Updates to LIBs #4615/4616 Screening Method for Veterinary Drug Residues using LC-HRMS: Validation in Shrimp

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ABSTRACT

A modified version of the HRMS screening method described in LIBs #4515/4616 was validated in the DENL for the qualitative analysis of targeted veterinary drugs and pesticide residues in shrimp. Modifications from the original LIBs include use of an optimized MS2 data acquisition and 30 additional analytes to the fortification standard mixture. The results of the validation demonstrate that this updated method can be used in a regulatory laboratory to screen shrimp samples for residues of these drugs at screening target testing levels (TTLs). The method validation data also met high resolution mass spectrometry confirmation of identity criteria for the targeted list of 98 residues.

For the validation study, one matrix extract fortified at the TTLs was designated as the 1X standard (designated 100% relative recovery) and the relative recoveries of the other replicates were determined by comparison to that single standard using an average response factor calculation. The precision was also determined. The overall average recovery for the positive ion compounds was 96% (14% RSD) and for the negative analytes the average recovery was 97% (13% RSD) for 21 shrimp sample replicates fortified at the 1X level.

Limits test threshold cutoff values were calculated from these data and it was determined that a threshold cutoff value of ≥ 50% TTL was reasonable for all analytes to be considered presumptive positive. Therefore, if a calculated amount of any analyte is ≥ 50% of that found in the designated 1X TTL extracted matrix standard and qualitative identification criteria are met, the sample is considered presumptive positive for that residue and more analysis may be required. This is consistent with the results reported in LIBs #4615/4616.

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INTRODUCTION

The Animal Drugs Research Center (ADRC) developed a screening method for veterinary drug residues in fish, shrimp and eel using LC-Q-Orbitrap HRMS. This original method was published as Laboratory Information Bulletins (LIBs) #4615/4616. [1, 2] The method has been used to analyze for drug residues in imported fish samples. Several new compounds including 2-amino mebendazole in eel and ofloxacin in croaker were detected and identified in addition to confirming findings of fluoroquinolone and sulfonamide residues in several fish. [3, 4] More recently, the method has been modified to test additional veterinary drug residues as well as other types of chemical contaminants (pesticides, human drugs) that may be expected to be present in farmed fish. [5, 6] In addition, several different data acquisition modes for the LC-Q-Orbitrap were evaluated to detect and identify both targeted and non-targeted chemical residues in fortified and imported eel samples. [7] Although these method improvements have been documented in the LIBs or peer-reviewed publications cited above, the objective of this LIB is to describe the updated screening method for targeted analytes as it was transferred to regulatory laboratories for validation and to report the results of the single laboratory validation performed by DENL for shrimp in response to high priority special assignment.

EXPERIMENTAL

Equipment

a. The instrument used was a Thermo Q-Exactive High Field (QE HF) Orbitrap high resolution mass spectrometer (HRMS) with a heated electrospray ionization (HESI) source coupled to a Thermo Vanquish LC system. Thermo XCalibur software (V.4.1)

b. Centrifuge: Programmable refrigerated centrifuge capable of speeds of 13,000 rpm or 28,900 RCF (g)

c. Mechanical Shakers: multi-tube vortex mixer

d. Pipettors: adjustable volume: 10-100 µL, 100-1000 µL, and 0.5-5 mL

e. Nitrogen evaporator: set at 55˚C with nitrogen flow up to 20 psi

f. Balance: capable of weighing 0.01 g

g. Solid phase extraction (SPE) cartridge: Waters Oasis PRiME HLB 6cc, 200 mg (Part # 186008057)

h. Centrifuge tubes – Polypropylene (PP), 50-mL and 15-mL; for example, Falcon Part Numbers 352070 and 352096

i. Column: Supleco Ascentis (Part # 53804-U), (7.5cm x 2.1 mm, 2.7 µm)

j. Low-volume polypropylene vials (0.6-mL volume) with pre-scored snap caps; for example, Part Numbers #69400-124 from National Scientific (vials) and #242775 from Wheaton (caps)

Reagents and Standards

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever is soonest.

Reagents

a. Methanol (MeOH) – Fisher Chemical, Optima LC/MS Grade

b. Acetonitrile (ACN) – Fisher Chemical Optima LC/MS Grade

c. Formic acid – Fisher Chemical Optima LC/MS Grade

d. Glacial Acetic Acid – Fisher

e. Water – Fisher Chemical Optima LC/MS Grade

f. p-Toluene sulfonic acid monohydrate (p-TSA), ACS regent > 98.5%, CAS #6192-52-5 (Sigma Aldrich)

Reagent Solutions

a. Extraction Solution: 0.2% p-toluene sulfonic acid monohydrate (w/v), and 2% glacial acetic acid (v:v) in 100% ACN, 1 year expiration. Weigh 1.96 grams of p-TSA to 800 mLs of ACN in a graduated cylinder. Added 20.0 mLs of glacial acetic acid, then file to the mark with acetonitrile, invert and mix until the p-TSA is dissolved.

b. Reconstitution Solution: 10% ACN in water. 100 mLs of acetonitrile is added to 800 mLs of water, dilute to 1000 mLs with water to the mark. Invert and mix.

c. LC-MS Mobile Phase:

   a) LC-MS Aqueous Mobile Phase: 0.1% Formic Acid in water

   b) LC-MS Organic Mobile Phase: ACN

Analytical Standards (Neat Materials)

a. All analytical standards were ordered from Sigma-Aldrich, specifically as Fluka products, USP, Toronto Chemicals, and Santa Cruz Biochem.

b. SPEX Premade Solutions: Alternatively, premade solution can be ordered from SPEX at the following concentration:
100 µg/mL for Sulfonamides/Potentiator/Hormones;
50/100 µg/mL for Quinolones/Fluoroquinolones;

Tetracyclines 500 µg/mL; and 50 µg/mL for Benzimidazoles.

Procedures

The procedures described are based on LIBs #4615/4616[1, 2] with the exceptions of updated data acquisition programs and the addition of several analytes included in the validation procedure.

Standard Solutions

Purity and counter-ions are taken into account when calculating standard concentrations. The stability timeframe of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever ends sooner. Standards can be used beyond the expiration date for qualitative work only.

Stock Standards

Individual stock standards were made in MeOH, except for β-Lactams which were dissolved in acetonitrile or acetonitrile/water depending upon the solubility. All stock standard solutions were made at approximately 100 µg/mL – 500 µg/mL, as the free base or acid. All stock solutions were stored at 4 °C for the stable and -20 °C for the unstable compounds. Stock standards expire 1 year from preparation date or sooner if the neat material expires. As this is a qualitative screening method, standards may be used outside expiration date. Exception: The tetracyclines and beta lactams expire 6 months from the preparation date.

Mixed Standard Spiking Solutions

Two different spiking standards mixes ("stable" and "unstable") were made as described in LIBs #4615/4616 from stock solutions or purchased standard mixtures. The "stable" standard spiking solution consists of the 76 compounds from the following classes of compounds: sulfonamides, a potentiators, a hormone, fluoroquinolones, quinolones, benzimidazoles, phenicols, macrolides, nitroimidazoles, preservatives, benzylureas, avermectins, and pesticides in acetonitrile. The solutions are made by pipetting the required volume of the stock solutions or purchased standard mixtures to yield a final specified concentration for each analyte in the mixed stable spiking standard in a final volume of 25.0 mL ACN. *Example procedure for preparing stable mixed standard spiking solution is shown in Appendix A*.

