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Analysis of Veterinary Drug Residues in Frog Legs and Other Aquacultured Species Using Liquid Chromatography Quadrupole Time-of-Flight Mass Spectrometry

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ABSTRACT: A liquid chromatography quadrupole time-of-flight (Q-TOF) mass spectrometry method was developed to analyze veterinary drug residues in frog legs and other aquacultured species. Samples were extracted using a procedure based on a method developed for the analysis of fluoroquinolones (FQs) in fish. Briefly, the tissue was extracted with dilute acetic acid and acetonitrile with added sodium chloride. After centrifugation, the extracts were evaporated and reconstituted in mobile phase. A molecular weight cutoff filter was used to clean up the final extract. A set of target compounds, including trimethoprim, sulfamethoxazole, chloramphenicol, quinolones, and FQs, was used to validate the method. Screening of residues was accomplished by collecting TOF (MS^1) data and comparing the accurate mass and retention times of compounds to a database containing information for veterinary drugs. An evaluation of the MS data in fortified frog legs indicated that the target compounds could be consistently detected at the level of concern. The linearity and recoveries from matrix were evaluated for these analytes to estimate the amount of residue present. MS/MS data were also generated from precursor ions, and the mass accuracy of the product ions for each compound was compared to theoretical values. When the method was used to analyze imported frog legs, many of these residues were found in the samples, often in combination and at relatively high concentrations (>10 ng/g). The data from these samples were also evaluated for nontarget analytes such as residue metabolites and other chemotherapeutics.

KEYWORDS: *LC quadrupole time-of-flight MS, veterinary drug residues, frog legs, aquaculture*

INTRODUCTION

The international trade in frog legs is a 40 million dollar per year business. Most of the product originates in Asian countries and >75% is imported to France, Belgium, and the United States.[1] Approximately 2280 t is imported into the United States each year.[2] The most common species imported into the Unites States are *Rana catesbeiana*, *Fejervarya cancrivora*, and *Limnonectes macrodon*. Frogs are susceptible to many types of bacterial and fungal infections, and contamination of frog legs with *Salmonella* is a significant problem.[3] The U.S. Food and Drug Administration (FDA) has issued an import alert for frog legs imported from Bangladesh as well as several firms from China and other countries because of extensive contamination with *Salmonella*.[4] Another issue of concern related to microbial contamination in food is the use of antibiotics to stem the growth of the bacteria. For that reason, the FDA has also included frog legs in an import alert for unapproved drug residues in aquacultured products.[5]

Fluoroquinolones (FQs), such as ciprofloxacin and enrofloxacin, have been commonly found at violative levels in frog legs using a LC−fluorescence method developed for other aquacultured species.[6] Chloramphenicol has also been confirmed in frog legs using analytical methods specific to that residue.[7] Because the farming of frogs for food is an industry that is not well controlled, it is important to have sensitive analytical methods to screen, quantify, and confirm the identity of a wide variety of veterinary drug residues that might be present in frog legs samples. There are several examples of methods for drug residues in fish and shrimp utilizing liquid chromatography−mass spectrometry (LC-MS) with specific parameters established for dozens of chemical residues in a single analytical procedure.[8,9] Although these methods allow for a large number of compounds to be monitored with excellent selectivity and sensitivity, they still detect only a discrete list of target residues. Using a mass spectrometer that collects data continuously over a wide mass range, rather than with preselected acquisition parameters corresponding to specific residues, virtually an unlimited number of compounds can be analyzed simultaneously. The ability of a time-of-flight (TOF) mass spectrometer to assign a mass to compounds with high accuracy (±0.005 Da) enables it to collect full scan data and still detect low levels (ng/g) of contaminants in complex food matrices with sufficient selectivity. For regulatory applications, this capability means that any given sample can be analyzed not only for the drugs on a target list but also for other residues or contaminants. This can be important in samples such as frog legs, in which a wide variety of antibiotics might be used to combat severe *Salmonella* contamination. Several examples of methods using TOF to monitor for residues in aquacultured commodities have been published.[10,11] A hybrid quadrupole TOF detector (Q-TOF) has the additional ability to obtain

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MS/MS spectra that can be used to further characterize drug residues. For example, LC Q-TOF MS methods have been used to analyze quinolones and FQ residues in fish.[12,13]

This paper describes the development of a multiresidue method for the analysis of veterinary drugs in frog legs using LC Q-TOF MS based on an extraction method developed previously for FQ residues in fish.[6] The presence of fluoroquinolone residues in these samples was corroborated using the LC Q-TOF MS to accurately measure the mass of the protonated molecule and other characteristic product ions. In addition, the full scan data were further evaluated against a database that contained accurate mass data for compounds known for their possible veterinary drug application. The method was validated for drug residues that were frequently found in the frog legs samples (target analytes).

MATERIALS AND METHODS

Reagents and Consumables. Acetonitrile, acetic acid, and sodium chloride used for sample preparation were of reagent grade (Fisher, Fair Lawn, NJ). Water, acetonitrile, and formic acid used to prepare the mobile phase were of LC-MS grade (Fisher Optima). Acetic acid solution (1% v/v) was prepared with water purified to 18.2 megaohms by a Millipore Corp. (Bedford, MA) filtration system. Formic acid solution (0.1% v/v) was prepared with LC-MS grade water. Ceramic homogenizer pellets were purchased from Agilent Technologies, Santa Clara, CA. Amicon Ultra 0.5 mL, 30000 Da molecular weight, cutoff filters were purchased from Millipore Corp. The LC column used was a YMC ODS-AQ (120 Å, 2 × 100 mm, 3 μm) purchased from Waters Corp., Milford, MA.

Standard Solutions. Standards of ciprofloxacin (CIP), enrofloxacin (ENR), trimethoprim (TMP), and chloramphenicol (CAP) were obtained from the U.S. Pharmacopeia (USP, Rockville, MD); oxolinic acid (OXO), nalidixic acid (NAL), and flumequine (FLU) were obtained from Sigma (St. Louis, MO), and sulfamethoxazole (SMOZ) was purchased from TCI America (Portland, OR). Separate stock solutions for each residue were prepared in methanol at concentrations ranging from 200 to 500 μg/mL. An intermediate mixed standard solution was prepared in methanol at concentration levels of 1000 ng/mL for TMP, SMOZ, NAL, FLU, and OXO; 500 ng/mL for CIP and ENR; and 100 ng/mL for CAP. LC Q-TOF MS standards were prepared by diluting the intermediate mixed standard to a final volume of 5 mL with 0.1% formic acid. Additional details of the LC Q-TOF MS standards preparation are given in Table 1. Stock solutions were stable for 1 year. Intermediate and LC Q-TOF MS standards were prepared monthly and daily, respectively.

