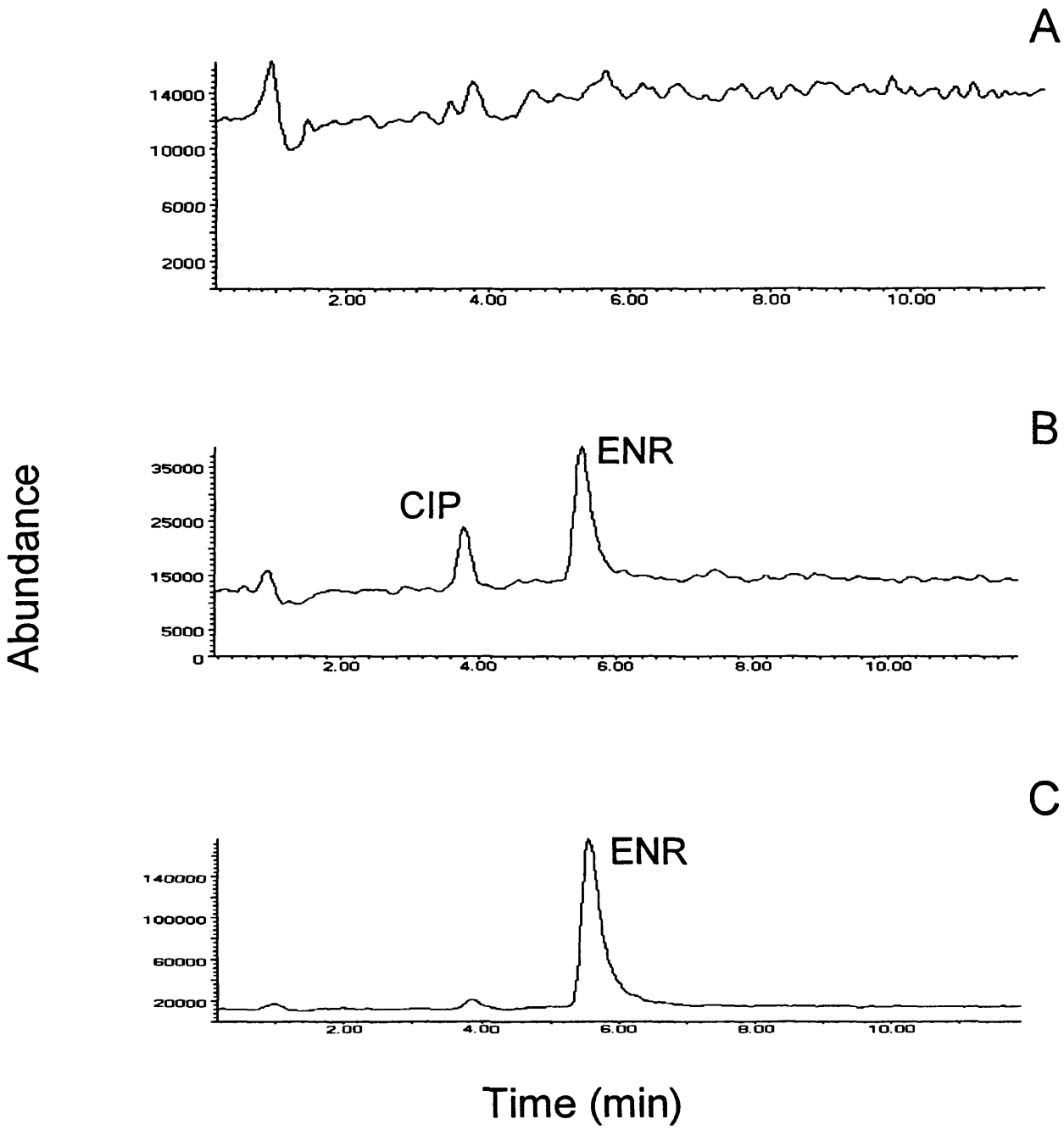

Figure 3. Combined ion chromatograms for CIP/ENR in extracts of (A) control catfish, (B) catfish tissue fortified with FQ at 10 ppb, and (C) catfish dosed with ENR at 5 mg/kg and sacrificed after 144 h.

