Confirmation of Fluoroquinolones in Catfish Tissue by Electrospray LC/MS

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Introduction

Fluoroquinolones (FQs) are antibacterial agents that are used in both human and veterinary medicine. Sarafloxacin is approved for use in poultry in the United States (1) and enrofloxacin, approved in the U.S. for non-food animals, is believed to be used extensively in cattle in Europe (2). Residues of fluoroquinolones in tissue of food animals are of concern due to reports of antibacterial resistance developing to these drugs; the FDA has recently banned the extra-label 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 matrices (5-12). Most quantitative methods utilize liquid chromatography with fluorescence detection and can measure residues down to 5-20 ppb. Two methods have recently been developed in our laboratory using a simple extraction procedure combined with LC/fluorescence for the determination of ciprofloxacin (CIP), enrofloxacin (ENR), sarafloxacin (SAR) and difloxacin (DIF) (Figure 1) in milk (10) and catfish tissue (11,12).

For regulatory purposes, unambiguous identification of the suspect residues found in the sample by the determinative methods is critical. Mass spectral analysis is the preferred technique for confirmation of suspect residues due to its inherent specificity and sensitivity. Several reports of mass spectral characterization and use of mass spectrometry to confirm fluoroquinolone or quinolone residues have been described. In early methods, quinolones were decarboxylated to form stable products for GC/MS analysis (13-15). More recent reports utilize electrospray (ES) LC/MS to characterize and confirm quinolones and fluoroquinolone antibacterial drugs (16-20). For example, Schneider et al. report the confirmation of danofloxacin in cattle and chicken liver using this technique (16). Chui and Lee characterized eight fluoroquinolones by ES and have applied this method to confirmation of residues in milk (17). There has also been previous work confirming FQ residues in catfish tissue. In a method developed in part for regulatory purposes, Schilling et al. used electrospray LC/MS for the confirmation of sarafloxacin in catfish tissue (18). This method utilized collisionally induced dissociation in a triple quadrupole instrument in order 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 fluoroquinolones into the mass spectrometer were reported. The goal of this work was to design multi-residue confirmation procedures complimentary to the determinative methods developed in our laboratory for CIP, ENR, SAR, DIF in catfish using a single quadrupole instrument at low ppb levels.

Method

Sample Preparation

The procedure used to extract the fluoroquinolones from catfish is essentially the same as what was developed for the determinative method (11,12) with a few modifications. An abbreviated description of the extraction procedure follows. A more detailed description of all reagents and apparatus used is available in previous references (10-12).

Accurately weigh 2.0 g of blended catfish muscle into a 50 mL polypropylene conical tube. Fortify control catfish muscle by adding 40 µL of fortification standard; wait several minutes. Add 18 mL of extracting solution (98:1:1 of absolute ethanol:water:glacial acetic acid) and homogenize for 20 s. Centrifuge for 5 min at 3000 rpm. Decant the supernatant into a 175 mL polypropylene tube. Add another 18 mL of extracting solution to the pellet and homogenize again. Centrifuge for 5 min at 3000 rpm and add the supernatant to the first portion. Add 20 mL of 1% glacial acetic acid to the combined extracts. Allow the extracts to sit in freezer for 30 minutes and then centrifuge 175 mL tubes at 2500 rpm for 10 min at 4 °C.

Attach a 75 mL reservoir (Varian, Harbor City, CA) containing a single 20 µm frit to a propyl sulfonic acid (PRS) solid phase extraction cartridge (Bond Elut LRC PRS, 500 mg , 10 mL, Varian) using a Bond Elut adaptor (Varian). Place the column on a vacuum manifold. Condition the column with approximately 2 mL of methanol followed by 2 mL of SPE-equilibrating solution (35:20 of extracting solution: 1% glacial acetic acid). Apply the extracts to the column and allow to flow through at a rate of 1-2/drops/s. After the extract has passed through the SPE cartridge, wash with 2 mL of methanol, 5 mL of water, and finally 2 mL of methanol. Allow the columns to dry for 30 s. Elute the FQs with 2 mL of eluting solution (1:4 of 30% ammonium hydroxide: methanol) into a disposable glass tube. The eluent is dried under nitrogen in a 50 °C water bath and reconstituted in 500 µL of mobile phase. The residues are then filtered using 0.45 µm syringe filters (Puradisk 25 PP, Whatman) into LC vials.

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 equivalent to 10.0 mg into individual 100 mL volumetric flasks. Dilute to volume with methanol. Fortification standards were made by aliquoting 2 mL of each stock solution into a 50 mL flask and bringing to volume with methanol for a 4 ng/ µL standard mix. This 4 ng/ µL standard was used to fortify tissue at 80 ppb. Serial two-fold dilutions with methanol were made to obtain 2, 1, 0.5 ng/ µL fortification solutions to fortify tissue at 40,20, and 10 ppb, respectively. An LC/MS working solution was made by aliquoting 1 mL of each stock solution into 100 mL volumetric and diluting with methanol to give a 1 ng/ µL solution. 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.