Sample Preparation. Frog legs and fish used for the validation study were purchased from a local store. Tissue was removed from the bones and homogenized with dry ice using a food processor. The homogenate was placed in a sterile sample bag, loosely sealed, and stored in the freezer (−25 °C) overnight to allow the carbon dioxide to dissipate and then sealed until the time of analysis. Validation samples were fortified by the adding an appropriate volume of intermediate mixed standard to the frozen tissue and allowing to sit for 15 min prior to proceeding with the extraction procedure. As an example, 0.025 mL of the intermediate mixed standard was added to tissue to fortify the sample at the 1*X* level for all residues.

Table 1. Preparation of LC Q-TOF MS Standards

			approx concn of LC Q-TOF MS stds (ng/mL) in solution (corresponds to ng/g in tissue)[a]		
level	vol of intermediate std (mL)	final vol (mL)	CIP, ENR	TMP, SMOZ, OXO, NAL, FLU	CAP
4*X*	0.5	5	50 (20)	100 (40)	10 (4)
2*X*	0.25	5	25 (10)	50 (20)	5 (2)
1*X*[b]	**0.125**	**5**	**12.5 (5)**	**25 (10)**	**2.5 (1)**
0.5*X*	0.0625	5	6.25 (2.5)	12.5 (5)	1.25 (0.5)
0.25*X*	0.03	5	3.1 (1.25)	6.25 (2.5)	0.625 (0.25)
0*X*	0	5	0 (0)	0 (0)	0 (0)

[a]The extraction procedure concentrates samples by a factor of 2.5. [b]Where *X* is the level of interest for the method. See Results and Discussion for justification for target level concentrations.

Extraction Procedure. A portion (2.5 ± 0.03 g) of ground frozen tissue was weighed into a 50 mL centrifuge tube; this should be done quickly to avoid moisture absorption. The samples were extracted by adding 5.0 mL of 1% acetic acid in water, 10 mL of acetonitrile, 2.0 g of sodium chloride, and a ceramic homogenizer pellet to each tube. The samples were placed on a multitube vortexer (Fisher) at maximum speed (2400 rpm) for approximately 5 min and then centrifuged at 4000 rpm (2730 rcf) at 5 °C for approximately 5 min. The upper organic layer was transferred with a Pasteur pipet into a clean 50 mL centrifuge tube. An additional 10 mL of acetonitrile was added to the original tissue and water mix. The tubes were again shaken on the multitube vortexer for approximately 5 min and centrifuged at 4000 rpm (2730 rcf) for 5 min. The acetonitrile layers were combined, and the acetonitrile phase was evaporated using a 24 position N-EVAP (Organomation Associates Inc., Berlin, MA) heated to 55 °C for approximately 20 min. The residue was reconstituted with 1.0 mL of 10% acetonitrile and 0.1% formic acid (overall) in water. After 5 min in a sonicator, the tubes were centrifuged at 4000 rpm for 5 min at ambient temperature. A portion of the extract was pipetted into an Amicon Ultra 0.5 mL 30K centrifugal filtration device already positioned in a concentrate collection tube. These tubes were then centrifuged at 13500 rpm (17000 rcf) for 15 min. The filtered extracts were transferred to LC vials (2 mL polypropylene vials with conical inserts) for analysis.

Instrumentation. An Agilent quadrupole time-of-flight (Q-TOF) 6530 mass spectrometer coupled to an Agilent 1290 liquid chromatograph utilized electrospray ionization with Agilent Jet Stream Technology in the positive ion mode for all analytes except CAP, which was detected by negative ion. The MS was calibrated daily according to the manufacturer's recommendations in both positive and negative ion modes. A reference mass solution was continuously introduced along with the LC stream for real-time mass accuracy calibration. Ions at *m/z* 121.05087 (purine) and *m/z* 922.00980 (hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine) were monitored when positive ion data were collected; the same solution was used for negative ion mode, but ions at *m/z* 112.985587 (trifluoroacetate) and 966.000725 (formate adduct of hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)phosphazine) were used as a reference. Typical resolution values obtained during calibration were 9000−15000 fwhm in the mass range monitored (*m/z* ∼ 300−1500). Source parameters in positive ion were optimized by making multiple injections of a solvent standard mixture containing the target analytes while making incremental changes in instrument values. These final parameters (listed in Table 2) were chosen on the basis of the best response for most compounds. Because of the higher sensitivity required for CAP, the instrument was optimized in the negative ion mode for this compound by infusing a standard of CAP via a syringe pump into the LC flow via a T-union. The TOF parameters were then varied, and the response of the deprotonated ion of CAP was monitored in real time. These instrument parameters are also listed in Table 2. Separate methods were used for obtaining positive and negative ion full scan TOF (MS^1) data and for positive and negative MS/MS analysis (the sample extracts were re-injected). It has been found that the instrument requires some time to stabilize between polarity modes. Therefore, it is recommended that a set of samples are all analyzed initially by either positive or negative ion. When the samples are re-injected for analysis using methods for the opposite polarity, several injections of a standard should be made before the sequence is continued.

