Expanding LIB 4615 and 4616 to include additional chemical contaminants in the analysis of tilapia, salmon, eel and shrimp using Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS)

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ABSTRACT

The Liquid Chromatography High-Resolution Mass Spectrometry (LC-HRMS) methodology described in LIBs 4615 and 4616 was developed by the Denver Animal Drugs Research Center to analyze for commonly used veterinary drugs that might adulterate different types of aquaculture products. In the LIB described here, this earlier procedure was further evaluated for its feasibility to detect several other classes of compounds that might also be a concern as possible contaminants in tilapia, salmon, eel and shrimp. Some chemicals could contaminate water sources used in aquaculture production through agricultural runoff. These compounds include several widely used triazine herbicides, organophosphate and carbamate pesticides, as well as various discarded human pharmaceuticals. Other possible contaminants investigated were selected disinfectants, some newer antibiotics, growth promoters, and various parasiticides. Most of the new compounds tested worked well using the rapid clean-up procedure and HRMS detection methodology described in LIB 4615 and 4616. Using exact mass identification criteria, most analytes had screening limit levels of between 0.5-10 ng/g in the matrices examined. The screening procedure was not successful for a few compounds for various reasons as detailed in the results and discussion section.

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INTRODUCTION

LIB 4615[1] described a rapid cleanup procedure for analyzing veterinary drug residues in fish, shrimp and eel using Liquid Chromatography connected to a Q-Exactive High Resolution Mass Spectrometer (LC-HRMS). The cleanup procedure mentioned used a newer gravity-drain SPE column specifically designed to remove phospholipids from biological sample extracts, thereby greatly improving MS detection. LIB 4616[2] details various Q-Exactive mass spectrometer optimization procedures used to detect those analytes listed in LIB 4615. These two LIBs together were used to detect a wide range of 60 "test" compounds (representing a variety of common veterinary drug classes) most likely to be illegally used in aquaculture products. The MS detection used high resolution Orbitrap technology. (These two LIBs were later combined and published in the *Journal of Agricultural and Food Chemistry*[3]). The procedure in these two LIBs is now being used to further analyze regulatory aquaculture samples that are found to be violative using FDA's current targeted-analysis triple quadrupole LC/MS method[4]. Since LIB 4615 and 4616 also proved to be successful[3,5] in analyzing for these initial test compounds (mostly antibiotics) in spiked and incurred aquaculture tissues, expanding the method for additional types of analytes was investigated.

HRMS instruments such as the Q-Exactive have the potential to analyze many compounds simultaneously because full-scan data are collected, instead of pre-selected ion transitions corresponding to specific compounds, which is the traditional approach of targeted triple-quadrupole MS methods. In theory, any sample injection's data file obtained using the Q-Exactive operating in a non-targeted fashion could be retroactively examined for other compounds of interest that might arise at some future date. This is possible because HRMS has the advantage of providing very accurate mass measurements at low levels with great selectivity. Although these features of HRMS are very useful and powerful, there are some limitations to retroactive searching for non-targeted compounds. The compound of interest may not ionize well with the MS source parameters used, and therefore have very poor sensitivity, or the compound may not chromatograph on the column and mobile phase chosen. In addition, the analyte may not be recovered in the cleanup procedure used. This might happen to a different degree with different biological matrices. For example, an eel sample with sauce is often a more difficult matrix than a less oily fish such as tilapia. Finally, any retroactive searching that produces a presumptive accurate mass and isotopic match for a specific analyte may still not be sufficient for assessing residue identity confirmation without additional retention time matching with a reference standard. This can especially be a problem for compounds with ubiquitous lower molecular weight masses. For these reasons, actual matrix spikes at the level of interest can be necessary when determining whether the analytical method can analyze various compounds when extending the method's applicability. This LIB describes the performance of such matrix spikes using additional classes of compounds beyond those included in LIB 4615 and 4616. Spikes of all compounds were made at levels of 100, 10 and 1 ng/g to test method performance and determine confirmatory screening limits for each added compound.

Table 1. New Compounds Tested in the Fish Screening Method

Compound	Description	Molecular Formula	Ret. Time (min)	Precursor	Product 1	Product 2	Product 3
Acriflavine 1 [a]	Antifungal dye	$C_{14}H_{14}ClN_3$	5.0	224.11822	209.09475	182.08385	
Acriflavine 2 [a]	Antifungal dye	$C_{14}H_{14}ClN_3$	5.3	224.11822	209.09475	182.08385	
Aldicarb	Carbamate insecticide	$C_7H_{14}N_2O_2S$	6.6	213.06682 *(Na^+)*	116.05285	89.04195	
Aldicarb Sulfone	Carbamate metabolite	$C_7H_{14}N_2O_4S$	3.6	245.05665 *(Na^+)*	166.07127	109.04981	
Aldicarb Sulfoxide	Carbamate metabolite	$C_7H_{14}N_2O_3S$	2.3	229.06173 *(Na^+)*	166.07127	109.04981	
Amitraz (as DPMF) [b]	Insecticide (amitraz) metabolite	$C_{10}H_{14}N_2$	3.9	163.12298	132.08078	122.09643	107.07295
Atenolol	Hypertension drug	$C_{14}H_{22}N_2O_3$	1.5	267.17032	190.08626	145.06479	
Atrazine	Triazine herbicide	$C_8H_{14}ClN_5$	8.2	216.10105	174.05410	96.05562	
Azadirachtin	Limonoid insecticide	$C_{35}H_{44}O_{16}$	8.5	743.25216 *(Na^+)*	725.24159		
Azamethiphos	Organophosphate antiparasitic	$C_9H_{10}ClN_2O_5P$	7.5	324.98093	182.99541	139.00558	
Baquiloprim	Diaminopyrimidine potentiator	$C_{17}H_{20}N_6$	0.9	309.18222	294.15875	123.06652	
Benzocaine	Topical anesthetic	$C_9H_{11}NO_2$	6.9	166.08626	138.0545	94.06513	77.03900
Benzylalkonium Cl C12 [c]	Disinfectant	$C_{21}H_{38}N$	9.7	304.29988	212.23728	91.05423	
Benzylalkonium Cl C14 [c]	Disinfectant	$C_{23}H_{42}N$	9.9	332.33118	240.02686	91.05423	
Caffeine	Stimulant	$C_8H_{10}N_4O_2$	4.1	195.08765	138.06619	110.07127	
Carbamazepine	Antiseizure drug	$C_{15}H_{12}N_2O$	7.6	237.10224	194.09643	192.08078	
Carbaryl	Carbamate Insecticide	$C_{12}H_{11}NO_2$	8.2	202.08626	159.04406	145.06477	
Carbofuran	Carbamate Insecticide	$C_{12}H_{15}NO_3$	7.8	222.11247	165.09101	123.04406	
Clarithromycin	Macrolide antibiotic	$C_{38}H_{69}NO_{13}$	7.6	748.48417	590.38990	158.11760	