The "unstable" working solution consist of 22 compounds from the following class of compounds: tetracyclines, β-Lactams, cephalosporin, dyes and baquiloprim in acetonitrile or water. The solutions are made by pipetting the required volume of the stock solutions or purchased standard mixtures to yield a final specified concentration for each analyte in the working standard in a final volume of 25.0 mL ACN or water. *Example procedure for preparing unstable mixed spiking standard solution is shown in Appendix B*.

Solvent System Suitability Standard

A 1X solvent standard was prepared by adding 100 µL of the stable standard mix and 50 µL of the unstable standard mix to 5.00 mLs of the reconstitution solution. (This assumes

an extracted scheme of 2g -> 10mLs, then 2 mLs -> 0.4 mLs). These standards are injected to determine if the instrument has retention time stability and adequate mass accuracy before an analytical batch is extracted and analyzed.

Extracted System Suitability Standard
A separate type of system suitability standard can be used in lieu of the 1X solvent standard before every batch. An extra 1X "Continuing Calibration Verification (CCV)" can be extracted with the analytical batch. This is preferred as the retention times for earlier eluting compound in matrix can vary from the solvent standards and the data analysis method then requires updating between the solvent standards and extracted standards.

Sample Preparation

Muscle tissue is chopped into pieces, homogenized with 1 to 2 blocks (1-2 kg) of dry ice in a food processor to produce a fine powder, and the powdered sample is loosely sealed in a whirl-pak bag for overnight storage. After the carbon dioxide has sublimed, the homogenized sample is stored at –20 °C or lower until analysis.

Extraction Procedure

The sample weights were recorded to at least three significant figures and calibrated pipettes/volumetric glassware were used.

1) Weigh out 2.0 ± 0.05 g of tissue into 50-mL polypropylene tube. *(Note: Two grams of sample were used for the validation data reported in this LIB. Additional experiments have shown that 4.0 ± 0.05 g of fish tissue can also be used successfully using this extraction procedure. The use of 4 g samples would be compatible with quantitative method used for residues in aquaculture described in LIB # 4653A.[8])*

2) For 2 g samples, add 40 µL of the stable mix and 20 µL of the unstable mix to the tissue to prepare the 1X - calibrator (CCV), 1X "Initial Calibration Verification (ICV)" standard, spike, and duplicate.

3) Add 8 mL of extraction solution to all samples.

4) Vortex samples for 30 minutes on a multi-tube vortexer set at 2500 rpm.

5) Centrifuge the tubes for 7 minutes at 4 °C at 10,000 rpm or 17,000RCF (g).

6) Transfer 3 mL of extractant into an Oasis PRiME HLB Extraction Cartridge with a 15-mL polypropylene tube underneath.

7) Allow the SPE to gravity drain for approximately 10 minutes.

8) Use a pipette bulb, gently push out the remaining few drops of extractant through the SPE cartridge.

9) The collection tube should contain approximately 2 mLs.

10) At this point, transfer 100 µL of eluent collected from the SPE into a limited volume conical HPLC vial for separate analytical injections of this acetonitrile initial extract (before drying and reconstitution) in the positive and negative modes.

11) Dry the remaining portion of the extract under a nitrogen stream at 55 °C under 15 psi nitrogen. Take to near dryness (~ 75-100 µL remaining in the tube is acceptable). Approximate drying time is 30 minutes.

12) Add 400 µL of the reconstitution solution to the tube, cap and vortex for two minutes on the multi-tube vortexer. *Note: used 400 µL of water for 4 g sample.*

13) Centrifuge tubes at 13,000 rpm or 28,900 RCF (g) for 7 minutes at 4 °C.

14) With a 1.00-mL variable pipette, carefully transfer 300 µL from the center of the 15-mL tube into a limited volume HPLC vial.

Example of Spike Concentration: 10 ng/g x (2grams/10 mLs) x (2mLs/0.4mLs) = 10 ng/mL in vial

Instrument Procedures

MS Parameters

The instrument was calibrated for mass accuracy according to the manufacturer's recommendations at least once a week. The tuning method optimized signals for a majority of the test compounds with the LC conditions described below. This tune file was used for both MS acquisition programs described below and the general parameters are as follows: spray voltage, 4kV (positive ion), 2.5kV (negative ion); S-Lens RF level, 50; capillary temperature, 350 ˚C; auxiliary gas temperature, 325 ˚C; gas flow rate (N2, arbitrary units): sheath, 50; auxiliary, 10; sweep, 0. Other general MS parameters include: acquisition time, 0-12.5 min; polarity, positive or negative; Lock mass, OFF.

Acquisition method using vDIA MS2 experiments:

The non-targeted acquisition method included a full scan MS1 followed by three variable data independent acquisition (vDIA) experiments for MS2. Initially a full MS1 scan (m/z 150-1000; Resolution 60,000) was acquired. With MS2 vDIA, all MS1 ions are selected for fragmentation using higher energy C-trap dissociation (HCD), but the width of the precursor isolation windows is varied to more efficiently isolate and detect ions. For this method, 11 vDIA MS2 scans were performed with variable isolation widths as shown in Table 1. The resolution for the MS2 experiments was 30,000 and normalized collision energies of 10, 30, and 50 were used. This approach was used for both positive and negative data acquisition (separate injections).

Table 1: vDIA MS2 Scanning Experiments Inclusion List

DIA Experiment	Loop	Scanning Event	Mass (m/z)	vDIA Scan Isolation Width, m/z	Range of Precursor Ions (m/z)
1	1	1	125	52	99-151
1	2	2	175	52	149-201
1	3	3	225	52	199-251
1	4	4	275	52	249-301
1	5	5	325	52	299-351
1	6	6	375	52	349-401
1	7	7	425	52	399-451
1	8	8	475	52	449-501
2	1	9	550	102	498-601
2	2	10	650	102	598-701
3	1	11	850	300	700-1000

Chromatography

LC separation was performed using a Supelco Ascentis Express C18 (7.5 cm x 2.1 mm, 2.7 µm) fused-core reversed-phase column. The mobile phase consisted of 0.1 % formic acid (A) and ACN(B) at a flow rate of 0.3 mL/min. The LC gradient program was initialized at 5% B and held for 1.5 min then ramped to 50% B from 1.5 to 8.5 min, followed by a ramp to 99% B from 8.5 to 9 min, and then was held at 99% B from 9 to 12 min. The mobile phase was returned to 5% B from 12 to 12.5 min and the column was re-equilibrated for an additional 2 minutes. The total LC runtime was 14.5 min; MS data were collected for 12.5 min (no divert valve was used). The column temperature compartment was kept at 30 ˚C, and the autosampler tray temperature was maintained at 10 ˚C. The LC injection volumes was 10 µL. Multiple injections of a high-level dye standard may be needed to condition a new column to detect low levels of leucocrystal violet and leucomalachite green.

Batch Analysis

The following were included with each batch of samples:

1) Solvent standards to determine system suitability. Test compounds should meet confirmation of identity criteria using vDIA data acquisition.
2) Two Blank control for each matrix.
3) Two extracted CCVs fortified at the 1X - level. One can be used as the system suitability in place of the solvent standard.
4) Extracted ICV at the 1X - fortification level.
5) Spike and duplicate at the 1X - fortification level.
6) Duplicate exacts of the sample. The duplicate extraction is to assist with additional data analysis including non-targeted data mining using larger exact mass databases. For each sample, both the final reconstituted extract and the initial acetonitrile extract are analyzed in both the positive/negative mode (four separate injections).

