LABORATORY INFORMATION BULLETIN

A Rapid Liquid Chromatography-Fluorescence Detection (UPLC/FLD) for the Quantitative Analysis of Avermectin Residues in Salmon and Trout (CARTS No. IR01392)

Christine R. Casey[1], Tara J. Nickel[1], Shanae W. Lanier[1], J. David Bradley[1], Sherri Turnipseed[2] and Patrick Ayres[1]

[1]U.S. Food and Drug Administration, ORA Denver Laboratory[1] and Animal Drugs Research Center[2], Denver Federal Center, Denver, CO 80225-0087

ABSTRACT

A high-throughput UPLC/FLD (Ultra Performance Liquid Chromatography/Fluorescence Detection) method was developed for the determination of avermectins in salmon and trout utilizing an extraction procedure previously published in LIB 4496 (FY11)[1] and LIB 4567 (FY14)[2]. Avermectins were extracted from ground fish with an acidified acetonitrile solution and defatted using an alumina solid-phase extraction column followed by a derivatization to form fluorescent analytes. The LC-MS/MS method described in LIB 4496 used an extracted matrix calibration curve, but this UPLC/FLD method can be performed using derivatized standards in solvent for the calibration curves. The method was validated at the 0.5VL, 1.0VL, and 2.0VL levels where the 1.0VL corresponds to 10 ng/g for all avermectins as specified in the FDA Chemotherapeutics in Seafood Compliance Program[3]. The method was validated for farm raised salmon and trout, wild-caught salmon and trout, and smoked salmon following the FDA OFVM (Office of Food and Veterinary Medicine) Level Two validation[4] criteria. Recoveries calculated for salmon at all validation levels were as follows: 88.9±15.8%, 73.9± 22.9%, 89.5%±12.7%, 88.9%±15.8% for ivermectin (IVR), emamectin (EMA), abamectin (ABA) and doramectin (DOR), respectively. The trout matrices demonstrated recoveries over the similar validation levels were 88.3% ± 10.1%, 74.5%± 10.9%, 87.4%± 10.1%, 87.1± 11.2 for IVR, EMA, ABA, and DOR, respectively. Method Detection Limits (MDL) were evaluated using salmon matrix by analyzing seven replicates at the 1.0 ng/g level following CFR Part 136[5]. The MDL's calculated using salmon were 0.187 ng/g for IVR, 0.957 ng/g EMA, 0.120 ng/g ABA, and 0.127 ng/g for DOR. Incurred salmon and trout samples were analyzed via the UPLC/FLD method and the results were compared to LIB 4496 as well as High Resolution Mass Spectrometry (HRMS)[6] screening methodology. This method is capable of extracting 20 samples and analyzing the data all within one workday.

INTRODUCTION

Ivermectin, emamectin, abamectin, and doramectin, collectively known as avermectins, are antiparasitic drugs known as macrocyclic lactones and are widely used for the control and treatment of parasitic diseases in food producing animals and aquaculture[7]. This method was initially validated by LC-MS/MS (LIB 4496) but due to limited resources and instrumentation, the method was successfully validated and transferred to the Waters Acquity UPLC/FLD systems. The use of LC/FLD systems for the determination of derivatized avermectins has been well documented in the scientific literature[7-9]. The derivatization procedure used was previously published by Van De Riet, et al.[9] using dilute 1-methylimidazole and anhydrous trifluoroacetic acid. This Laboratory Information Bulletin describes the successful platform transfer from a LC-MS/MS system to the UPLC/FLD method for the analysis of IVR, EMA, ABA, and DOR in salmon and trout tissue. The Denver Laboratory has analyzed incurred residues, proficiency samples, and over 100 samples (including salmon and trout) using this methodology.

METHOD AND MATERIALS

Equipment

a) UPLC/FLD Instrument – Water Acquity UPLC System autosampler, pumps, degasser, thermostat column compartment and detector.
b) UPLC column – Waters Acquity UPLC BEH C18 1.7um 2.1 mm x 50 mm (no guard column).
c) Solid-Phase Extraction (SPE) Cartridge – Alumina (P/N 714-0500-E; 25 ml column, Isolute 5 g AL-N, Biotage, Charlotte, NC, USA).
d) Centrifuge - refrigerated to 5 ºC, capable of accelerating 50-mL tubes to 4000 rpm (2725 x g).
e) Vortex mixer – Vortex Genie 2 (Scientific Industries, Bohemia, NY).
f) Sonicating bath – 8892 Ultrasonic Cleaner (Cole-Palmer, Vernon Hills, IL, USA).
g) Evaporator - TurboVap® LV nitrogen evaporator with thermostat water bath set to 50 ºC (P/N 103198, Zymark, Hopkinton, MA).
h) Shaker - 2000 Geno/Grinder (Spex Sample Prep, Metuchen, NJ, USA).
i) Food processor - RobotCoupe Blixer, homogenizer, 4-quart, model RS1BX4V (RobotCoupe USA, Inc., Ridgeland, MS).
j) Centrifuge tubes – 50 mL disposable, conical, graduated, polypropylene tubes with cap (Falcon® Blue Max™, 50 mL tubes P/N 352070, Becton Dickinson, Franklin Lakes, NJ). 1 mL disposable, micro-centrifuge tubes (VWR International Inc., West Chester, PA, USA, P/N 87003-296).
k) Syringes- disposable plastic, latex free, 1 mL (Cat#309602, Becton-Dickinson, Rutherford, NJ).
l) Teflon syringe filter- Pall Acrodisc 13 mm syringe filter with 0.2 micron PTFE membrane (VWR Cat. # 28143-392, Gelman through VWR).
m) Volumetric glassware and pipettors – 100.0 and 10.0 mL volumetric flasks, class A; adjustable volume pipettors with disposable polypropylene tips – 10-100 µL (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY), 200-1000 µL (Ulster Scientific, Inc., New Paltz, NY), and 1-5 mL (Wheaton Science Products, Millville, NJ).
n) Glassware and LC vials – disposable glass culture tubes (20 x 150 mm), disposable Pasteur pipettes; 2 mL glass LC vials with pre-scored snap caps.

