LABORATORY INFORMATION BULLETIN

Determination and Confirmation Analysis of Lufenuron Residues in Salmon and Trout Tissue by Liquid Chromatography-Tandem Mass Spectrometry Atmospheric Pressure Chemical Ionization (LC-MS/MS APCI) **(CARTS No. IR01362)**

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Abstract

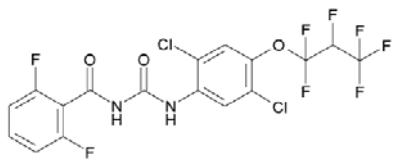
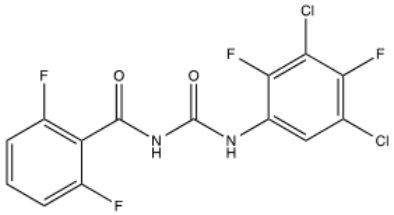
As part of its approval process, the US FDA Center for Veterinary Medicine, Office of New Animal Drug Evaluation (CVM/ONADE) requested ORA-Denver Laboratory (DENL) to validate a method to evaluate a drug sponsor's application for the use of lufenuron to control sea lice in salmonids. The FDA published LIB 4463[1] for the analysis of teflubenzuron in salmon, and since lufenuron is structurally similar to teflubenzuron, LIB 4463[1] was adapted and validated for lufenuron in salmon and trout tissue. The presumptive tolerance of lufenuron in salmon is 1.35 µg/g (1,350 ng/g). Due to the large difference in testing level for teflubenzuron (1.0 ng/g), the LIB 4463[1] method was modified by changing the sample size, eliminating concentration steps, diluting the extract, monitoring relevant multiple reaction monitoring (MRM) transitions, and changing the ionization source to atmospheric pressure chemical ionization (APCI). The validated calibration range for lufenuron was 2.50–60.0 ng/mL, corresponding to 188 ng/g – 4,500 ng/g in the sample. The method was validated in salmon and trout at the 0.5X, 1.0X, and 2.0X levels, where the 1.0X corresponds to 1300 ng/g, following criteria in the FDA OFVM Level Two validation[2] and FDA Guidance for Industry No. 3 (GFI #3)[3]. Recoveries were greater than 90% for lufenuron in salmon and trout with <10% CV. The method detection limit (MDL) was calculated to be 96 ng/g for lufenuron. In addition to validating the method with fortified salmon, DENL analyzed incurred salmon samples provided by the sponsor. The results from the DENL were evaluated by CVM/ONADE and determined to be statistically equivalent to the incurred residue values reported by the sponsor. This LIB describes the modification and validation of an LC-MS/MS method for the regulatory determinative and confirmatory analysis of lufenuron in salmon and trout tissue.

INTRODUCTION

In 2015, the US FDA Center for Veterinary Medicine, Office of New Animal Drug Evaluation (CVM/ONADE) determined that a drug sponsor was developing lufenuron for use in salmon and trout. Lufenuron is the marker residue in salmon tissue. Lufenuron is a pesticide and acts as a chitin synthesis inhibitor[1]. It is effective to treat sea lice infestations. As part of the veterinary drug approval process, the sponsor was required to develop and validate a method to serve as an official method for lufenuron residue in salmon tissue and trout tissue. For the sponsor's method to qualify as an official method, the proposed method is required to pass an inter-laboratory method trial that involves CVM/ONADE, the sponsor's reference laboratory, and sponsor-designated independent laboratories. Alternatively, CVM/ONADE could recommend a scaled-down method trial if there was a method available by an FDA laboratory that could be validated and bridged to the sponsor's method, using incurred tissues provided by the sponsor.

The FDA published LIB 4463[1] for the analysis of teflubenzuron, a similar benzylurea pesticide. For this study, LIB 4463[1] was adapted and validated for lufenuron in salmon and trout tissue. The CVM/ONADE requested specific parameters be met for the FDA analytical method following specific criteria stated in FDA OFVM[2] and the FDA Guidance for Industry No. 3 (GFI #3)[3]. Table 1 shows the characteristics of the Sponsor's method for lufenuron as well as LIB 4463 for teflubenzuron.

Table 1: Comparison of sponsor's method for lufenuron vs. teflubenzuron LIB 4463

Procedure	Sponsors Method (lufenuron)	LIB 4463 (teflubenzuron)
Structure		
Principle	1. Single analyte method 2. LC-MS/MS with ESI negative mode 3. Monitored ions: m/z 326,175, 202 (lufenuron) + internal standard 4. Calibration range: 2.5-200 ng/mL solvent standards (50-4000 ng/g tissue equivalent) 5. UPLC: Column CSH C18	1. Single analyte method 2. LC-MS/MS with ESI, negative mode 3. Monitored ions: m/z 379, 359, 339 (teflubenzuron) 4. Calibration range: 0.1-100 ng/mL solvent standard (0.4-400 ng/g tissue equivalent) 5. HPLC: Column C18
Extraction	1. Mix 0.5g sample + 5 mL acetonitrile 2. Centrifuge 3. C18 SPE cartridge clean-up 4. Elute with ACN 5. Filter supernatant through PVDF syringe filter 6. Extraction results in 20X dilution of starting tissue concentration	1. Modified QuEChERS: Mix 10 g sample + 5 mL water + 15 mL of acetonitrile + $MgSO_4$ + NaCl 2. Centrifuge 3. Filter supernatant through PVDF syringe filter 4. Extraction results in 4X dilution of starting tissue concentration
Performance	1. LOD: 5 ng/g Salmon and 6 ng/g Trout 2. LLOQ: 100 ppb 3. Application: Lufenuron in salmon and trout tissue	1. LOD: 0.12 ng/g Salmon 2. LLOQ: 0.40 ng/g Salmon 3. Application: Teflubenzuron in salmon tissue

For the DENL validation of lufenuron in salmon and trout, LIB 4463[1] was modified by reducing sample size to 2.00 grams, changing mass spectrometry MRM transitions monitored to those in the sponsors methodology, eliminating the internal standard, and using an APCI source. The calibration range for the DENL validation of lufenuron encompassed the Sponsor's method with a solvent calibration range prepared from 2.50 ng/mL to 60 ng/mL corresponding to a tissue 1equivalent of 188 ng/g to 4500 ng/g. This DENL method was validated by analysis of lufenuron

in fortified salmon and trout tissue samples. Blinded incurred salmon tissue samples provided by the sponsor were also evaluated and compared to the sponsor's results.

