Quantitative Determination of Oxytetracycline in Six Aquaculture Commodities by Rapid and Sensitive UHPLC-MS/MS

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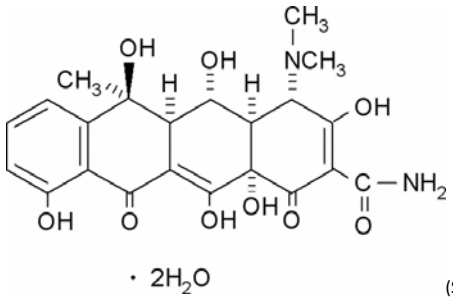
Abstract

A fast, efficient and sensitive method using ultra-high performance liquid chromatography tandem mass spectroscopy (UHPLC-MS/MS) was conducted as a single laboratory, level-one validation study for the confirmation and quantification of oxytetracycline (OTC) in shrimp and tilapia. The method was verified by extension to four additional commodities, including sea bass, catfish, salmon, and lobster. Two gram composites of each matrix were spiked at 100, 200, and 400 ppb, and then extracted twice with 10.0 mL of 0.1 M succinic acid in the presence of sodium chloride using the extraction protocol of Andersen et al. The supernatant was added to 5.0 mL of 0.01 M oxalic acid and 0.5 g alumina, mixed and centrifuged, and followed by HLB solid phase extraction (SPE) cleanup, and elution with methanol. The extracts were further diluted to 10.0 mL with 0.1% formic acid and analyzed by UHPLC-MS/MS. The average accuracy recoveries of OTC spiked in triplicate ranged from 57.5 to 83.1 %. The relative standard deviation ranged from 1.6-2.5 %, 1.6-4.4 %, 0-4.6 %, 1.3-8.3 %, 0.6-11 %, and 0.01 -0.03 % for shrimp, tilapia, catfish, salmon, bass, and lobster, respectively. The post-extracted matrix standard calibration curves were linear for all commodities with coefficients of determination (r^2) greater than 0.995. The overall accuracy, precision, and linearity recovery data may indicate method suitability for regulatory analysis of shrimp, tilapia, bass, catfish, salmon and lobster.

Introduction



(Source: Sigma-Aldrich)

Figure 1: Structure of Oxytetracycline (dihydrate), MW: 460.44 g/mole (as base)

Oxytetracycline (OTC) (Figure 1), known under various trade names such as Terramycin™, Tetroxy™, Oxymarine™, is a naturally-occurring tetracycline antibiotic derived from fungi, Streptomyces sp[1]. The family of tetracycline antibiotics, including tetracycline, chlortetracycline, doxycycline and oxytetracycline, are broad-spectrum antibiotics that are generally effective against Gram-positive and Gram-negative bacteria[2]. The therapeutic effectiveness of tetracycline antibiotics, however, has been limited due to apparent bacterial resistance that may be attributed to over usage of antibiotics in livestock, and particularly, in aquaculture farming[3].

Per Code of Federal Regulations (21 CFR 556.500(b)(1))[4], the combined tolerance for tetracycline antibiotic residues (oxytetracycline, chlortetracycline and tetracycline) in domesticated livestock, finfish and lobster is 2 parts per million (ppm) in muscle tissue. In aquaculture farming, OTC is allowed for use by the U.S. Food and Drug Administration (FDA) with residues not to exceed the 2 ppm level. In 2010, programs of the FDA were audited by the European Union (EU) Commission for U.S. domestic food commodities, including aquaculture farmed seafood, destined for exportation into the EU. Under EU Audit Field Assignment in 2012, the maximum allowable limit for OTC was established at 0.2 ppm (200 ppb), ten times lower than the total tolerance stated in the CFR.

Numerous analytical techniques of analysis for the determination of OTC in seafood are known, including FDA methods specified under the Chemotherapeutics in Seafood Compliance Program[5]. LC/UV/FLD detection[6,7] and LC/MS[8,9] methods were available for quantification of OTC at lower regulatory levels specified in the EU Audit Field Assignment Program. In 2012, Northeast Food and Feed Laboratory (NFFL, formally Northeast Regional Laboratory) validated a rapid and sensitive UHPLC-MS/MS method for OTC in shrimp, tilapia, catfish, bass, salmon and lobster using a modified sample extraction protocol reported previously in a publication by Andersen et al.[9] Summary of the single-laboratory method validation study, including spike recovery data, precision, accuracy, and linearity, is presented.

Experimental

1. Standard and Reagents

A. Analytical Standards

a) Oxytetracycline dihydrate ($C_{22}H_{24}N_2O_9.2H_2O$; CAS 6153-64-6, MW 496.46) purchased from MP Biomedicals for calibration curve preparation (CCV) and spiking.
b) Oxytetracycline dihydrate ($C_{22}H_{24}N_2O_9.2H_2O$; CAS 6153-64-6, MW 496.46) purchased from Sigma-Aldrich for preparation of independent calibration verification (ICV).

B. Solvents and Chemicals

a) Deionized water (DI), LC-grade, or equivalent
b) ACS grade oxalic acid ($CH_2H_2O_4.2H_2O$; CAS 6153-56-6, FW 126.07), Fisher Scientific

c) ACS grade sodium hydroxide (NaOH; 5N), Fisher Scientific
d) ACS succinic acid ($C_4H_6O_4$; CAS 110-15-6, FW 118.09), Acros Organics
e) Sodium chloride (NaCl; CAS 7647-14-5, FW 58.44, ACS grade), Fisher Scientific
f) Methanol, LC-MS grade, Fisher Scientific
g) Acetonitrile, LC-MS grade, Fisher Scientific
h) Formic acid, LC-MS grade (CH_2O_2; CAS 64-18-6, FW 46.03), Sigma-Aldrich
i) Alumina (80-200 Mesh), (Al_2O_3; CAS 1344-28-1, FW 101.96), Fisher Scientific

2. Preparation of Consumable Reagents

a) 0.1 M succinic acid extraction solution: dissolve 12.0 g of succinic acid and 7.0 mL of 5 M sodium hydroxide (pH 4) in 1 L of DI water
b) 0.01 M of oxalic acid: dissolve 1.26 g of oxalic acid in 1 L of DI water
c) 0.1% formic acid: 1.0 mL of formic acid diluted to 1 L with DI water

