LABORATORY INFORMATION BULLETIN

Analysis of Oxytetracycline, Chloramphenicol, and Florfenicol in Lobster and Crab Tissue Using Liquid Chromatography - Triple Quadrupole Mass Spectrometry (LC-MS/MS) (CARTS: IR01309)

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Abstract

In 2013, ORS tasked the Denver Laboratory to determine if domestic lobster could be analyzed for oxytetracycline and florfenicol. The Denver Laboratory had previously published a shrimp multi-residue method bulletin, LIB 4533[1], which included oxytetracycline as an analyte but did not include florfenicol. Another bulletin, LIB 4508[2], included florfenicol as an analyte with chloramphenicol (amphenicols) but did not contain oxytetracycline. Although ORS did not specify chloramphenicol, the analyte was included for this method validation. During method development, it was determined the ion trap mass spectrometer (MS) did not have adequate sensitivity for the amphenicols. To include all analytes, the method was changed to a triple quadrupole mass spectrometer method, similar to LIB 4508. Because this new method required changes to LIB 4533 and LIB 4508, to include: 6cc HLB SPE columns, the use of the triple quadrupole mass spectrometer, Atmospheric Pressure Chemical Ionization (APCI) versus ESI (Electrospray ionization) in the MS source, and a different analytical column, the validation was performed in accordance to FDA OFVM Level Two validation[3] criteria. The extraction procedure from LIB 4533 was followed due to the similar "fat-protein-carbohydrate[4]" composition of shrimp to lobster and the instrument method from LIB 4508 was also followed. The LIB 4533 testing level for oxytetracycline was 1000 ppb and the LIB 4508 testing level for amphenicols was 0.3 ppb. Due to the large range in validation levels between oxytetracycline and amphenicols in those LIBs, the validation levels in this lobster method were set to 100 ng/g (ppb) for oxytetracycline and to 0.5 ng/g for florfenicol (**see Footnote next page**). The testing levels were designated as "X" for each drug, and the method was validated at concentrations of 0.5X, 1X, and 2.5X. Due to ionization differences needs in the MS, each sample requires two analytical runs: one in positive ionization mode for oxytetracycline and one in negative ionization mode to analyze the samples for chloramphenicol and florfenicol.

Recoveries were calculated using extracted matrix-matched calibration curves for oxytetracycline. An extracted matrix-matched calibration curve with a stable isotopically labeled internal standard (Chloramphenicol-d5) was used for to calculate recoveries for both chloramphenicol and florfenicol. The average accuracy of fortified compounds in lobster ranged from 71.5% to 99.9%% at the target quantitative validation level. The method was also validated as a semi-quantitative screening method where all sample responses were compared to an extracted-matrix matched 1X standard and the average recoveries ranged from 75.7% - 115%.

After completion of the lobster validation, The Denver Laboratory was requested to perform the same method to determine oxytetracycline and amphenicols in samples of crab tissue. The Denver Laboratory performed an emergency matrix extension (Level 1; Table1 in the OFVM guidance)[3] was performed in accordance to FDA OFVM criteria. The method was validated as a semi-quantitative screen where all sample responses were compared to extracted matrix-matched 1X standards and the average recoveries ranged from 74.5% - 106% for all three analytes.

INTRODUCTION

Lobsters harvested from the waters near Maine have long been favorite seafood among the American consumers. The American lobster fishery is one of the most valuable fisheries in the United States with an average total catch of approximately 90 to 100 million pounds worth up to $400 million annually[4]. Millions of pounds of lobster are brought to Maine docks every year; however, they don't immediately find their way to a customer's plate or a processor's plant. In fact, most lobsters are stored for some period of time before reaching their ultimate destination. Those storage facilities range from the simple to the sophisticated but each has one goal: to keep the lobsters as healthy and vigorous as possible[5]. The antibiotic, oxytetracycline, is used to protect American lobsters held in tidal pounds in the United States and Canada from gaffkemia (caused by *Aerococcus viridans*), a fatal blood-borne disease that can spread rapidly among other lobsters[6]. For feed use, specifically oxytetracycline may only be used in salmonids, catfish, and lobster. The withdrawal times are: marking in pacific salmon, 7 days; disease control in salmonids, 21 days; catfish, 21 days; lobster, 30 days[7]. In accordance with the 21 CFR 556.500, Oxytetracycline tolerance in the flesh is 2.0 ppm (2000 ng/g)[8].

More recently, the Gram-negative bacterium *Photobacterium indicum* has been cultured from over 95% of morbid and dead lobsters held in Maine tidal impoundments, and may represent a new/emerging threat to lobster culture. Preliminary in vitro susceptibility data indicate that *Photobacterium indicum* is susceptible to florfenicol treatment. Aquaflor® is currently FDA-approved in the U.S. for specific FDA-approved claims for fish. Not surprisingly, Aquaflor® has become a strong candidate for other potential approved uses in U.S. aquaculture, including use to control mortality caused by bacterial diseases in cultured/captive lobsters[9].

Chloramphenicol (CAP) is another antibiotic that is prohibited for use in food producing animals, including aquacultured shellfish. To contain the potential health risk posed by veterinary drug residues found in lobster and crab meat, the Denver Laboratory validated a method to determine oxytetracycline, florfenicol, and chloramphenicol (tetracycline and amphenicol classes) in lobster and crab tissue at the ppb levels. The FDA has established a zero tolerance for chloramphenicol residues in shellfish with a target testing level of 0.30 ng/g[10]. To monitor this, the FDA analyzes hundreds of shrimp and crab samples every year to screen for illegal residues of chloramphenicol.

This liquid chromatography/triple-quadrupole mass spectrometry (LC-MS/MS) method was developed to screen, confirm and quantitate veterinary drug residues in lobster and crab tissue at parts-per-billion (ppb) levels. The extraction procedure consists of extracting homogenized lobster samples with 5% trichloroacetic acid, pH adjustment, SPE cleanup, and filtration with a 0.2 µm PTFE syringe filter. A 150 mm C_8 column separates the analytes prior to analysis with a triple quadrupole mass spectrometer that is interfaced to an electrospray ionization (ESI) source. Each sample requires two analytical runs: one in positive ionization mode for oxytetracycline and one in negative ionization mode to analyze the samples for both chloramphenicol and florfenicol.

FOOTNOTE:
LIB 4631 method was developed and validated prior to the establishment of current FDA target testing levels for florfenicol. The marker residue for florfenicol is the florfenicol amine metabolite. Moreover, the target testing level for florfenicol amine (or the florfenicol parent drug) is currently 1,000 ng/g.

METHODS AND MATERIALS

Equipment

a) LC-MS instrument ─ Thermo TSQ Quantum Access triple quadrupole mass spectrometer with Surveyor MS Pump (Thermo, San Jose, CA).
b) HPLC Column - Phenomenex® Luna 150 mm x 2.0 mm, 5 µm C_8(2) 100 Å (P/N 00F-4249-B0, Phenomenex Corp., Torrance, CA).
c) Food processor - RobotCoupe Blixer, homogenizer, 4 quart, model RS1BX4V (RobotCoupe USA, Inc., Ridgeland, MS) or equivalent.
d) Centrifuge – Sorvall TM RC 6+ (Cat. No. 46910), with FiberliteTM F13-14 x 50cy Fixed Angle Rotor (Thermo Fisher Scientific, Waltham, MA), capable of operating at 4000 rpm for 10 min with refrigeration to 4 ˚C, or equivalent.
e) Evaporator ─ TurboVap® LV nitrogen evaporator with thermostated water bath (#103198, Zymark, Hopkinton, MA), or equivalent.
f) Polytron grinder ─ Polytron PT DA 2112/2EC (Kinemetica AG, Switzerland), probe 1.1 cm, or comparable models
g) Vortex Genie 2, (Scientific Industries, Bohemia, NY) or equivalent.
h) Polypropylene (PP) tubes - 50 mL disposable, conical, graduated, with cap (#352098, Becton Dickinson, Franklin Lakes, NJ)
i) pH Meter – UltraBasic UB-10 (Denver Instrument, Bohemia, NY), or equivalent.
j) SPE Manifold – 24-port SPE vacuum elution manifold with aspirator and vacuum gauge (Cat. No. 26080, Restek, Bellefonte, PA).
k) Disposable glass tubes - 16 x 100 mm (#47729-576, VWR Int., West Chester, PA)
l) SPE cartridges ─ Oasis® HLB 6 cc (200 mg) extraction cartridge (#WAT106202, Waters Corp.) with 20 mL SPE reservoir and frit (#12131011 and 12131023, Varian Inc., Palo Alto, CA).
m) LC sample vials – 1.5 mL (#5183-2072, Agilent, Santa Clara, CA with pre-scored snap caps (#5182-0717, Agilent, Santa Clara, CA).

