Concurrent Determination of Four Fluoroquinolones; Ciprofloxacin, Enrofloxacin, Sarafloxacin and Difloxacin in Atlantic Salmon Tissue by LC with Fluorescence Detection

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A. INTRODUCTION

Fluoroquinolone antibacterials (FQs) have been shown to be very effective in combating various diseases in animal husbandry and aquaculture. This method focused on the detection of sarafloxacin (SARA), enrofloxacin (ENRO), difloxacin (DIFLX), and ciprofloxacin (CIPRO) because of their availability and very effective broad spectrum activity against many microbes. Although none of the FQ's have US approval for use as aquaculture therapeutants, the potential for the extra-label use of these FQ's is of concern. The interest indicated by the aquaculture industry in these drugs and potential for the emergence of drug resistant bacteria through their use has created a need for analytical methods to monitor for residues these drugs in both domestic and imported aquaculture products. The goal of this work was to develop a method which could analyze residues of these four FQs in aquaculture products, concurrently, in a timely manner suitable to a regulatory application.

B. PRINCIPLE

A liquid chromatographic (LC) method with fluorescence detection is presented for the concurrent analysis of four FQs, ENRO, CIPRO, SARA and DIFLX in Atlantic salmon. The procedure consists of extraction of fish tissue with acidified ethanol, isolation and retention on a cation exchange SPE column, elution with basic methanol and LC analysis with fluorescence detection. LC analysis is performed by isocratic elution utilizing Acetonitrile/2%Acetic Acid (16+84) mobile phase and a PLRP-S polymer column with fluorescence detection, EX-278nm and EM-450nm. A target level of 20 ppb for each of the four fluoroquinolones has been established for this method. Fortified and incurred fish sample results are based on a five point standard curve calculation (10-160 ppb). Overall percent recoveries (%RSD) from fortified Atlantic salmon were 56 (15), 93 (5.6), 61 (11.) and 87 (5.0) for CIPRO, ENRO, SARA and DILFX, respectively (see Table 1). Chromatograms of control salmon, fortified salmon and 20 ppb equivalent standard are shown in Figure 1.

Analysis of a set of samples can easily be performed in one day (8 hours). A set consists of 5 fortified or incurred samples and one control. Those facilities with autosample LC systems can performed at least two sets per day.

C. EQUIPMENT

As specified or equivalent

1. *Liquid chromatograph.*-- Hewlett-Packard, Model HP1090 with HP Vectra 486 HP Chem Station (Hewlett-Packard, Avondale, PA). Operating conditions: mobile phase flow, 0.9mL/min.; column temperature, 60°C; column pressure, 2300-2600 psi; volume injected, 50ml.

2. *Detector.*-- Hewlett-Packard Fluorescence programmable detector, Model HP1046A, Excitation (EX)= 278nm and Emission (EM)= 450nm with a 418nm cut-off filter (Hewlett-Packard, Avondale, PA).

3. *LC column.*-- PLRP-S polymer, 5 µm, 100Å, 250mm x 4.6mm id. (p/n 1512-5500 & s/n 5µ-PRS1-62B-89). With guard column consisting of cartridge holder (p/n1310-0016) and PLRP-S cartridge (p/n 1612-1801) of same packing, (Polymer Laboratories, Amherst, MA) or equivalent.

4. *Blender.*-- 5 speed, pulsed Oster Model 54841 (Baxter Scientific Products, McGraw Park, IL) or equivalent.

5. *Homogenizer.*-- Tissuemizer**7**, Model SDT1810 with Model SDT-18EN Probe (Tekmar, Cincinnati, OH) or equivalent.

6. *Food grinder.*-- Hobart, consisting of No.12 Brite-metal chopping end (P/N-119-860-3), No.12 stay-sharp blade (P/N290-339), 0.25 in. stay-sharp plates (P/N-16425-2), and No.12 stainless steel feed pan (P/N-120903) with plastic feed stomper (P/N-A-119922-1) (Hobart Corp., Denver, CO) or equivalent.

7. *Pipettors.*-- (a) Adjustable, 5mL pipette, cat. # 851350, with disposable polypropylene macrotips, 5mL capacity, cat. #851357; (b) Calibra**7** Digital micropipette, 10 - 100µL capacity, cat. #851164 with disposable polypropylene microtips, cat. # 851271 and (c) Calibra**7** Digital micropipette, 100 - 1000µL capacity, cat. # 851168 with disposable polypropylene microtips, cat. # 851276 (Wheaton Science Products, Millville, NJ) or equivalent.