3. Equipment

a) High-speed centrifuge refrigerated (Sorvall/ RC5c Plus), Thermo Fisher, or equivalent
b) Model Geno-Grinder 2010, SPEX
c) Vortex mixer Model # Vortex Genie 2, Scientific Industries
d) 50 mL polypropylene (pp) centrifuge tube (20 k RCF) with plug cap (SuperClear Ultra High Performance), VWR
e) 15 mL polypropylene (pp) centrifuge tubes, VWR
f) 3/8 in. (9.5 mm) stainless steel grinding balls (P/N: 2155, SPEX)
g) Mettler-Toledo PM 4800 Delta Range and Mettler-Toledo XP -204 (analytical balances)
h) Borosilicate glass LC vials, 2 mL
i) Hobart Robot Coupe food processor Model R10, or equivalent
j) AB SCIEX Model 5500 Q-Trap/Shimadzu Corporation Model XR-LC system, or equivalent
k) Liquid carbon dioxide tank equipped with dry ice aspirator cone
l) Oasis HLB 3cc (60 mg) Vac Cartridge (P/N: WAT094226, Waters Corporation)
m) 1.2 micron Acrodisc Syringe Filter with Glass Fiber (P/N:4523, Pall Corporation), or equivalent
n) Empty polypropylene SPE tubes (P/N: 57022, Sigma-Aldrich), or equivalent
o) Borosilicate Pyrex fiber glass wool (P/N: 11-388, Fisher Scientific)

4. Instrumental

A. Liquid Chromatography

a) Shimadzu XR LC installed with Shimadzu C-18, 50 X 2.1 mm, 1.9 µm UHPLC column, 40°C column oven temperature, coupled to AB SCIEX 5500 Q-Trap MS
b) Controller CBM-20A, Shimadzu Scientific
c) Auto sampler SIL-20AC-XR 1.5mL Cooled (15°C), Shimadzu Scientific
d) LC-20AD-XR chromatographic pumps, Shimadzu Scientific
e) Column oven CTO-20AC

f) Degasser, Shimadzu Scientific
g) Mobile phase A: 0.1% formic acid; mobile phase B: LC/MS grade acetonitrile
h) LC isocratic flowrate: 600 µl/min acetonitrile 0.1% formic acid (20+80)
i) LC runtime: 60 seconds (1 minute)
j) Injection volume: 10 µl

B. Mass Spectrometer MRM Parameters

a) AB SCIEX Q-Trap 5500 Linear Ion-Trap/Quadrupole MS/MS
b) Analyst 1.5.1 software
c) ESI voltage (positive): 5.5 kV
d) DP: 49
e) EP: 10
f) CXP: 20
g) Source temperature: 500 °C
h) CE (QUANT ION, 461.2>426.0) OTC1: 34 V. Refer to Table 1(a).
i) CE (CONFIRM ION, 461.2>444.2) OTC2: 26 V. Refer to Table 1(b).
j) CE (CONFIRM ION, 461.2>337.0) OTC3: 43 V. Refer to Table 1(c).
k) Retention time criteria: +/-5%
l) Confirmation Criteria (CVM Guidance for Industry 118[10]):

i. Peak area must be greater than determined method detection limit (MDL).
ii. The signal to noise ratio (S/N) must be ≥ 10:1 for the quantitation ion and ≥ 3:1 for each of the two confirmation ions.
iii. Retention time (t_R) must be ≤5% RSD of the average for the calibration curve.
iv. Ion ratios must be within ± 20% (absolute) of the average ion ratios of the standards in the calibration curve.

Table 1(a): MRM Transition for M/Z 426.0 (OTC1, QUANT ION)

Q1 Mass	Q3 Mass	Dwell (msec)	Parameter	Start	Stop	ID
461.2	426.0	200	DP(V)	49	49	OTC1
			EP(V)	10	10	
			CE (V)	34.00	34.00	
			CXP(V)	20	20	

Table 1(b): MRM Transition for M/Z 444.2 (OTC2, CONFIRM ION)

Q1 Mass	Q3 Mass	Dwell (msec)	Parameter	Start	Stop	ID
461.2	444.2	200	DP(V)	49	49	OTC 2
			EP(V)	10	10	
			CE (V)	26.00	26.00	
			CXP(V)	20	20	

Table 1(c): MRM Transition for M/Z 337.0 (OTC3, CONFIRM ION)

Q1 Mass	Q3 Mass	Dwell (msec)	Parameter	Start	Stop	ID
461.2	337.0	200	DP(V)	49	49	OTC 3
			EP(V)	10	10	
			CE (V)	43.00	43.00	
			CXP(V)	20	20	

5. Sample Preparation

Remove head, tail, shell, skin and scales from bass, catfish, lobster, salmon, shrimp, and tilapia. Grind sample with sufficient dry ice using a robot coupe or similar equipment until it becomes a powdery fine mixture. Transfer the composite into a plastic container and store in a freezer at -20°C with the lid loosely placed on top to allow the mixture to degas overnight.

6. Oxytetracycline Standard Preparation

a) 100 ppm Stock Solution (100 µg/mL, as base):

Weigh 10.0 mg of oxytetracycline standard into a 100.0 mL volumetric flask. Adjust to volume with methanol and stored at -20°C (Stability six months at -25°C)[15].

b) 4 ppm Intermediate Stock Solution (4 µg/mL):

Transfer 2.0 mL of the 100 ppm oxytetracycline standard into a 50.0 mL volumetric flask. Dilute to volume with 0.1% formic acid and stored at 4°C for one month.

c) 400 ppb Post-Extracted Matrix-Matched Intermediate Stock Solution (400 ng/mL):

Transfer 100 µl of the 4 ppm intermediate stock solution and add 900 µL negative sample control extract in an LC vial (400 ppb). Prepare the following standard calibration concentration range from the 400 ppb stock solution: 10 ng/mL, 20 ng/mL, 40 ng/mL and 80 ng/mL (sample equivalent to 50 ppb, 100 ppb, 200 ppb, and 400 ppb (ng/mL), respectively) into respective LC vials. Refer to Table 2 below. Prepared on day of use.

