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Application and evaluation of a high-resolution mass spectrometry screening method for veterinary drug residues in incurred fish and imported aquaculture samples

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Abstract

The ability to detect chemical contaminants, including veterinary drug residues in animal products such as fish, is an important example of food safety analysis. In this paper, a liquid chromatography high-resolution mass spectrometry (LC-HRMS) screening method using a quadrupole-Orbitrap instrument was applied to the analysis of veterinary drug residues in incurred tissues from aquacultured channel catfish, rainbow trout, and Atlantic salmon and imported aquacultured products including European eel, yellow croaker, and tilapia. Compared to traditional MS methods, the use of HRMS with nontargeted data acquisition and exact mass measurement capability greatly increased the scope of compounds that could be monitored simultaneously. The fish samples were prepared for analysis using a simple efficient procedure that consisted of an acidic acetonitrile extraction followed by solid phase extraction cleanup. Two different HRMS acquisition programs were used to analyze the fish extracts. This method detected and identified veterinary drugs including quinolones, fluoroquinolones, avermectins, dyes, and aminopenicillins at residue levels in fish that had been dosed with those compounds. A metabolite of amoxicillin, amoxicillin diketone, was also found at high levels in catfish, trout, and salmon. The method was also used to characterize drug residues in imported fish. In addition to confirming findings of fluoroquinolone and sulfonamide residues that were found by traditional targeted MS methods, several new compounds including 2-amino mebendazole in eel and ofloxacin in croaker were detected and identified.

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Compliance with ethical standards

Reference to any commercial materials, equipment, or process does not, in any way, constitute approval, endorsement, or recommendation by the U.S. Food and Drug Administration. In addition, the views expressed in this article are those of the author(s) and may not reflect the official policy of the Department of Health and Human Services, the U.S. Food and Drug Administration, or the U.S. Government.

The experimental protocol to generate the incurred samples was approved by the Animal Care and Use Committee at the FDA/CVM/OR, and all procedures were conducted in accordance with the principles stated in the Guide for the Care and Use of Laboratory Animals (2011) and the Animal Welfare Act of 1966 (P.L. 89–544), as amended.

Conflict of interest The authors declare they have no conflict of interest.

Keywords

High-resolution mass spectrometry; Screening method; Veterinary drug residues

Introduction

An important ongoing issue in food safety is adulteration with chemical contaminants. One example of this is the presence of veterinary drug residues in animal-based food products. According to a World Bank Report [1], by 2030 over 100 million tons of seafood will be consumed worldwide and over 60% is expected to be farmed. Judicious aquaculture practices will be important to sustain this food resource. One possible downside of fish farming is the potential use of chemotherapeutics, such as antibiotics, anthelmintics, and antifungals, to maintain a healthy population of animals housed in a high-density environment. Although some veterinary drugs have been approved in various countries for use in aquaculture [2], the use of unapproved drugs remains a problem. A primary concern with the use of antibiotics for food animals, including aquaculture, is the potential for increased antimicrobial resistance. Other chemicals used in fish farming, such as chloramphenicol or the triphenylmethane dyes used as antifungal agents, also have potential adverse human health effects and are prohibited for use in food animals. Nevertheless, residues of these compounds and other veterinary drug residues are continually found in fish and shellfish [3–5]. Monitoring for these residues in aquacultured products is critical to minimize human exposure to unintended veterinary drugs in this important food commodity.

Traditionally, analytical methods developed to monitor for drug residues in aquaculture were specific to a limited set of target compounds. Most recently, these methods utilize liquid chromatography coupled to tandem mass spectrometry using a triple quadrupole mass analyzer programmed to acquire data for selected ion transitions corresponding to the analytes of interest [6–8]. Although this technique can be very sensitive and selective, it does require preselection of the analytes prior to data acquisition and therefore can miss other compounds that could be present in fish.

A current trend in monitoring for veterinary drugs and other contaminants in food products is to use high-resolution mass spectrometry (HRMS). With HRMS, a potentially unlimited number of compounds can be analyzed simultaneously because full-scan data are collected, rather than preselected ion transitions corresponding to specific compounds. The selectivity and sensitivity required to detect and identify low levels of analytes in complex food matrices are achieved by taking advantage of the HRMS ability to provide very accurate exact mass measurements. This capability produces methods that can detect a wide range of residues and contaminants, allowing regulatory agencies to be more proactive in discovering possible adulteration of food, including aquacultured fish and shellfish [9–12].

Our laboratory recently developed and validated a screening method [13] for veterinary drug residues in fish, shrimp, and eel using LC with a quadrupole-Orbitrap HRMS. This screening method can monitor for over 300 veterinary drugs using an in-house compound database that includes exact mass measurements and retention times. The optimization and validation of this method, including the extraction procedure and HRMS acquisition

parameters, focused on 70 test compounds most likely to be found in aquaculture products. Target testing levels were determined for the 70 test compounds that were validated in this method; only qualitative screening was performed for the other compounds in the database as acceptable residue levels have not been established. The sample preparation consisted of an acidic acetonitrile extraction followed by solid phase extraction cleanup. Data were collected using both nontargeted and targeted acquisition. The amount of drug residues was estimated for these test compounds by comparing MS¹ signal to a one-point extracted matrix standard at a target testing level. Comparing samples to an extracted standard in the same seafood matrix corrected for any differences in recoveries and/or matrix effects between individual analytes. For screening, false-negative and false-positive rates for most test compounds were acceptable at less than 5%. With this method, it was possible to begin to look for additional unexpected compounds, including metabolites, to increase the scope of residue monitoring in farmed fish products.

In the current study, the application and evaluation of this wide-scope screening method was explored further. Specifically, the method was applied to the analysis of incurred and imported samples to determine what veterinary drug residues might be present. The screening procedure was used to analyze several species of fish that had been dosed with different classes of veterinary drugs, including difficult analytes such as avermectins, aminopenicillins, and dyes. In addition, the method was applied to imported fish samples to detect and identify drug residues that might be missed using traditional targeted analytical methods.