Clofibric acid	Herbicide	$C_{10}H_{11}ClO_3$		214.04022 *(NEG)*	126.99562		
Cypermethrin	Pyrethroid insecticide	$C_{22}H_{19}Cl_2NO_3$	ND[d]				
Dichlorvos	Organophosphate pesticide	$C_4H_7Cl_2O_4P$ 8.9	7.3	220.95318	144.98158	127.01547	78.99434
Diclofenac		$C_{14}H_{11}Cl_2NO_2$	10.0	296.02396	278.01340	250.01850	215.0496
Diltiazem	Hypertension drug	$C_{22}H_{26}N_2O_4S$	6.7	415.16860	370.11076	178.03211	150.0372
Diphenhydramine	Antihistamine	$C_{17}H_{21}NO$	6.4	256.16959	167.08553		
EtofenproxNSAID	Pyrethroid insecticide	$C_{25}H_{28}O_3$	ND				
Fipronil	Phenylpyrazole insecticide	$C_{12}H_4Cl_2F_6N_4OS$	10.1	434.93143 *(NEG)*	329.95954	249.95848	183.01756
Fipronil sulfone	Phenylpyrazole metabolite	$C_{12}H_4Cl_2F_6N_4O_2S$	10.2	450.92634 *(NEG)*	281.99256	243.98948	183.01756
Fluoxetine	Anti-depressant drug	$C_{17}H_{18}F_3NO$	7.5	310.14133	148.11208		
Gemfibrozil	Fibrate for lowering lipids	$C_{15}H_{22}O_3$	10.2	249.14962 *(NEG)*	121.06589		
Ibuprofen	NSAID	$C_{13}H_{18}O_2$	10.0	207.13796	166.09883		
Malathion	Organophosphate pesticide	$C_{10}H_{19}O_6PS_2$	10.0	331.04334	285.00148	127.03897	99.00767
Marbofloxacin	Fluoroquinolone antibiotic	$C_{17}H_{19}FN_4O_4$	4.3	363.14631	320.10410	205.03964	72.08780
Methylene Blue	Antifungal dye	$C_{16}H_{18}N_3S$	6.0	284.12159	268.09029	241.0794	
Naproxen	NSAID	$C_{14}H_{14}O_3$	9.1	231.10157	185.09610	170.0726	
Orbifloxacin	Fluoroquinolone antibiotic	$C_{19}H_{20}F_3N_3O_3$	5.0	396.15295	352.16310	295.1053	
Phoxim [e]	Organophosphate pesticide	$C_{12}H_{15}N_2O_3PS$	10.3	299.06138	216.99233	129.04478	
Praziquantel	Antiparasitic	$C_{19}H_{24}N_2O_2$	9.0	313.19105	203.11789	174.09134	83.08553
Proflavine[a]	Antifungal dye	$C_{13}H_{11}N_3$	4.9	210.10257	193.07602	166.06513	
Propazine	Triazine herbicide	$C_9H_{16}ClN_5$	9.3	230.11670	188.06975	146.02280	
Propranolol	Hypertension drug	$C_{16}H_{21}NO_2$	6.1	260.16451	183.08044	116.10699	
Quinalphos [e]	Organophosphate pesticide	$C_{12}H_{15}N_2O_3PS$	10.2	299.06138	242.99847	163.03245	147.05529
Quinoclamine	Naphthoquinone pesticide	$C_{10}H_6ClNO_2$	7.3	208.01598	172.03930	105.03349	
Ranitidine	Antacid	$C_{13}H_{22}N_4O_3S$	1.7	315.14854	270.09069	176.04882	130.05592
Rifampicin	Ansamycin antibiotic	$C_{43}H_{58}N_4O_{12}$	8.7	823.41240	791.38618		
Rifaximin	Ansamycin antibiotic	$C_{43}H_{51}N_3O_{11}$	9.8	786.35964	754.33342	362.11353	151.07536
Rotenone	Isoflavone pesticide	$C_{23}H_{22}O_6$	10.0	395.14891	241.08592	213.09101	192.07810
Roxithromycin	Macrolide antibiotic	$C_{41}H_{76}N_2O_{15}$	7.7	837.53185	679.43760	158.11760	

Simazine	Triazine herbicide	$C_7H_{12}ClN_5$	202.08540	132.0323	124.08692	96.05562
Simvastatin	Statin for lowering lipids	$C_{25}H_{38}O_5$	419.27920	285.18491	199.14813	
Sotalol	Antiarrhythmic drug	$C_{12}H_{20}N_2O_3S$	273.12674	255.11560	213.06882	133.07602
Sulfafurazole(sulfisoxazole)	Sulfonamide antibiotic	$C_{11}H_{13}N_3O_3S$ 7.0	268.07504	156.01140	108.04440	113.07090
Thiabendazole	Bendazole antiparasitic	$C_{10}H_7N_3S$ 10.5 1.4	201.03552	175.03250		
Trichlorfon	Organophosphate pesticide	$C_4H_8Cl_3O_4P$ 6.3	256.92985	220.95318	127.01547	
Triclocarban	Antibacterial agent	$C_{13}H_9Cl_3N_2O$	312.97077 *(NEG)*	159.97263	126.01160	
Triclosan	Antibacterial agent	$C_{12}H_7Cl_3O_2$ 3.8 5.0	286.94389 *(NEG)*	ND		
Trifluralin	Dinitroanaline herbicide	$C_{13}H_{16}F_3N_3O_4$ 10.3	336.11657	294.06847	236.02661	
Virginiamycin M1	Streptogramin antibiotic	$C_{28}H_{35}N_3O_7$ 10.3 10.6	526.25478	508.24420	337.11830	

[a] Commerically purchased acriflavine is a mixture of two acriflavine isomers with different retention times and proflavine (demethylated acriflavine)
8.5

[b] DPMF = N-(2,4-dimethylphenyl)-N-methylformamidine a breakdown product of amitraz was monitored as the marker of this compound.

[c] Benzylalkonium chloride is a mixture of analogs (C8-C16). The C12 and C14 analogs were the most abundant and were monitored in this method.

[d] ND = Not detected

[e] Phoxim and quinalphos are isomers and not completely chromatographically resolved in this method.

EXPERIMENTAL

Standard preparation and sample extraction

The compounds added to the method are those listed in Table 1. Individual, accurately weighed stock standards at approximately 100 µg/mL were made of the listed compounds. In addition, stock standards of compounds attempted but not recovered by the method (see results and discussion section) were also prepared. All stock standards were made in methanol (except for simazine which was prepared in 1:1 THF/methanol). All reference standards were obtained from Sigma or USP.