Data Analysis. The TOF data were initially evaluated against a database compiled in-house that contains molecular formulas and

Table 2. Instrumental Parameters

MS source	POS	NEG
fragmentor	150 V	150 V
nozzle	250 V	200 V
vcap	4000 V	2000 V
nebulizer	40 psig	40 psig
drying gas N_2	8 L/min	8 L/min
sheath gas N_2	11 L/min	11 L/min
MS[1] (TOF)	POS	NEG
acquisition rate	4 GHz to m/z 1700	4 GHz to m/z 1700
scan range	m/z 100−1200	m/z 100−1200
scan rate	1.08 spectra/s	1.08 spectra/s
data collected	centroid and profile	centroid and profile
MS/MS	POS	NEG
retention time window	±0.5 min	±.05 min
precursor ion isolation width	narrow (1.3 m/z)	medium (4 m/z)
time	200 ms/spectrum	200 ms/spectrum
collision energy	20 V (25 V for OXO)	15 V
LC		
column	YMC ODS-AQ, 2 × 100 mm, 3 μm	
mobile phase A	0.1% formic acid	
mobile phase B	acetonitrile	
flow rate	250 μL/min	
column oven	35 °C	
autosampler tray	4 °C	
injection volume	10 μL	
needle wash	50:50 (v/v) water/methanol, 3 s	
LC gradient		%B
0−2 min		5
2−12 min		5−50
12−13 min		50
13−16 min		50−100
16−18 min		100
18−18.5 min		100−5
postrun equilibration (3 min)		5

exact mass values for over 200 veterinary drugs. Retention times with current chromatographic conditions are known for 76 of these compounds, including all of the target residues in this study. Using Agilent's MassHunter *Find by Formula* algorithm, data files were compared to compounds in this database. For the target analytes, TOF data were searched for ions corresponding to $[M + H]^+$ or $[M - H]^-$ using windows of 10 ppm mass accuracy and 0.5 min retention time. When the purpose of sample analysis is primarily screening, that is, to rapidly evaluate if the residues of interest are present at a concentration that could be considered violative, then the abundance of the protonated molecule can be compared to that from a matrix extracted spike at 1*X*. For any residue to be considered presumptive positive (above the minimum threshold), the measured response in the unknown sample has be ≥50% of the signal from this "extracted 1*X* standard".

Evaluation of a separate data file containing MS/MS data was performed. MS/MS data were processed manually by extracting narrow window (±10 ppm) product ion scans. The extracted ion chromatograms for two or more structurally important product ions were summed together. Product ion spectra were evaluated by comparing mass assignments to theoretical values and ions ratios (relative abundances) to those from compounds in a solvent standard or fortified sample.

Quantification was performed using Agilent's *Q-Tof Quantitative Analysis* program. Extracted ion chromatograms were generated from $[M + H]^+$ or $[M - H]^-$ ions in the TOF data using a 10 ppm mass window. Calibration curves (nonweighted, linear) were made from the integrated peak areas of the solvent standards described in Table 1. In addition, observed matrix effects were estimated by comparing the response of an analyte in an extracted sample to a matrix-matched (postfortified) standard at the same concentration level.

Several strategies can be used to detect the presence of nontarget analytes. For example, the MassHunter *Find by Formula* routine can be used without requiring a retention time match. Large commercially available accurate mass databases, such as Agilent's *Personal Forensics and Toxicology Database* (n > 6500 compounds), can also be searched. In addition, the TOF data can be evaluated for potential metabolites if their molecular formulas are known. Alternatively, the MassHunter *Find by Molecular Feature* program will identify "features" or compounds by detecting related ions that appear as unique chromatographic peaks. These compounds can then be identified by comparing the calculated accurate mass data against the compounds in the databases. The number of compounds found by these programs will depend on the abundance threshold values and mass tolerance windows selected. A comparison of compounds found in unknown samples and matrix controls can also give an indication of which found analytes may be worthy of further investigation.

RESULTS AND DISCUSSION

Screening Validation with Target Compounds. The primary objective of this method was to develop a rapid screening method for veterinary drug residues in frog legs and other aquacultured samples by comparing data obtained by a LC Q-TOF MS to a compound database using accurate mass and retention times. The utility and limits of this approach were tested with target analytes. On the basis of the initial findings in imported frog legs samples along with information regarding which drugs are often used in aquaculture,[14−16] the compounds

Table 3. Screening Data for Frog Legs Fortified at the Level of Interest

compd	mol formula	level of interest[a] (ng/g)	ret time (min)	protonated molecule[b] (m/z)	av mass error[c,d] (Δ ppm)	av abundance[c,e] (counts)	abundance[e] % RSD
TMP	$C_{14}H_{18}N_4O_3$	10	5.9	291.1452	0.80 ± 0.51	259992	20
CIP	$C_{17}H_{18}FN_3O_3$	5	6.7	332.1405	1.19 ± 0.82	9878	24
ENR	$C_{19}H_{22}FN_3O_3$	5	7.1	360.1718	1.52 ± 0.92	19393	25
SMOZ	$C_{10}H_{11}N_3SO_3$	10	8.6	254.0594	0.84 ± 0.78	6623	40
OXO	$C_{13}H_{11}NO_5$	10	9.8	262.0710	0.93 ± 0.86	28161	26
NAL	$C_{12}H_{12}N_2O_3$	10	11.1	233.0921	1.89 ± 1.36	22467	22
FLU	$C_{14}H_{12}FNO_3$	10	11.5	262.0874	1.16 ± 1.14	14798	28
CAP	$C_{11}H_{12}N_2O_5Cl_2$	1	9.4	321.0051	0.81 ± 0.57	2849	20

[a]Levels of interest (1*X*) for this method. [b]Theoretical value rounded to fourth decimal place. $[M + H]^+$ measured; except for CAP $[M - H]^-$. [c]Average for residues in frog leg tissue (n = 20) fortified at the level of interest. Data were collected over 7 different days in a 2 month period. [d]Average and standard deviation of mass error (absolute value). Mass (not m/z) was used to calculate mass error. [e]Abundance and relative standard deviation (RSD) for peak height of protonated (or deprotonated) molecule in MS TOF scan.