LC/MS Conditions

A Hewlett-Packard 1090 LC was interfaced to 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 using an Inertsil Phenyl column (150 x 2.0 mm, 5 µm) purchased from Metachem Technologies (Torrance, CA). The mobile phase consisted of 86:14 2% Formic Acid (88%, Baker): acetonitrile 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 analyzing CIP and ENR residues ions m/z 332, 314, 288, and 231 were monitored from 0 to 4.5 min., and ions m/z 360, 342, 316, and 245 were selected after 4.5 min. The CapEx voltage varied using the instrument's ability to dynamically ramp this setting as follows: ions m/z 245 and below, CapEx = 202; m/z 288, 174; m/z 316, 178; m/z 332, 128; m/z 342, 184; m/z 342 and above, 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. In this program ions m/z 386, 368, 342, and 299 were monitored up to 7.6 min. After that time ions m/z 400, 382, 356, and 299 were selected. The CapEx voltage was optimized as with CIP and ENR and the following program was used: at m/z 299, CapEx = 220; m/z 342 and 382, 190; m/z 386 and above, 150. For both programs the dwell time for ions was set to 200 ms and the low resolution setting was used.

Results and Discussion

The LC/MS confirmation method for FQs in catfish described here is an extension of the LC/fluorescence determinative method which was developed in our laboratory for these residues (11,12). 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. This additional step expedited the flow of the extracts through the reservoirs and SPE columns. The final residue was also reconstituted in a smaller volume, 500 µL, instead of 1 mL as indicated in the original method. This allowed confirmation of the FQ at 10 ppb in catfish tissue, or ½X as defined by the determinative method, and still obtain adequate signal to noise for all monitored ions. Chromatographic conditions were modified in order to be compatible with the electrospray interface. A formic acid/acetonitrile mobile phase at 0.35 mL/min on a semi-micro phenyl column resulted in the best chromatographic performance and electrospray sensitivity. A polymer column (PLRP-S, Polymer Laboratories, MA) was tested later in method development and gave comparable results with the same mobile phase.

In order to unambiguously identify animal drug residues in a matrix, some structural information must be obtainable from the mass spectral analysis. Since electrospray is a soft ionization technique, some sort of secondary fragmentation is necessary. Many of the papers published previously for the confirmation of FQs utilized collisionally induced dissociation (CID) with a tandem mass spectrometer, usually a triple quadrupole system (16, 18). For this method, "in-source" CID was used to obtain similar results. For this instrument, higher CapEx voltages yield higher energy fields and therefore 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 for these FQs at increased values of CapEx is similar to what has been reported earlier (16-20). The mass spectrum of all four compounds contain ions representing MH^+, $[MH - H_2O]^+$, $[MH - CO_2]^+$. There is also a fourth ion available for monitoring in each mass spectrum. These ions correspond to: 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; DIF $[MH - CO_2 - NC_3H_7]^+$, m/z 299.

In order to adequately confirm these residues at low levels, CapEx must be optimized for each ion for each compound. This was accomplished by using the instrument's ability to dynamically ramp CapEx to different voltages for different m/z ions. It was not possible to optimize all four ions for all four residues simultaneously using this technique, so CIP and ENR were done together while DIF and SAR were optimized by another acquisition program. The separation of these residues into two groups was justified because while it is possible that CIP and ENR may occur together (CIP is a metabolite of ENR), it is highly unlikely that these residues would be found in the same tissue as SAR or DIF. In addition, a tentative identification of suspect residues should be possible from the determinative method retention times. At higher concentrations (40 ppb or higher) it was possible to confirm all four residues using a single acquisition program with CapEx set at 180V (data not shown).

With slight modifications to the determinative method extraction procedures and optimization of CapEx, it was possible to confirm all four FQs in fortified catfish tissue and also in incurred 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 , 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 seen for fortified and incurred tissue extracts. 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, an incurred sample containing 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 to those from a standard compound analyzed on the same day under the same conditions. Representative data from these calculations is shown for each residue 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 relative abundance for an ion in spectrum of standard is 40%, the relative abundance in the sample spectrum should be between 30 and 50%). All residues in a set of five samples fortified with each FQ at 10 ppb were also confirmed meeting this criteria (data not shown). 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 range from 0.993 to 0.999) in the 20-160 ppb range. For most residues recoveries values were approximately 60% at the 10 ppb levels. 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, as the response (and apparent recovery) was affected by the volume of extract injected. Despite this problem, the sensitivity was adequate to confirm all four residues at the 10 ppb level. The results from the confirmation analysis of incurred tissued support the data reported earlier from the LC/fluorescence method (11,12). Catfish incurred with 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 four fluoroquinolone residues in catfish tissue using a rapid, efficient extraction developed for an LC determinative method . The confirmation utilized in-source collisionally induced dissociation to obtain structurally significant fragment ions using a single quadrupole instrument. In future work, this confirmation method will be applied to fluoroquinolone residues in other matrices, such as milk and shrimp.