METHODS AND MATERIALS

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

Equipment

a) LC-MS/MS instrument – 5500 Q TRAP hybrid quadrupole linear ion trap mass spectrometer (AB Sciex, Foster City, CA, USA) utilizing a TurboV™ ion source with the TurboIonSpray® (i.e., APCI source) probe and APCI probe coupled to an Agilent 1200 Series binary pump, degasser, thermostated column compartment (Agilent Technologies, Santa Clara, CA, USA) and HTC PAL autosampler (CTC Analytics, LEAP Technologies, Carrboro, NC, USA). Analyst 1.6.2 software was used to acquire and analyze the data (AB Sciex).
b) LC column – 75 mm x 2.1 mm, 3.5 µm XSelect CSH C18 (Waters Milford, MA).
c) Centrifuge – refrigerated to 5 ºC, capable of accelerating 50 mL tubes to 4000 rpm (2725 x g) or equivalent.
d) Vortex mixer – Vortex Genie 2 (Scientific Industries, Bohemia, NY), or equivalent.
e) Sonicating bath – 8892 Ultrasonic Cleaner (Cole-Palmer, Vernon Hills, IL, USA), or equivalent.
f) Eppendorf pipettes – variable (5µL to 1000 µL $\pm$ 0.8%) volume (Brinkman Instruments, Inc., Westbury, NY), or equivalent.
g) Sonicator – Branson 8510 (Danbury, CT)
h) Syringe filters – Acrodisc® CR 13 mm syringe filter with 0.2 µm polytetrafluoroethylene (PTFE) membrane (P/N 4542, Pall Life Sciences) with 1-mL disposable syringe (P/N 309602, Becton Dickinson, Franklin Lakes, NJ), or 0.20 um PTFE Mini-UniPrep filters (Agilent, P/N 5190-1419)
i) Centrifuge tubes – 15 mL and 50 mL disposable, conical, graduated polypropylene tubes with cap (Falcon® Blue Max™, P/N:50 mL tubes 352070, 15 mL tubes 352097, Becton Dickinson, Franklin Lakes, NJ).
j) 2000 Geno/Grinder (Spex Sample Prep, Metuchen, NJ, USA) or equivalent
k) Food processor – RobotCoupe Blixer, homogenizer, 4 quart, model RS1BX4V (RobotCoupe USA, Inc., Ridgeland, MS) or equivalent.
l) Volumetric glassware and pipettors – 10.0 mL and 25.0 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), or equivalent.
m) Glassware and LC vials – disposable Pasteur pipettes; 2 mL glass LC vials with snap caps.

Reagents and Standards

a) Solvents –
    a. Acetonitrile – LC/MS Optima Grade (Fisher Scientific, Pittsburgh, PA, USA)
    b. Methanol – LC/MS Optima Grade (Fisher Scientific, Pittsburgh, PA, USA)
    c. Water – LC/MS Optima Grade (Fisher Scientific)
    d. Formic Acid – LC/MS Optima Grade (Fisher Scientific)
b) Reagents –
    a. Magnesium Sulfate ($MgSO_4$) – Sigma Aldrich (St. Louis, MO, USA)
    b. Sodium Chloride (NaCl) – Fisher Bioreagents (Fisher Scientific, Pittsburgh, PA, USA)
c) LC systems mobile phases – methanol, water, and formic acid used for LC-MS mobile phase preparation.
    a. Mobile phase A (0.05% formic acid in water) - was prepared by diluting 0.50 mL of formic acid in 1000 mL of water.
    b. Mobile Phase B - methanol

d) Analytical standards. –
   a. All analytical standards were ordered from Sigma-Aldrich, specifically as Fluka products, or obtained from the Sponsor.

e) Negative control – Salmon and trout were obtained from Denver Laboratory control tissue supplies and analyzed to ensure that lufenuron was not present above the stated method detection level (MDL).

Standard Preparation

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

a) Stock Standard Solutions (CCV): Accurately weigh approximately 3.00 ± 0.05 mg of reference material into a 50.0 volumetric flask and dilute to volume with acetonitrile. The weight is corrected for purity per the certificate of analysis and considering any counter ions. This solution is approximately 60,000 ng/mL and used as a spiking solution for method validation and for the working solution used to prepare the solvent curve.

b) Stock Standard Solutions (ICV): A second set of stock solutions is prepared as initial calibration verification (ICV) solutions, using the same neat source or a source from a different manufacture. These solutions were prepared in the same manner as the stock standard CCV.

c) Working Intermediate Solution (CCV – Solvent Standards): A 6,000 ng/mL working intermediate solution was prepared by adding approximately 2.00 mL of stock standard solution to a 20.0 mL volumetric flask and diluting to volume with acetonitrile.

d) Working Intermediate Solution (ICV – Solvent Standards): A second set of 6,000 ng/mL working intermediate ICV was prepared by adding approximately 2.00 mL of the ICV stock standard solution to a 20.0 mL volumetric flask and diluting to volume with acetonitrile.

e) Solvent Calibration Curve Preparation: The 6,000 ng/mL working intermediate is used to prepare solvent curve calibration standards and range from 2.50 ng/mL – 60 ng/mL (tissue equivalent: 188 ng/g – 4500 ng/g). Examples of these solvent standard preparations are given in Table 2.

f) ICV Solvent Curve: A solvent ICV is prepared from the working intermediate ICV solution, refer to Table 2 for an example of this preparation.

