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Analysis of veterinary drug residues in shrimp: A multi-class method by liquid chromatography–quadrupole ion trap mass spectrometry

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Abstract

A liquid chromatography–mass spectrometry (LC–MS) method was developed to screen and confirm veterinary drug residues in raw shrimp meat. This method simultaneously monitors 18 drugs of different classes, including oxytetracycline (OTC), sulfonamides, quinolones, cationic dyes, and toltrazuril sulfone (TOLS). The homogenized shrimp meat is extracted with 5% trichloroacetic acid. The extract is further cleaned using polymer-based SPE. A 50 mm phenyl column separates the analytes, prior to analysis with an ion trap mass spectrometer interfaced with an atmospheric pressure chemical ionization source. This method is able to confirm oxytetracycline residues at 200 ng/g, toltrazuril sulfone at 50 ng/g, sulfaquinoxaline at 20 ng/g, and the other 15 drugs at 10 ng/g or lower levels. An estimate of the level of residues can also be made so that only confirmed samples above action levels will be sent for quantitation. The method is validated with both fortified and incurred samples, using multiple shrimp species as well. This multi-class method can provide a means to simultaneously monitor for a wide range of illegal drug residues in shrimp.

Keywords: Shrimp; Multi-class; Multi-residue; High throughput; Veterinary drug; Confirmatory; Sulfonamide; Quinolone; Fluoroquinolone; Cationic dye; Oxytetracycline; Toltrazuril sulfone

1. Introduction

In recent years, shrimp has replaced tuna as the American consumer's favorite seafood [1]. A considerable percentage of the shrimp eaten is imported farmed shrimp. Recent surveys have found a widespread use of veterinary drugs in shrimp farming in various countries [2–4]. Drugs found to be used include sulfamethazine (SMZ), enrofloxacin (ENR), oxolinic acid, malachite green, oxytetracycline (OTC), along with many other unidentified antibiotics. These drugs are effective in treating bacterial or fungal infections in various animal species. However, no antibiotic or antifungal agent has been approved for use in shrimp farming by the U.S. Food and Drug Administration (FDA), and uncontrolled use of veterinary drugs in aquaculture may cause health, environmental, and drug-resistant microbial problems. Currently only a small percentage of both imported and domestically produced shrimp is monitored for drug residues due to a lack of high-throughput methods. The need exists for regulatory laboratories to be able to quickly establish if shrimp samples are free of drug residues and target suspect samples for further investigation. This problem is to be addressed by developing a suitable multi-class, multi-residue analytical method.

The rapid advancement of LC–MS–MS technology has led to increased use of the technique for the analysis of drug residues in animal derived food products [5–7]. Over the past years liquid chromatography–mass spectrometry (LC–MS) multi-residue methods have been developed for drug groups, such as sulfonamides [8,9], tetracyclines [10,11], quinolones [12], β-lactams [13], aminoglycosides [14], cephapirin [15], and cationic dyes [16]. However, multi-class residue methods are relatively scarce, compared to multi-matrix, single-class residues methods. The effective extraction of compounds of very different chemophysical properties is a significant challenge in multi-class residue method development. Notwithstanding, a few multi-residue and -class methods have been successfully developed [17–19].

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Considering the potential of the technique to enhance the ability for regulatory agencies to monitor illegal use of veterinary drugs, an LC–MS-based multi-class residue screening/confirmatory method for shrimp was therefore developed.

Quadrupole ion trap mass spectrometry (QIT-MS) is suitable instrumentation for unknown identification and confirmation. The ion trap can selectively store ions of a selected range of mass over charge ratio (*m*/*z*), and perform in-trap collision-induced disassociation (CID) to get fragments of precursor ion(s). Further fragmentation on selected product ion(s) can be done by employing additional rounds of CID. The higher order of MS–MS (MS^n) spectra can enhance the specificity of the analysis. A significant advantage of MS^n analysis is the reduction of background noise, thus both high specificity and sensitivity can be achieved. Recent studies have demonstrated the use of this type of mass spectrometry on confirmation of veterinary drugs [9,15,20,21]. Thus, LC–QIT-MS was chosen for this screening/confirmatory method.

The veterinary drugs included in this method are: sulfadiazine (SDZ), sulfamerazine (SMR), sulfamethazine, sulfachloropyridazine (SCP), sulfadimethoxine (SDM), sulfaquinoxaline (SQX), oxolinic acid (OXO), nalidixic acid (NAL), flumequine (FLU), enrofloxacin, sarafloxacin (SAR), difloxacin (DIF), malachite green (MG), gentian violet (GV), leucomalachite green (LMG), leucogentian violet (LGV), oxytetracycline, and toltrazuril sulfone (TOLS). Among these drugs, several of them have specific tolerances in meat products (including some aquaculture products other than shrimp) set by FDA to date. For example, OTC has a combined tolerance with chlorotetracycline and tetracycline in muscle of 2 part per million (ppm; μg/g in solid tissues or μg/mL in milk) in a variety of species. Sulfachloropyridazine, SDM, SMZ, and SQX all have 0.1 ppm tolerance in edible tissues from various farmed animals [22]. These tolerances can serve as references to help determine target monitoring levels for shrimp.

Our LC–QIT-MS^n-based method provides both confirmation of these drugs and a gross estimate on whether the incurred level is likely to exceed a pre-determined level to warrant further quantitation. Two fortified quality control samples (QC) are processed and analyzed along with unknowns to provide such information. In this study, appropriate target levels are set for each drug based on a joint consideration of the above regulatory reference and instrument capability.

2. Experimental

2.1. Standards

All standards were purchased with highest available purity from the following sources. From Sigma–Aldrich: OTC $2H_2O$, SCP, SQX (Na salt), LGV, LMG; from Sigma: SDZ (Na salt), SMR, SMZ, SDM, OXO, NAL, FLU, GV; from Aldrich: MG; from Bayer: ENR; from Abbott Laboratories: SAR (HCl salt), DIF (HCl salt). TOLS was a generous gift from Dr. P. Gowik at the Community Reference Laboratory/National Reference Laboratory (CRL/NRL), Berlin, Germany.

2.2. Stock solutions

About 10 mg of a standard (record to 0.1 mg) was weighed and dissolved in appropriate solvent(s) in a 10-mL volumetric flask, except OTC, SAR (50 mL), and LGV, LMG (25 mL). Some standards required use of acid or base and/or sonication for complete dissolution. The stock solutions were stored in 20-mL scintillation vials at <−6 °C. Long time exposure of stock solution to light should be avoided. The solutions can be used within one year for screening/confirmation purposes. A separate set of 100 μg/mL solutions for each drug were prepared from the above stock solutions. The mixed solution containing the 18 drugs was prepared by pooling the appropriate amount of the 100 μg/mL solutions of each drug, and diluting with 1:3 (v/v) methanol/water. The concentrations for SDZ, SMR, SMZ, SCP, SDM, ENR, SAR, DIF, OXO, NAL, and FLU are 500 ng/mL, SQX 1 μg/mL, LGV, LMG, MG, GV 200 ng/mL, OTC 20 μg/mL, and TOLS 5 μg/mL (18-mix).

2.3. Other chemicals

Acetonitrile and methanol were purchased from Burdick & Jackson (MI). HPLC grade water was generated in-house with Milli-Q Plus (Milli-Pore; resistance ≥ 18.2 MΩ). Sodium hydroxide (volumetric, 10 N), hydroxylamine hydrochloride (ACS), and formic acid (88%) were purchased from Mallinckrodt (AR); trichloroacetic acid (TCA; SigmaUltra), succinic acid (SigmaUltra), EDTA (disodium, dehydrate, 99.6%), and ascorbic acid (SigmaUltra), from Sigma (MO); ammonium formate (minimum 96%), from J.T. Baker (NJ); and concentrated HCl (36-37%), from Fisher Scientific (PA).

2.4. Liquid chromatography

The Agilent 1100 LC system consisted of the following components: quaternary pump, autosampler, and column compartment with heater. Injection volume: 50 μL. Flow rate: 0.70 mL/min. Compartment temperature was set at 30 °C. LC column: Waters YMC Phenyl, 4 mm × 50 mm, 3 μm, with guard column (YMC Phenyl S3 4 mm × 20 mm 120 Å). Mobile phase A: 5% (v/v) acetonitrile/water, with 0.1% formic acid; mobile phase B: 85% (v/v) acetonitrile/water with 0.05% formic acid. Two gradients were used. The 19-min LC method was set as follows (where t refers to time in min): t_0, $B = 0\%$; t_1, $B = 0\%$; t_{11}, $B = 44\%$; $t_{11.3}$, $B = 69\%$; $t_{14.5}$, $B = 100\%$; t_{16}, $B = 100\%$; t_{17}, $B = 0$; t_{19}, $B = 0\%$. The 10-min method was set as follows: t_0, $B = 0\%$; t_2, $B = 0\%$; $t_{2.5}$, $B = 50\%$; t_4, $B = 50\%$; $t_{4.5}$, $B = 100\%$; t_8, $B = 100\%$; t_9, $B = 0$; t_{10}, $B = 0\%$.

2.5. Mass spectrometry

Finnigan MAT LCQ Classic ion trap mass spectrometer controlled by Thermo/Finnigan XCalibur software v1.3 was used, with APCI as the ionization source. The following parameters were adopted for all 18 drugs: sheath gas 55 unit; auxiliary gas 0; evaporation chamber temperature 450 °C; heated capillary

Table 1
Time schedule and mass spectrometer parameters for acquisition of mass spectra

	Retention time (min)	Acquisition window (min)	Isolation width (Da)	Collision energy (%)	Activation time (ms)	Precursor ion(s) (*m/z*)	Scan range (*m/z*)
SDZ	6.0	5.5–6.5	1.6	32	30	251.1	140–190
SMR	6.8	6.0–7.5	1.6	34	30	265.1	140–205
SMZ	7.4	6.5–8.0	1.6	34	30	279.1	110–220
SCP	9.0	8.5–9.5	1.6	36	60	284.9	90–170
SDM	10.9	10.5–12.0	1.6	33	60	311.1	140–260
SQX	11.1	10.5–12.0	1.6/4.0	36/30	20/30	301.1/208.0[b]	130–240
ENR	7.8	7.0–8.5	1.6/4.0	36/40	30/30	360.1/316.2[b]	230–310
SAR	8.4	7.5–9.5	1.6/4.0	34/38	30/30	386.1/342.2[b]	270–335
DIF	8.6	8.0–9.5	1.6/4.0	36/40	30/30	400.1/356.2[b]	285–350
OXO	10.2	9.5–10.8	1.6/4.0	32/38	30/30	262.0/244.1[b]	200–270
NAL	11.4	10.8–12.0	1.6/4.0	36/33.5	30/30	233.1/215.1[b]	170–240
FLU	12.1	10.8–12.8	1.6/4.0	35/40	30/30	262.0/244.2[b]	190–270
LGV	6.5	5.5–7.5	1.6	44	30	374.2	225–365
LMG	10.2	9.5–10.8	1.6/4.0	38/40	30/30	331.2/316.2[b]	225–328
MG	13.6	12.8–14.5	1.6	48	100	329.3	195–330
GV	14.0	12.8–14.5	1.6	52	100	372.3	235–368
OTC	6.5	6.0–7.0	1.6/4.0	48/24	30/30	461.1/426.0[b]	215–435
TOLS[a]	6.8	4.5–8.0	1.6	30	45	388.1	275–360