Reagents and Standards

a) Solvents. –
    a. Acetonitrile –LC/MS Optima Grade (Fisher Scientific, Pittsburgh, PA, USA)
    b. Water – LC/MS Optima Grade (Fisher Scientific)
    c. Methanol – LC/MS Optima Grade (Fisher Scientific)
b) Extraction solution – 0.10% glacial acetic acid in acetonitrile. 1.00 mL glacial acetic acid diluted to 1000 mL with acetonitrile.

c) LC systems mobile phases –
   a. Mobile Phase A – 950 mL of water and 50 mL acetonitrile (95% H_20/5% ACN).
   b. Mobile Phase B – 100 % acetonitrile.
d) Purge Solution: 75:25 acetonitrile:water
e) Wash: 50:50 acetonitrile:water
f) Seal Wash: 90:10 water: methanol
g) Reagents-
   a. Sodium Chloride (NaCl) – Fisher Bioreagents (Fisher Scientific, Pittsburgh, PA, USA).
   b. Acetic Acid (glacial) – (EMD Chemicals, Inc., Gibbstown, NJ).
   c. 1-methylimidazole - Sigma Aldrich (Sigma Aldrich St. Louis, MO)
   d. Trifluoroacetic anhydride (TFAA) – Sigma Aldrich (Sigma Aldrich St. Louis, MO)
h) Derivatizing Reagent A (DR-A) (1:1 v/v 1-methylimidazole:acetonitrile). The derivatizing solution should be prepared just prior to use. Add 1 part acetonitrile to 1 part 1-methylimidazole.
i) Derivatizing Reagent B (DR-B): (1:1 v/v trifluoracetic anhydride: acetonitrile) Add 1 part acetonitrile to 1 part trifluoracetic anhydride.

Calculating the amount of DR-A and DR-B:

The derivatizing reagents need to be prepared daily at the time of use. The amount of derivatizing reagent needed is calculated based on the number of standards, QA/QC, and samples to be analyzed.

For example: If the following batch is to be analyzed: Reagent blank (1), standards (5), ICV (1), negative control (1), spike (1), duplicate (1), and samples (3), the amount of each derivatizing agent required: 0.200 mL x 13 = 2.60 mL each. The amount is rounded to 3.00 mL to insure enough solution is prepared. Hence, 1.50 mL of DR-A and 1.50 mL of acetonitrile is prepared and the same for DR-B, 1.50 mL and 1.50 mL of acetonitrile for a total volume of 3.00 mL each.

j) Analytical standards. –
   a. Neat Materials - All analytical standards were ordered from Sigma-Aldrich, specifically as Fluka products.
   b. SPEX CertiPrep - Alternatively, custom prepared solutions may be purchased from manufacturers such as SPEX Certiprep. Premix standards containing all 4 compounds at 100 μg/mL from SPEX CertiPrep Metuchen, NJ
k) Negative control – Farm raised salmon and trout, wild-caught salmon and trout, and smoked salmon were acquired from a local market or previous samples tested to determine that specific avermectins were not present above the stated method detection level (MDL).

Note: Equipment and reagent sources have been provided for information and guidance. Equivalent products may be substituted as appropriate.

Standard and Fortified Sample Preparation

Note: All stock standards solutions were transferred to 20 mL glass scintillation vials and stored at 4 °C. The CCV, ICV, and stock and mixed standard solutions are stable for 1 year[1].

a) Stock Standard Solutions (Continuous calibration verification CCV): Prepare individual stock standards at ~500 μg/mL in methanol, taking into account the content of the active substances (i.e., counter ions and purity) of the avermectins.

Table 1: Example of Stock Standard Solution from Neat Materials

Stock Standards	salt form (g/mol)	Non-salt (g/mol)	Wt. factor	Purity Factor	corr. Wt.	mg wt	Final Vol. (mL)	Conc µg/mL
Abamectin (ABA)	873.1	873.1	1.000	0.969	0.9690	5.125	10.0	496.6
Doramectin (DOR)	899.1	899.1	1.000	0.971	0.9710	4.980	10.0	483.6
Emamectin Benzoate (EMA)	1008.2	886.1	0.879	0.993	0.873	4.750	10.0	414.5
Ivermectin (IVR)	639.8	639.8	1.000	0.960	0.960	5.036	10.0	483.4

b) Stock Standard Solutions (ICV): A second set of stock solutions is prepared as initial calibration verification (ICV) solutions. These solutions were prepared in the same manner as the stock standard CCV.

c) Working Mixed Intermediate Solution (CCV - Standards & Spiking): Prepare one solution containing 1000 ng/mL of ivermectin, emamectin, abamectin, and doramectin. This was done by adding the compounds to a 25.0 mL volumetric flask and diluting to the mark with methanol.

d) Working Mixed Intermediate Solution (ICV- Standards & Spiking): A second Mixed Intermediate Solution was prepared from the ICV Stock Standard Solutions. This solution was prepared in the same manner as the Mixed Intermediate CCV.

