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Extended liquid chromatography high resolution mass spectrometry screening method for veterinary drug, pesticide and human pharmaceutical residues in aquaculture fish

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Abstract

A liquid chromatography high resolution mass spectrometry (LC-HRMS) screening method was developed previously to analyze for veterinary drug residues commonly found in different types of aquaculture products. This method has been further evaluated for its feasibility to detect several other classes of compounds that might also be a concern as possible contaminants in farmed tilapia, salmon, eel and shrimp. Some chemicals could contaminate water sources used in aquaculture production through agricultural run-off. 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. The sample preparation consisted of an acidic acetonitrile extraction followed by solid-phase extraction clean-up. Data were collected with a quadrupole-Orbitrap MS using both non-targeted and targeted acquisition. This rapid clean-up procedure and HRMS detection method described previously for veterinary drug residues also worked well for many other types of compounds. Most analytes had screening limit levels between 0.5–10 ng/g in the matrices examined using exact mass identification criteria. The strategy described in this paper for testing the performance of additional analytes will help expand the applicability of the HRMS procedure as aquaculture samples can now be analyzed for a wider range of contaminants.

Keywords

Aquaculture; chemical contaminants; screening method; high resolution mass spectrometry

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Disclosure Statement

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Introduction

Monitoring the global food supply for potentially harmful chemical contaminants is an important responsibility of international food control agencies. Recently, high resolution mass spectrometry (HRMS) has become an important tool in achieving this goal. HRMS instruments such as the hybrid quadrupole-Orbitrap (e.g. 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. This is possible because HRMS has the advantage of generating very accurate mass measurements which provide selectivity to detect analytes even at low concentrations in complex food matrices. HRMS methods that monitor for a wide scope of residues in foods, including veterinary drugs, pesticides and other human pharmaceuticals, have been reported (Gómez Pérez et al. 2014; Dasenaki et al. 2015; Masiá et al. 2016; Zhao et al. 2017; Kong et al. 2018), although very few methods monitor for all three of these categories of compounds simultaneously.

In one specific example, a screening method for veterinary drug residues in fish, shrimp and eel using liquid chromatography coupled to a Q-Exactive HRMS was developed in our laboratory (Turnipseed et al. 2017). This method was initially optimized and validated with 70 “test” compounds (representing a variety of common veterinary drug classes) of known use in aquaculture products. MS detection was performed with both non-targeted and targeted product ion data acquisition. The clean-up procedure used a newer gravity-drain SPE column specifically designed to remove phospholipids from biological sample extracts, thereby greatly improving MS detection (Storey et al. 2016). This method was used to analyze regulatory aquaculture samples which contained residues initially detected by targeted triple quadrupole LC-MS/MS analysis (Storey et al. 2014). Several additional analytes, including 2-amino-mebendazole in eel and ofloxacin in croaker fish, were discovered with this HRMS screening method (Turnipseed et al. 2018). There was a reasonable possibility that residues of ofloxacin might have been found in the croaker fish because this drug was present in the surrounding environment. As a result, there was an interest in expanding the scope of this HRMS screening method to include additional chemical contaminants that may result either from direct application to aquaculture ponds or from accidental environmental exposure. Since this method proved successful in analyzing for the initial test compounds (mostly antibiotics representing several different chemical classes) in fortified and treated aquaculture tissues, the goal of the current work was to investigate extending the method to additional chemical contaminants including pesticides, human drugs, and chemical disinfectants.

One approach to monitoring for additional contaminants is to take advantage of the fact that full scan HRMS has been acquired in a non-targeted fashion and perform retrospective analysis or post-analysis screening. This has been described for both food (Kaufmann and Walker 2013; Gómez Pérez et al. 2015; León et al. 2016) and environmental (Krauss et al. 2010; Hernández et al. 2011) analyses. Generally this is performed by comparing HRMS data to an expanded database consisting of exact mass data for potential contaminants and transformation products (metabolites and degradants) to generate a list of additional suspect