8. *Solid Phase (PRS-SPE) columns.*-- disposable, propylsulfonic acid-solid phase extraction column, PRS-SPE, 500mg, BondElut LRC**7** (P/N 1211-3038, Varian Associates, Harbor City, CA). Do not substitute PRS-SPE column.

9. *Reservoir.* --75mL polypropylene reservoir with 20 micron frit (P/N 1213-1018, Varian Associates, Harbor City, CA).

10. *Column connection adaptors* -- 12,20mL adaptor for PRS-SPE, Bond Elut column LRC extraction columns (g) (P/N 1213-1003, Varian Associates, Harbor City, CA).
11. *Centrifuge*.-- IEC Model PR-7000M, refrigerated, with temperature set at 4° C, with rotor # 825A for 50mL centrifuge tubes and /or rotor # 259 for 150mL centrifuge tubes (International Equipment Company, Needham Heights, MA) or equivalent.

12. *Centrifuge tubes*.-- 50mL and 150mL, Falcon Blue Max, disposable, conical, graduated, polypropylene with cap (Cat. Nos. 2070 and 2076, respectively, Becton/Dickinson, Lincoln Park, NJ) or equivalent.

13. *Test tube*.-- disposable, 13 x 100 mm borosilicate glass, culture tube (P/N 73500, Kimble Products, Vineland, NJ) or equivalent.

14. *Nitrogen Evaporator*.-- 12 position nitrogen evaporator, 50-55°C water-bath (P/N 11155, Organomation Associates, Inc, Berlin, MA) or equivalent.

15. *Syringes*.-- disposable plastic, latex free, 1 mL, (Cat.# 309602, Becton-Dickinson, Rutherford, NJ).

16. *Pasteur pipet*.-- disposable, glass, 5.75 in.

17. *Nylon Syringe Filter*.-- Whatman, syringe filter, GD/X disposable, 13mm, 0.45 micron, nylon filter media with glass filter pre-filter in polypropylene housing, (Cat.# 6870-1304, Whatman Inc., Clifton, NJ) or equivalent.

D. REAGENTS

As specified or equivalent

1. *Solvents*.-- Distilled-in-glass, HPLC and UV spectro-grade Methanol and Acetonitrile (Burdick & Jackson Laboratories, Inc., Muskegon, MI 49442) or equivalent.

2. *Water*.-- Deionized, purified to 18.2 MΩ-cm using a Milli-Q Plus water purification system (Cat.# ZD5211584, Millipore, Bedford, MA)

3. *Acetic acid (AA)*.-- ACS grade, glacial, aldehyde-free. Use to prepare 1% and 2% aqueous solutions.

4. *Ammonium Hydroxide, NH_4OH*.-- ammonuium hydroxide, 30%, Baker Instra-Analyzed Reagent (cat.# 9733-01, J.T. Baker, Inc., Phillipsburg, NJ).

5. *Absolute Ethanol*.-- Ethyl Alcohol, 200 proof, dehydrated alcohol, U.S.P., Punctilious (Quantum Chemical Corporation, USI Division Tuscola, IL).

6. *Mobile phase*.-- Acetonitrile+2% Acetic Acid, (16+84).

7. *Extracting Solution*.-- absolute Ethanol+H_2O+Acetic acid (98+1+1).
8. *SPE equilibration solution.*-- Extracting solution(g)+1% Acetic acid (35+20).

9. *Eluting solution.*-- NH_4OH+Methanol (1+3).

10. *Reference Standards*.

    a. *Ciprofloxacin HCl*.--858µg Ciprofloxacin/mg, Lot #Pt238870K, graciously provided by Bayer AG, Leverkusen, Germany.

    b. *Enrofloxacin*.--99.0%, Std #46.03, Lot# R-177-2, graciously provided by MILES Agriculture Division, Shawnee Mission, KS.

    c. *Sarafloxacin HCl*.--88.5%, Lot# 23-336-CE, graciously provided by ABBOTT Laboratories, Chemical and Agriculture Products Division, North Chicago, IL.

    d. *Difloxacin HCl*.--90.2%, Lot#36-776-CE, graciously provided by ABBOTT Laboratories, Chemical and Agriculture Products Division, North Chicago, IL.