A spiking mix (labeled as spiking mix A) containing all compounds at a level of 2 µg/mL as the free base or acid in acetonitrile was prepared. However, two compounds, marbofloxacin and orbifloxicin, were prepared in spiking mix A at a level of 0.1 µg/mL. These fluoroquinolone, or FQ compounds do have an FDA "target testing level or TTL" of 5 ng/g so they were added to the spiking mix A at a lower concentration. Using the extraction procedure of LIB 4615, 100 µL of spiking mix A was added to 2 g of tissue to make a 100 ng/g spike of the listed compounds (except for the FQs which would be at 5 ng/g). Spiking mix B (a 1:10 dilution of spiking mix A) and spiking mix C (a 1:10 dilution of spiking mix B) were also prepared in acetonitrile. Spikes of 10 ng/g (0.5 ng/g for the FQs) were prepared by adding 100 µL of spiking mix B to 2 g of tissue. Spikes of 1 ng/g (.05 ng/g for the FQs) were prepared by adding 100 µL of spiking mix C to 2 g of tissue. The spiking levels are summarized below in Table 2.

Table 2. Spiking Protocol

Spiking Mix	Analyte Concentration (µg/mL)	Volume added to 2 g tissue (µL)	Spiking Level (ng/g)
A	2 (0.1 for 2 FQs)	100	100 (5 for 2 FQs)
B	0.2 (0.01 for 2 FQs)	100	10 (0.5 for 2 FQs)
C	0.02 (0.001 for 2 FQs)	100	1 (0.05 for 2 FQs)

For each extraction batch, the relevant spiking standard mix was diluted 1:20 with 10% acetonitrile in water to make an equivalent solvent standard for that spiking level (100, 10 or 1 ng/g) tested. The (1 point) solvent standard was used to estimate analyte recovery. The solvent standard was also used as a continuing CCV standard. With each analytical batch, reagent blanks and blank matrix controls were also extracted. The matrix blanks were recent regulatory samples determined to be negative by Denver Laboratory's targeted testing method[4].

For a more detailed description of the experimental procedure, including information on sample and reagent preparation, refer to LIB #4615[1].

1. Weigh 2.0 ± .05 g of homogenized tissue into a 50 mL polypropylene tube.

2. Add spiking standard mixtures as appropriate.

3. Add 8 mL of extraction solution. The extraction solution consists of 0.2% p-toluene sulfonic acid monohydrate (w/v) and 2% glacial acetic acid (v:v) in 100% acetonitrile (ACN).

4. Vortex for 30 minutes (Fisher Multi-tube vortexer, speed setting 2500 rpm).

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

6. Transfer 3 mL of the extractant into an Oasis PRIME HLB 6cc (200mg) Extraction Cartridge (with an empty 15 mL polypropylene tube underneath). Allow to gravity drain (ca. 10 minutes). With a pipet bulb, gently push out the remaining few drops of extractant through the SPE tube. (This should give just over 2 mL of liquid at this point). This SPE does not have to be conditioned prior to adding the extract.

7. For salmon (see note below**), transfer 100 µL of the extractant into a limited volume conical HPLC vial for a separate ACN injection.

8. Dry the remaining portion of the extract at 55 °C under 15 psi nitrogen. Take to near dryness (a drop of liquid remaining in the 15 mL tube is acceptable).

** ACN injection was not needed for compounds tested in this LIB, but should be included to cover all the compounds validated in LIB #4615/4616.

Instrumentation

The LC-MS instrumentation is described in LIB #4616[2]. A Q-Exactive Orbitrap was coupled to a Dionex 3000 LC system. The chromatographic parameters are the same as described earlier[1,2]. As before, data were collected primarily by using the "All Ion Fragmentation" mode of acquisition in which a full scan MS is followed by a MS^2 scan where all precursors are allowed into the high collision dissociation (HCD) cell to form product ions simultaneously. Additionally, two other types of data acquisition programs were used to analyze the fortified samples. Data independent acquisition (DIA) was evaluated as an alternative nontargeted screening HRMS method. The DIA program allowed for segments of precursor ions to be filtered through the quadrupole before forming product ions in the HCD cell. For the analyses described in this LIB, the screening results for DIA were very similar to AIF (data not shown); this will be discussed in more detail elsewhere[6]. Instead of performing DDMS2 for more targeted analysis with precursor

isolation as described in LIB #4616, "Parallel Reaction Monitoring" or PRM was performed to obtain product ion spectra for isolated precursor ions from the analytes listed in Table 1. With PRM all analytes in the inclusion list were isolated in the quadrupole at the appropriate retention time window to form product ions in the HCD cell. MS acquisition parameters for both the positive and negative ion AIF and PRM are listed below. (Positive ion mode was injected separately from the negative ion mode.)

All Ion Fragmentation (AIF)

Full MS: 70K resolution, $3e^6$ automatic gain control target, maximum inject time 200 ms, m/z 150-1000 scan range

AIF (MS^2): 70K resolution, $3e^6$ automatic gain control target, maximum inject time 200 ms, m/z 80-1000 scan range, normalized collision energy 10, 30, 50

Parallel Reaction Monitoring (PRM)

Full MS: 70K resolution, $3e^6$ automatic gain control target, maximum inject time 200 ms, m/z 150-1000 scan range

PRM (MS^2): 17.5K resolution, $1e^6$ automatic gain control target, maximum inject time 50 ms, loop count of 5, isolation width 1 amu, inclusion list N=60 (POS) or N=12 (NEG) compounds, 1 min time windows, normalized collision energy 10, 30, 50

Validation and Data Analysis

A Thermo TraceFinder "Quantitative Method" was established to provide data for the test compounds listed in Table 1 using the extracted ion chromatograms (5 ppm window) of precursor ions from the MS1 scan. One extracted spike replicate was used as a single point calibration point for semi-quantitative analysis. Recoveries were estimated by comparing this extracted sample to a solvent standard at the same concentration. Table 3 shows the number of spikes performed at each level for each type of fish meeting FDA OFVM Chemical Method Validation Guidelines[7] for Level Two qualitative validation. The screening limit was determined by the fortification level for each compound that met criteria for identification using exact mass data[8] and was significantly higher than any amount of analyte that was calculated to be in the matrix blank (amount due to background or carryover needs to be < 10% of that found in spike at the screening limit).

