LABORATORY INFORMATION BULLETIN

Multiclass, Multiresidue Method for the Quantitation and Confirmation of over 110 Veterinary Drugs in Game Meat (Bison, Deer, Elk, and Rabbit) by Rapid Polarity Switching Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

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ABSTRACT

Veterinary drugs are widely used in animal food products to prevent diseases and are a complex set of drugs with very different chemical properties. The FDA requires a program for multiclass, multiresidue analysis to qualitatively and quantitatively determine veterinary drug compounds in domestic game meats. A validated LC-MS/MS residue screening method developed by the USDA Agricultural Research Services (ARS) for bovine muscle analysis was modified and applied to the analysis of muscle from bison, deer, elk, and rabbit to test for 112 veterinary drug residues from the following drug classes: thyreostats, corticosteroids/hormones, β-agonists, nitroimidazoles, phenicols, tetracyclines, β-lactams, fluoroquinolones, macrolides, sulfonamides, anthelmintics, sedatives, and anti-inflammatory drugs. The analytes were extracted from muscle tissue via a simple and quick procedure based on a solvent extraction with 80% acetonitrile/water, thorough mixing, and sample clean-up via dispersive SPE. The compounds of interest were separated using an Agilent 1260 liquid chromatography system with a Waters HSS T3 column and detected using a SCIEX 5500 QTrap mass spectrometry system with rapid polarity switching to detect both negatively and positively charged ions in a single injection. The method was validated for bison, deer, elk, and rabbit muscle according to FDA Foods Program Level Two validation criteria. Recoveries were calculated using extracted matrix matched calibration curves for each type of matrix. The average accuracy of fortified compounds in all the matrices ranged from 95.6% to 101% at the target quantitative validation level. The method was also validated as a qualitative method where all sample responses were compared to an extracted-matrix matched 1X standard, where the 1X level was generally at the concentration 5 ng/g for most compounds and 25 ng/g for other classes of compounds. Samples demonstrating a presumptive positive above the threshold value were extracted by a separate analyst using a five-point matrix matched extracted calibration curve. Since the beginning of this survey program in 2014, 360 samples have been analyzed for veterinary drug residues. Antibiotic and/or sedative residues have been identified in deer (chlortetracycline, haloperidol, and tulathromycin), and rabbit (sulfadiazine).

INTRODUCTION

While the global production of animal protein is dominated by domesticated animals (cattle, sheep, pigs, poultry) and seafood, game animals represent a growing contribution to the global meat supply.[1,2] In the United States, there were over 14,000 bison, deer, elk, and rabbit farms in 2017 to meet consumer demands for low-fat meat sources.[3,4]

Few veterinary drugs are approved by the US Food and Drug Administration (FDA) for therapeutic use in game animals. Ivermectin is approved for bison and reindeer, and lasalocid and sulfaquinoxaline are approved for rabbit.[5] Tissue residue tolerances are 15 ppb (15 ng/g) for ivermectin in bison or reindeer liver, and 0.7 ppm (700 ng/g) for lasalocid in rabbit liver.[6] FDA-approved veterinary drugs may be permitted for extralabel use in game animals under the supervision of a veterinarian according to the provisions of the Animal Medicinal Drug Use Clarification Act.[7] However there are few published studies in game animals to establish appropriate withdrawal periods after veterinary drug administration to minimize the presence of residues in edible tissues.[8] Without an established tolerance, the presence of a detectable concentration of the drug residue in the edible tissue of a treated animal is considered a violation regardless of appropriate extralabel use of the drug.

Due to the increasing prevalence of game meat products for consumers, and the wide range of veterinary drugs that may be appropriate under the extralabel use provision, analytical methodology is required to monitor a variety of drug residues in bison, deer, elk, and rabbit meat. Residue testing methods for ivermectin and lasalocid have been validated for game meats in analytical methodology for avermectin (LIB 4644[9]) and coccidiostat (LIB 4627[10]) drug classes, but methodology for other drug classes was required for the FDA regulatory game meat program including: thyreostats, resorcyclic acid lactones, β-agonists, phenicols, tetracyclines, β-lactams, fluoroquinolones, macrolides, sulfonamides, anthelmintics, sedatives, anti-inflammatory drugs, corticosteroids/hormones and other unauthorized drugs. Regulatory monitoring programs for veterinary drug residues in animal products have been increasingly dominated by methodology that combines generic techniques to extract many compounds with a wide range of chemical properties and sensitive and selective liquid chromatography mass spectrometry analysis.[11] Many extraction and analysis methods to quantify or screen residues of more than 100 veterinary drugs from multiple drug classes have been developed for meat (bovine, swine), fish, milk, and other animal products in recent years.[12-16] Few methods have been developed and validated specifically for the analysis of veterinary drug residues in game meats, and most are focused on determining tissue residue levels for one or only a few residues in a single matrix.

After a thorough review of the scientific literature and comparison of the merits of several methods intended for residue screening in muscle matrix samples, it was determined that none were ready-made for the necessary compounds in the game meat matrices. One method published by Geis-Asteggiante et al.[17] performed well for residue screening and quantitative analysis for most of the necessary compounds in bovine muscle matrix. The Geis-Asteggiente et al. method was initially tested for suitability in the game meat matrices, and then modified to improve performance. Method modifications to the sample preparation procedure included eliminating the liquid-liquid extraction step with hexane to remove fat hence reducing sample processing time and modifying the extraction and reconstitution step to improve method detection limits. The instrumental analysis was also modified because the Denver Laboratory (DENL) did not have a UPLC system; hence, different mobile phases were tested to improve the chromatography. Also, polarity switching was required so that data from both positively and negatively ionized analytes could be acquired in a single analytical run. Multiple deuterated surrogates were added to the extraction procedure to represent specific classes of compounds to monitor method accuracy. Finally, extracted matrix-matched calibrants were used to generate calibration curves for improved quantitative accuracy overall for the residues in the game meat matrices. These modifications led to the development of a faster extraction procedure, lower residue concentration detection capability, and single injection analysis to yield quantitative results for all 112 compounds in a single run.

The method was validated according to the requirements of the FDA Foods Program Validation[18] guidelines with validation levels (VL) set at 0.5VL, 1.0VL, and 2.0VL, where VL was equal to 5.0 ng/g for most compounds. The validation level 1.0 VL is equivalent to the concentration 1X, which is considered the target

testing level for each analyte in this method. In some cases, these levels were selected to harmonize with existing EU Maximum Residue Levels (MRLs) for some of the veterinary drug compounds in other animal muscle (non-game). In other cases, validation levels were set to investigate the detection limit capability of the method since there are not threshold residue levels in game for most of the compounds. In addition to quantitative analysis using a five-point extracted matrix-matched calibration curve, the method was also validated for use in routine screening by comparing residue responses in each matrix to a single extracted matrix-matched calibrant at the 1.0X (1.0 VL) level. This validated method is intended for ORA regulatory analysis to screen game meats for unapproved veterinary residues and to expand the scope of ORA veterinary drug residue monitoring. To date, the method has been used to analyze over 360 muscle samples of bison, deer, elk, rabbit for veterinary drug residues.

METHODS AND MATERIALS

Equipment

Note: Equivalent equipment may be substituted. Avoid glass contact surfaces if the Mini-UniPrep filter vials are substituted with syringe filters and autosampler vials. Substitutes must be checked for possible absorption of analytes.

a. Platform shaker – Fisher Scientific Multi-tube vortexer.
b. Centrifuge – Sorvall RC-6, Thermo IEC, capable of acceleration to 10,000 rpm.
c. Balance – Mettler Model X-205 Dual range capable of weighing 2.00 ± 0.01 g.
d. Nitrogen Evaporator – Turbovap LV Concentration Workstation –Biotage Corp.
e. Dispersive sorbent Bondesil – C18 40 µm or equivalent, Agilent.
f. Tissue Homogenizer-Tissumizer and 2000 Geno/Grinder SPEX Sample Prep
g. Centrifuge tubes – Polypropylene (PP), 50 mL, Falcon Part Number 352070 and – 15 mL, Falcon Part Number 352096
h. Glassware and LC vials –disposable Pasteur pipettes; 15-mL glass tubes, 20 mL glass scintillation vials, 2-mL LC vials with snap top, or equivalent.
i. Filter vials – Thompson Agilent Mini-UniPrep Syringless 0.20-micron PTFE, Cat. No. 5190-1419
j. Magnetic stirrer and stir bars, freezer, volumetric flasks, graduated cylinders, Pasteur pipettes, repeating pipettes and tips, beakers, bottles, weigh boats, spatulas, funnels, bottle top volumetric dispensers, and other miscellaneous items.

Negative Control Tissue

Negative control – Bison, deer (venison), elk, and rabbit were acquired from a local market and tested to ensure that specific veterinary compounds were not present above the stated method detection level.

Reagents and Solutions

Note: Equivalent reagents / solutions may be substituted. The stability time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever is soonest.

a. Methanol (MeOH) – Fisher Chemical, Optima LC/MS Grade
b. Acetonitrile (ACN) – Fisher Chemical Optima LC/MS Grade
c. Formic acid – Fisher Chemical Optima LC/MS Grade
d. Water – Fisher Chemical Optima LC/MS Grade
e. Ammonium Formate – Fisher Chemical Optima LC/MS Grade

Solutions

a. HPLC Aqueous Mobile Phase (5 mM ammonium formate, 0.1% formic acid in 100% water)

Weigh 0.36 grams of ammonium formate and add to a 1 L volumetric flask containing approximately 900 mL of water. Add 1.0 mL of formic acid to the flask. Bring to volume with Optima LC/MS water. Mix and transfer to the aqueous reservoir of the LC.

b. HPLC Organic Mobile Phase (5 mM ammonium formate, 0.1% formic acid in 100% methanol)

Weigh 0.36 grams of ammonium formate and add to a 1 L volumetric flask containing approximately 900 mL of Optima LC/MS methanol. Add 1.0 mL of formic acid to the flask and bring to volume with additional methanol. Mix and transfer to the organic reservoir of the LC.

c. Acetonitrile: Water (80:20)
Measure 800 mL of acetonitrile using a graduated cylinder and transfer to a 1 L volumetric flask. Measure 200 mL of deionized water using a graduated cylinder and add to the volumetric flask containing the acetonitrile. Mix this solution and transfer to a dispenser bottle.

d. Formic Acid in Water (0.10%)

Measure 80 mL of Optima LC/MS water into a 100-mL graduated cylinder. Add 100 µL of formic acid to the graduated cylinder and dilute to the 100 mL mark with additional water and mix well.

Analytical Standards

Note: Equivalent standards / solutions may be substituted. Purity and counter ions are taken into account when calculating standard concentrations. The stability time frame of the solution is dependent on the expiration date of the components used or the listed expiration date, whichever ends sooner.

a. All analytical standards were ordered from Sigma-Aldrich, specifically as Fluka products, USP, and Toronto Research Chemicals (Toronto, Canada).
b. All surrogates were ordered from Witega (Berlin, Germany), Cerillant, Cambridge Isotopes, and Toronto Research Chemical.
c. Analytical standard premade mixes were also purchased from SPEX and Lab Solutions (Italy).

Stock and surrogate solutions from neat materials were prepared from 250 µg/mL- 500 µg/mL and diluted with the appropriate solvent. In general methanol was used for most compounds with the exception of the β-lactam compounds, which were prepared in water. All stock solutions were stored at -20°C. Two working solutions consisting of 12 β-lactams/tetracyclines (2500 ng/mL) in water and 100 veterinary drugs (1000 ng/mL) in acetonitrile by pipetting the required volume of the stock solutions into the final volume of 25.0 mL. The following compounds were prepared at 5,000 ng/mL in the veterinary drug solution mentioned in the previous sentence: bacitracin, carprofen, mefenamic acid, tolfenamic acid, and meloxicam. Both working standard solutions are transferred to 2.00 mL polypropylene tubes and taken out for use no more than five times, reducing the degradation of the tetracyclines and β-lactam compounds. If the premade standard solutions were used, the final concentration in the two separate working solution were prepared in the same manner. Two working surrogates was prepared corresponding to 1000 ng/g containing ciprofloxacin-d8, clenbuterol-d9, albendazole-d3, dimetridazole-d3, flunixin-d3, sulfamerazine^{13}-C6, chloramphenicol-d4 and carprofen-d3 at 5,000 ng/mL in acetonitrile and the second surrogate solution of penicillin-d7 at 1000 ng/mL in water.

Extracted Matrix Calibrants and Recovery Control Checks for Regulatory Analysis

Extracted calibration standards were prepared by spiking 2.00 (± 0.10) grams of the appropriate negative control muscle tissue and taking the fortified tissue through the extraction procedure. The fortified extracted calibration standards were spiked at the beginning of the extraction to correspond to analyte concentrations of 1/4X, 1/2X, 1X, 2X, 5X, and 10X by adding the volume shown in Table 1, where X is equal to 5 ng/g for all compounds with the exception of tetracyclines, β-lactams, bacitracin, carprofen, mefenamic acid, tolfenamic acid, and meloxicam, refer to Table 3 for more details. The two surrogate working solutions (50 µL) were added to all samples and controls prior to extraction corresponding to a concentration of 25 ng/g for these surrogate compounds in the sample matrix. Deuterated surrogates were added to the beginning of the extraction procedure to monitor for losses during extraction and sample cleanup. Also, deuterated surrogates were used to monitor matrix enhancement and suppression for the specific classes of compounds.

Note: for routine regulatory sample analysis, only calibrant 3 at the 1X level was prepared for initial workflow qualitative determination. To quantify suspect positive samples, calibrants 2-6 would be prepared for a subsequent analysis as described in more detail later.

