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Analysis of Veterinary Drugs and Metabolites in Milk Using Quadrupole Time-of-Flight Liquid Chromatography−Mass Spectrometry

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ABSTRACT: A quadrupole time-of-flight (Q-TOF) liquid chromatography−mass spectrometry (LC-MS) method was developed to analyze veterinary drug residues in milk. Milk samples were extracted with acetonitrile. A molecular weight cutoff filter was the only cleanup step in the procedure. Initially, a set of target compounds (including representative sulfonamides, tetracyclines, β-lactams, and macrolides) was used for validation. Screening of residues was accomplished by collecting TOF (MS^1) data and comparing the accurate mass and retention times of found compounds to a database containing information for veterinary drugs. The residues included in the study could be detected in samples fortified at the levels of concern with this procedure 97% of the time. Although the method was intended to be qualitative, an evaluation of the MS data indicated a linear response and acceptable recoveries for a majority of target compounds. In addition, MS/MS data were also generated for the $[M + H]^+$ ions. Product ions for each compound were identified, and their mass accuracy was compared to theoretical values. Finally, incurred milk samples from cows dosed with veterinary drugs, including sulfamethazine, flunixin, cephapirin, or enrofloxacin, were analyzed with Q-TOF LC-MS. In addition to monitoring for the parent residues, several metabolites were detected in these samples by TOF. Proposed identification of these residues could be made by evaluating the MS and MS/MS data. For example, several plausible metabolites of enrofloxacin, some not previously observed in milk, are reported in this study.

KEYWORDS: Milk, quadrupole time-of-flight LC-MS, veterinary drug residues, metabolites

INTRODUCTION

Over the past decade, methods for monitoring veterinary drug residues in foods have changed significantly. Rather than monitoring for a single drug residue, or a specific class of drug residues, multiclass screening procedures with large numbers of analytes are now commonly reported. Generally these methods utilize LC-MS/MS instrumentation. There are several recent examples of methods for drug residues in milk using triple-quadrupole LC-MS/MS with multiple reaction monitoring (MRM) for each compound.[1−5] These methods have focused on detecting residues in milk near the level of concern. Although time-scheduled MRM allows for a large number of compounds to be monitored with excellent selectivity and sensitivity, these procedures detect only a discrete list of target residues.

LC-MS with a time-of-flight (TOF) detector is capable of obtaining high-resolution data. Virtually an unlimited number of compounds can be simultaneously analyzed with this instrument because full-scan data, rather than preselected ion transitions corresponding to specific residues, are collected. Therefore, in addition to looking for target analytes, any number of additional nontargeted compounds may also be detected.[6] Data can also be evaluated retrospectively for possible contaminants. This technique has been applied to monitoring veterinary drug residues in food, including quantification of residues.[7−10] There are at least two methods described for the analysis of drug residues in milk by TOF MS.[11,12] These procedures detected between 100 and 150 residues in milk utilizing LC-MS with TOF detection. Determination of residues was performed by filtering very narrow (±0.01−0.02 Da) ion chromatograms from the MS scan data. In one method,[11] the milk was extracted with acetonitrile followed by a solid-phase extraction cleanup. The other procedure[12] also includes extraction with acetonitrile, but filtration through a molecular weight cutoff filter was the only cleanup step. This idea was first proposed by van Rhijn for the analysis of sulfonamides in milk.[13] The data reported in these papers demonstrate that LC-MS with TOF detection works very well for screening and quantification of drug residues in milk. However, having only MS data for the protonated molecules, even with very accurate mass (±0.01 Da), was not considered suitable for confirmation of residue identity. In some TOF residue methods, in-source fragmentation was performed to generate fragment ions that could also be evaluated to provide more data for compound identification.[7,10,12] Although in-source collisions can generate accurate mass data for fragment ions, the process does not

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Table 1. Screening Data for Milk Samples Fortified at Level of Interest

compd	mol formula	level of interest[a] (ng/mL)	retention time (min)	protonated molecule (m/z)[b]	av mass error[c,d] (Δppm)	av abundance[c,e] (counts)	abundance[e] % RSD
TBZ	$C_{10}H_7N_3S$	50	5.6	202.0433	1.22 ± 0.98	31178	21
SPD	$C_{11}H_{11}N_3SO_2$	10	6.0	250.0644	0.82 ± 0.53	6531	18
SDZ	$C_{10}H_{10}N_4SO_2$	10	5.3	251.0597	1.29 ± 1.00	3288	21
STZ	$C_9H_9N_3S_2O_2$	10	5.9	256.0209	1.28 ± 0.82	3110	26
TRIP	$C_{16}H_{21}N_3$	20	6.9	256.1808	0.65 ± 0.50	65268	18
SMR	$C_{11}H_{12}N_4SO_2$	10	6.3	265.0754	9.09 ± 4.34[f]	9130	41
SMZ	$C_{12}H_{14}N_4SO_2$	10	7.1	279.0910	3.09 ± 2.01	10823	19
SCP	$C_{10}H_9N_4SO_2Cl$	10	8.3	285.0208	0.97 ± 0.46	2878	21
SQX	$C_{14}H_{12}N_4SO_2$	10	10.2	301.0754	1.43 ± 0.76	5063	35
SDM	$C_{12}H_{14}N_4SO_4$	10	10.2	311.0808	1.25 ± 0.66	11345	25
FLU-OH	$C_{14}H_{11}F_3N_2O_3$	2	13.0	313.0795	2.56 ± 2.44	1583	13
CIP	$C_{17}H_{18}FN_3O_3$	5	6.5	332.1405	0.77 ± 0.52	34100	22
PEN G	$C_{16}H_{18}N_2SO_4$	5	7.1	335.1060	1.08 ± 0.94	3509	19
AMP	$C_{16}H_{19}N_3SO_4$	10	5.8	350.1169	0.83 ± 0.46	12684	18
SAR	$C_{20}H_{17}F_2N_3O_3$	5	7.3	386.1311	0.56 ± 0.45	11934	20
CEPH	$C_{17}H_{17}N_3S_2O_6$	20	5.2	424.0631	1.72 ± 0.81	1072	20
CLOX	$C_{19}H_{18}N_3SO_5Cl$	10	13.2	436.0729	1.08 ± 0.69	1303	25
TC	$C_{22}H_{24}N_2O_8$	100	6.6	445.1606	0.58 ± 0.55	13158	22
DC	$C_{22}H_{24}N_2O_8$	100	8.2	445.1606	0.56 ± 0.43	22511	33
OTC	$C_{22}H_{25}N_2O_9$	100	6.3	461.1555	0.57 ± 0.34	12688	31
CTC	$C_{22}H_{23}N_2O_8Cl$	100	7.8	479.1216	0.94 ± 1.17	11153	14
VIR	$C_{28}H_{35}N_3O_7$	100	12.5	526.2548	0.87 ± 0.60	21460	42
ERY-H_2O	$C_{37}H_{67}NO_{12}$	50	10.1	716.4580	0.67 ± 0.62	12056	31
TIL	$C_{46}H_{80}N_2O_{13}$	100	8.2	869.5733	0.36 ± 0.30	48964	28
TYL	$C_{46}H_{77}N_2O_{17}$	50	9.8	916.5264	0.81 ± 0.70	12874	37

[a] Levels of interest (tolerances or safe levels) in milk from FDA/CFSAN.[21] Several drugs do not have tolerances for milk. Method target level was set at 100 ng/mL (tolerance in muscle) for TIL and VIR and at 5 ng/mL for CIP and SAR. For the tetracyclines, the actual tolerance is 300 ng/mL for the sum of residues; the method level of interest was set to 100 ng/mL each. [b] Theoretical value rounded to the fourth decimal place. [c] Average for residues in samples (n = 14) fortified at the level of interest (1X). Data were collected over 8 days in a 3 month period. [d] Average and standard deviation of mass error (absolute value). Mass (not m/z) was used to calculate mass error. [e] Abundance (peak height) of protonated molecule in MS TOF scan. [f] Using *Find by Molecular Feature* and then comparing to the veterinary drug database, the average mass error for SMR was found to be 3.99 ± 5.12 ppm.

provide the additional selectivity or sensitivity that is achieved by tandem mass spectrometry.