E. PROCEDURE

I. Standard Solutions

See **H. NOTES 1.**

a) *Stock Standards, 200µg/mL*.-- Accurately weigh an amount of each of four fluoroquinolone Reference Standards (CIPRO, ENRO, SARA & DIFLX) equivalent to 10.0 ±0.5mg (as the free base after correcting for purity) into individual 50mL volumetric flasks, dissolve in 25mL methanol, sonicate for 5 minutes and dilute to volume with methanol. Store in refrigerator. Properly sealed and stoppered, these solutions are stable 6 months.

b) *LC Mixed working standard, 2000ng/mL*.-- Place 1.0 mL of each of the above stock standards in a single 100 mL volumetric flask and dilute to volume with mobile phase. Store in refrigerator. Stable for at least 3 months.

c) *LC calibration standards*.-- Place 100 µL, 200 µL, 400 µL, 800 µL, and 1600 µL, respectively, of the LC working standard into five separate 10 mL volumetric flasks and bring to volume with mobile phase. This provides LC calibration standards in the concentration range of 20 to 320 ppb (ng/mL) for each FQ. These concentrations are equivalent to extracts (2 g) from tissues containing concentrations of 10-160 ppb (ng/g). Prepare daily with each assay set and use to generate the 5 point standard curve.

d) *4FQ Fortification Standards*.–Solution B, 4000 ng/mL Std mix. Place 1.0 mL of each of the four stock standard solutions into one 50 mL volumetric flask and dilute to volume with methanol. Solution D, 1000 ng/mL Std mix. Aliquot 25.0 mL of solution B to 100.0 mL volumetric flask and dilute to volume with methanol. See **H. NOTES 2.** For preparation of fortification standards for method validation purposes.

II. Sample Preparation

After collection, all samples should be immediately frozen for shipment. Upon receipt at analyzing laboratory, the samples should be placed in a freezer (-10 to -20°C) until analysis. Salmon samples are eviscerated, de-scaled, the head, tail and fins removed and discarded. The main torso, with skin, is cut into one inch thick fillets and then split in half. The fillets are ground and homogenized in Hobart food grinder (f). The homogenate is ground two more times by passing through food grinder. Place homogenate in whirl-pak bags for storage and store in freezer (-10 to -20°C). See **H. NOTES 3.**

III. Fortification

For analysis of unkown samples, one control and one fortified sample at 20 ppb should be analyzed with each set. Add 40 µL Solution D, 1000 ng/mL Std mix to a 2.0 g portion of tissue to yield a tissue fortification level of 20 ng/g of each fluoroquinolone, CIPRO, ENRO, SARA, DILFX.

IV. Extraction of Samples

a. Accurately weigh 2.0 ± 0.02 g of blended sample tissue into a 50 mL polypropylene conical tube.

b. For fortified samples, wait at least 2 minutes after fortification before proceding to step c.

c. Add 18 mL of extracting solution (**D.7.**) and homogenize at high speed for 20 sec. Rinse sides of probe with ethanol into 50mL centrifuge tube.

d. Cap tube and centrifuge at 3000 rpm (1870 rcf) for 5 minutes at 4°C.

e. Decant supernatant into a 150 mL centrifuge tube.

f. Repeat extraction by adding another 18 mL of extracting solution (**D.7.**) to tube containing the sample pellet. Cap tube and vortex vigorously, to breakup and mix pellet, for 20 sec.

g. Centrifuge capped tube at 3000 rpm (1870 rcf) for 5 minutes at 4° C.

h. Decant supernatant into150 mL centrifuge tube containing the first supernatant.

i. Add 40 mL of 1% Acetic acid to the combined extracts, cap and mix by swirling. **Caution Critical Step: See H. NOTES 4.**

j. Centrifuge at 3000 rpm (2420 rcf) for 5 minutes at 4° C.

k. Prepare PRS-SPE column by placing on vacuum manifold and conditioning with 2mL MeOH, 4mL equilibration solution(**D.8.**) with full vacuum on. Stop flow. Leave about 15-20mm of the equilibration solution(**D.8.**) above the PRS-SPE column bed. **Caution: do not allow to go dry.**

l. Using a BondElut adaptor, attach a 75 mL reservoir with 20 µm pore frit to PRS SPE column on the vacuum manifold. Decant entire sample extract contents of 150mL centrifuge tube into the 75mL reservoir attached to the PRS-SPE column.