Table 2: Preparation of Oxytetracycline Post-Extracted Calibration Standard Curve

Volume of 400 ppb Intermediate Standard (mL)	Volume of Extracted Negative Matrix Control (mL)	Standard Concentration, ppb (ng/mL)	Sample Equivalent, ppb (ng/g)
0.025	0.975	10	50
0.050	0.950	20	100
0.100	0.900	40	200
0.200	0.800	80	400

d) Sample Fortification at 200 ppb (ng/g):

Weigh 2.0 g of sample composite into a 50 mL polypropylene (20 k RCF) centrifuge tube. Fortify sample by adding 100 µL of the 4 ppm intermediate stock solution to produce a 200 ppb spike level and proceed with Extraction Protocol.

7. Extraction Protocol (modified from Ref. 9, Andersen et al.)

a) Weigh composite (2.0 ± 0.01 g) into 50 mL polypropylene centrifuge tube (20 k RCF). Note: For regulatory analysis, sample batch should include a method blank (2.0 ± 0.1 g of DI water), a matrix blank and two matrix spikes at 200 ppb.
b) Add 1.0 g of NaCl powder.
c) Add 10.0 mL of succinic acid extraction solution.
d) Vortex mixture for 30 sec. at 1500 rpm.
e) Add one steel ball and shake on a Geno-Grinder at 1000 cps for 1 minute.
f) Centrifuge at 7500 rpm and 4^0C for 10 minutes.
g) Carefully decant supernatant into a clean 50 mL polypropylene centrifuge tube containing 0.5 g alumina and 5.0 mL of 0.01 M oxalic acid without filtering.
h) Repeat steps (c-f) without addition of another steel ball and combine with supernatant from step (g).
i) Vigorously shake the polypropylene centrifuge tube containing the extracts on the Geno-grinder at 1000 cps for 10 sec.
j) Centrifuge extracts at 7500 rpm and 4^0C for 10 minutes.

k) Condition an Oasis HLB SPE cartridge sequentially with 4.0 mL of methanol, 4.0 mL DI water and 4.0 mL succinic acid extraction solution. Add an additional 3.0 mL of succinic acid extraction solution over the HLB SPE cartridge to serve as the solvent reservoir layer.
l) Position a connector on top of the HLB SPE extraction cartridge with a 1.2 µm microfiber filter and connect to an empty reservoir tube containing a small borosilicate glass wool plug.
m) Decant final supernatant into the SPE setup and pass under vacuum at approximately 2 drops per sec. and discard.
n) Rinse SPE tube with 4.0 mL DI water and let dry for 5 minutes under vacuum.
o) Elute with 4.0 mL methanol under gravity into 15 mL polypropylene centrifuge tubes.
p) Dilute and mix eluent to 10.0 mL with 0.1% formic acid. No evaporation required.
q) Pipet about 2.0 mL of the diluted eluent into an HPLC vial and inject for LC/MS analysis.

Validation Protocol

The goal of this study was to validate an ad-hoc method for the confirmation and quantification of OTC in aquaculture products at a concentration of 0.2 ppm (200 ppb) for routine regulatory analysis. This efficient method was validated per the criteria outlined in NRL-QMS.013[11] and ORA-LAB.5.4.5[12]. Pertinent method validation parameters include specificity, accuracy, precision, and linearity, as discussed below.

Validation Study Results

1. **Method System Suitability Requirements:** Typical system suitability consists of at least 6 replicate injections of a standard to demonstrate that the instrument is suitable to perform reproducible analysis. The % RSD of the peak area should be less than 5%. Seven replicate injections of the 200 ppb OTC standard (n=7) were performed prior to each batch to ensure the system suitability criteria were met.
2. **Selectivity:** Specificity of parent compound, OTC (m/z 461.2), is confirmed by the presence of three product ions, m/z 337.0, m/z 426.0, m/z 444.2, and their respective ion ratios to the QUANT ion (m/z 426.0). Figure 2(b) shows typical MRM scans of shrimp and tilapia post-extracted OTC standards (200 ppb, equiv.) of the three product ions monitored during this validation study. Additionally, matrix blanks devoid of OTC were taken through the entire extraction process and did not show any indication of interferences at the expected OTC elution retention time. Figure 2(a) presents typical MRM scans of tilapia and shrimp matrix blanks.
3. **Blank Analysis:** Sections 6.3.B.1 and 6.3.C.1.e of ORA-LAB.5.4.5[12] require that the blank be less than the determined method detection limits (MDL). For this study, method blank and negative controls were processed concurrently under identical conditions as samples taken through all steps of the extraction procedure. A typical multiple reaction monitoring (MRM) scan of method (reagent) blank, used to evaluate any possible OTC contamination in the laboratory environment or instrument and to demonstrate low system background, is shown in Figure 3. All blanks were less than the calculated method detection limits (MDL) determined by this study.
4. **Calibration Curve:** The calibration plot (QUANT ion, m/z 426.0) with a matrix prepared standard linear range 50, 100, 200, and 400 ppb (equivalent sample concentration) based on the least squares line fitting (LSLF) equation were satisfactory, as shown in Table 3. Coefficients of determination are satisfactory in accordance with ORA.5.4.5[12], the linearity acceptance criteria is given as $r^2>0.995$.

Table 3: Linear Range (50-100-200-400 ppb) Coefficient of Determination, r^2

Commodity (One Source)	Coefficients of Determination, r^2
Shrimp	0.9997
Salmon	0.9997
Lobster	0.9995
Tilapia	0.9998
Catfish	0.9988
Sea Bass	0.9993