compounds. There are some limitations to retroactive searching. As others have described (Krauss et al. 2010; Kaufmann and Walker 2013; Turnipseed et al. 2014), it can be difficult to balance false negative and false positive responses depending on data processing parameters such as allowable mass accuracy windows and response thresholds. Having data for expected product ions can greatly enhance the confidence of any analyte identification. There are a variety of data acquisition methods available on these instruments which generate product ion data in either a targeted (for specific analytes) or non-targeted (for all compounds in the sample) manner that have relative advantages and disadvantages for residue screening (Wong et al. 2018; Wu et al. 2019). Also, any retroactive searching that produces a presumptive accurate mass (including isotopic pattern) match for a specific analyte may still not be sufficient for assessing residue identity confirmation without additional retention time matching with a reference standard. It is also important to note that 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 clean-up procedure used. This might happen to varying degrees with different biological matrices. For example, with aquaculture samples, an eel sample packaged with sauce is typically a more difficult matrix than a less oily fish such as tilapia. This can especially be a problem for compounds with ubiquitous lower molecular weight ions. For these reasons, testing actual matrix spikes at the level of interest can be necessary when determining whether the screening method can analyze various compounds to extend the method's applicability.

The aim of this work was to assess the ability of this method to screen for additional classes of compounds beyond those included in the original method for veterinary drug residues (Turnipseed et al. 2017) including triazine herbicides, organophosphate and carbamate pesticides, human pharmaceuticals, selected disinfectants, growth promoters and some newer antibiotics. While other published LC-MS methods are available for monitoring aquaculture or wild fish for chemical contaminants including pesticides (Han et al. 2014; Song et al. 2018) or pharmaceuticals and personal care products (Ramirez et al. 2007; Chen et al. 2015), the goal of this study was to determine how well representative compounds that may be found in fish can be detected in a single HRMS screening method. 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. Retrospective analysis, or post-analysis screening, was also performed on data from historic samples for these additional chemical contaminants.

Materials and methods

Standard preparation and sample extraction

The compounds added to the method are 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-Aldrich (St. Louis, MO) or USP (North Bethesda, MD).

A spiking mix 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 orbifloxacin, were prepared at a level of 0.1 µg/mL. These fluoroquinolone (FQ) compounds have an FDA “target testing level” or TTL of 5 ng/g (FDA 2017) so they were added to the spiking mix at a lower concentration. To fortify the fish, 100 µL of the spiking mix 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). Tenfold serial dilutions of the spiking mix were used to prepare fish spikes at lower levels.

A 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 calibration) solvent standard was used to monitor instrument system suitability and also to estimate analyte recovery. Blank matrix controls were also extracted with each analytical batch. A more detailed description of the experimental procedure, including information on sample and reagent preparation, can be found in previous work (Storey et al. 2016; Turnipseed et al. 2017), but a summary of the extraction method is provided here:

Fish tissue (2 g) was extracted with 8 mL of a solution consisting of 0.2% *p*-toluene sulfonic acid monohydrate (w/v) and 2% glacial acetic acid (v:v) in 100% acetonitrile (ACN) by vortex mixing for 30 min followed by centrifugation (7 min at 4 °C at 10,000 rpm or 17,000 RCF (g)). A 3 mL portion of the extractant was transferred to an Oasis PRIME HLB 6cc (200 mg) extraction cartridge. The extractant was collected after passing through the cartridge and then taken to near dryness using a nitrogen evaporator (55 °C). The extract was then reconstituted in 400 µL of 10% ACN in water (v:v), vortexed for 2 min and then centrifuged (7 min, 4 °C, 13,000 rpm or 28,900 RCF (g)). A 300 µL aliquot was then transferred to a vial for LC-HRMS analysis. The sample equivalent in the final extracts was approximately 1.7 g/mL. A separate injection of the extract before the drying step (ACN injection) was required for some of the nonpolar analytes tested previously, but this step was not needed for compounds tested in this paper.

Instrumentation

The LC-MS instrumentation and chromatographic parameters have been described previously (Turnipseed et al. 2017). A Q-Exactive Orbitrap was coupled to an Ultimate 3000 LC system. As before, data were collected primarily by using the “All Ion Fragmentation” (AIF) mode of acquisition in which a full scan MS¹ is followed by a MS² scan where all precursors are allowed into the high collision dissociation (HCD) cell to form product ions simultaneously. With AIF acquisition, full scan MS¹ data are obtained initially (70K resolution, 3e⁶ automatic gain control target, maximum inject time 200 ms, m/z 150–1000 scan range). Product ion data are then collected without any isolation of precursor ions (70K resolution, 3e⁶ automatic gain control target, maximum inject time 200 ms, m/z 80–1000 scan range, normalized collision energy 10, 30, 50).