Table 3. Number of Spikes Analyzed

Spike Level	**Tilapia**	**Salmon**	**Shrimp**	**Eel**	**Total**
Matrix blank	6	4	4	4	**18****
1 ng/g	6	3	3	3	**15**
10 ng/g	6	6	6	6	**24**
100 ng/g	6	3	3	3	**15**
Total	**24**	**16**	**16**	**16**	**72**

** many additional matrix blanks of different tissues were also run earlier for LIB 4615[1] and 4616[2]

RESULTS AND DISCUSSION

Selection of new analytes

Recent published reviews[9,10] have discussed trends in aquaculture practices on farms around the world. Surveys have been done on many of these farms for several different internationally traded aquaculture species to gather information on the use of chemicals and other additives used in aquaculture production. Great effort is being given to using alternatives to antibiotics as increased monitoring for banned chemicals has resulted in increased commodity rejection. Developing bacterial resistance is also a concern for aquaculture farmers. Alternatively, helpful bacteria (probiotics such as *Nitrobacter* and *Bacillus*) are often now used to improve water quality for the stocking pond. One survey[9] reported that 31-97% of aquaculture farms used probiotics. This same survey reported that 24-50% of farms used naturally occurring feed additives and plant extracts to include medicinal herbs such as *Artemisia, Radix, Rheum* and others. These two groups of (non-toxic) additives are not applicable to LC-MS analysis. There are also reports of greater usage (5-78% of farms) of application of chemical disinfectants to stock ponds. Some of the disinfectants used are smaller inorganic molecules (such as iodine, hydrogen peroxide, hypochlorites and others) that are also not suitable for LC-MS detection. However, some disinfectants reported to be in use such as benzylalkonium chlorides (BAC), dichloro-dimethyl hydantoin (DCDMH), dibromo-dimethyl hydantoin (DBDMH), and trichloroisocyanuric acid were investigated for their recovery by the LC-HRMS procedure. Other surveys[11,12] have noted that various parasiticides have been used for parasites and fungal infections in fish. Other parasiticides have been used to kill unwanted organisms in culture ponds prior to stocking. Compounds in this group included phoxim, trichlorfon, trifluralin, azadirachtin, praziquantel and others. Other sources of unwanted chemicals that might contaminate aquaculture products are those chemicals derived from runoff from traditional agricultural farms. There are reports of such chemicals occurring in aquaculture ponds used for fish farming[12]. Compounds of concern in this regard include widely used

triazine herbicides (atrazine, simazine), carbamate pesticides including the toxic aldicarb and its metabolites, and organophosphate pesticides such as dichlorvos and malathion[12].

Although alternatives for aquaculture production are being utilized, antibiotics are still widely used. Newer antibiotics such as semi-synthetic "second generation" macrolides clarithromycin and roxithromycin were investigated for LC/MS analysis. Since fluoroquinolone residues (primarily ciprofloxacin and enrofloxacin) have been found in FDA regulatory aquaculture samples (both by the targeted LC/MS method[4], and also by LIBs 4615 and 4616), two additional fluoroquinolones, orbifloxacin and marbofloxacin were added. These two fluoroquinolone compounds were chosen because they have recently been added to the scope of analytes monitored in aquacultured products in Canada[13]. fipronil was added due to its unexpected recent use that resulted in the widespread contamination of millions of eggs around the world[14]. praziquantel was added because of its known widespread contamination of fresh water bodies throughout China[15]. The pesticide Azamethiphos was added, for although it is approved in the US (as the product Salmosan[16]) for use in farmed salmon for sea lice, its overuse is possible. The anesthetic Benzocaine can be used to keep some aquaculture species from fighting in confined ponds or to improve their successful transport[17]. Powerful growth promoters such as Virginiamycin may also be widely used. Misuse of rotenoids, pyrethroid derivatives, and Neem oil (azadirachtin) could also be a concern and they were added to the list. As illegal dye compounds, such as malachite green and crystal violet, are still occasionally found in FDA regulatory samples[4,18](primarily in eel), other dyes such as methylene blue, and acriflavine were investigated.

The possible occurrence of pharmaceuticals in aquaculture ponds could be yet another source of unwanted chemicals in aquaculture products. Extensive surveys[19,20] of rivers, estuaries, and wastewater streams throughout the US document the wide-ranging occurrence of pharmaceuticals and other unwanted chemicals in these water sources. This same problem is likely to occur in other countries, indicating a high potential for bioaccumulation of many of these compounds throughout the world. One recent study[21] of 12 fish species from a variety of families concluded that 65-86% of human drug compounds can accumulate in such tissue. Unfortunately, many of these compounds pass through wastewater treatment procedures. Over 4000 approved drug products are available in the US alone[22]. A few of the more widely used human compounds such as Zantac (Rantidine), Prozac (Fluoxetine), Ibuprofen, caffeine, Benadryl (diphenhydramine), propranolol, and statins were added to the screening protocol and assessed.

Screening Results

Spiking levels for all compounds were arbitrarily chosen at 100, 10, and 1 ng/g (for orbifloxacin and marbofloxacin the concurrent spiking levels chosen were 5, 0.5, and 0.05 ng/g). Table 3 shows how many replicate spikes were run for all compounds in each matrix investigated. Solvent standard mixtures were initially used to determine if the compounds would ionize sufficiently with the MS source parameters and if they would be retained on the chromatographic column used. Data including exact mass m/z values for precursor and

product ions, along with analyte retention times using the chromatographic method described in LIB #4616 are listed in Table 1. Compounds such as cypermethrin were unsuccessful at this point due to poor ionization in the dilute formic acid mobile phase while compounds such as the halogenated hydantoins and trichloroisocyanuric acid were found to break down in water making their MS analysis difficult.

Recoveries of different tissue matrices at the various spiking levels were estimated by comparison to an equivalent level (one point) solvent standard injected throughout each analytical injection sequence and are therefore estimates. However, the main reason for using AIF data collection in conjunction with the high resolution Orbitrap MS is as a "first pass" screening procedure to detect as many compounds (both targeted and non-targeted) as possible. In such a technique, precise quantitation is not the main goal-- instead, the procedure should detect the widest range of analytes at low levels. For this reason, the lowest confirmation limit level is more important to discern when adding new compounds to the procedure of LIBs #4615 and 4616. For any presumptive positive found by HRMS, a specific targeted-analyte triple-quadrupole MS technique can then be used if precise quantitation is desired. The lowest screening levels for each compound were determined to be the spiking level at which replicates met confirmation of identity criteria and had calculated abundances that were significantly higher than blank matrix. For exact mass detection using HRMS, FDA's confirmation criteria[8] require that the precursor ion of the unknown must agree within 5 ppm to the true precursor mass ion of the reference standard, one product ion must agree within 10 ppm to the reference standard, and the retention time must match (within 0.2 min) of the known standard. Because some compounds (e.g. benzyl alkyl chlorides, carbaryl) have significant signal in the blank matrices, a signal well above background (10X) was also required to define the screening limit for this LIB.

