Contents

A. INTRODUCTION

1. Summary of Procedure

   Animal drug residues are extracted from tissue using dispersive SPE for both extraction and sample clean up. The extracted residues are examined using UHPLC-MS-MS using a triple quadrupole mass spectrometer under electrospray ionization (ESI) conditions. Analytes are identified by comparison against matrix matched standards.

2. Applicability

   This method is suitable for the screening and confirmation of animal drug residues in beef, poultry, and porcine kidney and muscle tissue as well as equine muscle tissue at the levels listed in Tables 18 and 19 in Appendix J.3.

   Note: Refer to 21CFR for tolerance values set by FDA and 40CFR for tolerance values set by EPA.

B. EQUIPMENT

Note: Equivalent equipment may be substituted.

1. Apparatus

   a. Platform shaker - Cat. No. 6010, Eberbach

   b. Centrifuge - Thermo IEC, Sorvall RC-6 capable of 3720 rcf

   c. Balance - Mettler Top Loading Model PB300 Balance capable of weighing 2 ± 0.01 g

   d. Balance Analytical - Mettler Model X-205 Dualrange

   e. Turbovap LV Concentration Workstation - Biotage Corp

   f. C18 BakerBond Octadecyl (C18) 40 µm Prep LC Packing

   g. Centrifuge tubes - Polypropylene (PP), 50 mL, Falcon Part number 352070

   h. Centrifuge tubes - Polypropylene (PP), 15 mL, Falcon Part Number 352096

   i. Whatman Mini-UniPrep Syringless filter vials - VWR 0.2 micron, PVDF, Cat. No. 12000-524.

      Note: Avoid glass if the Mini-UniPrep filter vials are substituted with syringe filters and autosampler vials, and substitutes must be checked for possible retention of analytes.

   j. Magnetic stirrer and stirbars, freezer, volumetric flasks, graduated cylinders, Pasteur pipettes, repeating pipettes and tips, beakers, bottles, weigh boats, spatulas, funnels, bottle top volumetric dispensors, and other items.

k. LC vials with screw cap lids- Amber glass, 4 mL, Cat. No. 1232 P 49, Thomas Scientific

l. Plastic screw cap vials - Polypropylene, 4 mL, Cat. No. 1708 H 01, Thomas Scientific

2. Instrumentation

a. Waters UHPLC-MS/MS TQD system with MassLynx operating software.

b. UHPLC column - Waters UHPLC HSS T3, 2.1 x 100 mm, 1.8 µm column with VanGuard Precolumn UHPLC HSS T3 2.1 x 5.0 mm, 1.8 µm.

C. REAGENTS AND SOLUTIONS

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

1. Reagents

a. Hexane - HPLC Grade, Fisher Optima Cat. No. H303-4

b. Acetonitrile (ACN) - HPLC Grade, Spectrum Chemical Co. Cat. No. HP412

c. Formic acid - Sigma Chemical Co., Cat. No. F0507-500ML

d. Water - LC Grade, House deionized water passed through an ELGA Pure Lab Ultra Filtration System.

e. Sodium hydroxide (NaOH) - pellets, Fisher, Item No. S318-3.

2. Solutions

a. 80:20 Acetonitrile/Water:

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.

b. Hexane (saturated with acetonitrile):

Add 40 mL of acetonitrile to 1 L of hexane in a separatory funnel. Mix this solution vigorously. Allow the layers to separate and discard the acetonitrile layer. Transfer the saturated hexane to a dispenser bottle for storage. Mix this solution prior to use each day.

c. 0.1% Formic Acid in water:

Add 1.0 mL of formic acid to a 1 L volumetric flask. Dilute to volume with deionized water.

d. 0.03 M Sodium Hydroxide:

Add 0.12 g of NaOH to a 100 ml volumetric flask containing 80 mL of deionized water. Mix and allow solution to cool. Adjust to final volume using deionized water. Store in a plastic container.

e. UHPLC Aqueous Mobile Phase (5% ACN, 95% Water, 0.1% Formic Acid):

Measure 50 mL of acetonitrile and add to a 1 L volumetric flask. Add 1.0 mL of formic acid to the flask. Bring to volume with deionized water. Mix and transfer to the aqueous reservoir of the LC.

f. UHPLC Organic Mobile Phase (Acetonitrile, 0.1% Formic Acid):

Add 1.0 mL of formic acid into a 1 L volumetric flask. Bring to volume using acetonitrile. Mix and transfer to the organic reservoir of the LC.