Table 1: Fortified Extraction Calibrants

Calibrant	Control tissue weight (g)	Volume (µL) of working solution added	Concentration (ng/mL) working solution	Final Conc (ng/g) for most analytes	Final Conc (ng/g) Tetracyclines, β-lactam, Bacitracin, Carprofen, Mefenamic acid, Tolfenamic acid, and Meloxicam
1	2.00	25	100/500	1.25	6.25
2	2.00	50	100/500	2.50	12.5
3 (1X Level)	**2.00**	**10**	**1000/5000**	**5.00**	**25**
4	2.00	25	1000/5000	10.0	62.5
5	2.00	50	1000/5000	25.0	125
6	2.00	100	1000/5000	50.0	250

Sample Preparation

Samples collected fresh were kept cold before and during shipping to the laboratory. Once received at the laboratory, samples were frozen (< -10°C) prior to grinding, if they could not be prepared on the day of receipt. Once frozen, the sample was allowed to thaw, while keeping it as cold as possible. If the bison matrix contained an excessive amount of fat and connective tissue some was dissected from the sample to ensure homogeneity. The tissue was homogenized in a Robot Coupe food processor with dry ice until homogeneous consistency was achieved. Samples were stored frozen (< -10°C) prior to analysis for at least 12 hours for the dry ice to sublime.

Extraction Procedure

Homogenized matrix was weighed out (2.00 ± 0.10 g) into a 50 mL centrifuge tube and 50 µL of both surrogate solutions were added to all samples. Working standard solutions were added to the fortified samples, ACN/water (12 mL, 80:20) was added to each sample. Samples were shaken on the Geno Grinder for 5 minutes at 500 rpm, then centrifuged at for 5 min at 6000 rpm 5°C. The solution supernatant was decanted into a 50-mL graduated polypropylene centrifuge tube containing 500 mg of end-capped C18 dispersive sorbent. Samples were vortexed for 25 seconds, then centrifuged at 6000 rpm for 10 minutes at 5ºC. A portion (10mL) of the extract was transferred to glass tubes for evaporation under nitrogen flow at 50°C to a volume that is less than 0.70 mL. The extracts are not brought to dryness to prevent the loss of the tetracyclines and β-lactams. To the reduced extract, 100 µL of acetonitrile and 100 µL of 0.1% formic acid was added to all tubes and the volume was diluted to 1 mL with water (by weight, where 1 mL is equivalent to 1 gram). All tubes were vortexed for 5 seconds and 0.50 mL of the final extracts were transferred to the UniPrep filter. The filter was pressed to fill the sampling vial, then the vials were analyzed via LC-MS/MS.

Instrumentation

A SCIEX 5500 QTrap mass spectrometer coupled with an Agilent 1260 series liquid chromatography (LC-MS/MS) system was used for this analysis**.** Electrospray ionization was used with polarity switching, as 7 compounds required analysis in the negative mode. The selection of whether positive or negative mode was to be used for a specific compound was determined by reviewing previously published articles.[12,13,17,19,20] At the time of this initial work, there were not many articles using the SCIEX instrumentation hence; all compounds were infused to determine the optimal MS/MS parameters such as declustering potential, collision energies, and collision entrance potential. The compound optimization program was used, and each analyte was diluted with 50/50 of Mobile Phase A and B. The infusion solution included ammonium formate at 5 mM to evaluate if any ammonium adduct ions were formed with the use of the mobile phase. The analyte concentration for infusion varied from 10 ng/mL to 100 ng/mL. The section below describes the LC-MS/MS conditions used for the method validation and sample analysis.

a) *LC-MS/MS system* – The 5500 QTRAP hybrid quadrupole linear ion trap is a combination system in which the final quadrupole can operate as conventional mass filter or as linear ion trap with axial ion ejection. For the purpose of this method, the instrument was operated in triple quadrupole mode and calibrated per the manufacturer's instructions. The analyses were performed using electrospray ionization in both positive and negative mode (switching) using multi reaction monitoring with a 60-s retention time (t_R) window, 0.2-s target scan time, 5-ms pause, and 50-ms settling time. SCIEX Analyst 1.6.2 software was used for instrument control and Multi Quant 3.0 for data processing. The instrument conditions were as follows: ion spray voltage, +4500 V/-4500 V; curtain gas, 20 (arbitrary units); GS1 and GS2, 50 and 60, respectively; probe temperature, 400 ºC. The entrance potential (EP) was +10/-10 for all analytes. Nitrogen served as sheath gas and collision gas with a CAD gas setting of medium. MRM experiments allowed the maximum sensitivity to be obtained for the detection of the target molecules. The optimization of MS parameters declustering potential (DP), collision cell entrance potential (CEP) for precursor ions and collision energy (CE), collision cell exit potential (CXP) for product ions was performed by compound optimization. Appendix A shows the values of the parameters optimized and the MRM transitions used for the confirmation and quantification of veterinary drug residues.

b) *HPLC system* – The Agilent 1260 HPLC system was equipped with pump, solvent degasser, column oven, and CTC autosampler. A Waters XSelect HHS T3, 150 mm x 3 mm, 2.5 µm, 100A column was used and kept at 40ºC oven temperature. The pump was operated at a flow rate of 0.45 mL/min. A binary gradient system was used to separate analytes comprising mobile phase A, 5 mM ammonium formate and 0.1% formic acid in water, and mobile phase B, 5 mM ammonium formate and 0.1% formic acid in methanol. The gradient profile was: (1) 0-1.0min, 95% A; (2) 1.00-15.0 min, gradient to 5.0% A; (3) 15.0-25.0 min, hold at 5% A. The column was then re-equilibrated with the initial conditions for 5 minutes. The CTC injection volume was 10 µL and the autosampler temperature at 5ºC. A programmed needle wash with 95% water/5% acetonitrile followed by a wash with 5% water/95% acetonitrile was used to minimize injection carryover.

RESULTS and DISCUSSION

Determination of Analytes and Target Test Levels in Game Meats

Prior to the development of this method, the FDA did not have a multiclass, multiresidue analysis program to screen for veterinary drug compounds in domestic game meats (bison, deer, elk, and rabbit). Beginning in 2012, DENL worked directly with project leads at CFSAN and ORA/ORS to develop a list of suitable analytes, testing levels, and analytical strategies for the determination of the veterinary drugs in the different game meat matrices. The list of veterinary drug analytes to include in the method was based on combining analytes from single drug class regulatory methods. After reviewing the scientific literature and comparing the merits of several methods intended for residue screening in muscle matrix samples, it was found that none were intended for game meat analysis. DENL reviewed MRLs from the EU and Canada, the US tolerances, and the Codex Alimentarius to determine which if any analytes had required testing level in bison, deer, elk, and rabbit. At the time of this validation, required testing levels were not found for bison, deer, elk, or rabbit for any of the analytes validated in this method. Therefore, analyte validation levels were determined by method performance and monitored with multiresidue method.

Method Development

The objective of this project was to develop a sensitive and rapid method for the qualitative and quantitative determination of a wide range of veterinary residues in muscle from different types of game. The performance of the method was tested with respect to selectivity, linearity, recovery, repeatability, ruggedness, reproducibility, matrix effects, consistent retention times (t_R), and MS/MS product ion ratios during the method development process. Compared to the reference method,[17] specific parameters were modified to optimize the method for specific compounds and for the game meat matrix including: 1) mobile phase composition, 2) particle size and length of column, 3) removal of hexane cleanup, and 4) use of an extracted matrix-matched calibration curve. Geis-Asteggiante et al.[17] used a novel post-column infusion of 27 mM ammonium formate in MeOH:ACN (75:25) towards the end of the analytical run to enhance the

response for the anthelmintic class of compounds which compromised the response of earlier eluting compounds. This was attempted by DENL using the same conditions reported by Geis-Asteggiante et al.[17] Our method development initially included the avermectin anthelmintic compounds for bison liver and muscle matrices. Ivermectin has a tolerance of 15 ng/g in bison liver[6] but even with the post-column infusion, the lowest concentration in the solvent standard was 25 ng/mL while scanning for both the Na+ and NH_4+ adduct ions. The method was therefore not suitable to quantify ivermectin at the 15 ng/g tolerance level in bison liver. To increase the method sensitivity for ivermectin, other possible mobile phases were investigated. Water and methanol with 5 mM ammonium formate and 0.1% formic acid in both mobile phases were evaluated to determine if the response for the ammonium adduct ion for ivermectin could be increased. From these investigations, the use of methanol in the modified mobile phase improved the separation of the sulfonamide, fluoroquinolone, and quinolone compounds (Figure 1) permitting polarity switching and ensuring enough data points[18] were collected for each analyte for qualitative and quantitative determination. The improved chromatography and polarity switching permitted the use of one analytical run instead of two separate analytical runs for all 112 analytes (Figure 2). It was eventually determined that ivermectin could not be accurately determined at the 15 ng/g level. All of the avermectin compounds were removed from this multiresidue method and a different method was validated for the determination of avermectin class residues in bison liver and bison, deer, elk, and rabbit muscle (LIB 4644[9]).

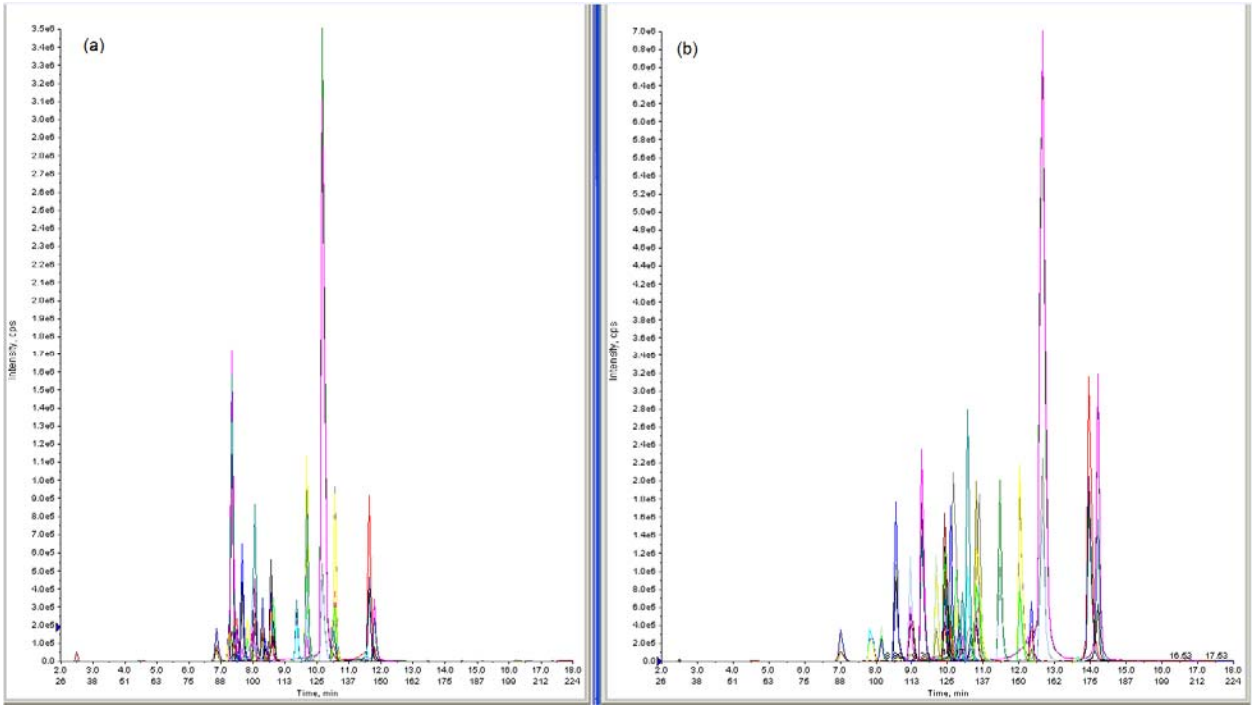

Figure 1: Example of the separation of sulfonamide, quinolone, and fluoroquinolone compounds using the (a) original mobile phase and the (b) new mobile phase. The original mobile phase was water/ACN with 0.1% formic acid and the new mobile phase was water/MeOH with 5 mM ammonium formate and 0.1% formic acid. The same gradient was used for both (a) and (b).

The Geis-Asteggiante et al.[17] method was based on using UHPLC system with column particle size of 1.8 µm, but DENL only had a traditional HPLC system available. DENL invested in an HPLC column with the same manufacturer and phase (HSS T3) with the particle size of 2.5 µm, but the column length was increased from 100 mm to 150 mm while the diameter was increased from 2.1 mm to 3.0 mm. The flow rate, gradient, and injection volume were optimized for the DENL modified column and HPLC system. The resulting chromatogram for the optimized HPLC separation is shown in Figure 2 with positive and negative analytes shown separately.

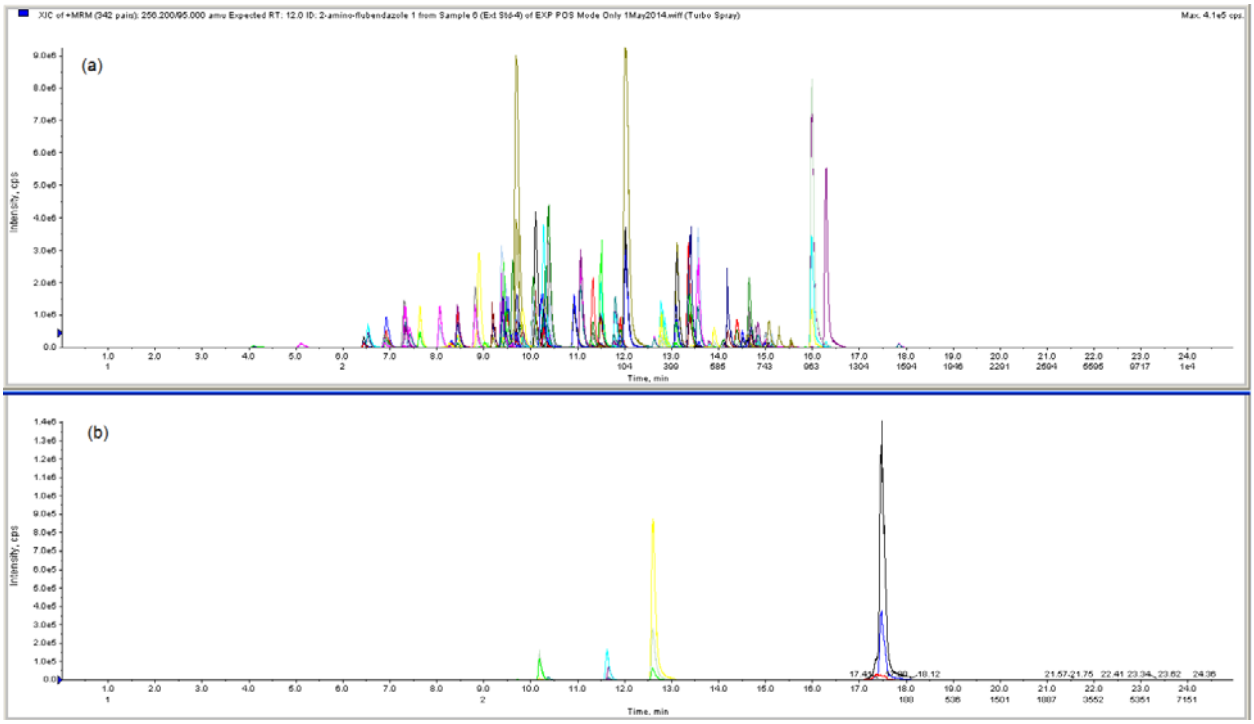

Figure 2: Chromatograms for the (a) positive mode and (b) negative mode analytes with the new mobile phase and HPLC column.