cial acetic acid [35 + 20]). Apply extracts to column, and let extracts flow through at rate of 1–2 drops/s. After extracts have passed through SPE cartridge, wash cartridge with 2 mL methanol, 5 mL water, and finally 2 mL methanol. Let column dry for 30 s. Elute FQs with 2 mL eluting solution (30% ammonium hydroxide–methanol [1 + 4]) into disposable glass tube. Dry eluate under nitrogen in 50°C water bath, and reconstitute in 500 µL mobile phase. Filter reconstituted residues, using 0.45 µm syringe filters (Puradisk 25 PP, Whatman), into LC vial.

Standard Preparation

For stock solutions accurately weigh an amount of each standard (CIP HCl, Bayer AG, Germany; ENR 99%, Miles Ag., Shawnee Mission, KS; SAR HCl 88.5%, Abbott Lab., North Chicago, IL; DIF HCl 90.2%, Abbott Lab.) approximately

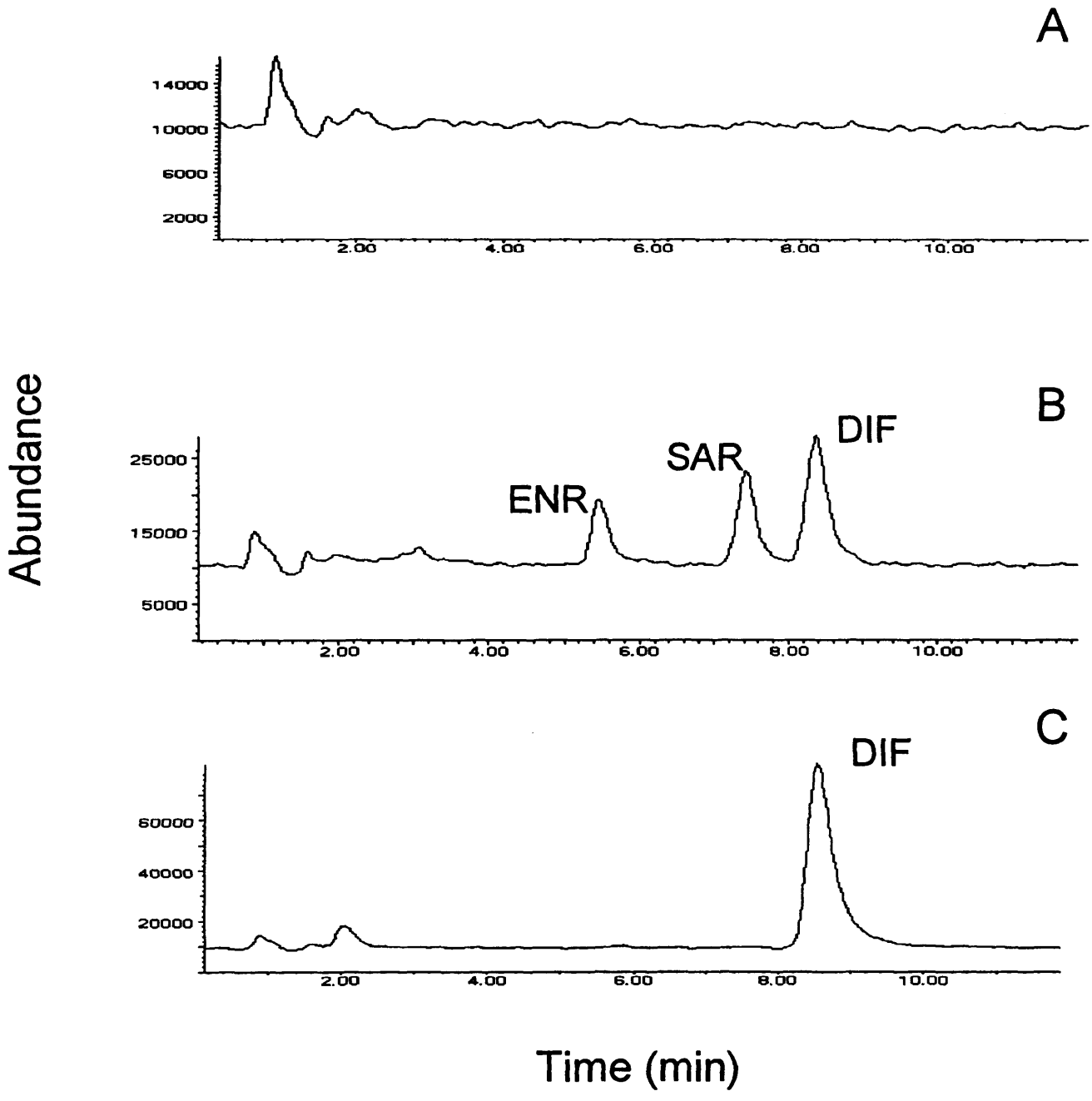

Figure 4. Combined ion chromatograms for SAR/DIF in extracts of (A) control catfish, (B) catfish tissue fortified with FQ at 10 ppb, and (C) catfish dosed with DIF at 5 mg/kg and sacrificed after 240 h.

equivalent to 10.0 mg into individual 100 mL volumetric flasks. Dilute to volume with methanol. Fortification standards were prepared by placing a 2 mL aliquot of each stock solution into a 50 mL volumetric flask and diluting to volume with methanol for a solution containing each standard at 4 ng/μL. This 4 ng/μL standard was used to fortify tissue at 80 ppb. Serial 2-fold dilutions with methanol were made to obtain 2, 1, and 0.5 ng/μL fortification solutions to fortify tissue at 40, 20, and 10 ppb, respectively. An LC/MS working solution was prepared by placing a 1 mL aliquot of each stock solution into a 100 mL volumetric flask and diluting to volume with methanol for a solution containing each standard at 1 ng/μL. The standard equivalent in concentration to a 10 ppb fortified sample extract (0.04 ng/μL in a final volume of 500 μL, assuming 100% recovery) was obtained by diluting 200 μL of this working solution to a final volume of 5 mL with LC/MS mobile phase.

