Sensitive and Accurate Multi-Class Veterinary Drug Analytical Method Validation for Shell Eggs Using Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT

A multi-residue analysis method was developed and validated for forty residues from multi-class veterinary drugs. The forty compounds included belong to eight different drug groups - sulfonamides, tetracyclines, fluoroquinolones, macrolides, ß-lactams, nitroimidazoles, ionophores and amphenicols. All compounds were extracted using acetonitrile, with phosphate buffer, and followed by a C18 solid phase extraction (SPE) cartridge clean up. The extracts were then analyzed in a single run on a newly developed liquid chromatography-tandem mass spectrometry (LC-MS/MS) method with matrix matched standard calibration. Electro-spray ionization (ESI) mode was used to collect two multi-reaction monitoring (MRM) ions for each of the forty veterinary drugs. This method was validated using three difference sources of shell eggs for sensitivity, accuracy, precision and linearity for the target levels of 0.3 ng/g for chloramphenicol, 2 ng/g for tetracyclines, fluoroquinolones and ß-lactams, and 1 ng/g for all other compounds.

KEY WORDS: Tetracycline antibiotics, multi-residue, LC-MS/MS, veterinary drugs

INTRODUCTION

Application of antibiotics or other veterinary drugs in livestock can prevent disease, increase efficiency of feed and regulate growth [1]. However, overuse of these drugs can lead to the accumulation of high levels of drug residues in final food products such as meat and eggs. This accumulation of drug residues is transferred to the public as we consume these animal-derived food products. It is also known that the long-term misuse of antibiotics can induce antibiotic-resistance in bacteria [2,3], whether by direct application or indirect accumulation such as by consumption. Antibiotic-resistant bacterial infections can be very difficult to treat with currently available drugs [4].

In 2011, the FDA decided to study the quality of domestic shell eggs for the presence of veterinary drug residues. The Pacific Southwest Lab (PSW) in conjunction with ORS (formerly DFS) and CVM/CFSAN evaluated available information on shell egg analysis, multi-veterinary drug residue analysis and similar programs established in other foreign countries, and determined a list of potential veterinary drugs to include in this study. The regulations established in the EU provided much of the information used to develop this study.

The result of the informational review were forty-four compounds of interest, belonging to nine drug classes -nitrofurans, sulfonamides, tetracyclines, fluoroquinolones, macrolides, ß-lactams, nitrimidazoles, ionophores and amphenicols. The forty-four compounds of interest are Dimetridazole, Metronidazole, Ronidazole, Sulfaguanidine, N-Acetylsulfanilamide, Sulfadiazine, Sulfapyridine, Sulfathiazole, Sulfamerazine, Sulfamethazine, Sulfamethiazole, Sulfamethoxypyridazine, Sulfamonomethoxine, Sulfachloropyridazine, Sulfamethoxazole, Sulfasoxazole, Sulfadimethoxine, Sulfaquinoxaline, Ciprofloxacin, Enrofloxacin, Sarafloxacin, Difloxacin, Amoxicillin, Cephapirin, Ampicillin, Penicillin-G, Cloxacillin, Oxytetracycline, Tetracycline, Isochlortetracycline, Chlortetracycline, Doxycycline, Erythromycin, Tylosin, Lasalocid, Monensin, Salinomycin, Narasin, Nicarbazin, Chloramphenicol and four Nitrofurans in the form of their metabolites (1-aminohydantoin (AHD), 3-amino-2-oxazolidinone (AOZ), semicarbazide (SC), and 3-amino-5-morpholinomethyl-2-oxazolidinone (AMOZ)).

Among the forty-four residues, the four nitrofurans of interest metabolize rapidly in biological systems and only their metabolites are available for analysis. The four nitrofuran metabolites need to be analyzed separately due to their unique requirement. The classical analytical methods [5] for these metabolites rely on a simultaneous extraction and derivatization step prior to analysis. Many of the remaining forty compounds would decompose under such derivatization conditions.

There were multi-residue analytical methods available in literature that individually studied some of the forty residues in eggs or other matrices. Stubbings et al. (2005) published a multi-residue method of 30 compounds from 6 drug classes [6]. Li et al. (2006) published a method for 18 compounds from 5 drug classes [7]. Heller et al. (2006) published a method for 29 compounds from 4 drug classes [8]. For efficiency, our scope for this study was to develop a single extraction and analytical method which could identify and quantify all forty non-nitrofuran compounds.

EXPERIMENTAL

The method validation was designed according to "Guidelines for the Validation of Chemical Methods for the FDA Foods Program", version 1.0, from the FDA Office of Foods [9]. The resulting data and technical details were evaluated with FDA's Center of Veterinary Medicine's (CVM) "Guidance for Industry, Studies to Evaluate the Metabolism and Residue Kinetics of Veterinary Drugs in Food-Producing Animals: Validation of Analytical Methods Used in Residue Depletion Studies" from FDA's Center of Veterinary Medicine (which was in draft in 2012) and various EU guidelines [11-13]. The CVM draft guidance document was finalized and introduced in March 2015 as CVM GFI #208, VICH GL49(R). [10]

From reviewing the maximum residue limits (MRL) of European Commission and U.S. regulations, this study set target levels which were sensitive enough to meet or exceed the interest and existing requirements of both governments. For chloramphenicol, the target level was 0.3 ng/g. For tetracyclines, fluoroquinolones and ß-lactams, the target level was 2 ng/g. For all other compounds, the target level was 1 ng/g. Six batches of experimental analyses were performed for the method validation using three different sources of organic eggs. Each brand of eggs was analyzed in duplicate batches. In each batch, three replicates of each of three levels of drug concentrations were fortified into homogenized eggs for spike-recovery studies. A matrix-matched five-point standard calibration with linear regression was used for quantitation. The parameters-linearity, selectivity, precision, accuracy, limit of quantitation (LOQ), limit of detection (LOD) and independently prepared ICV were evaluated in the validation. [10-13]

Sample Matrices:
Organic eggs were purchased from three different commercial sources. Each batch of eggs was individually blended using a homogenizer and served as control matrix. The three composites were then aliquoted in 2 g portions into 50 mL centrifuge tubes and stored frozen (-10 to -25 ºC) until used.

Reagents and Solutions:

1. DI Water, in-house Milli-Q water purification system, quality at 18MΩ
2. Methanol, Acetonitrile and Formic Acid, HPLC grade, Fisher Scientific, Hampton, NH
3. Isochlortetracycline was obtained from Santa Cruz Biotechnology Inc. (SCBI, Santa Cruz, CA). All other 39 veterinary drug compounds (Table 1) obtained from Sigma-Aldrich, St. Louis, MO
4. Sodium phosphate monobasic and sodium phosphate dibasic from Sigma-Aldrich, St. Louis, MO
5. Second source of standards for ICV (Table 1) from USP (Rockville, MD), Witega GmbH (Berlin, Germany), SCBI (Santa Cruz, CA) and Toronto Research Lab (TRC, Toronto, Canada)
6. SPE Cartridge, 500 mg, C18 from UCT Inc., (Bristol, PA), Agilent, (Lake Forest, CA) or equivalent
7. 0.2 M Phosphate Buffer – Dissolve 24.0 g of NaH_2PO_4 and 28.4 g of Na_2HPO_4 in 200 mL of DI water, sonicate 5 min.
8. Mobile Phase A, 0.1% Formic Acid (FA) in water – Dissolve 1 mL Formic Acid in 1 L of DI water in amber glass bottle, sonicate for 5 min.