Reagents and Standards

a) Solvents.-
    a. LC grade water ─ Generated in-house with Milli-Q Plus (Milli-Pore). Resistivity ≥18.2 MΩ-cm at room temperature (RT)
    b. Acetonitrile ─ Burdick & Jackson (Muskegon, MI), B&J Brand, #UN1648
    c. Methanol ─ Burdick & Jackson, B&J Brand, #UN1230
    d. Sodium hydroxide, 10 N ─ Mallinckrodt (Phillipsburg, NJ), volumetric (9.95-10.05 N), #H385
    e. Trichloroacetic acid ─ Sigma-Aldrich (St. Louis, MO), SigmaUltra 99.0+%, # T9159
    f. Ammonium formate ─ J. T. Baker, ≥96%, #M530-08
    g. Succinic acid ─ Sigma, SigmaUltra ≥ 99.0%
    h. EDTA, disodium, dihydrate ─ Sigma, 99.6%, #E4884 Ascorbic acid ─ Sigma, SigmaUltra, ≥99.0%
    i. Hydroxylamine hydrochloride ─ Mallinckrodt, AR (ACS)
    j. Formic acid, 88% ─ Mallinckrodt, AR (ACS), Cat. No. 2592
    k. pH buffers (4.00 and 7.00) ─ J&H Berge (South Plainfield, NJ)
    l. Concentrated HCl ─ Fisher Chemical (Fairlawn, NJ), 36-37%

b) LC system mobile phase –
    a) Oxytetracycline:
        a. Mobile Phase A: 0.1% Formic Acid LC Grade Water. Prepared by diluting 1.00 mL of formic acid in 1000 mL of water
        b. Mobile Phase B: in Acetonitrile
    b) Amphenicols:
        a. Mobile Phase A: LC Grade Water
        b. Mobile Phase B: Acetonitrile

b) Reagent Preparation
   a. TCA aqueous solution, 0.05 g/mL ─ Prepared by dissolving 50.0 ± 0.1 g TCA in water to make 1 L solution. Store at 2-8˚C under subdued light.
   b. Sodium succinate solution, 0.4 M, pH 6.3 ─ Dissolve 23.6 ± 0.1 g succinic acid in 400 mL water and 20 mL 10 N NaOH. Adjust to pH 6.30 ± 0.10 with additional 10 N NaOH. Transfer to a 500-mL volumetric flask, rinse beaker twice with water and combine with the aforementioned solution, and finally add water to mark. Store at RT.
   c. Ammonium formate buffer, 20 mM, pH 3.9 ─ Prepare by dissolving 1.26 ± 0.01 g ammonium formate in water to make 1 L solution, then adjust pH to 3.90 ± 0.10 with ~400 µL formic acid (88%). Store at RT.
   d. Hydroxylamine hydrochloride solution, 0.20 g/mL ─ Weigh 5.0 ± 0.1 g hydroxylamine hydrochloride and dissolve in 25 mL of water. Store at 2-8˚C.
   e. Ascorbic acid solution in methanol, 1.0 mg/mL ─ Weigh 100 ± 2 mg ascorbic acid and dissolve in 100 mL methanol (slow dissolution). Store at RT and avoid light.
   f. EDTA solution, 0.1 M ─ Weigh 18.6 ± 0.1 g EDTA (2Na, $2H_2O$), dissolve in ~ 450 mL water (heat or sonicate to facilitate dissolution) and transfer to a 500-mL volumetric flask. Wash beaker and combine with bulk solution. Add water to mark. Store at RT
   g. HCl, 1 N ─ Measure with glass pipette 4.00 mL concentrated HCl (36-37%, ~ 12 N) and transfer to a 50-mL graduated cylinder, and fill to 48 mL with water.

Note: All reagent solutions are stable for 6 months unless otherwise specified.

c) Negative Controls – Lobster and crab were acquired from a local market and tested to ensure that no compounds monitored for were present above the stated method detection limit (MDL).

d) Analytical Standards
   a. Chloramphenicol (CAP) – USP (Rockville, MD)
   b. Florfenicol (FF) – Sigma Aldrich (St. Louis, MO)
   c. Chloramphenicol-d5 (CAP-d5) (internal standard) – Cambridge Isotopes (Andover, MA)
   d. Oxytetracycline (OTC) – USP (Rockville, MD)

Standards Preparation

Note: All standards solutions were transferred to 20-mL glass scintillation vials and stored at < -6˚C. The CCV, ICV, and ISTD stock and mixed standard solutions are stable for 1 year.

a) Stock Standard Solutions and Continuing Calibration Verifications (CCVs): Prepare individual stock standards at 350 µg/mL for the amphenicols and 1000 µg/mL for oxytetracycline in methanol taking into account the content of the active substances (i.e., counter ions and purity). Store the individual stock standards in 20-mL scintillation vials at < -6˚C and avoid long-term exposure to light, especially for oxytetracycline. All solutions are stored at < -6˚C and are stable under these storage conditions for 6 months for oxytetracycline and one year for the amphenicols.

b) Stock Standard Solutions, Initial Calibration Verifications (ICVs): 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) Internal Standard Stock Solution (ISTD): The deuterated CAP D-5 is received with a nominal concentration of 100 µg/mL.

d) OTC Intermediate Solution 1: Prepare an intermediate solution containing 5000 ng/mL of oxytetracycline by adding 50 µL of the 1000 µg/mL stock solution in a final volume of 10.0 mL in methanol.

e) CAP/FF Intermediate Solution 1: Prepare an intermediate solution containing the amphenicols at 1000 ng/mL by adding 30 μL of each stock standard to a final volume of 10.0 mL and diluting with methanol.

f) CAP/FF Intermediate Solution 2: Prepare an intermediate solution 2 containing the amphenicols at 100 ng/mL by adding 1.00 mL of Intermediate Solution 1 to a final volume of 10.0 mL and dilute with methanol.

g) Intermediate ISTD 1: Prepare one solution containing the ISTD compounds at 10,000 ng/mL in methanol by adding 1.00 mL of the 100 μg/mL deuterated CAP D-5 into a final volume of 10.0 mL.

h) Intermediate ISTD 2: Prepare one solution containing the ISTD compounds at 1000 ng/mL in methanol by adding 1.00 mL of Intermediate ISTD 1 into a final volume of 10.0 mL. This solution is stable for 6 months when stored at <-10 °C.

Examples of the standards preparation are given in Tables 1-3.

Table 1: Oxytetracycline - Preparation of Stock and Intermediate Standard Solution

Stock Standard				**Intermediate Stock Solution**		
Std	Amt. Weighed (mg)	Final Vol. (mL)	Conc. (μg/mL)	Amt. of Stock (mL)	Final Volume (mL)	Conc. (ng/mL)
OTC	10.0	10.0	1000	0.050	10.0	5000

Table 2: Chloramphenicol and Florfenicol - Preparation of Stock and Intermediate Standard Solutions

Stock Standard				**Intermediate Solution 1**			**Intermediate Solution 2**		
Std	Amt. Weighed (mg)	Final Vol.(mL)	Conc. (μg/mL)	Amt. of Stock (mL)	Final Vol.(mL)	Int. Stock 1 (ng/mL)	Amt. of Int. Stock 1 (mL)	Final Vol.(mL)	Int. Stock 2 (ng/mL)
CAP	3.50	10.0	333	0.0300	10.0	1,000	1.00	10.0	100
FF	3.50	10.0	333						

Table 3: Chloramphenicol-d5 - Preparation of Intermediate Internal Standard Solutions

Stock Solution		**Intermediate Solution 1**			**Intermediate Solution 2**		
Std	Concentration (μg/mL)	Amt. of Stock (mL)	Final Vol.(mL)	Int. Stock 1 (ng/mL)	Int. Stock 1 (mL)	Final Vol.(mL)	Int. Stock 2 (ng/mL)
CAP-d5	100	1.00	10.00	10,000	1.00	10.0	1,000

Sample Preparation

Lobster and crab muscle tissues were acquired from a local market and tested before use as a negative control. All tissue used for this LIB was homogenized with dry ice using a Robot Coupe mixer. The homogenate was placed in a whirl-pak bag, loosely sealed and stored in a freezer (-25°C) overnight to allow the carbon dioxide to sublimate, and then sealed until the time of analysis.

Extracted Matrix Calibrant and Recovery Control Checks for Regulatory Analysis

Extracted calibration standards were prepared by spiking 4.00 (± 0.50) grams of the appropriate negative control muscle tissue and taking the fortified tissue through the extraction procedure. The fortified extracted matrix calibration standards were prepared at concentrations of 0.250, 0.5, 1.25, 2.50, and 5.00 ng/g by adding 10.0, 25.0, 50.0, 100, and 250 µL of the 100 ng/mL CAP/FF Intermediate Solution 2. Oxytetracycline was prepared by adding 25.0, 50.0, 100, 250, and 400 µL to the same 4.00 g of homogenized muscle tissue, respectively, as shown in Table 4. The ISTD working solution (80.0 µL of

1000 ng/mL) was added to all samples and controls prior to extraction, and corresponds to 20.0 ng/g in the sample matrix.

Example Calculations:
Concentration of CAP/FF = 100 ng/mL x 0.020 mL/4.00 g = 0.500 ng/g.
Concentration of OTC = 5000 ng/mL x 0.080 mL/4.00 g = 100.0 ng/g.

Table 4: Preparation of Extracted Standards and Fortified (Spiked) solutions (CAP/FF/OTC)

Extracted Calibrant	Control tissue weight (g)	Volume of CAP/FF (µL) Int. Stock Solution 1	Volume of CAP/FF (µL) Int. Stock Solution 2	Volume of OTC (µL) Int. Stock Solution	Volume of CAP-d5 (µL) ISTD 2	Concentration in Tissue (ng/g)	
						Conc. CAP/FF	Conc. OTC
1	4.00	--	10.0	20.0	80.0	0.250	25.0
2		--	20.0	40.0		0.500	50.0
3		--	50.0	80.0		1.25	100
4		--	100	200		2.50	250
5		20.0	--	320		5.00	400

Matrix-Free Solvent Standard

The solvent standard is used to establish system suitability and injected before each of the two separate analytical runs on the LC/MS system. This matrix-free standard solution is made the same day as the prepared samples are analyzed by LC/MS and prepared according to Table 5. The dilution scheme used is similar to the final concentration in the sample extract. The following solution is prepared the day of analysis and diluted with the following solution: 0.5 mL of 1 mg/mL ascorbic acid, 0.5 mL 0.1 M EDTA, and 8 mL 20 mM ammonium formate buffer, pH 3.9

Table 5: Preparation of Solvent Standard CAP/FF/OTC

Solvent Standard	Volume of CAP/FF (µL) Int. Stock Solution 2	Volume of OTC (µL) Int. Stock Solution	Volume of CAP-d5 (µL) ISTD 2	Volume of Solvent (mL)*	Concentration (ng/mL)			
					CAP	FF	CAP-d5	OTC
Suitability Standard	20	80	80	1.82	1.00	1.00	20.0	100

Extraction Procedure

The extraction procedure is based on the previously-published LIB 4533, with the change from the 3cc HLB to the 6 cc HLB due to the sample weight increase from 2.00 grams to 4.00 grams. Weigh out 4.0 (±0.05) grams of lobster/crab tissue into a 50-mL centrifuge tube, add ISTD (CAP-d5), add 17.5 mL TCA solution (0.05 g/mL in water) and 0.5 mL hydroxylamine hydrochloride solution (0.20 g/mL). Cap the samples and vortex them for 2-5 minutes on a multi-tube vortexer at high speed. Visually inspect tubes to ensure that all are mixed properly. Homogenize samples with a Polytron blender at medium speed for 1 minute each. Rinse the material on the grinding probe into the sample with a small amount of water. Inspect the tip of the probe for any tissue and remove. Wash the grinding probe with water, acetonitrile, water, and acetonitrile consecutively between samples by running the submerged probe for in each solvent for about 10 seconds. Vortex tubes on multi-tube vortexer for 10 min at medium speed and centrifuge at 3700 RPM (4000 RFC) and 4ºC for 15 min.