The Geis-Asteggiante et al. method[17] sample preparation procedure was modified by eliminating the liquid-liquid extraction step with hexane to remove fat. This modification reduced sample processing time and allowed modification of the extraction step to improve method detection limits. The fat and protein content of bovine muscle was compared to the four game meat muscle matrices (Table 2). Game meat muscle contains considerably less fat content than bovine, hence the removal of the hexane defatting step did not adversely affect matrix effects or analyte recovery.

The method workflow was also divided into two different processes: 1) a qualitative identification screening method using an extracted matrix-matched 1X calibrant and 2) a quantitative determination of presumptive positive samples resulting from workflow 1. This approach enabled the laboratory to screen out samples that were determined not to have any residue above a calculated limit test threshold. DENL was able to analyze more than one matrix in the same day, increasing the throughput of regulatory samples analyzed per day. For example, bison and elk could be analyzed in the same batch with each matrix having one extracted matrix-matched 1X calibrant, negative control, 4 QC samples, and at least 5 regulatory samples. This would result in a total of 22 extracts. The set of extracts for each matrix can be queued sequentially in the analytical run. This number of extractions could be accomplished in under 3 hours by one analyst.

Table 2: Calories, fat, and protein in muscle from various animals[21]

Meat (per 100 g)	Calories (kcal)	Total Lipid (g)	Protein (g)
Beef, ground	260	16.8	26
Bison	142	2.4	28
Venison	190	3.9	36
Elk	146	1.9	30
Rabbit	205	8.4	30

Compared to the Geis-Asteggiante et al. method,[17] a few analytes in that method demonstrated poor performance with the quantitation and qualifier ions not passing ion ratio criteria or the analytes had low to

no recovery at the specific level of validation. Furthermore, compounds such as 2-thiouracil, and sulfanilamide demonstrated poor chromatography due to early elution within the first 4 minutes of the chromatographic analytical run. The NSAID, vedaprofen, demonstrated poor recovery. Some compounds included in the reference method[17] were not included in ours because the standards were not readily available to DENL. Poorly performing analytes or unavailable compounds were not included in our validation.

Method Validation

Initially, our intent was to develop a fast screening method for the analytes listed in Table 3, with method performance being independent of the different muscle matrices. Hence, when screening samples, a general reference matrix such as bison, was investigated to generate data for the other three matrices in an approach similar to a technique used for semi-quantitative screening for veterinary drugs in aquaculture[22]. Initially, bison was selected as the reference matrix and testing was conducted with and without internal standards. Unfortunately, this approach did not work as demonstrated by the anthelmintic class compounds in rabbit muscle having 150% - 200% recovery when compared to the bison matrix. The reference matrix approach was abandoned in favor of matrix-matched calibrants.

The qualitative and quantitative method was validated according to FDA Foods Program[18]. The analyte concentration levels tested in the method validation were lower than those described in the published reference methods.[12,13,17,19,20] The 1.0 VL validation level concentration was set for each compound as described in Table 3, and the calibration levels ranged from 1.25 to 50 ng/g for most compounds. β-Lactams, tetracyclines, bacitracin, carprofen, mefenamic acid, meloxicam, and tolfenamic acid had higher concentration for the 1.0 VL and correspondingly higher calibration range from 6.25 to 250 ng/g (Table 3).

Three validation levels were tested corresponding to concentrations of 0.5 VL, 1.0 VL, and 2.0 VL. The lowest validation level for each analyte was selected based on sensitivity and quantification ability determined by the initial method development and using the FDA method performance criteria.[18,23] Validation studies were carried out on muscle tissue (bison, deer, elk, and rabbit). Negative control matrix sources were verified to be free of veterinary drug residues prior to validation. Matrix matched extracted calibration curves were analyzed for each of the four matrices tested. Over 900 calibration curves were analyzed for the full validation of the four matrices and each matrix was tested over a two-day period. All calibration curves were generated with the ABI MultiQuant software, with linear curve fit selected (not forced through zero). The correlation coefficients (r^2) ranged from 0.9915 to 1.000, which is excellent linearity compared to other multiclass, multiresidue veterinary drug methods. The method detection level (MDL), limit of quantification (LOQ), screening threshold, accuracy and precision for all four game meat matrices, (n=15 for each validation level) is summarized in Table 3. The MDL was calculated as the standard deviation of the replicates at the 0.5 VL concentration multiplied by the Student's t value at the 99% confidence level, and LOQ was the standard deviation multiplied by 10. Figure 3 is a graphical representation of the accuracy and precision for the bison, deer, elk, and rabbit validation data.

In addition to the 0.5 VL, 1.0 VL, and 2.0 VL concentration levels for the quantitative workflow, the method was also validated for the screening workflow at the 1X (1.0 VL) concentration level, Table 3. The 1X level is the standard testing level for most compounds in the game meat analysis program. Routine regulatory qualitative determination for veterinary residues in game meat samples is performed at the 1X testing level by the screening workflow as described in later sections.

Finally, additional method optimization was required to distinguish two of the corticosteroid compounds. Betamethasone and dexamethasone are epimers, where the only structural difference is the orientation of a methyl group. Under the current chromatographic conditions, these compounds coelute as the retention times did not vary by more than 0.5 minutes and they have similar fragmentation parameters. However, when determining the fragmentation of each compound during compound optimization, adjusting the collision energy for m/z 165 and m/z 171 yield different ion ratios. This provided a way to distinguish, to some degree, which corticosteroids was present. If a regulatory sample was positive and the specific analyte was required for betamethasone and dexamethasone, a more selective analytical method would be required to determine which corticosteroid was present.

Qualitative Screening and Identification

DENL employed the same qualitative screening identification criteria described by Geis-Asteggiante et. al.[17] and the FDA CVM 118[23]. As the goal of the testing program was to minimize the number of suspect positive samples reanalyzed by full quantitation, the below criteria must be met:

- The retention time for the analyte chromatographic peak in the sample is within ± 5% of the retention time chromatographic peak relative to that of the standard.
- The chromatographic peak should exceed a signal-to-noise (s/n) threshold of 3:1. The SCIEX MultiQuant software is used to calculate signal to noise, if required.
- Two ion ratios for the analyte in the sample are $\leq$ I20%I or one ion ratio is $\leq$ I10%I of the average ion ratios for the calibration standards analyzed in the same sequence.
- Negative control and reagent blanks do not contain a positive identification for the analyte (i.e. no lab contamination or carryover).

Even with these criteria, human review of the automated peak integration assisted in the elimination of false positive samples.[13] DENL observed similar possible interference for cimaterol in bison muscle as did Geis-Asteggiante et al.[17] for bovine muscle. Figure 3 shows the interference peak that the processing software can integrate for cimaterol in bison muscle. However, the second and third product ion transition did not meet the criteria to yield a presumptive positive.

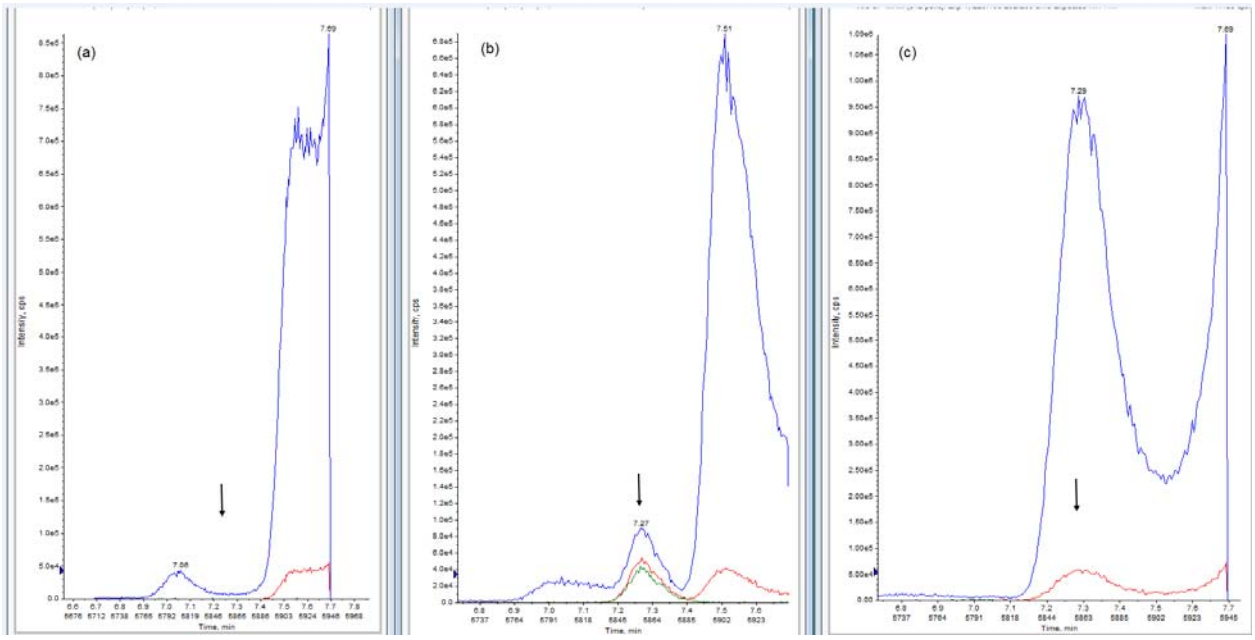

Figure 3: Bison matrix interference for cimaterol: (a) bison muscle negative control; (b) bison negative control fortified with cimaterol at 5 ng/g (three product ion transitions present); (c) regulatory bison sample with large matrix peak near the retention time for cimaterol. The matrix peak only has two of the three product ions present and not in the correct ratio).

In addition, to the criteria above, presumptive positive residues must have a response at or above the calculated threshold level. The threshold level for the screening workflow is determined by the limit test. Table 3 shows the screening threshold values (ng/g) that were determined at the 1.0 VL (1X) level from 10 replicates of fortified samples in each matrix. The qualitative threshold values were calculated from the quantitative product ion transition for each analyte in each matrix per the FDA Foods Program[18] by the following equation:

$$\text{Threshold value} = [\text{mean concentration} - (t * S)]$$

Where t = one-tailed student's t value for n=1 degrees of freedom at the 95% confidence level and as above, S is the standard deviation.

If a sample passes all the qualitative identification criteria and has an estimated concentration greater than or equal to the limit test threshold stated in Table 3, the sample will be re-extracted and analyzed with a full set of matrix-matched extracted calibrants for quantitative determination.

Quantitative Analysis

The main objective of this work was to develop a screening/identification workflow supported by quantitative determination to evaluate regulatory game meat samples for the presence of veterinary drug residues. The Geis-Asteggiante et al. method[17] included an atrazine surrogate that was added post extraction to monitor analyte loss from the filtration step. Two internal standards (ISTD) were added at the beginning of the extraction, sulfamethazine-$^{13}C_6$ and flunixin-d_3, but the ISTD were not used to calculated recoveries or to compensate for matrix effects. For the method developed by DENL, deuterated compounds for most drug classes were included and used as surrogates. These surrogates were used to evaluate extraction and cleanup for the specific veterinary drugs classes but were not used to correct recoveries or compensate for matrix effects. Table 4 and Appendix B lists the deuterated surrogates used for each specific veterinary drug class.

The USDA QC guidelines[13,17,19,20] allowed the following criteria for quantitative analysis: recovery range from 70%-120% with an average RSD_r<20% and RSD_R <25%. The USDA method was for bovine muscle analysis, including US tolerance levels and had the lowest validation level ranging from 5 to 500 ng/g. Most of the tolerance levels validated were near the 50 ng/g, an order of magnitude higher than the DENL 1X validation level at 5 ng/g. The game meats do not have residue tolerances; hence, the DENL method was validated at lower levels than specified in the USDA method. The recovery and precision criteria used by the USDA were too stringent for the game meat method since the method had sensitivity at least one order of magnitude lower for more than 85% of the compounds. Compounds with a USDA validation level similar to the DENL method were salbutamol, cimaterol, clenbuterol, chloramphenicol, and zeranol, however, these compounds did not meet the USDA performance criteria in bovine muscle.

The FDA method performance criteria[18,23] specifies recoveries based on the testing level, i.e. a 1 ppb (ng/g) testing level has an acceptable recovery ranges from 40%-120% with an RSD_r of 22%, and a 10 ng/g testing level has recoveries ranging from 60%-115%. The 1X validation target testing level was predominantly 5 ng/g, which is between these levels. Hence, the 40%-120% range was used to evaluate method performance. Some individual analytes were found to have broader variability than the desired repeatability precision of RSD_r < 22%.[18] Figure 4 is a graphical representation of the precision versus recovery (accuracy).

While method performance was usually within the desired criteria[18] for these analytes and matrices on a single day of analysis, the greater variability in accuracy and precision reported should be considered to be characteristic of the method performance. As these analytes do not have specified tolerances in the tested matrices, the reported accuracy and precision is sufficient for monitoring these residues in game meat samples.