Table 4 is a summary of screening results in aquacultured samples for the investigated analytes using data gathered by the Q-Exactive HRMS operating in AIF. The compounds were all analyzed in positive mode except for those listed in the lower portion of Table 4, which were acquired using negative ion mode. The second column in the Table shows the screening detection limit in ng/g for each tested analyte that met FDA's accurate mass confirmation criteria and had signal significantly above that observed in blank matrix. Most of the investigated analytes (45 of 60) worked well in the LC-MS procedure with confirmation detection limits in matrix of 1 or 10 ng/g (or 0.5 and 5 ng/g for orbifloxacin and marbofloxacin). Those compounds listed in bold in Table 4 also had >30% recoveries at the various spiking levels (when compared to an equivalent level solvent standard), and the majority had recoveries well above 60%. These include compounds from several classes such as organophosphate pesticides, triazine herbicides, macrolide antibiotics, dyes, etc.

The compounds shown in italics or strike-through font did not perform as well in the method due to low signal, high background in the blank or other issues as discussed below. Ibuprofen, carbaryl, naproxen and gemfibrozil were problematic due to high background and low signal. The benzyl alkonium chloride compounds had significant background signal which were worse in salmon and eel matrices. Other compounds, such as diltiazem,

showed minimal (<10% compared to screening limit) signal contribution due to either matrix background or analyte carryover. The disinfectants triclocarban and triclosan had low recoveries through the method but could be detected in fish using negative ionization at higher levels. No characteristic product ions were observed for triclosan, so preliminary detection would be based only on the precursor ion. Others[23] have used m/z 35 (Cl^-) as a product ion for triclosan, but that ion is below the MS^2 scan range used in this method. Cypermethrin and etofenprox did not ionize with this method (these compounds are usually detected and chromatographed as ammonium adducts using a buffered mobile phase in other published methods). The polypeptide antibiotic Colistin was also investigated, but it did not survive the extraction procedure. The detection limits for trifluralin, even in solvent standard, were very high. Phoxim and Quinalphos responded well, but these compounds are isomers which co-elute and therefore could not be separately measured in the MS^1 extracted ion chromatogram with this screening method. These two compounds do have unique product ions and could be distinguished in a sample using the MS^2 data.

Table 4 shows screening detection limits in the four matrices tested: tilapia, shrimp, salmon, and eel. In general, the screening limits are dependent on the analyte itself and the results are similar in the different types of aquacultured products. For some compounds, the screening limits are higher in eel which is not surprising as this matrix is significantly more complex with added sauce, etc. The estimated recoveries in Table 4 are given for tilapia at the 10 ng/g spike level, but are generally similar for the other matrices tested, although the amounts estimated for eel were lower for some compounds (data not shown). Also, although LIB #4615 described the use of an additional injection of the initial acetonitrile sample extract for very non-polar analytes especially in fatty fish such as salmon, this extra injection (although it was performed) was unnecessary for the compounds investigated in this LIB.

In addition to AIF, PRM data were also collected for the compounds in the spiked samples evaluated in this study. Because precursor isolation (1 m/z isolation window) is initially performed, PRM acquisition provides cleaner product ion spectra that can be used for library searching. We have found that using PRM generates more consistent product ion spectra for compounds when using a limited (N<100) inclusion list as compared to DDMS2 which did not always trigger product ion spectra for all the analytes. In general, the screening levels determined for these analytes using PRM data collection were similar to those found with AIF, but in some cases (noted in Table 4), a higher level of residue was required to detect fragment ions using PRM acquisition. For example, azadirachtin forms a sodium ion precursor which is difficult to fragment. A characteristic fragment ion was observed with AIF, but not using PRM.

Example ion chromatogram data are shown in Figures 1-4. Figure 1 shows the extracted ion chromatograms (5 ppm window) of the precursor ions for several human drugs spiked into tilapia at 10 ng/g as compared to the blank matrix. Extracted ion chromatograms for both the precursor ion and product ions collected using AIF for atrazine in solvent standard (1 ng/g), shrimp spike (1 ng/g) and shrimp blank matrix are included in Figure 2. A small amount (<5%) of signal for atrazine is observed in the MS1 trace, but nothing is detected

in the product ion chromatogram. A comparison of the product ion spectra generated using either AIF or PRM for azamethiphos in salmon spiked at 10 ng/g demonstrates how isolating the precursor ion results in a much cleaner spectrum, but that the characteristic ions can still be detected in the AIF MS2 data (Figure 3). Finally, Figure 4 illustrates that to detect the disinfectant triclocarban, a higher level of residue (100 ng/g) and negative ion detection were required (PRM data shown).

CONCLUSION

The HRMS screening procedure described in LIBs 4615 and 4616 performed well with many other classes of compounds that might be of a concern as potential emerging aquaculture contaminants. Data gathered on the new analytes investigated included their estimated recovery and their minimum confirmation screening limit levels. LC column retention times and product ion information were also established, which should expedite compound library searching. This will help expand the applicability of the HRMS procedure as more regulatory aquaculture samples are investigated for a wider range of contaminants in the future.

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Table 4. Screening Results for New Compounds

	Screening Limit[a] (ng/g)				Approximate Recoveries (for10 ng/g in tilapia)	Comments
COMPOUND	**TILAPIA**	**SHRIMP**	**SALMON**	**EEL**		
Acriflavine 1	1	1	1	1	> 60	
Acriflavine 2	1	1[b]	[b]	[b]	30-60	
Aldicarb	1	1	10	1[b]	> 60	
Aldicarb Sulfone	1	10	10	10	> 60	
Aldicarb Sulfoxide	100	100	100 1	100	<30	
Amitraz Degradant 1 (DPMF)	10	10 1	10	1	> 60	
Atenolol	1	10	1[b]	10	> 60	
Atrazine	1	1	1	1	> 60	
Azadirachtin	10[d]	[d]	10[d]	10[d]	<30	
Azamethiphos	1	1	1	1	> 60	
Baquiloprim	100	100	10	10	<30	noted retention time shifts
Benzocaine	1	1	10	10	> 60	
Benzylalkonium chloride C12	10010	100	100	100	high	background 2-3 ng/g
Benzylalkonium chloride C14	100	100	100	100	high	background 4-8 ng/g
Caffeine	1	10	10	10	> 60	
Carbamazepine	1	1	1	1	> 60	
~~Carbaryl~~	>100	>100	>100	>100	ND	high background, low signal
Carbofuran	1	1	1	1	> 60	
Clarithromycin	1	1	1	1	30-60	
~~Cypermethrin~~	>100	>100	>100	>100	ND	