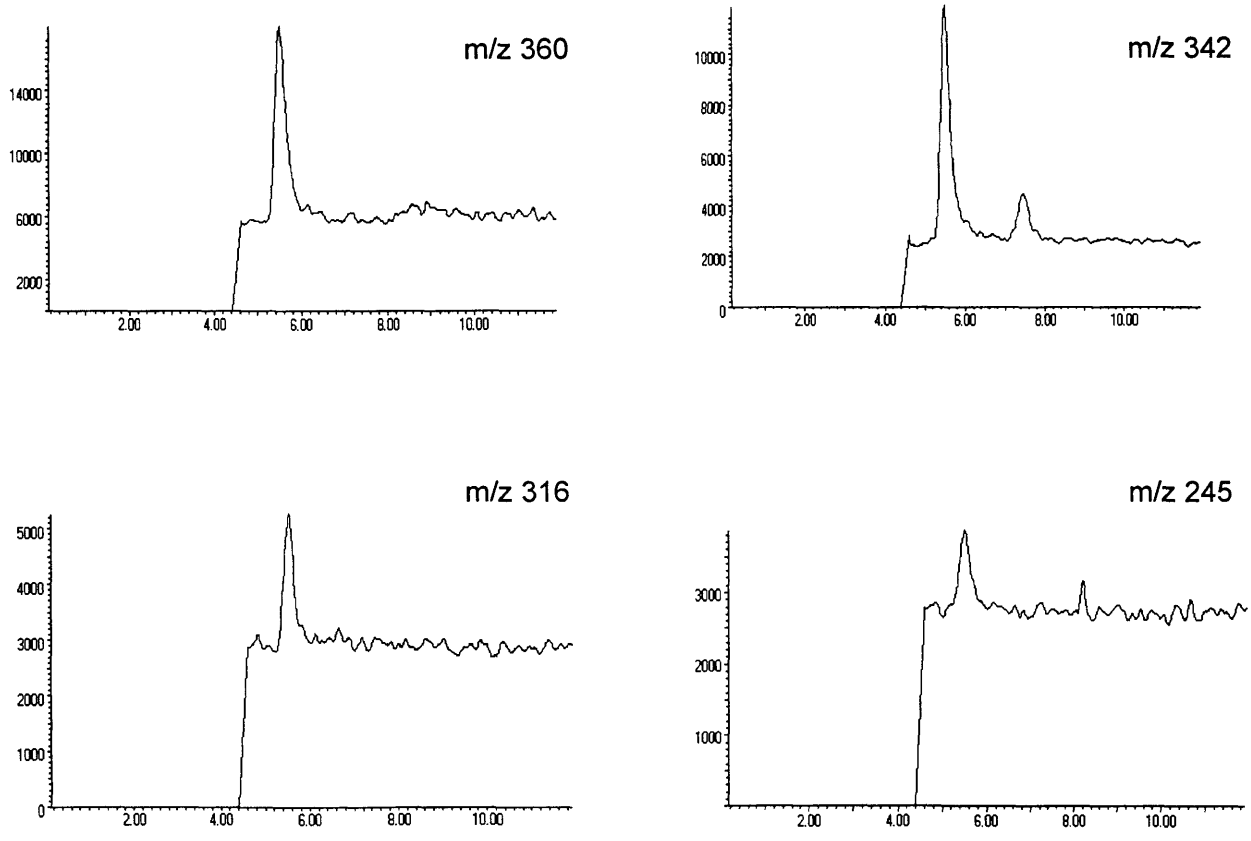

Figure 5. Selected ion chromatograms for ENR in extracts from catfish muscle fortified with FQ at 10 ppb.

LC/MS

A Hewlett-Packard 1090 liquid chromatograph was interfaced to a Hewlett-Packard 5989 mass spectrometer via a 59987A electrospray interface. A Windows-based Chemstation (Version B.02.05) was used to control the mass spectrometer.

The chromatography was performed by using an Inertsil Phenyl column (150 × 2.0 mm, 5 μm) purchased from Metachem Technologies (Torrance, CA). The mobile phase consisted of 2% formic acid (88%, Baker)–acetonitrile (86 + 14) at a flow rate of 0.35 mL/min. The column temperature was maintained at 40°C. Injections of 50 μL were made manually.

The electrospray interface was operated with the nitrogen drying gas at a flow setting of 40 and a temperature of 260°C. The nebulizing gas, also nitrogen, was run at 80 psi. Two different acquisition programs were used. For CIP and ENR residues, ions at *m/z* 332, 314, 288, and 231 were monitored from 0 to 4.5 min, and ions at *m/z* 360, 342, 316, and 245 were selected after 4.5 min. The CapEx voltage was varied by using the instrument's ability to dynamically ramp this setting as follows: ions at *m/z* 245 and below, CapEx = 202; ions at *m/z* 288, CapEx = 174; ions at *m/z* 316, CapEx = 178; ions at *m/z* 332, CapEx = 128; ions at *m/z* 342, CapEx = 184; ions at *m/z* 360 and above, CapEx = 128. These voltages were determined empirically by allowing a mixture of CIP and ENR to flow through the instrument and optimizing CapEx for each ion. The other acquisition program used was for DIF and SAR residues. A full explanation of why 2 different acquisition programs were needed is given in the *Results and Discussion* section. In this program the ions at *m/z* 386, 368, 342, and 299 were monitored up to 7.6 min. After that time the ions at *m/z* 400, 382, 356, and 299 were selected. The CapEx voltage was optimized in the same manner as it was for CIP and ENR, and the following program was used: ions at *m/z* 299, CapEx = 220; ions at *m/z* 342 and 382, CapEx = 190; ions at *m/z* 386 and above, CapEx = 150. For both programs the dwell time for ions was set to 200 ms, and the low resolution setting was used. For each day's analysis, a standard mixture was initially analyzed to determine the performance qualification, or system suitability, of the LC/MS instrument. The analytes needed to elute at the correct retention time (± 5% of the retention time measured previously for standards and within the time-dependent selected ion monitoring [SIM] window), show correct relative abundances (± 15% of what was observed for standards analyzed on previous days), and yield adequate signal-to-noise (S/N) ratios (>3:1) for all ions monitored.