Data Analysis

The sample extracts are acquired via the Xcalibur software and imported into TraceFinder (TF) for data analysis to determine if validated compounds are observed and pass the criteria for HRMS data. TF "Quantitative Methods" were established to provide data for the test compounds listed in Table 3 and Table 4, including metabolites or degradants from some compounds (e.g. penillic acid and dehydrated erythromycin). Data from full MS^1 scans were used for initial screening of test compounds using ion chromatograms of precursor ions extracted with 5 ppm window. The protonated molecules (MH^+) or deprotonated (MH-) were monitored depending on the polarity of the compounds, the exceptions were for eprinomectin, ivermectin, and doramectin which were analyzed as the sodium adducts. The TF quantitative method also checks for the presence of product ions.

Reports are generated into pdf and/or excel files and no "hand typing" of data was performed. Using Excel, the analyte responses in CCV, ICV, spikes, and presumptive positives detected in samples are normalized to the 1X CCV. As stated earlier, the CCV (extracted-matrix matched) is designated as "100%" and presumptive positive compounds are calculated at a % of the CCV (extracted). If a calculated amount is above "50%", more analysis maybe required.

RESULTS AND DISCUSSION

Method Validation

The objective of this work was to validate the method described in LIBs #4515/16 and other published articles for implementation in the regulatory laboratory. Due to a current survey for the determination of veterinary drugs in shrimp, this validation report only contains shrimp data. Other matrices will be validated on an "as-needed" basis.

The method was validated in accordance as outlined in the FDA Office of Food and Veterinary Medicine "Guidelines for the Validation for Chemical methods for the FDA FVM Program, 3rd Ed. [9] for "limit testing" with veterinary drugs from a variety of chemical classes in shrimp. Three different sources of shrimp were used in the validation study. The matrices were fortified at 0.5X, 1X, and 2X of the target testing level (TTL), and Table 2 shows the validation plan.

Table 2: Number of Validation Samples

		Source 1	**Source 2**	**Source 3**	**Total**
Blank		3	3	3	9
1X		7	7	7	21
½ X (0.5X)		3	3	3	9
2X		3	3	3	9
Total		16	16	16	**48**

Modification of LIBs #4615/4616: Data acquisition and additional analytes

In the referenced method LIBs #4515/16, several different data acquisitions methods were tested. In general, samples were initially analyzed using full scan MS1 and nontargeted MS2 data acquisition such as All Ion Fragmentation (AIF) or Data Independent Acquisition (DIA). The validation described in LIB #4616 utilized AIF and it was demonstrated that most of the analytes in the 1X spike could be detected in these samples at their target testing levels. Exceptions were the triphenyl methane dyes which were sometimes not detected and the avermectins which may not generate detectable product ions from the sodiated precursor. Analyzing the initial acetonitrile layer as described in LIB 4615 can increase the detectability of these difficult compounds. Recent publication by ADRC[7] demonstrated variable DIA (vDIA) methods produce similar results as AIF for the targeted compounds validated in LIBs #4515/16. Because cleaner MS2 spectra are produced, vDIA methods have some advantages when using data collected to search for non-targeted analytes using software programs such as Compound Discoverer and on-line databases. The validation data presented in this LIB used full scan MS1 and variable data independent acquisition (vDIA) to collect MS2 spectra.

The test compounds listed in Table 3 and 4 were a subset of analytes imported into TF "Quantitative Methods" from a larger in-house compound database (N ~500) which contains information for retention time and exact masses of product ions. There are 30 additional analytes in this validation study that were not part of the original method including 20 positive ion analytes (e.g. fluoroquinolones, benzimidazoles, pesticides) and ten negative ion compounds.

Table 3: Positive Targeted Compounds

Analyte	Class	RT (min)	Formula	MH^+	Product Ions		
Abamectin B1a	Avermectins	11.2	$C_{48}H_{72}O_{14}$	[a]895.48143	751.4028	184.0733	
Doramectin	Avermectins	11.3	$C_{50}H_{74}O_{14}$	[a]921.4971	449.2298	777.4126	
Emamectin B1a	Avermectins	9.6	$C_{49}H_{75}NO_{13}$	886.5311	82.0651	158.1176	302.1962
Eprinomectin B1a	Avermectins	11.0	$C_{50}H_{75}NO_{14}$	[a]936.50798	490.2883	352.1731	224.0893
Ivermectin B1a	Avermectins	12.0	$C_{48}H_{74}O_{14}$	[a]897.4971	609.3398	753.4184	
Moxidectin	Avermectins/Milbemycin	11.5	$C_{37}H_{53}NO_8$	640.3844	528.2956	498.2848	199.1117
Selemectin	Avermectins	12.0	$C_{43}H_{63}NO_{11}$	770.44739	None		
Albendazole	Benzimidazoles	7.0	$C_{12}H_{15}N_3O_2S$	266.0958	159.0427	191.0148	234.0696
Albendazole sulfone	Benzimidazoles	6.4	$C_{12}H_{15}N_3O_4S$	298.0856	159.0426	191.0325	224.0124
Albendazole sulfoxide	Benzimidazoles	5.0	$C_{12}H_{15}N_3O_3S$	282.0907	208.0175	240.0437	
Fenbendazole	Benzimidazoles	8.4	$C_{15}H_{13}N_3O_2S$	300.0801	159.0427	268.0539	
Fenbendazole sulfone	Benzimidazoles	7.3	$C_{15}H_{13}N_3O_4S$	332.0700	300.0437		
2-amino mebendazole	Benzimidazoles	5.3	$C_{14}H_{11}N_3O$	238.09749	105.0336		
5-hydroxy mebendazole	Benzimidazoles	5.8	$C_{16}H_{15}N_3O_3$	298.11862	266.0924		
Mebendazole	Benzimidazoles	7.5	$C_{16}H_{13}N_3O_3$	296.1030	264.0768	105.0336	
Thiabendazole	Benzimidazoles	3.8	$C_{10}H_7N_3S$	202.04334	175.0325		
Brilliant Green	Dyes	9.5	$C_{27}H_{33}N_2$	[b]385.2638	297.1386	341.2012	
Crystal violet	Dyes	9.0	$C_{25}H_{30}N_3$	[b]372.2434	251.1543	356.2121	
Leucocrystal violet	Dyes	5.6	$C_{25}H_{31}N_3$	374.2591	239.1543	253.1699	358.2278
Leucomalachite green	Dyes	8.2	$C_{23}H_{26}N_2$	331.2169	194.0964	239.1543	315.1856
Malachite green	Dyes	8.1	$C_{23}H_{25}N_2$	[b]329.2012	208.1121	313.1699	