Dichlorvos	1[b]		1	1	> 60	
Diclofenac	1	1	1	10	> 60	
Diltiazem	1	1	1	1	30-60	low level carryover
Diphenhydramine	1	1	1	1	> 60	
~~Etofenprox~~	>100	>100	>100	>100	ND	
Fluoxetine	1	1	1[b]	10[c]	> 60	
~~Ibuprofen~~	>100	>100	>100		ND	high background, low signal
Malathion	1	1	1	1[b]	> 60	
Marbofloxacin	5	5	5	5	> 60	
Methylene Blue	1	1	1	1	30-60	
Naproxen	100	100	100	>100	> 60	
Orbifloxacin	0.5	0.5	0.5	0.5	> 60	
Phoxim	1[b]		1[b]	[b]	> 60	
Praziquantel	1	1	1	1	> 60	
Proflavine	1	1[b]	[b]	[b]		
Propazine	1	1	1	10	> 60	
Propranolol	1	1	1	1	30-60	
Quinalphos	1	1	1	1	> 60	
Quinoclamine	1	1[b]	[b]	[b]	> 60	
Rantidine	1	1	1[b]	10	<30	noted retention time shifts
Rifampicin	1	1[b]		10	30-60	
Rifaximin	1	1[b]	[b]	1	> 60	
Rotenone	1	1	1[b]	10	30-60	
Roxithromycin	1	1	1	1	30-60	low level carryover
Simazine	1	1	1	1	> 60	
Simvastatin	10[b]		10	100	> 60	
Sotalol	1	1	1	1	> 60	
Sulfisoxazole (Sulfafurazole)	1	1	1	1	> 60	
Thiabendazole	1	1	1	1	> 60	

Trichlorfon	1	1	1	1	> 60	
~~Trifluralin~~	>100	>100	>100	>100	<30	
Virginiamycin M1	1	1	1	1	> 60	
NEGATIVE ION COMPOUNDS						
Clofibric acid	1	1	1	1	> 60	
Fipronil	1	1	1	1	> 60	
Fipronil sulfone	1	1[b]	[b]	[b]	> 60	
~~Gemfibrozil~~	?	100	10[c]	>100	ND	high background, low signal
Triclocarban	10[c]	100	10	10	<30	
Triclosan	10[c]	100	100[1]	>100	<30	

1

[a] Screening Limit is defined as fortification levels where confirmation of identity is met for accurate mass data and calculated amount of analytes in samples is >10X that found in blank matrix. Levels are given for data acquisition using AIF. Screening levels obtained with Parallel Reaction Monitoring (PRM) are the same unless indicated.

[b] Screening Limit with PRM is 10 ng/g

[c] Screening Limit with PRM is 100 ng/g

[d] Screening Limit with PRM is > 100 ng/g

Figure 1. Extracted ion chromatograms (EICs with 5 ppm window) for (A) caffeine, m/z 195.08765 (B) fluoxetine, m/z 310.14133 and (C) simvastatin, m/z 419.27920 in tilapia spiked at 10 ng/g (top) and blank tilapia matrix (bottom). Data from MS1 scan collected using all ion fragmentation (AIF) program.

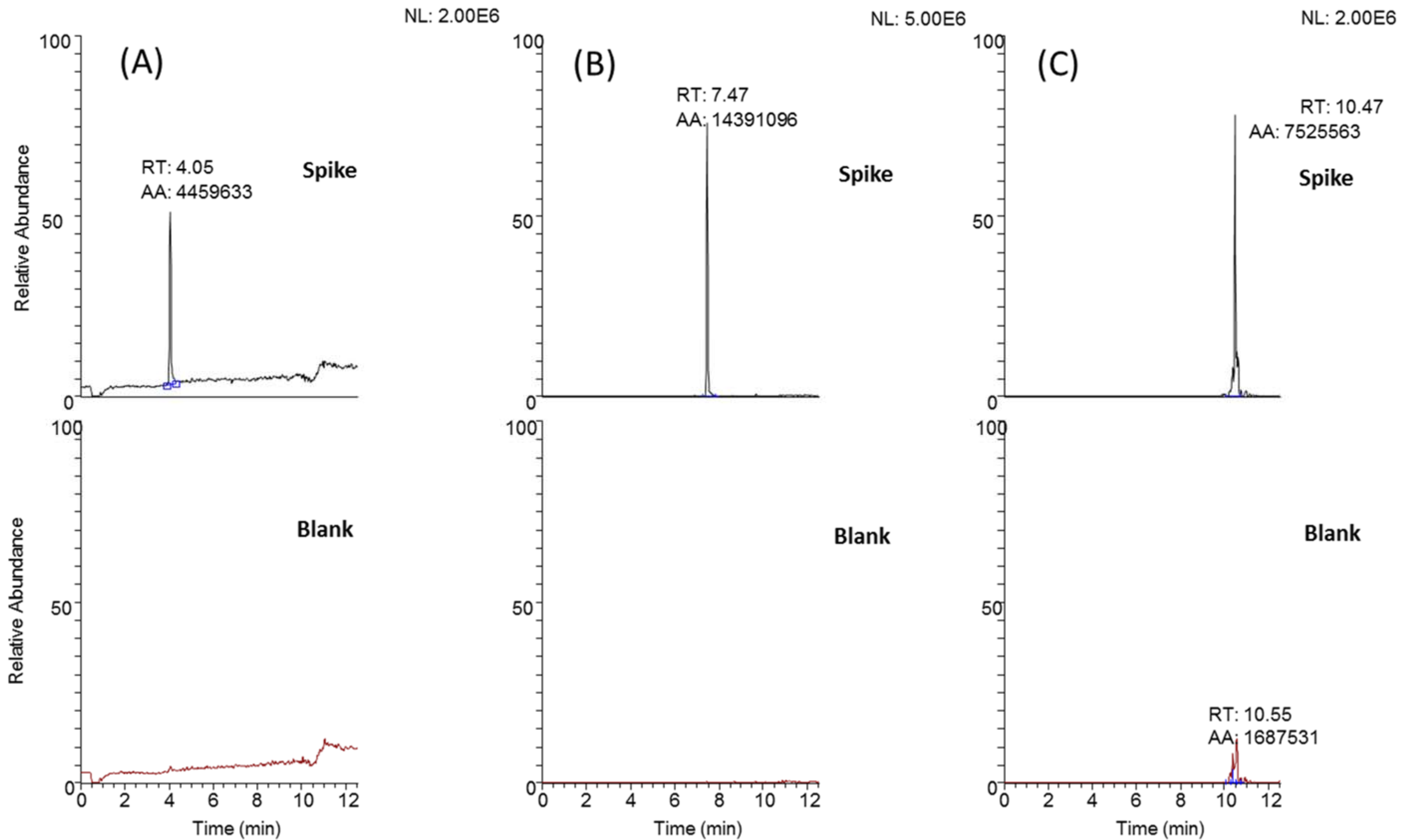


Figure 2. Atrazine solvent standard at 1 ng/mL (A), extract from shrimp spiked at 1 ng/g (B), and blank shrimp extract (C). Top traces are EICs from MS1 scan for m/z 216.10105 and bottom traces are EICs for characteristic product ions (m/z 174.05410, 96.05562) in the all ion fragmentation MS2 scan. All EICs have 5 ppm window.