The surrogates were used to assess extraction and cleanup for specific drugs classes and used as a QA/QC tool to ensure the recovery of analytes in the sample was sufficient. Table 4 is a summary of the performance statistics for the surrogates. The surrogates performed similarly to the native drug residues (Table 3), in some cases demonstrating high variability (RSD_r>22%). For this method, higher variability is considered acceptable method performance, and RSD<40% should be considered an upper limit for acceptability

Table 3: MDL, LOQ, screening threshold, accuracy and precision for all 4 game meat matrices (n=15 for each validation level).

		Bison						Deer						Elk						Rabbit					
Analyte	**1X ng/g**	**MDL ng/g**	**LOQ ng/g**	**Screening Threshold ng/g**	**0.5 VL**	**1.0 VL**	**2.0 VL**	**MDL ng/g**	**LOQ ng/g**	**Screening Threshold ng/g**	**0.5 VL**	**1.0 VL**	**2.0 VL**	**MDL ng/g**	**LOQ ng/g**	**Screening Threshold ng/g**	**0.5 VL**	**1.0 VL**	**2.0 VL**	**MDL ng/g**	**LOQ ng/g**	**Screening Threshold ng/g**	**0.5 VL**	**1.0 VL**	**2.0 VL**
β-Agonists (5)																									
Cimaterol		1.0	3.4	4.3	102 (13)	103 (9)	96 (7)	0.5	1.8	3.8	96 (8)	98 (16)	101 (17)	0.9	3.1	4.7	108 (9)	120 (8)	117 (7)	0.8	2.8	4.3	94 (13)	106 (9)	95 (6)
Clenbuterol		0.6	2.1	4.9	109 (7)	110 (8)	98 (8)	1.7	5.9	3.0	100 (24)	100 (25)	90 (19)	0.7	2.3	3.4	94 (7)	104 (16)	101 (12)	1.0	3.5	3.9	103 (15)	108 (10)	96 (9)
Ractopamine	5.00	0.4	1.2	5.2	101 (4)	107 (8)	93 (6)	1.3	4.5	4.2	99 (18)	105 (15)	89 (13)	0.7	2.6	2.9	77 (10)	99 (21)	103 (15)	1.0	3.0	4.0	126 (5)	121 (17)	104 (8)
Salbutamol		0.7	2.5	4.8	91 (11)	108 (8)	100 (5)	0.7	2.3	4.8	89 (10)	100 (6)	97 (8)	0.8	2.7	4.5	104 (8)	117 (9)	110 (7)	0.8	3.3	4.9	101 (3)	117 (13)	111 (17)
Zilpaterol		0.9	3.1	5.3	111 (11)	109 (8)	98 (6)	0.5	1.9	4.5	89 (9)	97 (8)	98 (13)	0.9	3.0	4.2	102 (7)	113 (7)	113 (7)	0.8	2.6	4.5	94 (15)	91 (12)	83 (5)
Anthelmintics (23)																									
Albendazole		0.9	3.1	3.9	93 (13)	105 (16)	107 (9)	1.5	5.4	2.8	89 (24)	90 (24)	90 (15)	0.8	2.7	3.3	98 (9)	92 (12)	110 (7)	1.1	3.7	3.4	89 (13)	118 (13)	113 (7)
Albendazole Sulfone		1.9	6.7	3.8	93 (29)	121 (22)	115 (23)	1.1	3.9	4.5	96 (16)	94 (9)	103 (11)	0.6	2.5	2.6	86 (7)	102 (14)	107 (10)	0.7	4.5	3.6	77 (18)	125 (22)	118 (19)
Albendazole Sulfoxide		0.6	2.0	5.0	99 (8)	107 (7)	94 (8)	1.3	4.8	3.7	96 (20)	98 (17)	94 (18)	1.3	4.4	4.0	89 (17)	111 (12)	106 (9)	0.8	3.5	4.2	92 (10)	107 (6)	98 (10)
Bithionol		0.8	1.8	1.8	96 (12)	76 (24)	82 (9)	1.1	3.9	4.2	93 (18)	91 (12)	77 (18)	0.7	2.4	3.2	80 (9)	88 (12)	94 (18)	0.9	3.1	3.1	86 (16)	82 (25)	88 (5)
Cambendazole		1.4	4.9	4.4	83 (24)	103 (11)	102 (11)	1.6	5.4	3.6	95 (23)	95 (17)	96 (23)	0.6	2.2	4.0	93 (7)	107 (10)	100 (14)	0.4	1.5	4.4	66 (9)	98 (11)	100 (10)
Carbendazim		0.5	1.7	5.1	98 (7)	111 (9)	104 (4)	1.0	3.4	3.9	101 (13)	96 (14)	97 (20)	0.8	2.7	3.6	100 (8)	110 (15)	100 (14)	0.7	2.7	4.2	100 (8)	110 (15)	100 (14)
Clorsulon		0.5	1.7	4.9	77 (9)	101 (8)	94 (9)	1.5	5.2	4.0	86 (24)	92 (9)	97 (18)	1.2	4.3	3.2	94 (14)	99 (17)	84 (16)	0.9	3.2	3.5	86 (15)	96 (10)	95 (7)
Closantel		0.6	2.0	1.8	74 (11)	95 (26)	80 (14)	0.9	3.2	4.2	102 (13)	99 (12)	87 (21)	0.5	1.9	3.7	88 (6)	107 (13)	92 (13)	0.7	3.4	4.2	83 (13)	95 (26)	87 (7)
Fenbendazole		1.3	4.6	4.8	96 (20)	97 (12)	91 (8)	0.6	2.0	4.3	90 (9)	95 (7)	92 (6)	0.5	1.8	4.6	92 (8)	98 (4)	98 (18)	0.4	1.3	3.7	93 (5)	97 (12)	91 (8)
Fenbendazole Sulfone		0.8	2.7	4.9	92 (12)	97 (11)	88 (7)	1.5	5.4	3.2	100 (22)	91 (21)	88 (21)	0.8	2.7	3.4	96 (9)	86 (13)	102 (8)	1.0	3.6	3.8	95 (15)	94 (18)	90 (5)
Flubendazole		1.0	3.7	5.1	84 (19)	93 (22)	92 (13)	1.6	5.5	3.1	91 (24)	82 (15)	88 (22)	1.3	4.2	4.2	87 (21)	90 (12)	93 (23)	1.3	4.5	3.1	89 (13)	87 (16)	90 (8)
Flubendazole Amine	5.00	1.0	3.4	4.7	79 (17)	109 (11)	97 (13)	1.2	4.4	3.1	89 (20)	89 (19)	96 (36)	1.2	5.3	4.6	84 (24)	93 (21)	93 (25)	0.8	3.4	3.2	90 (16)	110 (10)	116 (21)
Haloxon		0.5	1.9	3.7	76 (10)	92 (17)	103 (24)	1.4	4.9	3.7	98 (20)	95 (16)	96 (16)	0.6	2.1	3.7	101 (6)	103 (12)	97 (11)	0.8	3.0	3.7	95 (18)	96 (12)	87 (12)
Mebendazole		0.9	2.7	4.7	94 (12)	99 (13)	94 (8)	0.9	3.2	4.9	92 (14)	80 (18)	104 (9)	1.0	3.4	4.0	97 (11)	108 (10)	101 (8)	0.9	3.2	4.3	88 (8)	83 (19)	97 (12)
Morantel		0.8	2.9	4.9	105 (11)	111 (11)	98 (6)	1.6	5.8	3.6	101 (23)	93 (16)	96 (17)	0.7	2.3	3.5	90 (8)	99 (13)	102 (10)	1.0	3.7	3.9	94 (13)	96 (12)	94 (3)
Niclosamide		0.5	1.6	3.7	101 (6)	88 (14)	90 (10)	0.9	3.1	4.3	102 (12)	95 (9)	89 (20)	0.5	1.9	3.3	103 (6)	96 (13)	100 (11)	0.6	2.2	3.4	95 (5)	94 (13)	82 (12)
Nitroxynil		0.8	2.9	3.3	78 (15)	86 (15)	88 (22)	0.8	2.9	3.3	103 (11)	113 (25)	82 (13)	0.7	2.9	3.9	92 (8)	109 (12)	103 (17)	0.8	2.2	3.1	84 (11)	99 (22)	93 (10)
Oxfendazole		0.5	1.9	4.6	97 (8)	102 (6)	92 (8)	1.7	6.0	3.9	107 (22)	100 (16)	86 (14)	0.7	2.2	3.9	94 (7)	103 (9)	106 (9)	1.1	2.4	4.2	84 (6)	99 (5)	100 (16)
Oxibendazole		0.9	3.3	4.3	89 (15)	104 (13)	118 (13)	0.8	2.7	3.4	97 (11)	95 (19)	98 (11)	0.7	2.5	2.8	84 (9)	90 (20)	104 (14)	0.8	2.8	3.5	92 (12)	96 (18)	105 (7)
Oxyclozanide		0.5	1.6	3.9	77 (8)	79 (8)	100 (14)	0.7	2.4	4.0	89 (11)	97 (14)	89 (12)	1.3	4.6	4.1	107 (13)	112 (11)	89 (12)	0.8	2.9	4.4	92 (6)	94 (15)	94 (16)
Rafoxanide		0.5	1.7	4.9	84 (8)	99 (14)	107 (15)	0.7	2.6	4.4	95 (11)	96 (9)	94 (20)	0.6	2.0	3.8	94 (7)	109 (13)	119 (15)	0.6	2.1	4.0	78 (3)	89 (8)	101 (10)
Thiabendazole		0.5	3.0	5.2	101 (12)	116 (6)	110 (6)	1.1	3.8	4.4	99 (15)	108 (14)	102 (16)	0.8	2.6	4.0	98 (8)	113 (12)	110 (11)	0.8	2.9	4.9	84 (9)	105 (6)	96 (3)
Triclabendazole		0.8	3.2	3.3	70 (16)	80 (25)	111 (6)	1.3	4.5	3.9	96 (19)	91 (12)	97 (13)	0.7	2.4	3.8	95 (8)	100 (9)	116 (9)	0.9	2.8	3.7	81 (7)	84 (15)	103 (10)
Anti-inflammatory Drugs (12)																									
Carprofen	25.0	1.7	5.9	15	98 (6)	91 (12)	76 (12)	2.5	6.2	18	98 (6)	91 (12)	76 (12)	3.8	6.3	17	109 (7)	116 (8)	103 (17)	2.6	6.4	17	109 (7)	116 (8)	103 (17)
Diclofenac	5.00	1.2	4.4	3.7	90 (19)	84 (8)	70 (16)	1.0	3.6	3.2	87 (17)	92 (16)	92 (16)	1.3	2.5	3.6	101 (14)	104 (13)	104 (15)	1.2	3.7	3.5	93 (12)	92 (9)	83 (18)
Etodolac		1.3	4.2	4.5	84 (22)	93 (13)	80 (14)	1.0	3.4	3.1	97 (14)	90 (21)	87 (15)	1.2	4.2	3.4	90 (14)	101 (14)	96 (8)	1.2	4.0	3.7	90 (13)	94 (16)	86 (11)
Flunixin		0.5	1.8	4.9	84 (8)	112 (7)	116 (10)	1.0	3.6	4.2	93 (15)	103 (14)	94 (14)	0.4	1.4	3.5	90 (5)	103 (14)	109 (10)	0.6	2.3	4.2	86 (14)	105 (8)	101 (2)
Ketoprofen		0.7	2.8	4.4	94 (11)	91 (9)	83 (14)	1.3	4.7	3.4	96 (20)	91 (17)	93 (18)	1.2	4.0	4.5	103 (11)	115 (8)	109 (7)	1.1	3.8	4.1	91 (6)	94 (9)	87 (16)
Mefanamic Acid	25.0	2.0	5.3	4.6	89 (14)	83 (12)	92 (15)	2.3	8.1	3.0	94 (34)	79 (17)	86 (30)	0.8	2.8	3.3	94 (9)	107 (19)	108 (16)	1.7	5.4	3.9	76 (16)	85 (16)	68 (28)
Meloxicam		0.6	2.2	3.7	90 (10)	87 (11)	77 (6)	1.9	6.6	2.4	102 (26)	93 (29)	81 (9)	0.8	5.3	3.5	96 (14)	115 (8)	109 (7)	1.1	4.7	3.2	88 (11)	54 (10)	82 (6)
Naproxen	5.00	1.0	3.5	4.7	106 (13)	102 (10)	82 (19)	1.9	6.6	3.5	95 (29)	97 (19)	85 (13)	1.4	3.9	3.1	76 (12)	94 (15)	107 (9)	1.4	4.7	3.8	84 (17)	90 (15)	93 (16)
Niflumic Acid		0.5	1.7	4.9	80 (10)	98 (11)	102 (19)	1.6	5.8	5.4	101 (23)	98 (15)	96 (15)	0.6	2.4	4.8	99 (12)	92 (10)	89 (13)	0.9	3.3	5.0	97 (10)	96 (13)	91 (14)
Phenylbutazone		0.8	2.8	4.0	93 (12)	83 (15)	77 (17)	1.5	5.3	2.7	106 (20)	93 (26)	81 (13)	1.0	3.7	2.4	76 (15)	85 (21)	100 (10)	1.1	3.9	3.0	83 (9)	86 (12)	96 (17)
Suxibuzone		1.2	4.2	4.3	85 (20)	94 (20)	92 (20)	1.4	4.9	3.7	99 (20)	97 (16)	78 (11)	1.0	3.4	3.7	98 (11)	106 (13)	101 (20)	1.2	4.0	3.0	93 (16)	92 (22)	86 (12)
Tolfenamic Acid	25.0	0.8	3.0	3.3	93 (13)	83 (19)	86 (24)	1.4	5.0	3.4	93 (21)	87 (16)	97 (18)	0.6	2.2	3.7	87 (8)	96 (8)	97 (14)	1.1	3.7	3.5	101 (15)	80 (24)	93 (12)
Corticosteroids/Hormones (3)																									
Betamethasone		0.7	2.4	4.6	101 (9)	108 (9)	88 (9)	0.8	2.9	3.9	106 (11)	89 (23)	90 (11)	0.9	3.0	4.0	82 (11)	100 (8)	100 (21)	0.8	2.7	3.8	103 (7)	115 (5)	94 (6)
Dexamethasone	5.00	0.9	3.2	4.5	90 (14)	105 (11)	93 (11)	1.0	4.0	5.0	96 (15)	92 (12)	79 (15)	1.2	4.2	3.6	102 (13)	102 (13)	94 (21)	1.1	3.7	4.0	94 (13)	99 (16)	84 (6)
Prednisone		0.7	2.6	4.3	99 (11)	100 (10)	88 (6)	0.8	2.7	3.5	96 (12)	90 (16)	90 (15)	0.9	3.2	3.2	90 (11)	105 (10)	110 (7)	0.8	2.9	3.7	93 (16)	99 (10)	96 (12)
Fluoroquinolones (11)																									
Ciprofloxacin	5.00	0.5	1.8	5.1	104 (11)	103 (5)	93 (5)	1.1	3.9	2.6	85 (18)	90 (26)	82 (20)	0.8	2.8	3.2	93 (9)	100 (17)	89 (13)	0.8	2.2	3.6	98 (4)	105 (4)	96 (2)
Danofloxacin		0.4	1.5	5.2	99 (6)	110 (6)	99 (6)	1.9	6.7	3.1	92 (30)	93 (21)	81 (21)	1.1	3.9	3.0	89 (13)	99 (19)	87 (16)	1.5	4.2	3.8	93 (4)	96 (10)	85 (6)
Difloxacin		0.6	2.1	5.3	103 (8)	110 (6)	100 (5)	1.3	4.7	3.1	98 (19)	97 (23)	82 (15)	1.2	4.2	3.1	98 (13)	102 (19)	102 (12)	0.6	2.1	5.3	94 (6)	88 (3)	97 (2)
Enrofloxacin		0.6	2.2	4.9	105 (9)	113 (7)	100 (6)	1.2	4.1	4.5	96 (17)	94 (22)	93 (15)	1.0	3.5	3.6	106 (10)	110 (16)	106 (16)	0.9	3.3	3.5	100 (4)	93 (9)	96 (10)
Flumequine		0.6	2.1	5.5	95 (9)	110 (9)	102 (5)	1.5	5.2	3.5	93 (22)	100 (22)	85 (25)	1.2	4.0	3.4	94 (13)	106 (16)	96 (13)	0.8	3.9	3.8	96 (7)	105 (3)	89 (6)
Nalidixic Acid		0.5	1.7	4.9	100 (7)	111 (7)	102 (5)	0.8	3.0	3.6	97 (12)	96 (17)	94 (19)	0.8	2.8	2.8	101 (9)	109 (15)	108 (11)	0.8	2.9	3.5	97 (7)	105 (14)	97 (6)
Norfloxacin		0.3	1.0	4.6	101 (4)	106 (7)	95 (6)	1.1	3.7	4.1	93 (16)	100 (14)	86 (15)	0.8	2.8	3.2	90 (9)	100 (16)	91 (11)	0.7	2.5	4.0	98 (8)	96 (16)	94 (4)
Ofloxacin		0.6	2.2	4.9	106 (8)	103 (5)	97 (5)	0.5	1.6	4.2	109 (6)	100 (13)	90 (13)	0.9	3.0	3.6	104 (9)	97 (14)	93 (10)	0.7	2.2	4.2	102 (11)	95 (6)	96 (12)
Orbifloxacin		0.5	1.9	4.8	104 (7)	103 (6)	89 (6)	1.1	3.8	4.7	103 (15)	104 (9)	96 (15)	1.2	4.3	3.6	101 (13)	106 (14)	98 (11)	1.0	3.3	4.4	104 (7)	99 (6)	90 (6)
Oxolinic Acid		0.8	2.7	5.1	91 (12)	109 (11)	109 (7)	0.9	3.2	6.5	104 (9)	110 (23)	93 (24)	1.4	5.0	3.9	100 (15)	114 (13)	112 (12)	0.9	3.7	4.2	93 (8)	94 (18)	101 (9)
Sarafloxacin		0.7	2.3	5.1	102 (9)	107 (6)	94 (9)	1.1	3.9	4.2	98 (16)	102 (14)	90 (21)	0.9	3.0	2.8	91 (10)	97 (20)	96 (11)	0.8	3.1	4.1	99 (2)	93 (13)	89 (7)