Analyte	Class	RT (min)	Formula	MH^+	Product Ions		
Methylene Blue	Dyes	6.0	$C_{16}H_{18}N_3S$	284.12159	268.09029	241.0794	
Ciprofloxacin	Fluoroquinolones	4.6	$C_{17}H_{18}FN_3O_3$	332.1405	245.1085	288.1507	
Danofloxacin	Fluoroquinolones	5.0	$C_{19}H_{20}FN_3O_3$	358.1562	283.1241	314.1663	338.1499
Difloxacin	Fluoroquinolones	5.4	$C_{21}H_{19}F_2N_3O_3$	400.1467	299.0990	356.1569	
Enrofloxacin	Fluoroquinolones	5.1	$C_{19}H_{22}FN_3O_3$	360.1718	245.1085	316.1820	342.1612
Marbofloxacin	Fluoroquinolones	4.3	$C_{17}H_{19}FN_4O_4$	363.14631	320.1041	205.03964	72.0878
Norfloxacin	Fluoroquinolones	4.7	$C_{16}H_{18}FN_3O_3$	320.1405	276.1507	302.1299	
Ofloxacin	Fluoroquinolones	4.8	$C_{18}H_{20}FN_3O_4$	362.15106	318.1612	261.1034	
Orbifloxacin	Fluoroquinolones	5.0	$C_{19}H_{20}F_3N_3O_3$	396.15295	352.1631	295.1053	
Sarafloxacin	Fluoroquinolones	5.4	$C_{20}H_{17}F_2N_3O_3$	386.1311	299.0990	342.1413	
methyl testosterone	hormones	9.4	$C_{20}H_{30}O_2$	303.2319	97.0648	109.10118	
Azithromycin	Macrolides	5.4	$C_{38}H_{72}N_2O_{12}$	749.5158	158.1176	591.4210	
Erythromycin A	Macrolides	6.6	$C_{37}H_{67}NO_{13}$	734.4685	83.0491	158.1176	576.3742
Erythromycin dehyr	Macrolides	7.2	$C_{37}H_{65}NO_{12}$	716.4580	158.1176		
Lincomycin	Macrolides	3.8	$C_{18}H_{34}N_2O_6S$	407.2210	126.1277	359.2214	
Spiramycin	Macrolides	5.5	$C_{43}H_{74}N_2O_{14}$	843.5213	174.1125	540.3136	
Tilmicosin	Macrolides	6.0	$C_{46}H_{80}N_2O_{13}$	[c]435.2903	174.1125	522.3789	695.460
Tylosin A	Macrolides	7.0	$C_{46}H_{77}NO_{17}$	916.5264	174.1125		
Ketoconazole	Nitroimidazoles	7.2	$C_{26}H_{28}Cl_2N_4O_4$	531.1560	82.0525	489.1455	
Metronidazole	Nitroimidazoles	2.0	$C_6H_9N_3O_3$	172.0717	82.0525	128.04555	
Florfenicol Amine	Phenicols	0.67/1.33	$C_{10}H_{14}FNO_3S$	248.0751	104.0632	130.0651	230.0646
Ormetoprim	potentiators	4.6	$C_{14}H_{18}N_4O_2$	275.1503	123.0665	259.1190	
Trimethoprim	potentiators	4.3	$C_{14}H_{18}N_4O_3$	291.1452	123.0665	230.1162	
Baquiloprim	potentiators	0.9	$C_{17}H_{20}N_6$	309.18222	294.15875	123.06652	
Ethoxyquin	Preservatives	7.7	$C_{14}H_{19}NO$	218.1539	148.0757	176.1070	
Ethoxyquin Dimer	Preservatives	12.0	$C_{28}H_{36}N_2O_2$	433.2850	417.25365	216.13829	375.2067
Flumequine	Quinolones	7.82	$C_{14}H_{12}FNO_3$	262.0874	202.0299	244.0768	
Nalidixic Acid	Quinolones	7.6	$C_{12}H_{12}N_2O_3$	233.0921	187.0502	215.0815	
Oxolinic Acid	Quinolones	6.5	$C_{13}H_{11}NO_5$	262.0710	216.0291	244.0604	
Sulfacetamide	Sulfonamides	2.2	$C_8H_{10}N_2O_3S$	215.0485	92.0495	108.0444	156.0114
Sulfachloropyridazine	Sulfonamides	5.8	$C_{10}H_9ClN_4O_2S$	285.0208	92.0495	108.0444	156.0114
Sulfaclozine	Sulfonamides	6.9	$C_{10}H_9ClN_4O_2S$	285.0208	92.0495	108.0444	156.0114
Sulfadiazine	Sulfonamides	2.9	$C_{10}H_{10}N_4O_2S$	251.0597	92.0495	108.0444	156.0114
Sulfadimethoxine	Sulfonamides	7.0	$C_{12}H_{14}N_4O_4S$	311.0809	108.0444	156.0114	156.0768
Sulfadoxine	Sulfonamides	6.1	$C_{12}H_{14}N_4O_4S$	311.0809	92.0495	108.0444	156.0114
Sulfaethoxypyridazine	Sulfonamides	6.2	$C_{12}H_{14}N_4O_3S$	295.0859	92.0495	108.0444	156.0114
Sulfamerazine	Sulfonamides	4.1	$C_{11}H_{12}N_4O_2S$	265.0754	92.0495	108.0444	156.0114
Sulfamethazine	Sulfonamides	4.8	$C_{12}H_{14}N_4O_2S$	279.0910	92.0495	108.0444	156.0114
Sulfamethoxazole	Sulfonamides	6.1	$C_{10}H_{11}N_3O_3S$	254.0594	92.0495	108.0444	156.0114
Sulfamethoxypyridazine	Sulfonamides	5.1	$C_{11}H_{12}N_4O_3S$	281.0703	108.0444	126.0662	156.0114
Sulfamonomethoxine	Sulfonamides	5.6	$C_{11}H_{12}N_4O_3S$	281.0703	92.0495	108.0444	156.0114
Sulfapyridine	Sulfonamides	4.0	$C_{11}H_{11}N_3O_2S$	250.0645	108.0444	156.0114	184.0869
Sulfaquinoxaline	Sulfonamides	7.1	$C_{14}H_{12}N_4O_2S$	301.0754	92.0495	108.0444	156.0114
Sulfathiazole	Sulfonamides	3.9	$C_9H_9N_3O_2S_2$	256.0209	92.0495	108.0444	156.0114
Chlortetracycline	Tetracyclines	5.6	$C_{22}H_{23}ClN_2O_8$	479.1216	444.0845		
Doxycycline	Tetracyclines	5.9	$C_{22}H_{24}N_2O_8$	445.1605	154.0499	410.1234	428.1340
Oxytetracycline	Tetracyclines	4.7	$C_{22}H_{24}N_2O_9$	461.1555	154.0499	426.1183	
Tetracycline	Tetracyclines	4.8	$C_{22}H_{24}N_2O_8$	445.1605	154.0499	410.1234	
Amoxicillin	β-lactams	1.9	$C_{16}H_{19}N_3O_5S$	366.1118	114.0372	208.0427	349.0853
Amoxicillin Diketone	β-lactams	4.8	$C_{16}H_{19}N_3O_5S$	366.1118	160.0427	207.07616	
Ampicillin	β-lactams	4.2	$C_{16}H_{19}N_3O_4S$	350.1169	106.0651	114.0372	160.0427

Analyte	Class	RT (min)	Formula	MH^+	Product Ions		
Aspoxicillin	β-lactams	2.6	$C_{21}H_{27}N_5O_7S$	494.1074	160.0427	250.1186	366.1118
Cephapirin	β-lactams	3.4	$C_{17}H_{17}N_3O_6S_2$	424.0632	152.0165	292.0573	
Cloxacillin	β-lactams	9.0	$C_{19}H_{18}ClN_3O_5S$	436.0729	160.0427	277.0375	
Dicloxacillin	β-lactams	9.6	$C_{19}H_{17}Cl_2N_3O_5S$	470.0339	160.0427	310.9985	
Nafcillin	β-lactams	9.2	$C_{21}H_{22}N_2O_5S$	415.1322	199.0754	171.0441	115.0542
Oxacillin	β-lactams	8.5	$C_{19}H_{19}N_3O_5S$	402.1118	114.0372	160.0427	243.0764
Penicillin G later elute	β-lactams	7.5	$C_{16}H_{18}N_2O_4S$	335.1060	114.0372	160.0427	176.0706
Penillic acid	β-lactams	4.9	$C_{16}H_{18}N_2O_4S$	335.1060	128.0528	160.0427	289.0997
Penicillin V	β-lactams	8.3	C16H18N2O5S	351.10092	160.0427	114.0372	
Azamethiphos	Organophosphate antiparasitic	7.5	$C_9H_{10}ClN_2O_5P$	324.98093	182.99541	139.00558	
Dichlorvos	Organophosphate pesticide	7.3	$C_4H_7Cl_2O_4P$	220.95318	144.98158	127.01547	78.99434
Malathion	Organophosphate pesticide	10.0	$C_{10}H_{19}O_6PS_2$	331.04334	285.00148	127.03897	99.00767
Quinalphos	Organophosphate pesticide	10.2	$C_{12}H_{15}N_2O_3PS$	299.06138	242.99847	163.03245	147.05529
Trichlorfon	Organophosphate pesticide	5.3	$C_4H_8Cl_3O_4P$	256.92985	220.95318	127.01547	
Atrazine	Triazine herbicides	8.2	$C_8H_{14}ClN_5$	216.10105	174.0541	96.05562	
Propazine	Triazine herbicides	9.3	$C_9H_{16}ClN_5$	230.1167	188.06975	146.0228	
Simazine	Triazine herbicides	7.0	$C_7H_{12}ClN_5$	202.0854	132.0323	124.08692	96.05562