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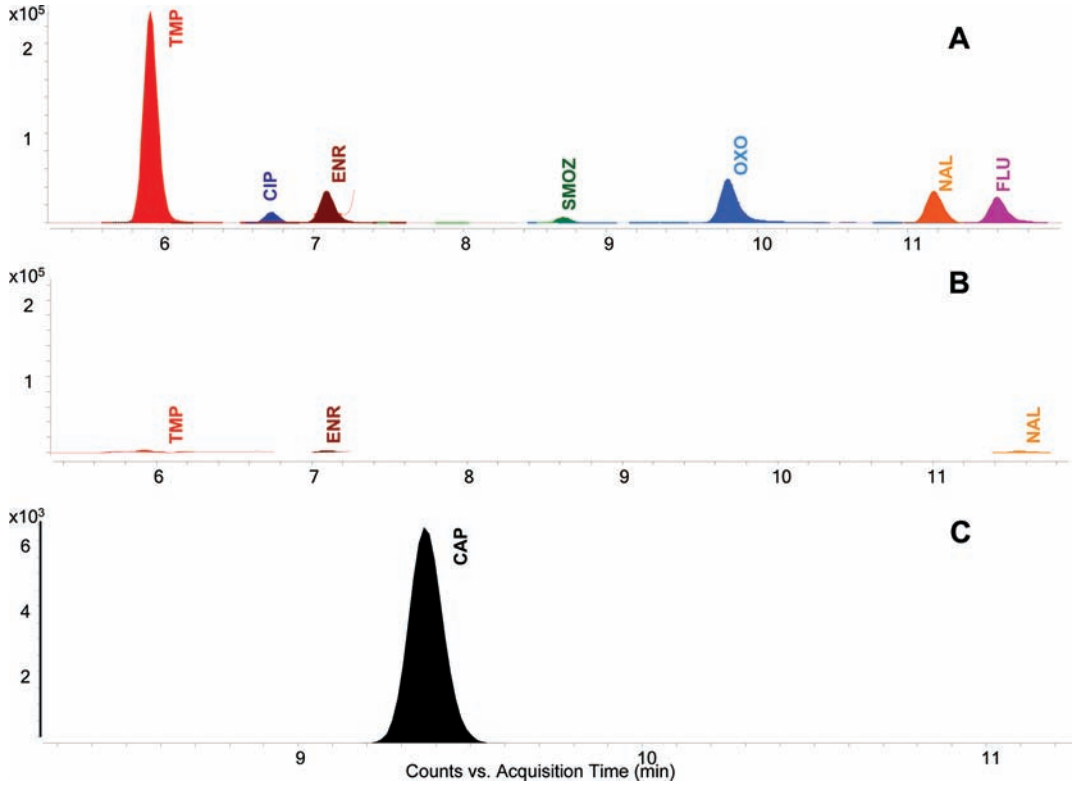

Figure 1. Overlaid extracted ion chromatograms for (A) $[M + H]^+$ ions of target compounds in frog legs fortified at the levels of interest (TMP, 10 ng/g; CIP, 5 ng/g; ENR, 5 ng/g; SMOZ, 10 ng/g; OXO, 10 ng/g; NAL, 10 ng/g; FLU, 10 ng/g), (B) $[M + H]^+$ ions of target compounds in control frog legs, and (C) $[M - H]^-$ ions of CAP in frog legs fortified at 1 ng/g. The mass window for extraction was set to 10 ppm and CIP, SMOZ, OXO, FLU, and CAP were not detected in the control frog legs.

chosen as target analytes were FQs (CIP and ENR), quinolones (OXO, NAL, and FLU), TMP, SMOZ, and CAP. This method was developed and validated to determine if these drug residues could be reliably detected at the level of interest. Because these drugs are not approved in the United States for use in aquaculture, there are no established tolerance levels. The levels of interest (1*X* levels) were set using current U.S. regulatory action levels for the FQs (5 ng/g) and quinolones (10 ng/g);[17] 10 ng/g was also chosen as the level of interest for TMP and SMOZ. This is well below the European Union (EU) maximum residue level for TMP of 50 ng/g in finfish.[18] Typically, the method performance level required for CAP has been below 1 ng/g. This Q-TOF multiclass screening method is not as sensitive as dedicated (single residue) triple-quadrupole LC-MS/MS methods.[7,19] However, even though the 1*X* level for CAP was set to 1 ng/g in this method, CAP was detected in samples (n = 6) fortified at half that level (0.5 ng/g).

Screening data for frog legs fortified with residues at 1*X* are shown in Table 3 and in Figure 1. For a residue to be detected using the MassHunter *Find by Formula* software routine, the mass accuracy error of the $[M + H]^+$ ion was set to be ≤10 ppm with a retention time window of 0.5 min. With these criteria, all of the target residues were detected in samples fortified at the level of interest (1*X*), as well as at half (0.5*X*) and twice (2*X*) that level. As can be seen in Table 3, the average mass error for these residues in frog legs fortified at the 1*X* level was measured to be within 1−2 ppm (~ ±0.001 Da).

Residue screening methods often establish a method threshold value to quickly determine if the concentration of a detected residue is near the level of interest using the signal response variability for samples fortified at the 1*X* level.[20−22] For example, with milk methods developed in our laboratory,[20,22] for residues to be detected with 95% confidence at the level of concern, the signal observed needed to be ≥50% of that from a positive control (1*X* extracted sample) analyzed that day. The variability of the data obtained for the target residues in frog legs indicates that this approach (setting the method threshold signal at ≥50% of 1*X* extracted sample) would also be acceptable for this LC Q-TOF MS method. The relative standard deviations of the measured peak abundances (heights) for the precursor ions of these residues (with the exception of SMOZ) was <30% for multiple days across several weeks (Table 3). In fact, for any set of samples, when an extracted 1*X* standard was used to set the ≥50% threshold, 98% of drugs in frog legs fortified at the 1*X* level would be considered presumptive positive. In addition, all of the residues added at the 2*X* levels (3 replicate samples) met this criteria, whereas approximately 54% of compounds fortified in frog legs at the 0.5*X* level (6 replicate samples) would be considered presumptive positive. Because these drugs are not approved for use (no tolerance levels set) in frog legs, it may be worthwhile to further investigate residues found below the level of interest as they could also be considered violative samples. Using these criteria, there were no presumptive positive residues in the frog legs matrix blanks (n = 9).

MS/MS Analysis of Target Residues. MS/MS data can also be collected using the LC Q-TOF MS. Mass assignments of product ions can be compared to theoretical values and relative abundances to those observed in solvent standards and fortified samples analyzed that day, and the fragmentation of target compounds to form product ions has been reported in

the literature. The molecular formulas and m/z values for the most important ions are shown in Table 4. There are published guidelines for the confirmation of identity of veterinary drug residues using mass spectrometry,[23,24] but criteria for accurate mass data have not yet been established. Several authors have suggested criteria giving additional weight to product ions if their measured mass is within 2 or 10 mDa[25] or 10 ppm[26] of the theoretical value. For the MS/MS data collected in this study, a majority of product ions from residues in frog legs at the target level have a mass accuracy of less than 10 ppm and 2 mDa, but some have a calculated mass error of 10−20 ppm (but still less than 5 mDa). Examples of MS/MS spectra obtained for residues in a solvent standard, fortified frog legs, and an imported sample are shown in Figure 2 (TMP) and 3 (CAP) and in Table 5. Adequate MS/MS spectra were obtained for a majority of residues in frog legs fortified at 1X (or at 0.5X for TMP and CAP). Higher concentration levels (>2X) of SMOZ and CIP were needed to obtain reasonable MS/MS spectra.