A hybrid quadrupole TOF detector (Q-TOF) has the ability to obtain true MS/MS spectra. Because a quadrupole ion filter is used between the source and the collision cell prior to the TOF analyzer, this instrument generates product ions from preselected precursors. This allows for additional structural characterization and analyte identification of target or nontarget analytes at parts per billion levels.[14] There are a few examples of Q-TOF analysis of pesticide metabolites.[15,16] Reports demonstrating the use of Q-TOF LC-MS for veterinary drug residue analysis include the monitoring of β-agonists,[17] quinolone,[18,19] and macrolide[20] residues in various foods. In the paper describing the analysis of macrolide residues, product ion spectra were collected to further characterize a degradation product of tylosin.[20]

In this study, we utilized a Q-TOF LC-MS to screen, quantify, and confirm veterinary drug residues in milk. The method was compared to a triple-quadrupole procedure[1] using 25 target compounds of interest. In addition, the ability to obtain accurate mass full-scan data with the Q-TOF instrument was used to elucidate probable metabolites of these drugs in incurred milk samples.

MATERIALS AND METHODS

Reagents and Consumables. Water, acetonitrile, and formic acid were of LC-MS grade (Fisher Optima, Fair Lawn, NJ). Formic acid solution (0.1% v/v) was prepared by pipetting 1.0 mL of formic acid into a 1000 mL graduated cylinder and diluting to the mark with water. Amicon Ultra centrifugal filters (0.5 mL, 3000 Da molecular weight cutoff) were purchased from Millipore Corp. (Bedford, MA).

Standard Solutions. The target analytes studied in this method were as follows: ampicillin (AMP), penicillin G (PEN G), cloxacillin (CLOX), cephapirin (CEPH), sulfamethazine (SMZ), sulfadiazine (SDZ), sulfadimethoxine (SDM), sulfathiazole (STZ), sulfaquinoxaline (SQX), sulfapyridine (SPD), sulfachloropyridazine (SCP), sulfamerazine (SMR), oxytetracycline (OTC), tetracycline (TC), chlortetracycline (CTC), doxycycline (DC), tylosin (TYL), tilmicosin (TIL), erythromycin (ERY), sarafloxacin (SAR), ciprofloxacin (CIP), 5-hydroxyflunixin (FLU-OH), thiabendazole (TBZ), virginiamycin (VIR), and tripelennamine (TRIP). Standards were obtained from the US Pharmacopeia (USP, Rockville, MD) except for virginiamycin (Sigma Aldrich, St. Louis, MO) and 5-hydroxyflunixin (Schering-Plough, Lafayette, NJ). Stock solutions were prepared in methanol or in water at a concentration of 100 μg mL^{-1} for each residue. Two intermediate mixed standards (one for β-lactams and one for the other compounds) were prepared at concentrations corresponding to 20 times the tolerance or safe levels[21] (listed in Table 1) of

these drug residues in milk. A more detailed procedure for the preparation of these standard solutions has been described previously.[1] For nontarget analysis, standards of pefloxacin and enrofloxacin were also obtained for comparison purposes from Sigma Aldrich.

Sample Preparation. Pasteurized whole milk was purchased from local retail stores for method development and fortification with target analytes. Incurred (raw) milk was obtained from the U.S. Food and Drug Administation's Center for Veterinary Research as cited previously.[1] Milk was aliquoted (1.0 mL) into 15 mL centrifuge tubes and frozen (−20 °C) until needed. For validation of target residue analysis, the store-bought samples were fortified as follows: 25, 50, or 100 μL of each intermediate standard was added to obtain levels of 0.5*X*, 1*X*, or 2*X* (where *X* is the level of interest), respectively. Fortified samples were allowed to sit for approximately 10 min; incurred milk samples were analyzed directly after thawing. To extract, 1.0 mL of acetonitrile was added and the samples were vortex mixed for 20 s and then centrifuged at 3250 rcf (*g*) and 4 °C for 10 min. One milliliter of the resulting supernatant (avoiding any visible fat layer) was transferred to a 16 × 150 mm disposable glass tube containing 0.5 mL of 0.1% formic acid. The solvent was evaporated in a water bath set at 40 °C using nitrogen (10−15 psi) until the volume was slightly less than 0.5 mL. Sample cleanup was performed with a 3000 Da molecular weight cutoff centrifuge filter. The filters were first prewashed by adding 0.5 mL of 0.1% formic acid to the filters and centrifuging at 17000 rcf (*g*) for 5 min. This wash was discarded, and the extracts were added to the cutoff filters. The volume was adjusted to 0.5 mL with 0.1% formic acid as needed. The samples were then centrifuged at 17000 rcf (*g*) for 15 min. The filtered extracts were transferred to LC vials (polypropylene with conical insert for low volume) for analysis.

Instrumentation. The instrument was an Agilent (Santa Clara, CA) quadrupole time-of-flight (Q-TOF) 6530 mass spectrometer coupled to an Agilent 1290 liquid chromatograph. Electrospray ionization with Agilent Jet Stream Technology was utilized. Source parameters were optimized by making multiple injections of a solvent standard mixture containing the target analytes while incremental changes in instrument values were made. These final parameters were chosen on the basis of the best response for most compounds: fragmentor (150 V), nozzle (250 V), Vcap (4000 V), nebulizer (40 psig), drying gas (N_2, 8 L/min, 325 °C), sheath gas (N_2, 11 L/min, 350 °C). The time-of-flight MS was calibrated daily according to the manufacter's recommendations. Typical resolution values obtained during calibration were 9000 (*m*/*z* 322) and 15000 (*m*/*z* 1522) fwhm. The instrument was operated in the high-resolution (4 GHz), lower mass range (<*m*/*z* 1700) positive ion mode. Data were collected in both centroid and profile formats. The TOF was scanned from *m*/*z* 100 to 1200 at a rate of 1.08 spectra/s. Reference masses at *m*/*z* 121.05087 (purine) and *m*/*z* 922.00980 (hexakis(1*H*,1*H*,3*H*-tetrafluoropropoxy)-phosphazine) were continually introduced along with the LC stream for accurate mass calibration. A separate method was used for MS/MS analysis (the sample extract was reinjected). For the latter method, a target list of the analytes of interest was generated with the precursor ion (protonated molecule), the retention time (±0.5 min), isolation width (4 *m*/*z*) and time (200 ms/spectrum), and the collision energy. The collision energy for each compound varied according to this formula: [3 × (mass/100)] + 10. For example, the collision energy for an ion with nominal *m*/*z* of 300 would be 19 V.

The LC program was the same as has been reported for the analysis of drug residues in milk.[1,2] The LC column was a YMC ODS-AQ, 120 Å, 2 × 100 mm, 3 μm (Waters Corp., Milford, MA). A mobile phase gradient (0.1% formic acid and acetonitrile) was used with a flow rate of 250 μL/min. The initial solvent composition was 95% aqueous for 2 min, followed by a linear gradient over 10 min to 50:50 (v/v) 0.1% formic/ACN. This 50:50 ratio was held for 1 min, followed by a second linear gradient to 100% ACN in 3 min. The column was held at 100% ACN for 2 min, then allowed to return (0.5 min) to the initial gradient conditions (95:5 (v/v) 0.1% formic acid/ACN). A postrun re-equilibration step (3 min) was added prior to the next injection. The column oven (35 °C) and autosampler tray (4 °C) temperatures were controlled. Injection volume was 10 μL, followed by a 3 s needle wash with 50:50 (v/v) water/methanol.