5. **Accuracy:** ORA-LAB.5.4.5 section 6.2.B1.aii[12] states that matrix spikes can be used to determine accuracy. Matrix spikes at three levels (100, 200 and 400 ppb) in triplicates were used in this method validation study to demonstrate accuracy. Figure 2(c) shows typical MRM scans of tilapia, shrimp and lobster spiked at 200 ppb. The validation recoveries demonstrate acceptable performance and excellent consistency across the board for all matrices. Average accuracy recoveries of OTC ranged from 74.8-83.1 % for bass, catfish, salmon, shrimp, and tilapia. The recoveries for lobster, however, were somewhat lower at the three spike levels with an average of 57.5 %. The overall recovery data are presented below in Table 4 for all six commodities.
6. **Precision:** Section 6.3.B.6, 6.3.B.7, and 6.3.C.1.d of ORA-LAB.5.4.5[12] states that duplicate matrix spikes with known concentrations of OTC will be used to access and demonstrate precision. Precision expressed as residual standard deviation (RSD), ranged from 0-11.0 % as shown in Table 4, indicating a satisfactory level of accuracy and reproducibility.
7. **Method Detection Limit (MDL):** Per 40 CFR, Part 136, Appendix B[13], the MDL is calculated by taking the standard deviation of the concentration obtained from 7 (n) independent spiked matrix samples and multiplying it by the Student's *t* value (n-1) at the 98% confidence level. In this study, we calculated a theoretical MDL for shrimp and tilapia selecting an OTC concentration of 100 ppb, which is half of the action level. See Table 5 for detailed result used to calculate the MDL in the tilapia and shrimp matrices. The calculated theoretical MDL for shrimp and tilapia are 11.62 ppb and 10.10 ppb, respectively. This is over 16-fold more sensitive than the action level of 0.2 ppm (200 ppb), thereby decreasing the chances of false positives and negatives when using this method of analysis.

Table 4: Accuracy and Precision Recovery Data

Aquaculture Commodity	Shrimp	Tilapia	Catfish	Salmon	Bass	Lobster
100 ppb Spiking Level (% Recovery in Triplicate)	Run 1: 74.8	Run 1: 89.0	Run 1: 91.1	Run 1: 75.2	Run 1: 84.2	Run 1: 50.4
	Run 2: 74.9	Run 2: 84.5	Run 2: 91.1	Run 2: 74.5	Run2: 83.5	Run 2: 51.6
	Run 3: 76.9	Run 3: 81.5	Run 3: 91.1	Run 3: 73.3	Run 3: 84.5	Run 3: 49.2
Average	**75.5**	**85.0**	**91.1**	**74.3**	**84.1**	**50.4**
RSD, %	**1.6**	**4.4**	**0.0**	**1.3**	**0.6**	**2.4**
200 ppb Spiking Level (% Recovery in Triplicate)	Run 1: 72.0	Run 1: 88.6	Run 1: 80.9	Run 1: 81.9	Run 1: 76.4	Run 1: 60.0
	Run 2: 72.4	Run 2: 85.7	Run 2: 77.5	Run 2: 70.0	Run 2: 61.3	Run 2: 59.5
	Run 3: 75.3	Run 3: 87.6	Run3: 79.6	Run 3: 72.9	Run 3: 70.4	Run 3: 58.3
Average	**73.2**	**87.3**	**79.3**	**74.9**	**69.4**	**59.3**
RSD, %	**2.5**	**1.7**	**2.2**	**8.3**	**11.0**	**1.5**
400 ppb Spiking Level (% Recovery in Triplicate)	Run 1: 75.0	Run 1: 76.1	Run 1: 79.9	Run 1: 74.3	Run 1: 81.7	Run 1: 65.5
	Run 2: 77.7	Run 2: 76.3	Run 2: 74.5	Run 2: 74.3	Run 2: 74.4	Run 2: 61.8
	Run 3: 74.6	Run 3: 78.3	Run 3: 73.4	Run 3: 79.5	Run 3: 76.6	Run 3: 61.3
Average	**75.8**	**76.9**	**75.9**	**76.0**	**77.6**	**62.8**
RSD, %	**2.2**	**1.6**	**4.6**	**4.0**	**4.8**	**3.3**

Table 5: Method Detection Limit (MDL) for Tilapia and Shrimp*

Sample ID	% Recovery of Spike at 100 ppb Tilapia	% Recovery of Spike at 100 ppb Shrimp
MDL1	81.05	83.63
MDL2	81.75	80.39
MDL3	76.14	82.28
MDL4	83.15	73.78
MDL5	83.15	76.89
MDL6	80.34	75.65
MDL7	86.66	76.27
STDV	3.21	3.70
****MDL (ppb)**	**10.10**	**11.62**

***MDL only conducted on shrimp and tilapia**
****MDL= STDV*t Value (3.142, n-1=6), at the 98% confidence level**

Discussion

Overview:

This method was originally developed in 2012 as a rapid quantitative UHPLC-MS/MS ad-hoc procedure for determination of oxytetracycline (OTC) in U.S. domestic food, including aquaculture seafood, destined for export to the European Union (EU). At the mandate of a memorandum of understanding between the EU and the U.S. Department of Agriculture and the Food and Drug Administration, food exported to the EU must meet specific tolerance guidelines. In the case of OTC, the Compliance Guide Program (CPG) 7304.018[5] states a tolerance of 2000.0 ppb (2.0 ppm) in muscle tissue based on 21 CFR 556.500 (b) (1) for finfish and lobster[4]. The guideline stated by the EU, however, is 0.2 ppm for aquaculture commodities. LC with UV/FLD-detection[6.7], as well as, LC-MS screening methods[8,9], were acceptable methods to meet the EU tolerance of 0.2 ppm. After reviewing sensitivity and chromatographic characteristics of the literature sources in 2012, chemists at NFFL decided to conduct a validation of the emergency ad-hoc procedure utilizing UHPLC-MS/MS/positive-mode ESI (electrospray ionization) and modified extraction protocol[9].

Ultra-High Performance Liquid Chromatography (UHPLC):

High-speed determination of OTC was conducted with the UHPLC system equipped with an ultra-fine 1.9 µm C-18 reversed-phase analytical column, coupled with ESI/Q-Trap MS/MS. Refer to section 4 (A) for UHPLC conditions. Under the specified isocratic chromatographic conditions of 0.1 % formic acid-acetonitrile (i.e., flowrate, column oven temperature), OTC was confirmed and quantified by MS/MS in approximately 30-35 seconds. Therefore, rapid sample throughput was maintained sample injection after sample injection. Considering LC runtime of about 1 minute, as many as 50 to 60 injections can be performed in one hour, while minimizing consumption of mobile phase. A one-minute sample injection recycling interval highlights the efficiency of the SPE cleanup and rapidity of determination of OTC by MRM. Another advantage of the modified extraction cleanup protocol[9] (discussed below) is the apparent minimization of sample matrix background that may contribute to matrix enhancement or suppression effects.