Table 4. Product Ions Used To Identify Residues by MS/MS

compd	precursor ion $(m/z)^a$	product ion formula	product ion $(m/z)^a$	refs
TMP	291.1452	$C_{12}H_{14}N_4O^+$	230.1162	28, 35
		$C_5H_7N_4^+$	123.0665	
CIP	332.1405	$C_{17}H_{17}FN_3O_2^+$	314.1299	12, 13, 22
		$C_{16}H_{19}FN_3O^+$	288.1507	
		$C_{14}H_{14}FN_2O^+$	245.1085	
ENR	360.1718	$C_{19}H_{21}FN_3O_2^+$	342.1612	12, 13, 22
		$C_{18}H_{23}FN_3O^+$	316.1820	
		$C_{14}H_{14}FN_2O^+$	245.1085	
SMOZ	254.0594	$C_6H_6NSO_2^+$	156.0114	36
		$C_6H_6NO^+$	108.0444	
		$C_6H_6N^+$	92.0495	
OXO	262.0710	$C_{13}H_{10}NO_4^+$	244.0604	8
		$C_{12}H_{10}NO_3^+$	216.0655	
		$C_{11}H_6NO_4^+$	216.0291	
NAL	233.0921	$C_{12}H_{11}N_2O_2^+$	215.0815	8
		$C_{10}H_7N_2O_2^+$	187.0502	
		$C_9H_7N_2O^+$	159.0553	
FLU	262.0874	$C_{14}H_{11}FNO_2^+$	244.0768	8
		$C_{11}H_5FNO_2^+$	202.0299	
CAP	321.0051	$C_{10}H_{10}N_2O_4Cl^-$	257.0335	19, 37
		$C_9H_6NO_3^-$	176.0353	
		$C_7H_6NO_3^-$	152.0353	

^{a}Theoretical m/z rounded to the fourth decimal place. Assumes ions are protonated (or deprotonated for CAP).

Quantitation of Target Residues in Frog Legs. Although the primary intended purpose of this procedure was qualitative screening for veterinary drug residues, some quantitative method performance characteristics for the target residues were also evaluated. The concentrations of drug residues present in fortified frog legs were determined using the TOF (MS^1) data. Standard curves were generated from solvent standards prepared as described in Table 1. The resulting curves were linear in the range of 0.2X−2X for all eight compounds with R^2 values ranging from 0.95 to 0.99 (R^2 < 0.99 for 75% of curves). In positive ion mode, the highest standard (corresponding to 4X) was not always in the linear range. An evaluation of the data indicates that this occurred after polarity switching and appears, therefore, to be due to inadequate stabilization of the instrument rather than saturation of signal.

Recoveries for most residues were acceptable (80−130% recovery with RSDs ≤ 30%) using solvent standard curves (Table 6). Exceptions were CIP with recoveries of approximately 50% and SMOZ with recoveries below 30% and very high RSDs. The original LC−fluorescence method also exhibited lower recoveries (67%) for CIP as compared to the other FQs.[6] The poor recoveries for SMOZ indicate that this method should be used for screening purposes only for this residue in frog legs.

Method detection limits (MDLs) were calculated from the standard deviation of residue concentrations in samples fortified at the 0.5X level. This is done by multiplying the standard deviation of those values times the t test value at the 99% confidence interval (MDL = standard deviation × 3.365 for one-tailed Student's t test, n = 6). These data are also shown

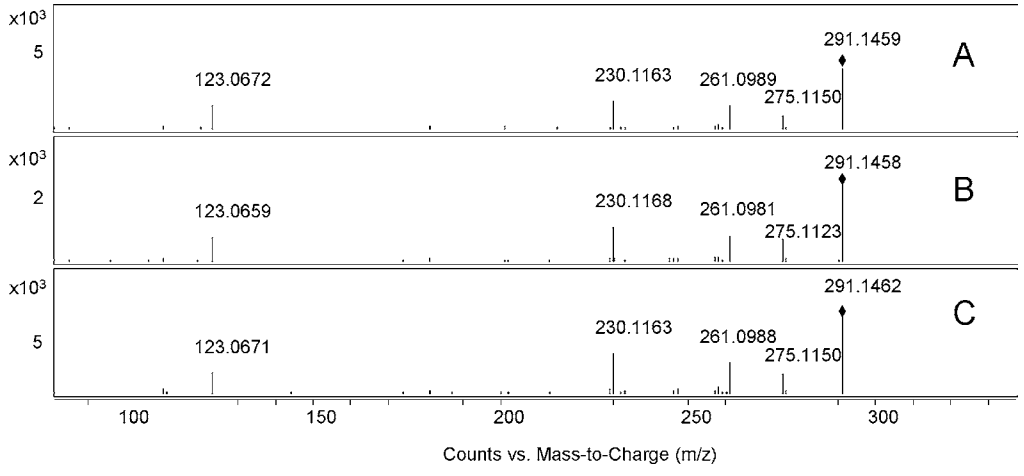

Figure 2. Product ion spectra of trimethoprim (TMP) in (A) frog legs sample 7, (B) frog legs fortified at 10 ng/mL (1X), and (C) solvent standard at 20 ng/g.

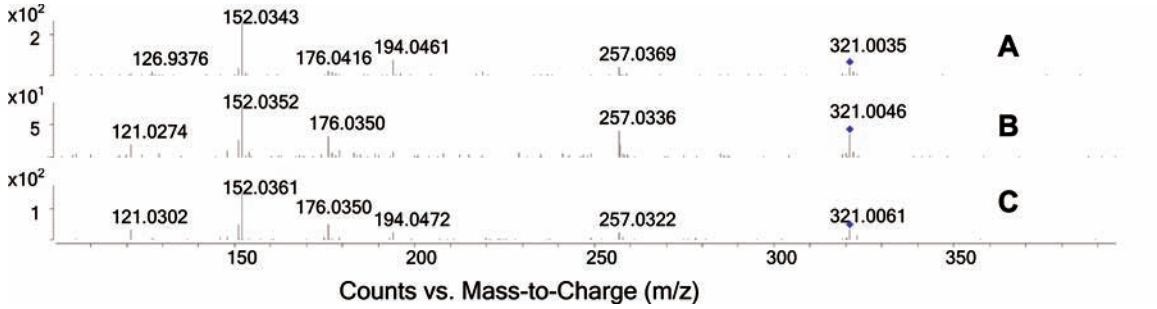

Figure 3. Product ion spectra of chloramphenicol (CAP) in (A) frog legs sample 7, (B) frog legs fortified at 1 ng/mL (1*X*), and (C) solvent standard at 2 ng/g.