Data Analysis. For target compounds, the TOF data were evaluated against a database compiled in-house that contained approximately 200 compounds known for their possible veterinary drug application. Retention times for the current chromatographic conditions had been determined for approximately one-third of these drugs, including all of the target residues in the validation study. Using Agilent's MassHunter *Find by Formula* algorithm, the data files were compared to the database using search windows of 10 ppm mass accuracy and a 0.5 min retention time for ions corresponding to the $[M + H]^+$ ion. Reports were generated that contained mass accuracy errors obtained by comparing experimental data to potential matching compounds in the database. The MS/MS data were processed by manually extracting narrow window (10 ppm) product ion scans. The extracted ion chromatograms for two structurally important product ions were summed together. Product ion spectra from the resulting extracted ion chromatograms were evaluated by comparing mass assignments to theoretical values and the ion ratios to those from compounds in a fortified (1*X* tolerance) milk sample. Quantification was performed using Agilent's *Q-Tof Quantitative Analysis* program. Extracted ion chromatograms were generated from $[M + H]^+$ ions in the MS^1 data using a 10 ppm mass window. Matrix-matched calibrations standards were prepared by extracting control milk and fortifying with intermediate mixed standards at levels corresponding to 0.1*X*, 0.25*X*, 0.5*X*, 1*X*, and 2*X* (where *X* is the level of concern) immediately before the filtration step.

To detect the presence of nontarget drugs or metabolites, the *Find by Formula* routine could be used without the retention time requirement. In addition, the MassHunter *Find by Molecular Feature* program could detect and assign mass values to ions that appear as unique chromatographic peaks. The *Find by Molecular Feature* program could be used to detect target and nontarget compounds. The number of compounds generated by this procedure depends greatly on the abundance threshold values allowed. Careful comparison between unknown and control samples can give an indication of which found compounds may be worthy of further investigation.

RESULTS AND DISCUSSION

Validation with Target Analytes. Sets of milk samples fortified with 25 target analytes at or near their level of concern were analyzed to determine method performance for screening, quantification, and identification of residues. The extraction procedure was simplified significantly as compared to the method developed and validated previously for a triple-quadrupole LC-MS/MS.[1] Solid-phase extraction was not utilized; only a molecular weight cutoff filter (3000 Da) was used to clean up the acetonitrile extracts as described by others.[12,13] With this approach, the background from milk matrix, as evaluated by the baseline noise levels observed in the total ion chromatograms, was not significantly different when the SPE was eliminated from the procedure. This is consistent with the results reported by Ortelli et al.[12] Steady MS signal response and LC back pressure indicated that instrument performance was not compromised by this rapid sample preparation. Recoveries of residues and matrix effects observed with this method are discussed below. Although UHPLC would also be a viable option, the chromatography was not modified from the original methods to facilitate rapid method transfer and comparison.

Screening, or the ability to quickly ascertain if a residue is present near the level of interest, was the primary objective of this method. Data obtained by TOF LC-MS can be rapidly evaluated

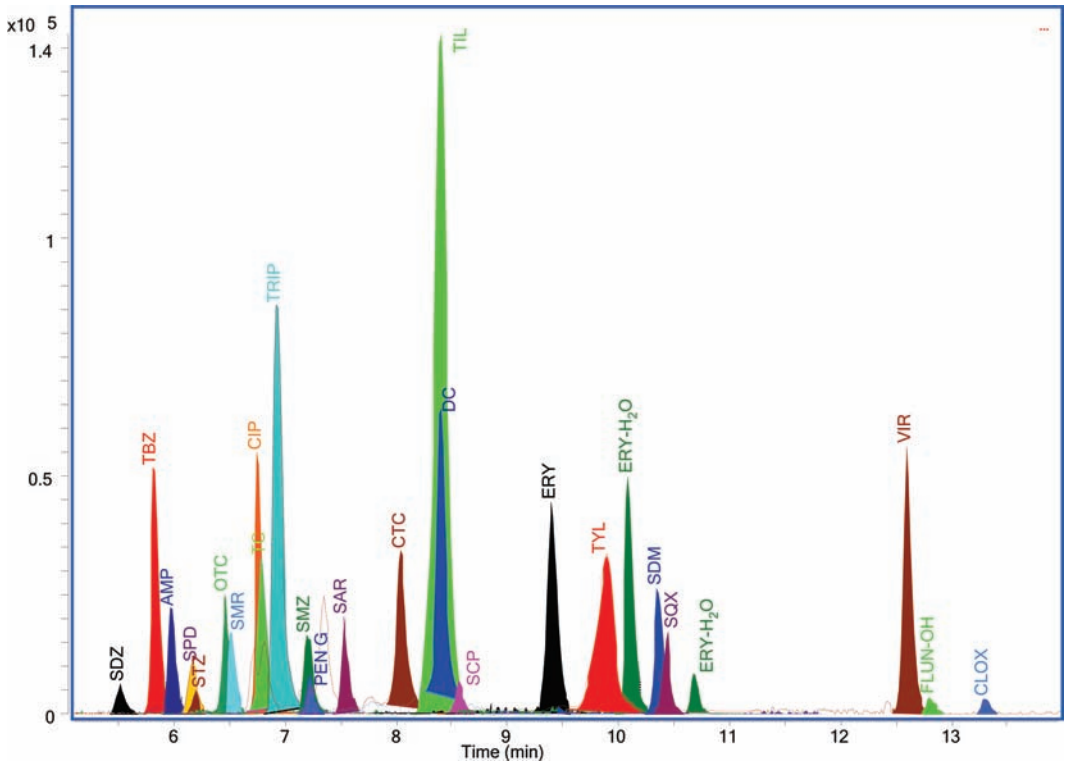

Figure 1. Overlaid extracted ion chromatograms for $[M + H]^+$ ions of target compounds in a milk sample fortified at the levels of concern. The mass window for extraction was set to 10 ppm.

for the presence of residues by comparing found compounds in a chromatogram to a veterinary drug database using accurate mass and retention time values. The utility and limits of this approach were tested with the target analytes. Screening data for milk samples fortified with residues at 1*X* (where *X* is the level of concern in ng/mL) are shown in Table 1 and 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 initially set to be ≤5 ppm with a retention time window of 0.5 min. In general, the mass error calculated for residues in the validation set data was <2 ppm for these compounds in milk extracts. One exception was sulfamerazine (SMR); this residue consistently had a mass error of −5 to −10 ppm in the milk matrix. This may be due to matrix interference, because an ion at m/z 265.0564 was observed in control milk samples close to (0.1 min earlier) the retention time of SMR ($[M + H]^+ = m/z$ 265.0754). A mass resolution approaching the limits of this instrumentation (approximately 14000) would be needed to separate these two coeluting compounds. The mass allowance for database matching in *Find by Formula* was changed to ±10 ppm to include results for SMR. Interestingly, when compounds were first detected using *Find by Molecular Feature* and then compared to the veterinary drug database, the mass error for SMR was found to be below 5 ppm. The difference for the calculated mass error for other compounds in milk fortified at the 1*X* level when the two software programs were compared was negligible (<0.5 ppm). It is possible that the algorithm utilized in the *Find by Molecular Feature* better compensates for coeluting interferences with very similar masses.

With these criteria, all of the target residues were consistently detected in samples fortified at the level of concern. For samples fortified at 1*X* (n = 350, 14 samples with 25 residues each) the number of false negatives was quite low (total of 4 residues, or approximately 1%) and was limited to CEPH (n = 1) and FLU-OH (n = 3). Over 80% of the target drugs were detected in milk samples fortified at half the level of interest (0.5*X*) or lower. The limits of detection, defined as the lowest fortification level at which a residue is found by comparison to the database with an acceptable (>3:1) signal-to-noise ratio for the peak in the extracted ion chromatogram, are shown in Table 2. The number of false positives found with this algorithm, using both mass (±10 ppm) and retention time (±0.5 min) requirements, was also reasonable. Typically, two or three compounds were "found" with the correct mass assignment and retention time, but the abundances for these compounds were less than a few hundred counts and the signal-to-noise ratio for the extracted ion chromatograms was unacceptable (<3:1). As has been observed previously,[1,20,22] some residues degrade in the dilute formic acid that was used as the final extract solution. Penillic acid, an isobaric analogue of penicillin G with a shorter retention time, was observed. In addition to the parent compound, two dehydrated degradation products of erythromycin were detected, a large peak at 10.1 min and a smaller one at 10.7 min.