Table 2: Example of solvent calibrant calibration curve and tissue equivalents

Lufenuron	ng/mL	mL	Final Vol (mL)	Calibrant Conc. ng/mL	Final Extract Total Vol (mL)	Tissue wt.(g)	Dilution (1mL extract /10 mL)	Tissue Conc. ng/g
Solvent Std-1	6000	0.0083	20.00	2.50	15.0	2.00	10X (10mL/1 mL)	188
Solvent Std-2		0.0167	20.00	5.00				375
Solvent Std-3		0.0417	20.00	12.5				938
Solvent Std-4		0.0833	20.00	25.0				1875
Solvent Std-5		0.2000	20.00	60.0				4500
Solvent ICV-3	6000	0.0417	20.00	12.5				938

Example of Solvent Curve:

$$6000\frac{ng}{mL}\ x\ \frac{0.0417\ mLs}{20.0\ mLs} = 12.5\ ng/mL$$

Example Calculation of Solvent Curve to tissue equivalents:

$$12.5\ \frac{ng}{mLs}\ x\ \frac{15.0\ mLs}{2.00\ grams}\ x\ 10x = 938\ ng/g$$

Incurred Tissues

The incurred samples for the validation study were prepared by the drug sponsor and shipped under appropriate storage conditions to the DENL. Prior to shipping, using a randomization procedure recommended by the CVM/ONADE study coordinator, the sponsor blinded the identity of the samples by assigning random numbers to the samples and controls and then assigning the samples identification numbers (IDs) based on the random numbers. Only the sample ID and weight were known to the analyst at the DENL. The sample batches were analyzed using both solvent and matrix fortified calibration standards and determined by LC-MS/MS-APCI.

Sample Preparation

Homogenization of the salmon and trout filet tissues with the skin were ground with dry ice in a food processor[4]. Muscle filet tissues, stored at −80 °C, were semi-defrosted and cut into small pieces (~1×1 cm). Half a cup of snow-like dry ice was added to pieces of tissue in a food processor and homogenized for ~30 s, producing a dry ice/tissue powder matrix. The dry ice/tissue matrix was transferred to sterile whirl-pak bags. The carbon dioxide was allowed to evaporate in a freezer overnight before tightly sealing the sample for storage at -80 ºC. Note: Fish tissue preparation should include the skin.

Spike Recovery Control Checks

Accurately weigh 2.00 g (±0.05 g) portions of homogenized negative control salmon tissue into 50 mL disposable centrifuge tubes and let thaw. Fortify thawed tissue at concentrations of 650, 1300 and 2600 ng/g by adding 21.7, 43.3 and 86.7 µL of the 60,000 ng/mL Stock Standard Solution (CCV), and equilibrate for 15 min.

Extraction Procedure

The extraction procedure is based on the previously-published LIB 4463[1] method with some modifications. Accurately weigh 2.00 g (±0.05 g) of homogenized salmon tissue containing lufenuron into 50 mL disposable centrifuge tubes and let thaw. Add 5.0 mL of water and 10.0 mL acetonitrile into the same tube, vortex 10 seconds and shake for 2 min on a SPEX 2000 Geno grinder at 500 stroke/min. Add 1.50 g (±0.1) of sodium chloride (NaCl) and 4.00 grams of anhydrous magnesium sulfate ($MgSO_4$) to each tube, vortex for 20 seconds or place on a Geno grinder at 500 stroke/min for 20 seconds. Centrifuge at 6000 rpm for 5 minutes at 5ºC and then transfer 1.00 mL of extract (top acetonitrile layer) to a 15 mL polypropylene tube and dilute to a final volume of 10.0 mL with acetonitrile. Vortex each sample and remove 500 µL of the extract and place into the PTFE filter vial or transfer to a syringe with filter. Analyze the filtrate by LC-MS/MS APCI system.

Instrument Conditions

An Agilent 1200 HPLC system with a Combi Pal autosampler coupled to an AB SCIEX 5500 QTrap mass spectrometer operated in the triple-quad mode with atmospheric pressure chemical ionization (APCI) source was used for this method. A Waters XSelect CSH column (C18 75 mm x 2.1mm x 3.5um) was used for the LC separation. The sample injection volume was 3 µL with the autosampler tray at room temperature. A divert valve directed column effluent to waste shortly before (0-2.5 minutes), after elution of the lufenuron (6.0-7.5 minutes), and during the system re-equilibration time. Column temperature was 50ºC and the flow rate was 0.500 mL/min. The mobile phase was 0.05% formic acid in water (A) and methanol (B), and the LC gradient is described in Table 3. The Combi Pal Injector wash solutions were used to minimize carryover, solution 1 was 95% water/5% acetonitrile and solution 2 was 95% acetonitrile/5% water.

Table 3: LC gradient for lufenuron

@Step	Total Time (min)	Flow Rate (µl/min)	A (%)	B (%)
0	0.00	500	50.0	50.0
1	3.00	500	1.0	99.0
2	5.00	500	1.0	99.0
3	5.50	500	50.0	50.0
4	7.50	500	50.0	50.0

The MS instrument was operated in the negative mode with the APCI source using MRM. AB Sciex 1.6.2 software was used for instrument control and Multi Quant 3.0 was used for data processing. Current gas was set to 20 psi, source gases 1 and 2 were set to 60 psi, collision gas was medium, nebulizer current was -2.0, and source temperature was 300ºC, Table 4 is a summary of the AB SCIEX acquisition parameter such as DP, CE, and ion transitions.

Table 4: Retention times (RT) and MS parameters: declustering potential (DP), for precursor ions and collision energy (CE), collision cell exit potential (CXP) and the resulting ion ratios for the product ions of lufenuron.

Analyte	RT (min)	Ion (m/z)	DP	CE	CXP	Ion Ratio (%)
lufenuron	4.12	509 → 326	-15	-28	-17	100
		509 → 202	-15	-52	-21	40
		509 → 175	-15	-30	-21	20

Results and Discussion

Method Development

Development of the lufenuron method was based on modifications of the teflubenzuron method described in LIB 4463[1]. Because the teflubenzuron testing level was significantly lower (1.0 ng/g) than the testing level for lufenuron, the sample size was reduced from 10 grams to 2 grams. The initial evaluation of the method using electrospray ionization (ESI) demonstrated a 60% increase in the calculated concentration for the lufenuron response, and a post fortified matrix sample also demonstrated the enhancement of the lufenuron response in the negative mode. The chromatographic conditions were modified as follows: the gradient was adjusted, the column flush was increased, and column was changed from C18 to CSH C18. The modified conditions reduced the negative mode enhancement from 60% to 30% but the signal enhancement was still higher than recommended required per the FDA Guidance for Industry No. 3 (GFI #3)[4] (80%-110%). The optimized conditions in the negative mode were also run in ESI positive mode to determine if the suppression in the positive mode would fall within the percent recovery requirements. The ion suppression was found to be similar to the enhancement in the negative mode. Other LC-MS/MS platforms were investigated to determine if instrument manufacture and source design had any influence on the negative mode enhancement. The same conditions were used on two separate LC-MS/MS platforms but similar results to the SCIEX instrumentation were demonstrated on these two other platforms.