Table 5. Exact Mass and Relative Abundance of Ions in MS/MS Spectra for TMP, ENR, and CAP

	2*X* solvent standard		frog legs fortified at 1*X*		imported frog legs sample 7	
theor *m/z*	*m/z* (Δ ppm)[a]	% rel abundance	*m/z* (Δ ppm)[a]	% rel abundance	*m/z* (Δ ppm)[a]	% rel abundance
TMP						
291.1452	291.1462 (3.43)	100	291.1458 (2.06)	100	291.1459 (2.4)	100
230.1162	230.1163 (0.43)	48	230.1168 (2.61)	40	230.1163 (0.43)	46
123.0665	123.0671 (4.88)	24	123.0659 (−4.88)	29	123.0672 (5.69)	38
ENR						
360.1718	360.1732 (3.89)	35	360.1692 (−7.22)	30	360.1676 (−11.66)	33
342.1612	342.1622 (2.92)	100	342.1596 (−4.68)	100	342.1616 (1.17)	100
316.1820	316.1828 (2.53)	108	316.1813 (−2.21)	81	316.1809 (−3.48)	98
245.1085	245.1087 (0.82)	54	245.1078 (−2.86)	32	245.1093 (3.26)	42
CAP						
321.0051	321.0061 (3.12)	19	321.0046 (−1.56)	44	321.0035 (−4.98)	16
257.0353	257.0322 (−12.06)	13	257.0336 (−6.61)	51	257.0369 (6.22)	16
152.0353	152.0361 (5.26)	100	152.0352 (−0.66)	100	152.0343 (−6.58)	100

[a]Δ ppm = difference from theoretical value.

Table 6. Quantitative Data for Fortified Frog Legs

		recoveries (RSDs) calculated from		
compd	level of interest (1*X*) (ng/g)	solvent std curve[a] (*n* = 20)	matrix std 1-point[b] (*n* = 13)	MDL[c] (ng/g)
TMP	10	99.5 (22)	90.6 (9)	1.8
CIP	5	47.6 (20)	58.0 (13)	0.5
ENR	5	105.9 (29)	77.8 (27)	0.8
SMOZ	10	27.0 (64)	76.5 (68)	2.5
OXO	10	128.7 (24)	104.6 (30)	3.9
NAL	10	79.3 (30)	128.8 (46)	0.9
FLU	10	89.7 (29)	87.0 (28)	1.4
CAP	1	109.9 (12)	117.2 (6)	0.2

[a]Recoveries based on solvent standard curve prepared as described in Table 1. [b]Recoveries based on comparison to a single point 1*X* matrix matched standard. For one day's analysis (corresponding to *n* = 6 replicates), the matrix-matched standards had to be diluted by an equal volume of 0.1% formic acid to obtain acceptable accurate mass match and quantification. [c]Method detection limit (MDL) = SD × 3.365, where SD is standard deviation from quantitative results of 0.5*X* spikes (*n* = 6); 1*X* spikes were used to calculate MDL for SMOZ.

in Table 6. Because SMOZ could not be quantified in samples fortified at the 0.5*X* level, the MDL was calculated using extracts from tissue fortified at 1*X* for this residue.

Matrix Effects. To provide some assessment of ion suppression caused by the matrix, recoveries from fortified samples were calculated using a one-point matrix-match (postfortified) standard at the level of interest (1*X*). These results (Table 6) indicate that the low recovery observed for SMOZ may be due to matrix ion suppression; less significant matrix effects, including signal enhancement, were observed for the other analytes. Matrix-matched (postfortified) standards were investigated as an alternative approach for quantification, but the responses for the lower concentration standards (0.2−0.5*X*) were quite variable, with matrix affecting both signal response and mass accuracy (see below), especially for the quinolone residues.

In addition to matrix components influencing compound ionization, in a few instances, the matrix also adversely affected the quality of the mass accuracy measurement. For all of the frog legs samples fortified at 1*X* prior to extraction, the mass accuracy was very good (Table 3). However, when the matrix was postfortified with the residues, the mass errors could be significantly larger (15−25 ppm). For one set of data, the matrix-matched standard had to be diluted 1:1 with 0.1% formic acid to obtain suitable (±10 ppm) mass accuracy. In another example, data from the LC Q-TOF MS analysis of one imported frog legs sample initially indicated no residues were present. The LC−fluorescence analysis, however, detected relatively high levels of both ENR (45 ng/g) and CIP (16 ng/g). When the extract was diluted 1:5 with 0.1% formic acid, ENR and CIP, as well as TMP, were detected using the LC Q-TOF MS method. Further investigation of the data from the original (nondiluted) extract showed that the compounds were present, but the calculated accurate mass error for all three compounds was 30−50 ppm. This error remained whether the data were analyzed in profile or centroid mode and regardless of whether the *Find by Formula* or *Find by Molecular Feature* algorithm was used. The mass accuracy for these residues in the diluted extract was <2 ppm. There were no apparent isobaric

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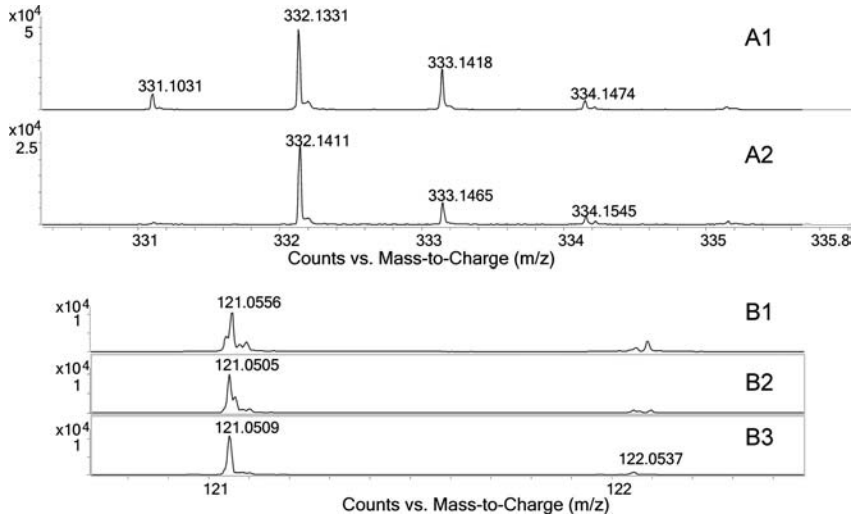

Figure 4. Profile spectra of CIP in undiluted (A1) and diluted (A2) extracts from frog legs sample 4. The exact mass for protonated ion of CIP is m/z 332.1405. Profile spectra of the reference compound purine (averaged over the chromatogram) in undiluted (B1) and diluted (B2) frog legs sample 4 are also shown along with the spectrum of this compound from a solvent standard analysis (B3). The exact mass for this protonated compound is m/z 121.0509.