Table 2: Example of Working Mix Intermediate Solution – Standards and Spiking

Working Mix Intermediate Solution	Stock Conc. (µg/mL)	mLs Added	Final Volume mL	Final conc. (ng/mL)
Abamectin (ABA)	496.6	0.0503	25	1000
Doramectin (DOR)	483.6	0.0517		
Emamectin (EMA)	414.5	0.0603		
Ivermectin (IVR)	483.4	0.0517		

Alternatively, if preparing from custom prepared solutions such as SPEX Certiprep.

a) Avermectin mix (100 µg/mL each component), cat# LC-FDACO-7. Store at 2-8°C.
b) Prepare the working mix solution to 1000 ng/mL in methanol by adding 250 µL of avermectin mix standard (100 µg/mL) into a 25.0-mL volumetric flask and diluted to volume with methanol.
c) Prepare the ICV working mixed solution at 1000 ng/mL as stated above, substituting a second set of solutions from a different lot or a different ampule of the same lot.

IMPORTANT: Sonicate all SPEX ampules for 15 minutes prior to taking an aliquot for dilution.

Sample Homogenization

Salmon and trout were obtained from local markets, in house samples, and/or from the FDA Center for Veterinary Medicine (CVM). The negative controls included the following: wild-caught salmon/trout, farmed salmon/trout, and smoked salmon. Controls from farmed and smoked salmon were used as negative controls because of the additional color added to the feed. These different controls were used to test the ruggedness of this method. Salmon and filets with the skin were ground with dry ice in a food processor based on the procedure in LIB 4496. Trout tissue was prepared in a similar manner as the salmon but without the skin. Muscle fillets, stored at −80 °C, were semi-defrosted and cut into small pieces (~1×1 cm). Approximately 65 grams of snow-like dry ice was added to pieces of tissue in a food processor and homogenized for ~30 seconds, producing

a dry ice/tissue powder matrix. The dry ice/tissue matrix was transferred to sterile whirl-pak bags. The carbon dioxide was evaporated in a freezer overnight before tightly sealing the sample for storage at -80 ºC.

Sample Preparation

This extraction procedure has been reported in LIB 4496 and LIB 4567. Three (3) grams (±0.05 g) of ground salmon or trout tissue was weighed directly into a 50-mL polypropylene centrifuge tube and allowed to thaw; fortified calibrant samples were equilibrated for 15 minutes after spiking. To each sample, 0.20 grams NaCl and 10 mL of 0.1% acetic acid in acetonitrile (v/v) was added. The sample was capped, vortexed for 10 seconds and mechanically shaken for 5 minutes. Samples were placed in a sonicating bath for 5 minutes and then centrifuged at 6000 rpm for 5 minutes at 4ºC. Alumina-N SPE cartridges were conditioned with 4.00 mL of acetonitrile without applying a vacuum or pushing air thru the cartridge and not allowed to go dry. The 10-mL sample extract was loaded onto the conditioned SPE and collected into glass culture tubes again, only by gravity. Cartridges were washed into the collection tubes by gravity with 4.00 mL of acetonitrile and collect followed by an additional 2.00 mL of acetonitrile. Sample eluates were evaporated to dryness at 50 ºC with initial 10 psi N_2 flow then increased to 15 psi for approximately 30-60 minutes until just dry. The samples were reconstituted with 2.60 mL acetonitrile, vortexed for 20 seconds, sonicated for 5.00 minute, and allowed to sit at room temperature for 15 minutes. The derivatizing agents were then added: 200 µL of DR-A and 200µL of DR-B. The extracts were lightly vortexed, covered with parafilm and incubated in the dark at room temperature for a minimum 30 minutes for the derivatization to be completed. Once derivatization has taken place, the extracts were lightly vortexed and filtered through 0.20 µm PTFE syringe filters into 2 mL autosampler vials and analyzed by UPLC/FLD.

(Note: moisture, methanol, and/or excessive matrix residue can interfere with the derivatization reaction.)

Solvent Matrix Calibrants

Solvent calibration standards were prepared at the following levels: 5.00, 10.0, 20.0, 40.0, and 80.0 ng/g for IVR, EMA, ABA, and DOR using the 1000 ng/mL mixed intermediate standard. The appropriate volumes of the standard were added to glass tubes (see Table 3). The methanol was then evaporated off by using a turbovap before adding the derivatization reagents and processing as indicated above.

Table 3: Preparation of Solvent Calibrants

	Amt added of 1000 ng/mL Working Standard (µL)	Final Volume (mL)	Conc. (ng/g)
Reagent Blank	n/a	3.00	
Solvent Std-1	15	3.00	5.00
Solvent Std-2	30	3.00	10.0
Solvent Std-3	60	3.00	20.0
Solvent Std-4	120	3.00	40.0
Solvent Std-5	240	3.00	80.0
Solvent/ ICV	30	3.00	10.0

Instrumentation

Chromatographic Conditions
To ensure the UPLC/FLD system is equilibrated and ready for sample analysis, system suitability is used to assess the instrument by inject at least five replicates of a mid-range standard used for the calibration curve. The RSD of the peak response and the retention time (R_t) should not be greater than 5%.

***(Note:** Due to the high pressure, the UPLC/FLD system is equilibrated at 0.500 mL/min with an oven temperature at 40 ºC. Once the column temperature has stabilized, the system is set to start conditions. The approximate pressure is 10,000 psi.)*

UPLC-FLD System – Acquity H-Class Flow Through Needle Instrument Conditions
Column: Waters BEH C-18, 2.1 x 50 mm, 1.8 µm
Column flow: 0.900 mL/min Stop time: 5.00 minutes Post time: 1.00 minutes
Mobile phase A: 95:5 water:acetonitrile Mobile phase B: acetonitrile

Table 4: UPLC Gradient Conditions

Time (min)	Flow Rate (mL/min)	%A (95% H_20/5% ACN)	%B (ACN)
Initial	0.900	25	75
1.00	0.900	25	75
3.00	0.900	5	95
4.00	0.900	5	95

Column temperature: 40 ºC
Flow rate: 900 µL/min
Injection volume: 3 µL
Purge Solution: 75:25 acetonitrile:water
Wash: 50:50 acetonitrile:water
Seal Wash: 90:10 water: methanol