Multi-Residue Monitoring (MRM) Mass Spectroscopy (Tuning and Optimization):

A highly specific and sensitive positive ion-ESI, triple quadrupole (Q-trap) in MRM scanning mode was utilized for the detection of OTC (mass to charge ratio, *m/z 461.2*). Based on the FDA reference, *Guidelines for Industry*[11], three specific product (fragment) ion transitions, namely, one (1) quantifier and two (2) additional confirmation product ions, were necessary for regulatory MS quantitative analysis. In the modification of this quantitative method, three (3) predominant product ion fragmentation pathways of OTC were selected: *m/z* 426.0 (-NH_4OH loss), *m/z* 444.2 (-NH_3), and *m/z* 337.0[14]. The most intense product ion, *m/z* 426.0 (QUANT ion), was used for quantitative analysis, and *m/z* 444.2 and *m/z* 337.0 (CONFIRM ions), were used for relative ion ratios for positive confirmation of OTC. All the fragments of OTC were obtained by preparing a 100 ppb standard in 0.1 % formic acid in methanol. The precursor ion was fragmented to obtain the quantitative product ion (m/z 426.0) and two qualifier product ions (m/z 444.2, 337.0). These product ion transitions were consistent with previous work of Andersen et al.[9] The mass spectrometer was tuned and optimized by ramping the collision energy (CE), declustering potential (DP), collision cell exit potential (CXP) and the entrance potential (EP) for each of the product ion transitions. Based on this MRM optimization procedure, the FDA criteria[10] on absolute ion ratio for these transitions for quantification and confirmation were met.

Sample Extraction (modified from ref. 9):

A simple extraction and cleanup procedure was based on a previously reported protocol[9]. The extraction procedure avoided hazardous organic solvents and employed an aqueous extraction solution acidified with succinic acid in the presence of sodium chloride. Efficient extraction of OTC in 2.0 g homogenized seafood tissue composites in aqueous extraction solvent was achieved with the use of steel balls in polypropylene (pp) centrifuge tubes placed on the Geno-Grinder apparatus at 1000 cps. Due to high impact of the steel ball on the centrifuge cap, it was necessary to obtain tubes capable of withstanding 20,000 g of force. The following steps in the protocol involved high-speed refrigerated centrifugation and acidification with oxalic acid in the presence of chromatographic grade alumina. The purification of the centrifuged extract was accomplished by a rapid pre-filtration setup consisting of an empty 60 mL pp SPE reservoir with glass wool plug attached to a 1.2 µm LC-disposable filter. Purification of the extract was achieved by passing 4.0 mL of methanol by gravity elution through the hydrophilic HLB solid phase extraction (SPE) column. The final extract was diluted to 10.0 mL with 0.1 % formic acid in DI water, equivalent to 0.2 g/mL (5X sample dilution), for LC/MS injection.

Efficiency and Recovery:

Post-extracted negative matrix controls were used for working LC calibration standards on day of use. In this approach, the post-extracted calibration curve was constructed for external standard quantitative determination. As such, the recoveries reported in Table 4, represent actual extraction efficiencies for each of the six aquaculture products, since matrix-extracted standards (i.e., calibrated recoveries over a specified linear range) were not used for the analysis during the EU Field Assignment. The recoveries as shown in the table clearly show satisfactory extraction efficiencies, with lower recoveries obtained for lobster, similar to the shrimp recovery results reported by Andersen et al[9.] Figures 2(a-c) present typical MRM scans for tilapia, shrimp, and lobster (negative controls, post-extracted standards, and spiked matrixes). Figure 3 presents a typical method reagent blank MRM scan. After the satisfactory validation of this rapid ad-hoc emergency method, we believe that future incorporation of matrix-extracted standards, and the utilization of a deuterated OTC internal standard, e.g., oxytetracycline-$^{13}C_2$-D_6 (AlsaChim), could enhance extraction efficiency across all aquaculture commodities.

Conclusion

The results of this validation study demonstrate that this reliable and sensitive method that may be suitable for the confirmation and quantification of OTC in aquaculture products bass, catfish, lobster, salmon, shrimp, and tilapia. The overall precision, accuracy, linearity and recovery results for OTC are acceptable for regulatory analysis based on our validation study. Additionally, this improved and dependable UHPLC-MS/MS quantitation method has been utilized for the analysis of OTC in regulatory analyses at the Northeast Food and Feed Laboratory.