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Table 1. Data for Confirmation of CIP in Catfish Tissue

		Relative Abundances			
Sample	**RT[1]**	**332**	**314**	**288**	**231**
10 ppb std	3.59	100	63	13	41
Control Fish	ND	--	--	--	--
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 [2]
10 ppb std	3.74	100	63	12	33
Control Fish	ND	--	--	--	--
10 ppb Fort	3.83	100	54	17	39
Incurred Fish A[3]-1	3.81	100	53	17	33
Incurred Fish A-2	3.82	100	61	21	33
Incurred Fish B[4]-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

[1] Retention time in minutes

[2] Each section separated by dotted line indicates single day's analysis

[3] Fish A was dosed orally with 5 mg/kg of ENR and sacrificed after 144 hr; 29 ppb of CIP was found by LC/Fluor (11).

[4] Fish B was dosed orally with 5 mg/kg of ENR and sacrificed after 144 hr; 34 ppb of CIP was found by LC/Fluor (11).

Table 2. Data for Confirmation of ENR in Catfish Tissue
Relative Abundances

Sample	RT[1]	360	342	316	245
10 ppb std	5.24	100	78	14	7
Control Fish	ND	--	--	--	--
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 [2]
10 ppb std	5.43	100	67	16	5
Control Fish	ND	--	--	--	--
10 ppb Fort	5.76	100	68	16	13
Incurred Fish C[3]-1	5.64	100	71	17	9
Incurred Fish C-2	5.65	100	74	16	9
Incurred Fish D[4]-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

[1] Retention time in minutes

[2] Each section separated by dotted line indicates single day's analysis

[3] Fish C was dosed orally with 5mg/kg of ENR and sacrificed after 144 hr. This tissue was diluted 1:4 with control; 45 ppb of ENR was found in the diluted tissue by LC/Fluor (11).

[4] Fish D was dosed orally with 5 mg/kg of ENR and sacrificed after 144 hr. This tissue was diluted 1:4 with control; 46 ppb of ENR was found in the diluted tissue by LC/Fluor (11).

Table 3. Data for Confirmation of SAR in Catfish Tissue

		Relative Abundances			
Sample	**RT[1]**	**386**	**368**	**342**	**299**
10 ppb std	7.14	100	55	22	32
Control Fish	ND	--	--	--	--
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 [2]
10 ppb std	7.33	100	54	30	36
Control Fish	ND	--	--	--	--
10 ppb Fort	7.68	100	52	24	38
Incurred Fish E[3]-1	7.69	100	58	26	34
Incurred Fish E-2	7.73	100	59	30	36
Incurred Fish F[4]-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

[1] Retention time in minutes

[2] Each section separated by dotted line indicates single day's analysis

[3] Fish E was dosed orally with 5 ng/kg of SAR and sacrificed after 144 hr; 30 ppb of SAR was found by LC/Fluor (11).

[4] Fish F was dosed orally with 5 mg/kg of SAR and sacrificed after 144 hr; 16 ppb of SAR was found by LC/Fluor (11).

Table 4. Data for Confirmation of DIF in Catfish Tissue
Relative Abundances

Sample	RT[1]	400	382	356	299
10 ppb std	8.06	100	73	27	24
Control Fish-	ND	--	--	--	--
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 [2]
10 ppb std	8.56	100	75	29	32
Control Fish	ND	--	--	--	--
10 ppb Fort	8.53	100	85	27	30
Incurred Fish G[3]-1	8.55	100	74	29	29
Incurred Fish G[4]-2	8.55	100	67	28	27
Incurred Fish H-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

[1] Retention time in minutes
[2] Each section separated by dotted line indicates single day's analysis
[3] Fish G was dosed orally with 5 mg/kg of DIF and sacrificed after 240 hr.; 27 ppb of DIF was found by LC/Fluor (11).
[4] Fish H was dosed orally with 5 mg/kg of DIF and sacrificed after 240 hr; 34 ppb of DIF was found by LC/Fluor (11).