For more targeted analysis with precursor isolation “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, regardless of the signal detected for the precursor in the MS¹ scan. For PRM data collection, a full scan MS¹ acquisition was obtained using the same conditions as described above for AIF. Product ion data for compounds on the inclusion list were then continuously collected (17.5K resolution, 1e⁶ 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). Negative ion data were also collected using AIF and PRM with similar acquisition parameters in a second injection.

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 calibration point for semi-quantitative analysis. Recoveries were estimated by comparing this extracted sample to a standard in solvent at the same concentration. A sufficient number of replicate spikes were performed at each level (100,10,1 ng/g) for each type of fish to meet the US FDA’s Chemical Method Validation Guidelines (FDA 2015b) 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 (FDA 2015a) which requires that the precursor ion of the unknown must agree within 5 ppm mass accuracy window to the theoretical precursor mass ion, one product ion must agree within 10 ppm, and the retention time must match (within 0.2 min) of the reference standard. Because some compounds (e.g. benzylalkonium chlorides) have significant signal in the blank matrices, area counts for the precursor ions in the MS¹ extracted ion chromatograms 10× greater than what was observed in matrix blanks were also required to define the screening limit for this method.

Results and discussion

Selection of new analytes

Recent published reviews (Rico et al. 2013; Li et al. 2016) have discussed trends in aquaculture practices on farms around the world. In order to avoid over exposure to antibiotics, some aquaculture farmers are experimenting with probiotics, medicinal herbs and/or inorganic disinfectants. Many of these compounds are not suitable for LC-MS detection. However, some disinfectants reported to be in use, such as benzylalkonium chlorides, triclocarban, and triclosan were investigated. Other surveys (Wan 2013; Chatterjee et al. 2016) have noted that various parasiticides have been used for parasites and fungal infections in fish or to kill unwanted organisms in culture ponds prior to stocking. Compounds in this group included phoxim, trichlorfon, trifluralin, azadirachtin and praziquantel. The pesticide azamethiphos has been approved (as the product Salmosan)

(Burridge et al. 2010) for treatment of sea lice with the US FDA establishing an import tolerance of 20 ng/g for residues in salmonids (FDA 2018).

Newer antibiotics such as clarithromycin and roxithromycin, as well as the growth promoter virginiamycin were investigated in this LC-HRMS method. Orbifloxacin and marbofloxacin were included because they have recently been added to the scope of analytes monitored in aquaculture products in Canada (Canadian Food Inspection Agency 2017). Fipronil, which was added due to its unexpected recent use that resulted in the widespread contamination of millions of eggs around the world (Stafford et al. 2018). Praziquantel was included because of its known widespread contamination of fresh water bodies throughout China (Lai et al. 2016). The anesthetic benzocaine can be used to reduce aggressive behavior or to improve successful transport of fish (Coyle et al. 2004). Misuse of rotenoids, pyrethroid derivatives, and Neem oil (azadirachtin) could also be a concern. Illegal dye compounds, such as malachite green and crystal violet, continue to be detected in aquaculture samples. In this HRMS method, other dyes such as methylene blue and acriflavine, which have been studied previously in fish (Plakas et al. 1997; Xu et al. 2009) were also investigated.

Other sources of contaminants are chemicals from runoff of traditional agricultural farms that have been reported in aquaculture ponds used for fish farming (Wan 2013). Compounds of concern include widely used triazine herbicides, carbamate pesticides including aldicarb and its metabolites, and organophosphate pesticides such as dichlorvos and malathion (Wan 2013). Although many LC-MS methods monitor for a wide scope of pesticides (Masiá et al. 2016), the intent of including this representative group was to extend the method developed for veterinary drugs to other compounds that might be found in aquaculture tissue. Specific pesticides are known to be used in aquaculture environments, for example crayfish farming in rice paddies (Song et al. 2018).

Human pharmaceuticals could be yet another source of unwanted chemical residues due to environmental exposure in aquaculture products. Extensive surveys (Ferrer and Thurman 2012; Meador et al. 2016; Bradley et al. 2017) 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 also is a global concern (Petrie et al. 2015; Sui et al. 2015; Zhao et al. 2016), indicating a high potential for bioaccumulation in aquaculture operations around the world. A recent study (Brown et al. 2014) of 12 fish species concluded that 65–86% of human drug compounds can accumulate in such tissues. Widely used human drugs such as ranitidine, fluoxetine, ibuprofen, caffeine, diphenhydramine, propranolol and statins were added to the screening protocol and assessed. Other drugs, including carbamazepine, diltiazem, and gemfibrozil which are commonly found in effluents and have been found to accumulate in fish (Ramirez et al. 2007; Chen et al. 2015; Meador et al. 2016) were also included.