Another important aspect of screening methods is the ability to quickly determine if the concentration of a detected residue is near the level of interest[1,3] by setting a threshold level. In our previous study utilizing SPE and triple-quadrupole LC-MS/MS, the signal response variability for samples fortified at the 1*X* level was used to set a threshold. In that case, for residues to be detected with 95% confidence at the level of concern (1*X*), the signal observed needed to be ≥50% of that from a positive control (1*X* milk extract) analyzed that day.[1] The data obtained with this rapid extraction Q-TOF LC-MS method show similar results in terms of variability. In fact, the relative standard deviations of the

Table 2. Limits of Detection and Identification for Target Compounds in Fortified Milk

	LOD[a]		LOI[b]	
compd	compared to 1X[c]	concn (ng/mL)	compared to 1X[c]	concn (ng/mL)
TBZ	0.1X	5	0.1X	5
SPD	0.5X	5	1X	10
SDZ	0.25X	2.5	2X	20
STZ	0.5X	5	2X	20
TRIP	<0.1X	<2	<0.1X	<2
SMR	1X	10	2X	20
SMZ	0.5X	5	0.5X	5
SCP	0.25X	2.5	0.5X	5
SQX	0.25X	2.5	0.5X	5
SDM	0.25X	2.5	0.5X	5
FLU-OH	1X	2	>2X	>4
CIP	0.1X	0.5	1X	5
PEN G	0.1X	0.5	1X	5
AMP	0.1X	1	0.5X	5
SAR	0.25X	1.25	0.5X	2.5
CEPH	1X	20	>2X	>40
CLOX	1X	10	0.5X	5
TC	0.1X	10	0.1X	10
DC	0.1X	10	0.25X	25
OTC	0.1X	10	0.25X	25
CTC	0.1X	10	0.1X	10
VIR	0.1X	10	0.5X	50
ERY-H_2O	0.1X	5	0.1X	5
TIL	0.1X	10	0.5X	50
TYL	0.5X	2.5	0.5X	2.5

[a] Limit of detection = lowest fortified milk sample at which residue is detected using MassHunter *Find by Formula* compared to database with mass (±10 ppm) and retention time (±0.5 min). The signal-to-noise ratio for the peak in the extracted ion chromatograms must also be >3:1. [b] Limit of identification = lowest level at which predominant product ions are observed above the noise in the MS/MS spectra with accurate mass within 10 ppm of theoretical value. [c] 1X = level of interest as defined in Table 1.

measured peak abundances (heights) for the $[M + H]^+$ ions of a majority residues was <30% for multiple days across several months. These data are included in the screening data shown in Table 1. There were residues that showed greater (30−42%) variability in their response. In general, however, the threshold standard set for presumptive positives previously (a response ≥50% of positive control at 1X level) should be adequate for screening with this method as well. Using this criterion, 97% of drugs fortified in milk at the 1X level (n = 14 replicates × 25 residues = 350) would be considered presumptive positives. In addition, 99% of residues added at the 2X levels (3 replicate samples) met this criteria, whereas approximately 34% of compounds fortified in milk at the 0.5X level (6 replicate samples) would be considered presumptive positives.

The Q-TOF LC-MS could also confirm the identity of residues. This was achieved by collecting and evaluating product ion spectra. The FDA's Center for Veterinary Medicine's guidance[23] for confirming identity from MS^n scan data states that a spectrum should "visually match the spectrum obtained from a contemporaneous standard". Using Q-TOF data, mass assignments of the product ions can also be compared to theoretical values for increased confidence. Table 3 shows example data for the identification of residues in a milk sample fortified with the target analytes at the level of interest. Theoretical m/z values for product ions (as protonated species) were calculated using postulated fragmentation patterns from the literature.[18,20,24−31] When experimental accurate mass values were compared to those calculated from the molecular formulas of these ions, the mass error observed was somewhat larger (average Δppm = 4.3) than what was observed for MS^1 ions. The mass accuracy was sufficient, however, to confirm the identity of these products and also provided additional clarification of the fragment ions for some compounds. For example, Vartanian et al. characterized the product ions of tetracylines using an ion trap MS.[30] At that time, it was not possible to distinguish whether the ion at nominal m/z 154 corresponded to $C_8H_{12}NO_2^+$ (m/z 154.0863) or $C_7H_8NO_3^+$ (m/z 154.0499). The data obtained in this study for that ion (m/z 154.0495) indicate that the latter is the correct assignment. It was also possible to resolve the two product ions from SDM at a nominal mass of m/z 156 corresponding to $C_6H_6NO_2S^+$ (m/z 156.0114) and $C_6H_{10}N_3O_2^+$ (m/z 156.0768), which has been observed by others.[32]

In addition to mass accuracy and retention time, the relative abundances of the product ions in the MS/MS spectra should match that of a known compound as described in published guidelines.[23] An example of ampicillin residue in a violative sample compared to a matrix-extracted milk standard is shown in Figure 2. Comparison of these spectra reveals that the relative abundances of the product ions in the MS/MS scan match within 20% of those obtained from matrix matched standards. Having accurate mass data for these product ions provides additional information that can be evaluated when the validity of compound identification is determined. As has been reported previously,[18] the strength of residue identification using product ion spectra with accurate mass data can also be calculated using the European Union identification point system.[33]

In general, the target drugs could be identified in milk extracts at their level of concern or below. For this study, the limit of identification was defined as the lowest fortification level where MS/MS spectra (at the correct retention times) visually matched a known standard and the predominant product ions were observed with an accurate mass within 15 ppm of the theoretical. Limits of identification for the target residues are also listed in Table 2. A few compounds could not be identified unless the fortification level was at least twice the level of concern (2X). These include FLU-OH, CEPH, SMR, SDZ, and STZ. For most residues evaluated with this method, the levels of identification were 5−10 ng/mL, or approximately twice the limits of detection defined earlier for MS^1 data.

Although the intended purpose of this method was more qualitative (screening and identification), the ability of this procedure to provide quantitative results was also investigated. Table 4 lists recoveries for residues fortified in milk at 0.5X, 1X, and 2X using matrix-matched calibration standards. Good linearity was observed for all compounds with R^2 values of ≥0.995. Recoveries for 18 of the drugs was adequate in the range of 60−95%. Recoveries for other residues (STZ, FLU-OH, ERY-H_2O, TC, OTC, and CTC) were 40−60%; SCP had 154% recovery. Even though erythromycin can degrade into several components, only the predominant peak (ERY-H_2O at 10.1 min) was measured. When compared to the previous method,[2] the recoveries for