m. With the aid of full vacuum, pass entire 75mL sample extract through PRS-SPE column at approximately 1-2 drops/second. After entire extract has passed through PRS-SPE column disconnect reservoir.

n. Sequentially wash the PRS-SPE column with 2 mL MeOH, 5 mL water and 2 mL MeOH. Remove excess MeOH by vacuum aspiration. Aspirate for 30 seconds after the last MeOH wash has just entered the column bed.

o. Elute FQ's from PRS-SPE column with 2.5mL Eluting solution (**D.9**) into disposable test tube.

p. Evaporate to dryness using nitrogen flow in water bath @ 50-55°C.

q. Dissolve residue in 1.0mL mobile phase. Vortex 20 seconds.

r. Using a Pasteur pipette, transfer the reconstituted sample to a 1mL disposable syringe (**C.15**) with nylon syringe filter (**C.17**) and filter into LC vial for analysis.

V. Liquid Chromatography

a. *Mobile phase flow rate*.--0.9 mL/min

b. *Column temperature*.--60°C

c. *Expected column pressure*.--2300-2600 psi

d. *Volume injected*.--50 µL

e. *Excitation (EX)*.--278nm

f. *Emission (EM)*.--450nm with a 418nm cut-off filter
g. *Run time*.--20 min

h. *Injection Sequence*.--Inject a mobile phase blank first then all five mixed LC calibration standards solutions, A through E, then the set of sample extracts. For samples containing > 160ppb it will be necessary to dilute sample extracts with mobile phase, so that the peak response of the analyte lies between highest and lowest point of standard curve (this dilution must be taken into account in calculation). Follow with an injection of the 40ppb standard to verify instrument performance. See **H. NOTES 5.** And **F. QUALITY CONTROL 8.**

i. The column should be flushed once a week or prior to a long term shutdown of the system. See **H. NOTES 6.**

VI. Calculations

Using the results from the analysis of mixed LC clibration standards solutions A through E, compute the linear regression data based on peak response (area or peak height/ppb) for each standard. The square of the correlation coefficient (r^2) of the standard curve should be ≥ 0.995. Determine the amount in the sample using the standard curve equation shown below (based on 2 g sample to final 1 mL volume as per method).

$$y = mx + b$$

Solving for x

$$y\text{-}b/m = x$$

where :

y = peak response (area or height) of analyte in the unknown
b = the y axis intercept (area or height)
m = slope of the line (area/ppb or height/ppb)
x = ppb of the analyte in the sample (ppb)

F. QUALITY CONTROL

1. Upon receipt at analyzing laboratory, the samples should be placed in a freezer (-10 to -20°C) until analysis (See **H. NOTES 7.**)

2. No stopping points are indicated because the analysis can easily be completed in one day. The application of the sample extract to the PRS-SPE column, the longest step in the procedure, takes approximately 45 minutes to one hour. During this time the analyst could, if so desired, begin the extraction of another set of samples assuming there is sufficient apparatus. An LC with an autosampler allows for overnight processing.

3. The stability of FQ's in sample extracts was not determined, see **F.2.** above.

4. When MS confirmation was required the samples in the LC vials were placed in freezer compartment of lab refrigerator. Sample extracts under these conditions are stable at least two weeks.

5. Under the LC conditions and parameters specified in the procedure, the retention times of CIPRO, ENRO, SARA and DIFLX were approximately 6, 8, 11.5 and 13 minutes, respectively. Over a period of 12 days of analysis these retention times varied (increased) about 0.2, 0.2, 0.5 and 0.6 minutes, respectively. During this same period the peak area response for the 40ppb standard varied by less than 2%.

6. No interfering peaks were noted in the chromatograms of any of the sample extracts. The background noise of known control extracts should be less than 10% of the peak height of the 40 ng/mL (equivalent to 20 ppb in tissue) standard. The fortified recoveries at 20 ppb in tissue should be at least 60% with a CV of less than 20.

7. If the peak response for 40ppb standard has varied by more 10% of initial peak response or retention time, the column should be flushed.