9. Mobile Phase B, 0.1% Formic Acid in 1:1 acetonitrile:methanol (v/v) – mix 500 mL acetonitrile, 500 mL methanol and 1 mL Formic Acid in 1 L glass bottle, sonicate for 5 min.
10. 90% acetonitrile in water (v:v) – mix 900 mL of acetonitrile with 100 mL of DI water. Sonicate for 5 min.
11. 60% methanol in water (v:v) – mix 600 mL of methanol with 400 mL of DI water. Sonicate for 5 min.

Equipment:

1. AB Sciex QTRAP 5500 Mass Spectrometry with Shimadzu AD20 series HPLC, Analyst 1.5.1 software from ABSciex, (Framingham, MA)
2. Geno Grinder 2000 from SPEX CertiPrep Group LLC, (Metuchen, NJ)
3. Centrifuge from Fisher Scientific, (Hampton, NH)
4. Turbovap from Caliper Life Sciences, (Hopkinton, MA)
5. YMC Phenyl 4.0 x 50 mm 3 µm cartridge column (Waters, Milford, MA) with a YMC Phenyl S-3 4.0 x 20 mm DC Guard Cartridge (Waters, Milford, MA)
6. Volumetric Pipette from Eppendorf North America, (Hauppauge, NY)
7. Ultra Turrax IKA T18 Basic Homogenizer from IKA Works, Inc, (Wilmington, NC)

Standard and QC Solution Preparation:

Stock Standards: Weigh 10 mg of each neat standard and dissolve each individually in 50 mL of appropriate solvent to make a 0.2 mg/mL stock standard solution (Table 1). Exception: Lasalocid was purchased as a 0.1 mg/mL solution in Acetonitrile and was directly used as a stock standard.

Intermediate Standard Mixture: Based on the drug class, seven intermediate standard mixture solutions were prepared from the eight drug groups. The seven groups are (1) nitroimidazoles group combined with chloramphenicol, (2) sulfonamides, (3) quinolones, (4) ß-lactams, (5) tetracyclines, (6) macrolides, and (7) ionophores. See Table 1 for the group listing of analytes. Each of the seven mixture solutions were prepared by delivering 0.5 mL of each standard stock (except chloramphenicol, which was 0.15 mL, and lasalocid, which was 1.00 mL) and diluted to 10.0 mL with acetonitrile to make 10 µg/mL of 39 of the analytes and 3 µg/mL for chloramphenicol.

Working Standards Mixture: The concentration of each compound in this working standard mixture was designed to be 100 times higher than the target level. The Working standard is prepared by delivering 0.1 mL of Intermediate Standard Mixtures (1), (2), (6) and (7), 0.2 mL of Intermediate Standard Mixtures (3), (4) and (5) altogether into one 10 mL volumetric flask and diluted to volume with Acetonitrile. The concentration of each group of drugs was: chloramphenicol: 30 ng/mL; tetracyclines, fluoroquinolones, ß-lactams: 200 ng/mL; sulfonamides, macrolides and ionophores: 100 ng/mL (Table 1).

The Stock Standards and Intermediate Standard Mixtures were stored in the freezer, except nicarbazin, which was stored at room temperature. The Working Standards mixture was stored in the refrigerator (2-8^{o}C). During the validation study, the Stock Standards and Intermediate Standard Mixtures were within 6 months old and the Working Standards mixture were used within 8 days.

Various literature indicated different storage conditions (refrigeration to -70ºC) and shelf-life for different combinations of these residues [14-16]. In general, frozen storage provided longer shelf-life. Caution is also noted for certain drug groups to minimize degradation caused by multiple freeze-thaw cycles [8].

Independent Calibration Verification (ICV): Using a different source of stock standard material where possible, independently prepare a second set of solutions as described above. This second Working Standard Mixture-ICV will be used to prepare the ICV for each batch analysis.

Sample Preparation:
A 2 g (+/- 0.05g) of sample homogenate was transferred into a 50-mL centrifuge tube. 10, 20 and 40 µL of the Working Standards Mixture was added to individual sample portions and prepared in triplicate at each level. In each batch, one sample portion was analyzed as the "matrix blank" to monitor for process contamination.

Calibration and quality control standards were all matrixed matched. The calibration standards were prepared by fortifying with 10µL to 100 µL of the Working Standards Mixture in clean matrix, followed by adding an appropriate amount of acetonitrile to adjust the total added volume in each tube to 100 µL to get 0, 0.5X, 1X, 2X, 3X and 5X level of calibration points. For Chloramphenicol, 1X level was 0.3 ng/mL, for tetracyclines, fluoroquinolones and ß-lactams, 1X level was 2 ng/mL; all other compounds, 1X level was 1 ng/mL (Table 2). ICV samples were fortified only at the target 1X concentration by fortifying with 20 µL of the independently prepared Working Standards Mixture-ICV solution in clean matrix.

All sample tubes were capped and vortexed for 20 sec. Eight mL of acetonitrile was added to each tube and shaken on a Geno Grinder for 4 min at 500 cycle/min. An aliquot of 0.5 mL of 0.2 M phosphate buffer was added to each tube, followed by shaking on a Geno Grinder for another 4 min at 500 cycle/min. Then the tubes were centrifuged at 3,000 g for 5 min.

The supernatants were transferred to individual 10 mL syringe barrels adapted to a 0.45 um nylon syringe filter followed by a C18 cartridge for clean-up. The C18 cartridge was not preconditioned. The extract was passed through the filtration set-up with positive pressure into a clean 15 mL centrifuge tube. Then, the cartridge was rinsed with 3 mL of 90% acetonitrile in water (v/v). The rinse solution was collected into the same 15 mL centrifuge tube and combined with the first filtrate. Then, the combined filtrate was evaporated at 40-45 ºC under nitrogen gas on a Speedvac to an approximate volume of 0.3 to 0.5 mL for solvent exchange. The concentrated extract was then diluted to 2 mL with 60% methanol in water for LC-MS/MS analysis.

The variation in the concentrated sample volume of 0.3 to 0.5 mL affects the ratio of methanol in final 2 mL solution, but it did not affect the quantitation results. 15-mL centrifuge tubes with graduation marks were used for the final 2-mL dilution and reliably gave reproducible results. Where desired, Class A K-D collection tubes can be used for definitive 2.0-mL measurement.

LCMS Analysis:
Liquid Chromatography-Tandem mass spectrometry (LC-MS/MS) with electro-spray ionization (ESI) mode was used to collect two multi-reaction monitoring (MRM) ions for each of the forty veterinary drugs. The mass spectrometry parameters for each compound are listed in Table 3.

Each extract vial was injected twice – once using positive ionization for 38 compounds, the other using negative ionization for 2 compounds. The LC conditions were the same for both ionization modes. The LC conditions are indicated below.