FLD parameters
Excitation Wavelength: 353nm
Emission Wavelength: 455nm

Table 5: Approximate Retention Time (R_t) based on order of elution

Analyte	Retention Time (min.)
Emamectin	3.15
Abamectin	3.52
Doramectin	3.76
Ivermectin	3.99

Results and Discussion

Method Validation

The objective of this LIB was to develop a sensitive and rapid method for the determination of avermectin residues in aquaculture tissue. The method was validated per the FDA OFVM Level Two Chemical Method Validation guidelines[2] and performed by two separate analysts over a couple of months. Three validation levels were tested corresponding to concentrations of 0.50 VL, 1.0VL, and 2.0 VL, were 1.0VL corresponds to 10 ng/g for all avermectins as specified in the FDA Chemotherapeutics in Seafood Compliance Program[3]. To generate validation data, 3.00 grams (0.05g) portions of homogenized salmon or trout tissue were fortified by spiking with 15, 30, and 60 µL of the 1000 ng/mL mixed intermediate standard corresponding to 5, 10, and 20 ng/g. Method accuracy and precision results from the solvent curve validation are summarized in Table 6 and include all types of salmon and trout matrices.

Method detection levels (MDLs) were calculated by analyzing seven replicates at 1.00 ng/g, where the MDL=t*s (“t” is the Student's t values at the 99% confidence level, and “s” is the standard deviation of the tested concentration), refer to Table 7. The calibration was lowered to 0.5 ng/g - 45 ng/g to encompass the concertation of the MDLs at 1.00 ng/g.

Recovery was also determined using an extracted curve prepared similar to the solvent curve but with the addition of 3.00 grams of negative control (salmon). When using an extracted salmon

curve, whether wild or farmed, trout matrix spikes demonstrated significant signal enhancement for emamectin. Since accuracy and precision meet the FDA guidelines using the solvent standard curves, extracted matrix-specific standard curves were not used.

Table 6: Solvent Standard - Analyte Recovery and Precision in Fortified Salmon and Trout

		% Recovery ± %RSD			
Conc. (ng g^{-1})	**Number of replicates (n)**	**Ivermectin**	**Emamectin**	**Abamectin**	**Doramectin**
Salmon					
1	7	113 ± 7.11	95.7 ± 9.87	108 ± 3.57	100 ± 4.01
5	11	89.2 ± 13.2	76.6 ± 11.4	89.6 ± 10.3	90.9 ± 12.6
10	15	84.6 ± 14.0	76.6 ± 14.8	86.3 ±1 0.1	86.1 ± 14.0
20	9	95.6 ± 7.68	76.0 ± 8.88	94.5 ± 11.2	97.8 ± 11.1
	Avg (n=42)	88.9 ±15.8	73.9 ± 22.9	89.5 ±12.7	88.9 ±15.8
Trout					
5	9	93.5 ± 8.73	76.5 ± 10.9	95.0± 6.23	90.4 ± 8.50
10	9	81.4 ± 10.5	73.4 ± 14.5	80.8 ± 10.9	79.5 ± 11.8
20	9	90.6 ± 5.96	73.8 ± 10.9	97.3 ± 6.38	91.7 ± 8.33
	Avg (n=18)	88.3 10.1	74.5 ± 10.9	87.4 ± 10.1	87.1 ± 11.2

Table 7: Method Detection Levels (ng/g) determined from 1.00 ng/g spike level

Analytical Replicate	**Ivermectin**	**Emamectin**	**Abamectin**	**Doramectin**
1	1.088	1.020	1.073	0.986
2	1.082	0.944	1.060	0.984
3	1.066	0.827	1.023	0.941
4	1.227	1.077	1.144	1.048
5	1.184	1.024	1.106	1.058
6	1.112	0.904	1.077	0.998
7	1.153	0.906	1.062	0.999
Mean	**1.13**	**0.957**	**1.08**	**1.00**
sd	0.0596	0.0869	0.0382	0.0400
%RSD	5.28	9.08	3.55	3.99
MDL	**0.187**	**0.273**	**0.120**	**0.126**

Incurred Tissues

Incurred Tissues Set 1

The incurred tissues initial analyzed by LIB 4496 (LC-MS/MS) were re-extracted and analyzed by the UPLC/FLD methodology to determine if the solvent curve demonstrated results similar to the extracted curve LC-MS/MS method. Table 8 summarizes the results and compared between the LC-MS/MS extracted curve and the UPLC/FLD analysis using a solvent curve. The results of the analysis of incurred tissues demonstrated similar performance, hence the UPLC/FLD solvent curve methodology can be used for the quantitation of avermectin in salmon and trout.

Table 8: Comparison of Incurred Results LIB 4496 (LC-MS/MS) vs UPLC/FLD

Fish #	LIB 4496 (n=3)	UPLC/FLD (n=3)	LIB 4496 (n=3)	UPLC/FLD (n=3)
	Emamectin ng/g (%RSD)		Ivermectin ng/g (%RSD)	
10-31-S-07	-	-	-	-
10-31-S-09	18 ±16	17.6 ± 15.2	-	-
10-31-S-12	15 ±10	13.7 ± 24.6	-	-
10-31-S-11	-	-	26 ± 11	25.3 ± 15.3
10-31-S-10	-	-	121 ± 18	117 ± 11.8

Note: Refer to LIB 4496[1] for detailed information on the incurred salmon

Incurred Tissues Set 2

A second set of incurred salmon and trout samples were analyzed via LIB 4496, UPLC/FLD, and by a HRMS screening method[6], Table 9 is a summary of all three methods for salmon and trout. The levels found were comparable, especially for salmon, considering that the UPLC/FLD and LC-MS/MS quantitative methods utilized a multi-point calibration curve. However, the HRMS results appeared not to be as consistent with the UPLC/FLD and LIB 4496 results. This can be easily explained by the fact that HRMS results were considered estimated, since only a one point calibration fortification level (200 µg/kg)[6] was used with the HRMS method. Each method was extracted and analyzed by a different analyst demonstrating the robustness of each method.