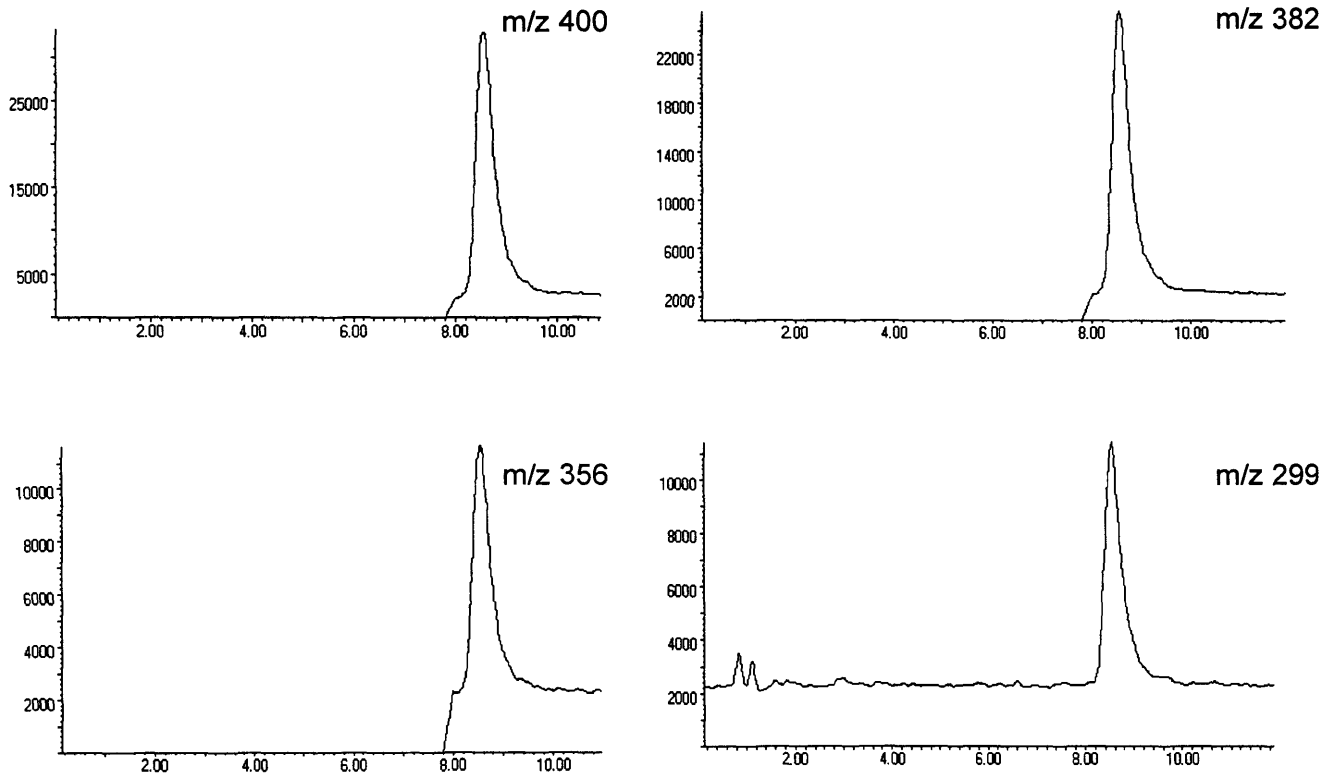

Figure 6. Selected ion chromatograms for DIF in extracts from catfish dosed with DIF at 5 mg/kg and sacrificed after 240 h.

Results and Discussion

The LC/MS confirmation method for FQs in catfish described here is an extension of the LC method with fluorescence detection that was developed in our laboratory for determination of these residues (11). The extraction method is virtually identical, with the exception of placing the extracts in the freezer and centrifuging them again before application to the SPE columns. The final residue was also reconstituted in a smaller volume, 500 µL, instead of 1 mL as indicated in the original method, to allow confirmation of the FQs at 10 ppb in catfish tissue (½X as defined by the determinative method) with an adequate S/N ratio for all monitored ions. Chromatographic conditions were modified to be compatible with the electrospray interface. A formic acid–acetonitrile mobile phase at 0.35 mL/min on a semimicro phenyl column resulted in the best chromatographic performance and electrospray sensitivity. A polymer column (PLRP-S, 150 × 2.1 mm, 5 µm, 100 Å, Polymer Laboratories, MA) was tested later in method development and gave comparable results with the same mobile phase.