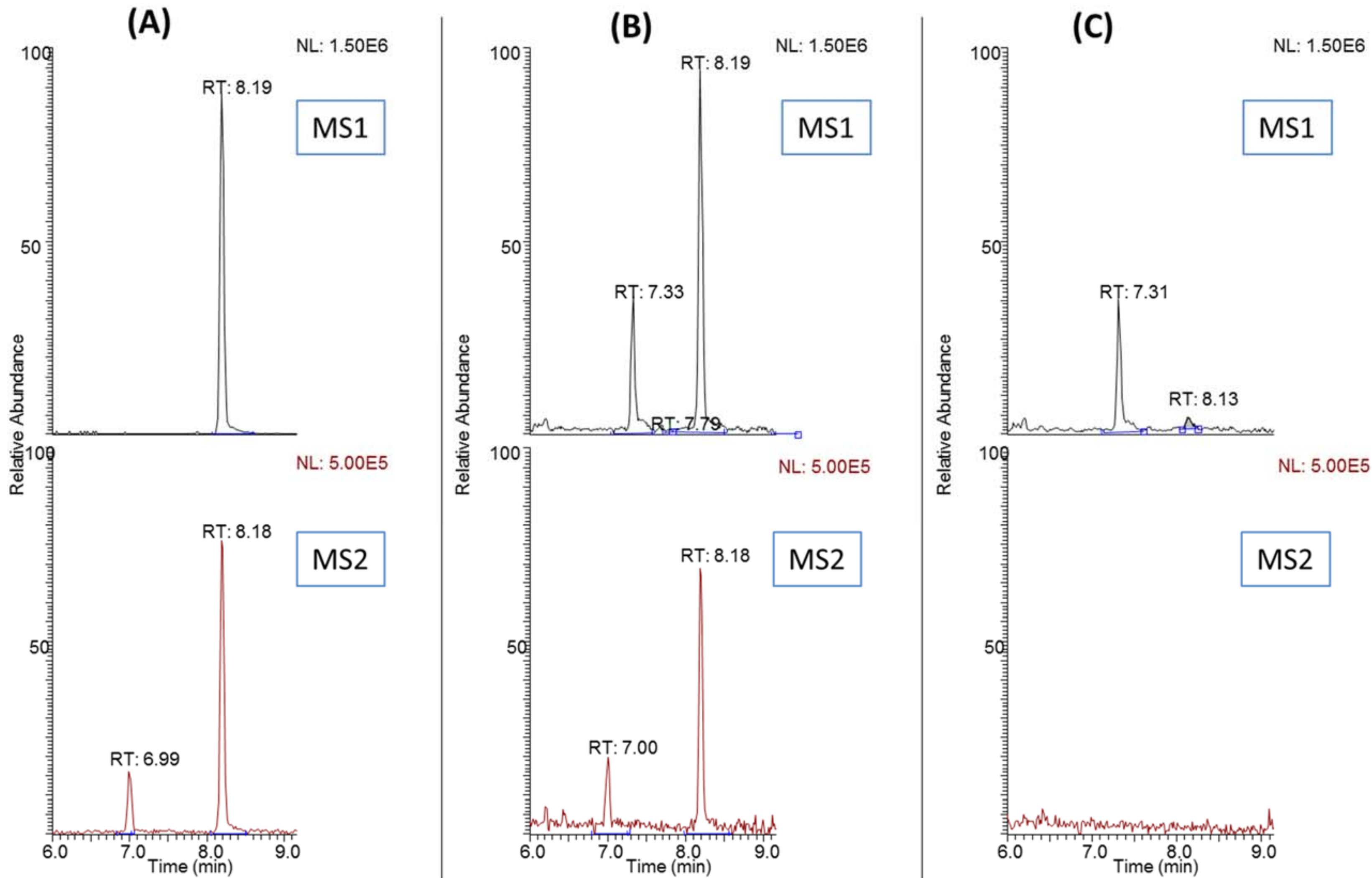


Figure 3. Azamethiphos fortified in salmon at 10 ng/g. EICs (5 ppm) for MS1 precursor ion (m/z 324.98093) and MS2 product ions (m/z 182.99541, 139.00558) as well as product ion spectra are shown for data collected by all ion fragmentation (left) and parallel reaction monitoring (right).