		Bison						Deer						Elk						Rabbit					
Analyte	**1X ng/g**	**MDL ng/g**	**LOQ ng/g**	**Screening Threshold ng/g**	**0.5 VL**	**1.0 VL**	**2.0 VL**	**MDL ng/g**	**LOQ ng/g**	**Screening Threshold ng/g**	**0.5 VL**	**1.0 VL**	**2.0 VL**	**MDL ng/g**	**LOQ ng/g**	**Screening Threshold ng/g**	**0.5 VL**	**1.0 VL**	**2.0 VL**	**MDL ng/g**	**LOQ ng/g**	**Screening Threshold ng/g**	**0.5 VL**	**1.0 VL**	**2.0 VL**
β-Lactams (8)																									
Amoxicillin	25.0	3.3	12	14	66 (14)	84 (6)	80 (10)	3.5	12	17	92 (13)	101 (12)	116 (12)	3.0	15	16	82 (22)	87 (16)	78 (18)	3.2	13	17	69 (11)	90 (11)	76 (5)
Ampicillin		2.4	9	14	88 (10)	80 (7)	78 (8)	4.2	15	11	87 (17)	94 (24)	98 (19)	3.0	11	13	94 (9)	91 (13)	81 (7)	3.2	11	13	75 (21)	88 (17)	82 (8)
Cloxacillin		3.2	11	14	69 (16)	77 (6)	69 (15)	3.2	12	12	81 (14)	103 (27)	88 (28)	3.2	11	14	93 (9)	98 (14)	94 (17)	3.2	11	13	71 (11)	82 (15)	75 (21)
Dicloxacillin		3.7	13	16	73 (18)	81 (14)	77 (8)	6.0	21	16	94 (23)	99 (16)	103 (16)	3.3	12	14	102 (9)	102 (14)	87 (11)	4.3	15	15	73 (18)	81 (14)	77 (8)
Nafcillin		3.1	11	18	87 (13)	106 (7)	74 (9)	4.4	15	18	81 (19)	105 (12)	100 (13)	3.7	10	13	100 (8)	90 (13)	108 (13)	3.7	12	16	92 (8)	100 (14)	74 (9)
Oxacillin		4.8	17	16	89 (19)	103 (12)	82 (7)	4.4	16	16	90 (18)	96 (14)	100 (12)	4.3	15	11	90 (13)	97 (20)	74 (14)	4.5	16	14	83 (14)	93 (9)	82 (7)
Penicillin G		5.0	18	15	91 (20)	94 (12)	84 (13)	6.9	25	13	98 (25)	94 (21)	107 (42)	3.5	12	15	95 (10)	105 (11)	113 (12)	5.1	18	14	87 (16)	90 (9)	99 (17)
Penicillin V		6.7	24	11	99 (24)	86 (18)	90 (14)	5.6	19	14	94 (21)	96 (19)	83 (11)	3.4	12	12	87 (11)	94 (17)	86 (13)	5.2	18	13	86 (20)	88 (14)	81 (16)
Macrolides (10)																									
Clindamycin	5.00	0.7	2.5	4.8	86 (12)	105 (9)	93 (6)	1.2	4.4	3.6	91 (20)	93 (16)	84 (18)	1.9	3.6	4.8	93 (18)	99 (12)	91 (7)	1.3	3.2	4.1	94 (9)	96 (12)	106 (13)
Dehydro-Erythromycin		0.5	1.8	4.8	106 (7)	113 (6)	93 (16)	3.7	2.1	7.3	86 (32)	84 (12)	87 (15)	0.8	2.8	3.7	99 (89)	105 (13)	111 (9)	1.6	2.2	5.3	92 (7)	95 (12)	96 (17)
Erythromycin A		1.3	4.6	3.2	81 (23)	80 (12)	68 (8)	1.6	5.6	3.9	96 (23)	103 (17)	82 (8)	0.9	3.1	2.8	84 (12)	84 (15)	97 (14)	1.5	3.7	3.3	96 (16)	85 (8)	76 (12)
Josamycin		0.7	2.5	4.4	96 (10)	102 (13)	97 (8)	1.1	3.8	4.4	91 (17)	93 (8)	96 (14)	0.7	2.6	3.2	92 (9)	91 (14)	102 (12)	0.8	3.0	3.4	82 (12)	94 (16)	101 (16)
Lincomycin		1.0	3.5	4.6	83 (17)	92 (14)	89 (6)	0.6	2.1	3.9	92 (10)	92 (12)	95 (20)	0.9	3.0	4.0	101 (9)	112 (12)	105 (7)	0.8	2.9	4.1	83 (17)	94 (14)	89 (6)
Spiramycin		0.8	2.8	4.5	82 (14)	96 (13)	82 (10)	1.1	4.0	4.1	105 (15)	103 (16)	87 (13)	0.4	1.5	3.3	72 (6)	86 (13)	81 (13)	1.7	2.8	3.8	90 (13)	94 (9)	88 (13)
Tilmicosin		0.7	2.6	4.9	103 (10)	108 (9)	88 (15)	0.7	2.5	3.6	100 (10)	98 (18)	85 (9)	0.8	3.0	3.5	88 (9)	94 (10)	96 (11)	0.7	2.5	1.0	94 (12)	102 (12)	93 (17)
Tulathromycin A		1.6	5.5	3.4	99 (22)	96 (13)	97 (19)	1.1	4.0	3.8	95 (17)	100 (17)	96 (15)	0.6	2.1	2.8	92 (7)	91 (17)	90 (8)	1.2	3.9	3.3	96 (17)	94 (10)	84 (16)
Tylosin		0.8	2.8	4.4	77 (15)	91 (6)	97 (19)	1.3	4.5	3.7	99 (18)	95 (16)	83 (8)	0.7	2.3	4.0	99 (7)	102 (8)	98 (8)	0.9	3.1	4.0	90 (10)	89 (14)	92 (9)
Virginiamycin		0.7	2.4	5.2	91 (11)	105 (10)	93 (13)	0.8	2.7	4.4	109 (10)	107 (13)	90 (8)	1.0	3.6	3.7	93 (13)	95 (8)	111 (19)	0.8	2.9	4.4	87 (8)	98 (11)	100 (15)
Nitroimidazoles (4)																									
Dimetridazole	5.00	0.4	1.4	4.4	102 (6)	106 (5)	95 (4)	1.0	3.5	3.6	89 (16)	91 (11)	85 (12)	1.0	3.3	3.9	99 (10)	108 (11)	95 (10)	0.8	2.7	4.0	102 (6)	106 (5)	95 (3)
Metronidazole		0.5	1.9	4.5	92 (8)	96 (5)	92 (4)	0.9	3.2	4.9	92 (14)	80 (18)	104 (9)	1.1	3.9	5.3	105 (14)	116 (9)	102 (14)	0.8	3.0	4.9	96 (7)	94 (8)	98 (4)
Ronidazole		0.2	0.9	4.9	100 (4)	101 (4)	93 (4)	0.5	1.8	4.5	97 (8)	99 (10)	101 (16)	1.0	3.4	2.9	84 (12)	99 (10)	101 (16)	0.6	2.0	4.4	87 (16)	95 (13)	96 (11)
Tinidazole		0.3	1.1	5.3	102 (4)	103 (4)	93 (3)	0.6	2.0	4.9	91 (9)	100 (6)	95 (17)	0.4	1.5	3.9	95 (5)	104 (9)	110 (7)	0.4	1.5	4.9	99 (6)	100 (11)	105 (7)
Phenicols (3)																									
Chloramphenicol	5.00	0.4	1.6	4.1	94 (7)	96 (11)	89 (6)	0.8	2.7	4.6	95 (11)	102 (9)	89 (14)	1.0	3.8	3.6	95 (13)	99 (11)	95 (18)	0.7	2.7	4.1	94 (7)	96 (11)	88 (6)
Florfenicol		0.9	3.2	5.4	95 (14)	110 (5)	92 (9)	0.6	1.6	4.8	98 (7)	102 (8)	96 (7)	1.0	12.0	2.8	89 (12)	98 (21)	88 (17)	0.8	5.6	4.3	90 (8)	88 (16)	97 (10)
Florfenicol Amine		1.0	3.4	4.7	79 (17)	109 (11)	97 (13)	0.6	2.2	2.3	84 (10)	90 (25)	95 (23)	1.0	3.5	4.0	94 (11)	105 (10)	98 (9)	0.9	2.7	3.9	79 (17)	99 (4)	97 (13)
Polypeptides (1)																									
Bacitracin	25.0	7.3	26	29	96 (15)	74 (11)	75 (8)	13.6	48.0	37	92 (21)	94 (16)	84 (14)	5.9	10.9	30	91 (11)	84 (9)	78 (9)	8.9	30	32	58 (13)	70 (17)	68 (14)
Sulfonamides (16)																									
Sulfacetamide	5.00	0.3	1.1	5.2	108 (4)	103 (6)	93 (7)	0.5	1.9	3.9	88 (9)	91 (11)	94 (23)	0.6	1.9	4.2	89 (7)	106 (8)	97 (12)	0.5	1.6	4.4	101 (6)	84 (8)	99 (5)
Sulfachloropyridazine		0.5	1.8	4.6	98 (7)	97 (8)	89 (4)	1.0	3.4	4.3	96 (14)	99 (12)	98 (12)	0.8	2.9	3.7	92 (10)	99 (10)	92 (13)	0.8	2.7	4.2	89 (8)	94 (5)	92 (7)
Sulfadiazine		0.4	1.3	4.8	101 (5)	102 (5)	92 (3)	0.3	0.9	4.8	92 (4)	99 (7)	97 (17)	0.8	2.9	3.7	87 (10)	101 (11)	94 (13)	0.4	4.5	4.5	96 (4)	100 (7)	93 (3)
Sulfadimethoxine		0.7	2.5	4.4	82 (12)	99 (7)	91 (9)	1.1	4.0	4.7	107 (15)	101 (9)	97 (11)	0.7	2.5	2.7	89 (4)	95 (22)	89 (8)	0.8	3.0	3.9	87 (9)	96 (8)	89 (3)
Sulfadoxine		0.6	2.0	4.3	89 (9)	103 (8)	95 (8)	1.2	4.2	4.4	100 (17)	86 (9)	89 (12)	1.0	3.4	2.7	90 (12)	94 (20)	93 (9)	0.9	3.2	3.8	95 (7)	90 (9)	96 (12)
Sulfaethoxypyridazine		0.4	1.3	4.5	94 (5)	104 (7)	94 (4)	0.7	2.3	4.6	101 (9)	96 (7)	91 (20)	1.3	4.4	3.2	83 (16)	94 (15)	84 (13)	0.8	2.7	4.0	86 (6)	97 (11)	93 (5)
Sulfamerazine		0.3	1.0	5.1	102 (4)	103 (6)	93 (4)	0.4	1.2	4.3	89 (6)	93 (8)	95 (17)	0.8	2.9	4.0	98 (9)	106 (99)	89 (11)	0.6	1.7	4.5	92 (3)	96 (9)	84 (2)
Sulfamethazine		0.4	1.2	4.9	94 (5)	103 (10)	95 (4)	0.6	2.6	4.9	87 (10)	97 (4)	97 (15)	1.0	2.5	3.8	97 (12)	105 (12)	87 (12)	0.8	2.0	4.5	91 (6)	95 (16)	83 (9)
Sulfamethizole		1.1	4.0	4.8	87 (18)	97 (7)	91 (3)	0.9	0.1	4.9	95 (9)	95 (10)	96 (16)	1.1	3.9	4.0	98 (12)	108 (11)	91 (15)	0.8	2.7	4.1	91 (4)	96 (5)	94 (7)
Sulfamethoxazole		0.5	1.9	4.4	96 (9)	99 (7)	91 (4)	0.9	3.1	4.9	92 (14)	96 (4)	98 (13)	1.0	3.6	3.8	98 (11)	108 (13)	89 (12)	0.5	1.9	4.4	96 (8)	89 (7)	91 (4)
Sulfamethoxypyridazine		0.5	1.7	4.9	96 (7)	104 (7)	94 (5)	1.0	3.6	4.6	95 (15)	94 (6)	93 (17)	0.9	3.1	3.7	95 (10)	102 (11)	87 (13)	0.7	2.7	4.3	90 (10)	102 (9)	92 (15)
Sulfanitran		0.5	1.9	4.4	108 (10)	99 (17)	90 (19)	0.5	1.9	4.3	103 (7)	102 (14)	97 (16)	0.6	2.8	3.8	86 (7)	108 (13)	98 (16)	0.6	2.1	4.1	98 (7)	97 (14)	92 (22)
Sulfapyridine		1.1	3.8	4.8	86 (17)	98 (6)	96 (6)	0.9	3.2	3.9	99 (13)	104 (17)	101 (22)	0.7	2.4	3.7	95 (8)	102 (11)	93 (14)	0.9	3.1	4.1	91 (14)	95 (9)	97 (12)
Sulfaquinoxaline		0.8	2.7	6.4	89 (12)	90 (11)	91 (3)	0.9	3.4	4.4	92 (15)	91 (7)	85 (8)	0.6	2.0	3.3	93 (7)	99 (15)	88 (7)	0.7	2.9	4.7	87 (10)	93 (15)	92 (10)
Sulfathiazole		1.1	4.0	4.8	87 (18)	97 (7)	91 (3)	0.6	2.2	2.3	95 (9)	95 (10)	96 (16)	1.0	3.8	3.9	98 (12)	108 (11)	91 (14)	0.9	3.3	3.7	92 (11)	98 (12)	94 (3)
Trimethoprim		0.3	1.1	5.1	99 (4)	107 (4)	99 (5)	1.2	1.1	5.1	97 (5)	105 (4)	97 (8)	1.0	3.4	3.5	98 (11)	103 (13)	94 (10)	0.8	1.9	4.5	85 (6)	89 (4)	102 (8)
Tetracyclines (4)																									
Chlortetracycline	25.0	2.0	7.0	13	133 (10)	82 (12)	77 (7)	6.6	23.0	15	96 (30)	82 (8)	90 (20)	5.6	20.0	15	103 (15)	110 (13)	78 (7)	4.7	14.0	15	103 (15)	110 (13)	78 (7)
Doxycycline		2.6	9.0	22	60 (15)	102 (24)	83 (6)	3.7	13.0	9.3	84 (15)	95 (22)	94 (30)	4.0	14.0	13	99 (11)	99 (14)	94 (11)	3.4	12.0	13	65 (18)	100 (27)	97 (15)
Oxytetracycline		4.2	15	17	109 (14)	109 (18)	84 (12)	4.3	15.0	17	89 (17)	103 (13)	92 (15)	2.7	9.6	13	96 (8)	99 (15)	88 (8)	3.7	13.0	17	93 (12)	102 (7)	92 (15)
Tetracycline		2.6	10	15	67 (14)	75 (9)	74 (8)	5.7	20.0	17	96 (21)	97 (12)	103 (18)	3.9	14.0	14	103 (10)	106 (15)	80 (8)	4.1	13.0	14	72 (17)	86 (19)	83 (11)
Thyreostats (3)																									
2-Mercaptobenzimidazole	5.00	0.5	1.7	5.4	89 (8)	100 (7)	94 (7)	1.2	4.2	4.7	92 (18)	100 (8)	111 (18)	0.7	2.5	3.3	103 (8)	96 (14)	79 (8)	0.8	2.8	4.5	103 (8)	96 (14)	79 (8)
6-Phenyl-thiouracil		0.6	2.0	4.8	98 (8)	101 (4)	89 (3)	1.2	4.3	3.5	92 (19)	95 (17)	98 (21)	1.0	3.9	4.1	101 (11)	108 (10)	98 (15)	0.9	3.4	4.1	103 (6)	101 (3)	89 (3)
6-Propyl-2-thiouracil		0.6	2.3	5.3	97 (10)	104 (7)	91 (12)	0.9	3.3	4.7	94 (14)	99 (7)	101 (20)	1.1	4.0	4.0	102 (12)	114 (13)	102 (10)	0.9	3.2	4.7	99 (12)	100 (8)	94 (9)
Tranquilizers (9)																									
Acepromazine	5.00	1.0	3.5	4.2	98 (14)	122 (25)	119 (17)	1.0	3.2	3.7	93 (14)	89 (14)	91 (8)	1.0	3.5	3.2	90 (12)	87 (11)	113 (6)	1.2	4.1	4.2	91 (18)	121 (26)	123 (13)
Azaperol		0.4	1.5	5.3	92 (6)	112 (13)	102 (10)	1.1	4.0	3.9	89 (14)	100 (16)	89 (20)	1.4	4.8	3.4	93 (16)	95 (12)	100 (13)	1.2	1.5	5.9	95 (7)	126 (4)	112 (4)
Azaperone		1.2	4.3	5.4	110 (16)	124 (13)	110 (10)	1.1	3.7	3.8	103 (15)	96 (15)	98 (17)	1.1	3.7	3.2	95 (13)	95 (12)	100 (13)	1.1	3.9	4.2	111 (15)	132 (8)	117 (6)
Carazolol		1.2	3.6	4.9	108 (13)	117 (11)	105 (11)	1.3	4.5	3.7	97 (19)	96 (16)	93 (26)	1.2	4.3	3.6	92 (14)	100 (12)	96 (22)	1.2	4.2	4.1	83 (23)	111 (16)	107 (10)
Chlorpromazine		1.4	5.1	3.3	87 (23)	89 (17)	118 (28)	1.3	4.8	4.6	109 (18)	103 (12)	98 (19)	1.0	3.7	3.3	103 (11)	101 (15)	110 (10)	1.3	4.5	3.7	91 (21)	99 (11)	123 (22)
Haloperidol		1.1	3.8	5.2	99 (15)	109 (4)	121 (25)	0.6	2.2	3.2	103 (9)	92 (20)	93 (15)	0.7	2.3	4.2	106 (7)	116 (12)	107 (13)	1.0	2.8	4.2	98 (15)	109 (4)	123 (22)
Promethazine		0.7	2.3	4.6	93 (10)	99 (14)	93 (9)	0.8	2.7	2.3	104 (10)	106 (24)	114 (14)	0.7	2.5	3.9	112 (9)	106 (11)	111 (5)	1.0	2.5	6.1	100 (10)	96 (8)	116 (13)
Triflupromazine		0.8	2.7	2.3	88 (12)	97 (34)	99 (14)	1.0	3.4	3.5	98 (14)	84 (14)	90 (18)	1.5	4.7	3.3	98 (15)	97 (13)	100 (15)	1.1	3.6	3.0	91 (8)	88 (7)	94 (10)
Xylazine		0.5	1.6	5.0	97 (7)	111 (8)	98 (8)	1.1	4.0	3.9	97 (17)	100 (16)	91 (9)	1.1	3.8	3.5	100 (12)	96 (12)	83 (14)	0.9	3.1	4.1	86 (10)	101 (6)	94 (3)