Prepare neutralizing solution in another set of 50-mL PP centrifuge tubes as follows: (to each tube) add 2.5 mL 0.4 M sodium succinate, pH 6.3, and 280 µL 10 N NaOH. Transfer supernatant from above into this set of 50 mL PP centrifuge tubes. All controls, samples, and QCs must have a final pH of 3.6 ± 0.1 (rounded to the nearest 0.1 pH unit), if not adjust pH to 3.6 ± 0.1 with 10 N NaOH or 1 N HCl solution.

Mount Waters HLB cartridges (200 mg, 6 cc) onto a vacuum chamber. Condition all cartridges with 3 mL MeOH, letting the cartridges drain just to dryness. Place 2.5 mL of ammonium formate buffer into each cartridge, stack an adapter then a reservoir with frit on top of it, and then load the extracts into the reservoirs. Draw liquid through to initiate the elution using a low vacuum, maintaining an elution rate of about 1 drop per second.

Critical: Stop vacuum when extract loading is complete.

Remove sample reservoirs and adapters. Rinse SPE cartridges with 3 mL 20 mM ammonium formate buffer followed by 3 mL Milli-Q water and discard these rinses. Prior to final elution with methanol, the excess water in cartridge is blown out briefly with a pipette bulb. Elute extracts with 2 mL MeOH followed by 1 mL 1:1 ACN/MeOH into 15-mL graduated PP centrifuge tubes, maintaining an elution rate of about 1 drop/sec.

Prepare a solution that contains 8 mL ammonium formate buffer, 0.5 mL 0.1 M EDTA, and 0.5 mL 1 mg/mL ascorbic acid. Add 0.5 mL of this solution to each 15-mL graduated PP centrifuge tube. Mix the solution using a vortexer for 10 seconds. Evaporate eluate under nitrogen flow at 45ºC until ~ 0.5 mL of solution is left.

Note: the evaporation to near-dryness generally takes about 30-40 min in a TurboVap with a nitrogen pressure setting of 12-15 psi.

Critical: Do not let the sample extract go to complete dryness.

Zero a balance with the same type of tube used in the evaporation step. Add 2 mL of the 1:1 acetonitrile/water to a tube and determine the weight. Add 1:1 acetonitrile/water to each tube containing remnant extract from evaporation until the same weight is reached as the 2 mL of the 1:1 acetonitrile/water. Mix using vortex mixer. Filter solution using a 0.2 µm PTFE syringe filter into 1.5-mL amber glass autosampler vials and seal. Extracts are stored at < -80ºC until they are ready to be analyzed.

Instrumentation

(a) Oxytetracycline *LC-MS/MS system* - A Thermo triple quadrupole mass spectrometer system with an electrospray ionization source was operated in the positive mode using selected reaction monitoring (SRM). The capillary temperature was 250°C, sheath gas flow was 50 arbitrary units, auxiliary flow 5.0 arbitrary units, and the spray voltage was 4.0 kV. Three MRMs were collected for oxytetracycline and was compared to the products ions generated by the ion trap in LIB 4533. Compared to the reference article, alternate qualitative product ion transitions were determined for oxytetracycline, Table 6. The procedure for optimizing parameters for tune files is as follows: using a "T" connector, infuse OTC 20.0 µg/mL into the mass spectrometer at a flow rate of 10 µL/min from the syringe pump. The automatic optimization process in the "Autotune" window provides the $(M+H)^+$ ion and save this tune file to the instrument method. Divert valve: set "to mass spectrometer source" from 2.00 to 11.00 min and "to waste" for other time segments.

(b) Oxytetracycline *HPLC system* – A Thermo Surveyor HPLC system equipped with pump, solvent degasser, autosampler, and column oven. Phenomenex® Luna 150 mm x 2.0 mm, 5 µm $C_8(2)$ was used and kept at 35ºC oven temperature. The pump was operated at a flow rate of 0.250 mL/min. A binary gradient system was used to separate analytes comprising mobile phase A, 0.1% formic acid in water, and mobile phase B was acetonitrile. The gradient profile was: (1) 0.0 min, 95.0% A; (2) 0-10 min, 50.0%A; (3) 11 min, 95.5% A and a 3.00 min post run was included to re-equilibrate the column to the initial conditions. The autosampler injection volume was 10 µL and a 5 second needle wash with methanol–water (50 + 50, v/v) was used to minimize carryover. Total HPLC run time is 13.0 minutes.

Table 6: Oxytetracycline - Retention times (RT) and MS parameters: precursor ions, collision energy (CE), and the resulting ion ratios for the product ions.

Compound	Retention Time (min)	Precursor Ion	Collision Energy (%)	Product Ions (*m/z*)	Median ion ratio, Qual/Quan %
OTC	6.5	461	20	426	100
			12	443	45
			26	337	17

(a) *CAP/FF/CAP-d5 LC-MS/MS system* – A Thermo triple quadrupole mass spectrometer system with an electrospray ionization source was operated in the negative mode using selected reaction monitoring (SRM). The capillary temperature was 320°C, sheath gas flow was 50 arbitrary units, auxiliary flow 5.0 arbitrary units, and the spray voltage was 3.0 kV. Three MRMs were collected for CAP/FF and the products ions were similar to the LIB 4508, two qualitative product ion transitions were determined for the CAP/FF, Table 7. The procedure for optimizing parameters for tune files is as follows: using a "T" connector, infuse CAP/FF 5.0 µg/mL into the mass spectrometer at a flow rate of 10 µL/min from the syringe pump. The automatic optimization process in the "Autotune" window provides the $(M+H)^-$ ion and save this tune file to the instrument method. The automatic "Autotune" window on the $(M+H)^-$ ion and save as the current tune file. Divert valve: set "to mass spectrometer source" from 4.00 to 9.00 min and "to waste" for other time segments.

(b) *CAP/FF/CAP-d5 HPLC system* – A Thermo Surveyor HPLC system equipped with pump, solvent degasser, autosampler, and column oven. Phenomenex® Luna 150 mm x 2.0 mm, 5 µm $C_8(2)$ was used and kept at 30°C oven temperature. The pump was operated at a flow rate of 0.250 mL/min. A binary gradient system was used to separate analytes comprising mobile phase A, water, and mobile phase B was acetonitrile. The gradient profile was: (1) 0.0 min, 95.0% A; (2) 0-4 min, 35%A; (3) 4-6min, 35%A, (4) 8-10 min 95.0% A. The autosampler injection volume was 10 µL and a 5 second needle wash with methanol–water (50 + 50, v/v) was used to minimize carryover. Total HPLC run time is 10.0 minutes. Divert valve is switched to MS at 3.6 min.

Table 7: CAP/FF/CAP-d5 - Retention times (RT) and MS parameters: precursor ions, collision energy (CE), and the resulting ion ratios for the product ions.

Compound	Retention Time (min)	Precursor Ion	Collision Energy (%)	Diagnostic Product Ions (*m/z*)	Median ion ratio, Qual/Quan
CAP	6.4	321	19	152	100
			15	194	36
			14	176	23
FF	6.3	356	10	336	100
			20	185	55
			12	219	6.5
CAP-d5	6.4	326	20	157	N/A

RESULTS AND DISCUSSION

Method Validation for OTC, FF, and CAP in Lobster Muscle

The objective of this research was to develop a sensitive method for the determination of oxytetracycline and amphenicols in lobster tissue. The method was validated according to the FDA OFVM Level Two Chemical Method Validation guidelines for lobster and an emergency matrix extension for crab tissue. The analyte concentration levels tested in the method validation were lower than those described in the LIB 4533 due to the addition of florfenicol and chloramphenicol. At the time of development, the levels of concern for oxytetracycline residues were 2000 ng/g and the florfenicol parent drug residue was 200 ng/g, with no tolerance for chloramphenicol residues (see **FOOTNOTE** in introduction section for current regulatory requirements). Due to the significant range in concentration for oxytetracycline and amphenicols, lower levels were validated so a single extraction procedure could be used in the laboratory. Thus, the 1VL validation level was set for each compound as shown in Table 8, and the extracted calibration levels ranged from 25 ng/g to 400 ng/g for oxytetracycline and 0.25 ng/g to 5.00 ng/g for the amphenicols.

Three validation levels were tested corresponding to concentrations of 0.5VL, 1VL, and 2.5VL and performed over three days by two separate analysts, Table 9. The lowest validation level for each analyte was selected based on sensitivity and quantification ability. Negative controls were verified to be free of

residues prior to validation studies, Figure 1-3. Quantification was performed by extracted external matrix match calibration curves for oxytetracycline, and the amphenicols were determined by internal standard corrected extracted curve standards.

Table 8: Lobster Three-Day Method Validation Protocol

Sample Type	Day 1	Day 2	Day 3
Negative Controls	n=3	n=3	n=3
Fortified @0.50X	n=3	n=5	n=5
Fortified @1.0X	n=3	n=5	n=5
Fortified @2.5X	n=3	n=5	n=5

An extracted single point standard was fortified at 1X (0.50 ng/g for the amphenicols and 100 ng/g for oxytetracycline) to evaluate the performance of calibration with a single-point standard (1X(CCV)). Tables 9 and 10 show the results for the lobster samples fortified at 0.5x, 1x, and 2.5x target level demonstrating the accuracy and precision of the quantitation method.