Table 9: **Results for Incurred Salmon and Trout HRMS, UPLC/FLD, and LIB 4496**

Incurred No.	Analyte Identified/Confirmed (ng/g)	HRMS Screen (ng/g)	UPLC/FLD (ng/g)	LIB 4496 LC-MS/MS (ng/g)
S9	Emamectin	10	6.77	10.1
S10	Ivermectin	31	20.88	20.5
S11	Doramectin	23	18.78	18.3
S12	Abamectin	N/A	17.67	17.4
T13	Emamectin	30	10.67	13.5
T14	Ivermectin	57	26.3	30.4
T15	Doramectin	38	16.23	16.2
T16	Abamectin	N/A	26.56	25.3

Note: Refer to HRMS screening method[6] for detailed information results and incurred salmon and trout

Quantitative Analysis

For routine regulatory sample analysis, it is labor intensive and inefficient to analyze a set of five matrix-matched extracted calibrants with every analysis, as required by LIB 4496 and other mass spectrometry methods. The burden increases if more than one type of matrix is present per batch (e.g., salmon and trout) and multiple sets of matrix-matched calibrants must be extracted and analyzed to perform the analysis. To reduce the number of calibration standards that must be extracted routinely, and if the method performance permits, a solvent calibration curve, used in this procedure, can be substituted for the extracted calibration curve in LIB 4496. All the avermectins demonstrate a linear curve with a correlation coefficient above 0.995, Table 10. Since this LIB is quantitative but does not confirm the identity of the avermectins, samples demonstrating a concentration at 10 ng/g or above are re-extracted and analyzed per LIB 4496 for MS/MS confirmation.

Table 10: Solvent Calibration Curve over 4 days, n=6

Analyte	Linear Range (ng/g)	Mean r^2	SD	%RSD
Ivermectin	5.0 - 80.0	0.9984	0.00222	0.222
Emamectin	5.0 - 80.0	0.9980	0.000857	0.0857
Abamectin	5.0 - 80.0	0.9990	0.000841	0.0842
Doramectin	5.0 - 80.0	0.9987	0.00214	0.214

Stability of Derivatized Extracts

The stability of the derivatives standards and samples extracts were evaluated over a period of three days. All extracts were stable in amber HPLC vials over 3 days with no apparent degradation. In the event that a batch might need to be reanalyzed due to instrument malfunction, derivatized extracts can be stored in the dark at room temperature for instrumental analysis the following day. In addition, standards and samples can be extracted and analyzed on a separate day but standards and samples must be extracted and analyzed contemporaneously.

CONCLUSION

A high-throughput UPLC/FLD method was developed and validated for the quantitative determination of avermectins in salmon and trout. This method can determine ivermectin, emamectin, abamectin, and doramectin in salmon and trout corresponding to 10 ng/g for all avermectins as specified in the FDA Chemotherapeutics in Seafood Compliance Program[3]. To date, the Denver Laboratory has analyzed over 100 salmon and trout samples and performed an external blind proficiency test via this LIB. The efficiency of the method permits the extraction, instrumentation, and data analysis to be performed in one workday.

REFERENCES

1. C.R. Casey, C.M. Karbiwnyk, W.C. Andersen, P. Ayres Liquid Chromatography-Tandem Mass Spectrometry Method for the Confirmation and Quantitative Analysis of Avermectin Residues in Salmon, FDA LIB#4496 (2011) http://inside.fda.gov:9003/downloads/PolicyProcedures/Laboratories/LaboratoryInformationBulletins/UCM273079.pdf

2. C.R. Casey, O. Idowu, W.C. Andersen, P. Ayres, Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination and Confirmation Analysis of Emamectin Residues in Salmon Tissue, FDA LIB#4567 (2014) http://inside.fda.gov:9003/downloads/PolicyProcedures/Laboratories/LaboratoryInformationBulletins/UCM408504.pdf

3. U.S. Food and Drug Administration, Chemotherapeutics in Seafood Compliance Program 7304.018, Compliance Program Guidance Manual, 2017. https://www.fda.gov/downloads/Food/ComplianceEnforcement/ucm073192.pdf

4. U.S. Food and Drug Administration, Office of Foods, *Guidelines for the Validation of Chemical Methods for the FDA Foods Program*, Version 2.0 2015. https://www.fda.gov/downloads/ScienceResearch/FieldScience/UCM298730.pdf

5. U.S. Code of Federal Regulations, Title 40, Chapter 1, Appendix B to Part 136, Definition and Procedure for the Determination of the Method Detection Limit, Revision 1.11. Government Printing Office, Washington DC, 2008.CFR Part 136 Reference, https://www.gpo.gov/fdsys/pkg/CFR-2011-title40-vol23/pdf/CFR-2011-title40-vol23-part136-appB.pdf

6. S.B. Turnipseed, J.M. Storey, I.-L. Wu, C. M. Gieseker, N.R. Hasbrouck, T.C. Crosby, W.C. Andersen, S. Lanier, C.R. Casey, R. Burger, M.R. Madson. Application and evaluation of a high resolution mass spectrometry screening method for veterinary drug residues in incurred fish and imported aquaculture samples. Anal. Bioanal. Chem. (2018)

7. M. Danaher, W. Radeck, Wolfgang, et al. Recent Developments in the Analysis of Avermectin and Milbemycin Residues in Food Safety and the Environment. Current Pharm. Biotech. (2012) 13:963-951

8. H.S. Rupp, S.B. Turnipseed, J.E. Roybal, C.C. Walker, A.R. Long, Determination of ivermectin in salmon muscle tissue by liquid chromatography with fluorescence detection, J. AOAC Int. (1998), 81: 549-553.