Screening validation 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). Standard in solvent 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 previously (Turnipseed et al. 2017) are listed in Table 1. Many of the compounds were analyzed in positive mode using the protonated molecule as a precursor ion, except for a few (indicated in Table 1) that were monitored as sodiated ions or in negative ion mode. The theoretical values for the product ions listed in Table 1 were determined by their exact mass, logical fragmentation patterns and consistency with previous reports (Nuñez et al. 2004; Ferrer and Thurman 2012; Gómez Pérez et al. 2014; Turnipseed et al. 2014; Sage et al. 2017; Wong et al. 2018). As purchased, the standard of acriflavine is a known mixture of the parent compound and the demethylated derivative, proflavine (Plakas et al. 1997). In addition to the peak for proflavine, two peaks with the same precursor and product ions but chromatographically separated were observed for acriflavine. The two chromatographic peaks observed for acriflavine could represent different free base and hydrochloride components that may both be present in a commercial mixture (Galliot 1934). This was not investigated further with purified compounds since it is most likely that a commercial product would be used in aquaculture practice (Plakas et al. 1997). Others (Amelin et al. 2017) have also observed two peaks for acriflavine, in addition to proflavine, but did not characterize the isomers.

Some analytes tested in this method could not be detected. Cypermethrin and etofenprox, were unsuccessful due to poor ionization in the dilute formic acid mobile phase. These compounds are usually detected and chromatographed as ammonium adducts using a buffered mobile phase in other published methods using LC-MS (Gómez Pérez et al. 2014; Wong et al. 2018). Disinfectants such as the halogenated hydantoins and trichloroisocyanuric acid were initially included but found to break down in water making their MS analysis difficult. The polypeptide antibiotic colistin was also investigated but did not chromatograph well using the described conditions. The detection limits for trifluralin, even in solvent standard, were also very high.

The compounds that could be detected in solvent standards using this HRMS instrument method were then tested in fortified fish, shrimp and eel. Their recoveries in different tissue matrices at the various spiking levels were determined by comparison to an equivalent level (one point) solvent standard injected throughout each analytical batch 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 is meant to 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. For any presumptive positive found by HRMS, a specific targeted triple-quadrupole MS method could then be used if accurate quantitation and maximum sensitivity is desired.

Table 2 is a summary of screening results in aquaculture samples for the investigated analytes using data gathered by the Q-Exactive HRMS operating in AIF. The data in Table 2

shows the screening detection limit in ng/g for the lowest fortification level that each tested analyte met FDA's accurate mass confirmation criteria and had area counts for the precursor ion in the extracted ion chromatogram greater than ten times 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 2 also had >30% recoveries in tilapia spiked at 10 ng/g (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 and dyes. Because this is a screening method, efforts were not made to distinguish between the contributions of extraction efficiency and matrix suppression to overall estimated recoveries. Instead, the precision of the method between replicate 10 ng/g spikes for tilapia was determined and included as relative standard deviations (RSDs) in Table 2. These data show, as was observed previously for veterinary drug residues in this screening method (Turnipseed et al. 2017), that consistent results are observed with RSDs for the analytes that worked well (in bold) ranging from 4 to 16%. These values could be used to calculate threshold cut-off values for limits test screening if target testing levels are established for these compounds in fish (FDA 2015b). Analytes with lower recoveries that only met screening criteria at higher fortification levels had higher RSDs.

Example ion chromatogram data from data collected using AIF are shown in Figures 1 and 2. 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 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.