Column: YMC Pack Phenyl (4.0X 50 mm 3µm) with YMC Phenyl S-3 DC Guard Cartridge (4.0 x 20 mm)
Column Temp: 30 °C
Injection volume: 5-10 µL
Autosampler Temperature: 15 °C
Mobile Phase A: 0.1% Formic Acid in DI Water
Mobile Phase B: 0.1% Formic Acid in 1:1 Methanol:Acetonitrile
Flow Rate: 0.8 µL/min

LC Gradient:		
Time (min)	Mobile Phase A	Mobile Phase B
0	95%	5%
1	95%	5%
2.5	88%	12%
3	84%	16%
7	72%	28%
9.5	40%	60%
10	20%	80%
12.5	15%	85%
12.6	0%	100%
13.9	0%	100%
14	95%	5%
17	95%	5%

The analytes were detected and quantified by comparing against matrix-matched standards. The concentrations were calculated based on non-weighted linear regression. Each compound had two fragment ion chromatogram peaks for quantitation and identification.

RESULTS AND DISCUSSION

Chromatography and Selectivity:

Figure 1 shows a typical overlaid LC-MS/MS chromatogram of the forty veterinary drugs. For each compound, the retention time shift was less than 1%; the signal to noise ratio was equal to or greater than 10.

The LC separation and mass spectral (MS) data acquisition was completed within 13 min. The combination of mobile phase modifier and LC gradient program was optimized to minimize the

overlap of the peaks. Chlortetracycline and doxycycline did show broader peaks than other compounds due to a compromised pH with other classes of drugs present in the mobile phase. However, using these LC conditions, no significant ion-suppression was observed and adequate quantitation results were obtained for each of the compounds in this study.

Recovery and Linearity:
This method generated highly accurate and reproducible results. Table 4-Table 6 provide data on linearity, retention times, LOD, LOQ, spike recoveries, CCV and ICV recoveries and are discussed as follows.

The calibration curves, with unfortified matrix as concentration zero and 5 calibration points at 0.5X, 1X, 2X, 3X and 5X, were linear, where $R^2 \geq 0.995$. An example of one set of regression data is shown in Table 5.

Figure 2 shows eight plots of linear regressions. One drug was chosen from each of the drug groups in a typical batch with the quantitation range used in this study. Figure 3 shows examples of confirmation of identification, using peak area ratios of characteristic MRM transitions, with a representative analyte from each drug class. The selected criteria were one precursor ion, two transition ions and the abundance deviation of ±20% for the second transition ion. Due the broad range of characteristics of the residues included in this method, the ion confirmation requirements are more aligned with European regulations [12-13].

Table 4 shows the pooled average recovery results of sample fortifications from six spike-recovery studies using three independent sources of shell eggs. Each of the studies included triplicate spike recoveries each at 0.5X, 1X, and 2X target level. The pooled average recoveries ranged between 80 to 120% for all forty compounds. Table 4 also shows the inter-relative standard deviation (inter %RSD) which were all less than the expected inter %RSD of 32%, as recommended in the CVM guideline, VICH GL49(R) [10]. While the data is not shown, the intra %RSD of the individual triplicates were less than 25% except for three analytes in the first study, where, for one spike level with each of those three analytes, their intra-%RSD were 27-28%.

The average recoveries of ICV spiked at 1X in matrix were between 90-110%. The average recoveries of CCV (at 1X) were 81-119% (Table 6). Table 6 also shows the inter-relative standard deviation (inter %RSD). The calculated inter %RSD for sample spike, CCV and ICV recoveries are consistent with the expected inter %RSD of 32% as recommended in the CVM guideline, VICH GL49(R) [10].

Limit of Quantitation (LOQ) and Limit of Detection (LOD):
The guideline VICH GL49(R) [10] provided several scientifically valid ways to determine LOD and LOQ. This validation uses the Student distribution t-value $t_{0.99}$ and standard deviation, SD, of 18 replicate spike-recovery tests at 0.5X target level to estimate the LOD and LOQ, as follows

$$LOD = t_{0.99} * SD$$
$$LOQ = 3 * LOD$$

Where, the replicate number, n, is 18, $t_{0.99}$ is 2.567, the LOD and LOQ results are shown in Table 5. Though the data is not provided in Table 5, the physically fortified 0.125X and 0.25X target level sample preparations supported the calculated LOD results.

Discussion:
The broad range of characteristics between all the analytes and the egg matrix created many challenges in the attempt to develop a single extraction and detection method. There were several critical factors to overcome during method development and are discussed below.

The acid dissociation constant, pK_a, and octanol-water partition constant, Log K_{ow}, of the analytes were reviewed and established that the forty compounds had the full range of acidic compounds to strong bases (pK_a from 0 to 12) and highly hydrophilic to hydrophobic compounds (Log K_{ow} from -2 to 10) [multiple sources, 17-19]. See Table 2. Egg matrix is inherently full of lipids and proteins, presenting a similar mix of characteristics as the target analytes. There was no obvious single or simple extraction and clean up. A staged approach was thus studied. The known process of using acetonitrile to precipitate the proteins was effective [20]. A 4:1 acetonitrile (volume in mL) to sample (grams) ratio was determined to successfully remove the proteins. The ratio of acetonitrile to sample between 7:2 and 4:1 were tested and showed no significant difference in protein removal.

The Log K_{ow} characteristics provided a guide for the extract clean-up step. A C18 solid phase extraction was used to remove lipids from the sample. However, the hydrophobic analytes were also retained on the C18 stationary phase. Rinsing the cartridge with 3 mL of 90% acetonitrile/water was successful in eluting these hydrophobic analytes while leaving behind the lipids.

After a successful clean-up, the final extract is still a soup of acidic compounds to strong bases and highly hydrophilic to hydrophobic compounds. The chromatography was relied upon to create the optimized conditions to elute all analytes. Extensive study of the chromatography method produced a busy elution profile but separated most of the compounds. Where a co-elution was deemed acceptable, the mass spectrometer was relied upon to differentiate the co-eluting compounds.

The chelation of drugs with metal ions is a well-known problem for tetracyclines and quinolones [21]. Many existing methods analyze the tetracyclines group alone, or 1-2 tetracyclines with other drugs. Any harsh conditions during sample extraction such as high temperature, high or low pH, or high concentration of salt (such as $MgSO_4$) could mask some residues and/or cause other residues to chelate or breakdown. A few buffers (EDTA-McIlvaine buffer, oxalic acid and succinic acid) were studied for potential metal ion chelation. Although these buffers may be suitable for ion chelation, they did not suitably recover all the compounds of interest, in addition to other factors that negatively affected the group of forty drugs. EDTA can enhance protein solubility which would result in a dirtier extraction [22]. The presence of EDTA in the final sample extract would be another co-eluting compound along with some of the analytes, although the instrument method does not monitor for EDTA. Another factor was the low pH would degrade the macrolide drug group. Thus, compared with the EDTA extraction, the extraction using phosphate buffer was cleaner and more efficient for tetracyclines, with less negative impact on the other analytes. The phosphate buffer was further optimized to 0.2 M concentration and requiring only 0.5 mL.

Several drugs are pairs of prodrug and drug compounds, such as Enrofloxacin-Ciprofloxacin, Difloxacin-Sarafloxacin, and Chlortetracycline-isoChlortetracycline. The consideration for the extraction method should minimize hydrolysis or other degradations of the pro-drugs. The use of

acetonitrile and phosphate buffer demonstrated to be gentle enough to prevent or minimize these conversions.