G. SAFETY

No special safety conditions are required other than observing normal laboratory safety precautions. Appropriate Personal Protection should be worn. Gloves should be suitable for use with the solvents used, acetornitrile, methanol, ethanol, and acetic acid. The Solid Phase Extractions should be done in a hood.

This procedure will generate waste streams of acetonitrile, methanol, ethanol, 1, 2 & 6% acetic acid. All should be handled, stored and disposed of in accordance to your local Hazardous Waste Management Program protocol.

H. NOTES

1. The method is designed for analysis of all four FQs. If the user does not need to assay for all four compounds, the user may prepare the standard solutions using only the compounds being assayed.

2. Only one of the five fortification solutions used for method validation are used for routine analysis. To minimize the number of solutions the user needs to prepare, an alternate preparation scheme is shown in section **E.I.d.** then that given in the original method. In order to keep the nomenclature used in the method consistent, these two solutions are still referred to as Solution B, 4000ng/mL, Std mix and Solution D, 1000ng/mL, Std mix. For validation of the method, all five fortification solutions should be prepared as follows: Solution A, 8000 ng/mL, Std mix. Place 2.0 mL of each of the four stock standard solutions into one 50 mL volumetric flask and dilute to volume with methanol. Solution B,

4000ng/mL, Std mix. Aliquot 25.0 mL of solution A to 50.0 mL volumetric flask and dilute to volume with methanol. Solution C, 2000ng/mL, Std mix. Aliquot 25.0 mL of solution B to 50.0 mL volumetric flask and dilute to volume with methanol. Solution D, 1000ng/mL, Std mix. Aliquot 25.0 mL of solution C to 50.0 mL volumetric flask and dilute to volume with methanol. Solution E, 500 ng/mL Std mix. Aliquot 25.0 mL of solution D to 50.0 mL volumetric flask and dilute to volume with methanol. For recovery determinations for validation purposes, aliquot 40 µL of fortification solutions A through E to separate 2.0g portions of tissue to yield tissue fortification levels of 160, 80, 40, 20, & 10 ng/g, respectively, of each fluoroquinolone CIPRO, ENRO, SARA, DILFX. Store in refrigerator. Stable for at least 3 months.

3. The grinding procedure described is that used by the methods developer. The user is not required to follow the grinding procedure as given. Alternate sample preparation procedures that yield uniform homogenates, such as grinding with dry ice may be used.

4. The method as developed by the author's called for use of 20 mL of 1% acetic acid at this step. During the method trial, the recoveries of SARA were unacceptably low at several of the laboratories. It was observed that increasing the amount of 1% acetic acid to 40 mL improved the recovery to an acceptable concentration.

5. If the peak response for 40ppb standard has varied by more 10% of initial peak response or retention time, the chromatographic column should be reconditioned by washing for 30 minutes with a Acetonitrile:6% Acetic Acid (1+1) at 1mL/min. After returning to mobile phase and allowing the column to equilibrate 10 minutes at 1 mL/min, the entire sequence, including all LC calibration standards and samples must be re-injected.

6. The column should be flushed once a week, usually at the end of the week prior to shut down. The same flushing sequence is used as for column reconditioning. Wash the column for 30 minutes with a Acetonitrile:6% Acetic Acid (1+1) at 1mL/min then return to mobile phase and allow the column to equilibrate 10 minutes at 1 mL/min prior to shutdown. For a long term, greater than two weeks, the column should be stored in accordance with the manufacturer's recommendations.

7. During the trial, several of the participants kept samples in ultralow (below -60°C) freezers. Both regular and ultralow freezers proved suitable for sample storage.

I. METHOD HISTORY

Submitted to CVM for Method Trial:

Method Trial Completed: June, 2002. A summary of the data for the fortified samples is shown in Table 2. Most of the control samples assayed during the method trial (n=17) did not have any significant background at the retention time of the compounds. One control sample had a peak at the retention time of SARA equivalent to about 4 ppb based on a ratio to the 20 ppb standard. At one laboratory, peaks were seen at the retention time of ENRO, however, all were equivalent

to less than 2 ppb based on the ration to the 20 ppb standard, and therefore within CVM guidelines.