The compounds shown in italics or strike-through font in Table 2 did not perform well in the method due to low signal, high background in the blank or other issues discussed below. Ibuprofen, carbaryl, naproxen and gemfibrozil were problematic due to high background and low signals. Better results for carbaryl could be obtained by monitoring the breakdown product at m/z 145.06477 [MH-C₂H₃NO]⁺ as reported previously (Wong et al. 2018). Because this method has a MS¹ scan range of m/z 150–1000, this ion could be only monitored in the AIF MS² trace. This degradant of carbaryl (1-naphthol) was detected in spiked samples at 100 and 10 ng/g, but not in corresponding blank matrices. However, product ion confirmation was not obtained for this compound. The benzylalkonium chloride compounds had significant background signal that was worse in salmon and eel matrices. Others have noted that background levels of these compounds can be quite significant and may even be due to disinfectants used in analytical reagents (Nuñez et al. 2004). Some compounds, such as roxithromycin, showed a small (<10% compared to screening limit) signal in solvent and matrix blanks due to instrument carryover, but this background was not enough to interfere with the screening method results. 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 (González-Mariño et al. 2009) 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. 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 2 also shows screening detection limits for the other matrices tested: shrimp, salmon, and eel. In general, the screening limits were mostly dependent on the analyte itself and the results were similar in the different types of aquaculture products. For some compounds, the screening limits are higher in eel which is not surprising as this matrix can be significantly more complex. Also, although the original method (Turnipseed et al. 2017) 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 study. Moving forward, the consistency of the screening limits across different types of aquaculture matrices supports evaluating new analytes in a single type of fish, such as tilapia, and then following up with more rigorous quantitation in corresponding matrix if an analyte is detected in the screening process.

In addition to non-targeted AIF acquisition, PRM data with targeted MS^2 data collection for the compounds in the spiked samples were also evaluated in this study. Because precursor isolation (1 m/z isolation window) is initially performed, PRM acquisition provides cleaner product ion spectra. Using the identification criteria of one precursor ion (within 5 ppm) one product ion (within 10 ppm) at the correct (within 0.2 min) retention time, the initial analysis with AIF data can produce false positives in complex matrices. Follow-up with a more targeted data acquisition can confirm or rule-out the presence of compounds found using AIF. We have found that PRM generates more consistent product ion spectra for compounds when using a limited ($N < 100$) inclusion list as compared to the $DDMS^2$ acquisition method used previously (Turnipseed et al. 2017) which did not always trigger product ion spectra for all the analytes. 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 using PRM, but that the characteristic ions (m/z 182.9954, 139.0058) were also detected in the AIF MS^2 data (Figure 3). 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 2), a higher level of residue was required to detect fragment ions using PRM acquisition. For example, azadirachtin forms a sodium ion precursor that is difficult to fragment. A characteristic fragment ion for this compound was observed with AIF, but not using PRM.

Retrospective analysis of imported fish and eel samples

Several imported samples including eels, many of which had been found to be contaminated with 2-amino-mebendazole, were analyzed to determine if any of the additional compounds evaluated in this study were also present in those samples. In addition, data from imported fish and eel samples that had been analyzed previously (Turnipseed et al. 2018) using this

HRMS screening method, were also retrospectively evaluated to determine if the analytes listed in Table 1 could be detected. Overall, 22 imported samples of eel (15), tilapia (4), yellowtail (1), croaker (1) and salmon (1) were evaluated. In general, significant levels of the additional analytes described in this paper were not detected in these samples. Varying amounts of benzylalkonium chloride C14 were detected and met identification criteria, particularly in eel samples, but the levels observed were not above the screening limit that was set at 100 ng/g because of the higher background contamination observed with this compound. Trace levels (< 1 ng/g) of diltiazem were also found when retrospectively evaluating data that had been collected earlier for an eel sample. Diltiazem is a hypertension drug that has been detected in surface waters and fish tissues (Ramirez et al. 2007; Meador et al. 2016).

A presumptive positive for the first chromatographic peak of acriflavine was observed at reasonably high levels (up to 80 ng/g compared to eel fortified at 10 ng/g) in several current and previously analyzed eel samples using AIF data acquisition. The precursor ion, one product ion and retention time criteria were met. However, the predominant product ion for this compound was not detected in the AIF MS² trace of these samples. In addition, when these samples were analyzed using a PRM method that included acriflavine in the targeted inclusion list, no fragment ions corresponding to acriflavine were detected. One of these eel samples was spiked with the fortification mix at 10 ng/g, and the extracted ion chromatogram for acriflavine (m/z 224.1182) showed an additional peak from the matrix that was chromatographically separated (eluting 0.12 min earlier) from the peaks associated with acriflavine (Figure 4). The product ion spectrum from precursor ion m/z 224.1182 for this peak consisted of an ion at m/z 133.0363 as well as a small ion at m/z 91.0542 which could correspond to C₇H₇ N₃⁺ and C₇H₇⁺, respectively. These ions would be consistent with a benzyl benzimidazole amine, but additional work would be required to further characterize this compound. As others have noted (Delaunay et al. 2018), this practice of standard addition can be very useful for screening methods when the matrices can be highly variable. The use of a more targeted data acquisition, such as PRM, can also be a very useful in evaluating the veracity of results obtained with retrospective analysis of data collected using non-targeted MS². However, PRM does require reanalysis of the final extract to include precursor ions of interest, and standard addition requires re-extraction of the sample itself. Analysis of aquaculture samples using this HRMS method to monitor for these and other chemical contaminants will be ongoing, but this example illustrates the need to thoroughly verify suspect positives found by screening methods.