In solvent, the tetracyclines have the tendency to epimerize to an epimer form which has the same MRMs but elute at a different retention time [23]. We did not monitor the tetracycline epimers. In addition, Beta-lactams have ions that tend to form adducts with methanol, which also has a different retention time from the proton adduct form [24]. We monitored the proton adduct only.

A single scheduled MRM (sMRM) method with positive-negative mode polarity switching was tested. It was capable of analyzing the forty compounds in a single LC-MS/MS run. However, the overall sensitivity was decreased. Therefore, two runs with separate positive and negative mode analysis were used for the validation study and to analyze the daily samples. If higher target levels are acceptable to the user, using a single sMRM method with polarity switching will be more efficient.

CONCLUSIONS

This newly developed and validated LC-MS/MS method was highly effective in detecting and quantifying forty veterinary drugs in a single extraction method with a single LC-MS/MS elution and detection method. The effective target levels are 0.3 ng/g for chloramphenicol; 2 ng/g for tetracyclines, fluoroquinolones, and ß-lactams; and 1 ng/g for nitroimidazoles, sulfonamides, macrolides, and ionophores. The forty veterinary drugs include all the compounds considered either as mandatory or optional as listed in European Commission regulations. These forty compounds also include five tetracyclines and four ß-lactams, both groups of which are technically difficult as they can easily degrade or convert to other chemical forms during sample extraction. The sample extraction protocol was also proven to be free of matrix interferences and was simple, rapid and cost effective. The LC-MS/MS method appears robust, with potential to be applied to other suitable matrix extracts. The extraction method is anticipated to be suitable for other matrices, but may require some adjustments, such as for high sugar matrices. Suitable matrix extension or validation studies should be performed to establish application of this method to other matrices.

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Table 1. Sources of forty veterinary drugs, concentration of the working standard, and the intermediate standard mixtures, with their mixture group number

Drug Name	Drug Class	STD Supplier	ICV Supplier	CAS Number	Solvent for Stock Standard	Intermediate Standard mixture, mg/mL (Group #)	Working Standard, ng/mL
Amoxicillin	Beta-Lactam	Sigma	USP	26787-78-0	1:1 MeOH:water	0.010 (4)	200
Ampicillin	Beta-Lactam	Sigma	USP	69-53-4	methanol	0.010 (4)	200
Cephapirin	Beta-Lactam	Sigma	USP	21593-23-7	methanol	0.010 (4)	200
Chloramphenicol	Amphenicol	Sigma	TRC	56-75-7	methanol	0.003 (1)	30
Chlortetracycline	Tetracycline	Sigma	USP	61-73-2	methanol	0.010 (5)	200
Ciprofloxacin	Fluroquinolone	Sigma	TRC	21593-23-7	1:1 EtOH: 0.1% FA in H2O	0.010 (3)	200
Cloxacillin	Beta-Lactam	Sigma	USP	61-72-3	methanol	0.010 (4)	200
Difloxacin	Fluroquinolone	Sigma	TRC	98106-17-3	1:1 EtOH:water	0.010 (3)	200
Dimetridazole	Nitroimidazole	Sigma	TRC	551-92-8	methanol	0.010 (1)	100
Doxycycline	Tetracycline	Sigma	USP	17086-28-1	methanol	0.010 (5)	200
Enrofloxacin	Fluroquinolone	Sigma	TRC	93106-60-6	1:1 EtOH:water	0.010 (3)	200
Erythromycin	Macrolide	Sigma	USP	114-07-8	methanol	0.010 (6)	100
Isochlortetracycline	Tetracycline	Sigma	TRC	514-53-4	1:1 MeOH:water	0.010 (5)	200
Lasalocid	Ionophore	Sigma	SCBI	25999-31-9	acetonitrile	0.010 (7)	100
Metronidazole	Nitroimidazole	Sigma	SCBI	443-48-1	Methanol	0.010 (1)	100
Monensin	Ionophore	Sigma	TRC	22373-78-0	methanol	0.010 (7)	100
N-acetylsulfanilamide	Sulfonamide	Sigma	Sigma	144-80-9	methanol	0.010 (2)	100
Narasin	Ionophore	Sigma	SCBI	55134-13-9	methanol	0.010 (7)	100
Nicarbazin	Ionophore	Sigma	SCBI	330-95-0	DMSO	0.010 (7)	100
Oxytetracycline	Tetracycline	Sigma	USP	79-57-2	methanol	0.010 (5)	200
Penicillin-G	Beta-Lactam	Sigma	USP	61-33-6	methanol	0.010 (4)	200
Ronidazole	Nitroimidazole	Sigma	Witega	7681-76-7	methanol	0.010 (1)	100
Salinomycin	Ionophore	Sigma	Sigma	53003-10-4	methanol	0.010 (7)	100
Sarafloxacin	Fluroquinolone	Sigma	TRC	98105-99-8	1:1 EtOH:water	0.010 (3)	200
Sulfachloropyridazine	Sulfonamide	Sigma	SCBI	80-32-0	methanol	0.010 (2)	100
Sulfadiazine	Sulfonamide	Sigma	TRC	68-35-9	methanol	0.010 (2)	100
Sulfadimethoxine	Sulfonamide	Sigma	SCBI	122-11-2	methanol	0.010 (2)	100
Sulfaguanidine	Sulfonamide	Sigma	SCBI	57-67-0	methanol	0.010 (2)	100
Sulfamerazine	Sulfonamide	Sigma	TRC	127-79-7	methanol	0.010 (2)	100
Sulfamethazine	Sulfonamide	Sigma	TRC	57-68-1	methanol	0.010 (2)	100
Sulfamethiazole	Sulfonamide	Sigma	USP	144-82-1	methanol	0.010 (2)	100
Sulfamethoxazole	Sulfonamide	Sigma	TRC	723-46-6	methanol	0.010 (2)	100
Sulfamethoxypyridazine	Sulfonamide	Sigma	SCBI	80-35-3	methanol	0.010 (2)	100
Sulfamonomethoxine	Sulfonamide	Sigma	TRC	1220-83-3	methanol	0.010 (2)	100
Sulfapyridine	Sulfonamide	Sigma	TRC	144-83-2	methanol	0.010 (2)	100
Sulfaquinoxaline	Sulfonamide	Sigma	SCBI	59-40-5	methanol	0.010 (2)	100
Sulfasoxazole	Sulfonamide	Sigma	SCBI	127-69-5	methanol	0.010 (2)	100
Sulfathiazole	Sulfonamide	Sigma	TRC	72-14-0	methanol	0.010 (2)	100
Tetracycline	Tetracycline	Sigma	USP	60-54-8	methanol	0.010 (5)	200
Tylosin	Macrolide	Sigma	USP	1401-69-0	methanol	0.010 (6)	100