[a] MNa^+ [b] M^+ [c] MH_2^{2+}

Table 4: Negative Targeted Compounds

Analyte	Class	RT (min)	Formula	$[M- H]^-$	Product Ions		
Chloramphenicol	Phenicols	6.5	$C_{11}H_{12}Cl_2N_2O_5$	321.00505	152.0353	176.0353	
Florfenicol	Phenicols	6	$C_{12}H_{14}Cl_2FNO_4S$	355.99319	185.0278	335.987	
Thiamphenicol	Phenicols	4.6	$C_{12}H_{15}Cl_2NO_5S$	353.99752	185.0278	290.0259	
Toltrazuril	Toltrazuril	10	$C_{18}H_{14}F_3N_3O_4S$	424.05843	316.98132	404.97665	
Toltrazuril Sulfone	Toltrazuril	9.9	$C_{18}H_{14}F_3N_3O_6S$	456.04826	None		
Toltrazuril Sulfoxide	Toltrazuril	9.2	$C_{18}H_{14}F_3N_3O_5S$	440.05335	371.05781		
Diflubenzuron	Benzylureas	10	$C_{14}H_9ClN_2O_2F_2$	309.02478	242.98601	289.0184	
Lufenuron	Benzylureas	10.4	$C_{17}H_8Cl_2F_8N_2O_3$	508.97115	174.95972	325.9591	
Hexaflumuron	Benzylureas	10.5	$C_{16}H_8Cl_2F_6N_2O_3$	458.9743	438.96811	275.9623	174.96086
Teflubenzuron	Benzylureas	10.3	$C_{14}H_6Cl_2F_4N_2O_2$	378.96697	195.95378	338.95451	

Determination of Limit Test

Semi-quantitative limit testing determines if a residue is present at or above the concentration of interest, so it is important to measure the variance of the signals generated from residues present in samples at this concentration. A threshold cutoff value can then be set to determine when to call a sample "presumptive positive". The shrimp matrix was validated with three separate sources at the 0.5X (n=9), 1.0X (n=21), and 2.0X (n=9), where 1X is the screening TTL spike level. Samples were analyzed over three days. The extracted CCV was designated as the 1X standard (100% relative recovery) and the relative recoveries of the other replicates were determined by comparison to that single standard using an average response factor calculation (Tables 5,6). The precision was also determined. The overall average for the positive compounds was 96% (14%RSD) and for the negative analytes 97% (13%) at the 1X level.

For example, ciprofloxacin in the shrimp samples fortified at the TTL (5 ng/g) had an average recovery of 106% with a standard deviation of 22%. The limit threshold cutoff value for that compound in shrimp calculates to 68%. This means any shrimp sample with a signal for ciprofloxacin greater than 68% of the signal in a 1X CCV matrix-extracted TTL standard could (with 95% confidence) contain ciprofloxacin at a concentration at or above the TTL. For some residues, however, the standard deviations were higher, and the threshold cutoff values were therefore lower. Table 5 and 6 are the results at the 1X level for the limit test determination. To avoid false negatives and simplify data analysis by treating all test compounds the same, a threshold cutoff value of ≥ 50% TTL seemed reasonable to be considered presumptive positive for all analytes. The validation data at the 0.5X demonstrate the threshold cutoff value ≥ 50% was reasonable. This is also consistent with previous results. [2, 4]

Limit test threshold = [average recovery – (t * standard deviation)] where t = one-tailed value for n-1 degrees of freedom at 95% confidence level

Table 5: Limits Test Thresholds (% of the 1X TTL) for Positive Ion Analytes

Analyte [a,b]	Class	Screening TTL (ug/Kg)	Ave % Rec	SD	Limits test threshold (% compared to TTL)
Abamectin B1a	*Avermectin*	200	114	16	86
Doramectin			117	9	102
Emamectin B1a			114	13	91
Eprinomectin B1a			123	23	78
Ivermectin B1a			113	12	113
Moxidectin	*Avermectin/Milbemycin*		147	47	66
~~*Selemectin*~~	*Avermectin*				
Albendazole	Benzimidazole	50	114	24	73
Albendazole sulfone			99	12	78
Albendazole sulfoxide			102	14	78
Fenbendazole			118	41	47
Fenbendazole sulfone			101	12	80
2-amino mebendazole			99	12	77
5-hydroxy mebendazole			96	12	75
Mebendazole			107	15	81
Thiabendazole			96	12	76
Brilliant Green	Dye	1	105	17	75
Crystal violet			103	25	61
~~*Leucocrystal violet*~~					
~~*Leucomalachite green*~~					
Malachite green			112	43	37
Methylene blue			96	24	55
Ciprofloxacin	Fluoroquinolone	5	106	22	68

Analyte [a,b]	Class	Screening TTL (ug/Kg)	Ave % Rec	SD	Limits test threshold (% compared to TTL)
Danofloxacin			103	15	76
Difloxacin			101	14	78
Enrofloxacin			106	10	88
Marbofloxacin			101	13	78
Norfloxacin			99	15	73
Ofloxacin			100	15	75
Orbifloxacin			98	13	76
Sarafloxacin			102	19	70
Methyl testosterone	Hormone	0.8	115	31	62
Azithromycin			114	22	71
Erythromycin A			87	15	61
Erythromycin dehyr			95	13	72
Lincomycin	Macrolide	50	101	17	71
Spiramycin			109	22	60
Tilmicosin			100	16	72
Tylosin A			101	14	76
Ketoconazole	Nitromidazole	10	105	22	68
Metronidazole			98	12	78
Florfenicol Amine	Phenicol	50	105	9.7	88
Ormetoprim			99	12	78
Trimethoprim	Potentiator	10	98	13	76
Baquiloprim			102	17	73
Ethoxyquin	Preservative	50	89	38	24
~~Ethoxyquin Dimer~~					
Flumequine			106	13	83
Nalidixic Acid	Quinolone	10	107	13	85
Oxolinic Acid			107	13	86
Sulfacetamide			107	23	67
Sulfachloropyridazine			103	12	82
Sulfaclozine/ Sulfachloropyrazine			98	14	74
Sulfadiazine			98	15	72
Sulfadimethoxine			101	11	82
Sulfadoxine / Sulphadoxine	Sulfonamide	10	101	15	75
Sulfaethoxypyridazine			102	13	79
Sulfamerazine			103	16	76
Sulfamethazine / Sulfaimidine			99	12	78
Sulfamethoxazole			99	13	76
Sulfamethoxypyridazine			102	15	75