9. Van De Riet , N.N. Brothers, J.N. Pearce, B.G. Burns. Simultaneous Determination of Emamectin and Ivermectin Residues in Atlantic Salmon Muscle by Liquid Chromatography with Fluorescence Detection J. AOAC Int.(2001) 84:1358-1362.M.D. Hernando, J.M. Suárez-Barcena, J.M., M.J.M Bueno, J.F. Garcia-Reyes, A.R. Fernández-Alba, A.R., J. Chrom. A (2007) 1155:62-73.

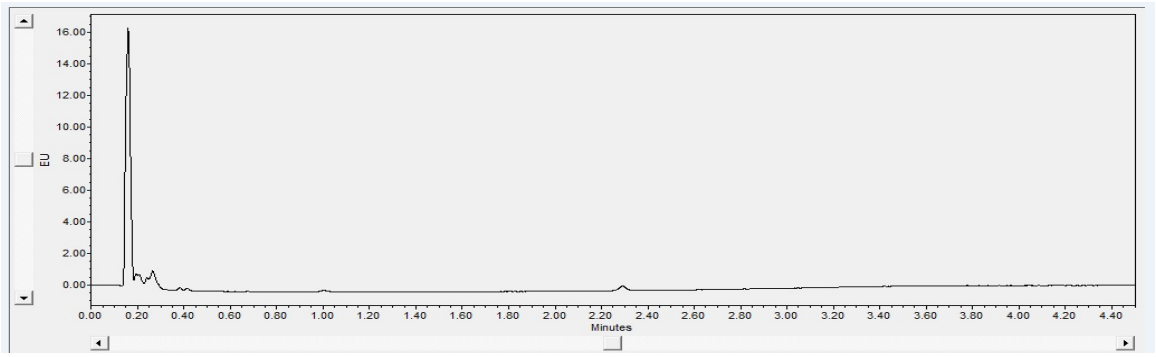

Figure 1: Salmon Negative Control

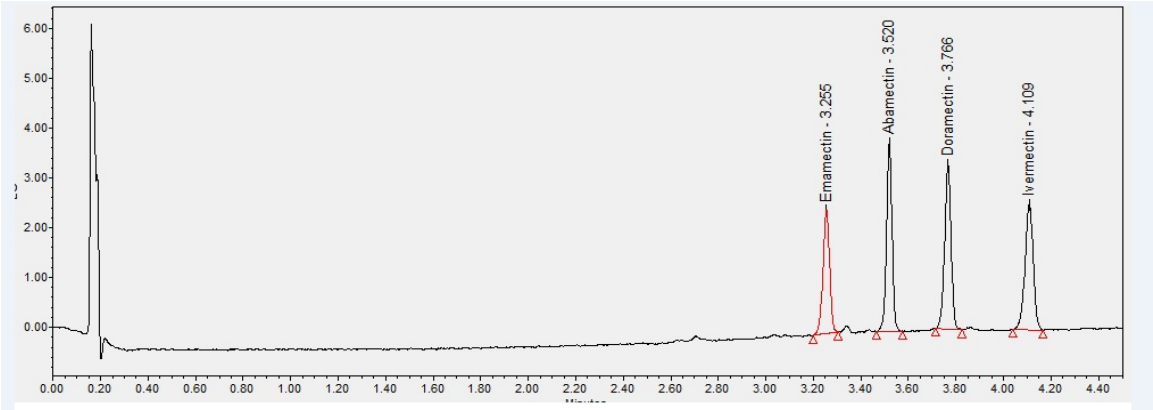