Figure Captions

Figure (1). Structures of Fluoroquinolones

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

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

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

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

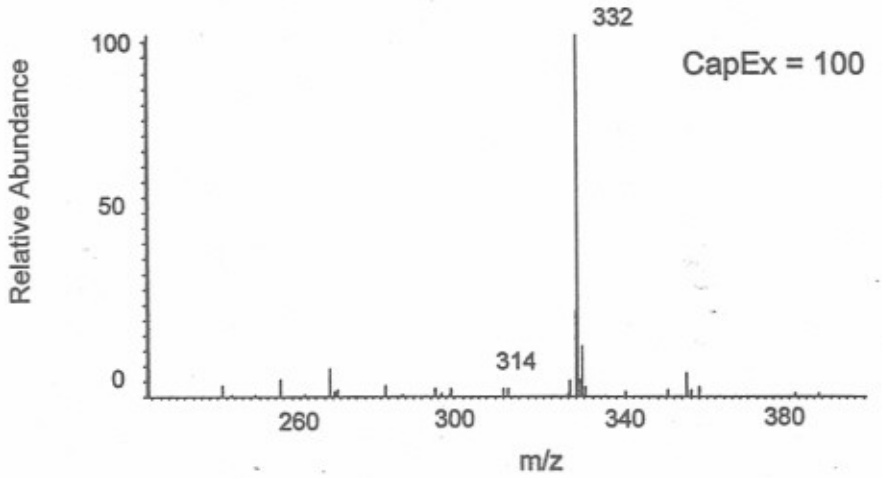
Figure (6). Selected ion chromatograms for DIF in extracts from catfish dosed with 5 mg/kg DIF and sacrificed after 240 hr.

Enrofloxacin (ENR)

Ciprofloxacin (CIP)

Difloxacin (DIF)