Table 2. pK_a and Log K_{ow} of forty veterinary drugs and target levels, X

Drug Name	Abbreviation	pK_a	Log K_{ow}	Target Level, X, ng/mL
Amoxicillin	AMOX	2.44	-2.54	2
Ampicillin	AMPI	2.44	-2.3	2
Cephapirin	CEFA	2.67	-0.34	2
Chloramphenicol	CAP	11.03	1.03	0.3
Chlortetracycline	CTC	4.5	-0.62	2
Ciprofloxacin	CIP	6.43	-1.27	2
Cloxacillin	CLOX	2.44	2.3	2
Difloxacin	DIF	6.17	0.25	2
Dimetridazole	DMZ	2.8	0.2	1
Doxycycline	DOXY or DX	4.79	-0.2	2
Enrofloxacin	ENR	6.43	-0.72	2
Erythromycin	ERY	8.88	3.06	1
Isochlortetracycline	ICTC	3.1	-	2
Lasalocid	LAS	5.8	6.74	1
Metronidazole	MNZ	0	-0.34	1
Monensin	MON	7.95	5.43	1
N-acetylsulfanilamide	NASA	10.6	-0.8	1
Narasin	NAR	7.9	8.64	1
Nicarbazin	DNC	-	3.6	1
Oxytetracycline	OTC	4.5	-1.22	2
Penicillin-G	PENG	2.45	1.12	2
Ronidazole	RNZ	12.99	-0.3	1
Salinomycin	SAL	4.4	8.53	1
Sarafloxacin	SAR	6.17	0.04	2
Sulfachloropyridazine	SCP	5.9	1.37	1
Sulfadiazine	SDZ	6.81	0.31	1
Sulfadimethoxine	SDM	1.4	0.31	1
Sulfaguanidine	SG	1.88	-1.02	1
Sulfamerazine	SMR	7.35	0.14	1
Sulfamethazine	SMZ	7.89	0.88	1
Sulfamethiazole	SMTZ	5.5	0.9	1
Sulfamethoxazole	SMX	5.81	0.71	1
Sulfamethoxypyridazine	SMPD	1.83	0.7	1
Sulfamonomethoxine	SMM	6.67	0.85	1
Sulfapyridine	SP	8.43	0.9	1
Sulfaquinoxaline	SQX	1.86	1.68	1
Sulfasoxazole	SSX	5	0.9	1
Sulfathiazole	STZ	7.2	0.05	1
Tetracycline	TC or TET	4.5	-0.9	2
Tylosin	TYL	7.1	3.41	1

Table 3. Tandem MRM method parameters for forty veterinary drugs

Drug Name	Ionization Mode	RT (min)	Q1 (m/z)	DP (volt)	Q3 (m/z)	CE (volt)	Q3 (m/z)	CE (volt)
Amoxicillin	ESI Positive	1.7	366	40	114	27	349	13
Ampicillin	ESI Positive	4.2	350	40	106	19	160	25
Cephapirin	ESI Positive	4.1	424	40	292	23	152	33
Chloramphenicol	ESI Negative	6.8	321	-110	152	-22	257	-18
Chlortetracycline	ESI Positive	6.5	479	40	444	31	154	39
Ciprofloxacin	ESI Positive	5	332	66	288	27	231	55
Cloxacillin	ESI Positive	10.5	436	80	178	33	220	25
Difloxacin	ESI Positive	6.7	400	60	356	27	299	41
Dimetridazole	ESI Positive	2.7	142	55	96	22	81	35
Doxycycline	ESI Positive	7.3	445	80	428	27	267	49
Enrofloxacin	ESI Positive	5.9	360	150	316	27	245	37
Erythromycin	ESI Positive	9.3	735	130	158	29	116	71
Isochlortetracycline	ESI Positive	5.8	479	100	462	29	197	59
Lasalocid	ESI Positive	11.7	613	100	377	47	359	49
Metronidazole	ESI Positive	2.3	172	40	128	20	82	40
Monensin	ESI Positive	11.8	693	80	675	57	461	73
N-acetylsulfanilamide	ESI Positive	2.9	215	100	156	15	92	31
Narasin	ESI Positive	12.1	788	80	431	67	531	63
Nicarbazin	ESI Negative	10.7	321	-90	137	-10	107	-52
Oxytetracycline	ESI Positive	4.4	461	116	426	27	443	19
Penicillin-G	ESI Positive	9.5	335	100	160	21	176	19
Ronidazole	ESI Positive	3.1	201	60	140	20	55	30
Salinomycin	ESI Positive	11.9	774	80	531	67	755	46
Sarafloxacin	ESI Positive	6.4	386	100	342	27	299	37
Sulfachloropyridazine	ESI Positive	6.3	285	116	156	21	92	37
Sulfadiazine	ESI Positive	4	251	60	156	20	92	37
Sulfadimethoxine	ESI Positive	8.5	311	60	156	27	92	43
Sulfaguanidine	ESI Positive	1.3	215	20	156	21	92	31
Sulfamerazine	ESI Positive	4.7	265	60	92	41	110	31
Sulfamethazine	ESI Positive	5.3	279	60	186	24	124	35
Sulfamethiazole	ESI Positive	5.6	268	60	156	23	92	37
Sulfamethoxazole	ESI Positive	6.7	254	60	156	23	92	33
Sulfamethoxypyridazine	ESI Positive	5.7	281	60	156	23	92	25
Sulfamonomethoxine	ESI Positive	6.1	261	60	156	21	92	23
Sulfapyridine	ESI Positive	4.4	250	60	156	23	92	35
Sulfaquinoxaline	ESI Positive	8.9	301	60	108	35	156	27
Sulfasoxazole	ESI Positive	7.4	268	60	156	19	92	41
Sulfathiazole	ESI Positive	4.5	256	60	156	23	92	37
Tetracycline	ESI Positive	4.9	445	40	410	29	154	37
Tylosin	ESI Positive	9.6	917	85	174	47	772	47

Table 4. Average recovery results pooled from sample fortifications: 3 sources of egg matrix, each source had 2 replicates of recovery test, each replicate had 3 fortification levels-low (half of target level, 0.5X), medium (target level, 1X) and high spiked level (twice the target level, 2X), each fortification level prepared in triplicate.

	Low Spike, (n=18)		Medium Spike, (n=18)		High Spike, (n=18)	
Drug Name	**Ave %Rec**	**%RSD**	**Ave %Rec**	**%RSD**	**Ave %Rec**	**%RSD**
Amoxicillin	105.1	21.1	95.0	12.2	98.2	9.9
Ampicillin	100.6	14.8	100.0	6.8	99.3	3.6
Cephapilin	102.2	15.0	100.0	6.9	97.8	4.2
Chloramphenicol	106.6	11.7	103.5	6.7	100.9	7.7
Chlortetracycline	109.7	6.3	101.0	11.2	92.8	11.3
Ciprofloxacin	104.6	13.8	97.5	10.4	97.1	7.1
Cloxacillin	104.5	10.7	103.0	5.8	99.9	3.6
Difloxacin	106.1	10.8	100.7	7.1	98.8	5.7
Dimetridazole	103.0	11.2	98.5	6.9	97.5	3.6
Doxycycline	115.5	10.0	101.6	11.9	97.7	8.9
Enrofloxacin	101.2	18.2	99.5	9.8	98.3	4.9
Erythromycin	107.0	12.3	102.3	6.3	101.0	3.3
isoChlortetracycline	99.6	13.5	99.7	8.5	97.9	7.3
Lasalocid*	103.5	14.0	103.2	11.4	95.0	13.4
Metronidazole	103.1	12.7	98.1	4.0	97.6	3.3
Monensin	104.9	12.3	99.8	12.1	100.0	5.9
N-acetylsulfanilamide	103.0	15.7	99.5	7.0	97.1	4.2
Narasin	109.0	11.5	97.5	8.1	99.3	6.8
Nicarbazin	103.1	16.4	101.9	6.3	102.2	4.1
Oxytetracycline	114.7	11.9	103.5	9.9	97.1	6.8
PenicillinG	100.7	17.3	100.1	7.1	99.1	3.5
Ronidazole	102.3	16.1	99.1	5.7	98.6	3.6
Salinomycin	105.1	12.1	100.6	7.0	99.8	5.6
Sarafloxacin	104.4	18.4	99.3	10.5	101.1	4.6
Sulfachloropyridazine	106.2	19.3	101.1	9.4	99.5	3.8
Sulfadiazine	101.2	15.4	98.8	7.4	98.1	4.3
Sulfadimethoxine	105.0	15.4	99.4	7.7	98.6	3.7
Sulfaguanidine	105.9	14.3	98.9	11.5	98.9	3.2
Sulfamerazine	104.9	12.9	100	6.5	98.3	4.6
Sulfamethazine	104.5	14.7	100.9	6.6	98.7	3.9
Sulfamethiazole	102.5	17.9	99.6	8.7	96.9	4.3
Sulfamethoxazole	100.9	14.7	100.8	12.0	99.8	6..6
Sulfamethoxypyridazine	104.3	13.9	101.6	7.3	99.3	4.3
Sulfamonomethoxine	101.9	13.3	100.1	6.7	98.4	4.3
Sulfapyridine	100.0	14.2	99.0	7.3	97.2	3.8
Sulfaquinoxaline	101.2	16.8	99.8	8.2	98.4	3.4
Sulfasoxazole	99.1	16.4	97.0	11.0	97.1	3.9
Sulfathiazole	105.1	15.8	100.9	9.1	96.9	4.1
Tetracycline	104.1	14.4	100.5	10.6	90.9	10.6
Tylosin	106.5	11.9	101.4	10.0	99.3	5.6