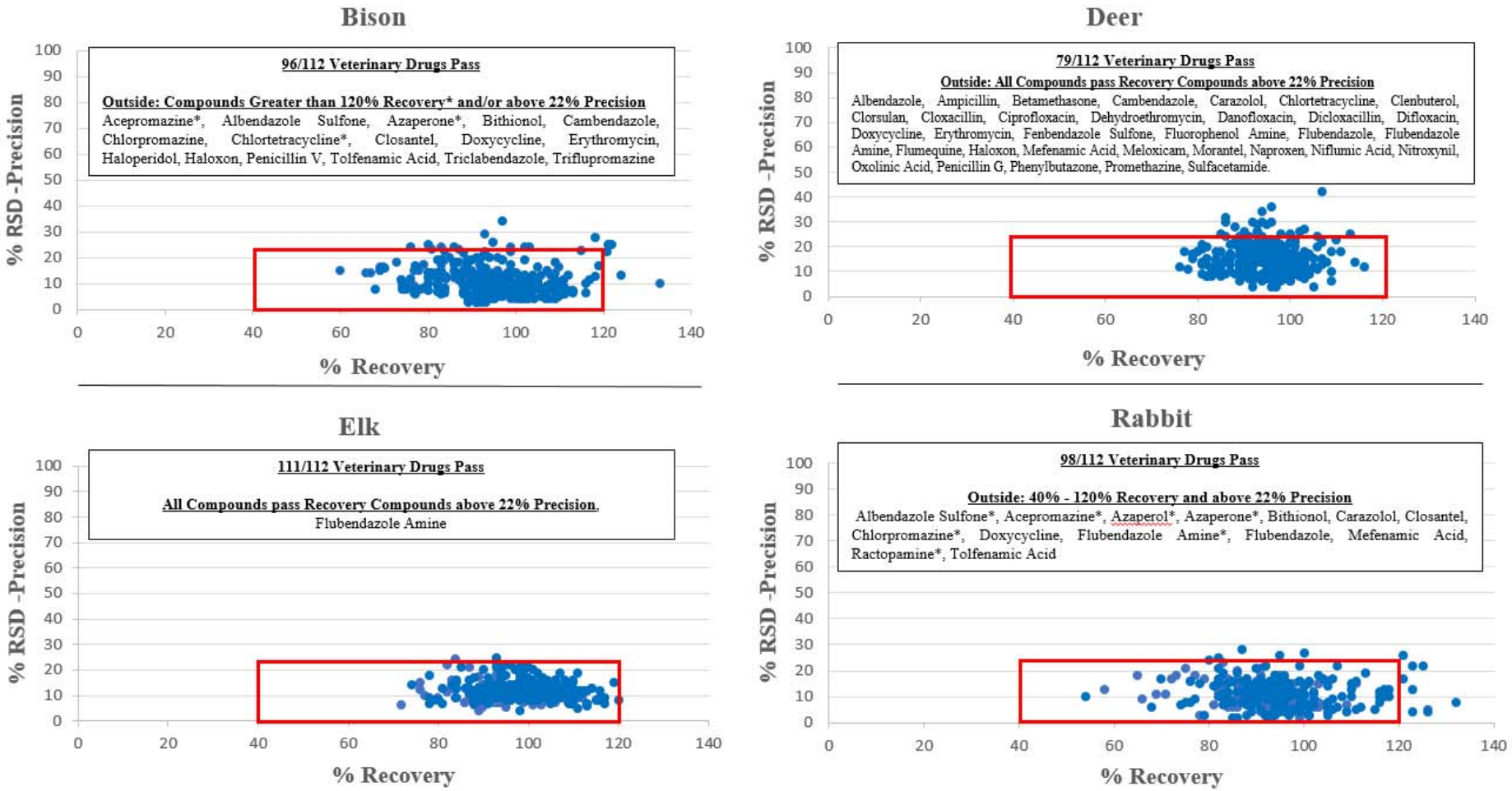

Figure 4: Overall method accuracy for interday precision for 112 drugs fortified at three levels in bison, deer, elk, and rabbit where n=5 for each level over 2 days.