Sarafloxacin (SAR)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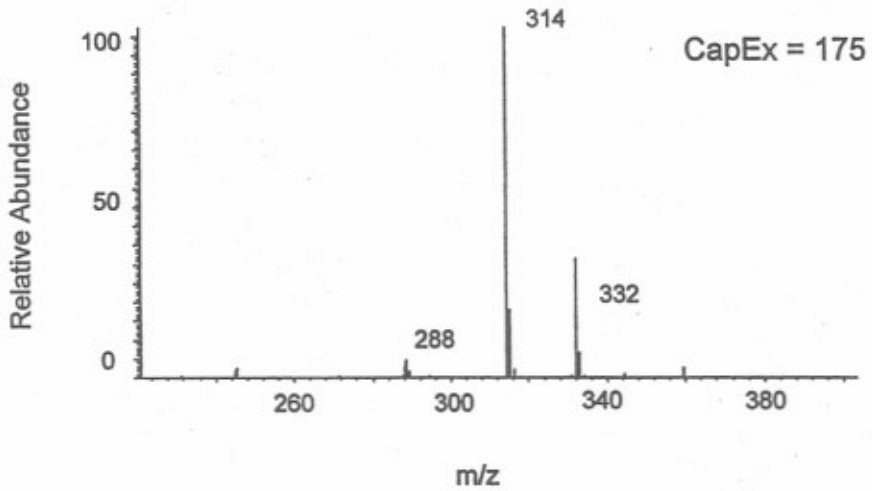
m/z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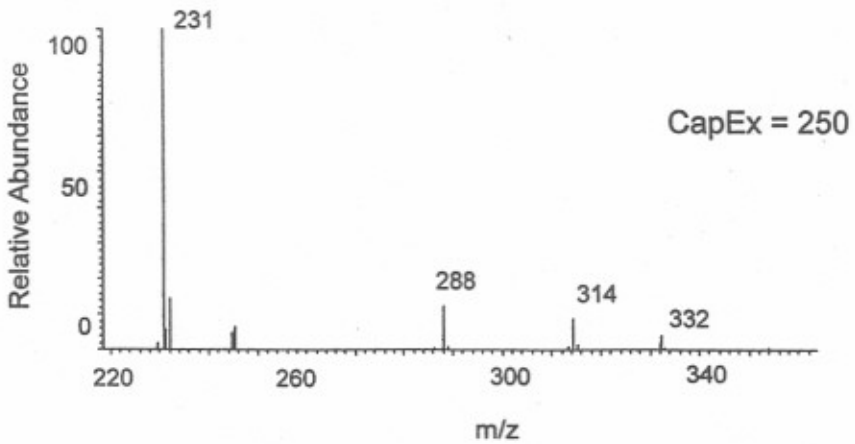
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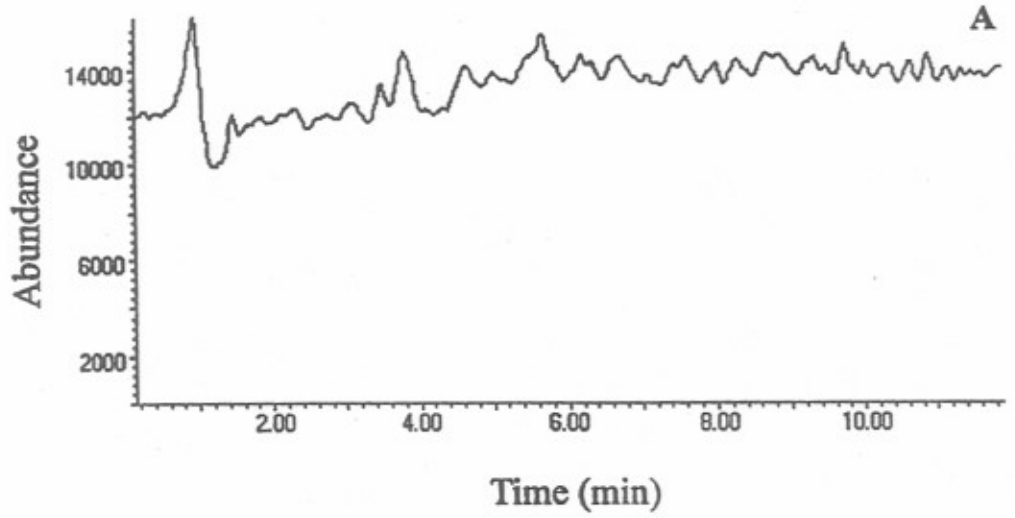
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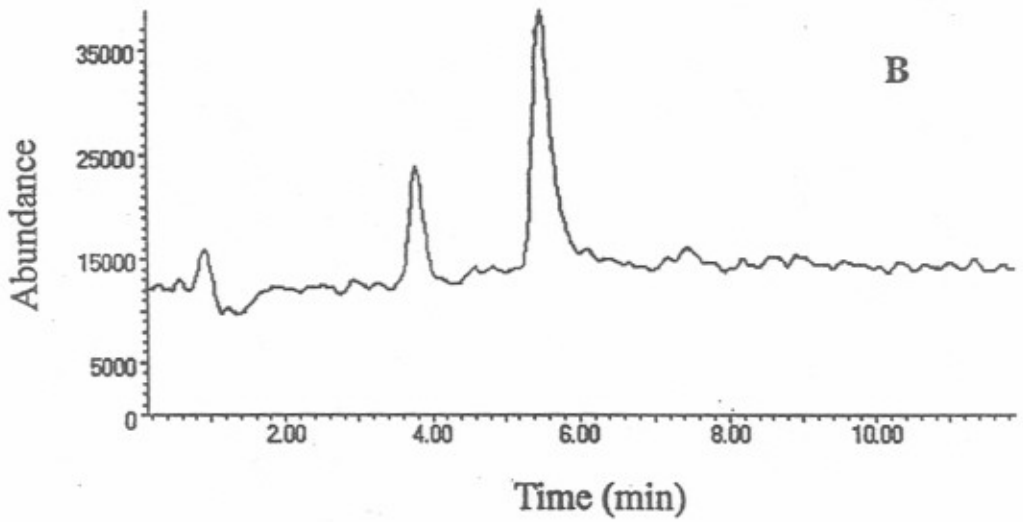