Table 9: Lobster Accuracy and precision (n=13)

	Level	**Trueness (% Recovery) ± %RSD**			
Analytes	**1VL:1X**	**0.5VL**	**1VL**	**2.5VL**	**1X (CCV)**
oxytetracycline	100	79.5 ± 19.7	89.4 ± 16.2	71.5 ± 30.2	96.5 ± 20.2
chloramphenicol	0.500	94.1 ± 18.8	84.0 ± 10.4	92.2 ± 11.0	91.3 ± 15.7
florfenicol	0.500	99.9 ± 22.5	93.0 ± 14.7	97.6 ± 10.2	89.4 ± 22.4

Method detection levels (MDLs) were calculated from the quantitative product ion transition for each analyte in lobster muscle and are reported in Table 10. The method detection limit was evaluated by analyzing thirteen replicates fortified at the 0.5VL:0.5X concentration, where 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. The values reported in the column “Linear Curve” in table 10 were calculated with the full extracted 5 point calibration curve and the values reported in the “1X(CCV)” were calculated using a single extracted calibration point.

Table 10: Lobster Method Detection Limit (MDL) in ng/g (standard deviation), n=13

Analytes	**Level (ng/g)**	**Concentration Calculated from Linear Curve (ng/g)**	**Concentration Calculated from 1X(CCV) (ng/g)**
oxytetracycline	50.0	20.9 (0.044)	28.6 (28.6)
chloramphenicol	0.250	0.118 (0.069)	0.185 (0.069)
florfenicol	0.250	0.150 (0.056)	0.155 (0.058)

Emergency Matrix Extension for Crab

After the lobster method was validated, the Denver Laboratory received crab samples for the analysis of oxytetracycline. As this was a one-time event, the Denver Laboratory performed an emergency matrix extension for crab meat. The 1X CCV calibrant was used for the matrix extension and five negative controls were spiked at the 1X CCV level. Table 11 demonstrates the results for the crab samples

fortified at 1x CCV level, demonstrating the accuracy and precision for this matrix extension. Overall, the crab matrix performed similarly to the lobster matrix.

Table 11: Crab Accuracy and precision (n=5)

	Level	Trueness (% Recovery) ± %RSD
Analytes	**1VL:1X (CCV) (ng/g)**	
oxytetracycline	100	74.9 ± 14.6
chloramphenicol	0.500	99.4 ± 4.80
florfenicol	0.500	106 ± 11.3

Routine Screening Protocol at the 1X Level

For routine regulatory sample analysis, it is labor intensive to analyze a set of five matrix-matched extracted calibrants with each analytical batch. The burden increases if more than one type of matrix is present in the set of regulatory samples (e.g., lobster and crab) 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, it is common practice to screen samples against a single matrix-matched calibrant prepared at an established testing level target testing level. Samples that exceed the threshold response value and that also pass qualitative identification criteria, are considered presumptive positive, and would require re-extraction and analysis against a full set of extracted matrix-matched calibration standards.

Table 12 shows the threshold values (ng/g) determined at the 1X level from 13 replicates of fortified samples (figures 7-9) in lobster and four replicates in crab tissue. The qualitative threshold values were calculated from the quantitative product ion transition for each analyte in each matrix per the FDA OFVM guidance[3] by the following equation:

Threshold value = [mean concentration] – (t *S)

Where t = one-tailed student's t value for n=1 degrees of freedom at the 95% confidence level and as above, S is the standard deviation.

Table 12: Threshold Values (ng/g) determined at the 1X level for each matrix

Analyte	Lobster	Crab
Oxytetracycline	58.0	35.4
Florfenicol	0.456	0.479
Chloramphenicol	0.448	0.144

Qualitative Identification

The FDA CVM 118 guidance[10] provides identification criteria to determine if a residue can be identified. It states that the LC-MS analysis yields a chromatographic peak within ± 5% retention time of the chromatographic peak relative to the standard and the chromatographic peak should exceed a signal-to-noise (s/n) threshold of 3:1. In addition, two qualitative product ion ratios must be within ± 20% (absolute) of the ion ratios from the 1X extracted calibrator standard analyzed in the same sequence (or the average of the ion ratios when more than one standard is analyzed), or one product ion ratio must be within ± 10% (absolute) of the standard.

Discussion

The LC-MS/MS method presented in this document provides both quantitative and confirmatory data for three drug residues in lobster and crab tissue. An extraction procedure for multiple analytes from two drug families is described and uses either extracted standard curves, which are commonly used in tissue methods to improve recovery, or a single point extracted standard for semi-quantitation. Although all the analytes were used to fortify tissue and were extracted simultaneously, detection by MS/MS was accomplished using two separate LC-MS/MS analytical methods (one in positive-mode and one in negative-mode). To streamline the two instrumental methods, a quaternary HPLC pump system was

used. Each reservoir was purged for an extended period of time, especially the water channel for the negative mode. The ThermoFisher software used was "LCQUAN" and two separate workbooks were setup for the negative mode and positive mode. The negative LCQUAN workbook was queued first and the analytical sequence consisted: a solvent blank (50% ACN/50% H_2O), solvent standard - suitability (at least two injections), another solvent blank, a 1X CCV extracted standard (1X), extracted ICV, method blank, negative control, spike, spike duplicate, and samples. The positive mode was queued up following the negative mode allowing all analytical runs to be performed overnight for time savings, due to lack of need to change the column and solvent reservoirs. The positive mode "LCQUAN" contained 5 injections at the beginning of the sequence to ensure the entire volume of the column mobile phase contained 0.1% formic acid in water. The negative and positive LCQUAN workbook processed the data and generated the reports separately.

Approximately 30 lobster (figures 10-12) and 10 crab (figures 13-16) regulatory samples have been screened by this method. Oxytetracycline and the amphenicols were not detected in these samples above the stated MDL.

CONCLUSION

A triple quad LC-MS/MS method has been validated for simultaneous confirmation and quantitation of 3 antibiotic compounds in lobster and crab tissue at the following validation levels: 100 ppb for OTC, 0.5 ng/g for florfenicol, and 0.50 ppb for CAP. This method may be routinely used for surveillance of the targeted drug residues in lobster. FDA guidelines indicated in the **FOOTNOTE** in the introduction section of this Laboratory Information Bulletin should be observed for current regulatory action levels and marker residues.

Acknowledgement

The authors would like to thank Dr. Hui Li, Office of Research, Center for Veterinary Medicine, Food and Drug Administration, Laurel, MD for technical discussions and review of this LIB.

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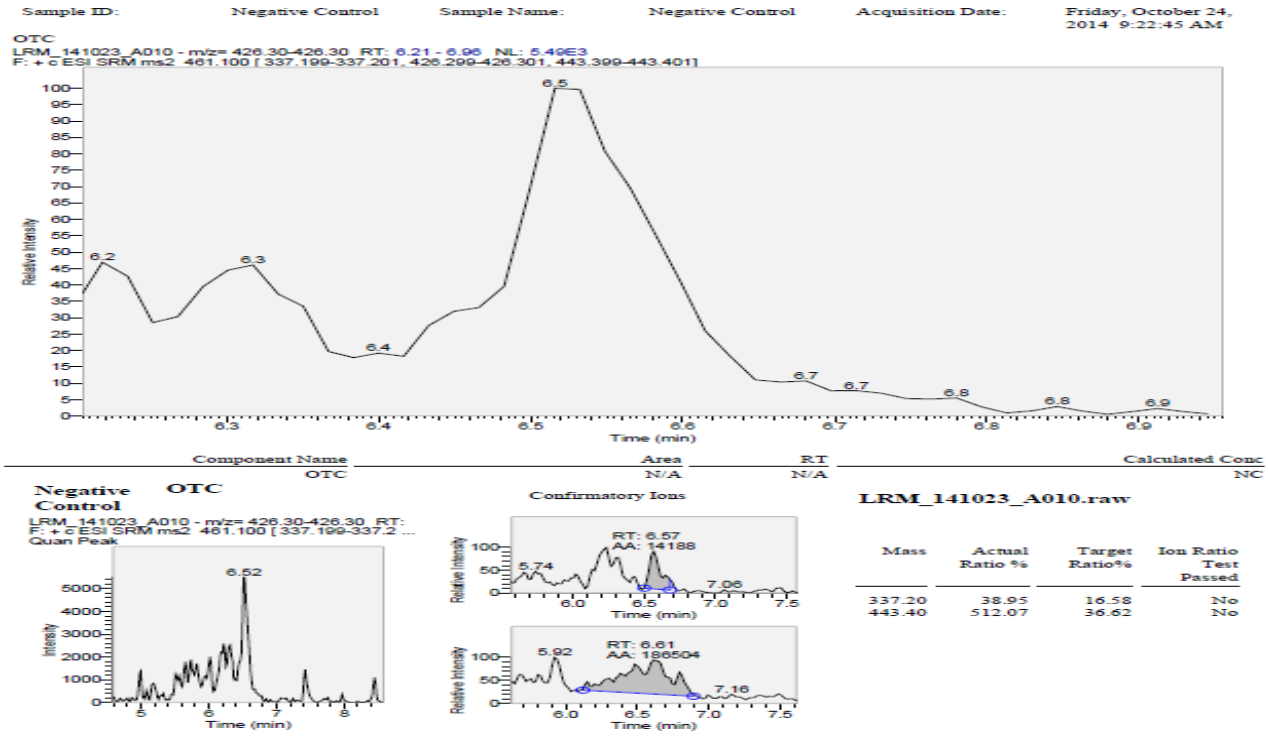


Figure 1: Lobster negative control sample monitored for Oxytetracycline: a) Reconstructred ion chromtogram (RIC); b) Ion ratio report