Materials and methods

Incurred fish samples

Channel catfish (*Ictalurus punctatus*), rainbow trout (*Oncorhynchus mykiss*), and Atlantic salmon (*Salmo salar*) were obtained from commercial or government sources as juveniles and grown to market size in recirculating aquaculture systems at the US Food and Drug Administration, Center for Veterinary Medicine, Office of Research (FDA/CVM/OR; Laurel, Maryland). The fish were fed commercial fish feed appropriate for each species (Zeigler Bros. Inc., Gardners, Pennsylvania) and were not administered any drug or chemical treatments prior to this study. To administer treatments, the fish were held in tanks supplied with a constant flow of the same water used for the holding systems at a similar temperature (23 °C—channel catfish; 13 °C—rainbow trout and Atlantic salmon) or in a static bath (bath incursions).

Capsule feeding—Eleven trout and 11 salmon (1 fish/drug) were tube fed capsules containing individual fluoroquinolones sarafloxacin (SAR), difloxacin (DIF), norfloxacin (NOR), or a combination of ciprofloxacin (CIP) and enrofloxacin (ENR); quinolones flumequine (FLU), oxolinic acid (OXO), and nalidixic acid (NAL); and avermectins ivermectin (IVR), doramectin (DOR), emamectin (EMA), and abamectin (ABA). The fluoroquinolone and quinolones were given at a dosage of 0.5 mg/kg body weight. Fish dosed with SAR, DIF, NOR, or ENR/CIP were sacrificed 6 days after dosing and fish dosed with FLU, OXO, or NAL were sacrificed 1 day after dosing. The avermectins were given at

0.1 mg/kg body weight and sacrificed 3 days after dosing. The different sacrifice dates were selected to achieve a target tissue level of approximately 1 to 10 µg/kg for each compound.

In a separate round of dosing, catfish, trout, and salmon (2 fish/drug; 4 fish/species) were tube fed capsules containing either amoxicillin (AMOX) or ampicillin (AMP) as the trihydrated forms at a dose of 100 mg/kg body weight. All of the catfish and one trout given AMOX were sacrificed 6–8 h after dosing. The remaining trout and salmon were sacrificed 1 day after dosing since we found undigested AMOX powder in the upper GI tract of the trout sampled at the earlier timepoint. The dosing targeted a tissue level of 200–500 µg/kg. Two control fish not dosed with a drug were sacrificed for each species.

Bath incursion—Trout and salmon (1 fish/species) were immersed in a water bath containing a combination of the triphenylmethane dyes brilliant green (BG), crystal violet (CV), and malachite green (MG) at a concentration of 4 µg/L each. After 1 h, the fish were moved to a bath containing only water (no dyes) for 1 h before being sacrificed. Similarly, one trout and one salmon were immersed in a water bath containing 0.1 mg/L of isoeugenol (ISOEUG) following previously described methods [14]. After 30 min, the fish were immersed in a separate bath of clean water for another 30 min before being sacrificed. The targeted tissue levels were 1 µg/kg for each dyes and 200 µg/kg for isoeugenol.

Dosed feed—Amoxicillin-containing fish food was prepared at a concentration of 20 g/kg by topcoating fish food with amoxicillin trihydrate. The commercial fish feed used to grow each species (500 g feed) was first mixed with cod liver oil (1 mL/25 g feed) using a benchtop mixer for 10 min at low speed following previous published methods [15]. The drug was then shaken on the feed/oil mixture using fine-mesh sieve while the mixer was running at low speed. Afterwards, the feed was mixed for 10 min. The pellets were spread on foil and allowed to air dry for 1–2 h at room temperature. After drying, the feed was placed in sterile plastic bags and stored at approximately 4 °C until use. Trout, salmon, and catfish (4 fish/species) were fed at a rate of 0.5% body weight once a day for 5 days. The fish were sacrificed 6 h or 1 day after the last feeding (2 fish/ withdrawal time) to achieve a target tissue level of 200–500 µg/kg. The feeding resulted in a dosing level of 100 mg feed/kg body weight. (Note—the two salmon sacrificed 1 day after feeding were fed medicated feed for 6 days due to inclement weather.)

Imported fish samples

Imported fish samples of eel, yellow croaker, and tilapia were collected according to the US FDA Chemotherapeutics in Seafood Compliance Program 7304.018 [16] and analyzed with validated LC-MS/MS triple quadrupole procedure [6] prior to analysis with the current LC-HRMS screening method.

Preparation of fish samples

Fish muscle tissue (fillets) was homogenized to a fine powder with dry ice. Fish skin was included in the sample preparation except for catfish, where it was removed prior to processing. Fish samples are generally prepared as they would be marketed to the consumer (with or without skin). Tissues were stored at – 20 °C until used. The samples were

extracted according to a method previously described [13]. Briefly, homogenized tissue (2.0 g) was extracted with 8 mL of extraction solution consisting of 0.2% *p*-toluene sulfonic acid monohydrate (*w/v*) and 2% glacial acetic acid (*v/v*) in 100% acetonitrile. The samples were vortex mixed for 30 min then centrifuged (17,000×*g*) for 7 min. For cleanup, a portion (3 mL) of the extract was allowed to pass through an Oasis PRIME HLB 6 cm³ (200 mg; Waters Corp.) extraction cartridge. The sorbent material in the Oasis PRIME HLB cartridge is designed to eliminate phospholipid interferences without severe loss of analyte recovery. The sample portion was allowed to gravity drain through the cartridge, and the last few drops of extractant was gently pushed through with a pipet bulb resulting in approximately 2 mL of liquid.

To analyze the more nonpolar analytes including the avermectins and leuco metabolites of the triphenylmethane dyes, especially in salmon tissue, 100 µL of the solid phase extraction eluent was transferred into a vial for a separate LC-MS injection. The remaining portion of the extract was taken to near dryness under nitrogen and then reconstituted with 400 µL of 10% acetonitrile in water (*v/v*), mixed, and centrifuged (28,900×*g*) for 7 min. An aliquot of 300 µL was transferred into an LC vial for analysis.

For each batch of incurred or imported samples, negative and positive control samples prepared with fish of the same species were also analyzed. The positive control sample was fortified with the test compounds used previously to validate the screening method [13]. These included 70 analytes representing a wide range of analytes and target testing levels. If the response for one of these test compounds found in an incurred or imported sample was at least 50% of that in the positive (1× target testing level, TTL) control and met identification criteria [17], then the sample was considered to be presumptive positive for that compound. The threshold cutoff level of 50% as compared to a positive control was established previously based on the measured variance of the response for analytes fortified in fish at the 1× TTL level [13]. Each test sample was extracted and analyzed in duplicate.