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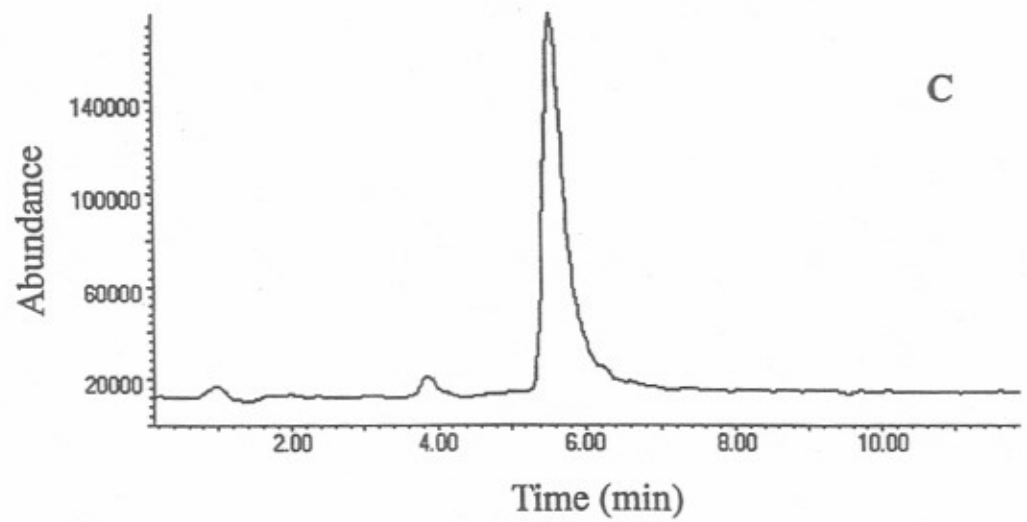
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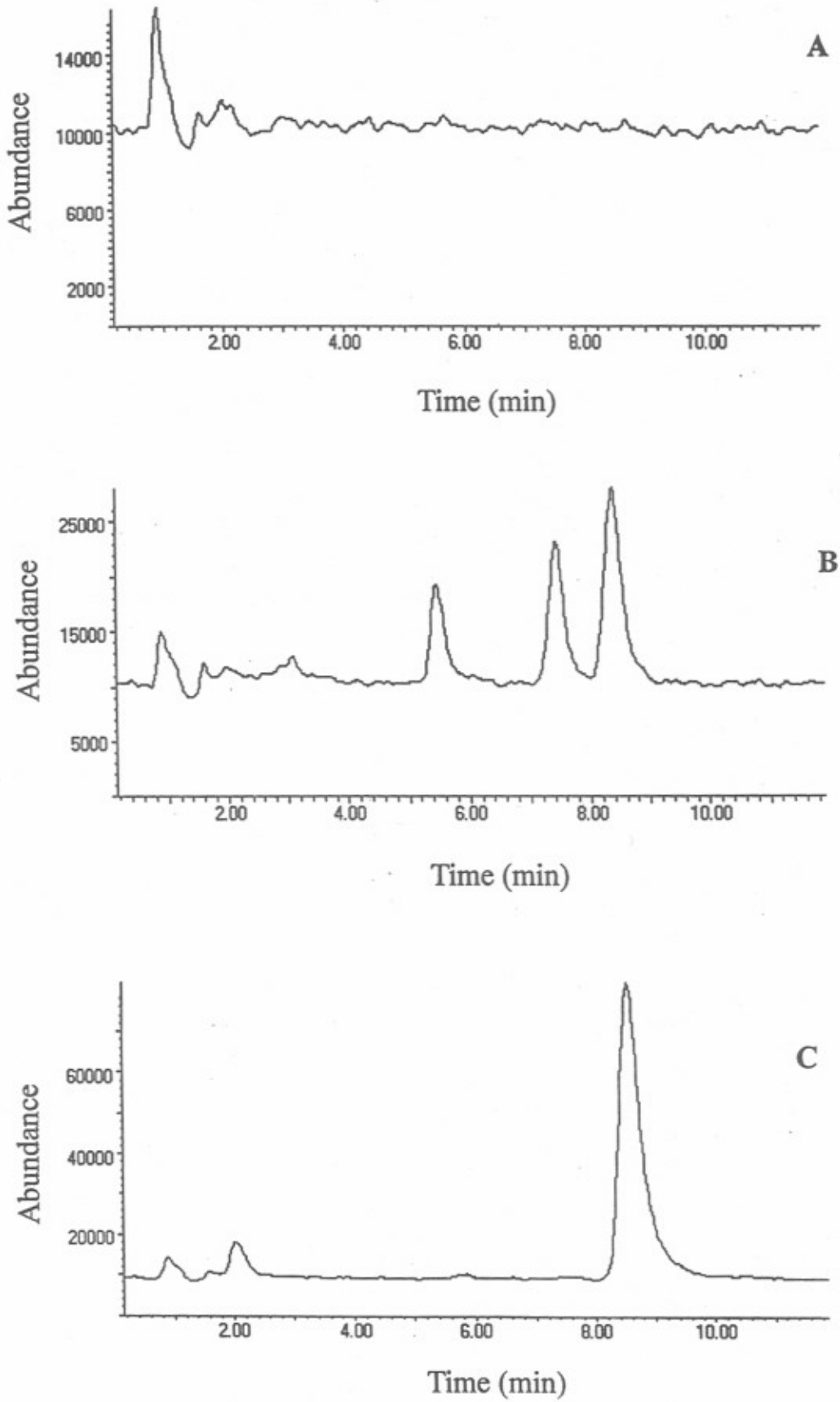
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Time (min)
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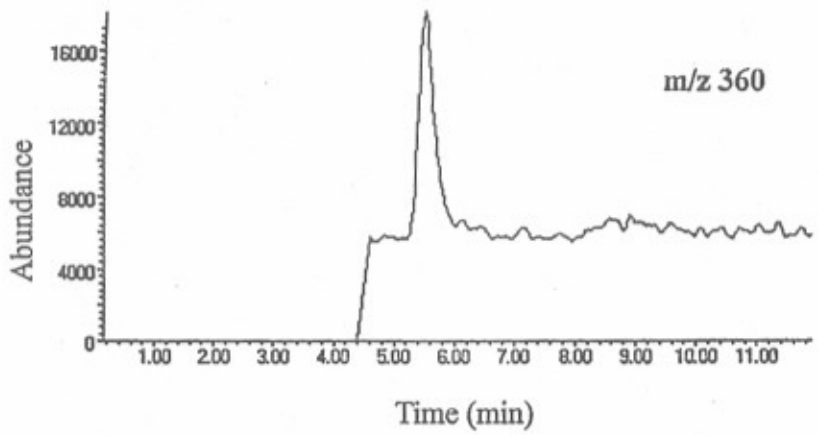