Conclusion

The HRMS screening procedure described and evaluated previously for veterinary drug residues (Turnipseed et al. 2017, 2018) 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 recoveries and their minimum confirmation screening limit levels. LC column retention times and product ion information were also established, which should expedite compound library searching. It is anticipated that additional analytes will be added to this screening method using the general

strategy described in this paper. 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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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 fortified at 10 ng/g (top) and blank tilapia matrix (bottom). Data from MS^1 scan collected using all ion fragmentation (AIF) program.

Figure 2.

Atrazine in mixed solvent standard at 1 ng/mL (a), extract from shrimp fortified 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.

(a) Extracted ion chromatogram (5 ppm window) for MS¹ of m/z 224.1182 (top) and total ion chromatograph for MS² of m/z 224.1182 (bottom) obtained with PRM data acquisition for imported eel sample fortified with analyte mix at 10 ng/g. Peaks at 4.80 min and 5.16 min correspond to isomers of acriflavine and the peak at 4.69 min is observed from the imported eel samples. (b) MS² spectrum of peak at 4.8 min. (c) MS² spectrum of peak at 4.69 min.

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 ₁₃ N ₃	4.8	224.11822	209.09475	182.08385	
Acriflavine 2 ^a	Antifungal dye	C ₁₄ H ₁₃ N ₃	5.2	224.11822	209.09475	182.08385	
Aldicarb	Carbamate insecticide	C ₇ H ₁₄ N ₂ O ₂ S	6.6	213.06682 (Na ⁺)	116.05285	89.04195	
Aldicarb Sulfone	Carbamate metabolite	C ₇ H ₁₄ N ₂ O ₄ S	3.6	245.05665 (Na ⁺)	166.07127	109.04981	
Aldicarb Sulfoxide	Carbamate metabolite	C ₇ H ₁₄ N ₂ O ₃ S	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 ₂ O ₃	1.5	267.17032	190.08626	145.06479	
Atrazine	Triazine herbicide	C ₈ H 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		
Azamephosphos	Organophosphate antiparasitic	C ₉ H ₁₀ ClN ₂ O ₄ PS	7.5	324.98093	182.99541	139.00558	
Baquioprim	Diaminopyrimidine potentiator	C ₁₇ H ₂₀ N ₆	0.9	309.18222	294.15875	123.06652	
Benzocaine	Topical anesthetic	C ₉ H ₁₁ NO ₂	6.9	166.08626	138.0545	94.06513	77.03900
Benzylalkonium Cl C12 ^c	Disinfectant	C ₂₁ H ₃₈ N	9.5	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 ₈ H ₁₀ N ₄ O ₂	4.1	195.08765	138.06619	110.07127	
Carbamazepine	Antiseizure drug	C ₁₅ H ₁₂ N ₂ O	7.6	237.10224	194.09643	192.08078	
Carbaryl	Carbamate Insecticide	C ₁₂ H ₁₁ NO ₂	8.2	202.08626	159.04406	145.06479	
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	
Clofibrate acid	Herbicide	C ₁₀ H ₁₁ ClO ₃	8.9	213.0324 (NEG)	126.99562		
Cypermethrin	Pyrethroid insecticide	C ₂₂ H ₁₉ Cl ₂ NO ₃	ND ^d				
Dichlorvos	Organophosphate pesticide	C ₄ H ₇ Cl ₂ O ₄ P	7.3	220.95318	144.98158	127.01547	78.99434
Diclofenac	NSAID	C ₁₄ H ₁₁ Cl ₂ NO ₂	10.0	296.02396	278.01340	250.01850	215.0496
Diltiazem	Hypertension drug	C ₂₂ H ₂₆ N ₂ O ₄ S	6.7	415.16860	370.11076	178.03211	150.0372
Diphenhydramine	Antihistamine	C ₁₇ H ₂₁ NO	6.4	256.16959	167.08553		