Table 3. Example of Identification of Residues in Milk Sample Fortified at Level of Interest by MS/MS

compd	precursor ion $(m/z)^a$	collision energy (V)	product ion formula	product ions $(m/z)^a$	product ions measured $(m/z)^b$	Δppm^c	ions above noise?	ref
TBZ	202.0433	16	$C_{10}H_8N_3S^+$	202.0433	202.0427	3.22	yes	24
			$C_9H_7N_2S^+$	175.0325	175.0316	5.07	yes	
SPD	250.0644	17	$C_{11}H_{10}N_3^+$	184.0869	184.0881	6.24	yes	25
			$C_6H_6NSO_2^+$	156.0114	156.0109	2.95	yes	
SDZ	251.0597	17	$C_6H_6NSO_2^+$	156.0114	156.0087	16.9	yes	25
			$C_6H_6NO^+$	108.0444	108.0445	1.21	yes	
STZ	256.0209	18	$C_6H_6NSO_2^+$	156.0114	156.0132	11.55	no	25
			$C_6H_6NO^+$	108.0444	108.0442	1.71	no	
TRIP	256.1808	18	$C_{14}H_{15}N_2^+$	211.1230	211.1225	2.18	yes	26
			$C_7H_7^+$	91.0542	91.0543	1.27	yes	
SMR	265.0754	18	$C_6H_6NSO_2^+$	156.0114	156.0110	4.34	yes	25
			$C_6H_6N^+$	92.0495	92.0489	6.61	yes	
SMZ	279.0910	18	$C_6H_8N_3SO_2^+$	186.0332	186.0329	1.32	yes	25
			$C_6H_6NSO_2^+$	156.0114	156.0114	0.3	yes	
SCP	285.0208	18	$C_6H_6NSO_2^+$	156.0114	156.0113	0.54	yes	25
			$C_6H_6N^+$	92.0495	92.0507	13.25	yes	
SQX	301.0754	19	$C_6H_6NSO_2^+$	156.0114	156.0116	1.34	yes	25
			$C_6H_6NO^+$	108.0444	108.0443	0.71	yes	
SDM	311.0808	19	$C_6H_6NSO_2^+$	156.0114	156.0113	0.34	yes	25, 32
			$C_6H_{10}N_3O_2^+$	156.0768	156.0762	3.28	yes	
			$C_6H_6NO^+$	108.0444	108.0441	2.35	yes	
FLU-OH	313.0795	19	$C_{14}H_{10}F_3N_2O_2^+$	295.0689	295.1470	>20	no	27
			$C_{13}H_7F_3N_2O_2^+$	280.0454	ND	NA	no	
PEN G	335.1060	20	$C_{15}H_{17}N_2SO_2^+$	289.1005	289.1022	5.66	yes	28
			$C_{10}H_{10}NO_2^+$	176.0706	176.0686	11.43	yes	
AMP	350.1169	20	$C_{10}H_8NO_2^+$	174.0550	174.0543	3.73	yes	28
			$C_7H_8N^+$	106.0651	106.0643	7.77	yes	
SAR	386.1311	21	$C_{20}H_{16}F_2N_3O_2^+$	368.1205	368.1210	1.29	yes	18
			$C_{19}H_{18}F_2N_3O^+$	342.1413	342.1412	0.35	yes	
CEPH	424.0631	23	$C_{13}H_{14}N_3S_2O^+$	292.0573	292.0598	8.54	no	28
			$C_7H_6NSO^+$	152.0165	152.0158	4.16	no	
CLOX	436.0729	23	$C_{13}H_{10}N_2O_3Cl^+$	277.0375	277.0379	1.75	yes	28
			$C_6H_{10}SNO_2^+$	160.0427	160.0438	6.76	yes	
TC	445.1606	23	$C_{22}H_{20}NO_7^+$	410.1234	410.1247	3.1	yes	29, 30
			$C_7H_8NO_3^+$	154.0499	154.0492	4.64	yes	
DC	445.1606	23	$C_{22}H_{22}NO_8^+$	428.1340	428.1342	0.47	yes	29, 30
			$C_7H_8NO_3^+$	154.0499	154.0497	1.17	yes	

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Table 3. Continued

compd	precursor ion $(m/z)^a$	collision energy (V)	product ion formula	product ions $(m/z)^a$	product ions measured $(m/z)^b$	Δppm[c]	ions above noise?	ref
OTC	461.1555	23	$C_{22}H_{20}NO_8^+$	426.1183	426.1187	0.86	yes	29, 30
			$C_7H_8NO_3^+$	154.0499	154.0495	2.56	yes	
CTC	479.1216	24	$C_{22}H_{21}NO_8Cl^+$	462.0950	462.0981	6.61	yes	29, 30
			$C_{22}H_{19}NO_7Cl^+$	444.0845	444.0889	9.68	yes	
VIR	526.2548	26	$C_{28}H_{34}N_3O_6^+$	508.2442	508.2404	7.56	yes	31
			$C_{19}H_{17}N_2O_4^+$	337.1183	337.1168	4.27	yes	
ERY-H_2O	716.4580	31	$C_{29}H_{48}NO_7^+$	522.3425	522.3413	2.67	yes	20
			$C_8H_{16}NO_2^+$	158.1176	158.1165	6.96	yes	
TIL	869.5733	36	$C_{38}H_{66}NO_{10}^+$	696.4681	696.4700	2.1	yes	20
			$C_8H_{16}NO_3^+$	174.1125	174.1109	9.03	yes	
TYL	916.5264	37	$C_{39}H_{66}NO_{14}^+$	722.4478	722.4424	7.28	yes	20
			$C_8H_{16}NO_3^+$	174.1125	174.1116	5.13	yes	

[a] Theoretical value rounded to the fourth decimal place. Assumed ions are protonated. [b] Measured value rounded to the fourth decimal place. [c] Mass (not m/z) was used by software to calculate the absolute mass error.

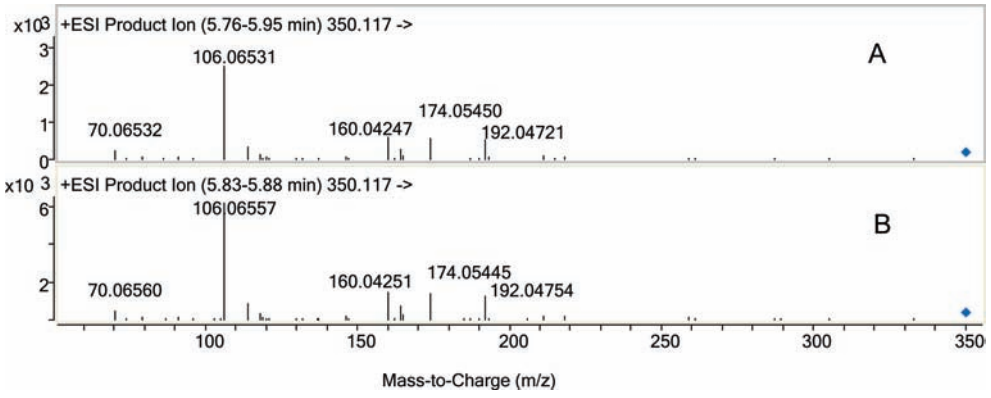

Figure 2. Product ion spectra of ampicillin residues from (A) milk fortified at 20 ng/mL (2X) and (B) violative milk sample.

many residues were similar (within 20%). The results for the β-lactams, however, were considerably better (~80% in this method compared to <50% previously), and recoveries for other residues, including tetracyclines, were 30−40% lower. The relative standard deviations for the recovery values are also listed in Table 4 and are acceptable for a semiquantitative method with values of <20% for 18 of the 25 residues. Some residues had higher RSDs due to the low response (CLOX) or degradation of compounds in acidic solution and/or matrix effects. Comparing solvent and matrix-matched (postfortified) standards provided a method to determine whether matrix effects were more significant for some compounds than for others (Table 4). Many compounds exhibited 10−40% ion suppression with more severe effects observed for the larger, later eluting, macrolide residues. A few sulfonamides, SCP and STZ, had enhanced signal in the presence of matrix. Bacitracin, a small peptide antibiotic, was included in the triple-quadupole methods,[1,2] but its response was extremely variable (RSD ~ 90%) with this method. It is possible that the large molecule (1422 Da) may not reproducibly pass through the 3000 Da molecular weight cutoff filter. Although this procedure could still screen for bacitracin at very high levels, data for this compound were not included in the validation study.

Overall, the results from validation with target compounds indicated that this method is suitable for the monitoring of drug residues in milk. Although the triple-quadrupole method using MRM for each compound generally gave lower limits of confirmation,[2] the estimated difference for all but one of the residues was less than an order of magnitude. The sensitivity for sulfamerazine was >20 times better for the triple-quadrupole method as compared to the TOF; this is most likely related to the difficulty in assigning accurate mass for this residue as discussed above. These results are consistent with other studies that have made similar comparisons,[10,20] and the Q-TOF method limits of detection are at or below the levels of concern for these drugs in milk. Elimination of the SPE step in the extraction allows for rapid preparation of extracts and higher throughput of samples.