Time (min)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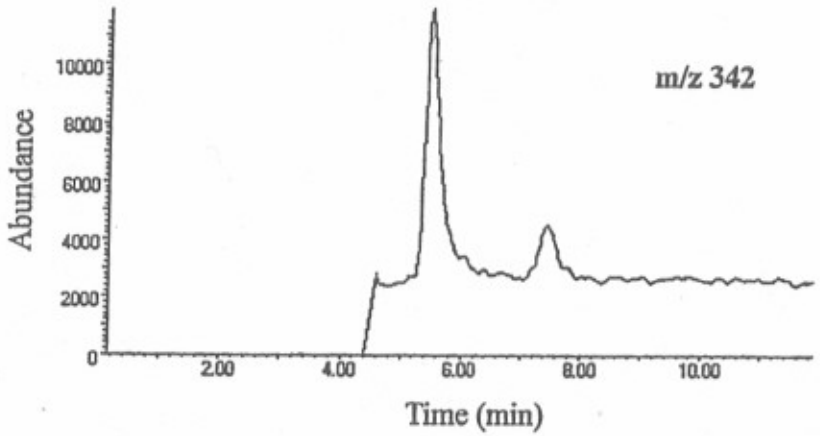
Time (min)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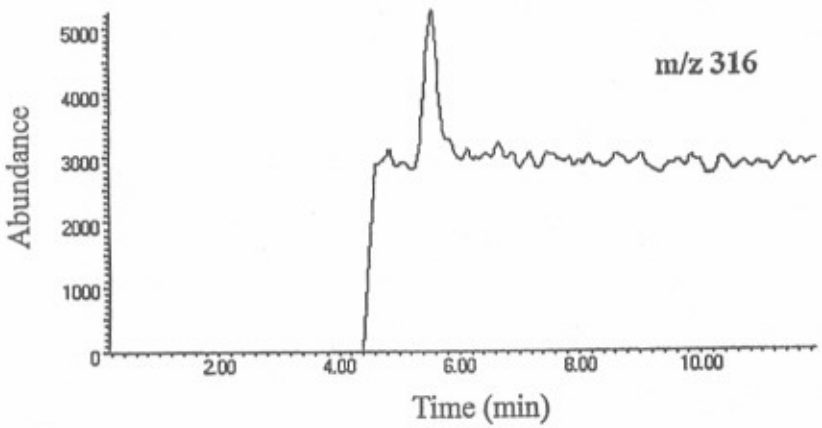
Time (min)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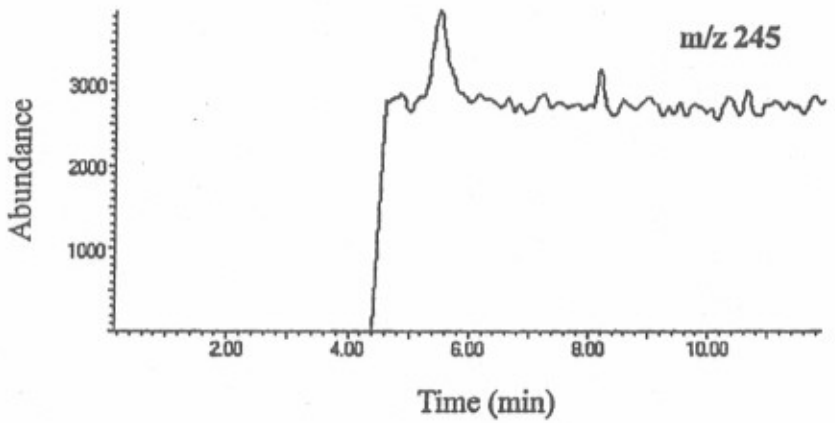