Instrumental method

The instrument used was a Thermo Q-Exactive Orbitrap high-resolution mass spectrometer (HRMS) with a heated electrospray ionization source and a Thermo Ultimate 3000 LC system. Thermo XCalibur (V.4.0) and TraceFinder software (V.4.1) were used to collect and analyze data.

The LC column was a Supelco Ascentis Express C18 (7.5 cm × 2.1 mm, 2.7 µm). The mobile phase (0.3 mL/ min) consisted of 0.1% formic acid in water (A) and acetonitrile (B). The LC gradient program was held at 5% B for 1.5 min then ramped to 50% B from 1.5 to 8.5 min, followed by 99% B from 8.5 to 9 min, and held at 99% B from 9 to 12 min. The column was re-equilibrated at 5% B for 2 min before the next injection. Other LC parameters included oven temperature at 30 °C, sample tray at 10 °C, and injection volumes of 10 or 20 µL depending on the data acquisition program (see below).

Two different MS acquisition programs were used to analyze the fish extracts. Both acquisition programs included a MS¹ (*m/z* 150–1000) scan followed by either a nontargeted All Ion Fragmentation (AIF) MS² scan or a more targeted data-dependent MS² (DDMS²)

data collection. AIF acquisition was initially used to screen the samples using a 10- μ L injection of fish extract. With AIF, all precursor ions are introduced into the high collision dissociation (HCD) cell to form product ions simultaneously. A separate injection of fish extract (20 μ L) was performed using DDMS² data acquisition where MS² data were collected only when a precursor ion from a predefined “inclusion list” was detected above a set threshold. When that occurs, the quadrupole filters the precursor ion into the HCD cell using a limited m/z window to produce fragment ions related to that compound. A more detailed description of these MS methods have been described previously [13]. Due to an upgrade in software from the original method, some settings, particularly for DDMS², were changed slightly. The following MS settings were used in this study: AIF full MS (70K resolution, 3e⁶ automatic gain control (AGC) target, maximum inject time 200 ms, m/z 150–1000 scan range) AIF MS² (70K resolution, 3e⁶ AGC target, maximum inject time 200 ms, m/z 80–1000 scan range, normalized collision energy 10, 30, 50); DDMS² full MS (70K resolution, 5e⁶ AGC target, maximum inject time 200 ms, m/z 150–1000 scan range) DDMS² (17.5K resolution, 1e⁶ AGC target, maximum inject time 50 ms, loop count 3, isolation width 4 m/z , normalized collision energy 10, 30, 50) dd settings (intensity threshold 2e⁴, Apex trigger 0.1–6 s, dynamic exclusion 6 s).

Each incurred fish sample was extracted in duplicate and analyzed (positive ion only) using AIF and DDMS² acquisition programs. For the analysis of fish that had been dosed with amoxicillin or ampicillin, the DDMS² data acquisition method was modified to use an inclusion list containing only β -lactam compounds and metabolites. The imported fish samples were analyzed by AIF and DDMS² in both positive and negative ion modes.

Data analysis

The data were initially analyzed using a quantitative data analysis program established for the 70 validated compounds to determine which residues met presumptive positive criteria as described previously [13]. In order for one of these analytes to be presumptive positive, a residue had to meet qualitative confirmation of identity criteria [17] and have a peak area of 50% as compared to matrix-extracted standard at 1 $\times$ TTL (positive control). The Thermo TraceFinder data analysis “Screening Method” was used to search for additional residues beyond the validated test compounds by comparison to a compound database containing ~ 350 potential veterinary drug residues, including metabolites and minor components. Criteria used in the “Screening Method” were 3 ppm mass accuracy tolerance for the precursor ion, signal-to-noise ratio > 100, and a signal of > 5000 counts for initial detection. In order to confirm a residue using the TraceFinder “Screening Method” software, a retention time window match within 60 s and a minimum of one fragment ion with intensity threshold of > 500 counts and mass tolerance within 10 ppm were selected. The isotope pattern match option was also used to filter out false detects. In addition, positive findings were further evaluated for validity according to the following more subjective criteria: signal intensity (preferably peak areas > 1e⁵), Gaussian chromatographic peak shape, repeatability (compound detected in all replicates), and uniqueness (peak found in test samples only, rather than in all samples and controls).

Results and discussion

This paper describes the application of a wide-scope screening method for veterinary drugs to real-life samples including fish incurred with various drugs and imported seafood samples to determine how well the method performs and if additional, unexpected, drug residues could be identified.

Incurred fish

Testing analytical methods with tissues from animals that have been administered veterinary drugs, as opposed to just fortifying control tissue with compounds, is critical in determining how effective these procedures will be for monitoring real samples. In this study, several species of fish (trout, salmon, and catfish) were treated with a variety of veterinary drugs. The goal for the fish dosing study was to generate tissues that had residue levels near the target testing levels (1–200 µg/kg depending on the analyte). It was not necessarily meant to mimic dosing protocols that might be used in the aquaculture industry as these drugs are not approved for use in the USA. The initial dosing study was to determine if the HRMS screening method could detect a variety of different compound in representative fish, and only one individual salmon or trout was dosed with each drug. Subsequent dosing studies were meant to be a more in-depth study of aminopenicillins with more types and numbers of fish since these compounds are particularly challenging analytes. The HRMS method performed well with these incurred fish samples by isolating and detecting the resulting residues in the tissues. This was also an opportunity to verify appropriate marker residues and/or characterize additional metabolites indicative of drug use. The results for residues found in incurred fish are shown in Tables 1, 2, and 3.

Quinolones and fluoroquinolones—The ability to detect residues of quinolones and fluoroquinolones is important because these synthetic antibiotics are often used in aquaculture and are found in fish at violative levels [3]. The transfer of antimicrobial resistance from food animals to humans has also been documented with this class of drugs [21]. In this study, both trout and salmon were dosed with these compounds at relatively low levels (0.5 mg/kg body weight). In general, these results were as expected with significant amounts of the parent compounds detected in the fish muscle. The amounts of SAR, DIF, FLU, OXO, and NAL found were in the 10–200 µg/kg range (Tables 1 and 2), and the identity of all compounds was confirmed by comparing retention times and the mass accuracy of the precursor and at least one product ion to known values according to the FDA guidelines [17]. The residue levels in salmon and trout were similar with the exceptions that higher levels of OXO and FLU were found in trout, and the residue levels of SAR were higher in salmon. Small amounts of SAR were also found in both species of fish that had been dosed with DIF; the metabolism of DIF to form SAR in fish has been reported in earlier work [22]. In the fish dosed with both ENR and CIP, primarily ENR (~ 100 µg/kg) was found with small amounts of CIP (4 µg/kg) also detected. Desethylene ENR, a metabolite of ENR that has been observed previously [13], was not detected in these fish. The dosing level for the current study (0.5 mg/kg) was ten times lower than in the previous work with subsequently lower residue levels.