Characterization of Metabolites. The Q-TOF can also screen for nontargeted compounds, thereby increasing the monitoring capability. Samples of milk from healthy cows dosed with various drugs had been generated previously.[1] In this study, nine individual cows were given a single dose of a veterinary drug (SDM, SMZ,

Table 4. Quantitative Data for Fortified Milk Samples

compd	level of interest (X)[a] (ng/mL)	matrix-matched calibration curve data[b]	recovery[c] (%)	RSD[c] (%)	matrix effect[d] (%)
TBZ	50	$y = 484702x - 16692$	69	9	−35
SPD	10	$y = 89652x + 1126$	70	9	−33
SDZ	10	$y = 51898x - 1996$	72	9	−18
STZ	10	$y = 38251x - 816$	54	9	88
TRIP	20	$y = 964361x - 3923$	64	9	−30
SMR	10	$y = 129456x + 1021$	80	16	−4
SMZ	10	$y = 150162x - 2460$	76	14	−15
SCP	10	$y = 30773x + 57012$	154	30	25
SQX	10	$y = 89701x + 1843$	78	33	−41
SDM	10	$y = 196309x + 4630$	72	17	−21
FLU-OH	2	$y = 51333x + 5122$	48	16	−37
CIP	5	$y = 469506x - 2255$	69	14	−8
PEN G	5	$y = 62132x - 2475$	80	8	−50
AMP	10	$y = 171926x - 3630$	79	5	−37
SAR	5	$y = 187590x + 1910$	61	8	−24
CEPH	20	$y = 11783x - 1395$	88	17	−30
CLOX	10	$y = 20265x - 697$	80	33	−16
TC	100	$y = 443621x + 11579$	49	8	−10
DC	100	$y = 809584x - 3727$	61	16	−10
OTC	100	$y = 281282x - 11216$	58	13	−15
CTC	100	$y = 454504x - 6489$	42	11	−13
VIR	100	$y = 335258x - 8582$	77	29	−38
ERY-H_2O	50	$y = 502494x + 3533$	47	27	−72
TIL	100	$y = 226226x - 11002$	95	28	−73
TYL	50	$y = 217167x - 3028$	80	33	−80
av			72	17	

[a] See Table 1 for explanation. [b] Curves based on matrix-matched standards at 0.1X, 0.25X, 0.5X, 1X, and 2X. [c] For set of milk samples fortified at 0.5X, 1X, and 2X (n = 4−6). [d] Ion suppression or enhancement based on concentration of 1X matrix-matched standard concentraton calculated from solvent based standard curve.

FLU, OTC, ENR, CEPH, PEN G, CLOX, or AMP). Milk was initially collected 8 h after dosing followed by collections at 8−16 h time intervals. These samples were analyzed with the Q-TOF, and the results were consistent with those obtained using the triple-quadrupole method[1] (data not shown). In addition to monitoring for the parent compounds, analysis using the Q-TOF enables the characterization of other metabolites and/or degradation products of these drugs. As an example, additional compounds were detected and further characterized in the milk from cows that had been dosed with either SMZ, CEPH, FLU, or ENR.

Sulfamethazine-Incurred Milk. Whereas the parent compound is the predominant residue in milk incurred with SMZ, previous work has reported the presence of other metabolites.[34,35] In the early 1990s, Paulson et al. completed a thorough study of the metabolism of SMZ in lactating dairy cows. Two of the predominant metabolites found in this study were the N^4-acetyl metabolite and the lactose conjugate of SMZ. In our study, milk collected from a cow that had been dosed with SMZ (100 mg/kg intravenously) was analyzed with the Q-TOF LC-MS method. The parent compound was found in milk samples collected from 8 to 144 h after administration. Additional information could then be obtained by specifically extracting ions corresponding to the metabolites found in the earlier studies. By extracting for ions at m/z 321.1016 ($C_{14}H_{17}N_4SO_3^+$) and 603.1967 ($C_{24}H_{35}N_4SO_{12}^+$) with a ±10 ppm window, the compounds corresponding to the acetylated and lactose metabolites were detected in milk samples up to 56 h after collection. Figure 3A shows the extracted ion chromatogram for the parent compound, as well as for ions matching the acetyl metabolite and lactose conjugate in milk collected after 24 h of withdrawal. Product ion spectra were also subsequently collected, and these data are shown in Figure 3B. The predominant product ions in the MS/MS spectra of the proposed SMZ−lactose conjugate corresponded to loss of sugar molecule at m/z 441.1439 ($C_{18}H_{25}N_4SO_7^+$, Δppm = 0.36), the N^4-acetyl molecule 321.1016 (Δppm = 0.44), and SMZ at m/z 279.0910 (Δppm = −0.44), as well as fragments of SMZ. The MS/MS of the ion corresponding to the acetyl metabolite showed product ions at m/z 255.1240 ($C_{14}H_{15}N_4O^+$, Δppm = −1.93) and 186.0332 ($C_6H_8N_3SO_2^+$, Δppm = 3.07). The signal abundance of the ions corresponding to the N^4-acetyl and lactose metabolites were 7 and 18% of the protonated SMZ, respectively, in the milk collected 24 h after dosing. These compounds were not found in control milk. Other possible metabolites, including the lactose conjugate of the N^4-acetyl derivative were not detected. An analysis of the compounds in the incurred milk using the *Find by Molecular Feature* software program, compared to those found in milk collected from this cow prior to administration of SMZ, did not detect any other unknown compounds of significant abundance. This example illustrates the capability of the Q-TOF instrument to obtain a more complete metabolic profile of veterinary drugs without the need for extensive radiolabeled studies.

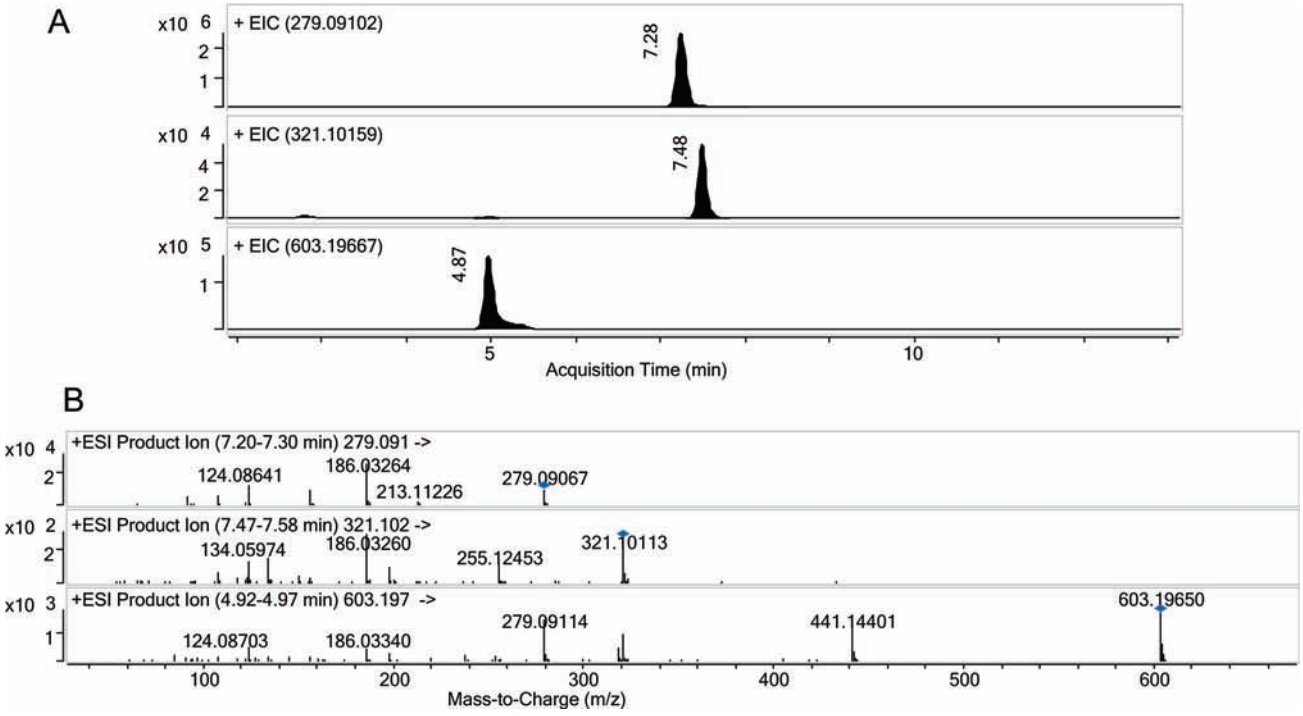

Figure 3. (A) Extracted ion chromatograms (±10 ppm) for $[M + H]^+$ ion of sulfamethazine (m/z 279.09102) and proposed N^4-acetyl (m/z 321.1016) and lactose (m/z 603.19650) metabolites. (B) Product ion spectra for these compounds.