Figure 2: Solvent Standard at 10 ng/mL

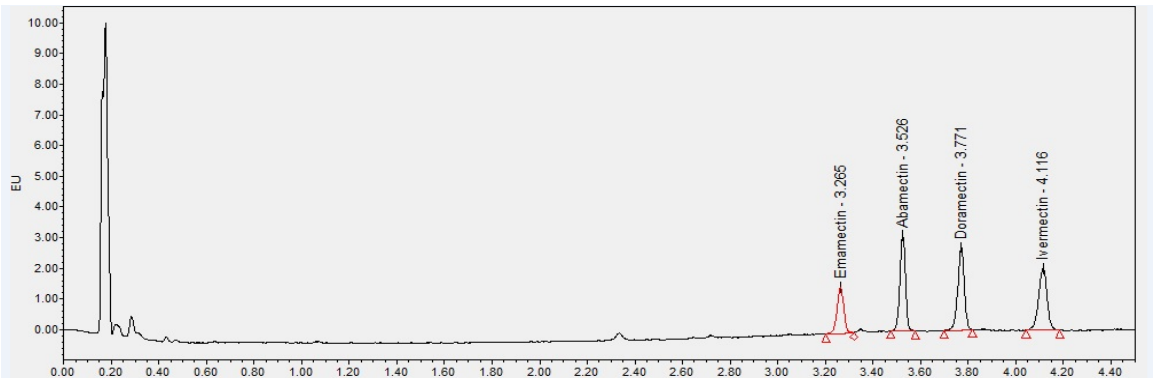

Figure 3: Salmon Spike at 10 ng/g

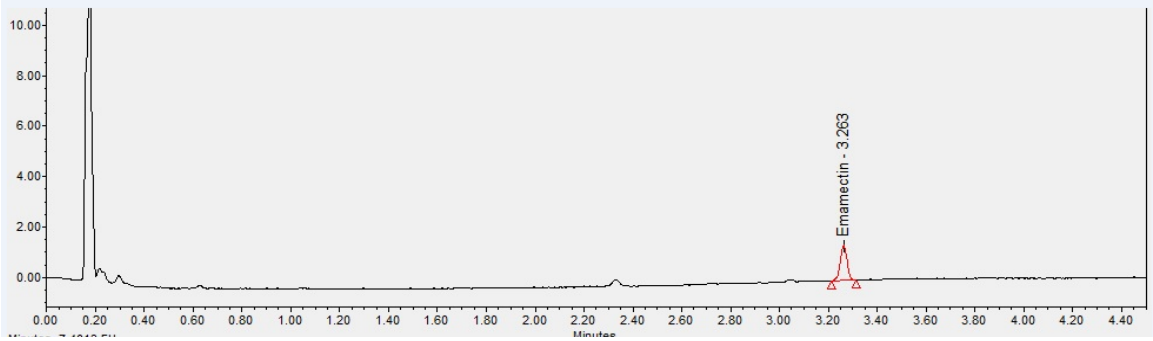

Figure 4: Salmon Incurred S9 with Emamectin

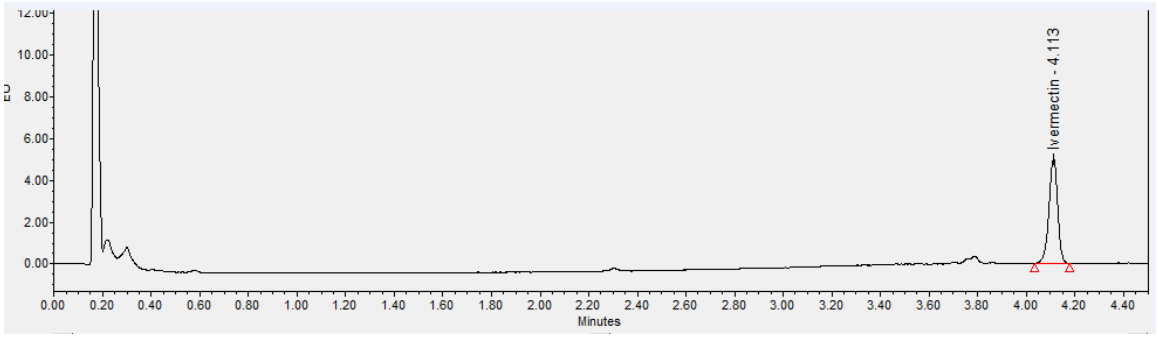

Figure 5: Salmon Incurred S10 with Ivermectin

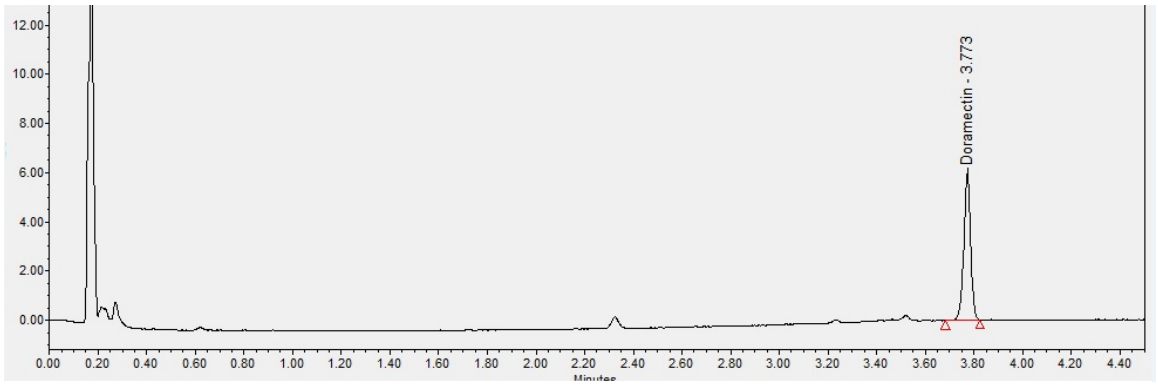

Figure 6: Salmon Incurred S11with Doramentin

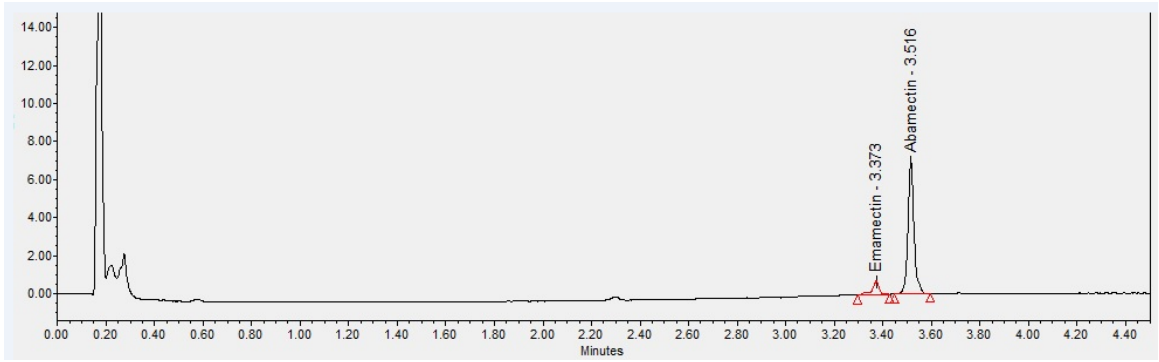

Figure 7: Salmon Incurred S12 with Abamectin