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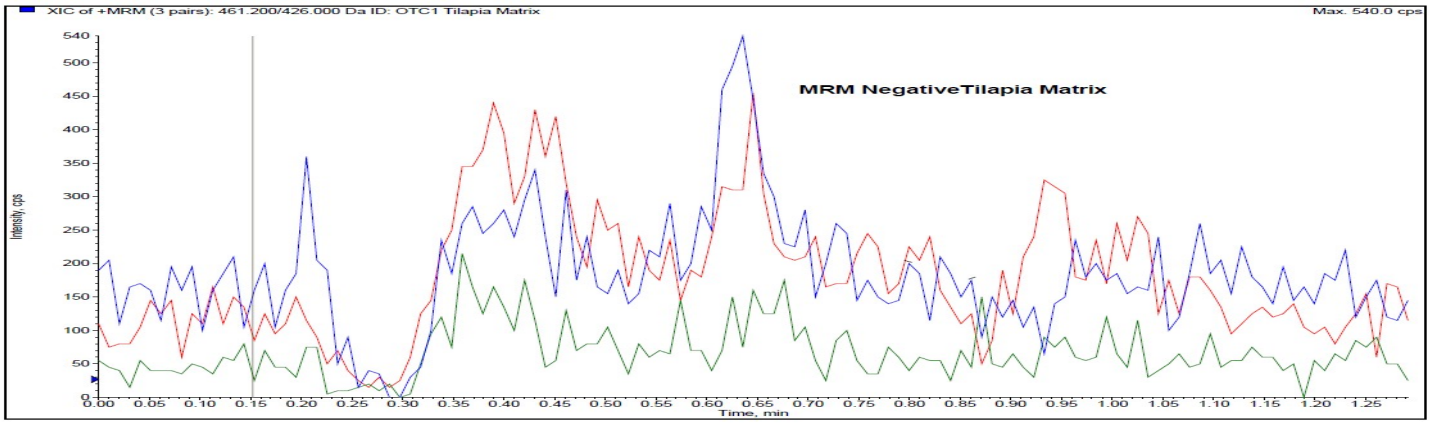
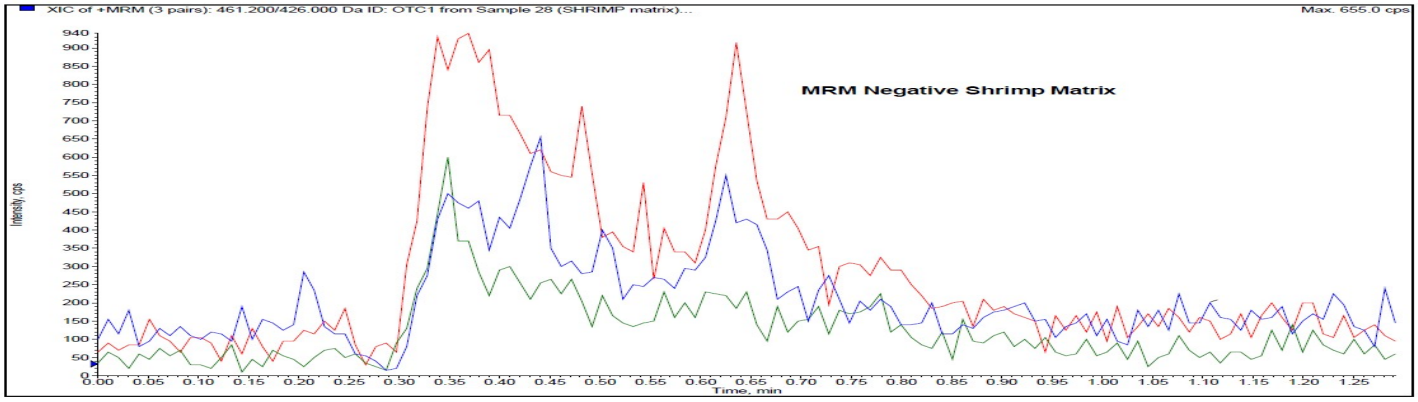
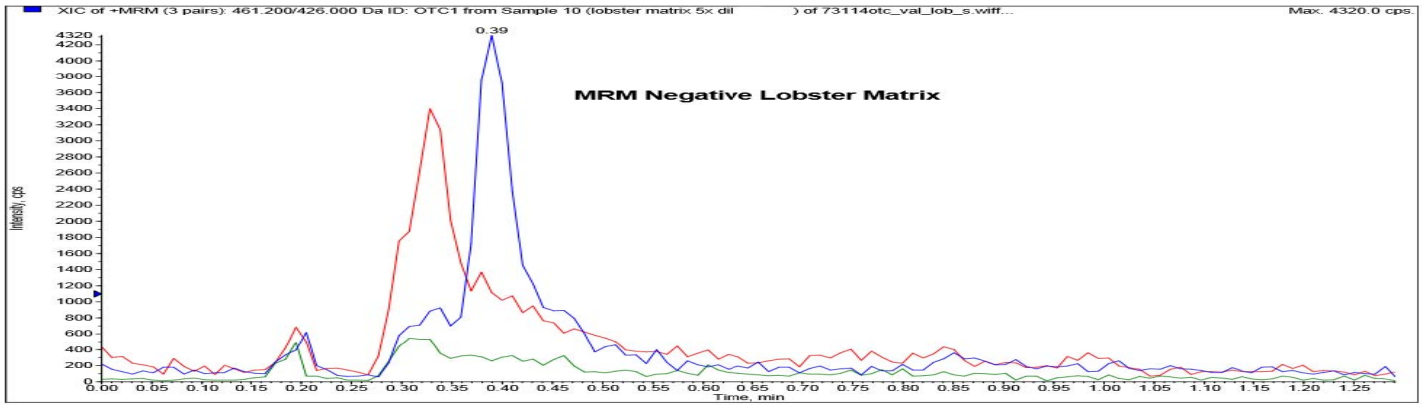
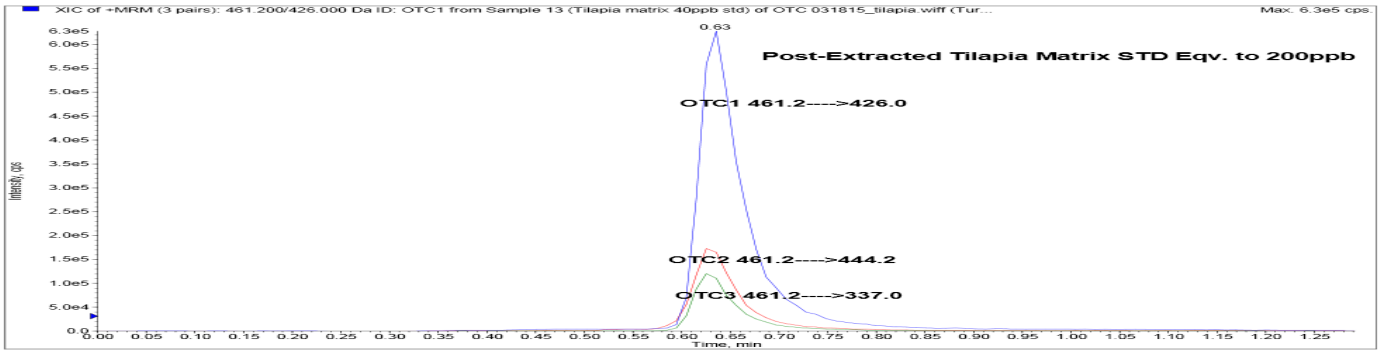
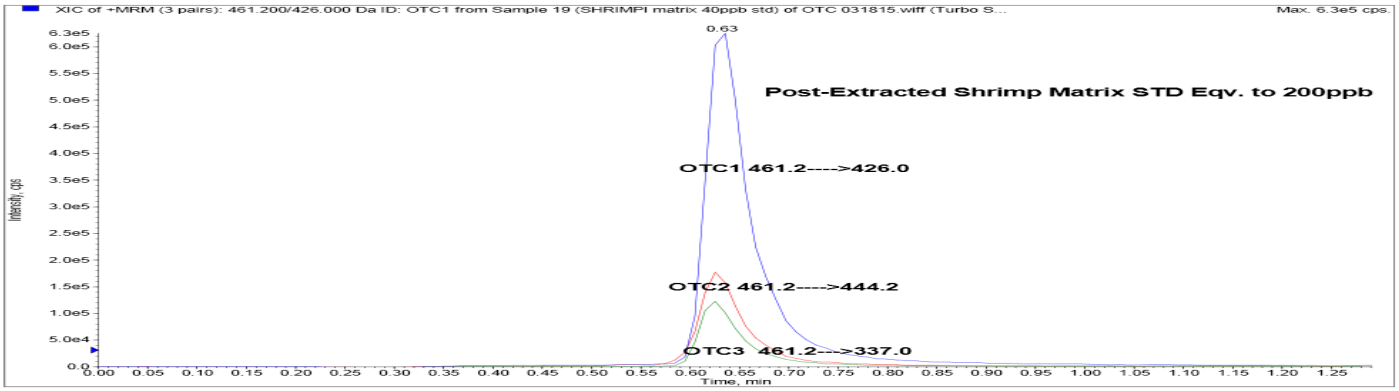