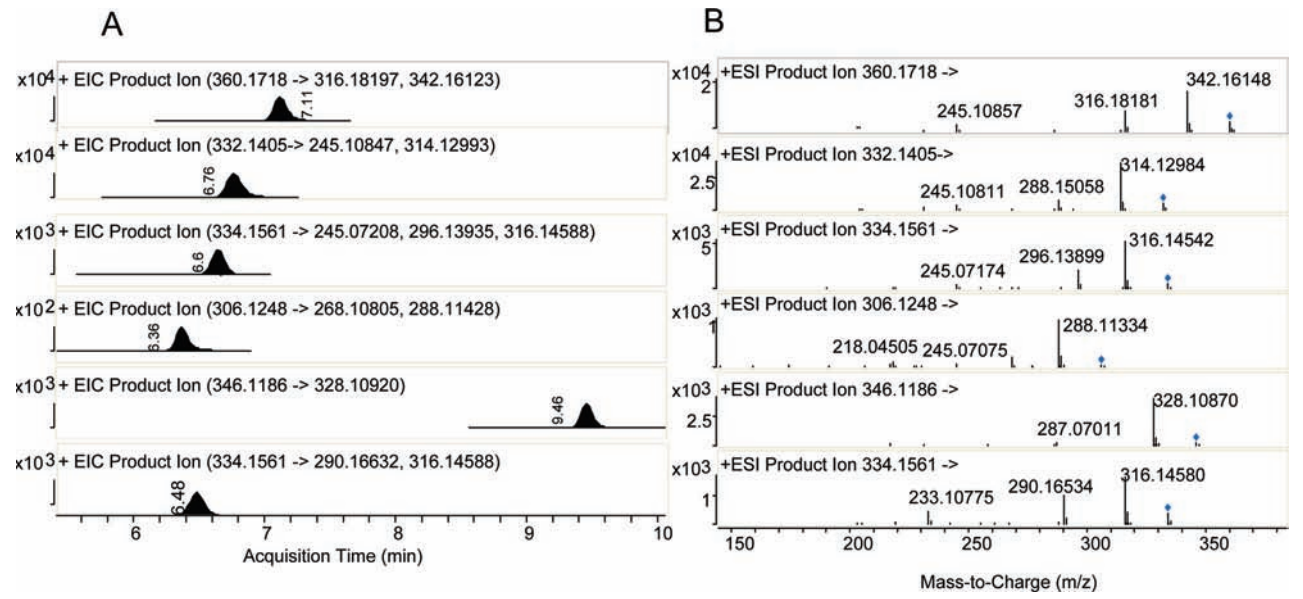

Figure 4. (A) Extracted MS/MS ion chromatograms for compounds in an incurred enrofloxacin milk sample. From the top, traces for enrofloxacin, ciprofloxacin, and the proposed metabolites desethylene enrofloxacin, desciprofloxacin, and oxociprofloxacin are shown. For comparison, the bottom MS/MS chromatogram is for a standard of pefloxacin. (B) Product ion spectra for these compounds.

Cephapirin-Incurred Milk. Cephapirin is a member of the cephalosporin family, a second generation of β-lactam drugs. Desacetyl cephaprin (DAC) is a known degradant[36] and metabolite[37] of CEPH. When milk samples that had been dosed with CEPH (200 mg/quarter intramammary) were analyzed after an 8 h withdrawal, the parent compound was initially found at very high levels (>15000 ng/mL when compared to 1*X* matrix-extracted standard) and then decreased rapidly after that. This is consistent with our previously reported data on these samples.[1] If scan data were then compared to the compound database using only mass (not retention time) matching requirements, DAC was found at levels comparable to those of the parent compound. However, DAC was also found with similar or greater abundance than CEPH in extracts of fortified milk samples, and even solvent standards, that contained CEPH, so although this compound is present at appreciable levels, it cannot be determined how much might be due to metabolism in the animal as compared to simple degradation of the parent compound. In addition,

Table 5. Product Ions Observed for Metabolites Found in Milk from Cow Dosed with Enrofloxacin

compd	mol formula	precursor ion $(m/z)^a$	product ion formula	corresponds to loss of	product ions $(m/z)^a$	product ions measured $(m/z)^b$	Δppm^c
ENR	$C_{19}H_{22}FN_3O_3$	360.1718	$C_{19}H_{21}FN_3O_2^+$	H_2O	342.1612	342.1615	0.72
			$C_{18}H_{23}FN_3O^+$	CO_2	316.1820	316.1818	0.51
			$C_{14}H_{14}FN_2O^+$	$C_5H_9NO_2$	245.1085	245.1086	0.41
CIP	$C_{17}H_{18}FN_3O_3$	332.1405	$C_{17}H_{17}FN_3O_2^+$	H_2O	314.1299	314.1298	0.29
			$C_{16}H_{19}FN_3O^+$	CO_2	288.1507	288.1506	0.29
			$C_{14}H_{14}FN_2O^+$	$C_3H_5NO_2$	245.1085	245.1081	1.46
Des-ENR	$C_{17}H_{20}FN_3O_3$	334.1562	$C_{17}H_{19}FN_3O_2^+$	H_2O	316.1456	316.1454	0.55
			$C_{17}H_{18}N_3O_2^+$	$H_2O + HF$	296.1394	296.1390	1.29
			$C_{13}H_{10}FN_2O_2^+$	$C_4H_{10}NO$	245.0721	245.0717	1.55
Des-CIP	$C_{15}H_{16}FN_3O_3$	306.1249	$C_{15}H_{15}FN_3O_2^+$	H_2O	288.1143	288.1133	3.19
			$C_{15}H_{14}N_3O_2^+$	$H_2O + HF$	268.1081	268.1066	5.46
Oxo-CIP	$C_{17}H_{16}FN_3O_4$	346.1190	$C_{17}H_{15}FN_3O_3^+$	H_2O	328.1092	328.1087	1.51
PEF	$C_{17}H_{20}FN_3O_3$	334.1562	$C_{17}H_{19}FN_3O_2^+$	H_2O	316.1456	316.1458	0.70
			$C_{16}H_{21}FN_3O^+$	CO_2	290.1663	290.1653	3.30
			$C_{13}H_{14}FN_2O^+$	$C_4H_7NO_2$	233.1085	233.1078	2.93

^{a} Theoretical value rounded to the fourth decimal place. Assumed ions are protonated. ^{b} Measured value rounded to the fourth decimal place. ^{c} Mass (not m/z) was used by software to calculate the mass error.

standards for DAC are not available, so it may not be an ideal marker compound for the misuse of cephapirin. This method, however, does provide the ability to monitor for DAC along with CEPH.