Table 4: Accuracy and precision of surrogate compounds at 25 ng/g (n=15)

Surrogate	Drug Class	Trueness (% Recovery ± % RSD)			
		Bison	Deer	Elk	Rabbit
Albendazole-D3	Anthelmintic	93 ± 16	87 ± 14	69 ± 35	70 ± 18
Clenbuterol-D9	β-Agonist	97 ± 26	103 ± 8	84 ± 17	91 ± 22
Chloramphenicol-D4	Phenicol	79 ± 19	95 ± 16	86 ± 20	102 ± 25
Ciprofloxacin-D8	Fluoroquinolone	95 ± 11	91 ± 9	77 ± 14	97 ± 23
Dimetridazole-D3	Nitroimidazole	89 ± 13	101 ± 4	93 ± 11	101 ± 22
Carprofen-D3	Anti-inflammatory	73 ± 7	81 ± 13	71 ± 26	98 ± 25
Flunixin-D3	Anti-inflammatory	101 ± 22	95 ± 13	93 ± 11	101 ± 16
Penicillin-G-D7	β–Lactam	81 ± 15	85 ± 14	84 ± 19	100 ± 19
Sulfamerazine-^{13}C6	Sulfonamide	87 ± 13	98 ± 6	91 ± 10	105 ± 24

Regulatory Testing

The workflow screening was used to analyze over 360 game meat regulatory samples. The qualitative limit test was performed and when a presumptive positive sample was identified, the full quantitation method was performed by a different analyst, using a different instrument, set of standards, and reagents. Presumptive positive samples with a residue above threshold value were reextracted by the different analyst and analyzed using a matrix matched extracted five-point calibration curve. Generally, the results of the 1X single matrix matched extracted calibrant result was similar to the calculated concentration using the five-point matrix matched extracted calibration curve, as shown in Table 5. The rabbit sample that contained sulfadiazine did demonstrate a significant different result between the two workflows. Despite this, all QA/QC criteria passed from both sets of data (screening vs. quantitative workflow) including spikes, duplicate spikes, independent calibration verification (ICV), and the end of the analytical run continuing calibration verification (CCV) passed. Since the screening result was much higher than the 1X level, this could explain the reason for the variation in the screening and quantitative data values. A total of 360 regulatory game meat samples consisting of 180 bison samples and 60 samples each of deer, elk, and rabbit have been tested by this method. Antibiotic and/or sedative residues have been identified in deer (chlortetracycline, haloperidol, and tulathromycin), and rabbit (sulfadiazine), Table 6. Figures 5 through 8 are chromatograms demonstrating the negative control, extracted matrix matched calibrant, and the sample that was found to be positive.

Table 5: **Results for qualitative and quantitative determination of positive samples**

Matrix	Analyte	1X Screening (ng/g)	Quantitative Results (ng/g)
Bison	N/A	N/A	N/A
Deer	Chlortetracycline	65	62.2
	Haloperidol	5	9.7
	Tulathromycin	35	37.4
	Tulathromycin	12	10.3
Elk	N/A	N/A	N/A
Rabbit	Sulfadiazine	160	62.9

An external quality control rabbit sample was received by DENL and analyzed using the workflows described in this LIB. Again, separate analysts, instruments, columns, standards, and reagents were used for the 1X qualitative screening and the full 5-point calibration quantitative workflow. Both the screening and the quantitated results yield acceptable residue concentrations within the limit of the ± 2z-score (Table 6). The quantitative workflow concentration results were within 10% of the reported value for the two sulfonamide residues. The testing of an external QC sample further demonstrates this method can qualitatively identify and quantitate veterinary drug residues in the rabbit matrix.

Table 6: Comparison results from external quality control rabbit sample

Analyte	QC Value Reported (n=20) ng/g	Range for Izl < 2	Screening Workflow at 1X (n=1) ng/g	Quantitative Workflow (n=1) ng/g
Sulfadimethoxine	117	65-168	131	126
Sulfamethazine	115	65-168	85	121

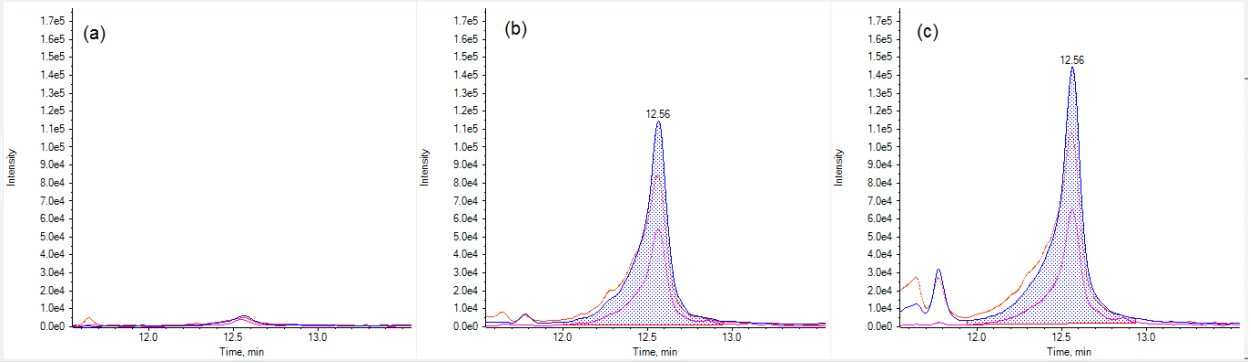

Figure 5: Extracted ion chromatograms for quantitative and confirmatory analysis of chlortetracycline residue in deer muscle: (a) negative control deer, b) extracted calibrant in deer matrix at 50 ng/g, and (c) positive deer regulatory sample with chlortetracycline residue concentration of 62.2 ng/g

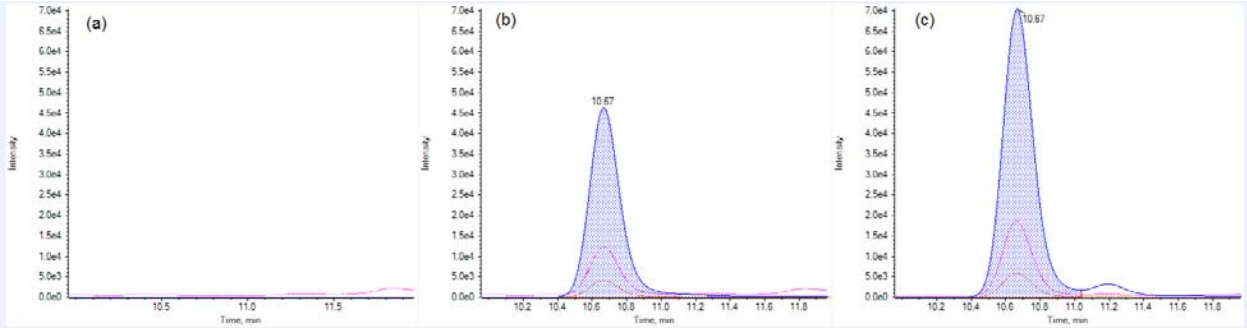

Figure 6: Extracted ion chromatograms for quantitative and confirmatory analysis of tulathromycin residue in deer muscle: (a) negative control deer, (b) extracted calibrant in deer matrix at 12.5 ng/g, and (c) positive deer regulatory sample with tulathromycin residue concentration of 37.4 ng/g.

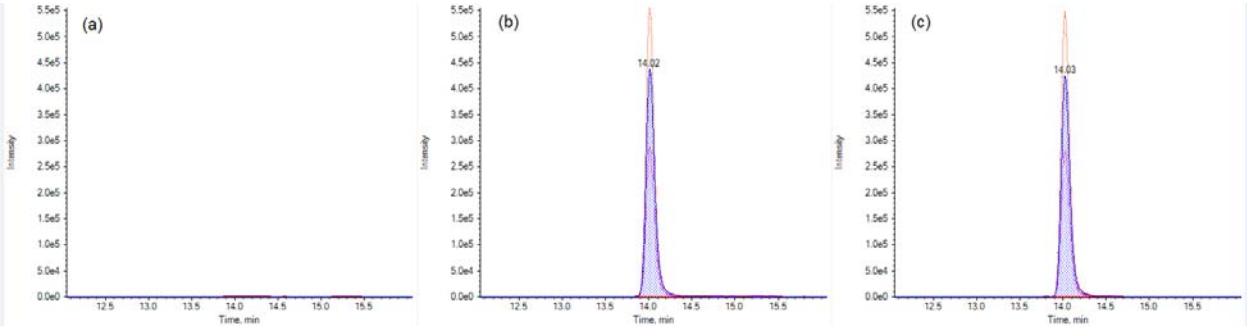

Figure 7: Extracted ion chromatograms for quantitative and confirmatory analysis of haloperidol residue in deer muscle: (a) negative control deer, (b) extracted calibrant in deer matrix at 5.0 ng/g, and (c) positive deer regulatory sample with haloperidol residue concentration of 9.7 ng/g.

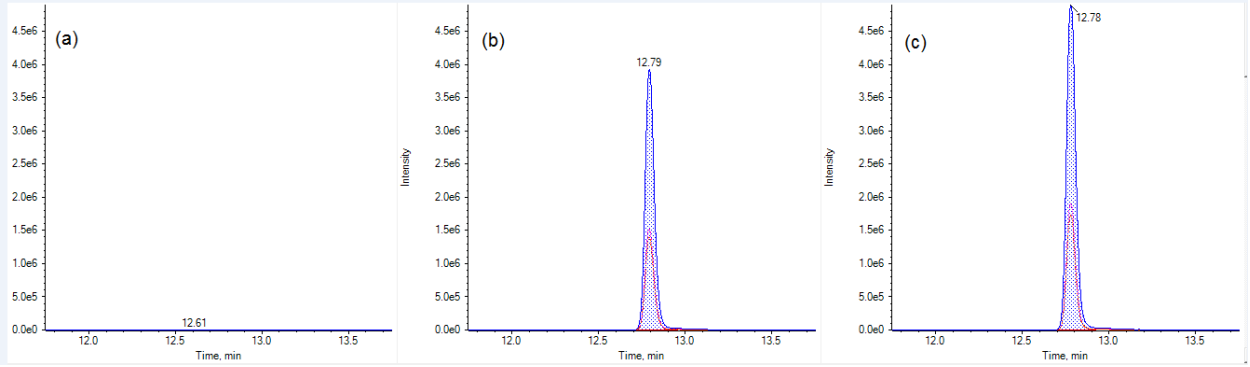

Figure 8: Extracted ion chromatograms for quantitative and confirmatory analysis of sulfadiazine residue in rabbit muscle: (a) negative control rabbit, (b) extracted calibrant in rabbit matrix at 50 ng/g, and (c) positive rabbit regulatory sample with sulfadiazine residue concentration of 62.9 ng/g.

CONCLUSION

DENL optimized and validated a multiclass, multiresidue method to develop a regulatory program to qualitatively identify and quantify 112 veterinary drug residues in game meats. The method workflows included an initial qualitative identification screening method using an extracted matrix-matched 1X calibrant and a quantitative determination of presumptive positive samples resulting from workflow 1. The screening and quantification workflows yielded acceptable results for 5 ng/g and 25 ng/g testing levels for most of the analytes. The method can be used as a semi-quantitative screen for the validated compounds by analyzing regulatory samples against a single matrix-matched extracted calibrant prepared at the 1X testing level (5 ng/g for most compounds). Samples demonstrating a presumptive positive were re-extracted with a full matrix-matched extracted calibration curve for accurate quantitative determination. The method is advantageous because of the speed and throughput of the sample preparation procedure. Samples are analyzed in a single 25.0 min chromatographic run, which allows the analysis of both positive and negative charged ions in a single injection. Recently, novel solid phase extraction products such as PRiME, Capitiva, and/or EMR have been used for the analysis of multiple veterinary drug residues in different types of muscle matrixes. Also, these methods included coccidiostats, avermectin, and other veterinary drug classes. Future work will explore these sample preparation techniques, UPLC, and the combination of analytes from this LIB, LIB 4627,[10] and LIB 4644[9] into a single multiresidue veterinary drug analysis method for game meats and bison liver.

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APPENDIX A

Supporting Information Table S1: List of drug analytes with retention time (t_R), MS/MS conditons including DP, CE, and CXP. Analytes acquired in the negative mode are noted (-).

Analyte	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	Relative Ion Ratio	DP (V)	CE (V)	CXP (V)
Florfenicol Amine	3.90	248	230	100	51	17	18
			130	69		31	10
			91	10		59	12
Amoxicillin	5.90	366	349	100	66	13	12
			114	34		27	10
			208	19		19	12
Sulfacetamide	6.00	215	156	100		13	18
			92	49	81	33	10
			108	42		27	10
Cimaterol	6.10	220	202	100		13	16
			160	66	41	23	16
			143	61		31	14
Salbutamol	6.70	240	148	100		25	12
			222	53	51	15	16
			166	34		19	14
Zilpaterol	6.70	262	244	100		19	8
			185	62	26	33	16
			202	34		27	16
Ronidazole	6.80	201	140	100		17	16
			55	21	56	29	16
			66	3		67	10
Sulfadiazine	6.90	251	156	100		21	18
			92	65	41	31	14
			108	56		35	14
Metronidazole	7.00	172	128	100		19	12
			82	49	51	35	8
			56	6		29	6
Sulfathiazole	7.20	256	156	100		19	12
			92	38	56	33	20
			108	35		31	20
Sulfapyridine	7.70	250	156	100		27	12
			184	58	61	27	12
			92	101		35	10
Dimetridazole-D3 (Surrogate)	7.80	145	99	n/a	31	23	18
Sulfamerazine	8.00	265	156	100		23	14
			92	9	36	19	12
			108	58		31	20
Tinidazole	8.00	248	121	100		23	12
			82	23	31	47	10
			128	29		29	12

Analyte	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	Relative Ion Ratio	DP (V)	CE (V)	CXP (V)
Sulfamerazine-^{13}C6 (Surrogate)	8.00	271	162	n/a	50	23	16
Dimetridazole	8.10	142	96	100		23	10
			95	47	66	31	10
			81	31		37	14
Trimethoprim	8.40	291	230	100		31	16
			261	62	66	33	10
			123	65		31	10
Lincomycin	8.50	407	126	100		35	14
			359	120	71	25	17
			124	4		39	12
Ofloxacin	8.80	362	261	100		27	10
			318	65	46	37	12
			205	35		59	16
Sulfamethoxypyridazine	8.90	281	108	100		33	10
			92	112	66	37	12
			156	172		23	10
Norfloxacin	9.00	320	302	100		29	10
			231	27	81	53	16
			276	14		23	20
Sulfamethizole	9.00	271	156	100		15	8
			92	52	25	33	10
			108	32		30	12
Sulfamethazine	9.00	279	186	100		25	14
			124	55	61	33	12
			108	26		29	20
Ampicillin	9.10	350	106	100		23	10
			192	41	56	21	18
			160	13		10	10
Ractopamine	9.10	302	164	100		23	12
			107	77	61	51	12
			121	78		31	12
Ciprofloxacin-D8 (Surrogate)	9.10	340	322	n/a	66	31	10
6-Propyl-2-thiouracil	9.10	171	112	100		25	12
			154	139	60	23	14
			86	32		33	10
Ciprofloxacin	9.20	332	231	100		47	20
			245	13	106	33	20
			288	20		25	20
Carbendazim	9.30	192	160	100		27	14
			132	25	46	43	12
			105	19		51	10