To unambiguously identify animal drug residues in a matrix, structural information must be obtained from the mass spectral analysis. Because electrospray is a soft ionization technique, some sort of secondary fragmentation is necessary. Many of the papers published previously for the confirmation of FQs used CID with a tandem mass spectrometer, usually a triple quadrupole system (15, 16, 19). For this method, "in-source" CID was used to obtain similar results. For this instrument, higher CapEx voltages (CapEx voltage is the voltage difference between the capillary exit and the first skimmer cone within the electrospray source) yield more fragmentation via CID with the nitrogen-drying gas. The effect of different CapEx voltages on the electrospray mass spectrum of CIP is shown in Figure 2.

The resulting fragmentation of these FQs at increased values of CapEx is similar to what has been reported earlier (15–19). The mass spectra of all 4 compounds contain ions representing MH^+, $[MH - H_2O]^+$, and $[MH - CO_2]^+$. A fourth ion is also available for monitoring in each mass spectrum. These ions correspond to the following: CIP $[MH - H_2O - C_3H_4 - NC_2H_5]^+$, *m/z* 231; ENR $[MH - CO_2 - NC_4H_9]^+$, *m/z* 245; SAR $[MH - CO_2 - NC_2H_5]^+$, *m/z* 299; and DIF $[MH - CO_2 - NC_3H_7]^+$, *m/z* 299.

For adequate confirmation of these residues at low levels, CapEx must be optimized for each ion of each compound. This was accomplished by using the instrument's ability to dynamically ramp CapEx to different voltages for ions at different *m/z* values; as the *m/z* of the ion monitored in a SIM experiment changes, CapEx immediately changes to the optimum voltage

Table 1. Data for confirmation of CIP in catfish tissue[a]

Sample	RT[b]	Relative abundance			
		m/z 332	*m/z* 314	*m/z* 288	*m/z* 231
10 ppb std	3.59	100	63	13	41
Control fish	ND[c]	—	—	—	—
Control fish	ND	—	—	—	—
80 ppb fort.	3.64	100	68	17	42
40 ppb fort.	3.63	100	66	18	37
20 ppb fort.	3.65	100	53	14	28
10 ppb fort.	3.64	100	57	11	30
10 ppb std	3.62	100	66	16	32
10 ppb std	3.74	100	63	12	33
Control fish	ND	—	—	—	—
10 ppb fort.	3.83	100	54	17	39
Incurred, fish A[d]-1	3.81	100	53	17	33
Incurred, fish A-2	3.82	100	61	21	33
Incurred, fish B[e]-1	3.81	100	62	18	35
Incurred, fish B-2	3.82	100	61	18	35
10 ppb std	3.79	100	64	16	31
10 ppb std	3.84	100	55	15	36
Control fish	ND	—	—	—	—
10 ppb fort.	3.83	100	50	15	31
Incurred, fish A-3	3.84	100	57	18	31
Incurred, fish B-3	3.80	100	60	19	31
10 ppb std	3.80	100	61	21	31

[a] Each section indicates a single day's analysis.
[b] Retention time in min.
[c] ND = none detected.
[d] Fish A was dosed orally with ENR at 5 mg/kg and sacrificed after 114 h; 29 ppb CIP was found by LC with fluorescence detection (11).
[e] Fish B was dosed orally with ENR at 5 mg/kg and sacrificed after 114 h; 34 ppb CIP was found by LC with fluorescence detection (11).