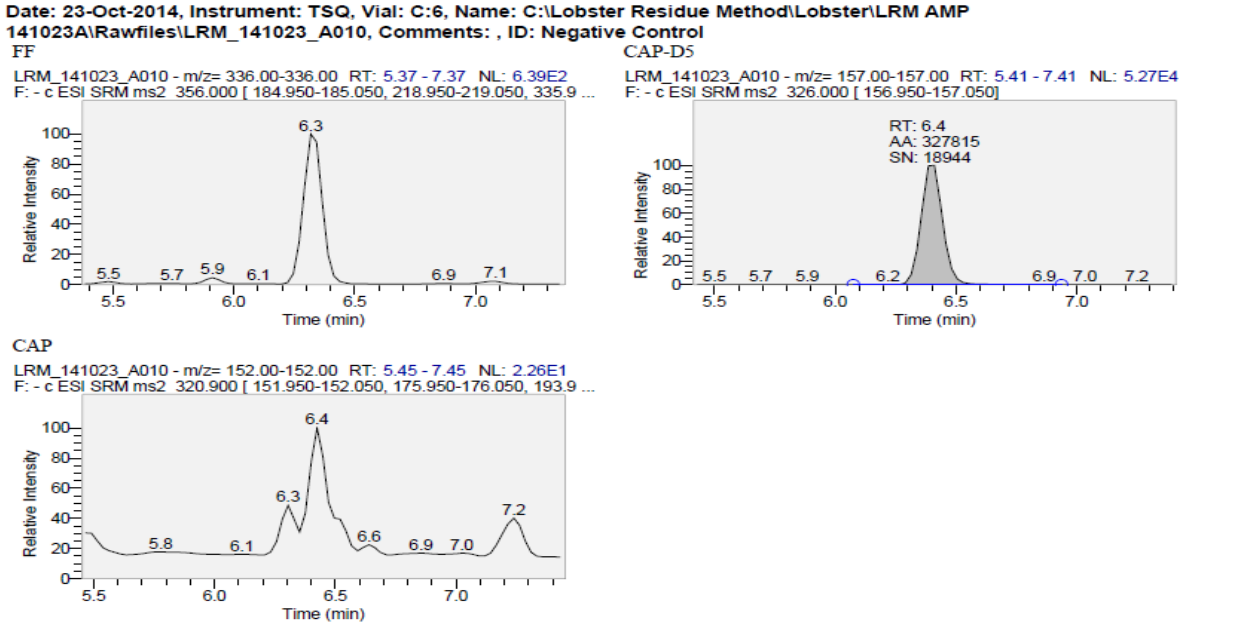


Figure 2: Lobster negative control sample monitored for Amphenicols (florfenicol, chloramphenicol-d5, chloramphenicol): reconstructed ion chromtograms (RIC)

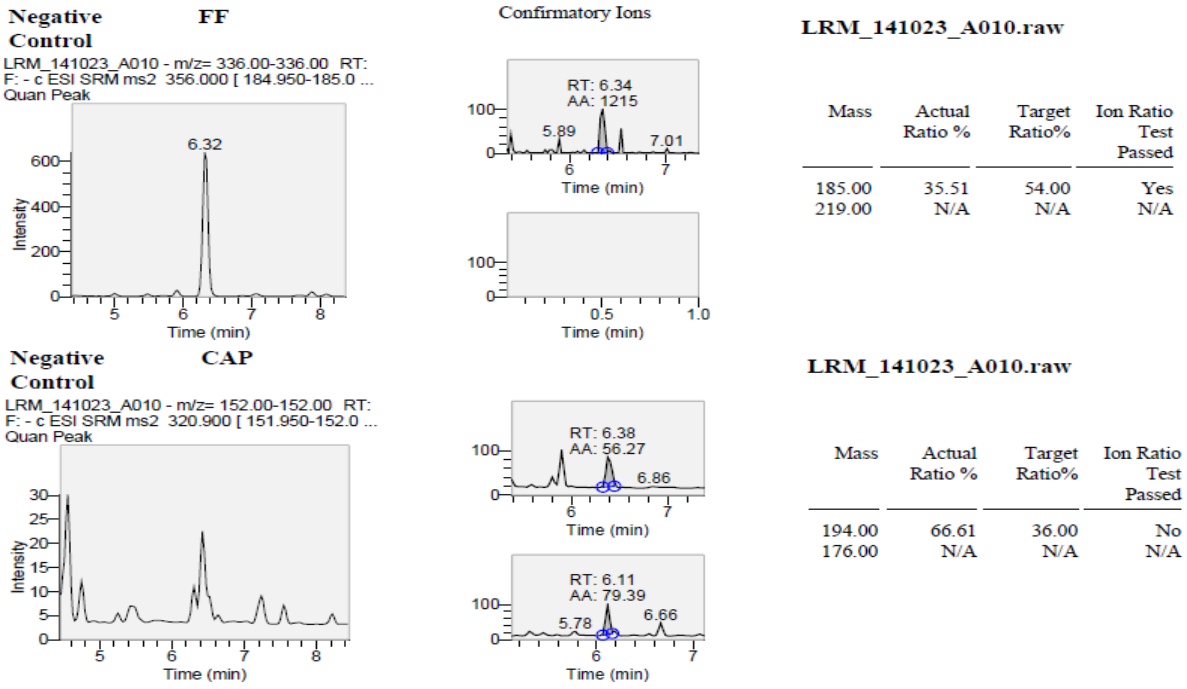


Figure 3: Lobster negative control sample monitored for Amphenicols (florfenicol, chloramphenicol): ion ratio report

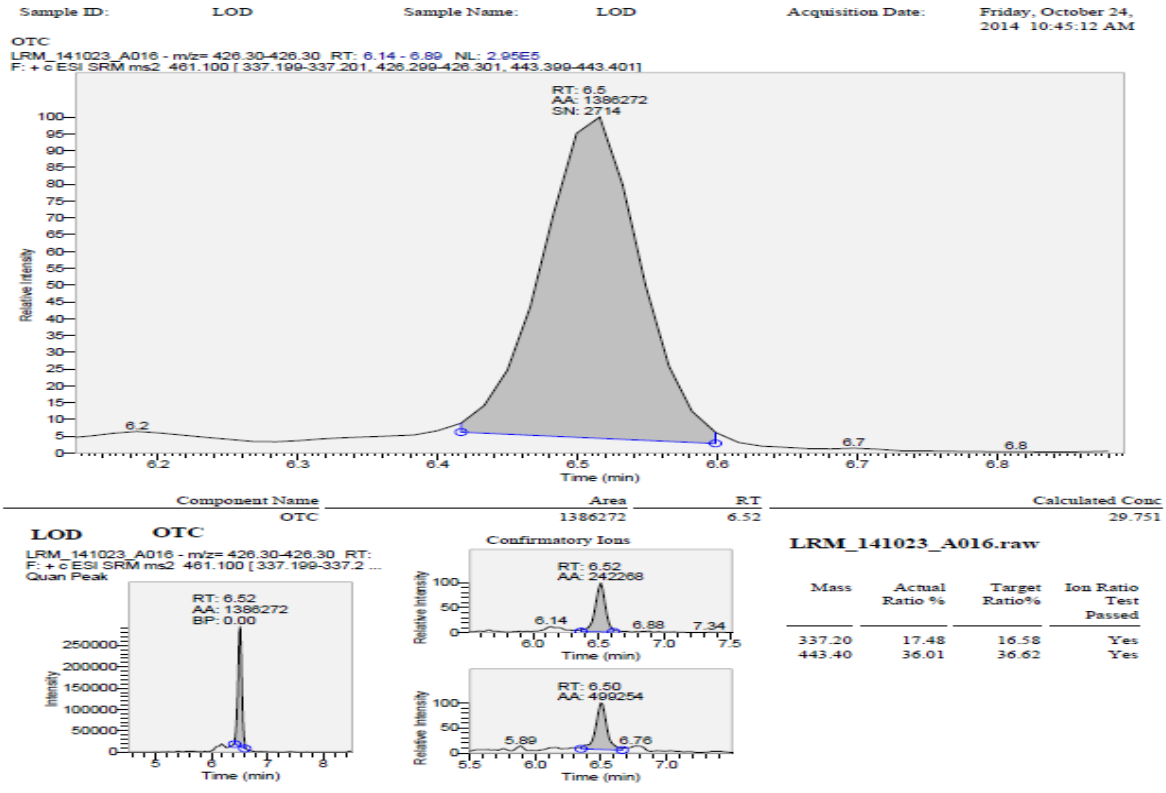
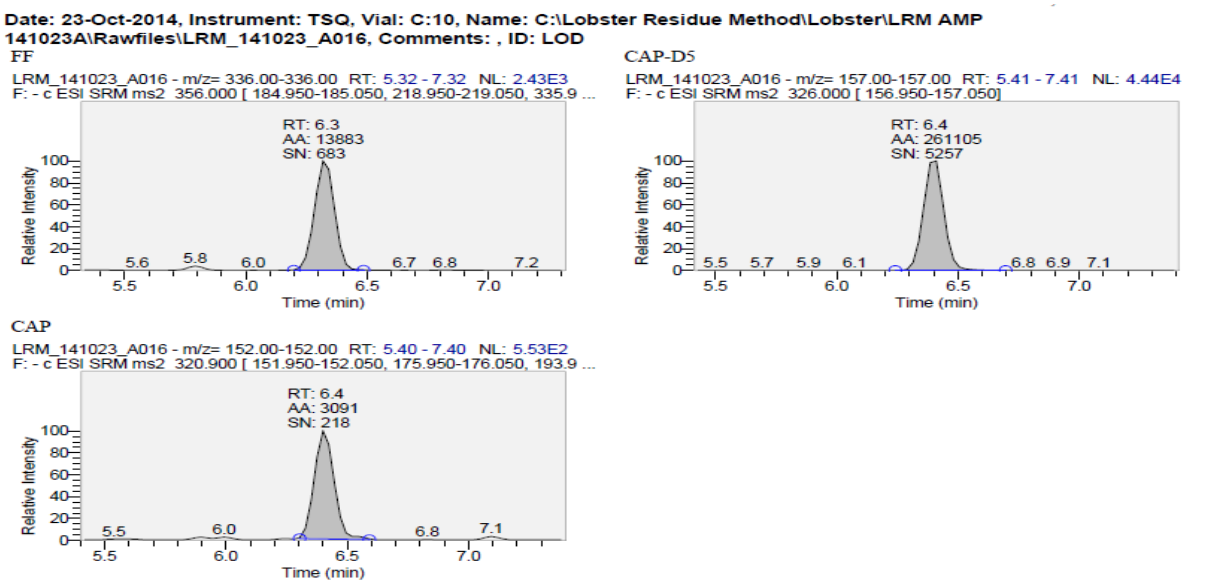
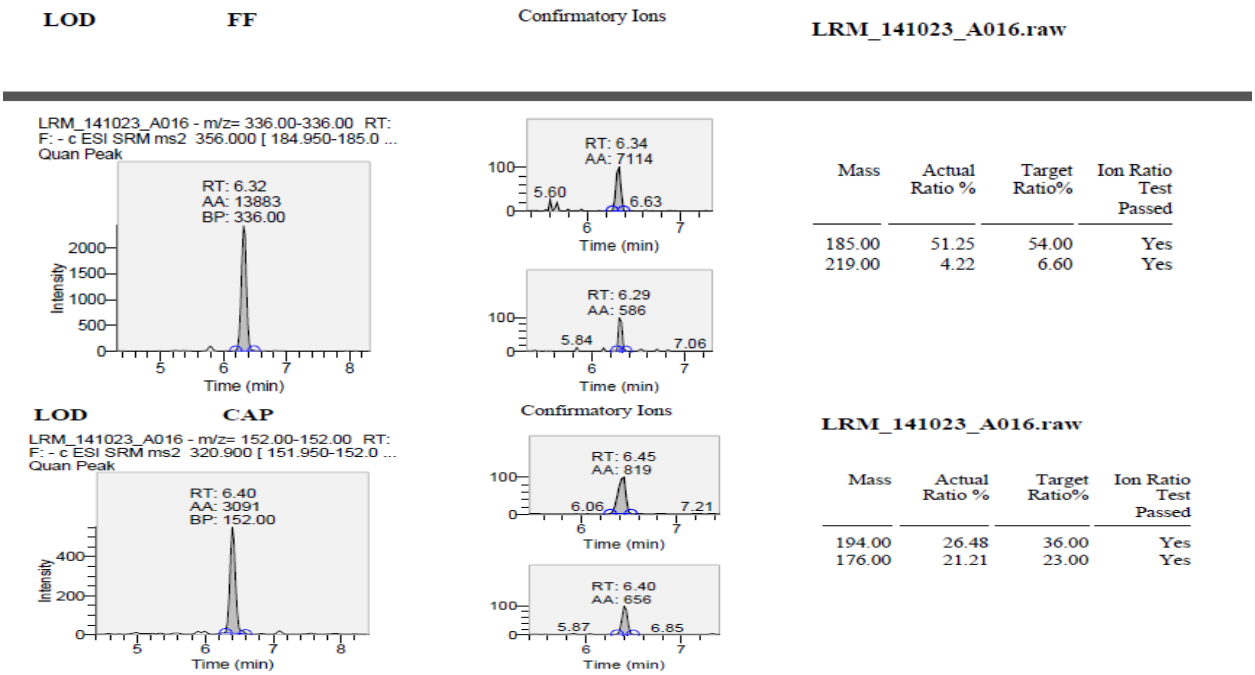