[a] From the 10-min method.
[b] Precursor ions for MS^2 and MS^3, respectively.

temperature 225 °C; 1 microscan/scan for MS^2 and MS^3 modes; automatic gain control on; inject waveform off; in-source CID off. For practicality, the 19-min method was divided into 16 segments, such that all drugs were monitored around their respective retention time. One of the three tune files based on three of the screened drugs were used for each time segment, i.e., OTC (5.5–7 min; segments 2–4, containing SDZ, SMR, LGV, and OTC), SAR (7–12.8 min; segments 5–14, containing all drugs except SDZ, OTC, MG, and GV), and MG (12.8–14.5 min; segment 15, containing MG and GV). The first and last segments were only used for diverting matrix-rich eluate to waste. On the other hand, the 10-min method is very simple: TOLS was used for tuning and there were only three segments. The four tune files have different parameters: OTC and SAR, discharge current 5 μA, maximum isolation time 200 ms, with MS^n target count of 2×10^7; MG, same as these two except the discharge current was zero; and TOLS, discharge current 1.5 μA, maximum isolation time 100 ms, with MS^n target count of 1×10^7. Parameters for each analyte are shown in Table 1.

2.6. Incurred and commercial shrimp

2.6.1. Incurred shrimp

Shrimp treated with one of the following drugs, OTC, OXO, NAL, and SDZ, at two concentrations each, were provided by Dr. Rodney Williams, University of Arizona, Tucson, AZ.

2.6.2. Control shrimp

Shrimp that were free of antibiotics, were obtained from Bowers Shrimp farm (Bowers), Palacios, TX. Two other shrimp samples (~2 lb. each) were purchased from a local grocery store and were treated as unknown samples. They are labeled as Raw Key West Pink shrimp (RKWP) and Black Tiger shrimp (BT), respectively.

2.7. Target levels for each drug

The following target levels have been set for this method: OTC 400 ng/g, TOLS 100 ng/g, SQX 20 ng/g, SDZ, SMR, SMZ, SCP, SDM, ENR, SAR, DIF, OXO, NAL, and FLU 10 ng/g. MG, GV and their metabolites, LMG and LGV are set to 4 ng/g. The fortified extracts were prepared at one of the three levels for each drug set, i.e., 0.5×, 1×, and 2.5 × target levels, denoted as L1, L2, and L3, respectively. For LGV however, the actual extraction and LC separation conditions developed for this collection of drugs renders a high noise background, such that its lowest validated level was 10 ng/g. Compared to those drugs that do have a tolerance in other types of meat, these levels can be regarded as relatively conservative.

2.8. Sample homogenisation

The shrimp samples were homogenized according to a published procedure [23]. Briefly, after whole shrimp samples were thawed at room temperature (RT), the chitin shell, tail, legs and fins were removed. The shrimp meat was mixed with pre-grinded dry ice in a food processor and was blended till no chuck larger than 1/4 in. was left. After dry ice sublimed in a −20 °C freezer from the ground shrimp meat overnight, portions of 2.0 ± 0.1 g of shrimp paste were weighted into 50-mL polypropylene centrifuge tubes. The blended shrimp samples were stored in −80 °C freezer until use.

2.9. Fortification of samples

A 2 g blended shrimp sample was thawed, and 100 μL of standard solution mixture (18-mix or 18-mix with appropriate dilutions) was applied to the surface. The sample was allowed to

stand at RT for approximately 30 min prior to further processing while covered with aluminum foil to avoid light.

2.10. Extraction and clean-up

Trichloroacetic acid (TCA) (17.5 mL; 0.05 g/mL) and hydroxylamine hydrochloride (0.5 mL; 0.20 g/mL) were added to each 50-mL polypropylene centrifuge tubes containing 2 g blended shrimp. The tubes were capped and vortexed for 5 min on multi-tube vortexer at high speed. The shrimp paste was then homogenized with a Polytron blender at medium speed for 1 min. The tubes were vortexed for 10 min at medium speed, and centrifuged at approximately 4000 rcf at 4 °C for 15 min. The supernatant was decanted into a neutralizing solution consisting of 2.5 mL 0.4 M sodium succinate (adjusted to pH 6.3 with NaOH) and 280 μL 10 N NaOH. The pH of the extracts should be 3.6 ± 0.1, otherwise pH was adjusted to this range with NaOH or HCl solution. The extracts were drawn through a set of pre-conditioned Waters Oasis HLB cartridges (60 mg, 3 mL) by applying about 15 in. vacuum. The SPE cartridges were then washed with 3 mL ammonium formate buffer (20 mM, adjusted to pH 3.9 with formic acid) followed by 3 mL Milli-Q water. The cartridges were vacuum-dried for 2 min. The retained drugs were eluted with 2 mL MeOH followed by 1 mL 1:1 CH_3CN/MeOH into 15-mL graduated polypropylene centrifuge tubes. The elution flow rate was kept at about 1 drop/s. The following reagent solutions were added to each collected eluate: 0.8 mL ammonium formate buffer (20 mM, pH 3.9), 50 μL EDTA (0.1 M), and 50 μL ascorbic acid (1 mg/mL in methanol). The combined solution was evaporated under nitrogen flow at 45 °C till about 0.8 mL is left. An aliquot of 1:1 water/acetonitrile (v/v) was then added to fill to 1-mL mark. After vortexing, each extract solution was poured into a 1.5-mL snap-cap polypropylene centrifuge tube, and was centrifuged at 14,000 rpm (rcf 16,000 × g) for 10 min using a bench-top centrifuge. The middle portion of the centrifuged solution (~0.8 mL) was transferred into a 1.5-mL amber glass autosampler vial for analysis.

2.11. Confirmation criteria

Chromatographic peak recognition was done using XCalibur's QualBrowser software. A set of pre-determined peak-detection parameters, including algorithm (ICIS), baseline window, noise factors, peak height restriction, and tailing factor, were applied on each of the analytes. For each drug, the reconstructed ion chromatogram (RIC) from one of the major CID transitions (or the sum of several) was designated as "primary RIC". Other RICs from less abundant fragments (MS^2 or MS^3 alike) were treated as "secondary RICs". When all required chromatographic peaks were recognized for any of the drugs from a given sample, the co-elution of all RICs (not applicable to TOLS, SCP, and SQX), retention time (t_R) of primary RIC, and full scan mass spectra were examined either numerically or visually to confirm positive samples. The two intra-day QCs (fortified extracts) were used as the basis for comparison. These criteria for mass spectra are in line with FDA/Center for Veterinary Medicine's published guideline on confirmation by mass spectrometry [24]. Particularly in this method, to confirm the presence of a drug residue in a given sample, t_R of secondary RICs must be within ± 1% that of the primary RIC, which should be within ±1% that of the corresponding RIC in QCs (2% allowance for SDZ and OTC in both cases). Visual inspection of the full scan mass spectra was performed to tell if an unknown matches QC. An example for ENR is shown in Fig. 1.

2.12. Additional criteria for screening purpose

To extend the use of this method, an additional restriction was applied to those samples with confirmed presence of drugs, to determine if further quantitation is needed. The height of the primary RIC peak in mass spectra was compared with its counterpart in QCs' spectra, to see whether drug residues are likely to exceed designated level. For many drugs included in this method, the LC–ion-trap-MS is sensitive enough to confirm residues well below target levels. Thus, a 5% of the weighed average (same way as "weighed peak area" is calculated, except using height in place of area; see next paragraph) peak height of the primary RIC for each drug in QCs was used as an arbitrary threshold. This "fit-to-purpose" screening was not specifically designed for a typical screening test, which requires certain criteria to be met. However, considering the intended use and high throughput feature of this method, it is worthwhile to include a preliminary screening step before quantitation.

2.13. Semi-quantitation

To evaluate whether this method is suitable for estimating residue levels at or close to L2, using two fortified QCs at 2.5 × L2 as reference points, all fortified extracts were processed according to the following algorithm: the major RIC's peak area (as $Area_{X\text{-}Y}$ below) was integrated using XCalibur's QuanBrowser, with 5 or 7-point smoothing depending on peak shape quality. The residue level was estimated with the following formula: residue level of drug X in sample Y = [$Area_{X\text{-}Y}$] × [QC's fortification level for drug X]/[$Area_{\text{weighted average of X-QCs}}$]; $Area_{\text{weighted average of X-QCs}} = \{[Area_{X\text{-}QC\#1}] \times (N_{QC\#2} - N_{\text{sample Y}}) + [Area_{X\text{-}QC\#2}] \times (N_{\text{sample Y}} - N_{QC\#1})\}/\{N_{QC\#2} - N_{QC\#1}\}$, where $N_{QC\#1}$, $N_{QC\#2}$, and $N_{\text{sample Y}}$ are the injection numbers of the first QC, second (last) QC, and sample Y within a sequence. A 3-point check (blank, L2 and L3) on linearity for some of the drugs in fortified BT and RKWP shrimps revealed reasonably good correlation, even in absence of internal standard.