Fish dosed with NOR, ENR/CIP, FLU, OXO, and NAL were also analyzed using a LC-MS/MS triple quadrupole method; these results are also shown in Tables 1 and 2. In general, the residue levels determined for these analytes using a triple quadrupole screening method compared well to the amounts estimated using the HRMS method. It is interesting to note that residues of NOR were not detected in either salmon or trout dosed with this drug regardless of whether the HRMS or triple quadrupole screening methods was used, even though NOR was adequately recovered in fish fortified with this compound. Because full scan data were collected by HRMS, it was possible to look for degradants (e.g., loss of water or CO₂) and potential metabolites of NOR, including acetylation products that have been described [23, 24]. None of these compounds were detected in the extracts from trout or salmon that had been dosed with NOR. Previous dosing studies with NOR in other fish (sea bream, sea perch) [25] did show measurable amounts of NOR in muscle tissue, but the dosing levels were much higher (30 mg/kg for 5 days) than in this current study. NOR was detected at a low level (5 µg/kg) in one imported fish analyzed by the HRMS method. Nevertheless, the fact that NOR residues were not detected in the dosed salmon or trout, in contrast to the fish that were dosed at the same level with the other fluoroquinolones in the current study, may be worth further investigation.

Avermectins—Avermectins including IVR, DOR, EMA, and ABA are macrocyclic lactone compounds used as anti-lice agents in fish, particularly in salmon [26]. Emamectin benzoate, known commercially as Slice®, has been approved in several countries for this purpose [27]. As described previously [13], these compounds are difficult to detect in a multi-class residue methods because they are more nonpolar and tend to form very stable sodiated ions. In the initial study [13], it was determined that the most effective way to monitor for these compounds was to analyze the acetonitrile extract prior to evaporation and reconstitution. The levels needed for the compounds that form sodium adducts (IVR and DOR) to consistently meet identification criteria were relatively high (200 µg/kg), although the precursor ions for these compounds could often be detected at lower (20 µg/kg) levels. When analyzing the fish that had been dosed with avermectins for this study, the precursor (MNa⁺) and one known product ion for DOR (*m/z* 449.2513) were detected in dosed salmon and in one of the dosed trout at the correct retention time with concentrations corresponding to 25–40 µg/kg using AIF data acquisition. The precursor ion for IVR (MNa⁺) was also detected with similarly high concentrations (30–60 µg/kg) in both trout and salmon that had been treated with IVR, but product ions of IVR were not detected using either AIF or DDMS² data acquisition. For EMA, however, the HRMS screening method was able to detect and confirm the identity of EMA with product ions from the MH⁺ precursor ion in both trout and salmon (Fig. 1). The concentrations found for EMA were 10–30 µg/kg when compared to the matrix extracted positive control at 1× TTL (200 µg/kg).

The estimated amounts of EMA, DOR, and IVR found by this HRMS screening method were compared to the values calculated using avermectin-specific quantification methods. The levels found were comparable, especially for salmon, considering that the UPLC/fluorescence [18] and LC-MS/MS [19] quantitative methods utilized a multi-point calibration curve, and the HRMS values were estimated using a single point extracted standard at a higher fortification level (200 µg/kg). The HRMS method overestimated the

amounts of avermectins in trout, but fulfilled the purpose of the screening method to detect and identify the residues so additional quantification could be performed. The residue levels from these methods are included in Tables 1 and 2. Abamectin was not included in the fortification standard used for the positive control (matrix-extracted standard), but when data from trout and salmon dosed with this compound were compared to the veterinary drug database, the sodiated ion of ABA B1_a was detected at the correct retention time. Product ion data could also be collected using DDMS² and several ions corresponding to this compound were observed in the dosed trout and salmon samples. The MS¹ response for sodiated DOR and ABA at the same concentration in a solvent standard were very similar, so the amount of ABA in the incurred fish was estimated using peak areas from DOR at 200 µg/kg in the matrix-extracted standard.

Dyes—Triphenylmethane dyes are a class of veterinary drugs used as antifungal agents in aquaculture. They are important to monitor due to their potential carcinogenicity, but they can also be challenging to include in a multi-class screening method due to their unique chemical properties and need for low (1 µg/kg) detection limits [28]. Previously, it was shown that this HRMS screening method [13] could detect dyes in catfish and salmon that had been exposed to these compounds. Although the measured levels were somewhat lower than those found using the quantitative AOAC method [20], the estimated amounts were appropriate to determine the samples were presumptive positive and should be processed for further testing. The results from the current work are similar (Tables 1 and 2). For both trout and salmon dosed in a bath containing MG, CV, and BG, this method detected and identified residues of MG, the leuco metabolite of MG (LMG), and BG at levels higher than the screening threshold (> 50% of the target testing level of 1 µg/kg). The leuco metabolite of crystal violet (LCV) was also detected in trout. Residues were confirmed with product ions acquired by AIF. With the exception of BG in trout, DDMS² product ion spectra were also obtained. These results were compared to both a LC-MS/MS screening method [6] and to the official AOAC method for quantification of triphenylmethane dyes [20] in seafood products. In general, the amounts of the parent compounds (BG, MG, and CV) found by the HRMS screen were similar to those from the AOAC method in salmon and somewhat lower values estimated for trout. CV was not detected using the HRMS screening method, and the amount of CV found by the AOAC method was very low (0.1 µg/kg). The HRMS screening method underestimated the amounts of leuco metabolites, but the amounts found were enough to be considered presumptive positive for samples that had > 1 µg/kg of these analytes as determined by the AOAC method. The HRMS screening method (comparing samples to a matrix-extracted standard in the same fish species) estimated amounts of BG and MG in these tissues that were closer to those found by the AOAC method as compared to the LC-MS/MS triple quadrupole screening method where residue concentrations were compared to a tilapia fortified matrix-extracted standard.