Analyte [a,b]	Class	Screening TTL (ug/Kg)	Ave % Rec	SD	Limits test threshold (% compared to TTL)
Sulfamonomethoxine			103	12	83
Sulfapyridine			99	14	75
Sulfaquinoxaline			99	14	75
Sulfathiazole			100	13	78
Chlortetracycline	Tetracyclines	100	92	13	70
Doxycycline			94	12	73
Oxytetracycline			88	11	69
Tetracycline			85	9	69
Amoxicillin	β-lactam	100	88	12	68
Amoxicillin Diketone		25	82	11	64
Ampicillin			89	10	72
Aspoxicillin			89	10	71
Cephapirin			85	13	63
Cloxacillin			92	12	72
Dicloxacillin			93	21	57
Nafcillin			77	17	48
Oxacillin			84	8.3	70
Penicillin G[c]			61	14	29
Penillic acid		NA - metabolite	97	15	71
Penicillin V		25	90	14	66
Azamethiphos	Organophosphate antiparasitic	10	102	12	81
Dichlorvos	Organophosphate pesticide		111	17	82
Malathion			103	22	66
Quinalphos			113	16	86
Trichlorfon			104	15	78
Atrazine	Triazine herbicide		100	11	81
Propazine			102	9	87
Simazine			99	12	79

[a] Compounds in blue italics are evaluated with the ACN injection
[b] Compounds with strikethrough did not perform well and/or did not produce fragment ions
[c] Penicillin G degraded to penillic acid which was used as the marker compound in this method

Table 6: Limits Test Thresholds (% of the 1X TTL) for Negative Ion Analytes

Analyte	Class	Screening TTL (ug/Kg)	Ave % Rec	SD	Limits test threshold (% compared to TTL)
Chloramphenicol	Phenicol	0.3	114	15	88
Florfenicol		5	114	11	95
Thiamphenicol			116	14	92
~~Toltrazuril~~	Toltrazuril	50			
~~Toltrazuril Sulfone~~					
Toltrazuril Sulfoxide			101	9.9	84
Diflubenzuron	Benzylurea	50	109	9	94
Lufenuron			106	14	92
Hexaflumuron			111	11	92
Teflubenzuron			102	10	84

[a] Compounds in blue italics are evaluated with the ACN injection
[b] Compounds with strikethrough did not perform well and/or did not produce fragment ions

Compound Identification

In order to be qualitatively identified in the TraceFinder program, the precursor ions must be present (signal-to-noise >3) and match theoretical exact mass within a 5 ppm mass tolerance. The data analysis program searched for residues within a time window of 60 s (30 s each side of specified retention time), but a narrower retention time match (0.1 min) to a standard injected the same day was typically observed. For most compounds, the most abundant peak in each time window was monitored rather than the peak with the closest retention time. Product ion detection was also required with at least 1 fragment with 500 count minimum intensity threshold within a 10 ppm maximum mass deviation window. These criteria are consistent with the FDA acceptance criteria for "Confirmation of Identity of Chemical Residues Using Exact Mass Data for the FDA foods and Veterinary Medicine Program". [10] The isotope match feature was also enabled for all compounds (except for leucocrystal violet because ions from other analytes or matrix components interfered with that isotope pattern) with a 70% fit threshold, 5 ppm mass deviation, and 10% intensity deviation allowance. A sample would be considered presumptive positive for a test compound if the qualitative criteria were met, and the signal was ≥ 50% as compared to the matrix-extracted standard fortified at the TTL. Table 7 shows the number of analytes that met these identification criteria at each fortification level over the three-day validation. The number of analytes that met qualitative identification criteria was used to corroborate the limit test calculation (i.e. analytes generally met identification criteria at or above the limits test threshold).

Table 7: Shrimp Validation: Confirmation of Identity Data at Each Level

			0.5X Qual Results		1X Qual Results		2X Qual Results	
			n=9		n=21		n=9	
Drug	**Screening TTL, ng/g**	**RT**	**Final Extract**	**ACN injection**	**Final Extract**	**ACN injection**	**Final Extract**	**ACN injection**
Positive Mode								
2-amino mebendazole	5	5.7	9/9		21/21		9/9	
5 hydroxymebendazole	5	5.8	9/9		21/21		9/9	
Abamectin (Avermectin B1a)	200	11.2	9/9	9/9	21/21	21/21	9/9	9/9
Albendazole	50	7.35	9/9		21/21		9/9	
Albendazole sulfone	50	6.35	9/9		21/21		9/9	
Albendazole sulfoxide	50	5.2	9/9		21/21		9/9	
Amoxicillin	100	1.4	9/9		21/21		9/9	
Amoxicillin Diketone	25	4.8	9/9		21/21		9/9	
Ampicillin	25	4.4	9/9		21/21		9/9	
Aspoxicillin	25	2.2	9/9		21/21		9/9	
Atrazine	10	8.4	9/9		21/21		9/9	
Azamethiphos	10	5.8	9/9		21/21		9/9	
Azithromycin	50	5.8	9/9		21/21		9/9	
Baquiloprim	50	1.5	9/9		7/7		3/3	
Brilliant Green	1	9.9	5/9	9/9	5/21	9/9	7/9	9/9
Cefapirin (Cephapirin)	25	2.9	8/9		21/21		9/9	
Chlortetracycline	100	5.8	9/9		31/21		9/9	
Ciprofloxacin	5	4.9	9/9		21/21		9/9	
Cloxacillin	25	9.1	9/9		21/21		9/9	
Crystal violet (Gentian Violet)	1	9.35	2/9	8/9	12/21	19/21	9/9	9/9
Danofloxacin	5	5.1	9/9		21/21		9/9	
Dichlorvos	10	7.6	9/9		21/21		9/9	
Dicloxacillin	25	9.75	6/9		20/21		9/9	
Difloxacin	5	5.6	9/9		21/21		9/9	
Doramectin	200	11.5	6/9	9/9	20/21	21/21	9/9	9/9
Doxycycline	100	6.2	9/9		21/21		9/9	
Emamectin B1a	200	9.9	9/9	9/9	21/21	21/21	9/9	9/9
Enrofloxacin	5	5.2	9/9		21/21		9/9	
Eprinomectin B1a	200	11	9/9	9/9	21/21	21/21	9/9	9/9
Erythromycin A	50	7	9/9		21/21		9/9	
Erythromycin dehydrated	50	7.9	9/9		21/21		9/9	
Ethoxyquin	50	7.9	9/9		21/21		9/9	
Ethoxyquin Dimer	50	12	2/9	9/9	10/21	7/21	0/9	0/9
Fenbendazole	50	8.6	9/9		21/21		9/9	