3. Results and discussion

As stated above, the positive identification of the presence of any of the 18 drugs in shrimp is based on the information from both ion chromatograms and mass spectra. The criteria on chromatography here are more restrictive than the aforementioned FDA Guidance, in which the acceptable range for retention time matching for LC–MS is 5%, and co-elution of

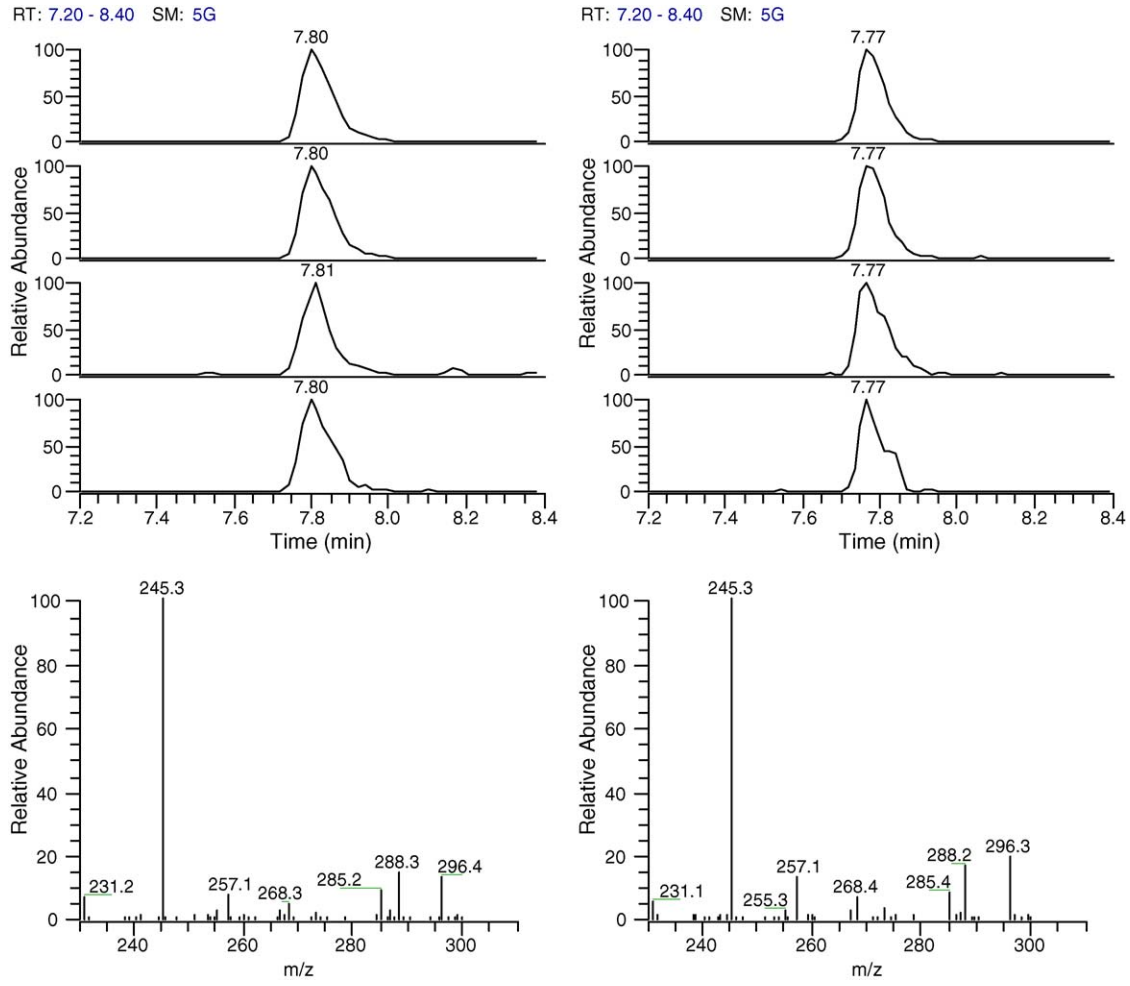


Fig. 1. Confirmation of enrofloxacin residue in an unknown shrimp sample: (left panels) ion chromatographs (primary and secondary RICs) and mass spectrum for fortified Bowers shrimp at L3 level (25 ng/g) and (right panels) ion chromatographs (primary and secondary RICs) and mass spectrum for a shrimp sample (Black Tiger) purchased from local store (estimated at 20 ng/g).

all RICs is not required. For mass spectral matching, at least two structurally specific product ions for each drug are verified, which is in line with the Guidance. Having identity confirmation established, subsequent analysis only needs to give quantitative information, as can be accomplished by less specific instruments such as LC–UV or LC–fluorescence. Fig. 2 shows the ion chromatograms and mass spectra (the co-elution of RICs is not shown) for all 18 drugs, including standards, blank controls, fortified samples, and for some drugs incurred ones. To better compare relative abundance of the ion traces, the primary RICs of each drug have the same fixed *Y*-scale (NL number) except the standard's.

The results of validation are listed in Table 2. For Bowers shrimp, all drugs but LGV can be confirmed at L1 and L2 levels for at least 5 out of 6 (L1) or 11 out of 12 (L2) analyses. All drugs in all 26 repeats can be confirmed at L3 level. All incurred samples are found positive as well, including the two samples that were diluted by a factor of 4 with drug-free shrimp tissue. Data is less comprehensive for RKWP and BT shrimps, but it is largely comparable to Bowers shrimp. The evaluation of the extraction efficiency at L2 level for these two shrimp species should be adequate. ENR was found positive in the BT shrimp sample at an estimate of 20 ng/g.

3.1. Sample preparation

Shrimp is a matrix high in protein and lipids. Attempts to use aqueous buffers of pH greater than 3 for extraction gave a jelly-like mixture, which was impossible to elute through SPE cartridges. Extraction with acetonitrile gave a solution rather high in fatty substance, and various attempts to remove it caused significant loss of certain drugs. Extraction with a mixture of acetonitrile and aqueous buffer gave enhanced recovery in general, but some of the more polar components such as SDZ and OTC "break through" the HLB cartridges due to a moderate amount of acetonitrile in the extracting solution. Other SPE cartridges, such as MCX, PRS, and C8 were tested. However, the HLB SPE cartridge gave the best overall performance and was deemed to be the most suitable when all 18 drugs are considered.

A gross estimate of absolute recoveries of the drugs was obtained by dividing the ion chromatogram peak area of fortified samples (standards added to blended shrimp before extraction)

by that of spiked samples (standards added to shrimp extracts when final reconstitution was made). It was used to evaluate the effectiveness of the various methods that had been tried. The 5% aqueous TCA extraction is effective for OTC, sulfonamides, some quinolones, and LGV. Interestingly, LMG has a rather low recovery despite its structural similarity to LGV. OTC appears stable in this acidic extraction buffer for the first ~1.5 h before neutralization. The comparison of the ion chromatograms between fortified and spiked samples (not exposed to acidic media) indicates a high recovery of OTC (>80%).

Recoveries of OTC, LGV and LMG are sensitive to pH at the SPE clean-up step. Higher recoveries for the two leuco-dyes were found at pH 3.2, as compared to pH 4.7. On the other hand, OTC cannot be retained on SPE if pH is below 3. A compromise at pH 3.6 was found to be optimal for all these drugs. Other drugs are insensitive to pH within this range. The low recoveries for MG, GV, and TOLS are probably due to either strong adsorption to shrimp tissue, or poor solubility in aqueous medium. Hexane washing does not significantly improve the cleanness of the extract, but adversely affects the recovery of the leuco-dyes, unless the washing is performed at low pH.

Estimated recoveries of the 18 drugs from Bowers shrimp are listed in Table 3. Several of the drugs have very low recoveries but can still be confirmed by this method at or below target levels. There is also a qualitative correlation between the t_R and recovery. Separate data indicate that HLB cartridge can retain