Table 7. Quantitative Data for Other Fish Fortified at the 1*X* Level

	tilapia (n = 3) recoveries (RSDs) calculated from		shrimp (n = 3) recoveries (RSDs) calculated from		catfish (n = 2) recoveries calculated from	
compd	solvent std curve[a]	matrix std 1-point[b]	solvent std curve[a]	matrix std 1-point[b]	solvent std curve[a]	matrix std 1-point[b]
TMP	104 (1)	89 (1)	117 (5)	89 (5)	73, 77	83, 88
CIP	66 (3)	62 (3)	96 (20)	89 (20)	66, 66	67, 67
ENR	110 (8)	84 (8)	113 (15)	87 (15)	182, 196	87, 93
SMOZ	77 (14)	85 (14)	100 (9)	99 (9)	50, 52	103, 107
OXO	101 (7)	95 (7)	100 (17)	95 (17)	72, 82	95, 109
NAL	117 (6)	96 (6)	120 (16)	88 (16)	62, 76	92, 114
FLU	96 (6)	93 (6)	98 (19)	85 (19)	57, 83	80, 115
CAP	105 (4)	101 (4)	106 (4)	103 (4)	140, 163	85, 98

[a]Recoveries based on solvent standard curve prepared as described in Table 1. [b]Recoveries based on comparison to a single point 1*X* matrix-matched standard.

matrix interferences near the retention time of these residues in this sample. However, there was an interfering matrix peak at the mass of the lower mass calibration standard (purine, m/z 121.05087) that shifted the calculated mass of this reference ion, most likely affecting the accurate mass measurements of all other compounds (Figure 4). The signal from this interference was reduced when the sample was diluted. This particular sample had deteriorated due to improper storage conditions, so the matrix components may have been significantly different from most frog legs samples. Others have also reported matrix effects on accurate mass measurements using a Q-TOF instrument.[27]

The use of solid phase extraction and other sizes of molecular cutoff filters was investigated during method optimization. However, these modifications did not improve overall method performance. It is also important to note that the primary purpose of this method is to screen for a wide variety of residues. Whereas better recoveries, reductions in ion suppression, and even improved mass accuracy measurements could possibly be obtained with more rigorous sample cleanup for these specific residues, the additional sample handling might exclude other residues from being detected. However, to avoid false-negative results, it would be important to realize that severe matrix effects are occurring and causing large errors in mass assignments. An internal standard or surrogate analyte could be added to monitor for severe shifts in mass accuracy due to matrix. If the mass error for the internal standard is larger than expected, further investigation (i.e., diluting the extract) may be required. Also, delivering a higher concentration of reference ions could mitigate the problem observed with the decomposed frog legs sample illustrated in Figure 4.

Application to Other Aquacultured Species. The method was expanded to other fish species, including shrimp, tilapia, and catfish, with a limited number of samples. At the level of interest, all target residues were detected (using *Find by Formula*). The mass accuracy for the protonated molecules matched the theoretical value within 1−2 ppm with the exception of ENR in fortified shrimp, for which the measured mass difference was 6−7 ppm. In general, these matrices gave better quantitative results (higher recoveries and lower RSDs) than was observed for frog legs. These results are shown in Table 7. The recoveries and matrix suppression for SMOZ, in particular, were much more acceptable in these fortified fish samples. The matrix effects from tilapia and shrimp on the analytes' response were also less than what was observed for frog legs. With catfish, however, ion suppression for SMOZ and ion enhancement for CAP and ENR were observed. MS/MS

data were also collected for these samples, and reasonable product ion spectra were observed.

Targeted Residues Found in Imported Frog Legs Samples. The drug residues found in imported frog legs samples are shown in Table 8. As expected from previous (LC−fluorescence) analysis, FQs were frequently found in these samples. ENR was found in most imported samples with concentrations varying from <2.5 to 40 ng/g. CIP, a known metabolite of ENR, was found in 6 of the 11 samples. The samples with the highest concentration of CIP (4−7 ng/g) were the same samples that contained higher levels of ENR. In general, the concentrations of FQs measured in these samples with the Q-TOF were comparable to the amounts found by LC−fluorescence methods (data not shown). In addition, high levels (~70 ng/g) of flumequine, an older generation fluoroquinolone, were also detected and confirmed in an imported sample (sample 6) using this method.

Table 8. Residues Found in Imported Frog Legs Samples

	concn of specific residues found (ng/g) in imported frog legs by TOF[a]				
sample	TMP	ENR	CIP	SMOZ	CAP
1	70	32	4	ND	NA[b]
2	7	5	<2.5	ND	14
3	98	40	4	106	86
4[c]	42	37	7	ND	ND
5	11	<2.5	ND	<5	ND
6	81	<2.5	<2.5[d]	ND	ND
7	11	6	<2.5	ND	3
8	30	5	ND	ND	<0.5
9	9	7	ND	ND	ND
10	27	<2.5[d]	ND	<5[d]	ND
11	15	ND	ND	ND	ND

[a]Calculated with solvent standard curve. [b]This sample was not analyzed in negative ion mode. [c]Sample diluted 1:5 (v/v) with 0.1% formic acid to obtain accurate mass. [d]Adequate MS/MS spectra not obtained.