Figure 4: Oxytetracycline spiked in lobster muscle at 50 ng/g (MDL): a) reconstructred ion chromtogram (RIC); b) Ion ratio report



Component Name	Area	ISTD Area	Response Ratio	RT	Calculated Conc
FF	13883	261105	0.053	6.32	0.210
CAP-D5	261105	N/A	N/A	6.41	N/A
CAP	3091	261105	0.012	6.40	0.212

Figure 5: Amphenicols (florfenicol, chloramphenicol-d5, chloramphenicol) spiked in lobster muscle at 0.25 ng/g (MDL) for FF and CAP, and 20 ng/g for chloramphenicol-d5: reconstructed ion chromtogram (RIC).



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
185.00	51.25	54.00	Yes
219.00	4.22	6.60	Yes

Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
194.00	26.48	36.00	Yes
176.00	21.21	23.00	Yes

Figure 6: Amphenicols **(**florfenicol, chloramphenicol-d5, chloramphenicol) in lobster at 0.25 ng/g (MDL) for FF and CAP, and 20 ng/g for chloramphenicol-d5: reconstructed ion chromtogram (RIC).

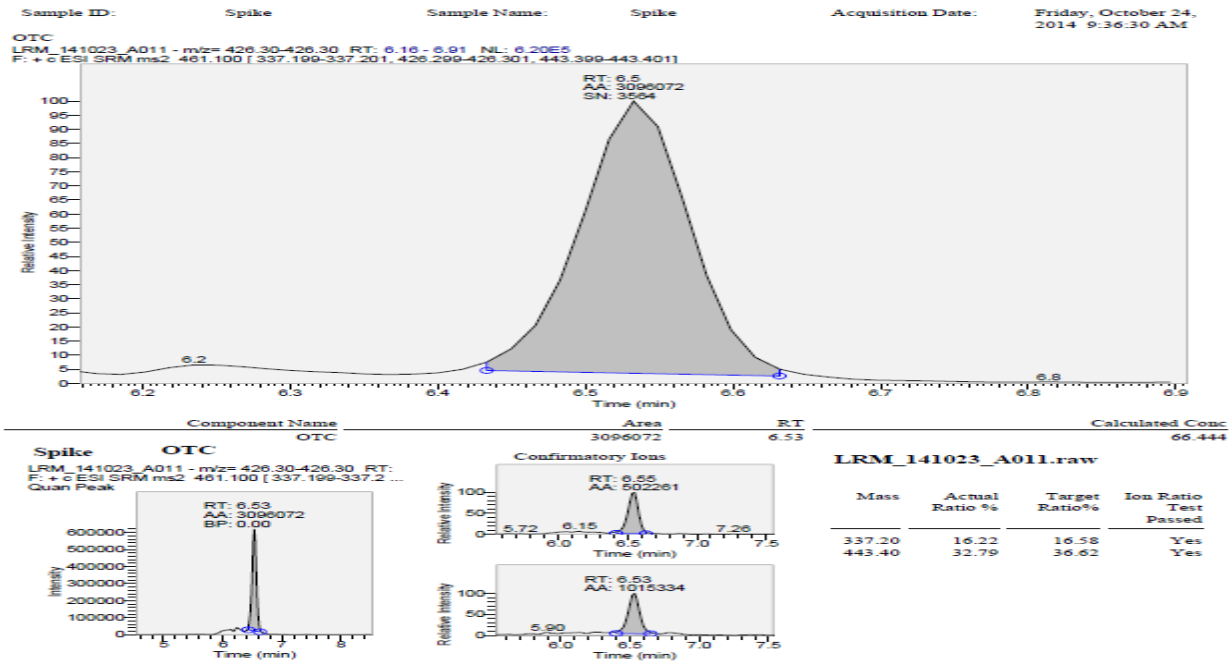


Figure 7: Oxytetracycline spiked in lobster muscle at 100 ng/g (1X CCV): a) Reconstructred ion chromtogram (RIC); b) Ion ratio report

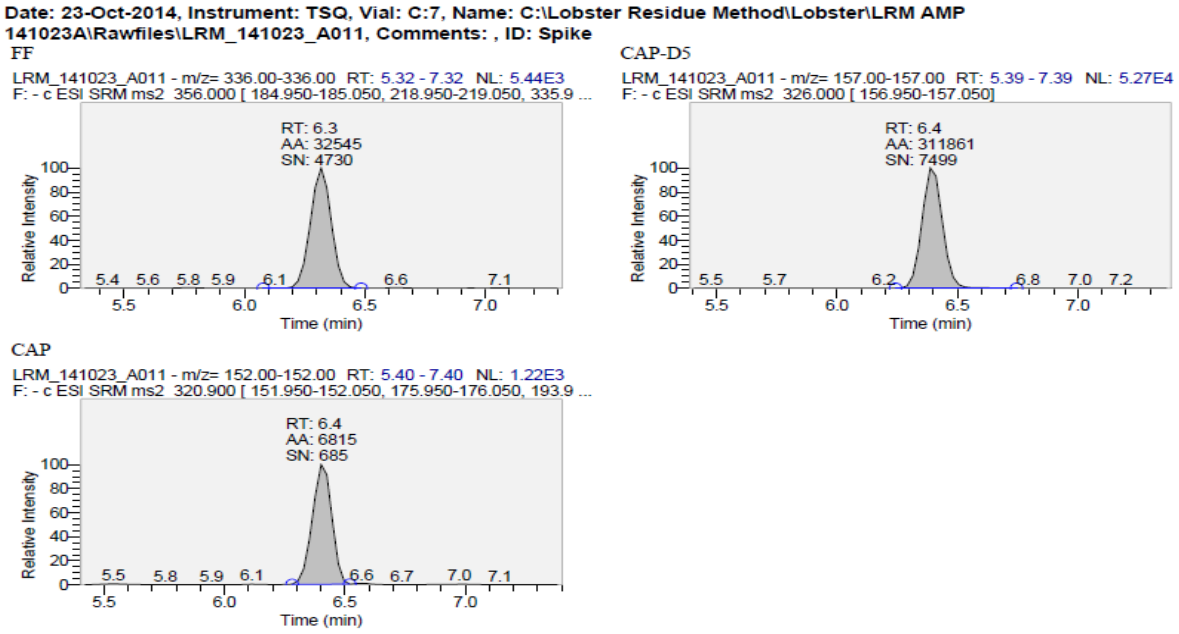


Figure 8: Amphenicols (florfenicol, chloramphenicol-d5, chloramphenicol) spiked in lobster muscle at 0.5 ng/g (1X CCV) for FF and CAP, and 20 ng/g for chloramphenicol-d5: reconstructed ion chromtogram (RIC)