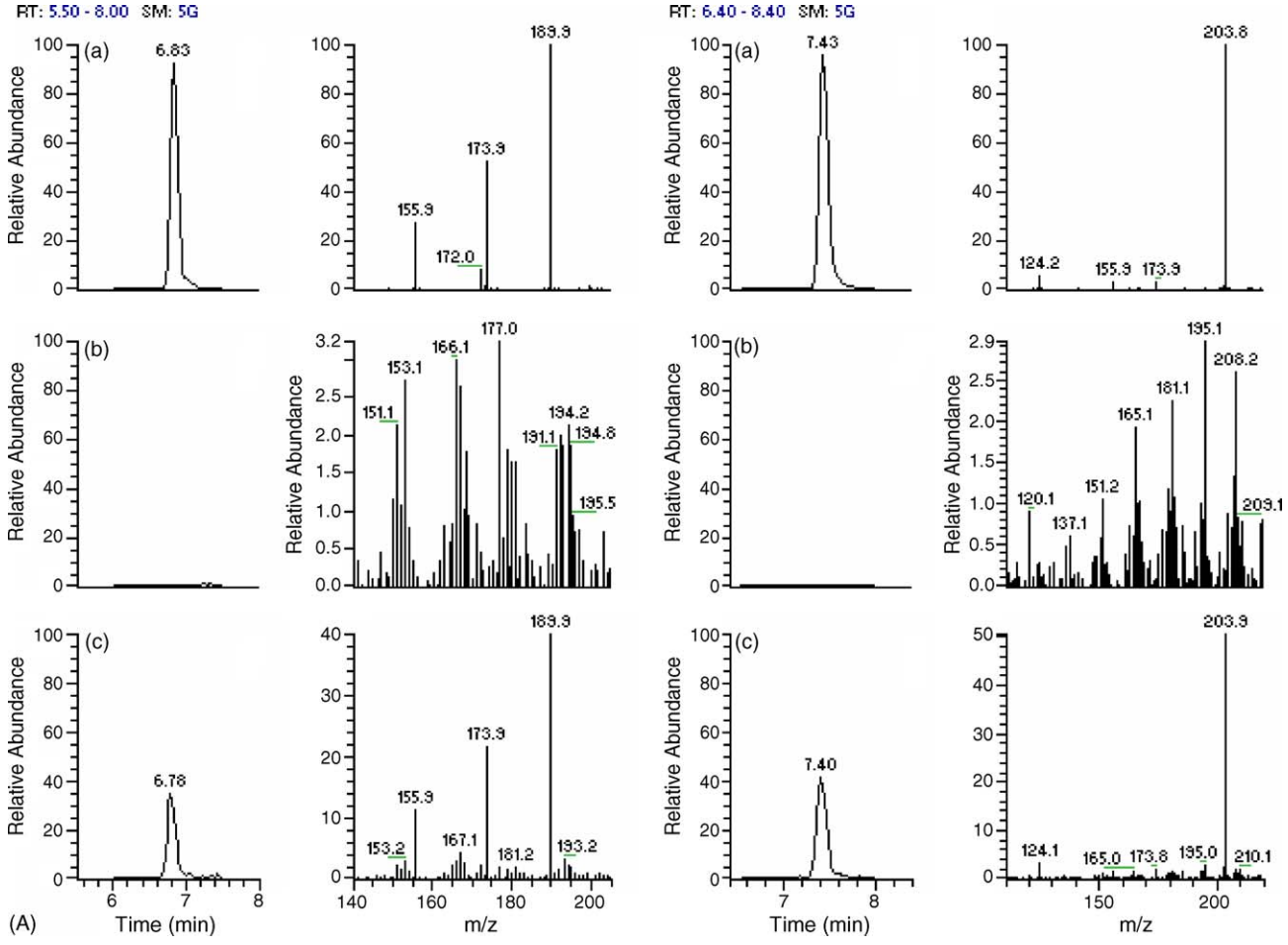


Fig. 2. Ion chromatograms and mass spectra for: (A) sulfamerazine (left) and sulfamethazine (right): (a) 100 ng/mL standard solution; (b) extract from control Bowers shrimp; and (c) fortified shrimp with 25 ng/g SMR or SMZ. (B) Sarafloxacin (left) and difloxacin (right): (a) 100 ng/mL standard solution; (b) extract from control Bowers shrimp; and (c) fortified shrimp with 25 ng/g SAR or DIF. (C) Sulfachloropyridazine (left) and sulfadimethoxine (right): (a) 100 ng/mL standard solution; (b) extract from control Bowers shrimp; and (c) fortified shrimp with 25 ng/g SCP or SDM. (D) Flumequine (left) and sulfaquinoxaline (right): (a) 100 ng/mL FLU and 200 ng/mL SQX standard solution; (b) extract from control Bowers shrimp; and (c) fortified shrimp with 25 ng/g FLU or 50 ng/g SQX. (E) Toltrazuril sulfone: (a) 1 μg/mL standard solution; (b) extract from control Bowers shrimp; and (c) fortified shrimp with 250 ng/g TOLS. (F) Leucogentian violet (left) and leucomalachite green (right): (a) 40 ng/mL standard solution; (b) extract from control Bowers shrimp; and (c) fortified shrimp with 10 ng/g LGV or LMG. (G) Malachite green (left) and gentian violet (right): (a) 40 ng/mL standard solution; (b) extract from control Bowers shrimp; and (c) fortified shrimp with 10 ng/g MG or GV. (H) Sulfadiazine: (a) 100 ng/mL standard solution; (b) extract from control Bowers shrimp; (c) fortified shrimp with 25 ng/g SDZ; and (d) incurred shrimp raised with 38 mg/kg SDZ in feed; 0.5 g sample diluted with 1.5 g Bowers shrimp tissue. (I) Oxolinic acid (left) and nalidixic acid (right): (a) 100 ng/mL standard solution; (b) extract from control Bowers shrimp; (c) fortified shrimp at 25 ng/g; (d) incurred shrimp raised with 10 mg/kg OXO or 5 mg/kg NAL in feed; and (e) incurred shrimp raised with 5 mg/kg OXO or 2.5 mg/kg NAL in feed. (J) Enrofloxacin (left) and oxytetracycline (right). ENR: (a) 100 ng/mL standard solution; (b) extract from control Bowers shrimp; (c) fortified shrimp with 25 ng/g ENR; (d) unknown Black Tiger shrimp sample bought from grocery; and (e) same unknown sample fortified with 10 ng/g ENR. OTC: (a) 4.0 μg/mL standard solution; (b) extract from control Bowers shrimp; (c) fortified shrimp at 1.0 μg/g; (d) incurred shrimp raised with 2.5 g/kg OTC in feed; and (e) incurred shrimp raised with 5.0 g/kg OTC in feed; 0.5 g sample diluted with 1.5 g Bowers shrimp tissue.

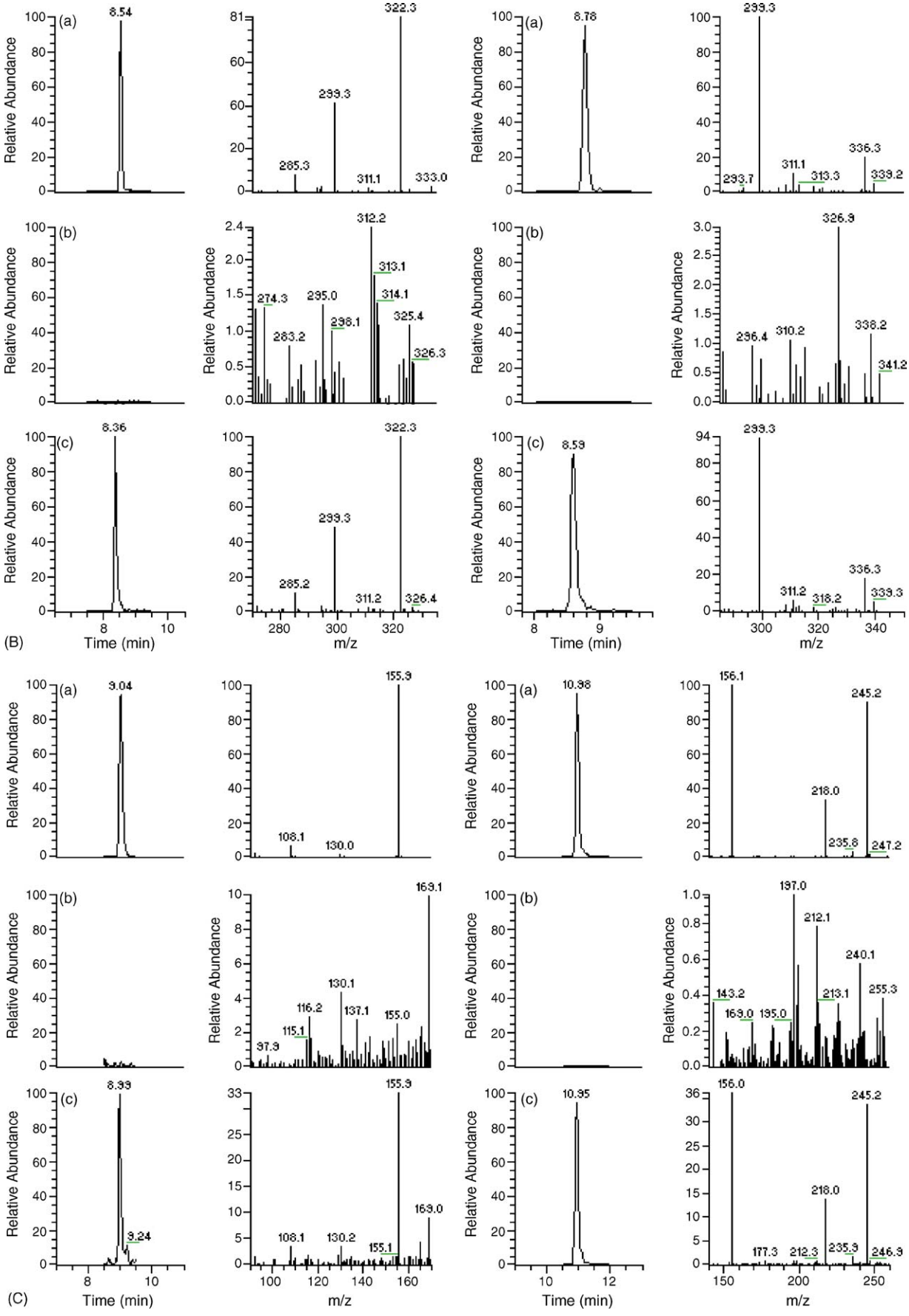


Fig. 2. (*Continued*)

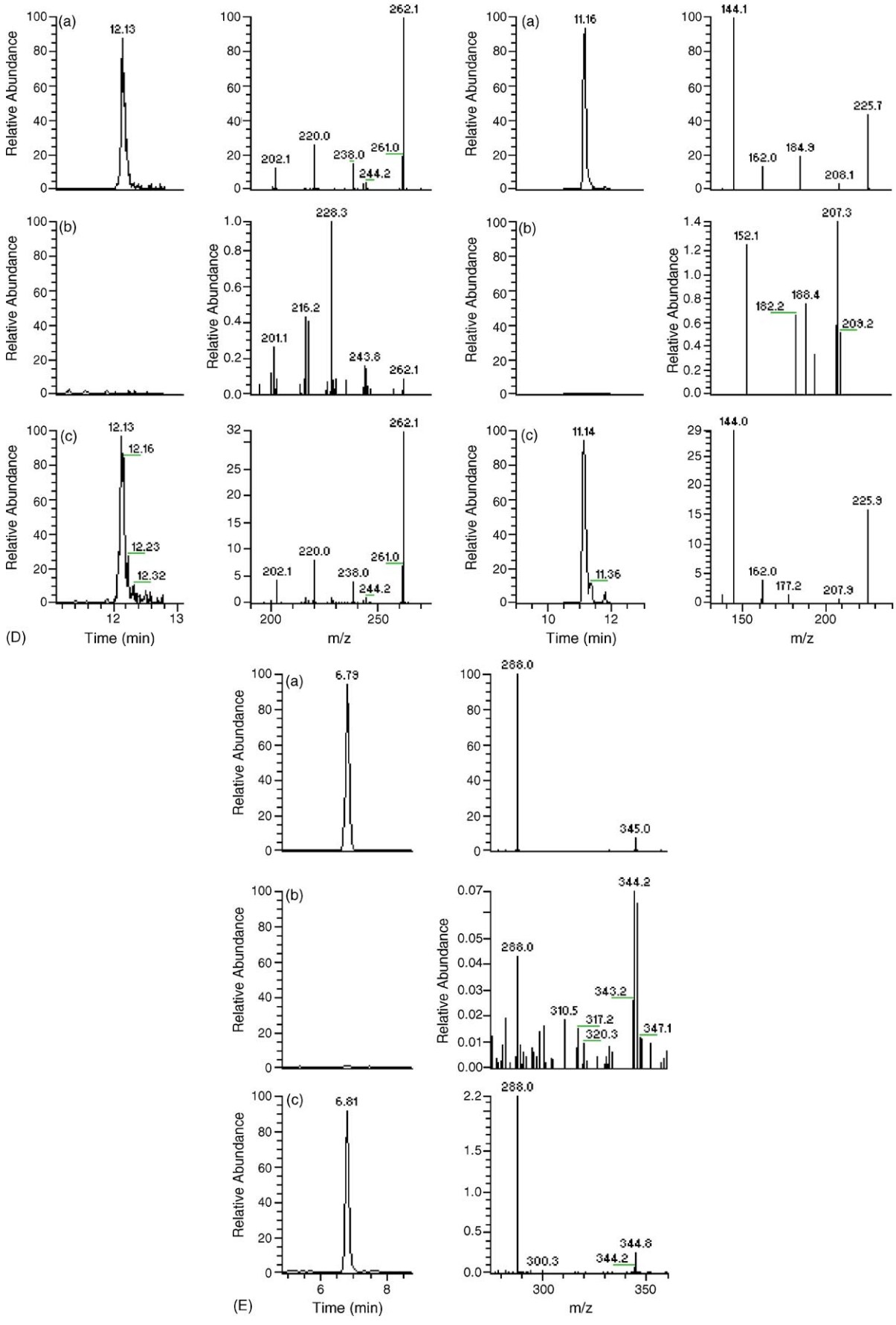
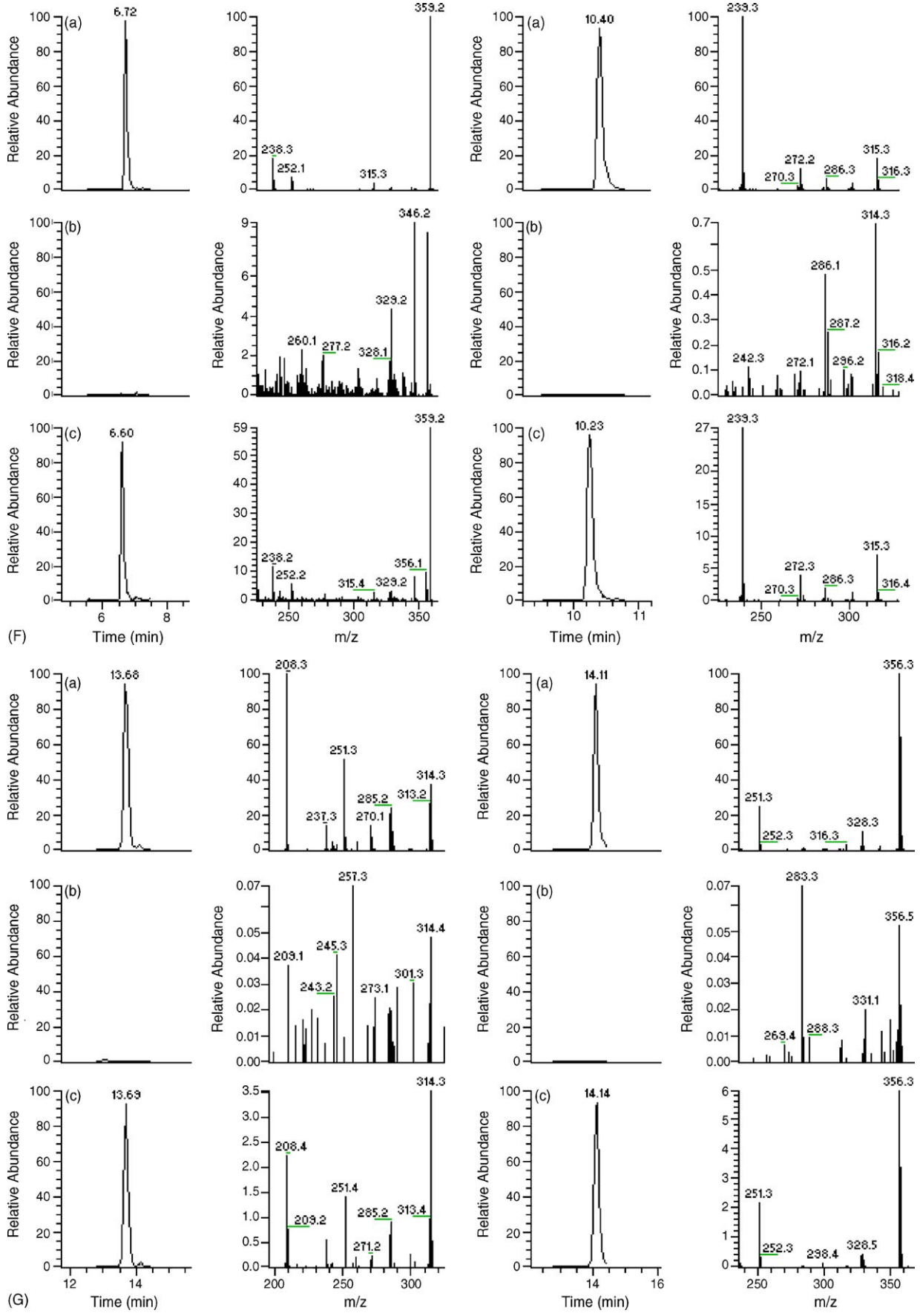