Flunixin-Incurred Milk. Flunixin is a nonsteroidal anti-inflammatory drug. Abuse of this drug in cattle has been reported.[38] Bovine metabolism studies of flunixin indicated that the 5-hydroxy metabolite (FLU-OH) could be monitored as the marker residue.[27,39] These studies also showed that some amount of the parent compound was still present in the milk. The Q-TOF analysis of milk from cows that had been dosed with FLU (22 mg/kg intraveneously) corroborates these findings. In milk collected 8 h after dosing, both FLU-OH and FLU were detected. The signal for FLU-OH was >20 times that observed in the 1*X* milk spike, corresponding to a concentration of >40 ng/mL. The amount of flunixin detected (based on the $[M + H]^+$ response of these two compounds) in the 8 h incurred milk was approximately 25% of the total (FLU + FLU-OH). These compounds depleted rapidly in subsequent milk samples. The formation of a glucuronidase conjugate of flunixin in milk has also been suggested,[40] but this compound was not detected in the incurred milk samples by searching for the corresponding protonated ion. These results confirm earlier findings indicating that FLU-OH should be used as a marker residue and that even this metabolite depletes rapidly from the milk when FLU is administered intraveneously.

Enrofloxacin-Incurred Milk. Like flunixin, the marker residue from cows dosed with enrofloxacin is not the drug itself, but a metabolite. In this case, ciprofloxacin, another fluoroquinolone that differs from ENR by an ethyl group, is the primary residue found in milk.[41] CIP, along with lower levels of the parent compound, was found in milk from cows dosed with enrofloxacin (7.5 mg/kg subcutaneously) by LC−fluorescence and LC-MS/MS with triple-quadrupole detection, as well as by this Q-TOF LC-MS procedure. LC-FL analysis of ENR-incurred milk contained additional peaks in the chromatograms. The full-scan MS trace collected with the Q-TOF for the 8 h enrofloxacin sample was investigated to determine if additional compounds could be detected. A search of the data using the *Find by Molecular Feature* program indicated several peaks present in large abundance that were not detected in the control milk. One compound that eluted 0.1 min before CIP had an accurate mass calculated at 333.1489 (without protonation), which corresponded to a molecular formula of $C_{17}H_{20}N_3O_3F$. Searching an online database[42] for fluoroquinolone compounds with that formula led to a few possibilities, including the known compound pefloxacin. A standard of pefloxacin was obtained and analyzed using this method. The accurate mass and retention time of the potential ENR metabolite matched pefloxacin within ±2 ppm and ±0.5 min. However, when the MS/MS spectrum was obtained for the compound in the incurred milk extract and compared to the product ions of pefloxacin, there were significant differences (Figure 4 and Table 5). Specifically, whereas both compounds showed loss of water (m/z 316.1459), the metabolite had ions corresponding to the loss of HF and $C_4H_{10}NO$. The lower mass product ions from pefloxacin were assigned to the loss of CO_2 and $C_4H_7NO_2$. These data would indicate that the unknown peak was not pefloxacin.

Another compound that is more consistent with the MS/MS spectra of the enrofloxacin metabolite is shown, along with structures of the other fluoroquinolones, in Figure 5. This chemical (CAS Registry No. 149091-97-4)[43] is a known metabolite of ENR in fungi[44] and results from the loss of ethylene from ENR (Des-ENR). Further analysis of the TOF data also detected compounds that match other known metabolites of CIP[45] in poultry, including desethylene-CIP (Des-CIP) and oxidized CIP (Oxo-CIP). The product ion spectra for these compounds are

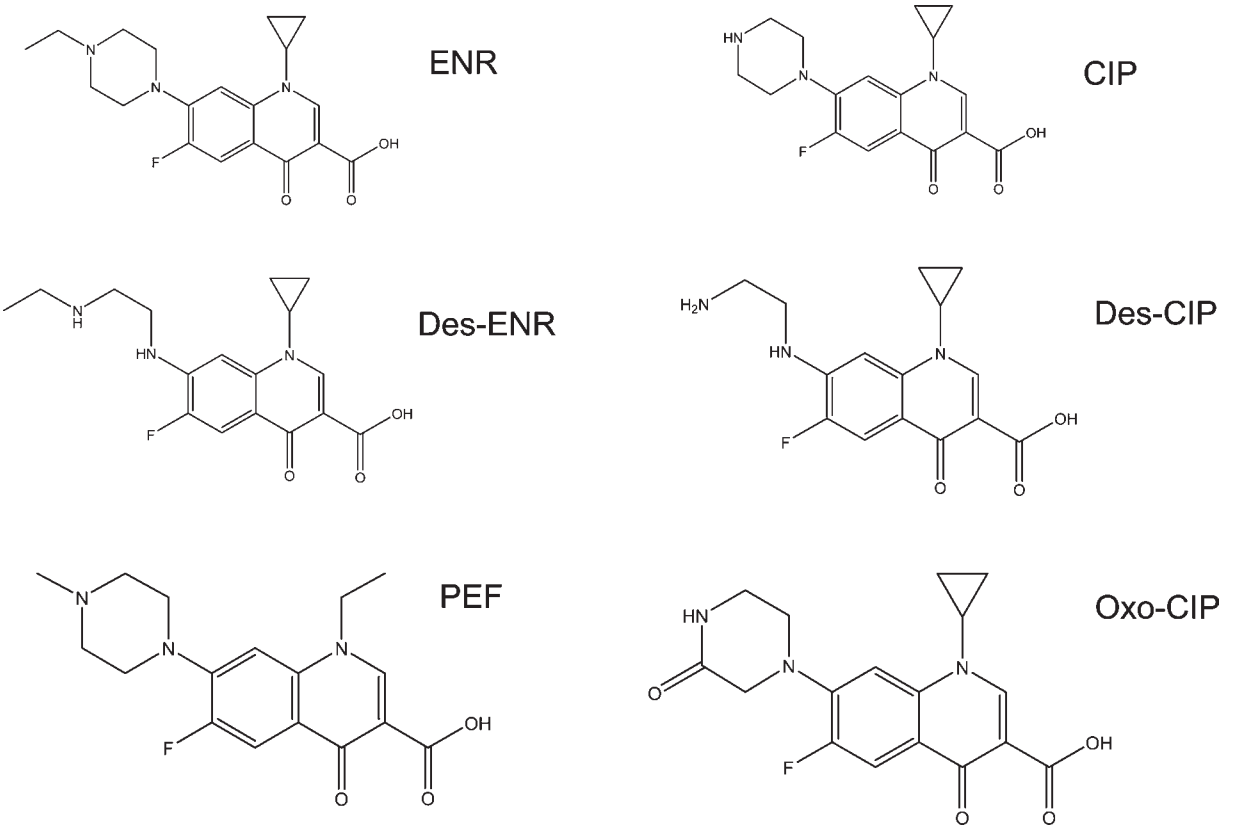

Figure 5. Structures of fluoroquinolones and proposed metabolites.

also shown in Figure 4 and described in Table 5. Ions corresponding to these compounds were not observed in either solvent or extracted matrix standards of ENR (or CIP). Other reported fluoroquinolone metabolites, such as the sulfa and formyl derivatives of CIP or Oxo-ENR,[45] were not detected in these samples. Although standards of these metabolites were not available for comparison, the MS and MS/MS data, in combination with literature references, provide strong evidence for the presence of these particular metabolites in the milk sample.

Although CIP was the predominant metabolite, the ion corresponding to the Des-ENR compound (m/z 334.1562) was present in higher abundance and persists longer than the ENR itself. This example shows the ability of the Q-TOF to detect and identify additional compounds, in this case potential metabolites that have not been described previously in the milk of dairy cows. Furthermore, it illustrates the importance of collecting MS/MS data to avoid misidentifying compounds on the bases of molecular formula and retention time alone.

In conclusion, several TOF methods have been reported previously for screening large numbers (>100) of residues.[8,9,11,12] Procedures utilizing the Q-TOF with product ion data collected to identify compounds have generally been limited to a single class of veterinary drugs.[17−20] This method illustrates the feasibility of using the instrument for screening, quantifying, and confirming drug residues in a multiclass method. In addition, nontargeted analytes can be detected and characterized by this method. Several plausible metabolites of veterinary drugs in milk, some not previously observed, were reported using Q-TOF LC-MS in this study.

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