* for Lasalocid, n=12 because 1 matrix source (used in two studies) was contaminated with lasalocid.

Table 5. Results for retention time, linearity, LOD, and LOQ. The maximum retention time difference, ΔRT_{max}, was used to calculate the percentage of RT shift, where the RT was the average retention time. An example of linearity is shown (R^2). LOD was calculated using the Student's t-distribution and the 18 replicates of the 0.5X concentration spiked samples (Table 4-low spike data). SD, LOD, LOQ are in units of X target level (Table 2).

	RT (min)	RT %Shift	Regression	SD	LOD	LOQ
Drug Name	**Ave (n=18)**	**ΔRT_{max}/RT**	**R^2, an example**	**unit of X**	**unit of X**	**unit of X**
Amoxicillin	1.73	0.60%	0.976	0.11	0.29	0.87
Ampicillin	4.19	0.30%	0.999	0.07	0.19	0.56
Cephapilin	4.15	0.40%	0.998	0.08	0.2	0.59
Chloramphenicol	6.77	0.30%	0.999	0.1	0.26	0.77
Chlortetracycline	6.54	0.40%	0.975	0.06	0.15	0.44
Ciprofloxacin	4.99	0.30%	0.997	0.1	0.25	0.74
Cloxacillin	10.5	0.00%	0.998	0.07	0.17	0.51
Difloxacin	6.72	0.60%	0.995	0.06	0.16	0.47
Dimetridazole	2.7	0.20%	0.998	0.06	0.15	0.44
Doxycycline	7.31	0.60%	0.995	0.1	0.26	0.79
Enrofloxacin	5.93	0.70%	0.996	0.13	0.32	0.96
Erythromycin	9.29	0.40%	0.999	0.06	0.16	0.48
isoChlortetracycline	5.82	0.50%	0.999	0.1	0.24	0.73
Lasalocid	11.68	0.30%	0.995	0.1	0.27	0.81
Metronidazole	2.26	0.50%	0.996	0.07	0.17	0.5
Monensin	11.77	0.40%	0.997	0.06	0.17	0.5
N-acetylsulfanilamide	2.93	0.40%	0.999	0.08	0.21	0.63
Narasin	12.12	0.30%	0.997	0.07	0.17	0.5
Nicarbazin	10.7	0.00%	0.999	0.09	0.22	0.67
Oxytetracycline	4.38	0.40%	0.999	0.11	0.28	0.83
PenicillinG	9.49	0.80%	1	0.09	0.23	0.68
Ronidazole	3.1	0.30%	0.999	0.08	0.21	0.63
Salinomycin	11.92	0.30%	0.999	0.06	0.16	0.49
Sarafloxacin	6.35	0.50%	0.998	0.08	0.22	0.65
Sulfachloropyridazine	6.3	0.30%	0.998	0.08	0.22	0.65
Sulfadiazine	4.02	0.20%	1	0.08	0.2	0.59
Sulfadimethoxine	8.55	0.30%	0.999	0.08	0.21	0.62
Sulfaguanidine	1.35	1.00%	0.995	0.09	0.23	0.69
Sulfamerazine	4.67	0.30%	1	0.07	0.17	0.52
Sulfamethazine	5.26	0.30%	0.998	0.08	0.2	0.59
Sulfamethiazole	5.61	0.30%	0.996	0.1	0.25	0.76
Sulfamethoxazole	6.71	0.30%	0.999	0.07	0.17	0.52
Sulfamethoxypyridazine	5.72	0.30%	0.999	0.07	0.18	0.55
Sulfamonomethoxine	6.11	0.30%	0.997	0.07	0.17	0.52
Sulfapyridine	4.37	0.20%	0.999	0.07	0.18	0.54
Sulfaquinoxaline	8.87	0.30%	0.999	0.08	0.22	0.65
Sulfasoxazole	7.41	0.20%	0.997	0.08	0.21	0.63
Sulfathiazole	4.52	0.20%	0.997	0.08	0.21	0.64
Tetracycline	4.88	0.30%	0.999	0.11	0.27	0.81
Tylosin	9.65	0.30%	0.995	0.07	0.19	0.56

Table 6. CCV and ICV recovery was calculated from quantitation at the 1X spiked target level, in matrix. The data shown are calculations for inter-batch average and precision.

	ICV at 1X in Matrix		CCV at 1X in Matrix	
Drug Name	**Ave %Rec (n=6)**	**Inter %RSD**	**Ave %Rec (n=6)**	**Inter %RSD**
Amoxicillin	104.8	10.3	99	6.2
Ampicillin	106.4	6.7	100.7	8.8
Cephapilin	103.6	9	103.2	8.9
Chloramphenicol	109.5	14.5	106.8	9.5
Chlortetracycline	108.8	15.7	104.7	7.3
Ciprofloxacin	96.5	17.1	96.5	9.8
Cloxacillin	104.2	4.5	103.6	7.6
Difloxacin	107.7	10.8	103.8	6.3
Dimetridazole	102.9	7.2	99.7	9.3
Doxycycline	112.2	10.3	102.5	9.4
Enrofloxacin	106	8.9	101.8	8.2
Erythromycin	105.5	4.4	103	6.5
isoChlortetracycline	102	13.6	100.3	11.2
Lasalocid	103.8*	15.9	106.9*	16.4
Metronidazole	106.3	5.4	100.7	6.1
Monensin	108.7	8.1	102.7	8.8
N-acetylsulfanilamide	107.6	12.2	99.1	7.8
Narasin	108.8	9.1	103.8	7.3
Nicarbazin	100.9	8.8	102.8	8.7
Oxytetracycline	113.8	9.5	102.1	9.2
PenicillinG	104.6	6.5	101	9.3
Ronidazole	103.7	5.9	101.5	7.7
Salinomycin	103.8	12.8	103.7	7.9
Sarafloxacin	102.4	5.3	100.5	7.9
Sulfachloropyridazine	110.6	14	103	14.2
Sulfadiazine	104.2	5	100	11.3
Sulfadimethoxine	104.6	5.4	101.5	9.8
Sulfaguanidine	98.1	12.8	99.1	12.3
Sulfamerazine	102.5	9.3	102	5.6
Sulfamethazine	103.8	6.4	102.3	7.8
Sulfamethiazole	102.8	6.2	100.1	10.3
Sulfamethoxazole	106	10.6	99.8	16.1
Sulfamethoxypyridazine	105.1	21.3	99.6	17.1
Sulfamonomethoxine	108.5	8.4	103.3	10.4
Sulfapyridine	101.3	6.2	101.7	9.1
Sulfaquinoxaline	102.6	6.2	101.7	9.3
Sulfasoxazole	103.7	6.5	101.5	12
Sulfathiazole	105.9	8.6	101.4	10.1
Tetracycline	113.8	6.8	101.3	17.6
Tylosin	104.3	9.6	103.3	9.7