Due to the tight precision guidelines for %CV of $\leq$10% on LC-MS/MS instrumentation, other extraction techniques were evaluated in order to reduce matrix interference. Per discussion with CVM/ONADE and the requirements in (GFI #3)[4], the method was requested to use solvent-based calibration standards and no internal standard correction. To meet method requirements, DENL explored newer technologies for sample cleanup such as: dispersive solid phase extraction (dSPE), Agilent Bond Elut Enhanced Matrix Removal-Lipid (EMR-Lipid)[6], and Waters Oasis PRiME SPE[7].

These technologies were developed to remove phospholipids and fats from tissue extracts to optimize performance, analytical column performance, and minimize matrix effects in mass spectrometry.

All sample cleanups were evaluated using the sample extract from LIB 4463[1], which is a modified QuEChERS procedure. Negative controls were spiked at the 1.0 X level (1300 ng/g) in triplicate and recoveries were calculated against the solvent curve using negative mode ESI (Table 5).

Table 5: Comparison of extract cleanup, n=3 at 1300 ng/g lufenuron in salmon

Cleanup Method	4463 Original Method – no cleanup	dSPE	dEMR	PRiME
mean	102%	94.6%	75.1%	53.6%
sd	7.20	4.01	22.6	2.61
%RSD	7.09	4.03	30.1	4.86

The initial results demonstrate that the dEMR %RSD was higher than the 10% required by the FDA guidelines and both the dEMR and the PRiME SPE had low recoveries. The initial techniques used for PRiME did not include a wash of the cartridge after the sample was eluted and collected. Further investigation demonstrated lufenuron required a wash of the PRiME column to ensure that lufenuron eluted from the column. The dSPE demonstrated the best recovery but the extraction technique was longer and tedious. Validation data on the recovery of lufenuron from fortified salmon tissue is shown in Table 6. The fortified tissue samples were prepared at three different concentration, using control salmon tissue from 3 different sources. Five replicates of each of the three different fortifications were analyzed on each of three different days. Initial inspection of the data, demonstrated that it was within the criteria of FDA Guidance for Industry No. 3 (GFI #3)[4], but further analysis showed recoveries at 2.0X level for salmon Source 2 had recoveries less than 80% and a mean recovery of 81.3% (Table 6). The second issue was that the solvent standard injected throughout the analytical run had a response that increased and exceeded the 110% system suitability limit for a CCV (Table 6).

Table 6: Recovery of lufenuron from fortified salmon tissue using QuEChERS PRiME SPE with ESI LC-MS/MS

Fortification (ng/g)[a]	650 ng/g (0.5X)	1300 ng/g (1.0X)	2600 ng/g (2.0X)
Recovery (%)	98.0 (4.05%)	94.4 (3.34%)	85.2 (4.24%)

[a]n = 15 at each fortification
CCV through run = 107 (±8.48) % (n =11)

Although many different instrument conditions and sample extraction and/or cleanup techniques were explored, it was not possible to satisfy the requirements[2]. Finally, a change of the ionization source from ESI to APCI was evaluated to resolve the problems. The final method selected for optimized performance and validation was the modified LIB 4463[1] extraction procedure with QuEChERS cleanup, followed by analysis by LC-MS/MS with APCI in the negative ion mode.

Method Validation

Validation of DENL Method for the Determinative and Confirmatory Analysis of Lufenuron in Salmon Tissue using a LC-MS/MS system with APCI

After ensuring that the LC-MS/MS system meets system suitability test (SST) criteria, the DENL validated the lufenuron method by replicate analysis of salmon and trout tissue fortified with lufenuron at 650 ng/g (0.5X), 1300 (1X) ng/g, and 2600 ng/g (2X), where X is 1300 ng/g (the presumptive tolerance of lufenuron). The sponsor analyzed salmon tissue fortified at similar concentrations. In both laboratories, each analysis set included a solvent calibration curve prepared in the respective ranges of 2.51 ng/mL to 60 ng/mL lufenuron (tissue equivalent: 188 to 4500 ppb

in salmon tissue) for the DENL method, and 2.5 to 200 ng/mL (tissue equivalent: 50 to 4000 ppb) for the sponsor's method. Based on FDA Guidance for Industry No. 3 (GFI #3)[3], the acceptance criteria for the determinative procedure are as follows:

1. The method recovery of the fortified control samples should range from 80 to 110%.
2. The precision of the method as measured by the coefficient of variation (%CV) of replicates at different tissue fortification levels should be ≤ 10%.
3. For the calibration curve, the linear regression should have a coefficient of determination (r^2) ≥ 0.990, and back-calculated concentrations of the calibration standards should be ±10% of the theoretical values (± 15% at LOQ).

DENL and the sponsor validated the accuracy of their respective methods by replicate analyses of control salmon tissue fortified with lufenuron at 650 ng/g (0.5X), 1300 ng/g (1X) and 2600 ng/g (2X). The accuracy data (percent recovery) and precision are summarized in Table 7. The data in Table 7 demonstrate that both laboratories obtained acceptable precision for their respective methods when applied to the analysis of lufenuron in fortified salmon tissue. The accuracy was acceptable for the DENL analysis but slightly outside the acceptance limit (>110%) for the 613 ng/g fortification analyzed by the sponsor's method, as the recovery is out of the acceptance range, 80%-110%. Chromatograms for the reagent blank, negative control salmon, and a lufenuron solvent standard are shown in Figures 1-3.

Table 7: Accuracy (% recovery) and precision of analysis of lufenuron in fortified salmon tissue using the DENL and sponsor methods

DENL		Sponsor	
Fortification (ng/g) n=9	Recovery (%), (CV %)	Fortification (ng/g)	Recovery (%), (CV %)
650	92.0, (6.0)	613	111, (5.9)
1300	92.0, (5.5)	1530	105, (4.3)
2600	94.4, (6.8)	3060	104, (3.6)

Analyses of Lufenuron in Incurred Salmon Tissue

The reference concentrations of the incurred samples, as determined by the sponsor, were 651 ng/g, 1310 ng/g and 2600 ng/g. Thus, the assessments of the accuracy and precision of the DENL and sponsor's methods, as applied to analysis of lufenuron in incurred salmon tissue, are based on comparison of the DENL and the sponsor's concentration data with respect to the reference concentrations. A compilation of the concentration data obtained in the two laboratories for blinded incurred samples having concentrations at approximately 0.5X, 1X, and the 2X are summarized in Table 8-10, respectively.