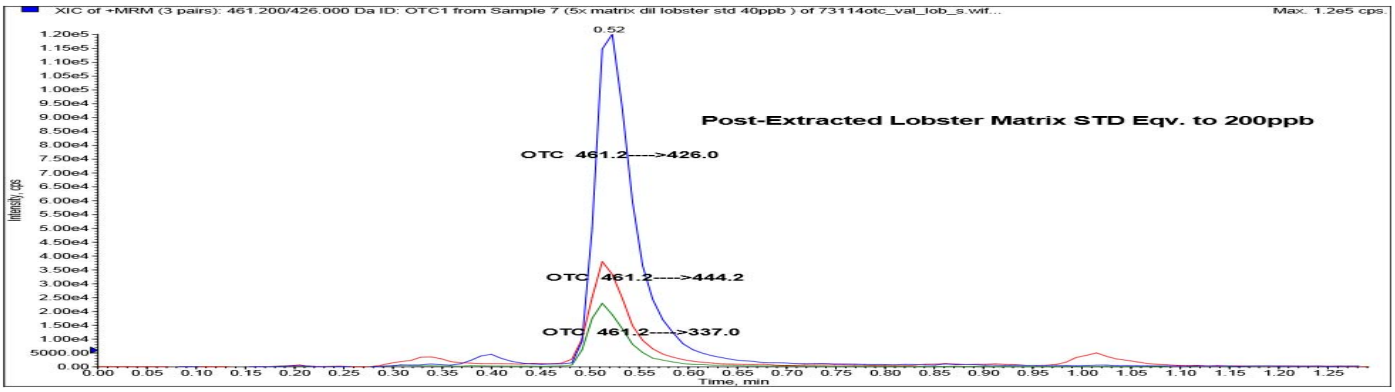




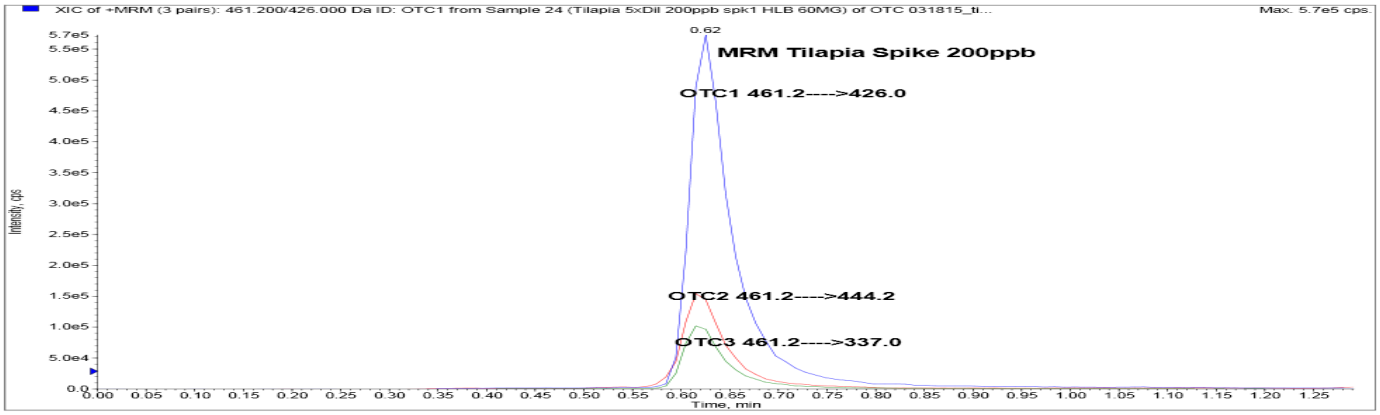
Figures 2(a): Typical MRM negative tilapia (upper figure), shrimp (middle figure), and lobster (lower figure) matrixes, demonstrates absence of OTC (<MDL). Product ions monitored: OTC1 m/z 461.2→426.0 (QUANT Ion), OTC2 m/z 461.2→444.2 and OTC3 m/z 461.2→337.0.