Table 2. Data for confirmation of ENR in catfish tissue[a]

Sample	RT[b]	Relative abundance			
		m/z 360	*m/z* 342	*m/z* 316	*m/z* 245
10 ppb std	5.24	100	78	14	7
Control fish	ND[c]	—	—	—	—
Control fish	ND	—	—	—	—
80 ppb fort.	5.24	100	74	14	8
40 ppb fort.	5.28	100	73	14	8
20 ppb fort.	5.26	100	80	16	8
10 ppb fort.	5.30	100	80	13	9
10 ppb std	5.26	100	73	16	7
10 ppb std	5.43	100	67	16	5
Control fish	ND	—	—	—	—
10 ppb fort.	5.76	100	68	16	13
Incurred, fish C[d]-1	5.64	100	71	17	9
Incurred, fish C-2	5.65	100	74	16	9
Incurred, fish D[e]-1	5.66	100	73	16	9
Incurred, fish D-2	5.71	100	71	16	8
10 ppb std	5.70	100	75	13	8
10 ppb std	5.59	100	69	13	10
Control fish	ND	—	—	—	—
10 ppb fort.	5.59	100	79	13	7
Incurred, fish C-3	5.55	100	68	16	9
Incurred, fish C-3	5.46	100	81	16	10
10 ppb std	5.54	100	75	17	9

[a] Each section indicates a single day's analysis.
[b] Retention time in min.
[c] ND = none detected.
[d] Fish C was dosed orally with ENR at 5 mg/kg and sacrificed after 144 h. The tissue was diluted 1:4 with control; 45 ppb ENR was found in the diluted tissue by LC with fluorescence detection (11).
[e] Fish D was dosed orally with ENR at 5 mg/kg and sacrificed after 144 h. The tissue was diluted 1:4 with control; 46 ppb ENR was found in the diluted tissue by LC with fluorescence detection (11).

for that ion. Because it was not possible to optimize all 4 ions for all 4 residues simultaneously by using this technique, CIP and ENR were optimized together, whereas DIF and SAR were optimized by another acquisition program. The separation of these residues into 2 groups was justified because although it is possible for CIP and ENR to occur together (CIP is a metabolite of ENR), it is highly unlikely that these residues would be found with SAR or DIF in the same tissue. In addition, a tentative identification of suspect residues should be possible from the retention times used in the determinative method. At higher concentrations (≥40 ppb) it was possible to confirm all 4 residues by using a single acquisition program with CapEx set at 180 V (data not shown).

With slight modifications to the extraction procedures of the determinative method and optimization of CapEx, it was possible to confirm all 4 FQs in fortified catfish tissue and also all 4 incurred residues in tissue. These results are shown in Figures 3–6 and in Tables 1–4. Figures 3 and 4 show combined ion chromatograms for CIP and ENR and for SAR and DIF, respectively. These figures illustrate that the control tissue extract shows no appreciable signal at the retention times of the residues and demonstrate the retention times and response obtained for incurred residues and residues from fortified tissues. The chromatogram in Figure 4 contains 3 peaks because all 4 FQs were used to fortify the tissue, and ENR and SAR share a common ion (*m/z* 342). Figures 5 and 6 are examples of selected ion chromatograms for individual residues. In Figure 5, ENR was confirmed in a 10 ppb fortified tissue extract. Likewise, incurred DIF was confirmed by monitoring the appropriate ions shown in Figure 6. For all samples, the relative abundance of each ion (calculated by integrating each ion chromatogram) was compared with those for a standard compound analyzed on the same day under the same conditions. Representative data for each residue are shown in Tables 1–4.

This method was used to confirm residues in tissues fortified in the 10–80 ppb range. All relative abundances were within ±10% of the values calculated for standard compounds (i.e., if the relative abundance for an ion in a spectrum of the standard

Table 3. Data for confirmation of SAR in catfish tissue[a]

Sample	RT[b]	Relative abundance			
		m/z 386	*m/z* 368	*m/z* 342	*m/z* 299
10 ppb std	7.14	100	55	22	32
Control fish	ND[c]	—	—	—	—
Control fish	ND	—	—	—	—
80 ppb fort.	7.15	100	62	27	32
40 ppb fort.	7.13	100	59	25	33
20 ppb fort.	7.14	100	63	24	33
10 ppb fort.	7.11	100	60	24	32
10 ppb std	7.12	100	52	26	39
10 ppb std	7.33	100	54	30	36
Control fish	ND	—	—	—	—
10 ppb fort.	7.68	100	52	24	38
Incurred, fish E[d]-1	7.69	100	58	26	34
Incurred, fish E-2	7.73	100	59	30	36
Incurred, fish F[e]-1	7.74	100	57	27	38
Incurred, fish F-2	7.75	100	61	27	35
10 ppb std	7.71	100	54	20	40
10 ppb std	7.44	100	57	26	37
Control fish	ND	—	—	—	—
10 ppb fort.	7.63	100	56	25	36
Incurred, fish E-3	7.64	100	56	29	37
Incurred, fish F-3	7.61	100	58	25	32
10 ppb std	7.56	100	51	21	34