Table 8 demonstrates that the DENL achieved acceptable within-laboratory recovery and precision for the analysis of lufenuron in blinded incurred salmon tissue having a reference concentration of 651 ppb. The recoveries obtained with the sponsor's method for half of the incurred samples at that concentration (Sample IDs A3, B3, and AF3) are outside the acceptance range. The inter-day precision of the concentration data obtained by the two laboratories are well below the acceptance limit (10%).

Based on the recovery data for the fortified samples at the 0.5X concentration (Table 7) and for the low concentration incurred samples (Table 8), it was observed that the sponsor's method fails to yield acceptable results (recovery= 80%-110%) for low concentrations of lufenuron in salmon.

The data obtained from the two laboratories for blinded incurred samples having concentrations near the lufenuron presumptive tolerance (1X) is shown in Table 9. Table 9 shows that both the DENL and the sponsor's methods yield lufenuron concentration data with acceptable accuracy and precision. Likewise, data in Table 10 show that both the DENL and the sponsor's methods yield

lufenuron concentration data with acceptable accuracy and precision in salmon with a lufenuron concentration equivalent to approximately 2 times the lufenuron estimated tolerance (2X).

The 80-110 % recovery recommended in GFI #3[3] corresponds to an acceptable deviation range of -20% to +10%. Tables 8, 9, and 10 each contain a row labeled "% Difference from reference conc.", which summarizes the deviation from the mean concentration data obtained with the DENL and sponsor methods, respectively. Except for the sponsor's results for the 0.5X samples (Table 8), all results are within the acceptance range for the three reference concentrations of the incurred samples. A chromatogram for lufenuron in incurred salmon at 1300 ng/g is shown in Figure 4.

Table 8: Measured concentration of lufenuron in incurred salmon tissue having a reference concentration of 651 ng/g (approximately 0.5X), using the DENL and sponsor methods[a]

Laboratory	Batch No.	Sample ID	Measured conc. (ppb)[b]	Accuracy (%)
DENL	1	A1	644	98.9
		B1	624	95.9
		C1	636	97.7
		F1	623	95.7
		G1	587	90.1
	2	M1	598	91.8
		R1	644	98.8
		S1	640	98.3
		Mean	**624**	**95.9**
		STDEV	22	
		CV %	3.5	
% Difference from reference conc.			-4.18	
Sponsor	1	A3	741	114
		B3	806	124
	2	O3	709	109
	3	Y3	718	110
		AC3	653	100
		AF3	729	112
		Mean	**726**	**112**
		STDEV	50	
		CV %	6.8	
% Difference from reference conc.			10.90	

[a]DENL method: LC-MS/MS with APCI; Sponsor method: LC-MS/MS with ESI
[b]Each DENL concentration is reported as the mean of duplicates, except for the A1 data, which is the mean of analysis triplicate.

Table 9: Measured concentration of lufenuron in incurred salmon tissue having a reference concentration of 1310 ppb (approximately 1X), using the DENL and Sponsor's methods[a]

Laboratory	Batch No.	Sample ID	Measured conc. (ng/g)[b]	Accuracy (%)
DENL	1	I1	1188	90.7
	2	K1	1156	88.3
		P1	1268	96.8
		U1	1281	97.8
		W1	1273	97.1
	3	Y1	1249	95.3
		Z1	1321	101
		AC1	1286	98.2
		Mean	1253	**95.6**

Laboratory	Batch No.	Sample ID	Measured conc. (ng/g)[b]	Accuracy (%)
		STDEV	54	
		CV %	4.3	
% Difference from reference conc.			-4.48	
Sponsor	1	F3	1380	105
	2	I3	1340	102
	2	K3	1250	95.4
	2	Q3	1320	101
	2	R3	1300	99.2
	3	AA3	1340	102
		Mean	**1322**	**101**
		STDEV	44	
		CV %	3.3	
% Difference from reference conc.			0.89	

[a]DENL method: LC-MS/MS with APCI; Sponsor method: LC-MS/MS with ESI.
[b]Each DENL concentration data is the mean of duplicate analysis.

Table 10: Measured concentration of lufenuron in incurred salmon tissue having a reference concentration of 2600 ppb (approximately 2X), using the DENL and Sponsor's methods[a]

Laboratory	Batch No	Sample ID	Measured conc. (ppb)[b]	Accuracy (%)
DENL	1	D1	2137	82.2
	1	E1	2299	88.4
	1	H1	2378	91.5
	2	J1	2389	91.9
	2	O1	2442	93.9
	2	T1	2474	95.1
	3	AD1	2538	97.6
	3	AE1	2568	98.8
		Mean	**2403**	**92.4**
		STDEV	138	
		CV %	5.8	
% Difference from reference conc.			-7.88	
Sponsor	1	E3	2490	95.8
	1	G3	2650	102
	1	H3	2550	98.1
	2	P3	2440	93.8
	3	V3	2520	96.9
	3	W3	2720	105
		Mean	**2562**	**98.5**
		STDEV	105	
		CV %	4.1	
% Difference from reference conc.			-1.49	

[a]DENL method: LC-MS/MS with APCI; Sponsor method: LC-MS/MS with ESI.
[b]Each DENL concentration data is the mean of duplicates.

To further determine if the DENL incurred results where statistically different from the sponsor's results, a functional relationship estimation by maximum likelihood (FREML) analysis was applied. For FREML analysis, it was assumed that the standard deviation of the concentration data for replicates of the incurred tissues is an approximation of the standard deviation of the concentration data obtainable from repeated analysis of each of the replicates. For example, repeated analysis

of one sample such as A1 should yield concentration data with standard deviation that should not be significantly different from the standard deviation (21.61659) obtained from the analysis of the other identical incurred samples B1, C1, F1, G1, M1, R1 and S1, as shown in the resulting FREML in Table 11.

A FREML regression slope of 1.0 would indicate that there is no significant difference between the set of all the lufenuron concentration data obtained with the DENL and sponsor's methods. As shown in Table 11, the range of the slope of the FREML regression equation includes 1. Thus, it can be concluded that the FREML regression slope (and slope range) shown in Table 11 demonstrates that there is no statistically significant difference between the concentration data obtained with the DENL and sponsor's methods from analysis of lufenuron in all the samples of blinded incurred salmon tissue.