Time (min)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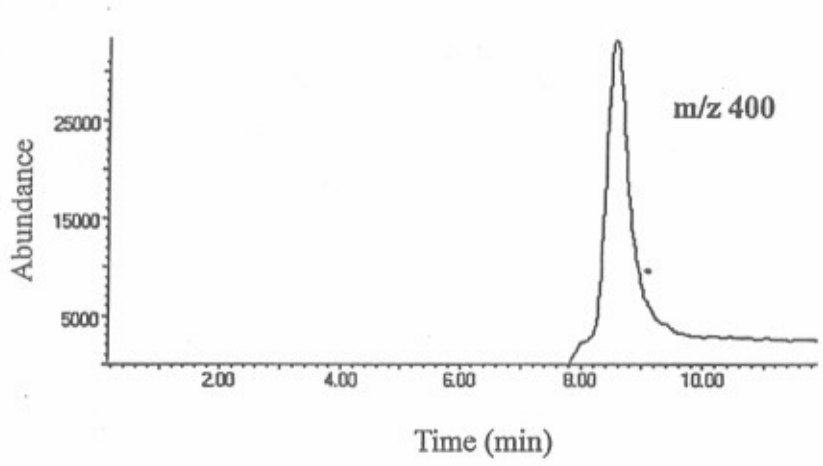
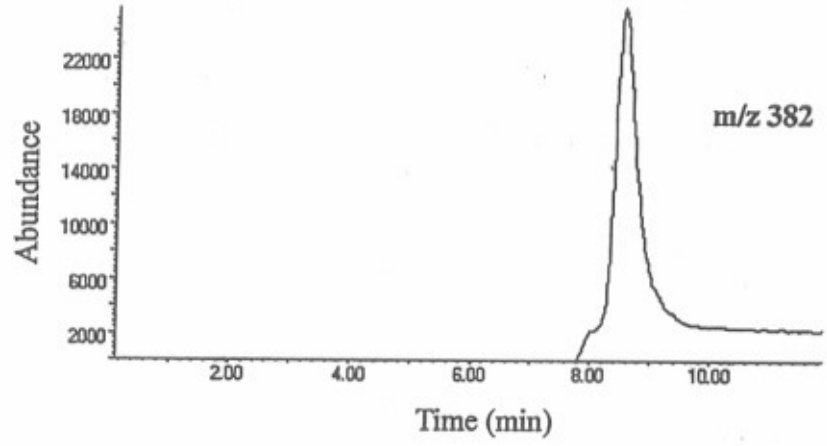
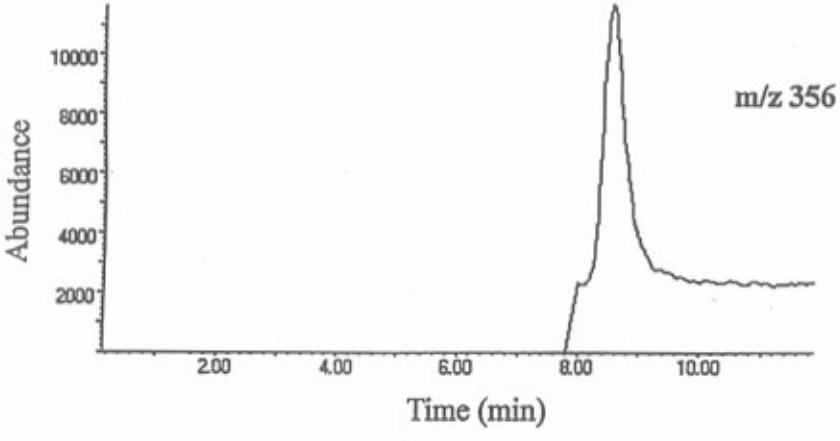
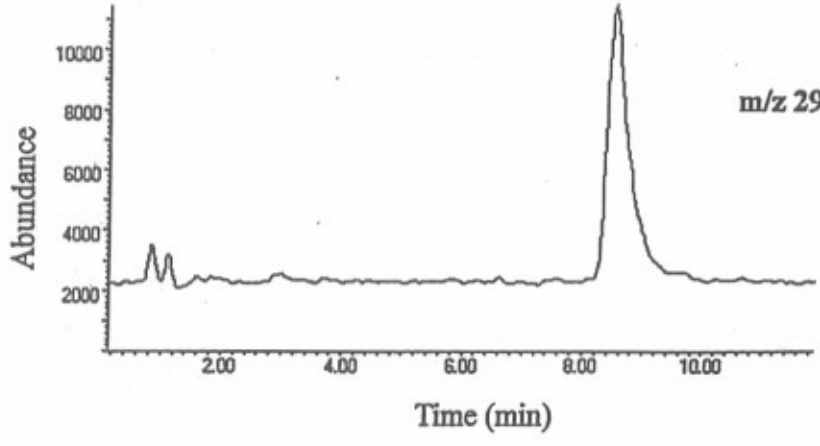
Time (min)









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LB #4 (10)
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