			0.5X Qual Results		1X Qual Results		2X Qual Results	
			n=9		n=21		n=9	
Drug	**Screening TTL, ng/g**	**RT**	**Final Extract**	**ACN injection**	**Final Extract**	**ACN injection**	**Final Extract**	**ACN injection**
Fenbendazole sulfone	50	12.2	9/9		21/21		9/9	
Flofenicol amine	5	1.2	9/9		21/21		9/9	
Flumequine	10	8.1	9/9		21/21		9/9	
Ivermectin B1a	10	12.2	5/9	9/9	16/21	21/21	9/9	9/9
Ketoconazole	10	7.6	9/9		21/21		9/9	
Leucocrystal violet (leucogentian violet)	1	5.4	0/9	0/9	0/21	0/21	0/9	0/9
Leucomalachite green	1	8.6	2/9	3/9	14/21	2/21	8/9	4/9
Linocmycin	50	3.5	9/9		9/9		9/9	
Malachite green	1	8.5	6/9		21/21		9/9	
Malathion	10	10.2	9/9		21/21		9/9	
Marbofloxacin	5	4.5	9/9		21/21		9/9	
Mebendazole	5	7.5	9/9		21/21		9/9	
methyl testosterone	0.8	10	9/9		21/21		9/9	
Methylene Blue	10	6.5	9/9		21/21		9/9	
Metronidazole	10	1.6	9/9		21/21		9/9	
Moxidectin	200	11.5	0/9	7/9	2/21	19/21	2/9	9/9
Nafcillin	25	9.2	9/9		21/21		9/9	
Nalidixic Acid	10	7.8	9/9		21/21		9/9	
Norfloxacin	5	4.8	9/9		21/21		9/9	
Ofloxacin (racemic mix) or Levofloxacin (L-)	5	4.8	9/9		21/21		9/9	
Orbifloxacin	5	5.2	9/9		21/21		9/9	
Ormetoprim	10	4.7	9/9		21/21		9/9	
Oxacillin (Oxocillin)	25	8.65	9/9		21/21		9/9	
Oxolinic acid	10	6.7	9/9		21/21		9/9	
Oxytetracycline	100	4.7	9/9		21/21		9/9	
Penicillin G degrad- Penillic acid	25	4.9	8/9		21/21		21/21	
Penicillin G later elute	25	7.7	8/9		21/21		21/21	
Penicillin V	25	8.3	2/3		21/21		21/21	
Propazine	10	9.6	9/9		21/21		21/21	
Quinalphos	10	10.4	9/9		21/21		21/21	
Simazine	10	7.3	9/9		21/21		9/9	
Spiramycin	50	8.5	9/9		21/21		9/9	
Sulfacetamide	10	2	8/9		21/21		9/9	
Sulfachloropyridazine	10	5.8	9/9		21/21		9/9	
Sulfaclozine/Sulfachloropyrazine	10	7.05	9/9		21/21		9/9	
Sulfadiazine	10	2.4	9/9		21/21		9/9	

			0.5X Qual Results		1X Qual Results		2X Qual Results	
			n=9		n=21		n=9	
Drug	**Screening TTL, ng/g**	**RT**	**Final Extract**	**ACN injection**	**Final Extract**	**ACN injection**	**Final Extract**	**ACN injection**
Sulfadimethoxine	10	7.1	9/9		21/21		9/9	
Sulfadoxine (Sulphadoxine)	10	6.1	9/9		21/21		9/9	
Sulfaethoxypyridazine	10	6.3	9/9		21/21		9/9	
Sulfamerazine	10	3.9	9/9		21/21		9/9	
Sulfamethazine (Sulfadimidine)	10	4.7	9/9		21/21		9/9	
Sulfamethoxazole	10	6.2	9/9		21/21		9/9	
Sulfamethoxypyridazine	10	5.1	9/9		21/21		9/9	
Sulfamonomethoxine	10	5.7	9/9		21/21		9/9	
Sulfapyridine	10	3.6	9/9		21/21		9/9	
Sulfaquinoxaline	10	7.2	9/9		21/21		9/9	
Sulfathiazole	10	3.5	9/9		21/21		9/9	
Tetracycline	100	5	9/9		21/21		9/9	
Thiabendazole	10	3.8	9/9		21/21		9/9	
Tilmicosin	50	6.4	9/9		21/21		9/9	
Trichlorfon	10	5.3	9/9		21/21		9/9	
Trimethoprim	10	4.3	9/9		21/21		9/9	
Tylosin A	50	7.3	9/9		21/21		9/9	
Negative Mode								
Chloramphenicol	0.3	6.7	9/9		21/21		9/9	
Diflubenzuron	50	10.3	9/9	9/9	21/21	21/21	9/9	9/9
Florfenicol	5	6.3	9/9		21/21		9/9	
Hexaflumuron	50	10.5	9/9	9/9	21/21	21/21	9/9	9/9
Lufenuron	50	10.65	9/9	9/9	21/21	21/21	0/9	9/9
Teflubenzuron	50	10.5	9/9	9/9	21/21	21/21	9/9	9/9
Thiamphenicol	5	4.8	9/9		21/21		9/9	
Toltrazuril	50	10.3	4/9		11/21		4/9	
Toltrazuril sulfone	50	10.2	0/9		0/21		0/9	

Variation of Sample Size

Although this validation was performed with 2.0 g of shrimp tissue as described in LIBs #4615/4616, additional experiments were performed with an initial sample size of 4.0 g. This was done to determine if extracts prepared with 4.0 g (such as described in LIB #4653R[8]) would be suitable for HRMS analysis using this method. Four gram shrimp samples (n=3) were spiked at the 1X TTL level (doubling volume of fortification standards) and taken through the extraction procedure analyzed by this HRMS method. The only modification to the extraction procedure as written was to use 400 µL water rather than 400 µL reconstitution solution in step 12. Regardless of the sample size, 84 of the 88 positive ion analytes met identification criteria (dyes and some avermectins were not identified).

Limitations of the Method

Almost all of the analytes included in this validation study were consistently detected and identified at the levels of interest. Below are the compounds that did not perform well and/or did not produce fragment ions in each acquisition:

Positive mode final extract: Ethoxyquin Dimer (recoveries were variable)

Positive mode acetonitrile before drying and final extract: Leucocrystal violet, Leucomalachite green, and Selemectin were not detected, or recoveries were variable

Negative mode final extract: Toltrazuril and Toltrazuril sulfone did not generate product ions

Negative mode acetonitrile before drying: all analytes evaluated in this extract met criteria

CONCLUSION

LIBs #4515/4616 was validated in the DENL for the qualitative analysis of targeted veterinary drugs in shrimp. The results of the validation were consistent with the stated method performance and with results obtained by previous publications. This validation studies also demonstrate that LIBs #4615/16 can be used to screen samples at the stated screening TTL (ug/kg). The method validation meets the criteria for high resolution mass spectrometry confirmation of identity for residues of the targeted list of veterinary drugs.

Overall, the validation performed similar results to LIBs #4615/4616 and other scientific articles published by ADRC. In addition to the 1X data shown, 94% at 2X of the ~100 analytes in 9 reps (900), all but 53 met all criteria. At the 0.5X spike level, the number of analytes that met these presumptive positive criteria varied depending on the analyte. That indicates some additional analysis may be needed if a residue is present near the 0.5X level, but there should be very few false negatives for residues near the TTL. There were no presumptive positives for any analytes in the control matrix samples that were analyzed.