Fig. 2. (*Continued*)



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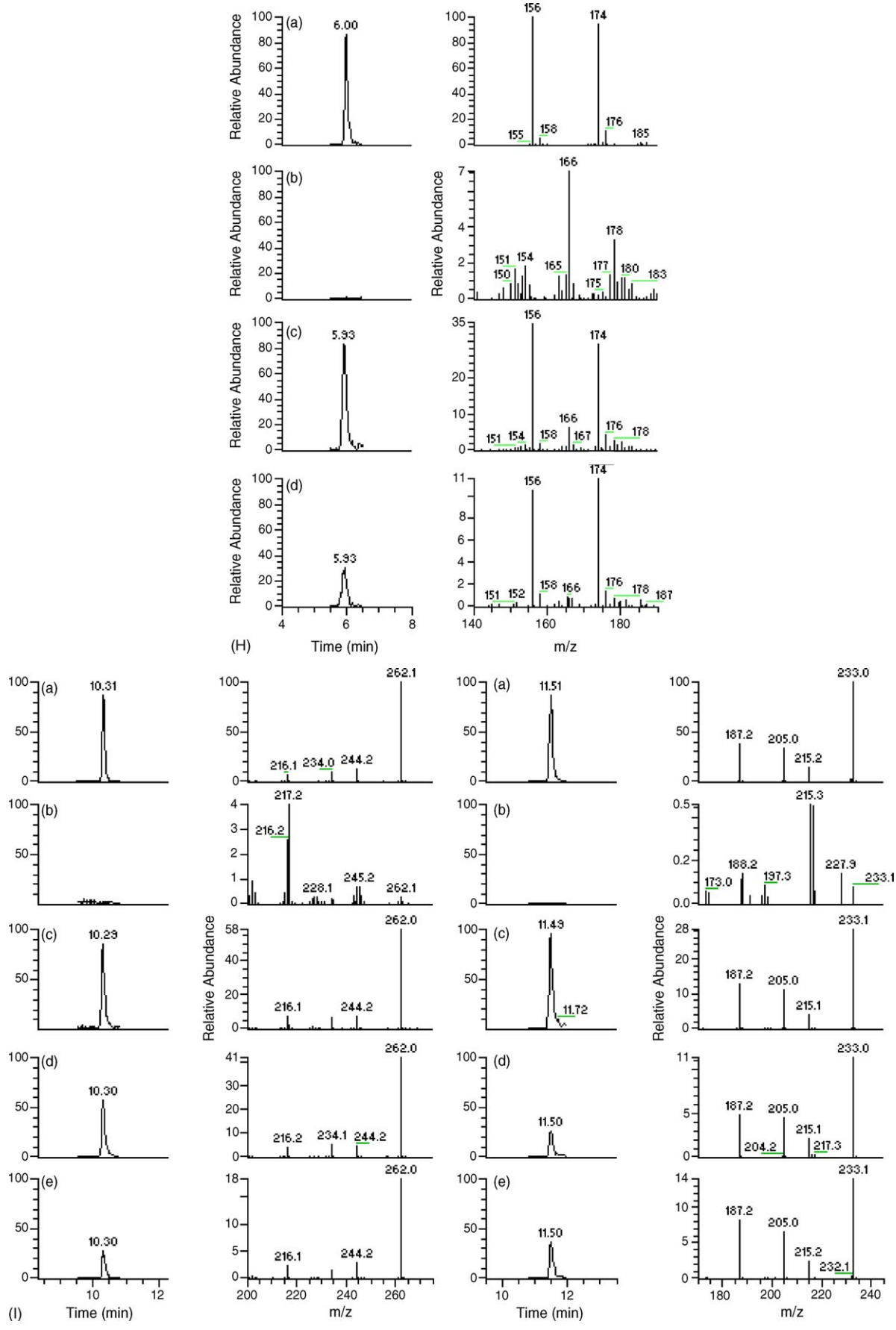


Fig. 2. (*Continued*)

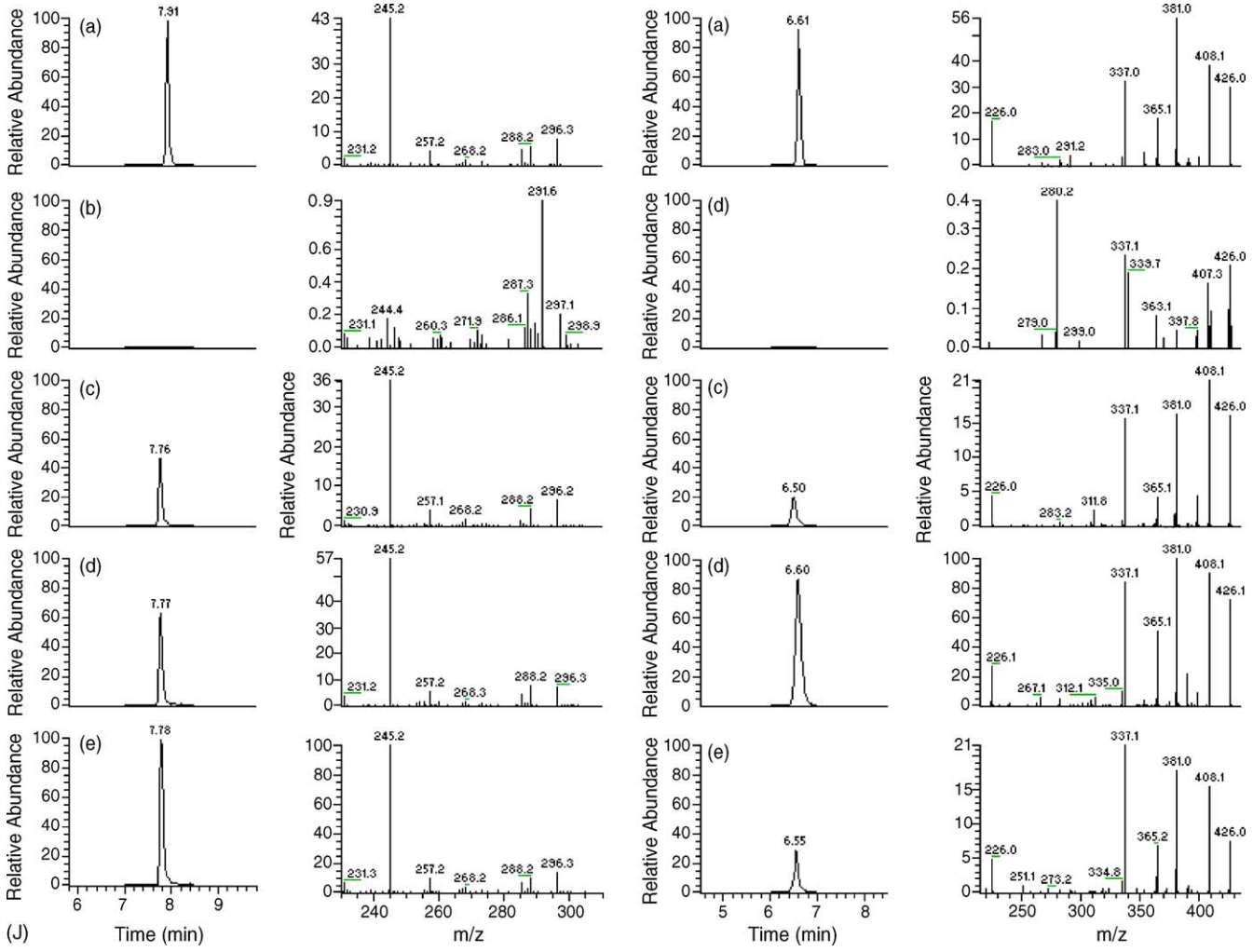


Fig. 2. (*Continued*).

and release all drugs relatively well, suggesting the likelihood that extraction efficiency is the key factor determining the overall recovery. Using fortified QCs as reference, the residue levels in five of the seven incurred samples, i.e., SDZ-38, NAL-2.5, NAL-5, OXO-10, and OXO-20, were estimated at 40 ng/g (SDZ), 5 ng/g (NAL), 6 ng/g (NAL), 7 ng/g (OXO), and 16 ng/g (OXO), respectively, error (R.S.D.%; replicates of 6) ranging from 11 to 30%. No estimate was made for OTC-doped samples.