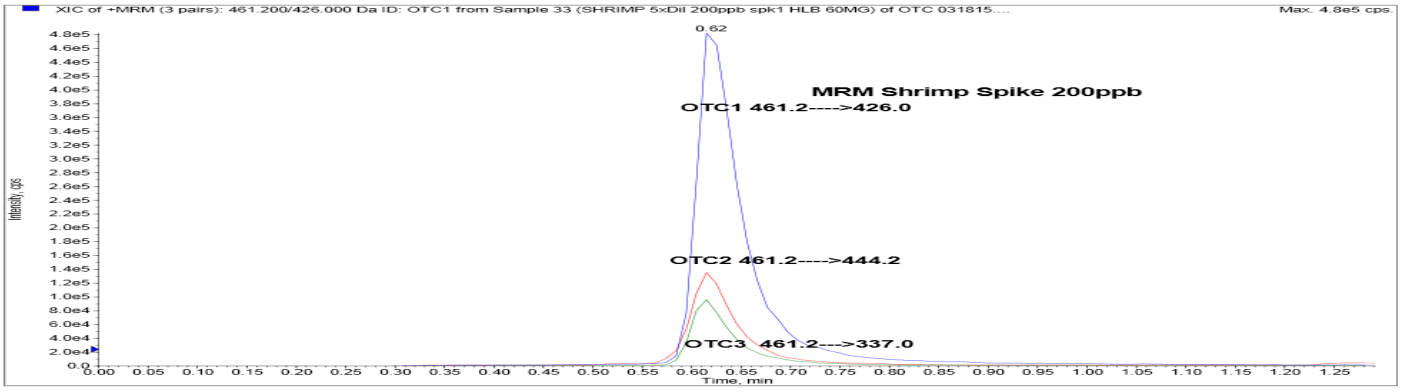


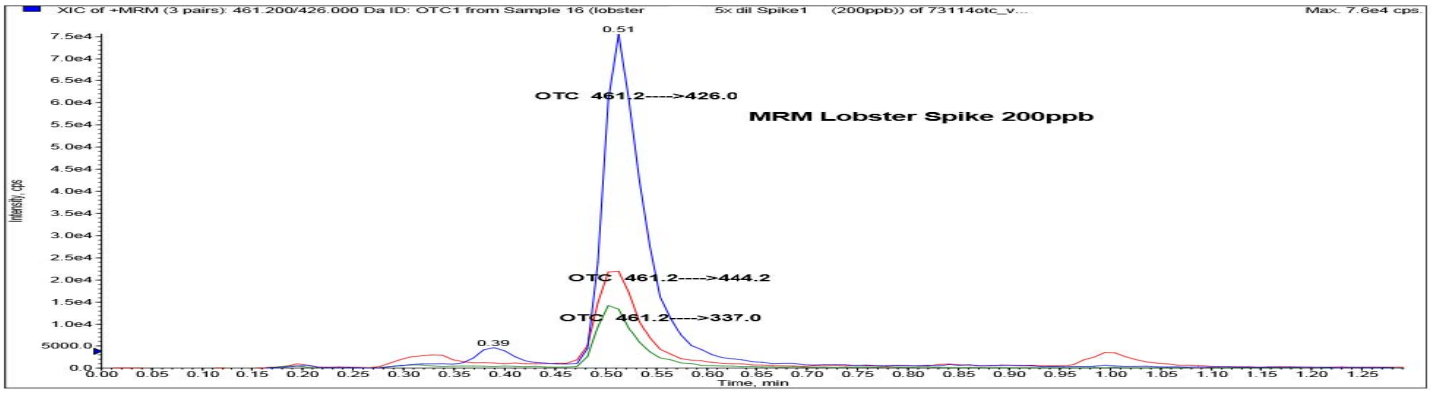




Figures 2(b): Typical standard MRM scans for tilapia (upper figure), shrimp (middle figure), and lobster (lower figure) post-extracted matrix standards, equivalent to 200 ppb, of the product ions monitored: OTC1 m/z 461.2→426.0 (QUANT Ion), OTC2 m/z 461.2→444.2 and OTC3 m/z 461.2→337.0.







Figures 2(c): Typical 200 ppb OTC spike recovery MRM scans for tilapia (upper figure), shrimp (middle figure), and lobster (lower) matrixes. Product ions monitored: OTC1 m/z 461.2→426.0 (QUANT Ion), OTC2 m/z 461.2→444.2 and OTC3 m/z 461.2→337.0.

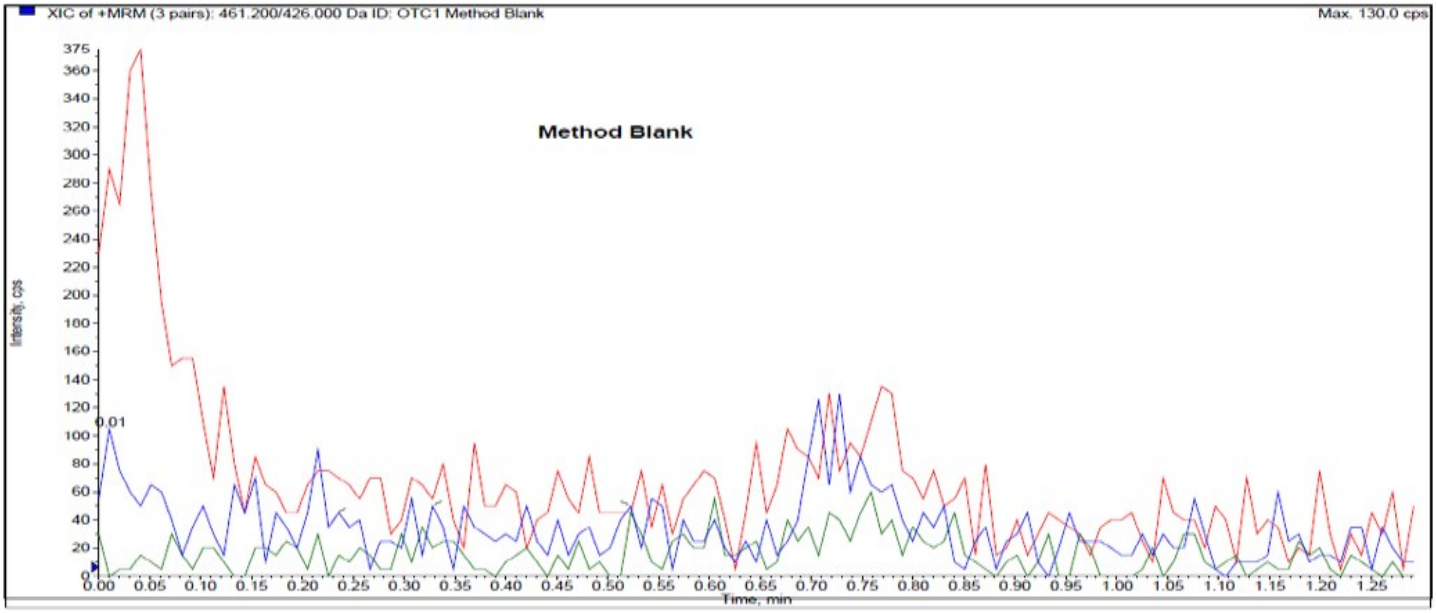


Figure 3: Typical method (reagent) blank MRM scan demonstrates the absence of OTC (<MDL).