D. STANDARD(S)

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

1. Standard Information

Table 1 – Standard information

Analyte Name	Manufacturer	Catalog number	Analyte Name	Manufacturer	Catalog number
2-Quinoxaline carboxylic acid (QCA)	Absolute Standards	91819	Ractopamine	Sigma Aldrich	34198
Amoxicillin	US Pharmacopeia	1031503	Salbutamol	Sigma Aldrich	S8260
Ampicillin	Sigma Aldrich	A1593	Sarafloxacin	Abbott Labs[5]	Not applicable
Beta/ Dexamethasone	MP Biochemicals	154853	Sulfachloro pyridazine	Sigma Aldrich	46778
Cefazolin	Sigma Aldrich	C5020	Sulfadiazine	Sigma Aldrich	S8626
Chloramphenicol	Sigma Aldrich	31667	Sulfadimethoxine	Sigma Aldrich	46794
Chlortetracycline	US Pharmacopeia	1129007	Sulfadoxine	US Pharmacopeia	1626500
Cimaterol	Tocris Bioscience	0435	Sulfaethoxy pyridazine	Fluka	02743
Ciprofloxacin	US Pharmacopeia	1134313	Sulfamerazine	Sigma Aldrich	S8876
Clindamycin	Sigma Aldrich	C5269	Sulfamethazine	Sigma Aldrich	S6256
Cloxacillin	US Pharmacopeia	1142005	Sulfamethizole	Sigma Aldrich	S5632
Danofloxacin	Pfizer[1]	Not applicable	Sulfamethoxazole	Sigma Aldrich	S7507

Analyte Name	Manufacturer	Catalog number	Analyte Name	Manufacturer	Catalog number
DCCD	Pfizer[1]	Not applicable	Pirlimycin	Pfizer[1]	Not applicable
Desethylene Ciprofloxacin	Bayer Healthcare[2]	Not applicable	Prednisone	Sigma Aldrich	P6254
Dicloxacillin	US Pharmacopeia	1189009	Sulfamethoxy pyridazine	Sigma Aldrich	S7257
Difloxacin	Abbott Labs[5]	Not applicable	Sulfanilamide	US Pharmacopeia	1632004
Enrofloxacin	Bayer Healthcare[2]	Not applicable	Sulfanitran	Sigma Aldrich	46882
Erythromycin A	Sigma Aldrich	E0774	Sulfapyridine	Sigma Aldrich	S6252
Florfenicol	Sigma Aldrich	F1427	Sulfaquinoxaline	Sigma Aldrich	45662
Flunixin	US Pharmacopeia	1274607	Sulfathiazole	Sigma Aldrich	S9876
Gamithromycin	Hovione Farma[3]	Not applicable	Tetracycline	US Pharmacopeia	1651009
Lincomycin	Sigma Aldrich	L6004	Tilmicosin	Lilly[4]	Not applicable
Melengestrol Acetate	MP Biochemicals	158952	Tulathromycin A	Pfizer[1]	CP-472,295
Nafcillin	US Pharmacopeia	1450007	Tylosin	Sigma Aldrich	T6134
Norfloxacin	Sigma Aldrich	N9890	Zeranol (B-Zearalanol)	Sigma Aldrich	Z0417
Oxacillin	US Pharmacopeia	1481000	Flunixin-d3	Sigma Aldrich[6]	34083 (Vetranal)
Oxyphenylbutazone	Toronto Research	0876950	13C6 Sulfamethazine Phenyl	Sigma Aldrich[6]	32519 (Vetranal)
Oxytetracycline	Sigma Aldrich	O5875	d7 Penicillin G	Toronto Research[6]	B288600
Penicillin G	US Pharmacopeia	1502508			
Phenylbutazone	MP Biochemicals	153567			

[1] - Pfizer, Groton, CT

[2] - Bayer Healthcare, AG Business Group Pharma, PH-GDD-PT, Clinical Supplies Ops , Wuppertal, Germany.