EDTA was added in later steps of the extraction to increase sensitivity for OTC and some of the quinolones. Both OTC and quinolones are known to have high affinity to metal ions. EDTA in buffer competes with these analytes for metal cations in solution to shift chemical equilibrium towards protonation. Both higher signal intensity and better repeatability were observed for their protonated ions. Hydroxylamine and ascorbic acid was also added to the extraction or reconstitution solutions to prevent oxidation and/or de-methylation of the two dyes or their leuco-dyes [25]. An ultra-centrifugation step was utilized to remove debris in the final reconstituted solutions. Use of PVDF Acrodisc Syringe Filter to remove small particles in solution caused significant loss of MG and GV from a low organic content solution, such as 20% acetonitrile/H_2O (v/v). Filters made of other materials were not tested.

3.2. Liquid chromatography

A short Waters YMC column was chosen based on previous results within our lab. An LC gradient elution schedule of 29-min was developed to analyze all 18 drug in a single LC run. The YMC Phenyl column was found to only separate GV and TOLS by less than 0.4 min apart, under a variety of mobile phase combinations and gradient schedules. However, these two analytes demand opposite polarity for ionization and analysis, while the LCQ Classic mass spectrometer is not particularly suitable for a rapid switch of polarities. Thus, a set of two shorter LC runs were used instead, one for positively charged analytes (the 19-min method) and another for negatively charged species (the 10-min method). This adds to the flexibility of the entire screening method, as more target compounds may be added into the list in the future without substantial change to current method. In fact, chloramphenicol can be analyzed with the 10-min method along with TOLS, with only minor modification.

To prevent matrix from accumulating on the column and the MS interface, the first and last few minutes of an LC run are diverted to waste, and a blank run with 100 μL injection of acetonitrile is performed between every two or three sample runs. In Table 4a it is apparent that the standard error for intra-day

Table 2
Confirmatory results of validated samples

Sample ID[a]	Drug(s) confirmed (number of positive samples)	Repeats for each sample
Blank control (Bowers)	None	26
18-L1 (fortified with 18-mix at L1 level)	SMZ/SDM/ENR/SAR/DIF/NAL/FLU/LMG/MG/OTC/TOLS (6), SDZ/SMR/SCP/SQX/OXO/GV (5), LGV (0)	6
18-L2 (fortified with 18-mix at L2 level)	All (6) except SQX (5), LGV (2)	6
18-L3 (fortified with 18-mix at L3 level)	All (26)	26
6S-L2 (fortified with 6-sulfonamide-mix at L2 level)	SDZ/SMR/SMZ/SCP/SDM/SQX (6)	6
6Q-L2 (fortified with 6-(fluoro)quinolone-mix at L2 level)	ENR/SAR/DIF/OXO/NAL/FLU (6), GV (1)[b]	6
4D-L2 (fortified with 4-(leuco)dye-mix at L2 level)	LMG/MG/GV (6), LGV (0)	6
O/T-L2 (fortified with OTC and TOLS at L2 level)	OTC/TOLS (6)	6
SDZ-38 (Incurred with SDZ; diluted × 4)	SDZ (6)	6
OXO-10 (Incurred with OXO; #1)	OXO (6)	6
OXO-20 (Incurred with OXO; #2)	OXO (6)	6
NAL-2.5 (Incurred with NAL; #1)	NAL (6)	6
NAL-5 (Incurred with NAL; #2)	NAL (6), SDZ (1)	6
OTC-2500 (Incurred with OTC; #1)	OTC (6)	6
OTC-5000 (Incurred with OTC; #2; diluted × 4)	OTC (6)	6
RKWP (Raw Key West Pink shrimp from market)	None	6
RKWP-L2 (Raw Key West Pink shrimp fortified at L2 level)	All (6) except SQX (5), GV (4), LGV (2)	6
BT (Black Tiger shrimp from market)	ENR (6)	6
BT-L2 (Black Tiger shrimp fortified at L2 level)	All (6) except SDZ/GV (5), MG(4); LGV (2)	6

[a] 18-L1, 18-L2, 18-L3: clean shrimp (Bowers) fortified with all 18 drugs at 3 levels, respectively (target × 0.5, target, and target × 2.5). 6S-L2, 6Q-L2, 4D-L2, O/T-L2: clean shrimp (Bowers) fortified with one of four groups of drugs (6S: SDZ, SMR, SMZ, SCP, SDM, SQX; 6Q: ENR, SAR, DIF, OXO, NAL, FLU; 4D: LGV, LMG, GV, MG; O/T: OTC, TOLS) at target levels, respectively. RKWP-L2, BT-L2: two other species of shrimps purchased from local grocery store, i.e., Raw Key West Pink (RKWP) and Black Tiger (BT), with no prior knowledge of drug residues. Besides direct extraction (RKWP, BT), a separate set of samples were fortified with 18 drugs at their target levels to confirm the extraction effectiveness (RKWP-L2, BT-L2). Incurred shrimp samples generated by outside provider. SDZ-38: shrimp raised with 38 mg/kg SDZ in feed; OXO-10, OXO-20: shrimp raised with 10 and 20 mg/kg OXO in feed, respectively; NAL-2.5, NAL-5: shrimp raised with 2.5 and 5.0 mg/kg NAL in feed, respectively; OTC-2500, OTC-5000: shrimp raised with 2.5 and 5.0 g/kg OTC in feed, respectively.
[b] False positive.

t_R is very small. The drift of average inter-day t_R for each of the drugs is also minimal during the 13-batch validation period, containing more than 300 matrix-bearing sample runs without column regeneration.

Matrix effect on liquid chromatography is more observable for a few drugs than others. The chromatographic peak shape for the early-eluting drugs, SDZ and OTC, was somehow compromised by co-eluting matrices, compared to matrix-free standards. As can be seen in Table 4b, the t_R for ENR, SAR, DIF, and LMG in fortified samples are consistently lower than that of standards by more than 0.1 min. Also, the t_R for these drugs in fortified RKWP shrimp is different to that in Bowers and Black Tiger shrimp, but to a smaller extent. This indicates that the elution of these few compounds on a phenyl column are affected by matrices, and the confirmation criteria on t_R should take this into consideration.

3.3. APCI ionization

All sulfonamides, quinolones, leucodyes, and OTC were analyzed in their protonated form. MG and GV are in ionic form without protonation, and TOLS only gave signals under negative mode. Both ESI and APCI have been evaluated for sensitivity and suitability. Preliminary results indicated that ESI and APCI have comparable sensitivity towards most of these drugs, except that ESI is much more sensitive for OTC. However, OTC has a rather high tolerance in meat (2 μg/g total tetracyclines), so the Finnigan LCQ with APCI interface is more than adequate for confirming OTC. As for OXO, NAL, and FLU, it is interesting to find that the ion-trap-CID-generated first-order product ion, $(M + H\text{-}H_2O)^+$, is more abundant from ESI source than from APCI. The sensitivity of MG and GV in presence of matrix was not compared for the two ion sources. For APCI source,

Table 3
Estimated recovery of 18 drugs from 5% TCA extraction[a]

Antibiotic	Estimated recovery (%)	Antibiotic	Estimated recovery (%)	Antibiotic	Estimated recovery (%)
SDZ	>75	ENR	>60	LGV	>75
SMR	>90	SAR	>40	LMG	>40
SMZ	>85	DIF	>40	MG	<10
SCP	>75	OXO	>55	GV	>10
SDM	>65	NAL	>50	OTC	>80
SQX	>40	FLU	>40	TOLS	∼10

[a] Estimation based on comparing primary RIC's peak area of fortified and end-spiked extracts. Repeat number is 2. The fortification levels are 50 ng/g (first 16 drugs) and 1000 ng/g (OTC and TOLS).

Table 4a
Retention times for inter-day LC runs (min). All are analyzed by the 19-min method except TOLS by the 10-min method

	Standard (no matrix)	Number of repeat	Fortified and incurred	Number of repeat[a]
SDZ	5.97 ± 0.02	26	5.93 ± 0.02	61
SMR	6.82 ± 0.02	26	6.78 ± 0.02	55
SMZ	7.42 ± 0.02	26	7.39 ± 0.01	56
SCP	9.01 ± 0.02	26	8.99 ± 0.02	55
SDM	10.96 ± 0.02	26	10.94 ± 0.01	56
SQX	11.15 ± 0.02	26	11.13 ± 0.01	53
ENR	7.90 ± 0.02	26	7.79 ± 0.02	62
SAR	8.54 ± 0.02	26	8.41 ± 0.02	56
DIF	8.77 ± 0.03	26	8.64 ± 0.02	56
OXO	10.32 ± 0.02	26	10.30 ± 0.01	67
NAL	11.50 ± 0.02	26	11.48 ± 0.01	68
FLU	12.15 ± 0.02	26	12.13 ± 0.01	56
LGV	6.68 ± 0.04	26	6.60 ± 0.03	32
LMG	10.36 ± 0.05	26	10.24 ± 0.04	56
MG	13.69 ± 0.02	26	13.69 ± 0.01	54
GV	14.10 ± 0.03	26	14.09 ± 0.02	53
OTC	6.59 ± 0.03	26	6.52 ± 0.02	68
TOLS	6.80 ± 0.01	26	6.80 ± 0.01	56

[a] Not including fail-to-confirm samples.

the sensitivity was much higher when the corona discharge current is turned off as was reported [26]. This is probably due to reduction of "space charge effect" or defocusing.