Compound	Description	Molecular Formula	Ret. Time (min)	Precursor	Product 1	Product 2	Product 3
Etofenprox	Pyrethroid insecticide	C ₂₅ H ₂₈ O ₃	ND				
Fipronil	Phenylpyrazole insecticide	C ₁₂ H ₄ Cl ₂ F ₆ N ₄ OS	10.1	434.93143 (NEG)	329.95954	249.95848	183.01756
Fipronil sulfone	Phenylpyrazole metabolite	C ₁₂ H ₄ Cl ₂ F ₆ N ₄ O ₂ S	10.2	450.92634 (NEG)	281.99256	243.98948	183.01756
Fluoxetine	Anti-depressant drug	C ₁₇ H ₁₈ F ₃ NO	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 ₆ PS ₂	10.0	331.04334	285.00148	127.03897	99.00767
Marbofloxacin	Fluoroquinolone antibiotic	C ₁₇ H ₁₉ FN ₄ O ₄	4.3	363.14631	320.10410	205.03964	72.08780
Methylene Blue	Antifungal dye	C ₁₆ H ₁₈ N ₃ S	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 ₃ N ₃ O ₃	5.0	396.15295	352.16310	295.1053	
Phoxim ^e	Organophosphate pesticide	C ₁₂ H ₁₅ N ₂ O ₃ PS	10.3	299.06138	216.99233	129.04478	
Praiquantel	Antiparasitic	C ₁₉ H ₂₄ N ₂ O 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 ₃ H 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 ₂ O ₃ PS	10.2	299.06138	242.99847	163.03245	147.05529
Quinoclamine	Naphthoquinone pesticide	C ₁₀ H ₆ ClNO ₂	7.3	208.01598	172.03930	105.03349	
Ramitidine	Antacid	C ₁₃ H ₂₂ N ₄ O ₃ S	1.7	315.14854	270.09069	176.04882	130.05592
Rifampicin	Ansamycin antibiotic	C ₄₃ H ₅₈ N ₄ O ₁₂	8.7	823.41240	791.38618		
Rifaximin	Ansamycin antibiotic	C ₄₃ H ₅₁ N ₅ O 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 ₂ O ₁₅	7.5	837.53185	679.43760	158.11760	
Simazine	Triazine herbicide	C ₇ H ₁₂ ClN ₅	7.0	202.08540	132.0323	124.08692	96.05562
Simvastatin	Statin for lowering lipids	C ₂₅ H ₃₈ O ₅	10.5	419.27920	285.18491	199.14813	
Sotalol	Antiarrhythmic drug	C ₁₂ H ₂₀ N ₂ O ₃ S	1.4	273.12674	255.11560	213.06882	133.07602
Sulfafurazole(sulfisoxazole)	Sulfonamide antibiotic	C ₁₁ H ₁₃ N ₃ O ₃ S	6.3	268.07504	156.01140	108.04440	113.07090
Thiabendazole	Benzazole antiparasitic	C ₁₀ H ₇ N ₃ S	3.8	202.04334	175.03250		

Compound	Description	Molecular Formula	Ret. Time (min)	Precursor	Product 1	Product 2	Product 3
Trichlorfon	Organophosphate pesticide	$C_4H_8Cl_3O_4P$	5.0	256.92985	220.95318	127.01547	
Triclocarban	Antibacterial agent	$C_{13}H_9Cl_3N_2O$	10.3	312.97077 (NEG)	159.97263	126.01160	
Triclosan	Antibacterial agent	$C_{12}H_7Cl_3O_2$	10.3	286.94389 (NEG)	ND		
Trifluralin	Dinitroaniline herbicide	$C_{13}H_{16}F_3N_3O_4$	10.6	336.11657	294.06847	236.02661	
Virginiamycin M1	Streptogramin antibiotic	$C_{28}H_{35}N_3O_7$	8.5	526.25478	508.24420	337.11830	

^a Commercially purchased acriflavine is a mixture of two acriflavine isomers with different retention times and proflavine (demethylated acriflavine)

^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.

Table 2.

Screening results for new compounds.