* N=5 for Lasalocid

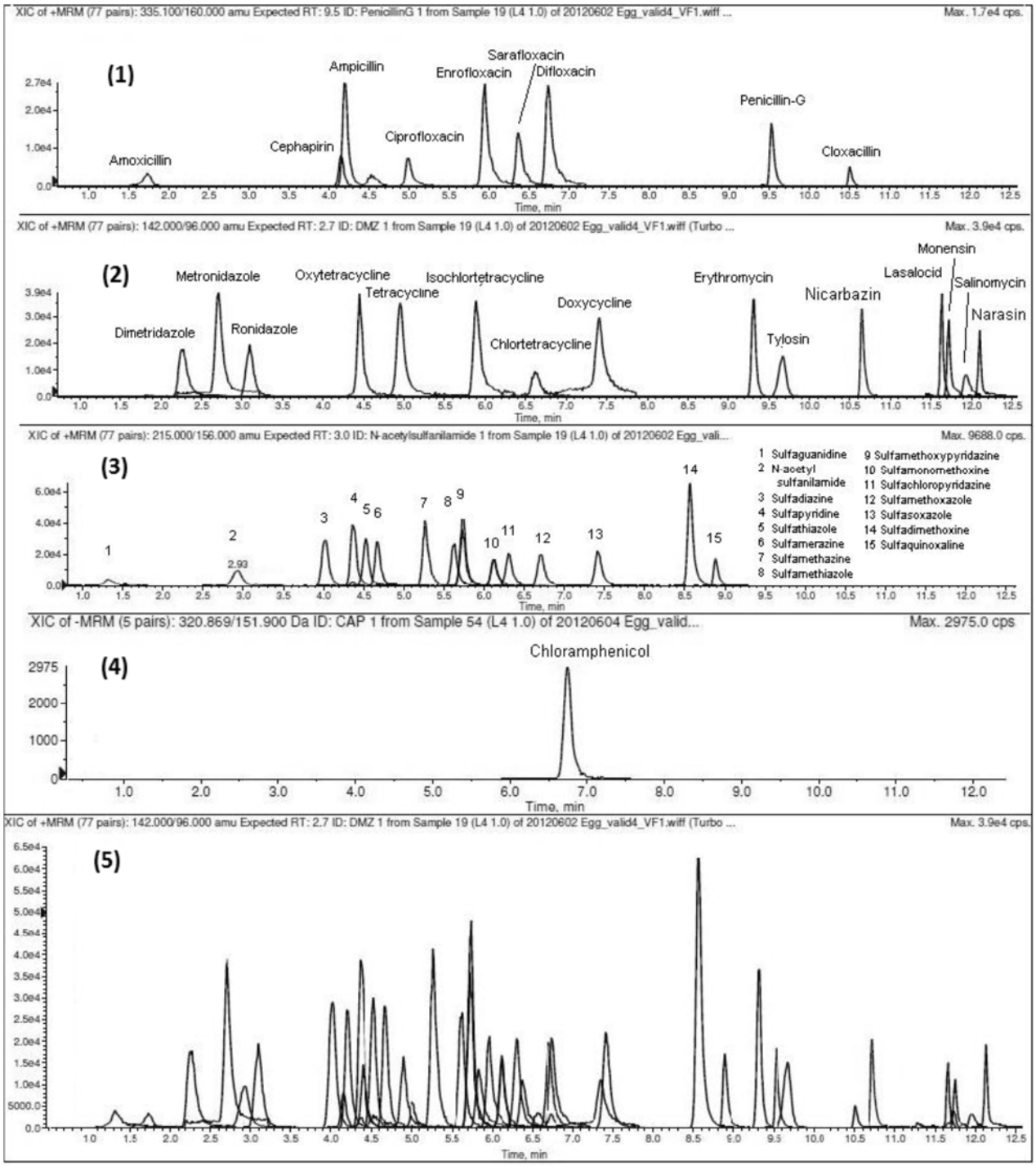

Figure 1. LC-MS/MS MRM chromatograms of 40 drugs, (1) fluoroquinolones and beta-lactams, (2) nitroimidazoles, tetracyclines, macrolides and ionophores, (3) sulfonamides, (4) chloramphenicol, (5) overlay with all 40 drugs, including 38 positively ionized and 2 negatively ionized compounds.