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Appendix A. Example of Stable Mixed Standard Spiking Solution (1X TTL CCV and ICV) from Purchased Mixes and Individual Stock Solutions

Unique Identifier: W-E47-17-03-01-CCV **Prepared:** 12/12/2020 **Expired:** 11/1/2021
HRMS Stable Mix- CCV **Analytes:** CRC **Solvent:** Acetonitrile

Compounds	Unique Identifier	stock conc. (ug/mL)	Vol. used (uL)	Final Vol. (mL)	final conc (ng/mL)	uL Added	Grams of Matrix	TTL - Spike Leve ng/g
Sulfonamides (16)/ potentiator (2)/Hhormone (1)								
Sulfapyridine	SPEX: LCMS-FDACO-51 LIMS: 277107 Lot No. GS201113004 Exp: 11/13/2021	100	125.0	25.0	500	40	2.00	10.0
Sulfadiazine								
Sulfathiazole								
Sulfaquinoxaline								
Sulfadimethoxine								
Sulfachloropyridazine								
Sulfamerazine (Sulfadimidine)								
Sulfamethazine								
Sulfamethoxazole								
Trimethoprim								
Sulfamethoxypyridazine								
Sulfaethoxypyridazine								
Sulfadoxine								
Sulfanitran								
Sulfamonomethoxine								
Sulfacetamide								
Methyl Testosterone		8.0			40			0.800
Sulfaclozine	S-E47-14-08-16	389.3	32		500			10.0
Ormetoprim	S-E47-15-01-45	568.5	22					
Fluoroquinolones/Quinolones (12)								
Ciprofloxacin	SPEX: LCMS-FDACO-52 LIMS: 276529 Lot No. AA201109014 Exp: 11/09/2021	50	125	25.0	250	40	2.00	5.00
Danofloxacin								
Difloxacin								
Enrofloxacin								
Flumequine		100			500			10.00
Nalidixic Acid								
Norfloxacin		50			250			5.00
Ofloxacin								
Oxolinic Acid		100			500			10.0
Orbifloxicin		50			250			5.00
Sarafloxacin								
Marbofloxacin	S-E47-14-08-15	446.6	14		250			5.00
Benzimidazole/Anthelmintics (10)								
Mebendazole	SPEX:LCMS-FDACO-25 LIMS: 275088 Lot No.GS201020023 Exp: 10/20/2021	50.0	125.0	25.0	250	40	2.00	5.00
Mebendazole amine								
5-hydroxymebendazole								
Albendazole	S-E47-14-08-04	341.9	183		2500			50.0
Albendazole Sulfoxide	S-E47-14-08-05	262.7	238					
Albendazole Suphone	S-E47-14-08-06	246.5	254					
Fenbendazole	S-E47-14-08-10	392.6	159					
Fenbendazole Sulfone	S-E47-14-08-11	330.8	189					
Thiabendazole	S-E47-15-01-47	653.9	19		500			10.0
Phenicols (4)								
Chloramphenicol	I-E47-15-07-05	4.99	75	25.0	15	40	2.00	0.300
Florphenicol	S-E47-15-01-41	279.3	22		250			5.00
Florphenicol Amine	S-E47-15-01-42	169.1	370		2500			50.0
Thiamphenicol	I-E47-15-07-06	99.9	63		250			5.00
Macrolids (6)								
Azithromycin	S-E47-14-08-07	263.3	237	25.0	2500	40	2.00	50.0
Erthromycin	LIMS 257092- Liquid	1000	63					
Lincomycin	S-E47-14-08-13	322.4	194					
Spiramycin	S-E47-14-08-17	250.2	250					
Tilmicosin	S-E47-14-08-18	377.9	165					
Tylosin	S-E47-14-08-19	270.0	231					
Nitromidazole (2)								
Metronidazole	S-E47-14-08-14	363.1	34	25.0	500	40	2.00	10.0
Ketoconazole	S-E47-15-01-43	468.4	27					
Preservative (2)								
Ethoxyquin	S-E47-14-08-09	1187.2	53	25.0	2500	40	2.00	50.0
Ethoxyquin dimer	S-E47-15-01-40	126.6	494					

Compounds	Unique Identifier	stock conc. (ug/mL)	Vol. used (uL)	Final Vol. (mL)	final conc (ng/mL)	uL Added	Grams of Matrix	TTL - Spike Level ng/g
Benzylurea (4)								
Diflubenzuron	S-E47-15-01-39	571.5	109	25.0	2500	40	2.00	50.0
Hexaflumuron	S-E47-15-04-27	568	110					
Lufenuron	S-E47-15-01-44	496.1	126					
Teflubenzuron	S-E47-15-01-46	708.1	88					
Avermectins (7)								
Abamectin	S-E47-14-10-04	610.5	410	25.0	10000	40	2.00	200
Doramectin	S-E47-14-10-05	470.4	531					
Emamectin	S-E47-14-10-06	678.8	368					
Eprinomectin	S-E47-14-10-07	484.8	516					
Ivermectin	S-E47-14-10-08	616.8	405					
Moxidectin	S-E47-14-10-09	454.0	551					
Selemectin	S-E47-14-10-10	407.6	613					
Pesticides (8)								
Atrazine	S-E47-15-01-35	523.3	24	25.0	500	40	2.00	10.0
Azamethiphos	S-E47-15-01-36	537.1	23					
Dichlorvos	I-E47-15-07-07	101	124					
Malathion	I-E47-15-07-08	104.5	120					
Propazine	S-E47-15-04-29	443.8	28					
Quinalphos	S-E47-15-04-28	315.9	40					
Simazine	S-E47-15-04-31	526.4	24					
Trichlorfon	S-E47-15-04-32	505.9	25					
Toltrazuril (3)								
Toltrazuril	S-E47-15-01-49	479.7	130	25.0	2500	40	2.00	50.0
Toltrazuril Sulfone	S-E47-15-01-50	280.9	222					
Toltrazuril Sulfoxide	S-E47-15-01-51	133.0	470					

TTL = Target Testing Level

Example Calculation:

Spiking Solution = Dichlorvos = 100.6 *0.1243 /25 = 500 ug/mL

Spike level = 500 ng/mL X 0.04 mLs/ 2.00 grams = 10 ng/g

Appendix B. Example of Unstable Mixed Standard Spiking Solution (1X TTL CCV and ICV) from Purchased Mixes and Individual Stock Solutions

HRMS: CCV Unstable Mix in Water **Date:** 12/12/2020 **Expired:** 6/1/2021

Unique ID: W-E47-17-04-01 CCV **Prepared:** Christine R. Casey

Compounds	Unique ID, SOLN	Stock Conc (ug/mL)	Vol stock used uL	Final Vol (mL)	Final conc (ng/mL)	uL Added	Grams of Matrix	Spike Level
Tetracyclines (4)								
Chlortetracycline	LIMS: 277040 LCMS-FDACO-31 Lot No.GS201111005 Exp. 11/11/2021	500.0	500	25.00	10,000	20	2.00	100
Doxycycline								
Oxytetracycline								
Tetracycline								
Beta- Lactams (10)								
Amoxicillin	S-E47-17-01-24	387.0	646	25.00	10,000	20	2.00	100
Ampicillin	S-E47-17-01-25	512.0	122	25.00	2,500	20	2.00	25
Cloxacillin	S-E47-17-01-26	376.2	166					
Dicloxacillin	S-E47-17-01-27	388.7	161					
Nafcillin	S-E47-17-01-28	464.1	135					
Oxacillin	S-E47-17-01-29	347.9	180					
Penicillin G	S-E47-17-01-30	524.8	119					
Penicillin V	S-E47-17-01-31	487.2	128					
Aspoxicillin	S-E47-15-04-24	441.9	141					
Amoxicillin Diketopiperazine	S-E47-15-01-34	160.5	389					
Cephalosporin (1)								
Cefapirin (Cephapirin)	S-E47-15-01-37	407.4	153	25.00	2,500	20	2.00	25
Potentiator (1)								
Baquiloprim	S-E47-15-04-25	274.7	91	25.00	1,000	20	2.00	10
Dyes (6)								
Malachite Green	LIMS:276107 GO-FDACO-21 Lot No. GS201103016 Exp. 11/03/2021	10.00	250	25.00	100	20	2.00	1.00
Crystal Violet								
Brilliant Green								
Leuco Malachite Green								
Leuco Crystal Violet								
Methylene Blue	I-E47-15-07-09	10.1	248	25.00	100	20	2.00	1.00

All solution are added one 25.00 volumetric flask