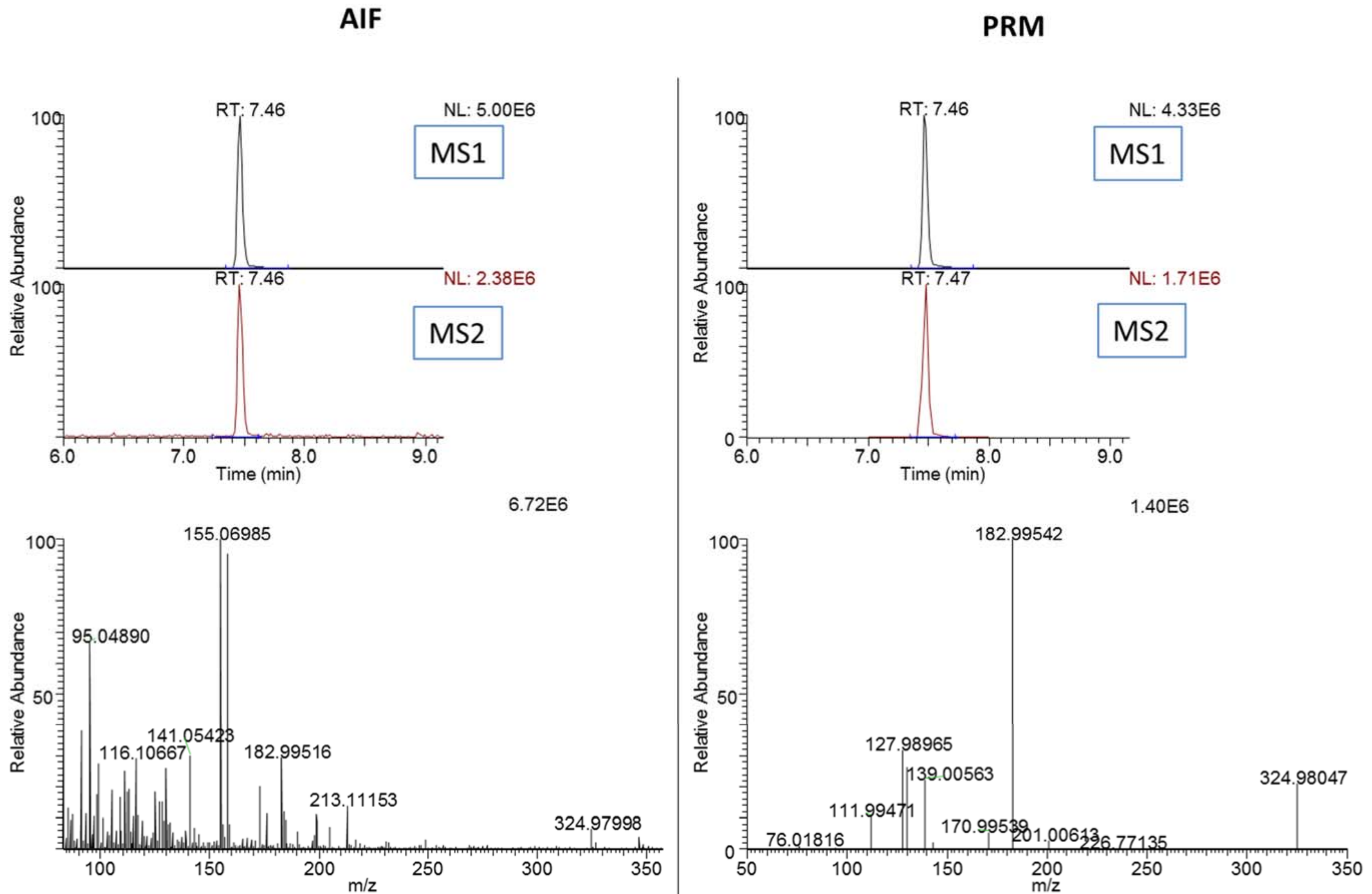
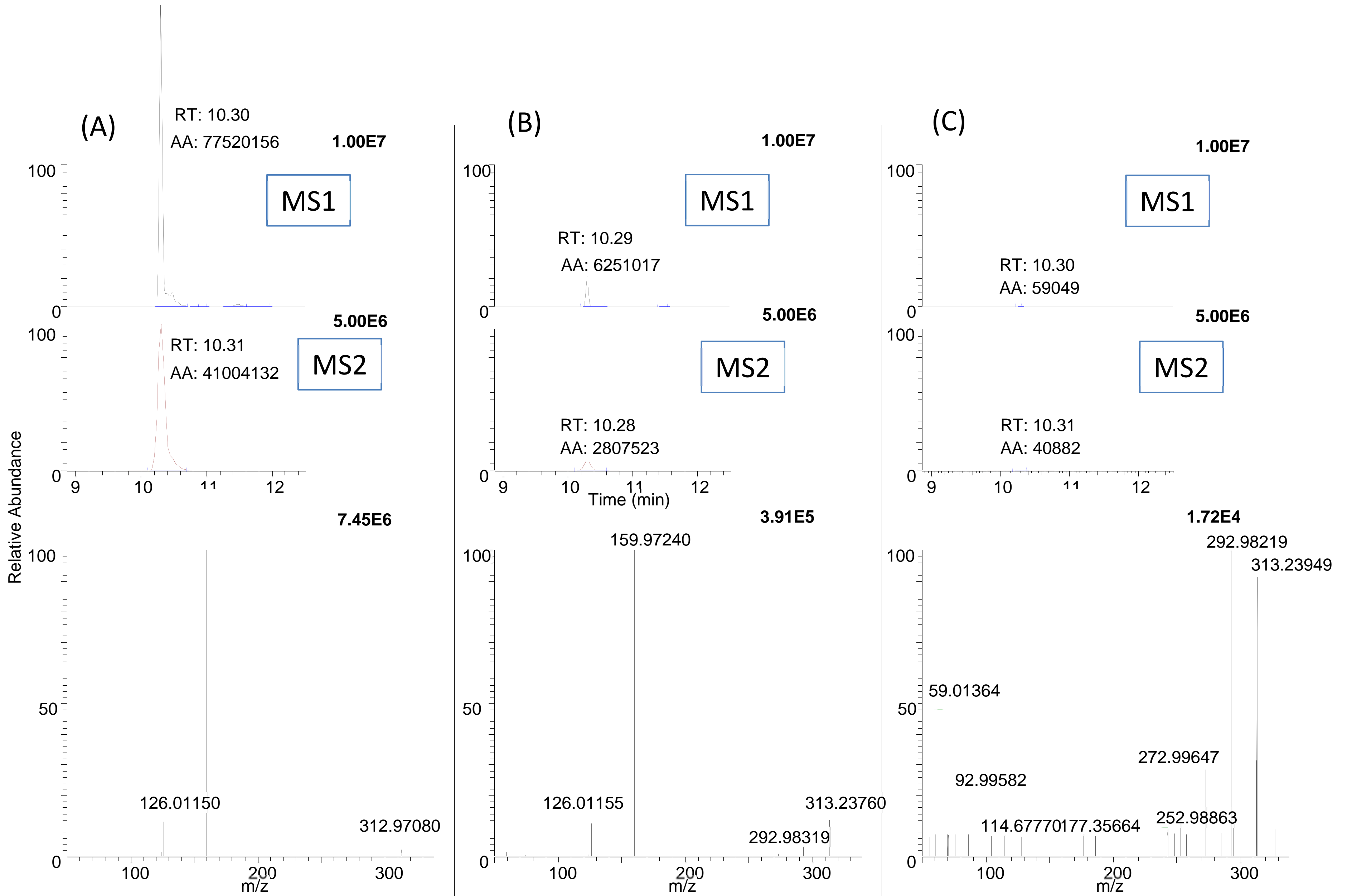


Figure 4. Triclocarban solvent standard at 100 ng/mL (A), extract from tilapia spiked at 100 ng/g (B), and blank tilapia extract (C). Top traces are EICs from MS1 scan for m/z 312.97077 and middle traces are EICs for characteristic product ion (m/z 159.97263) in the PRM MS2 scan. All EICs have 5 ppm window. The PRM product ion spectra are shown below each chromatogram.


(A)
RT: 10.30
AA: 77520156
1.00E7
MS1
5.00E6
RT: 10.31
AA: 41004132
MS2
7.45E6
126.01150
312.97080
(B)
1.00E7
MS1
RT: 10.29
AA: 6251017
5.00E6
MS2
RT: 10.28
AA: 2807523
Time (min)
3.91E5
159.97240
126.01155
313.23760
292.98319
(C)
1.00E7
MS1
RT: 10.30
AA: 59049
5.00E6
MS2
RT: 10.31
AA: 40882
1.72E4
292.98219
313.23949
59.01364
272.99647
92.99582
114.67770177.35664
252.98863
Relative Abundance
m/z