Table 11: FREML table of the incurred samples concentration data obtained using the DENL and Sponsor methods[a,b]

DENL Measured concentration (ng/g)	DENL STDEV	Sponsor Measured concentration (ng/g)	Sponsor STDEV
643.9	21.61659	741	49.63064
624.4		806	
636.1		709	
622.9		718	
586.7		653	
597.6		729	
643.5		726	
640.0		726	
1188	54.23826	1380	44.00758
1156		1340	
1268		1250	
1281		1320	
1273		1300	
1249		1340	
1321		1322	
1286		1322	
2137	1138.415	2490	104.5785
2299		2650	
2379		2550	
2390		2440	
2442		2520	
2474		2720	
2538		2562	
2568		2562	

[a]FREML Regression equation: y = 87.28 +1.006 ± (0.03155) * x
[b]Slope range: 0.974 - 1.038

Validation of the Confirmatory Procedure

DENL validated the confirmatory procedure using salmon and trout tissue fortified with lufenuron and the samples of blinded incurred salmon tissue provided by the sponsor. The FDA CVM 118 guidance[8] provides identification criteria to determine if a residue can be identified. Three MRM product ion transitions from lufenuron are monitored in this method: m/z 509 → m/z 326 (reference

ion), m/z 509 → m/z 202, and m/z 509 → m/z 175. For positive identification, the sample must meet all the three following criteria based on comparison of incurred salmon tissue sample extracts with replicates of solvent standards, including standards having concentration at the presumptive tolerance of lufenuron (1300 ppb):

1) **Ion ratios**
The relative abundance ion ratios of the qualifying ions to the reference ion (m/z 175:m/z326 and m/z 202:m/z 326) for the sample must be within ± 20% of the average ion ratio for each qualifying transition for the solvent standards. The ratios are calculated as a percentage according to the following formula:

$$\text{Ion ratio} = \frac{\text{peak area of qualifying ion}}{\text{peak area of reference ion}} \times 100$$

2) **Retention time**
The retention time of the product ion transition peaks from the sample must be within ±5% of the average retention time of the ion peaks from the standards.

3) **Sensitivity**
The signal-to-noise (S/N) ratio of the qualifying ions (m/z 509->202 and m/z 509-> 175) must be >100. Although GFI #3 recommends an S/N ratio of 5, we have found during several method trials that a higher S/N ratio criterion is advisable to ensure that the negative control extracts will fail to confirm by not meeting the S/N ratio criterion.

The confirmation data obtained by DENL, using APCI LC-MS/MS, are summarized in Table 12. Lufenuron was confirmed in the blinded incurred samples at all the tolerance-related concentrations, while the blinded negative control samples failed to confirm. The determinative analysis results from both DENL and the sponsor's suggest that some of the blinded control samples may have been contaminated with low, detectable concentrations of lufenuron that are below the respective calibration ranges for the two methods. Five of such control salmon tissue samples were analyzed by DENL and were shown to contain approximately 15-22 ng/g of lufenuron. The concentration data for the contaminated negative control samples are not valid, because they are below the calibration range of the method. These contaminated negative control samples failed also to confirm ion ratios and S/N ratio <100.

Table 12: Confirmation data[a] (mean ± SD) for lufenuron in fortified and incurred salmon[b]

Sample	Peak area ratio (m/z175:m/z326)	Peak area ratio (m/z202:m/z326)	RT (m/z 175)	RT (m/z 202)	S/N (m/z 175)	S/N (m/z 202)
Standards (acceptance limits)	30.6 - 50.7	9.22 - 29.2	3.91 - 4.32	3.90 - 4.32	> 5	> 5
Standard (1125 ng/g)[c]	40.8 ± 0.64	19.1 ± 0.30	4.12	4.12	19500 ± 500	9190 ± 259
Fortified control (650 ng/g)	40.7 ± 0.37	19.1 ± 0.33	4.12	4.12	121000 ± 681	5710 ± 332
Fortified control (1300 ng/g)	40.6 ± 0.54	19.3 ± 0.27	4.12	4.12	24200 ± 1440	11500 ± 681
Fortified control (2600 ng/g)	40.8 ± 0.700	19.2 ± 0.34	4.11	4.12	48700 ± 2860	23000 ± 1490
Blinded control	0	0	0	0	N/A	N/A
Blinded incurred (651 ng/g)[d]	40.4 ± 0.0100	18.5 ± 0.0036	4.12	4.12	15500 ± 963	7110 ± 537
Blinded incurred (1310 ng/g)	40.1 ± 0.0051	18.5 ± 0.0042	4.14	4.14	31000 ± 2990	14400 ± 1450
Blinded incurred (2600 ng/g)[e]	40.6 ± 0.0031	18.7 ± 0.0042	4.13	4.13	57900 ± 6470	26600 ± 3230

[a]Tissue analysis was performed using LC-MS/MS system with APCI, RT: Retention Time (min);
[b]Sample size is n=15; [c]n= 8; [d]n =17; d: [e]n = 16

In addition to investigating the use of ESI for the analysis of lufenuron in the incurred salmon and trout tissues, DENL investigated the use of ESI in combination with calibration curves prepared

with tissue matrix (post-extraction fortified ESI version). The APCI version of the DENL method makes use of calibration standards prepared in neat solvent. The FREML table of the concentration data obtained by the APCI and the 'post-extraction fortified ESI version' of the DENL method is shown in Table 13.

Based on the FREML regression equation, the lufenuron concentration data obtained with the 'post-extraction fortified ESI version' of the DENL method is significantly different from the lufenuron concentration data obtained with the APCI version of the method. It was concluded that the lufenuron concentration data obtained with the 'post-extraction fortified ESI version' of the DENL method is about 36 to 42 % lower than the lufenuron concentration data obtained with the APCI version of the method. The GF#3[2] guideline recommends the use of calibration standards prepared in neat solvent. Thus, we consider the 'post-extraction fortified ESI' version of the DENL method not to be favorable for the analysis of lufenuron in incurred salmon tissue.