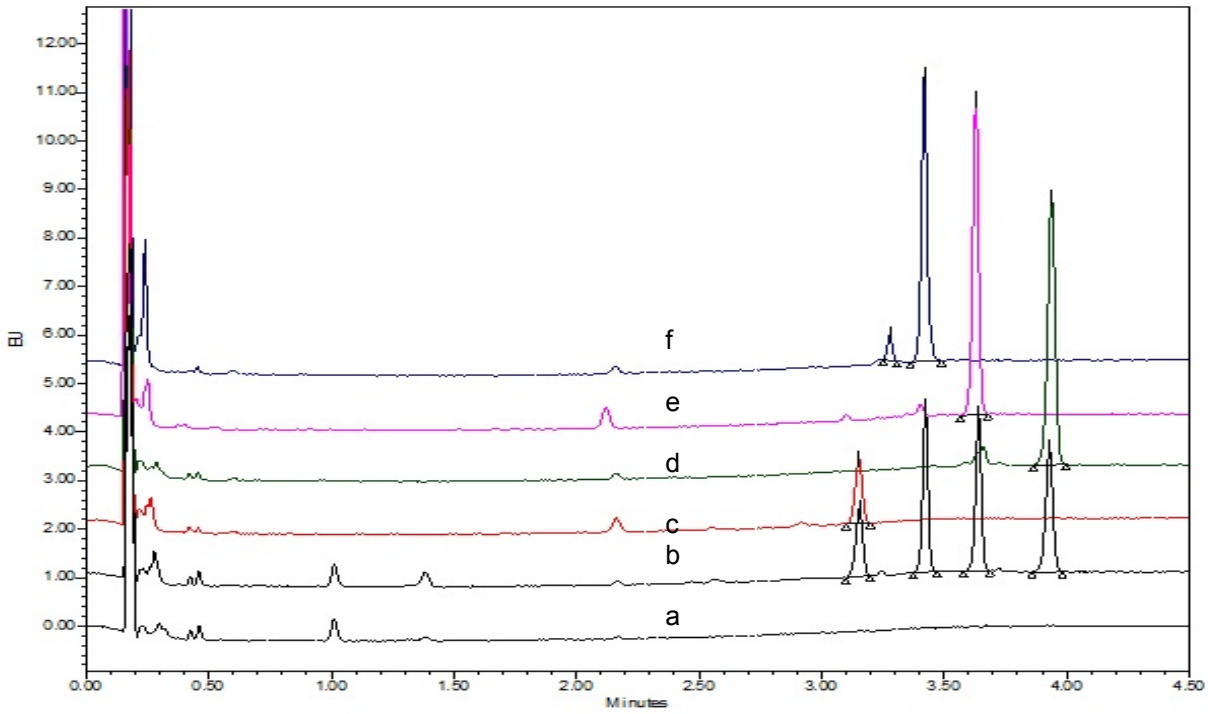

Figure 8: Overlay of Salmon Chromatograms: a) salmon negative control, b) 10 ng/g salmon spike, c) S9 incurred emamectin, d) S10 incurred ivermectin, e) S11 incurred doramentin f) S12 incurred abamectin

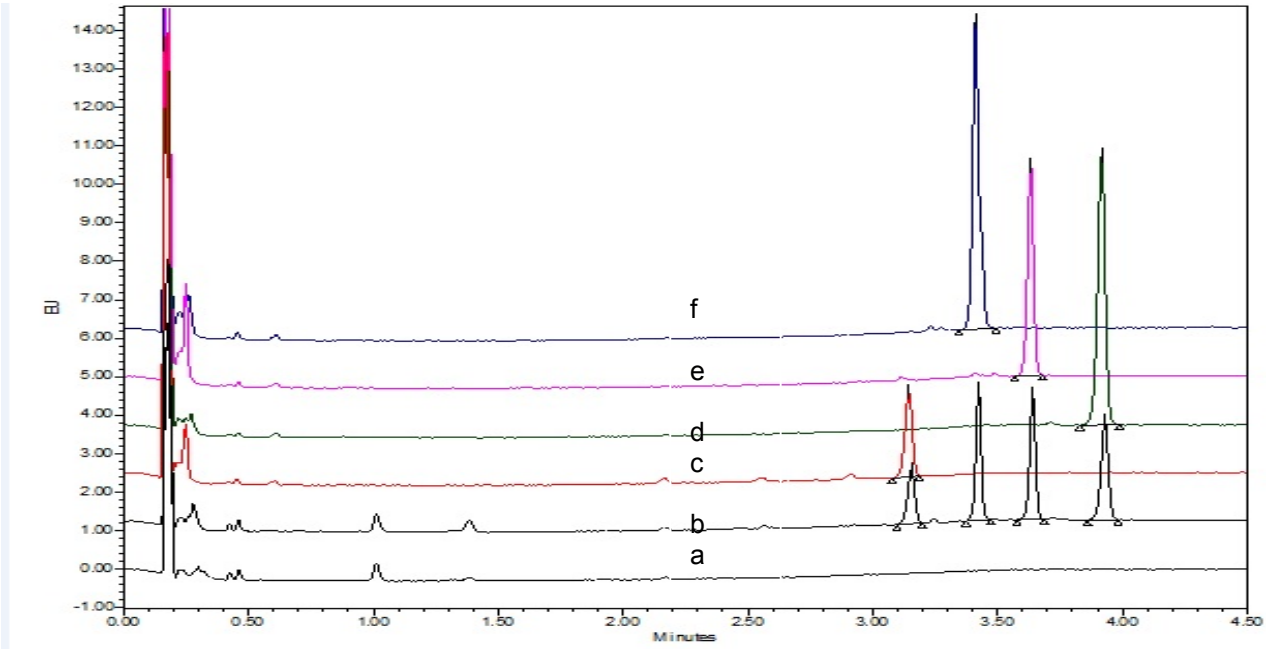

Figure 9: Overlay of Trout Chromatograms: a) salmon negative control, b) 10 ng/g salmon spike, c) T13 incurred emamectin, d) T14 incurred ivermectin, e) T15 incurred doramentin f) T16 incurred abamectin