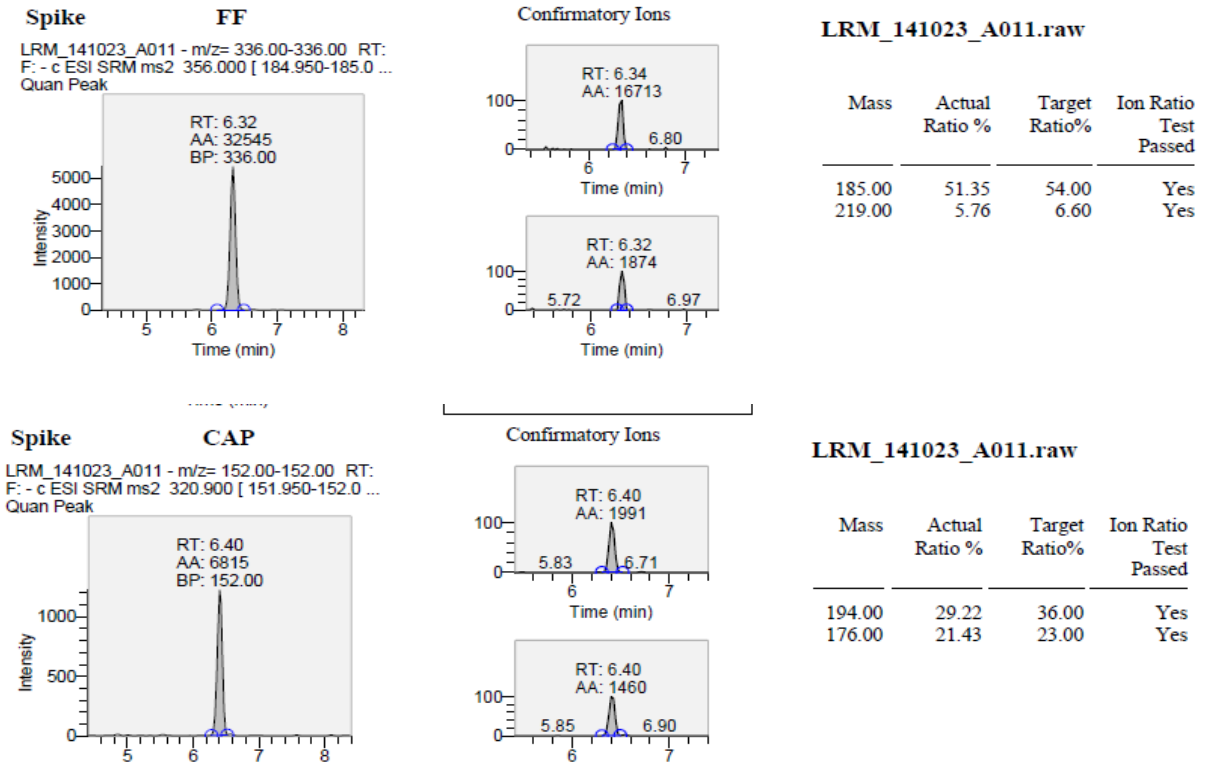


Figure 9: Amphenicols (florfenicol, chloramphenicol) spiked in lobster muscle at 0.5 ng/g (1X CCV): ion ratio report

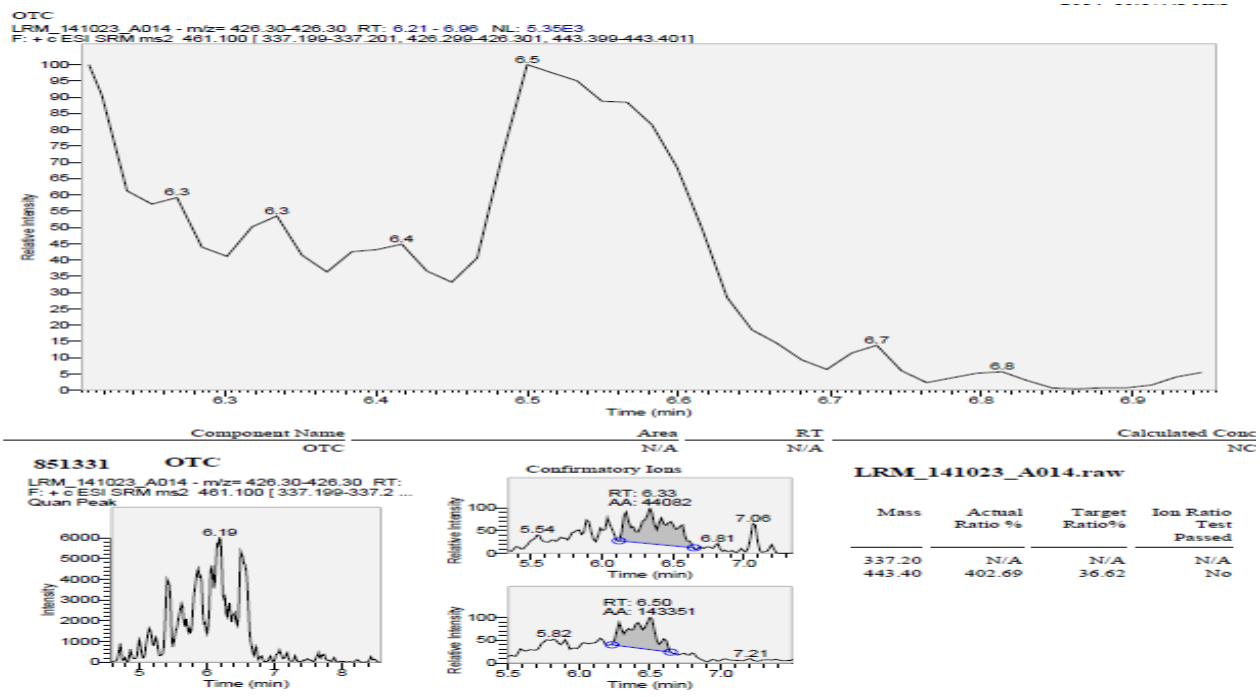
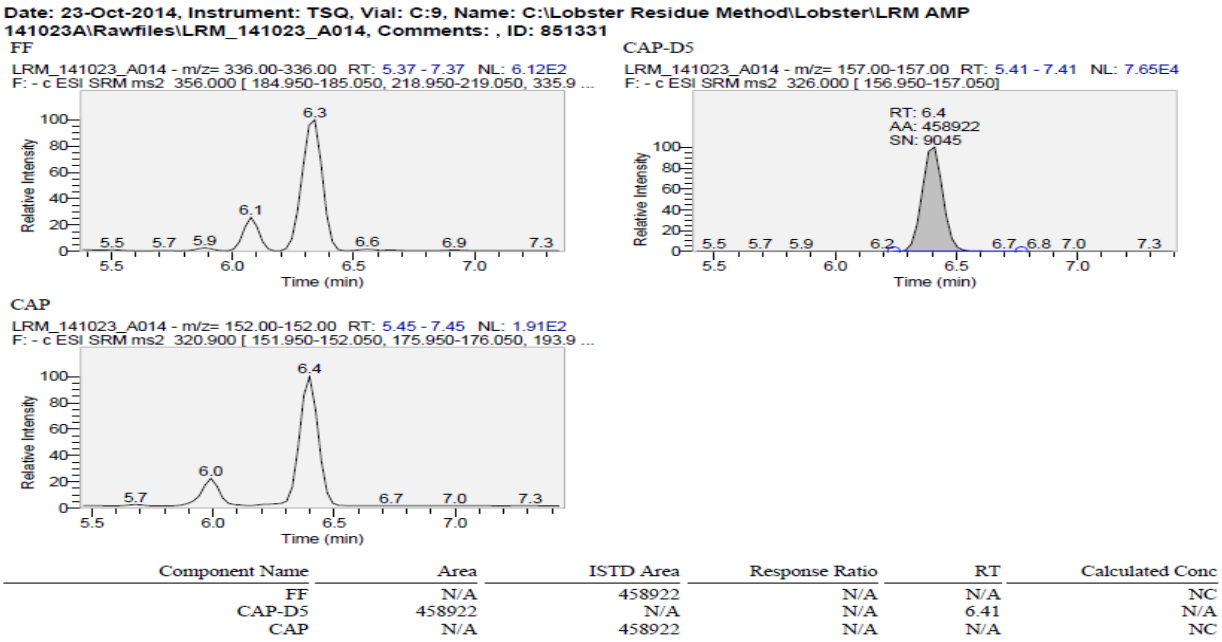
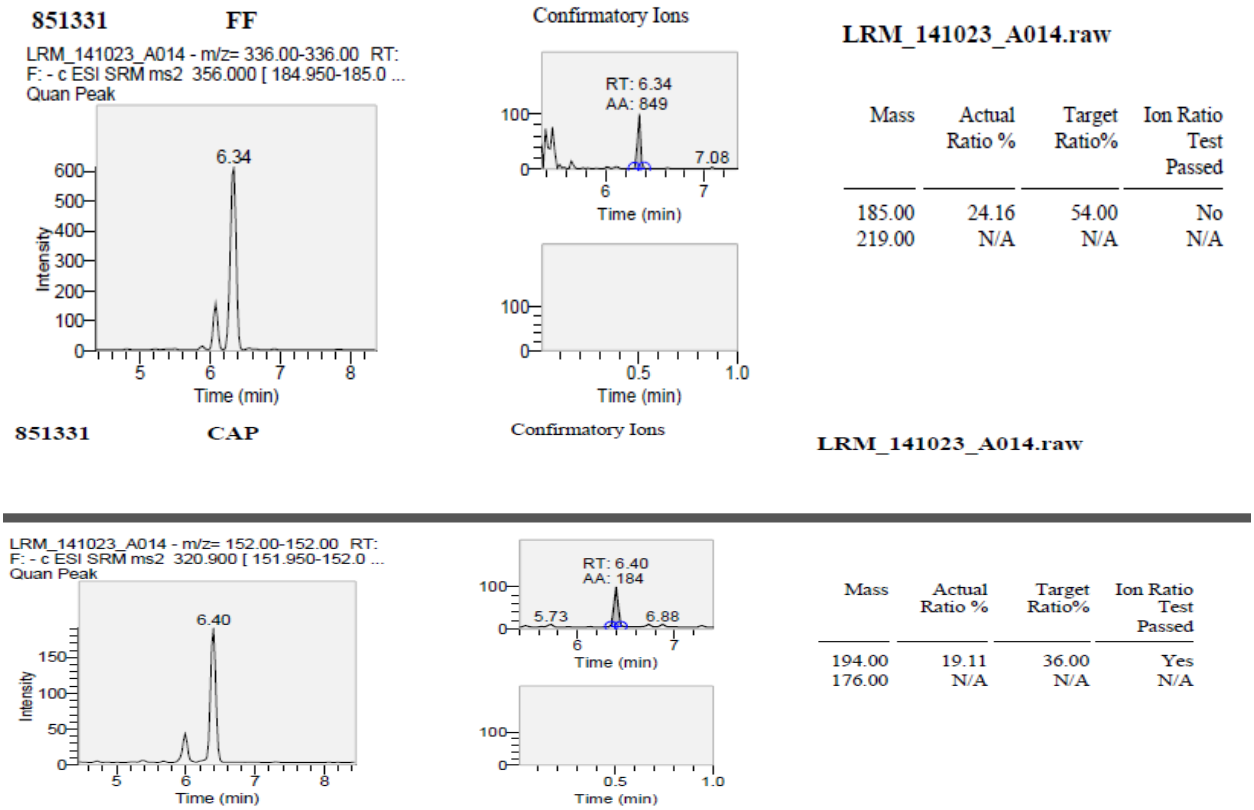