However, APCI is a far better ionization source for confirming TOLS than ESI using the LCQ. TOLS has two major ions from the APCI source in negative mode. The signal at 455 *m/z* is from the deprotonated molecule, and the other one at 388 *m/z* is probably from the loss of CF_3 group, in the form of anion, radical, or radical anion. This species is likely the result from an electron capture disassociation (ECD) mechanism, as reported for other molecules with high electron affinity [27]. It was found that the 455 *m/z* ion is very difficult to break down by CID, from either ESI or APCI sources, and on either QIT-MS (helium as collision gas) or triple quadrupole MS (argon as collision gas). A product ion from $(M\text{-}H)^-$, 398.5 *m/z*, is probably from loss of a methyl isocyanate molecule, but it is too weak and not very reproducible. On the other hand, the 388 *m/z* species easily disassociates by collision to give two major fragments at 288 and 345 *m/z*. In contrast, ESI does not generate the 388 *m/z* ion at all. All factors considered, APCI was chosen as the ionization source for both 19-min and 10-min LC–MS methods.

For this confirmatory/screening method, matrix effect on ionization efficiency is a lesser concern than matrix interference. The false positive rates for all drugs in fortified samples are virtually zero based on about 80–90 negative samples (including blank controls and fortified or incurred shrimp with drugs other than the designated one). The only two exceptions are: one was found SDZ-positive from a NAL-incurred sample, and one was found GV-positive from a 6-quinolone-fortified sample. It is not clear whether it is due to true drug residue in shrimp meat or contamination during extraction. Overall, this rate is satisfactory for a surveillance program. From APCI source, matrix effect on ionization is more significant to some of the analytes than others, especially OTC, ENR, SAR, and DIF. Interestingly, these are also the ones whose retention times are most affected by matrix. Matrix effect on another drug, MG, is species-dependent, as the ion signal from fortified RKWP shrimp is much more intense than from fortified Black Tiger and Bowers shrimp.

3.4. Ion trap mass spectrometric analysis

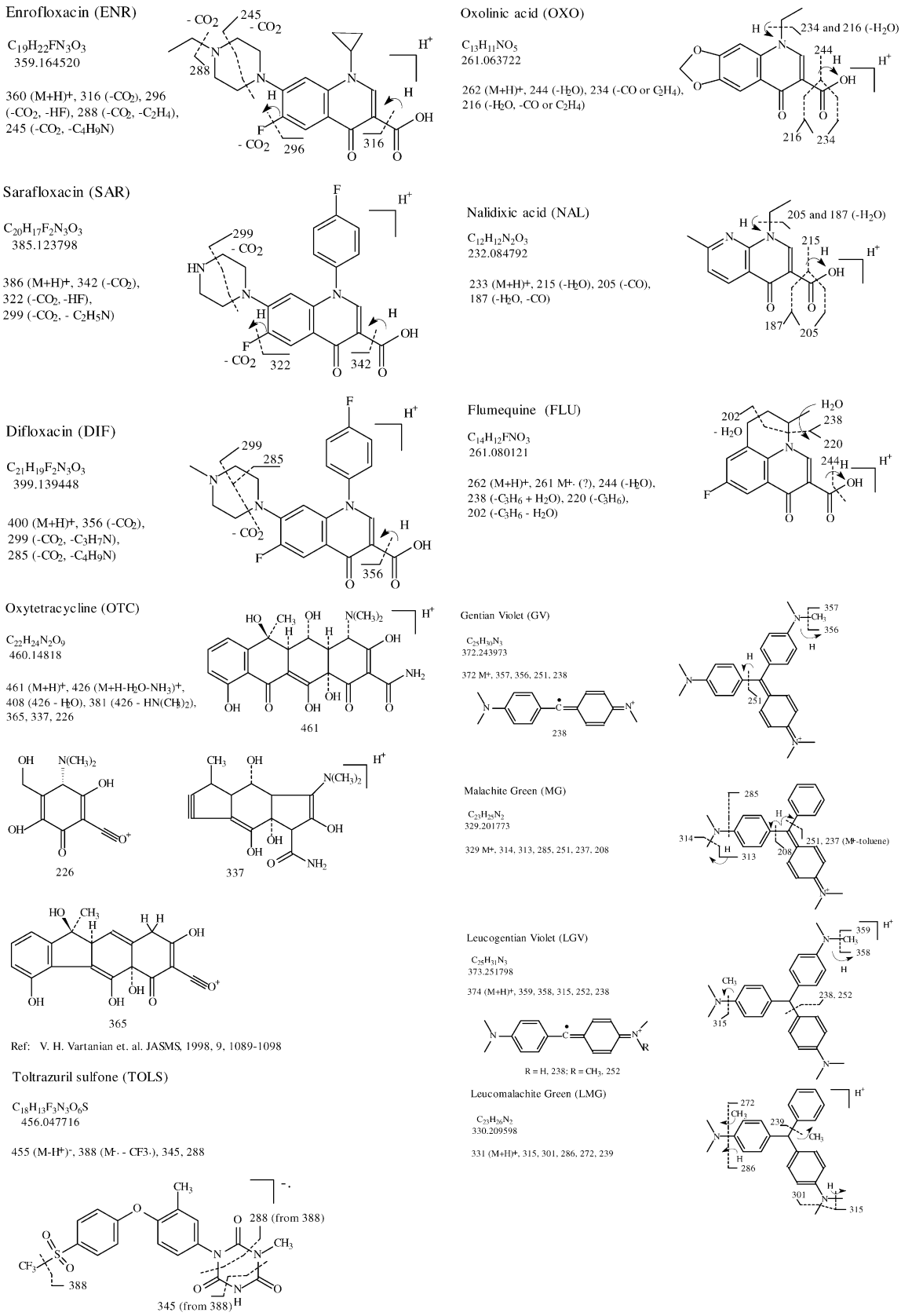
Eight of the drugs are confirmed with MS–MS, and nine are confirmed with MS–MS–MS. Fragmentation of TOLS can be considered as in-source-dissociation followed by in-trap-CID. For the MS^3 group the first order product ions are from unspecific transformations such as loss of water or CO_2. To achieve optimum spectra for confirmatory purposes, each drug's fragmentation parameters (CE% and activation time) have been optimized to obtain the most stable ion chromatograms. All spectra are acquired in full scan mode. The fragmentation pathways of these compounds can be found either in existing literature [9,28] or as proposed in Fig. 3.

Oxolinic acid, NAL, and FLU undergo dehydration to yield a primary fragment, $(M + H\text{-}H_2O)^+$, which undergoes a second CID process to give back the $(M + H)^+$ ion. This is apparently due to reaction with residual water in the ion trap [29]. Interestingly, the "backward reaction" is so predominant that the MS^2 process can only give a small percentage of dehydration product, a major portion of which transforms back to $(M + H)^+$ in the MS^3 stage. The ion ratios for all analytes, e.g., 174 *m/z* over 156 *m/z* for SDZ, are also evaluated. The overall relative standard deviation (R.S.D.%) is from 10 to 44%. In general, ions from MS^2 give narrower variation than MS^3, and those having intensity closer to base peaks exhibit less variability. Neat standards of higher residue level have lower R.S.D.% than fortified/incurred extracts, except OTC.

Table 4b
Retention time for selected drugs at different levels or eluted with different matrices

	18-L3	18-L2	18-L1	6Q-, 4D-, or O/T-L2	RKWP-L2	BT-L2	Incurred #1[a]	Incurred #2[b]
ENR	7.79 ± 0.02	7.79 ± 0.03	7.79 ± 0.03	7.78 ± 0.01	7.85 ± 0.02	7.78 ± 0.01	7.78 ± 0.01	
SAR	8.41 ± 0.03	8.41 ± 0.04	8.41 ± 0.05	8.40 ± 0.02	8.47 ± 0.02	8.40 ± 0.01		
DIF	8.63 ± 0.03	8.64 ± 0.02	8.63 ± 0.04	8.63 ± 0.01	8.71 ± 0.02	8.63 ± 0.01		
LMG	10.24 ± 0.04	10.24 ± 0.04	10.23 ± 0.04	10.23 ± 0.05	10.27 ± 0.05	10.20 ± 0.04		
OTC	6.51 ± 0.02	6.51 ± 0.03	6.51 ± 0.03	6.52 ± 0.01	6.53 ± 0.02	6.49 ± 0.02	6.57 ± 0.02	6.54 ± 0.02

All figures are average of six runs (inter-day) except for 18-L3 (26).
[a] Incurred #1: incurred shrimp samples SDZ-38, tissue-diluted four times and OTC-2500 in corresponding rows.
[b] Incurred #2: OTC-5000, tissue-diluted four times.



Proposed fragmentation pathways for selected veterinary drugs by ion-trap CID.

Fig. 3. Proposed fragmentation pathways for selected veterinary drugs by ion-trap CID.

3.5. Validation

This method was validated by analyzing both fortified and incurred samples. Eight incurred samples containing one of the four drugs (OTC, SDZ, NAL, and OXO) at one of two levels were generated under controlled conditions, while seven of them were extracted and analyzed. Due to the number of drugs included in the study, it is not practical to have dosed shrimp for every drug. As none of these drugs have been reported to covalently bond to endogenous substances, it is relatively safe to assume that the results from fortified samples are comparable to incurred ones. The dilution of incurred samples close to target level was done by simply combining calculated amount of dosed shrimp meat and drug-free shrimp (both in blended form) in a 50-mL PP centrifuge tube. The mixing of two kinds of unblended meat by blending was not performed due to the limited quantity of incurred samples and the stickiness of pre-blended shrimp tissue upon rising temperature, which prevented homogenous mixing. In addition to these known incurred samples, two unknown shrimp samples purchased from groceries were included. Because blank controls for these two unknown samples were not available to us, the unknown samples were also fortified with known amount of drugs to verify the identity of positive hits and extraction efficiency. As one of them was found ENR-positive, they altogether represent four of the drug classes out of the six.

Bowers shrimp is fortified in two schemes: one set of fortification was done by adding all 18 drugs at three different levels (L1, L2, and L3), respectively; another set was prepared by spiking the shrimp blend with one of the four following drug groups at the L2 level: the six sulfonamides, the six quinolones, the four (leuco)dyes, and OTC/TOLS. The fortified-by-drug-group samples showed that there was no interference among different classes of drugs. Two sets of fortified shrimp extract (in addition to those generated for validation) were sent to another FDA lab in Denver (CO) for inter-lab check. The LC–MS part of the method was slightly modified to adapt to the Finnigan LCQ DECA XP Plus ion trap system. The result is very similar to that obtained in our laboratory.