Analyte	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	Relative Ion Ratio	DP (V)	CE (V)	CXP (V)
Danofloxacin	9.30	358	340	100		31	10
			314	4	96	25	10
			283	2		33	10
Enrofloxacin	9.30	360	342	100		31	12
			245	21	36	41	20
			316	42		29	12
Sulfachloropyridazine	9.30	285	156	100		25	12
			92	66	56	35	10
			108	52		33	12
Sulfamethoxazole	9.30	254	108	100		35	20
			92	40	66	21	10
			156	42		21	16
Tetracycline	9.30	445	410	100		27	12
			154	42	61	35	14
			427	46		17	14
Florfenicol	9.30	358	340	100		55	12
			130	6	61	62	20
			241	4		25	10
Oxytetracycline	9.50	461	426	100		27	14
			201	14	56	51	14
			337	12		41	14
Penicillin G	9.50	335	289	100		35	10
			176	18	100	34	10
			160	49		31	10
2-mercaptobenzimidazole	9.55	151	93	100		23	10
			118	16	66	47	20
			92	45		21	10
Tulathromycin	9.60	807	577	100		33	18
			72	30	56	115	8
			158	17		53	12
Orbifloxacin	9.70	396	352	100		25	12
			295	42	76	25	10
			267	54		47	12
Difloxacin	9.80	400	382	100		31	12
			299	28	81	35	20
			356	32		27	20
Morantel	9.89	221	164	100		37	14
			123	62	66	47	14
			150	35		39	14
Sarafloxacin	9.90	386	368	100		31	12
			299	19	56	37	10
			342	23		27	12
Sulfadoxine	9.90	311	156	100		25	12
			108	38	26	33	10
			92	36		39	10

Analyte	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	Relative Ion Ratio	DP (V)	CE (V)	CXP (V)
Albendazole Sulfone	9.91	298	266	100		45	10
			159	32	26	32	8
			131	25		50	12
Xylazine	9.92	221	164	100		35	20
			90	200	41	29	10
			147	44		31	12
Azaperol	10.00	330	121	100		33	16
			109	31	61	75	10
			149	25		39	10
Clenbuterol	10.00	277	203	100		23	14
			132	38	46	41	12
			140	12		67	12
Clenbuterol-D9 (Surrogate)	10.00	288	206	n/a	51	23	20
Thiabendazole	10.10	202	175	100		35	16
			131	64	76	45	12
			65	14		59	20
Clorsulon (-)	10.35	378	342	100		-18	-11
			242	5	-95	-32	-25
			135	5		-32	-17
Sulfaethoxypyridazine	10.50	295	156	100		23	12
			108	56	51	39	14
			92	62		41	10
6-phenyl-2-thiouracil	10.80	205	103	100		35	12
			188	87	51	27	20
			77	35		59	20
Azaperone	10.90	328	165	100		31	14
			95	64	56	85	10
			123	93		49	14
Carazolol	10.90	299	116	100		27	10
			222	39	66	27	20
			194	21		41	14
Chlortetracycline	11.00	479	444	100		29	14
			154	47	36	35	14
			462	83		25	14
Chloramphenicol-D4 (Surrogate)	11.10	329	281	n/a	71	21	10
Spiramycin	11.20	422	174	100		27	14
			101	69	51	23	10
			142	31		19	10
Sulfadimethoxine	11.30	311	156	100		27	22
			108	49	71	37	14
			92	188		41	10
Chloramphenicol	11.30	323	275	100		21	14
			165	77	91	35	20
			305	95		11	14

Analyte	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	Relative Ion Ratio	DP (V)	CE (V)	CXP (V)
Sulfaquinoxaline	11.40	301	156	100		23	14
			92	49	51	41	10
			108	16		23	20
Albendazole Sulfoxide	11.50	282	240	100		19	10
			208	76	51	33	14
			159	71		53	12
Flubendazole Amine	11.60	256	123	100		37	12
			95	35	116	53	10
			133	14		51	12
Oxolinic Acid	11.60	262	244	100		23	14
			216	19	16	41	20
			160	13		49	20
Doxycycline	11.70	445	428	100		25	12
			154	6	51	37	12
			410	5		27	12
Oxfendazole	12.40	316	159	100		45	16
			191	66	66	29	16
			131	23		65	10
Tilmicosin	12.40	869	697	100	16	57	22
			174	74		57	16
Clindamycin	12.50	425	126	100	41	73	12
			377	27		27	12
Fenbendazole Sulfone	12.50	332	300	100		33	10
			159	69	56	51	16
			104	16		81	10
Penicillin-D7 (Surrogate)	12.50	342	160	n/a	101	17	12
Sulfanitran	12.60	336	156	100		19	14
			108	51	121	35	12
			93	45		49	12
Nitroxynil (-)	12.66	289	127	100		-42	-13
			162	20	-90	-26	-19
			116	10		-38	-15
Cambendazole	12.80	303	217	100		39	20
			261	89	70	25	20
			190	27		55	16
Haloperidol	12.80	376	123	100		55	14
			95	63	46	99	12
			165	12		33	12
Nalidixic Acid	13.00	233	187	100		33	26
			159	42	26	41	18
			104	43		55	10
Flumequine	13.20	262	244	100		27	12
			202	59	46	40	12
			126	28		63	14
Prednisone	13.10	359	147	100		41	12

Analyte	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	Relative Ion Ratio	DP (V)	CE (V)	CXP (V)
			115	101	56	125	12
			91	121		79	20
Acepromazine	13.30	327	86	100		27	20
			222	17	41	51	20
			254	19		33	20
Oxacillin	13.30	402	144	100		37	20
			243	52	56	33	10
			160	18		33	10
Oxibendazole	13.30	250	218	100		27	20
			176	99	66	39	14
			148	30		49	14
Promethazine	13.40	285	86	100		23	8
			198	56	50	20	10
			240	25		32	10
Bacitracin	13.50	712	199	100		51	20
			86	63	46	111	20
			110	16		111	20
Cloxacillin	13.60	436	160	100		19	14
			277	122	51	19	10
			114	63		53	12
Penicillin V	13.60	351	160	100		19	12
			114	60	111	47	10
			192	7		15	16
Tylosin	13.70	916	174	100		47	14
			772	31	31	41	24
			101	5		89	10
Erthromycin	13.80	735	158	100		39	18
			576	33	66	27	12
			116	13		61	10
Mebendazole	13.90	296	264	100		31	10
			105	36	116	45	10
			130	3		62	12
Dicloxacillin	14.00	470	160	100		19	16
			311	65	66	23	10
			114	53		57	12
Dehydro Erythromycin	14.10	716	559	100		23	18
			158	74	66	37	12
			116	12		57	12
Flubendazole	14.10	314	282	100		31	10
			95	34	41	63	10
			123	60		49	14
Meloxicam	14.10	352	115	100		27	22
			141	56	46	29	12
			184	6		23	14
Nafcillin	14.20	415	199	100		19	14

Analyte	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	Relative Ion Ratio	DP (V)	CE (V)	CXP (V)
			171	54	61	49	14
			115	39		93	12
Betamethasone	14.30	393	373	100		13	12
			147	30	66	39	10
			171	19		37	16
Dexamethasone	14.5	393	**373**	100		13	12
			165	12	41	109	20
			147	38		43	12
Virginiamycin	14.40	526	508	100		17	16
			355	52	141	25	10
			109	16		45	10
Chlorpromazine	14.50	319	86	100		27	20
			214	18	41	57	16
			152	8		101	16
Josamycin	14.60	828	174	100		43	14
			229	30	41	41	18
			600	26		37	18
Ketoprofen	14.60	255	105	100		31	20
			209	228	91	19	16
			103	25		45	10
Haloxon	14.80	415	211	100		49	16
			273	116	136	45	20
			353	99		33	12
Naproxen	14.80	231	185	100		19	14
			141	24	46	23	24
			115	56		77	12
Albendazole	14.90	266	234	100		27	18
			191	47	66	45	14
			159	28		53	14
Triflupromazine	14.90	353	86	100		27	20
			248	20	66	55	18
			280	18		35	20
Albendazole-D3 (Surrogate)	14.90	269	234	n/a	81	27	20
Suxibuzone	15.30	439	321	100		19	10
			160	87	121	44	14
			309	109		29	10
Phenylbutazone	15.50	309	160	100		27	12
			120	120	26	27	10
			106	40		37	12
Flunixin	15.60	297	279	100		31	22
			264	33	76	45	22
			259	12		41	22
Flunixin-D3 (Surrogate)	15.60	300	282	n/a	51	31	10
Niflumic Acid	15.80	283	265	100	50	35	20

Analyte	t_R (min)	Precursor ion (m/z)	Product ion (m/z)	Relative Ion Ratio	DP (V)	CE (V)	CXP (V)
			245	3		25	20
Carprofen	15.90	274	228	100		25	20
			193	79	26	43	24
			191	29		73	18
Etodolac	15.90	288	172	100		19	16
			144	20	61	45	14
			143	38		53	16
Carprofen-D3 (ISTD)	15.90	277	231	n/a	41	25	20
Diclofenac	16.00	296	215	100		27	20
			176	22	16	75	16
			151	24		82	12
Oxyclozanide (-)	16.51	398	176	100		-38	-15
			195	82	-60	-34	-13
			202	97		-34	-13
Mefenamic Acid	16.70	242	224	100		21	20
			209	38	66	39	10
			180	34		57	18
Triclabendazole	16.80	361	346	100		39	10
			274	59	66	51	10
			171	23		69	12
Fenbendazole	16.90	300	268	100		25	12
			159	32	80	26	11
			131	18		50	7
Tolfenamic Acid	17.00	262	244	100		30	15
			209	31	66	30	15
			229	6		30	15
Niclosamide (-)	17.40	325	171	100		-30	-15
			289	80	-50	-16	-13
			135	97		-46	-21
Bithionol (-)	17.92	353	161	100		-32	-15
			192	71	-45	-36	-21
			125	9		-58	-13
Closantel (-)	18.04	661	345	100		-52	-13
			127	155	-40	-96	-23
			315	85		-48	-29
Rafoxanide (-)	20.13	624	127	100		-92	-11
			345	54	-20	-48	-25
			513	11		-50	-15

APPENDIX B

Drug Class, Surrogates, Group[a], and Compounds

Drug Class/*Surrogate*	Group	Compounds			
Sulfonamides/*Sulfamerazine-^{13}C6*	B1	Sulfachloropyridazine	Sulfadiazine	Sulfaquinoxaline	Sulfathiazole
		Sulfadimethoxine	Sulfadoxine	Sulfaethoxypyridazine	Sulfamerazine
		Sulfacetamide	Sulfamethazine	Sulfanitran	Sulfamethoxazole
		Sulfamethoxypyridazine	Sulfapyridine	Sulfamethizole	Trimethoprim
Fluoroquinolones/*Ciprofloxacin-D8*		Ciprofloxacin	Enrofloxacin	Norfloxacin	Ofloxacin
		Sarafloxacin	Danofloxacin	Oxolinic Acid	Orbifloxacin
		Flumequine	Nalidixic Acid	Difloxacin	
β-Lactams/*Penicillin-D7*		Amoxicillin	Ampicillin	Oxacillin	Penicillin-G
		Cloxacillin	Dicloxacillin	Nafcillin	Penicillin-V
Tetracyclines/*Penicillin-D7*		Oxytetracycline	Tetracycline	Chlortetracycline	Doxycycline
Macrolides/*NA*		Erythromycin	Tylosin	Tilmicosin	
		Clindamycin	Josamycin	Virginiamycin	
		Lincomycin	Spiramycin	Tulathromycin	
Polypeptides/*NA*		Bacitracin			
Anthelmintics/*Albendazole-D3*	B2a	Albendazole Sulfone	Albendazole Sulfoxide	Fenbendazole Sulfone	Morantel
		Albendazole	Flubendazole	Haloxon	Clorsulon
		Fenbendazole	Carbendazim	Nitroxynil	Oxyclozanide
		Thiabendazole	Oxfendazole	Niclosamide	Bithionol
		Cambendazole	Mebendazole	Closantel	Rafoxanide
		Oxibendazole	Flubendazole Amine	Triclabendazole	
Sedatives/*NA*	B2d	Azaperol	Carazolol	Xylazine	Acetopromazine
		Propionylpromazine	Aceromazine	Triflupromazine	
		Chlorpromazine[b]	Haloperidol		
Anti-inflammatory/ *Flunixin-D3 and Carprofen-D3*	B2e	Naproxen	Meloxicam	Etodolac	Mefenamic Acid
		Ketoprofen	Flunixin	Tolfenamic acid	Phenylbutazone
		Niflumic acid	Carprofen	Diclofenac	
Corticosteroids and Hormones/*N/A*	B2f	Betamethasone	Dexamethasone	Prednisone	
Thyreostats/*N/A*	A2	2-Mercaptobenzimidazole	6-Phenyl-thiouracil	6-Propyl-2-thiouracil	
β-Agonists/*Clenbuterol-D9*	A5	Cimaterol	Clenbuterol	Ractopamine	Salbutamol
		Zilpaterol			
Nitroimidazoles/*Dimetridazole-D3*	A6	Dimetridazole	Metronidazole	Ronidazole	Tinidazole
Phenicols/*Chloramphenicol-D4*	A6[c]	Chloramphenicol	Florfenicol	Florfenicol Amine	

[a]Annex I to Directive 96/23/EC, OJ L 125, 23.5.1996, p.10.

[b]Chlorpromazine is a group A6 prohibited substance.

[c]Chloramphenicol is a group A6 prohibited substance; other phenicol compounds are not listed as prohibited.