Table 13: FREML analysis of incurred samples APCI and ESI using post-extraction fortified matrix calibration[a]

APCI[b] Conc. (ng/g)	APCI STDEV	Post-extraction Conc. (ng/g) fortified ESI	Post-extraction fortified ESI STDEV
643.9	21.61659	559.4	30.9367
624.4		599.4	
636.1		586.2	
622.9		586.0	
586.7		557.6	
597.6		642.4	
643.5		543.5	
640.0		618.2	
1188	54.23826	633.4	
1156		579.7	
1268		615.4	
1281		570.0	
1273		617.3	
1249		1138	
1321		1178	28.42569
1286		2343	
2137	138.4175	1757	472.5541
2299		2413	
2379		2206	
2390		2326	
2442		2220	
2474		1160	
2538		1240	
2568		2352	
2403		2256	

[a]Regression equation: y = 143.45 +0.6089(±0.03128) * x
Slope range: 0.5777 - 0.6402
[b]APCI version of the method makes use of solvent calibration curves

Method Limit of Detection (LOD) and Limit of Quantitation (LOQ)

Based on the calibration data obtained by DENL during analysis of the incurred samples, we estimated the LOD and LOQ of the APCI and post fortified ESI versions of the DENL methods

using the procedure described in VICH GL49 annex 2[5], using standard error of the intercept as the surrogate for blank signal. The estimates of the LODs and LOQs are shown in Table 14.

Table 14: Limit of Detection (LOD) and Limit of Quantitation (LOQ) of the APCI and ESI versions of the DENL method

Method Version	LOD (ng/g)	LOQ (ng/g)
APCI	96	291
ESI	117	354

Based on the estimate of the LOQ of the APCI version of the method, it is recommended that the concentration of the lowest calibration standard be 291 ng/g (equivalent solvent calibration standard concentration: 4 ng/mL based on the 75-fold total dilution involved in the extraction procedure).

Analysis of Lufenuron in Fortified Trout Tissue

DENL also investigated the analysis of lufenuron in fortified trout tissue using the three versions of the method described above. Chromatograms for lufenuron in negative control and fortified at the 1.0X level are shown in Figures 5 and 6. Accuracy (% recovery) and precision data obtained by DENL from analysis of trout tissue fortified with lufenuron, using the APCI, ESI and 'post-extraction fortified ESI' versions of the methods are summarized in Table 15.

Table 15: Accuracy (% recovery) and precision of analysis of lufenuron in fortified trout tissue using different versions of the DENL method

Method version Fortification (ng/g)	**APCI** Recovery (%), (CV %)	**ESI** Recovery (%), (CV %)	**'Post-extraction fortified ESI'** Recovery (%), (CV %)
650	91.7 (8.8)	96.0 (5.1)	99.6 (4.7)
1300	94.3 (7.8)	98.9 (3.3)	102 (4.6)
2600	95.3 (6.7)	99.1 (3.4)	102 (4.9)

The data in Table 15 demonstrates that the three versions of the DENL method all yield lufenuron concentration data with acceptable accuracy and precision for fortified trout tissues. The sponsor's validation analysis did not include incurred trout tissue. The methods are suitable for regulatory analysis based on the DENL validation.

CONCLUSION

CVM/ONADE concluded that there was no statistically significant difference between the concentration data obtained with the sponsor's method and the DENL APCI method for the analyses for of lufenuron in the same sets of incurred salmon tissue. CVM/ONADE also concluded that, lufenuron was qualitatively identified in the incurred samples at concentrations corresponding to approximately 0.5X (651 ppb), 1X (1310 ppb) and 2X (2600 ppb), while the negative control samples failed to confirm. The DENL APCI LC-MS/MS method is suitable for the regulatory determinative and confirmatory analysis of lufenuron in salmon and trout tissue.

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Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calc Conc. (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Lufenuron 1	4.11	4.81	968.52	214971		
Lufenuron 2	**4.11**	**4.81**		86623	**40.3% (40.5%)**	✓
Lufenuron 3	**4.11**	**4.78**		40477	**18.8% (19.1%)**	✓

Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions

Lufenuron 1

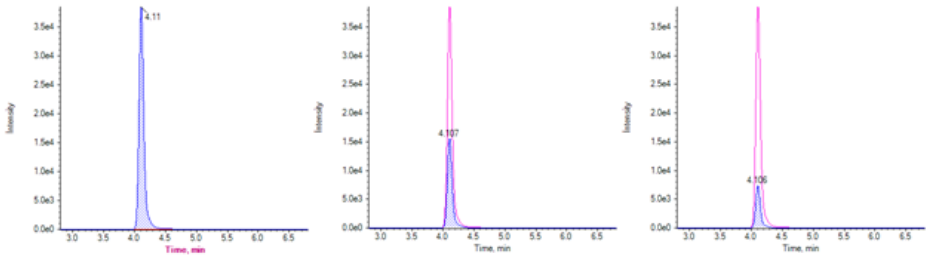

Figure 1: Lufenuron Report 12.5 ng/mL Solvent Standard equivalent to 938 ng/g in sample:
Lufenuron 1 quantitation ion (m/z 509 → 326),
Lufenuron 2 Confirmation (m/z 509→ 202) & Lufenuron 3 (m/z 509 → 175)

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calc Conc. (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Lufenuron 1	N/A	4.81	N/A	N/A		
Lufenuron 2	N/A	4.81		N/A	0.0% (40.0%)	
Lufenuron 3	**N/A**	**4.78**		N/A	**0.0% (17.9%)**	✓

Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions

Lufenuron 1

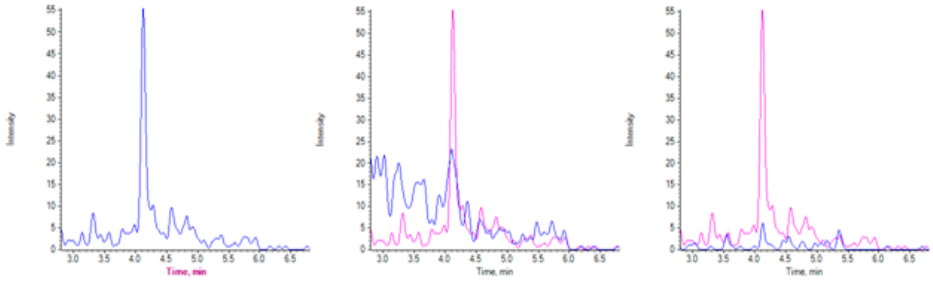

Figure 2: Lufenuron Multi Quant Report Reagent Blank:
Lufenuron 1 quantitation ion (m/z 509 → 326),
Lufenuron 2 Confirmation (m/z 509→ 202) & Lufenuron 3 (m/z 509 → 175)