COMPOUND	Screening Limit ^a (ng/g)				Relative Standard Deviations ^b for 10 ng/g in tilapia	
	TILAPIA	SHRIMP	SALMON	EEL		
Acriflavine 1	1	1	1	1		7
Acriflavine 2	1	1 ^c	1 ^c	1 ^c		8
Aldicarb	1	1	10	1 ^c		5
Aldicarb Sulfone	1	10	10	10		6
Aldicarb Sulfoxide	100	100	100	100		40
Amitraz Degradant 1 (DPMF)	10	10	10	1		7
Atenolol	1	10	1 ^c	10		7
Atrazine	1	1	1	1		5
Azadirachtin	10 ^e	10 ^e	10 ^e	10 ^e		7
Azamethiphos	1	1	1	1		6
Baquioprim	100	100	10	10		30
Benzocaine	1	1	10	10		7
Benzylalkonium chloride C12	100	100	100	100		6
Benzylalkonium chloride C14	100	100	100	100		3
Caffeine	1	10	10	10		9
Carbamazepine	1	1	1	1		6
Carbaryl	>100 ^f	>100 ^f	>100 ^f	>100 ^f		ND
Carbofuran	1	1	1	1		13
Clarithromycin	1	1	1	1		6
Cypermethrin	>100	>100	>100	>100		ND
Dichlorvos	1 ^c	1	1	1		7
Diclofenac	1	1	1	10		9
Diltiazem	1	1	1	1		6
Diphenhydramine	1	1	1	1		7
Etofenprox	>100	>100	>100	>100		ND

COMPOUND	Screening Limit ^a (ng/g)				Relative Standard Deviations ^b for 10 ng/g in tilapia	
	TILAPIA	SHRIMP	SALMON	EEL		
Fluoxetine	1	1	1 ^c	10 ^d		6
Heptofen	>100	>100	>100			ND
Malathion	1	1	1	1 ^c		7
Marbofloxacin	5	5	5	5		15
Methylene Blue	1	1	1	1		16
Naproxen	100	100	100	>100		11
Orbifloxacin	0.5	0.5	0.5	0.5		5
Phoxim	1 ^c	1	1 ^c	1 ^c		10
Praziquantel	1	1	1	1		5
Proflavine	1	1 ^c	1 ^c	1 ^c		8
Propazine	1	1	1	10		8
Propranolol	1	1	1	1		7
Quinalphos	1	1	1	1		10
Quinoclamine	1	1 ^c	1 ^c	1 ^c		6
Rantidine	1	1	1 ^c	10		14
Rifampicin	1	1 ^c	10	10		6
Rifaximin	1	1 ^c	1 ^c	1		7
Rotenone	1	1	1 ^c	10		13
Roxithromycin	1	1	1	1		7
Simazine	10 ^d	1	1	1		7
Simvastatin	1	10	10	100		10
Sotalol	1	1	1	1		5
Sulfisoxazole (Sulfafurazole)	1	1	1	1		8
Thiabendazole	1	1	1	1		9
Trichlorfon	1	1	1	1		10
Trifluralin	>100	>100	>100	>100		ND
Virginiamycin M1	1	1	1	1		4

COMPOUND	Screening Limit ^a (ng/g)				Relative Standard Deviations ^b for 10 ng/g in tilapia	
	TILAPIA	SHRIMP	SALMON	EEL		
NEGATIVE ION COMPOUNDS						
Clofibric acid	1	1	1	1	5	
Fipronil	1	1	1	1	6	
Fipronil sulfone	1	1 ^c	1 ^c	1 ^c	7	
Genfibrozil	100	100	10 ^d	>100	30	
Triclocarban	10 ^d	100	10	10	30	
Triclosan	10 ^d	100	100	>100	23	

^aScreening 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 All Ion Fragmentation (AIF). Screening levels obtained with Parallel Reaction Monitoring (PRM) are the same unless indicated.

^bRelative standard deviations are calculated as *standard deviation/average amount found in fortified samples* × 100 (N = 6). All compound fortified at 10 ng/g in tilapia except marbofloxacin and orbifloxacin (0.5 ng/g).

^cScreening limit with PRM is 10 ng/g

^dScreening limit with PRM is 100 ng/g

^eScreening limit with PRM is > 100 ng/g

^fIf the MS² ion at m/z 145.0649 is monitored instead of MH⁺, then carbaryl can be detected at 10 ng/g and above in these matrices