The fact that residues other than FQs were detected in these samples was the impetus for further evaluating and validating the extraction for additional compounds in frog legs. One residue that was consistently found in the imported frog legs was trimethoprim. The amount of TMP in the frog leg samples ranged from 7 to approximately 100 ng/g. TMP is a drug used in combination with sulfonamides to potentiate the effectiveness of these antibiotics,[28] and TMP and sulfamethoxazole are often formulated and administered in combination. In addition to finding TMP in all of the frog samples analyzed, SMOZ was detected in 3 and adequate MS/MS spectra were obtained in 2 of the 11 samples. It is not known why the levels of TMP found were, in general, much higher than the SMOZ, even allowing for differences in method performance for the two drugs. Limited information regarding the metabolism of these drugs in aquatic species indicates the depletion should not be significantly slower for TMP as compared to the sulfonamides.[29,30] In addition, tissues from catfish that had been dosed with the typical formulation (5:1 SMOZ/TMP ratio) were obtained and analyzed with this method. The concentrations of residues found in these fish were 290 ng/g SMOZ and 32 ng/g TMP and 56 ng/g SMOZ and 1 ng/g TMP after 12 and 24 h of depletion, respectively.

Chloramphenicol was detected in the frog legs samples analyzed using the LC Q-TOF MS in 4 of 11 samples analyzed using negative ion at levels ranging from <1 to >80 ng/g. Chloramphenicol residues are of human health concern because this drug is known to cause aplastic anemia in susceptible individuals. Because of this, the global consensus has been that analytical methods should be able to detect and confirm the presence of chloramphenicol in food at below ng/g levels. Using this Q-TOF method, the method detection limit for CAP was calculated to be 0.2 ng/g, but a concentration of 0.5 ng/g or higher was needed to obtain acceptable product ion spectra.

A modification of the extraction method described here has been validated[31] for use with a triple-quadrupole LC-MS/MS to quantitate and confirm multiple residues in frog legs and fish species. The triple-quadrupole method includes the target analytes found in frog legs using this Q-TOF method and incorporates the use of internal standards for improved quantitative reporting. In addition to the samples described in Table 8, additional frog legs samples that were prepared for and analyzed by the triple-quadrupole method were also analyzed by this LC Q-TOF MS procedure with similar results. In general, the triple-quadrupole method provides better sensitivity for product ions with selected reaction monitoring. A reasonable strategy for residue analysis may be to use the LC Q-TOF MS to continue to look for unexpected, or nontargeted, residues. Although this paper demonstrates that the Q-TOF can also successfully verify the identity of the analyte and determine the amount present, the triple-quadrupole may be better suited for quantification and confirmation of residues that are found routinely.

Nontargeted Residues Found in Imported Frog Legs Samples. Data from the imported frog legs samples were analyzed to look for additional nontarget compounds. Both the *Find by Formula* and the *Find by Molecular Feature* software algorithms were utilized. Additional residues that have been detected in frog legs samples at low levels include norfloxacin (<1 ng/g in two samples), crystal violet (<1 ng/g in one sample), florfenicol (~1−15 ng/g in three different samples), and dehydrated erythromycin (<5 ng/g in one sample). These findings were verified by comparison to standards using retention time, MS, and MS/MS data. When the results were compared to both the in-house database for veterinary drugs as well as a larger (>6000 entries) forensic and toxicology database, additional large abundance chromatographic peaks in the frog legs samples matched compounds (including ethopabate, dexamethasone, netilmicin, and nifurdazel) based on accurate mass alone. However, some of these "hits" were also observed in negative controls. Ethopabate and dexamethasone were ruled out after comparison to an authentic standard. Reference standards for the other compounds were not available, so further investigation is required. The retention time and product ion data obtained for netilmicin, however, do not match what would be expected for an aminoglycoside. Most likely these peaks correspond to matrix components that are isobaric with compounds in the databases.

In addition to searching for other veterinary drug residues, the TOF data can also be used to monitor for known metabolites. For example, it is well-known that sulfonamides can be converted to the N^4-acetyl derivatives.[32] The frog legs data were searched for the ions corresponding to N^4-acetyl SMOZ ($C_{12}H_{14}N_3O_4S^+$, m/z 296.0700) to determine if this could be the marker compound for this residue. This

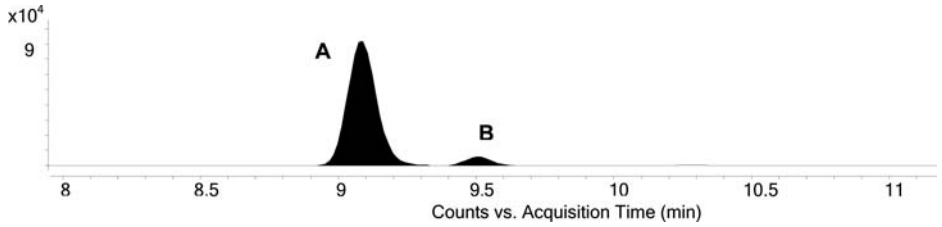

Figure 5. Combined extracted chromatograms for (A) SMOX, m/z 254.0594, and (B) N^4-SMOZ, m/z 296.0700, from frog legs sample 3. The mass window for extraction was set to 10 ppm.

compound was found and characterized in several frog legs samples (Figure 5), but only when SMOZ was also detected. In general, the peaks for the ion corresponding to the N^4-acetyl compound were ~10% of that for SMOZ. Therefore, it appears that the parent compound is the appropriate marker residue for these samples. It has also been postulated that CAP can form glucuronide conjugate ($C_{17}H_{20}N_2O_{11}Cl_2$) in tissue.[33,34] The presence of this compound was searched for using both positive (m/z 499.0517) and negative (m/z 497.0371) TOF data, but it was not detected in any of the imported frog legs samples. Additional data mining using more sophisticated differential analysis software programs, such as Mass Profiler Pro, is ongoing and may result in detection of additional analytes.

Summary. This method demonstrates the successful application of a LC Q-TOF MS instrument to the analysis of drug residues in frog legs and other aquaculture samples. Many of these frog legs contained residues in combination; for example, several samples contained residues of enrofloxacin, trimethoprim, and chloramphenicol. Although it is the goal of multiresidue methods to detect a wide spectrum of compounds, it is uncommon to detect residues from different chemical classes in the same sample. This underscores the importance of broad spectrum residue monitoring, as the indiscriminate use of chemotherapeutics in the farming of frogs is clearly evident. The presence of these additional drug residues, in addition to the fluoroquinolone residues targeted in the established method, would not have been detected with a MS procedure that limits data acquisition.

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Notes

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