Isoeugenol—Isoeugenol is approved as a sedative for fish in several countries, but is not allowed in the USA due to concerns of carcinogenicity [29]. This compound was not included in the initial HRMS screening method validation [13], but fish that had been dosed in a bath of isoeugenol were analyzed to see if this analyte, or related compounds, could be detected. Only trace amounts (with poor signal-to-noise and no product ions) of isoeugenol

were observed in these dosed fish as compared to control tissue. Therefore, this screening method would not be suitable for monitoring residues of isoeugenol. This is not unexpected as the routine monitoring method for isoeugenol in fish requires derivatization prior to LC-MS/MS analysis due to poor ionization of the native compound [29]. No other compounds were observed in the full scan total ion chromatogram of these fish extracts when compared to data from control animals.

Ampicillin and amoxicillin—Several fish dosing studies were performed to test the effectiveness of this HRMS screening method for monitoring ampicillin (AMP) and amoxicillin (AMOX). Analogs of penicillin are difficult to analyze at residue levels due to their high polarity and instability. Although these drugs are not approved for use in aquaculture in the USA, there is some concern they may be used for control of infection in farmed fish [15, 30–33]. AMOX is approved for use in fish in other countries. Initially, salmon, trout, and catfish were dosed with capsules containing either AMP or AMOX at a dose of 100 mg/kg body weight with a depletion time of either 6 or 24 h. The results from this dosing study using the HRMS method to analyze the tissues are found in Table 3. The amount of AMP in the dosed salmon and trout was estimated to be 100–200 µg/kg as compared to matrix-extracted standards fortified with AMP at 1× TTL (25 µg/kg). The levels of AMP found in catfish were lower (10–20 µg/kg). All residues of AMP in the dosed fish were confirmed using US FDA guidance for compound identification [17] using exact mass data for both AIF and DDMS² analyses. The data were investigated to look for other known metabolites of AMP (addition of water, loss of CO₂, etc.), but these compounds were not observed. The levels of AMP found in fish at these time points are consistent with a previous study where AMP was detected after 18 h at 63 µg/kg after IV dosing striped bass (10 mg/kg) [33].

The same species of fish dosed with capsules containing AMOX were also analyzed (Table 3). When the dosed samples were compared to a positive control of the same species containing AMOX fortified at 100 µg/kg, the levels of AMOX varied depending on the individual fish with catfish generally lower (15–20 µg/kg). Higher amounts were found in salmon tissue with almost 90 µg/kg of AMOX estimated in one fish and approximately 500 µg/kg in the other salmon sample. The tissue from one fish (Sc4) that had been dosed with AMP also had appreciable levels of AMOX. There was a problem with this fish needing to be given an additional capsule after rejecting one dose, so it could be that both drugs were inadvertently given to this particular fish.

A second dosing study for AMOX in salmon, catfish, and trout was performed by giving fish food that contained this drug. The results of this study are also shown in Table 3 and are similar to those from the fish dosed with capsules in that the catfish levels are generally lower and the estimated numbers vary between individual fish. The levels of AMOX found vary more dramatically between individual fish than between the fish sacrificed at 6 or 24 h. The greater variance between fish could be due to differences between feeding habits among the individual fish and/or the instability of the analyte.

In addition to AMOX, another chromatographic peak at later retention time was observed (Fig. 2) with an MH⁺ of *m/z* 366.1118 (isomeric with AMOX) in fish extracts dosed with

this drug either by capsule or with medicated feed. It was assumed that this compound corresponds to the amoxicillin diketopiperazine (AMOX DIKETONE) as has been observed by others in poultry [34–36], but has not been reported previously in fish. Subsequently, a standard of AMOX DIKETONE was purchased and analyzed. In addition to matching retention time, the fragment ions (m/z 207, 160, 114) found in the DDMS² product ion spectrum of the AMOX DIKETONE standard were also observed in the late-eluting peak in the fish extract (Fig. 3). These ions also matched those reported previously for this diketone metabolite [34–37]. Other ions (m/z 322, 237) observed in the DDMS² spectrum from the m/z 366 ion in the incurred fish samples were also seen at high levels in the control fish and are believed to be matrix-related rather than generated from the AMOX DIKETONE. The area counts for the later eluting peak were typically 2–5× more than those from AMOX in the capsule dosed fish extracts (Table 3). The peaks for amoxicillin diketone were also detected with area counts two to eight times higher than amoxicillin itself in the sample extracts from fish dosed with AMOX in the feed. It is difficult to estimate the relative amount of these two compounds because when the diketone standard was analyzed, it gave a much higher (6–10×) response in the extracted ion chromatogram at m/z 366.1118 as compared to a standard of AMOX at the same concentration. It does appear, however, that the AMOX DIKETONE may be present at similar levels to the parent compound in the fish. This may indicate that the diketone, in addition to AMOX, should be monitored as another marker residue for AMOX use. This diketone compound was not identified in previous studies that analyzed fish dosed with AMOX. This could be because the detection of residues was less specific, using either microbial inhibition [31] or fluorescence detection of a hydrolyzed formaldehyde derivative that may not distinguish between AMOX and the diketone metabolite [30, 38]. In general, the levels of AMOX found in these previous studies are similar. For example, in one study [30], four out of five catfish had AMOX residues at 40–65 µg/kg in tissue 6 h after an oral dose of 110 mg/kg body weight; a fifth catfish had markedly higher AMOX concentration of 300 µg/kg. Because this HRMS method is a semi-quantitative screen (limits test), a more quantitative method for these compounds with extraction and chromatographic procedures more tailored for penicillin compounds may be developed for fish [39]. Another compound at 1.2 min was also observed in the extracted ion chromatograms for m/z 366.1118 (Fig. 2). This peak is present in the control salmon and is believed to be an isobaric compound present in the matrix. The measured mass of the m/z 366 ion at 1.2 min is almost 5 ppm different than the theoretical m/z of amoxicillin (the peaks at 1.8 and 4.7 min are within 1 ppm), and no fragment ions associated with amoxicillin are observed at 1.2 min in the product ion scans.