Final Modified SOP prepared by CVM non-NADA Method Trials Coordinator: October 2003

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REFERENCES

Anadón, A., Martinez-Larrañaga, M.R., Daiz, M.J., Velez, C. and Bringas, P. (1990) *Ann. Rech. Vét.*, **21** (suppl. 1), 137s-144s.

Food Chemical News (1995) "Year in Review", 55-57.

Granneman, G. R., Snyder, K. M. and Shu, V. S. (1986) *Antimicrob. Agents Chemother.*, **30** (5), 689-693.

Granneman, G. R. and Sennello, L. T. (1987) *J. Chromatogr.*, **413**, 199-206.

Hammer, P. and Heeschen, W. (1995) *Milchwissenschaft*, **50** (9), 513-514.

Horie, M., Saito, K. Nose, N. and Nakazawa, H. (1993) *Shokuhin Eiseigaku Zasshi*, **34** (4), 289-293.

Horie, M., Saito, K. Nose, N. and Nakazawa, H. (1994) *J. Chromatogr., B: Biomed. Appl.*, **653**, 69-76.

Hormazabal, V. and Yndestad, M. (1994) *J. Liq. Chromatogr.*, **17** (17), 3775-3782.

Jain, R. and Jain, C.L. (1992) *LC/GC*, **10** (9), 707-708.

Kaartinen, L., Salonen, M., Älli, L. and Pyörälä, S. (1995) *J. Vet. Pharmacol. Therap.*, **18**, 357-362.

Munns, R. K., Turnipseed, S. B., Pfenning, A. P., Roybal, J. E., Holland, D. C., Long, A. R. and Plakas, S. M. (1995) *JAOAC Int.*, **78** (2), 343-352.
Tarbin, J. A., Tyler, D. J. and Shearer, G. (1992) *Food Addit. Contam.*, **9** (4), 345-350.

Tyczkowska, K., Hedeen, K. M., Aucoin, D. P. and Aronson, A. L. (1989) *J. Chromatogr.*, **493**, 337-346

Tyczkowska, K.L., Voyksner, R. D., Anderson, K. L. and Papich, M. G. (1994) *J. Chromatogr., B: Biomed. Appl.*, **658** (2), 341-348

Wilson, J. C. and MacMillan, J. R. (1989) *J. Aquat. Anim. Health*, **1**, 222-226.

Table 1: Average Recovery (%) of Four Fluoroquinolones from Fortified Salmon from method developer validation

FORTIFICATION LEVEL[A]	AVERAGE % RECOVERY [B]			
	CIPRO	ENRO	SARA	DIFLX
10 ppb	45	90	61	86
20 ppb	50	91	57	85
40 ppb	56	92	61	86
80 ppb	60	94	60	86
160 ppb	67	98	68	91
Overall %Recovery (n = 25)	56	93	61	87
Overall %RSD (n = 25)	15	5.6	11	5.0

[A] five (5) replicates of mixed FQ each level
[B] average of five(5) individual analysis (determinations, n=5)

Table 2: Summary of Results for Fortified Salmon Samples from the Three Laboratory Method Trial

	Ciprofloxacin			Enrofloxacin			Sarafloxacin			Difloxacin		
	A	B	C	A	B	C	A	B	C	A	B	C
10 ppb												
Average ppb	8.6[1]	5.5	5.0	11.0	8.4	8.0	8.5	7.1	6.0	10.1	7.5	6.9
Std. Dev	0.6	0.5	0.6	0.9	0.6	0.9	1.5	0.5	0.8	.3	0.5	1.0
% Recovery	86	54	52	110	85	81	85	71	57	101	82	76
CV	6	9	12	8	7	11	18	7	14	3	7	15
20 ppb												
Average ppb	16.8	12.4	8.1	21.2	17.1	13.3	17.1	15.2	11.7	19.4	15.2	13.7
Std. Dev	1.3	1.1	1.8	1.9	1.5	3.2	2.2	1.4	2.6	1.5	1.4	3.2
% Recovery	84	60	42	106	86	66	86	76	55	97	84	70
CV	8	9	22	9	9	24	13	9	22	9	9	22
40 ppb												
Average ppb	32.0	27.3	20.4	38.1	36.8	27.5	31.3	33.1	27.0	35.2	32.8	29.2
Std. Dev	2.0	2.3	2.6	1.6	2.9	2.3	3.9	2.7	2.4	1.8	2.5	2.2
% Recovery	80	68	53	95	92	69	78	83	64	88	90	76
CV	6	8	13	4	8	8	13	8	9	5	8	8