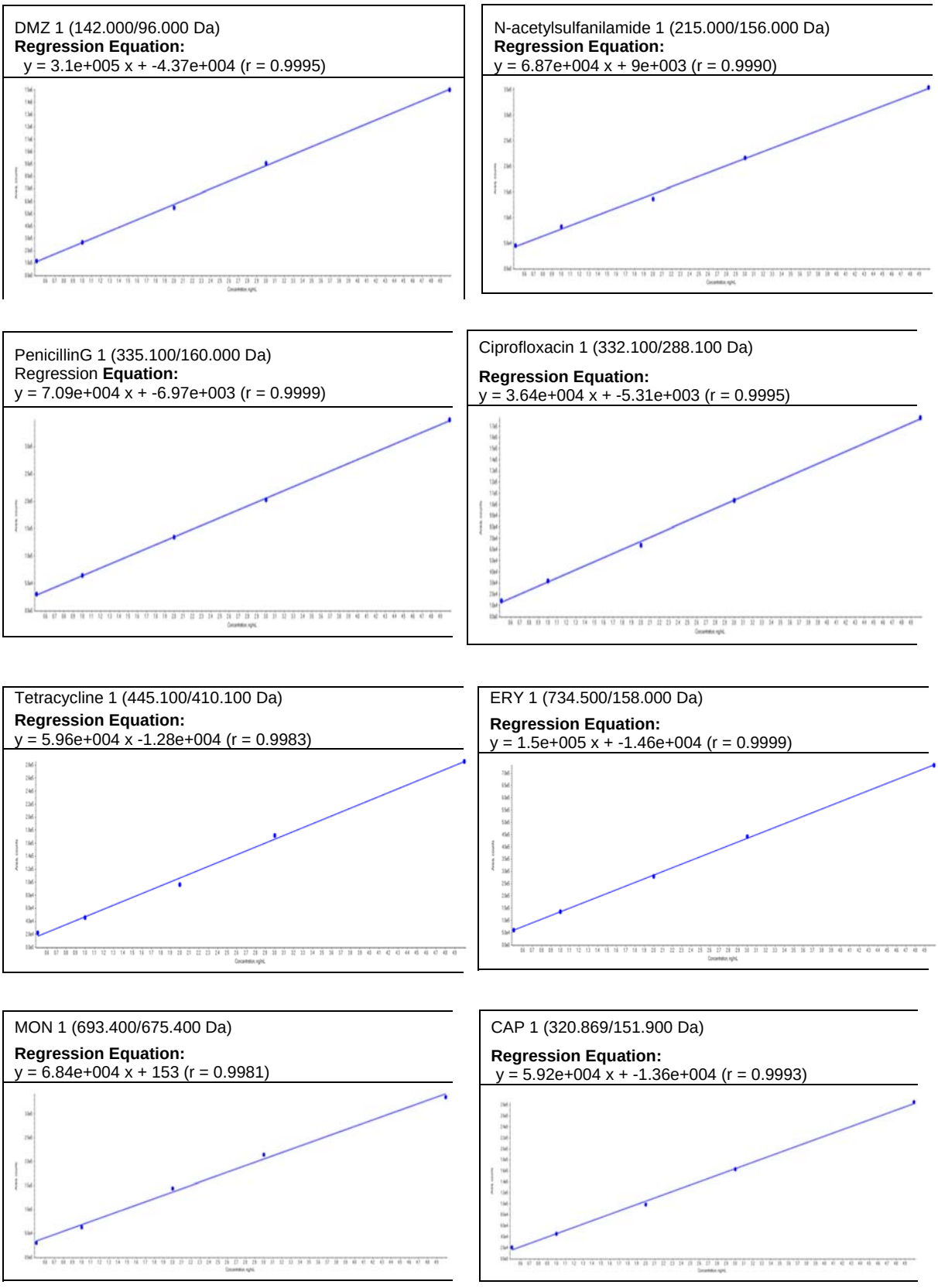

Figure 2. Matrix matched calibration curve with non-weighted linear regression, one representative compound from each class of drugs

Analyte Peak	Analyte RT, min	Expected RT, min	Analyte Peak Area		Calculated Ratio (expected ratio)	Ion Ratio Confirm
			Quant ion	Confirm ion		
Dimetridazole	2.70	2.71	2.65e+05	1.02e+05	38.4% (35.2%)	✓
N-acetylsulfanilamide	2.91	2.93	8.27e+04	4.90e+04	59.8% (59.1%)	✓
PenicillinG	9.51	9.49	7.19e+04	6.51e+04	90.5% (93.9%)	✓
Ciprofloxacin	5.00	4.98	4.66e+04	3.83e+04	82.2% (74.0%)	✓
Tetracycline	4.88	4.88	9.35e+04	3.24e+04	34.7% (45.6%)	✓
Erythromycin	9.29	9.25	1.59e+05	7.98e+04	50.2% (50.3%)	✓
Monencin	11.70	11.70	7.31e+04	3.46e+04	47.3% (49.1%)	✓
Chloramphenicol	6.74	6.74	2.37e+04	1.24e+04	52.3 % (63.0%)	✓

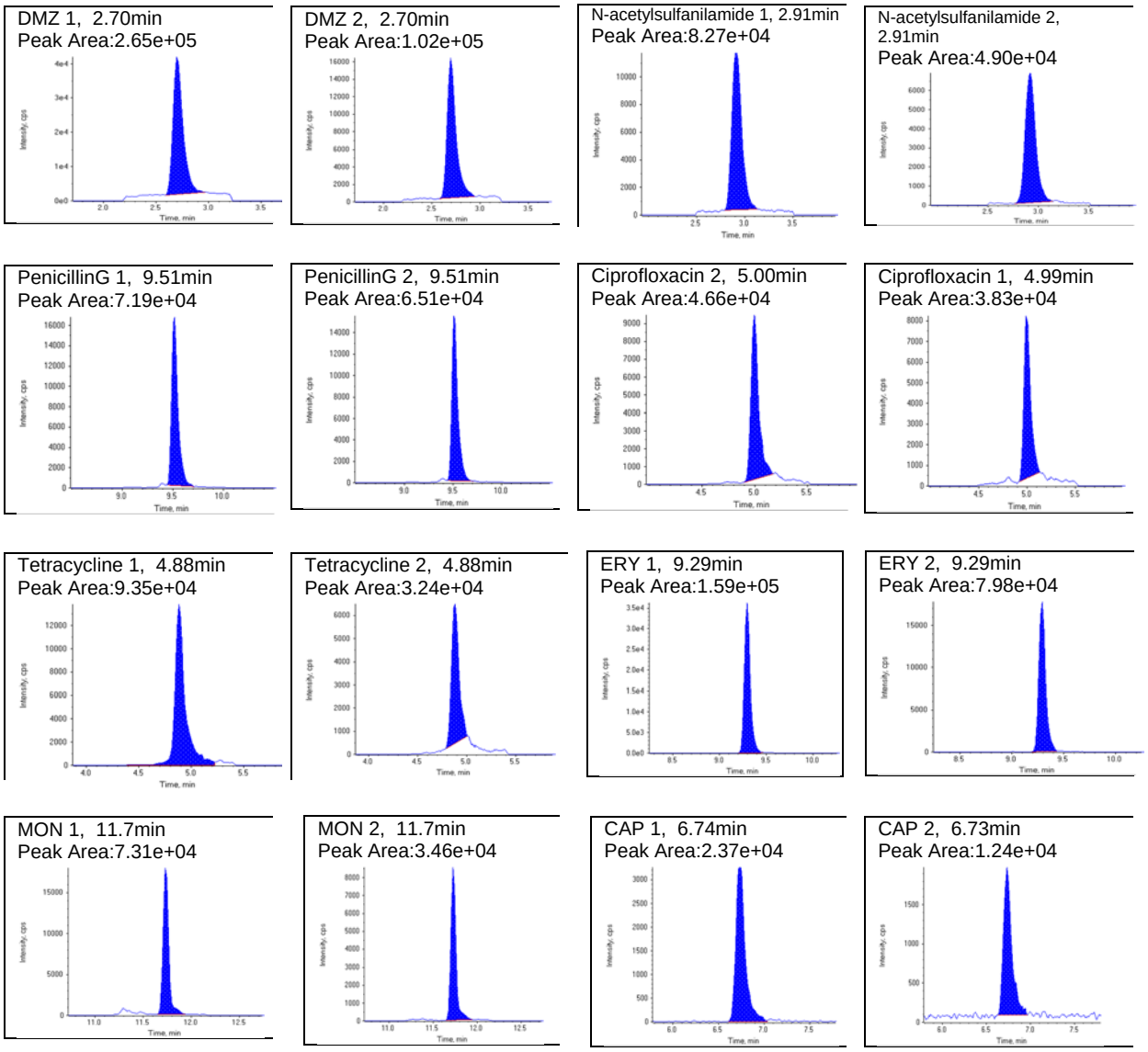

Figure 3. Examples of peak area ratio calculation between two fragment ions: One representative compound from each class of drugs