Imported fish samples

This HRMS screening method was previously used successfully [13] to analyze imported aquaculture samples for several veterinary drug residues including sulfonamides, quinolones, and their metabolites. In this study, the screening method was applied to additional imported fish samples. A summary of the compounds found is shown in Table 4. A more thorough data analysis of samples analyzed previously (Eel#1, Eel#2, Tilapia#1) using an expanded veterinary drug database was also performed retrospectively. These particular samples were chosen for additional analysis after they had been found to contain violative amounts of drug residues using the LC-MS/MS screening method [6], and initially

the HRMS method was used to confirm these results. For example, with the new samples analyzed, sulfonamides and trimethoprim were detected in Eel sample #3 (330 µg/kg sulfamethazine, 270 µg/kg trimethoprim) and Tilapia #2 (20 µg/kg of sulfamethoxazole, 7 µg/kg trimethoprim). These levels were similar to those initially found by the LC-MS/MS method. Quinolones and fluoroquinolones that were initially found using the targeted method were also found at similar levels using the HRMS screen in eel, croaker, and tilapia samples. Low levels (below 50% of the target testing levels) of other drug residues included in the positive control mix found with the HRMS method include azithromycin, ethoxyquin, erythromycin, florfenicol amine, lincomycin, and oxytetracycline in eel. When these current and older samples were searched against the larger veterinary drug compound database, additional analytes were found in several of these samples including ethoxyquin dimer and the corresponding *N*-acetyl metabolites of several of the sulfonamide compounds. Of particular interest, two veterinary drug residues, 2-amino mebendazole and ofloxacin, that would have been missed with traditional targeted methods were detected and identified at relatively high levels in some of these imported fish samples using this HRMS screening procedure.

2-Amino mebendazole—In Table 4, many of the residues in Eel #1 and #2 were reported previously [13], but the data from these samples were reanalyzed retrospectively to characterize additional analytes. This evaluation showed the potential presence of several additional compounds at appreciable area counts. These compounds included haloperidol in a negative ion scan and 2-amino mebendazole in positive ion chromatograms collected using AIF acquisition. These standards were obtained and analyzed. Haloperidol was ruled out as this analyte only forms positive ions and elutes at a significantly different retention time. The standard of 2-amino mebendazole, on the other hand, matched the retention time of the peak found in the positive ion MS¹ chromatogram of the eel extract. In addition, the primary product ion observed in the 2-amino mebendazole standard (m/z 105.0335, C₇H₅O⁺) was also detected in the AIF MS² scan from the imported eel samples (Fig. 4). This compound was present at high levels (compared to a 100 ng/mL solvent standard) in Eel sample #1 and at low levels in Eel sample #2. The 2-amino mebendazole analyte was also found in two other eel samples analyzed recently (Table 4) and one imported tilapia sample analyzed previously. It was not detected in three other imported eel samples or any other of the fish tested (data not shown). The presence of this compound in eel is reasonable as it has been previously reported that mebendazole is an effective anthelmintic for eels [40] and 2-amino mebendazole is a primary metabolite [41]. This compound has since been included in the routine monitoring of aquacultured products using the LC-MS/MS triple quadrupole platform. Although acceptable levels for 2-amino mebendazole have not been determined for fish, there is a maximum residue level established in the EU for mebendazole and its metabolites of 60 µg/kg in sheep and goat muscle, so 60 µg/kg or lower may be a reasonable target testing level for these residues in eel and other aquacultured products [42].

Ofloxacin—Another example of an imported fish sample where this HRMS screening method detected an unexpected drug residue, ofloxacin, was a yellow croaker fish that had been collected from the Fujian region of China. Croaker is a fish that lives along the coast in Asia and is known to be cage-farmed by small family operations [43]. This sample was

originally analyzed using the LC-MS/MS method and very high levels of enrofloxacin (> 5000 µg/kg) and ciprofloxacin (~ 500 µg/kg) were found. Subsequent analysis by HRMS found, in addition to high levels of these compounds, small amounts of trimethoprim and erythromycin. When searching against the larger veterinary drug compound database, large amounts of the metabolite desethylene enrofloxacin and another fluoroquinolone, ofloxacin, were detected (Fig. 5). An estimated amount of ofloxacin was not available because this compound was not included in the standard mixture fortified for the positive control, but the area counts were similar to those of ciprofloxacin in this sample extract. A solvent standard of ofloxacin was analyzed using both AIF and DDMS² data acquisition. In addition to the peak in the croaker sample matching the retention time and precursor accurate mass of the ofloxacin standard, the product ion spectrum of the compound observed in the croaker sample matched the analytical standard (Fig. 6).

Although this compound matched all confirmation criteria for ofloxacin, existing literature [44, 45] was reviewed to determine if this compound could be another metabolite of enrofloxacin rather than the parent compound ofloxacin resulting from fish treatment with a second veterinary drug. Of the over 130 metabolites identified for enrofloxacin in an extensive study [45], only two matched the elemental formula of ofloxacin. One of those metabolites appeared to be much more nonpolar based on the relative retention time compared to enrofloxacin and ciprofloxacin and is therefore likely not a match for the compound detected in the croaker sample. The other metabolite (formyl derivative) could not be completely ruled out as the structure indicated that its product ions could be similar to those from ofloxacin. A standard of this metabolite was not available, so the exact retention time in this method could not be determined and compared to the peak in the sample, although it had a similar retention time to enrofloxacin and ciprofloxacin in the published study [45]. However, it is important to note that no compounds similar to ofloxacin were found in fish dosed with enrofloxacin in this study or in previous studies done in our laboratory [13] or by others [46]. In fact, one paper [46] describes the use of ofloxacin as an internal standard to determine the amounts of enrofloxacin and ciprofloxacin in a residue depletion study. In all of the other imported samples analyzed using this method, ofloxacin was only found in one other sample at relatively small amounts in Eel sample #3 (< 1% of a 100 ng/mL solvent standard). This sample contained sulfonamide and trimethoprim residues at high levels, but only low levels of CIP (15 µg/kg) and ENR (2 µg/kg) were present. Because ofloxacin is not a residue that was expected to be found in aquacultured fish, the source of this compound in the croaker sample is not known. Formulations of ofloxacin are available online for poultry feed. Ofloxacin has also been found at high concentrations in surface and sewage water in China and other parts of the world [47–49], so environmental contamination is also a possibility.

Overall, using HRMS screening methods has the advantage over traditional targeted MS method to expand the scope of monitoring food for additional chemical contaminants such as unexpected veterinary drug residues. Novel analytes, for example ofloxacin in croaker fish and 2-amino mebendazole in eel, have been detected and identified with this HRMS screening method. Additional metabolites that can further characterize drug administration, such as amoxicillin diketone, were also found in dosed fish. This paper demonstrates the

application of this HRMS technology to survey fish and shellfish for routine use, thereby better protecting the safety and integrity of this important commodity.