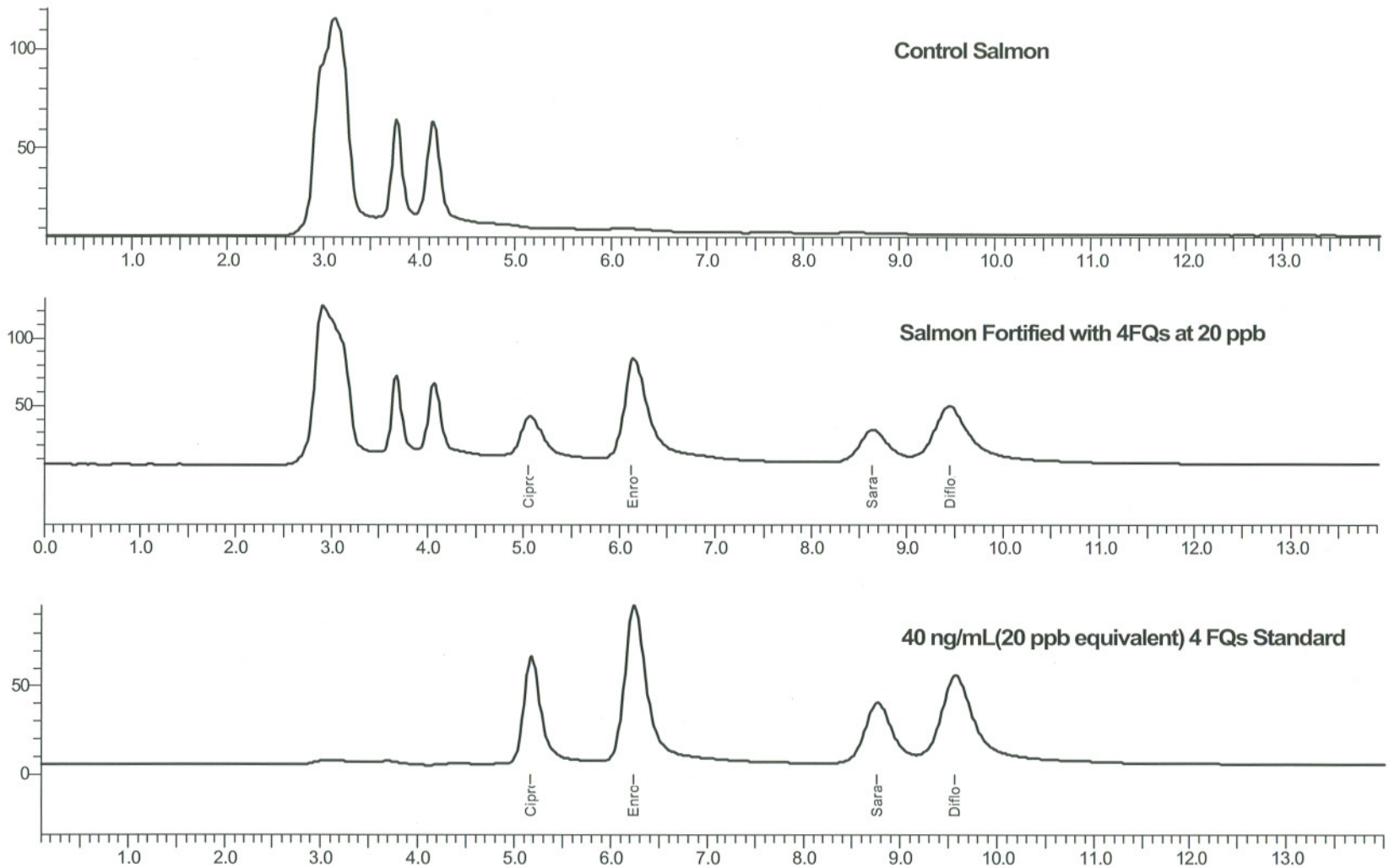

Figure 1: Chromatograms from the analysis of control salmon; salmon fortified with 20 ppb of ciprofloxacin (Cipro), enrofloxacin (Enro), sarafloxacin (Sara), and difloxacin (Diflo); and 40 ng/mL 4FQs standard.