Figure 10: A lobster–regulatory sample monitored for Oxytetracycline: a) Reconstructred ion chromtogram (RIC); b) Ion ratio report



Component Name	Area	ISTD Area	Response Ratio	RT	Calculated Conc
FF	N/A	458922	N/A	N/A	NC
CAP-D5	458922	N/A	N/A	6.41	N/A
CAP	N/A	458922	N/A	N/A	NC

Figure 11: A lobster–regulatory sample monitored for Amphenicols (florfenicol, chloramphenicol) with 20 ng/g for chloramphenicol-d5 spiked: reconstructed ion chromtogram (RIC).



Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
185.00	24.16	54.00	No
219.00	N/A	N/A	N/A

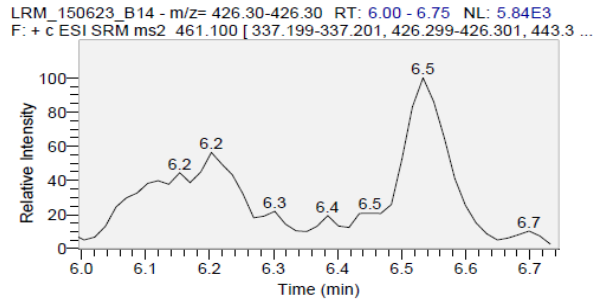
Mass	Actual Ratio %	Target Ratio%	Ion Ratio Test Passed
194.00	19.11	36.00	Yes
176.00	N/A	N/A	N/A

Figure 12: A lobster-regulatory sample monitored for Amphenicols (florfenicol, chloramphenicol): ion ratio report

Sample ID:	Negative control	Sample Name:	Negative control	Acquisition Date:	Tuesday, June 23, 2015 8:10:45 PM

Date: 23-Jun-2015, Instrument: TSQ, Vial: C:6, Name: C:\Documents and Settings\Quantum\Desktop\LIB 4508\Lobster\LRM OTC 150623\Rawfiles\LRM_150623_B14, Comments: , ID: Negative control

OTC



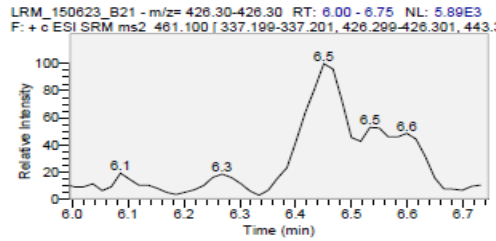
Component Name	Area	RT	Calculated Conc
OTC	N/A	N/A	NC

Figure 13: A Crab-negative control sample monitored for Oxytetracycline: a) Reconstructred ion chromtogram (RIC)

Sample ID:	907135	Sample Name:	907135	Acquisition Date:	Tuesday, June 23, 2015 9:47:20 PM

Date: 23-Jun-2015, Instrument: TSQ, Vial: C:13, Name: C:\Documents and Settings\Quantum\Desktop\LIB 4508\Lobster\LRM OTC 150623\Rawfiles\LRM_150623_B21, Comments: , ID: 907135

OTC

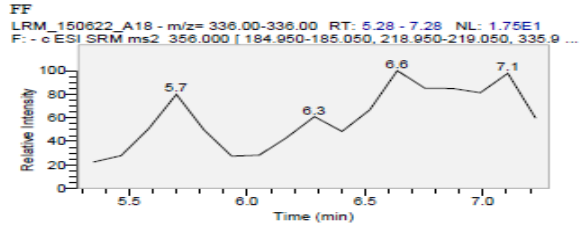


Component Name	Area	RT	Calculated Conc
OTC	N/A	N/A	NC

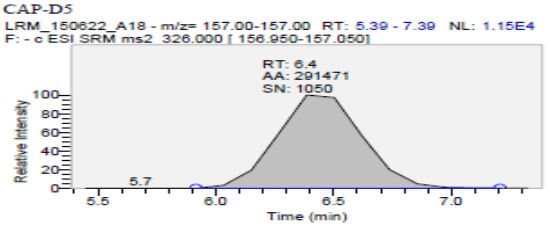
Figure 14: A Crab-regulatory sample monitored for Oxytetracycline: Reconstructred ion chromtogram (RIC)

Date: 23-Jun-2015, Instrument: TSQ, Vial: C:12, Name: C:\Documents and Settings\Quantum\Desktop\LIB 4508\Lobster\LRM AMP 150623\Rawfiles\LRM_150622_A18, Comments: , ID: 911834

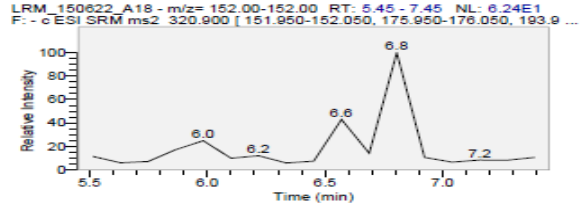
FF



CAP-D5

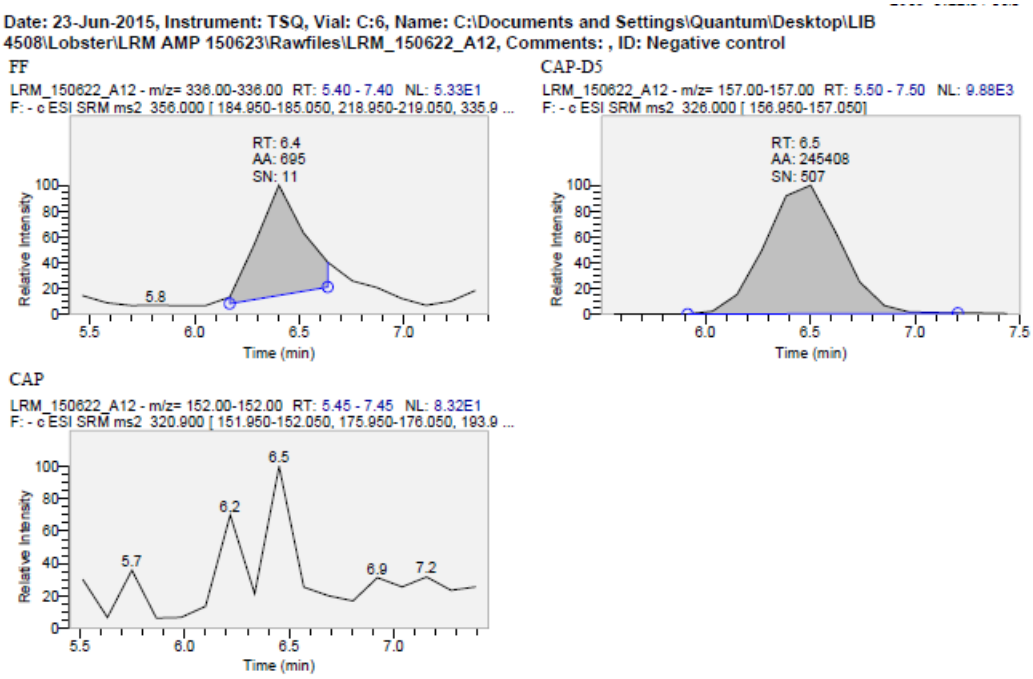


CAP



Component Name	Area	ISTD Area	Response Ratio	RT	Calculated Conc
FF	N/A	291471	N/A	N/A	NC
CAP-D5	291471	N/A	N/A	6.39	N/A
CAP	N/A	291471	N/A	N/A	NC

Figure 15: A Crab-negative control monitored for Amphenicols (florfenicol, chloramphenicol): Reconstructred ion chromtogram (RIC)



Component Name	Area	ISTD Area	Response Ratio	RT	Calculated Conc
FF	695	245408	0.003	6.40	0.022
CAP-D5	245408	N/A	N/A	6.50	N/A
CAP	N/A	245408	N/A	N/A	NC

Figure 16: A Crab-regulatory sample monitored for Amphenicols (florfenicol, chloramphenicol): Reconstructred ion chromtogram (RIC)