[a] Each section indicates a single day's analysis.
[b] Retention time in min.
[c] ND = none detected.
[d] Fish E was dosed orally with SAR at 5 mg/kg and sacrificed after 144 h; 30 ppb SAR was found by LC with fluorescence detection (11).
[e] Fish F was dosed orally with SAR at 5 mg/kg and sacrificed after 144 h; 16 ppb SAR was found by LC with fluorescence detection (11).

Table 4. Data for confirmation of DIR in catfish tissue[a]

Sample	RT[b]	Relative abundance			
		m/z 400	*m/z* 382	*m/z* 356	*m/z* 299
10 ppb std	8.06	100	73	27	24
Control fish	ND[c]	—	—	—	—
Control fish	ND	—	—	—	—
80 ppb fort.	8.04	100	87	29	23
40 ppb fort.	8.02	100	81	23	24
20 ppb fort.	8.04	100	77	25	23
10 ppb fort.	7.99	100	82	32	25
10 ppb std	8.01	100	80	25	21
10 ppb std	8.56	100	75	29	32
Control fish	ND	—	—	—	—
10 ppb fort.	8.53	100	85	27	30
Incurred, fish G[d]-1	8.55	100	74	29	29
Incurred, fish G-2	8.55	100	67	28	27
Incurred, fish H[e]-1	8.58	100	71	31	27
Incurred, fish H-2	8.52	100	70	29	28
10 ppb std	8.55	100	74	29	26
10 ppb std	8.44	100	77	28	24
Control fish	ND	—	—	—	—
10 ppb fort.	8.65	100	81	28	25
Incurred, fish G-3	8.68	100	74	32	28
Incurred, fish H-3	8.70	100	70	31	29
10 ppb std	8.61	100	76	24	27

[a] Each section indicates a single day's analysis.
[b] Retention time in min.
[c] ND = none detected.
[d] Fish G was dosed orally with DIF at 5 mg/kg and sacrificed after 240 h; 27 ppb DIF was found by LC with fluorescence detection (11).
[e] Fish H was dosed orally with DIF at 5 mg/kg and sacrificed after 240 h; 34 ppb DIF was found by LC with fluorescence detection (11).

is 40%, the relative abundance in the sample spectrum should be between 30 and 50%). All residues in a set of 5 samples fortified with each FQ at 10 ppb were also confirmed by meeting this criterion (data not shown). In addition to obtaining good agreement between samples and standards analyzed on the same day, a review of the data shows that the relative abundances of ions obtained by in-source CID on different days is also very reproducible. Although this method is meant to be qualitative and not quantitative, it is possible to estimate approximate recoveries by comparing integration data from MH^+ ion chromatograms of sample and standards. Fortified standard curves calculated in this manner show linearity (correlation coefficients of 0.993–0.999) in the 20–160 ppb range. For most residues, recoveries were approximately 60% at the 10 ppb level. The exception was CIP; recovery values for this residue were abnormally low, approximately 35%. It may be that the poor response of CIP in tissue extracts was due to ion suppression from matrix components, and the volume of extract injected affected the response (and apparent recovery). Despite this problem, the sensitivity was adequate to confirm all 4 residues at the 10 ppb level. The results from the confirmation analysis of tissue with incurred residues support the data reported earlier from the LC method with fluorescence detection (11). Catfish with incurred ENR showed small amounts of CIP in addition to the ENR residues. Only the parent compound was confirmed in catfish dosed with SAR or DIF.

In summary, this method describes the confirmation of 4 FQ residues in catfish muscle by using a rapid, efficient extraction developed for an LC determinative method. The confirmation uses in-source CID to obtain structurally significant fragment ions with a single quadrupole instrument. In future work, this confirmation method will be applied to FQ residues in other matrixes, such as milk and shrimp.

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