The arbitrary "5% cut-off rule" for screening was retrospectively checked against validation data. Note that for simplicity in data processing, primary RIC peak height was used instead of peak area integration. The target false positive rate was set at 1% ($N = 44$; 2-tail distribution), and false negative rate was set at 5% ($N = 12$ for L2 level; 2-tail distribution). Since negative samples do not necessarily have a primary RIC recognized at the right t_R, in these cases either zero or the nearest peak was utilized. If these criteria were applied alone, 13 out of the 18 drugs could statistically meet both requirements. Toltrazuril sulfone failed both, possibly because MS^3-level product ions are much less reproducible in intensity. Sulfachloropyridazine and OXO failed the false-positive test due to the high background for primary product ions. Leucogentian violet and GV failed the false-negative test because of either low instrument response or relatively high standard deviation at L2 level. Nonetheless, the joint confirmatory/screening criteria gave satisfactory results, as described before.

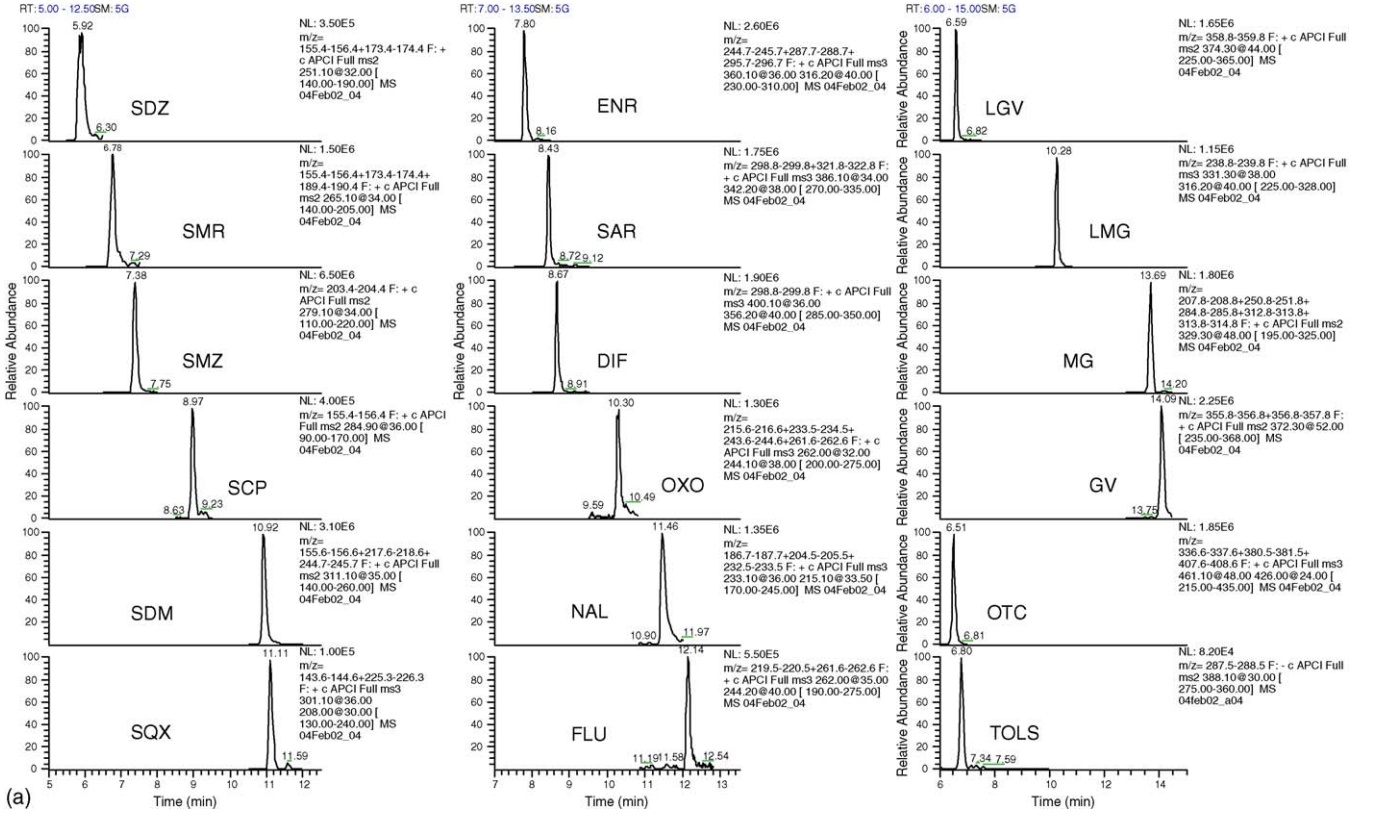


Fig. 4. Reconstituted ion chromatograms of: (a) one extracted sample fortified with all 18 drugs at target levels. The *Y*-scale is set close to 100%-normalization for each drug and (b) one unknown sample. The *Y*-scale for each chromatograph is the same (fixed) as the corresponding one in (a).

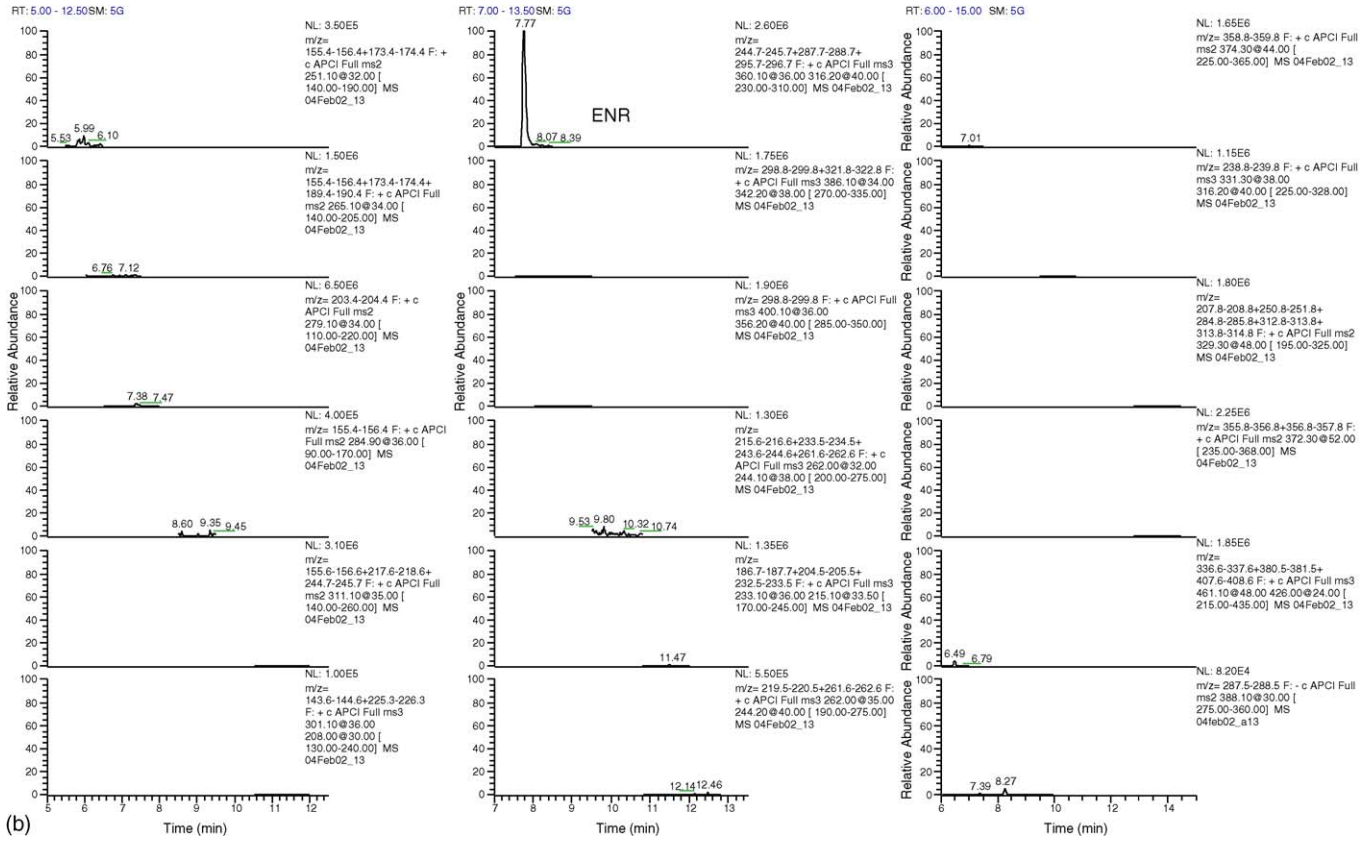


Fig. 4. (*Continued*).

3.6. Throughput

This method is able to process eight unknown samples (plus two controls and two fortified QCs) in about 6 h and the instrument can be set for overnight unattended analysis. It is possible to extract more unknowns within a day if a 24-channel vacuum manifold is used. The sample extraction and cleanup is the most time-consuming step. However, considering that 18 target analytes are screened from one extraction, and the potential that more drugs can be included in the screen list, the per drug per sample throughput rate is a significant improvement over many existing methods. The scanning speed of the quadrupole ion trap mass spectrometer is adequate to analyze the 18 drug in a time-scheduled fashion. A fixed-scale presentation for the RIC of all 18 drugs is shown in Fig. 4a. Fig. 4b gives the result of an unknown BT sample, in which ENR's presence is easily identifiable (about 20 ng/g by semi-quantitation). Thus visual examination of screening can preclude a lot of negative samples prior to the more elaborate confirmatory evaluation.

3.7. Semi-quantitative evaluation

Although this ion-trap based method primarily serves the purpose of screening and confirmation, an estimate of quantity of positive hits can be used for further actions, i.e., a dedicated analysis to obtain the exact concentration, which could help avoid unnecessary work when a drug is only present at lower-than-action level residue. As the response in our LC–MS system normally drifts upwards within a 21-injection-sequence (about 7 h for the 19-min method), possibly due to an accumulation of matrix on LC column, the "weighed average" algorism was applied to adjust for this effect. Therefore, two fortified QC samples must be each put before and after all unknown samples in a sequence to serve as both suitability and semi-quantitative controls.

4. Conclusion

A sensitive, multi-class, high throughput confirmatory/screening method for 18 antibiotic residues in shrimp has been developed and validated. All drugs at respective levels of interest can be confirmed by two injections from the same extract. Positive samples from this procedure should be subject to quantitative analysis. Development of automatic data processing would enhance throughput, especially in wake of the tremendous amount of information generated by the multi-residue method. Considering the overall amount of data and complexity of criteria, the current version of XCalibur provided limited automation in data processing. Software with dynamic library matching algorithm based on "fresh" QCs, coupled with a fixed set of criteria including both LC and MS aspects as afore-mentioned, may greatly shorten the time needed to review results, and reduce discrepancies between analysts. In addition, a clear presentation of result in a user-friendly format is also critical to save time.

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