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Fig. 1.

Extracted ion chromatogram for emamectin MH^+ precursor ion (m/z 886.5311) with 5 ppm mass extraction window in trout fortified at 200 µg/kg (**A1**) and trout incurred with emamectin (**B1**). The product ion spectra from m/z 886.5311 collected using DDMS² data acquisition are also shown for trout fortified at 200 µg/kg (**A2**) and trout incurred with emamectin (**B2**)

Fig. 2.

Amoxicillin and amoxicillin diketone in salmon. Extracted ion chromatograms for m/z 366.1118 (5 ppm mass extraction window) for control salmon (A), salmon fortified at target testing level 100 µg/kg for amoxicillin (B), incurred salmon #Sf1 (C), and incurred salmon #Sf4 (D)

Fig. 3. Product ion spectra collected using DDMS² data acquisition of precursor ion m/z 366.1118 for amoxicillin in solvent standard (**A1**) and incurred salmon #Sf4 (**A2**) and for the amoxicillin diketone in solvent standards (**B1**) and from incurred salmon #Sf4 (**B2**)

Fig. 4.

Extracted ion chromatograms for 2-amino mebendazole using AIF data acquisition for MH + (1, m/z 238.0975) and primary product ion (2, m/z 105.03349) with a 5 ppm mass extraction window from 100 ng/mL solvent standard (A), negative control eel tissue (B), and imported eel sample #1 (C).

Fig. 5. Compounds found in imported croaker sample. Extracted ion chromatograms (5 ppm mass extraction window) for enrofloxacin, m/z 360.1718 (**A**), ciprofloxacin, m/z 332.1405 (**B**), desethyleno enrofloxacin, m/z 334.1562 (**C**), and ofloxacin, m/z 362.1511 (**D**)

Fig. 6. Product ion spectra collected using DDMS2 data acquisition for ofloxacin (precursor ion m/z 362.1511) from 100 ng/mL solvent standards (**A**) and imported croaker sample (**B**)

Table 1

HRMS screening results for incurred salmon samples

Drug dosing (mg/kg body weight)	Depuration time	Test compounds found presumptive positive AIF		Spectra obtained by DDMS ² for		Compared to other methods	
		Analyte	(µg/kg) ^a	Analyte	(µg/kg)	Analyte	(µg/kg)
SAR (0.5)	6 days	SAR	30	SAR	Not analyzed		
NOR (0.5)	6 days	Not detected (ND)		None	ND ^b		
DIF (0.5)	6 days	DIF	104	DIF, SAR	Not analyzed		
ENR, CIP (0.5)	6 days	SAR	2				
		ENR	102	ENR, CIP	ENR	136 ^b	
		CIP	4		CIP	5 ^b	
Isoeugenol (bath)	30 min	ND		None	Not analyzed		
FLU (0.5)	24 h	FLU	79	FLU	FLU	79 ^b	
O XO (0.5)	24 h	O XO	12	O XO	O XO	11 ^b	
NAL (0.5)	24 h	NAL	122	NAL	NAL	121 ^b	
EMA (0.1)	3 days	EMA	10 ^c	EMA	EMA	6.8 ^d , 10.1 ^e	
IVR (0.1)	3 days	IVR	31 ^c	None	IVR	20.9 ^d , 20.5 ^e	
DOR (0.1)	3 days	DOR	24 ^c	DOR	DOR	18.8 ^d , 18.3 ^e	
ABA (0.1)	3 days	ABA	32 ^{c,f}	ABA	ABA	17.7 ^d , 17.4 ^e	
BG, MG, CV (bath)	1 h	BG	3.8 ^c	BG	BG	0.5 ^b , 4.9 ^g	
		MG	2.3 ^c	MG	MG	0.1 ^b , 2.9 ^g	
		LMG	0.8 ^c	LMG	LMG	0.5 ^b , 1.6 ^g	
		CV	ND ^c		CV	ND ^b , 0.1 ^g	
		LCV	ND ^c		LCV	0.1 ^b , 0.6 ^g	

^a Amount calculated compared to one-point matrix extracted standard at target testing level, average of duplicate extracts^b Amount from LC-MS/MS screening method [6] compared to one-point matrix extract standard in tilapia at 1× target testing level^c Residues detected in acetonitrile layer prior to evaporation (in extracts from dosed fish and samples fortified at 1× target testing level) [13]

g_g AOAC 2012.25 LC-MS/MS method for triphenylmethane dyes, average of triplicate analyses [20]

f_f Compared to DOR in one-point matrix extracted standard at target testing level (200 µg/kg)

p_p LC-MS/MS method for avermectins [19]

p_p UPLC-fluorescence method for avermectins [18]

Table 2

HRMS screening results for incurred trout samples

Drug dosing (mg/kg body weight)	Depuration time	Test compounds found presumptive positive AIF		Spectra obtain by DDMS ² for		Compared to other methods	
		Analyte	(µg/kg) ^a	Analyte	(µg/kg)	Analyte	(µg/kg)
SAR (0.5)	6 days	SAR	16	SAR	Not analyzed	Not analyzed	
NOR (0.5)	6 days	Not detected (ND)		None	ND ^b	ND ^b	
DIF (0.5)	6 days	DIF	131	DIF, SAR	Not analyzed	Not analyzed	
ENR, CIP (0.5)	6 days	SAR	1				
		ENR	116	ENR, CIP	ENR	117	
		CIP	4		CIP	4 ^b	
Isoeugenol (bath)	30 min	ND		None	Not analyzed	Not analyzed	
FLU (0.5)	24 h	FLU	207	FLU	FLU	211 ^b	
OXO (0.5)	24 h	OXO	166	OXO	OXO	208 ^b	
NAL (0.5)	24 h	NAL	127	NAL	NAL	131 ^b	
EMA (0.1)	3 days	EMA	30 ^c	EMA	EMA	10.7 ^d , 13.5 ^e	
IVR (0.1)	3 days	IVR	57 ^c	None	IVR	26.3 ^d , 30.4 ^e	
DOR (0.1)	3 days	DOR	38 ^c	DOR	DOR	16.2 ^d , 16.2 ^e	
ABA (0.1)	3 days	ABA	90 ^{c,f}	ABA	ABA	26.6 ^d , 25.3 ^e	
BG, MG, CV (bath)	1 h	BG	4.2 ^c	BG	BG	0.9 ^b , 7.3 ^g	
		MG	2.7 ^c	MG	MG	0.1 ^b , 4.4 ^g	
		LMG	1.1 ^c	LMG	LMG	1.6 ^b , 2.8 ^g	
		CV	ND ^c	LCV	CV	ND ^b , 0.1 ^g	
		LCV	0.7 ^c		LCV	1.2 ^b , 2.6 ^g	