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calc Conc. (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Lufenuron 1	N/A	4.81	N/A	N/A		
Lufenuron 2	N/A	4.81		N/A	0.0% (40.5%)	
Lufenuron 3	**N/A**	**4.78**		N/A	**0.0% (19.1%)**	✔

Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions

Lufenuron 1

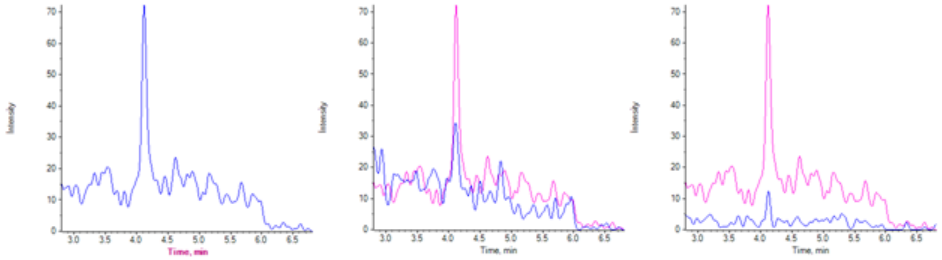

Figure 3: Lufenuron Multi Quant Report Negative Control Salmon:
Lufenuron 1 quantitation ion (m/z 509 → 326),
Lufenuron 2 Confirmation (m/z 509→ 202) & Lufenuron 3 (m/z 509 → 175)

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calc Conc. (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Lufenuron 1	4.12	4.81	1200.02	313394.0		
Lufenuron 2	**4.12**	**4.81**		126439	**40.3% (40.0%)**	✔
Lufenuron 3	**4.12**	**4.78**		57039	**18.2% (17.9%)**	✔

Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions

Lufenuron 1

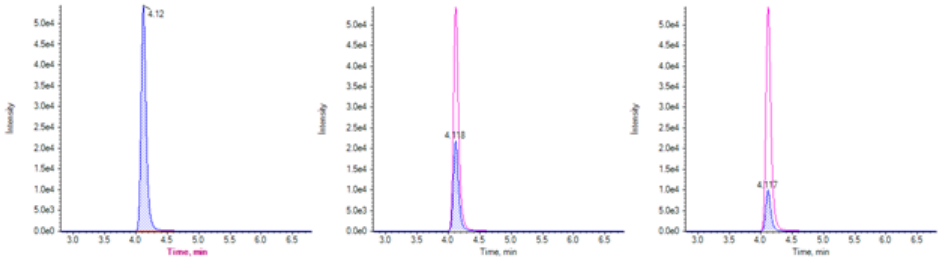

Figure 4: Lufenuron Multi Quant Report Incurred I1-AR1 Salmon:
Lufenuron 1 quantitation ion (m/z 509 → 326),
Lufenuron 2 Confirmation (m/z 509→ 202) & Lufenuron 3 (m/z 509 → 175)

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calc Conc. (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Lufenuron 1	N/A	4.81	N/A	N/A		
Lufenuron 2	N/A	4.81		N/A	0.0% (39.3%)	
Lufenuron 3	**N/A**	**4.78**		N/A	**0.0% (18.7%)**	✔

Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions

Lufenuron 1

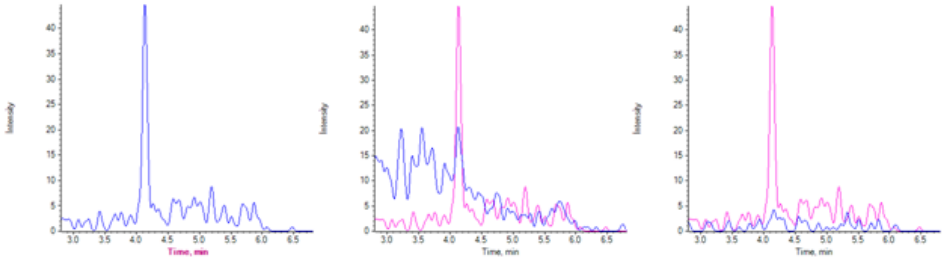

Figure 5: Lufenuron Multi Quant Report Negative Control Trout:
Lufenuron 1 quantitation ion (m/z 509 → 326),
Lufenuron 2 Confirmation (m/z 509→ 202) & Lufenuron 3 (m/z 509 → 175)

Results Summary

Analyte Peak Name	Analyte RT	Expected RT	Calc Conc. (ng/g)	Analyte Response	Calculated Ion Ratio (Expected Value)	Ratio Confirms
Lufenuron 1	4.13	4.81	1330.38	372672.0		
Lufenuron 2	**4.13**	**4.81**		149033	**40.0% (40.9%)**	✔
Lufenuron 3	**4.13**	**4.78**		67699	**18.2% (18.9%)**	✔

Chromatograms – Bars on peaks are expected ion ratio +/-10% or 20% depending on number of conf. ions

Lufenuron 1

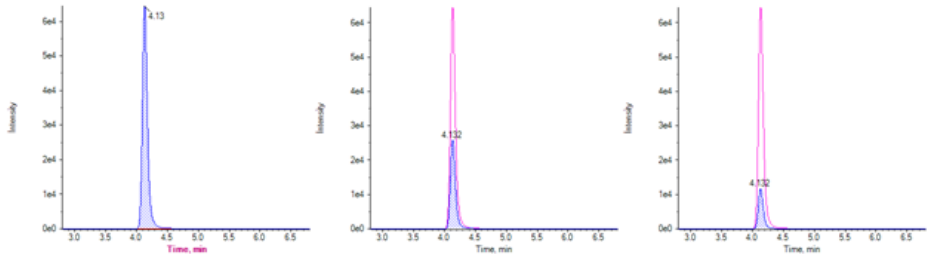

Figure 6: Lufenuron Multi Quant Report Validation 1.0X 1300 ng/g Trout:
Lufenuron 1 quantitation ion (m/z 509 → 326),
Lufenuron 2 Confirmation (m/z 509→ 202) & Lufenuron 3 (m/z 509 → 175)