^a Amount calculated compared to one-point matrix extracted standard at target testing level, average of duplicate extracts^b Amount from LC-MS/MS screening method [6] compared to one-point matrix extract standard in tilapia at 1× target testing level^c Residues detected in acetonitrile layer prior to evaporation (in extracts from dosed fish and samples fortified at 1× target testing level) [13]

g_g AOAC 2012.25 LC-MS/MS method for triphenylmethane dyes, average of triplicate analyses [20]

f_f Compared to DOR in one-point matrix extracted standard at target testing level (200 µg/kg)

p_p LC-MS/MS method for avermectins [19]

p_p UPLC-fluorescence method for avermectins [18]

Table 3
HRMS screening results for amoxicillin and ampicillin in fish dosed with capsules

Species	Dosing	Sample number	Drug (100 mg/kg body weight)	Depuration time (h)	AMP ($\mu\text{g/kg}$) ^a	AMOX ($\mu\text{g/kg}$) ^a	Relative response of AMOX DIKETONE ^b
Catfish	Capsule	Cc1	AMOX	6-8	ND	14	2.2×
Catfish	Capsule	Cc2	AMOX	6-8	ND	18	4.2×
Catfish	Capsule	Cc3	AMP	6-8	11	ND	ND
Catfish	Capsule	Cc4	AMP	6-8	12	ND	ND
Trout	Capsule	Tc1	AMOX	6-8	ND	46	2.1×
Trout	Capsule	Tc2	AMOX	22-24	ND	14	5.6×
Trout	Capsule	Tc3	AMP	22-24	129	ND	ND
Trout	Capsule	Tc4	AMP	22-24	104	ND	ND
Salmon	Capsule	Sc1	AMOX	22-24	ND	493	2.2×
Salmon	Capsule	Sc2	AMOX	22-24	ND	88	4.4×
Salmon	Capsule	Sc3	AMP	22-24	126	ND	ND
Salmon	Capsule	Sc4	AMP ^c	22-24	286	89	2.1×
Catfish	Feed	Cf1	AMOX	6	ND	22	6.6×
Catfish	Feed	Cf2	AMOX ^d	6	ND	7	7.8×
Catfish	Feed	Cf3	AMOX ^d	23	ND	21	5.4×
Catfish	Feed	Cf4	AMOX ^d	23	ND	16	6.6×
Trout	Feed	Tf1	AMOX ^d	6	ND	232	2.8×
Trout	Feed	Tf2	AMOX ^d	6	ND	150	2.9×
Trout	Feed	Tf3	AMOX ^d	23	ND	680	2.0×
Trout	Feed	Tf4	AMOX ^d	23	ND	67	3.2×
Salmon	Feed	Sf1	AMOX ^d	6	ND	85	5.4×
Salmon	Feed	Sf2	AMOX ^d	6	ND	65	5.1×
Salmon	Feed	Sf3	AMOX ^d	23	ND	64	4.5×
Salmon	Feed	Sf4	AMOX ^d	23	ND	309	4.0×

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^b Average of duplicate extractions. Compared to one-point matrix-extracted standard at $1 \times$ TTL (25 $\mu\text{g/kg}$ AMP; 100 $\mu\text{g/kg}$ AMOX)

^c Area counts for AMOX DIKETONE peak divided by area counts of AMOX peak in m/z 366.1118 extracted ion chromatogram (5 ppm window)

^d Fish may have also been exposed to AMOX

^e Fish were dosed at 100 mg/kg body weight for 5 days

Table 4

HRMS screening results for imported fish samples

	Eel #1 ^a	Eel #2 ^a	Eel #3	Eel #4	Croaker	Tilapia #1 ^a	Tilapia #2	Tilapia #3	Tilapia #4
Amount of residues found by HRMS compared to matrix-extracted standard (LC-MS/MS quantitative method ^b), µg/kg									
Ciprofloxacin	48^c (26)	52 (90)	15 (30)		467 (500)				
Enrofloxacin	9 (8)	72 (12)	2 (2)	1 (< 5)	3231 (6200)		10 (10)		6 (12)
Norfloxacin		2 (< 5)	4 (6)						
Oxolinic acid				170 (189)					
Sulfadiazine (SDZ)			3 (3)			3 (5)			
Sulfamethazine (SMZ)	1 (< 10)	104 (120)	330 (327)						
Sulfamethoxazole (SMX)	0.1 (nd)						20 (21)		
Trimethoprim	270 (597)	23 (30)	270 (303)		1 (< 10)		7 (< 10)		
Amount of other residues found by HRMS only and compared to matrix-extracted standard, µg/kg									
Azithromycin			13						
Ethoxyquin		114	1	25	1	1	6		
Erythromycin					3				
Florfenicol Amine			9						
Lincomycin		11	1						
Oxytetracycline		2	2	25					
Additional residues detected by searching veterinary drug database and found at high levels in at least one sample									
2-Amino mebendazole	HIGH ^d	LOW	LOW	HIGH		LOW			
		LOW	LOW		HIGH				
Desethylene-enrofloxacin									
Ethoxyquin dimer	LOW ^d	LOW	LOW	HIGH	HIGH	LOW		LOW	
N ⁴ acetyl metabolite	N ⁴ SMX (LOW)	N ⁴ SMZ (LOW)	N ⁴ SMZ (HIGH)				N ⁴ SMX (LOW)		
			N ⁴ SDZ						

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	Eel #1 ^a	Eel #2 ^a	Eel #3	Eel #4	Croaker	Tilapia #1 ^a	Tilapia #2	Tilapia #3	Tilapia #4
Ofloxacin			LOW		HIGH				

^aSome residues in these samples were reported previously [13]

^bQuantitation performed using matrix-extracted standard calibration curve [6]

^cAnalyte amounts in bold met criteria for presumptive positive

^dHIGH indicates MS¹ extracted ion chromatogram area counts > 10⁷; LOW indicates area counts < 10⁷