[3] - Hovione FarmaCiencia SA, Sete Casa, Loures, Portugal

[4] - Lilly Corporate Center, Indianapolis, Indiana

[5] - Abbott Labs, Chicago, IL

[6]- Internal Standard (IS) – optional

2. Preparation of Standard Solution(s)

a. Animal Drug Stock Solutions and internal standard stock solutions

Prepare animal drug stock solutions and internal standard stock solutions at approximately 1.0 mg/mL when adequate material is available. Other concentrations are used based on two criteria:

i. Solubility of the drug in the solvent

ii. Cost and availability of the drug

For each stock solution, calculate the amount of base material needed (ex. accounting for purity and/or water and sulfate content) to prepare at the concentration listed below using the appropriate solvent listed.

Table 2 – Stock standard concentrations

Standard Analyte	Category	Solvent used	Stock Standard Concentration (ng/µL)
2-Quinoxaline Carboxylic Acid (QCA)	Acetonitrile Mix	Purchased	15
Cimaterol	Acetonitrile Mix	Acetonitrile	1000
Ractopamine	Acetonitrile Mix	Water	1000
Salbutamol	Acetonitrile Mix	Acetonitrile or methanol	1000
Chloramphenicol	Acetonitrile Mix	Acetonitrile	1000
Florfenicol	Acetonitrile Mix	Acetonitrile	1000
Ciprofloxacin	Acetonitrile Mix	0.03 M NaOH	1000
Danofloxacin	Acetonitrile Mix	0.03 M NaOH	1000
Difloxacin	Acetonitrile Mix	50%ACN/MeOH	500
Enrofloxacin	Acetonitrile Mix	Acetonitrile	500
Norfloxacin	Acetonitrile Mix	Acetonitrile	1000
Sarafloxacin	Acetonitrile Mix	Methanol	1000
Desethylene Ciprofloxacin	Acetonitrile Mix	0.03 M NaOH	300
Sulfachloropyridazine	Acetonitrile Mix	Acetonitrile	1000
Sulfadiazine	Acetonitrile Mix	Acetonitrile	1000
Sulfadimethoxine	Acetonitrile Mix	Acetonitrile	1000
Sulfadoxine	Acetonitrile Mix	Acetonitrile	1000
Sulfaethoxypyridazine	Acetonitrile Mix	Acetonitrile	1000
Sulfamerazine	Acetonitrile Mix	Acetonitrile	1000
Sulfamethazine	Acetonitrile Mix	Acetonitrile	1000

Standard Analyte	Category	Solvent used	Stock Standard Concentration (ng/µL)
Sulfamethizole	Acetonitrile Mix	Acetonitrile	1000
Sulfamethoxazole	Acetonitrile Mix	Acetonitrile	1000
Sulfamethoxypyridazine	Acetonitrile Mix	Acetonitrile	1000
Sulfanilamide	Acetonitrile Mix	Acetonitrile	2000
Sulfanitran	Acetonitrile Mix	Acetonitrile	500
Sulfapyridine	Acetonitrile Mix	Acetonitrile	1000
Sulfaquinoxaline	Acetonitrile Mix	Acetonitrile	500
Sulfathiazole	Acetonitrile Mix	Acetonitrile	1000
Clindamycin	Acetonitrile Mix	Acetonitrile	1000
Erythromycin A	Acetonitrile Mix	Acetonitrile	1000
Gamithromycin	Acetonitrile Mix	Acetonitrile	500
Lincomycin	Acetonitrile Mix	50%ACN/MeOH	500
Pirlimycin	Acetonitrile Mix	50%ACN/MeOH	1000
Tilmicosin	Acetonitrile Mix	Acetonitrile	1000
Tulathromycin A	Acetonitrile Mix	Acetonitrile	1000
Tylosin	Acetonitrile Mix	Acetonitrile	1000
Chlortetracycline	Acetonitrile Mix	Methanol	500
Oxytetracycline	Acetonitrile Mix	Methanol	1000
Tetracycline	Acetonitrile Mix	Methanol	500
Flunixin	Acetonitrile Mix	Methanol	1000
Phenylbutazone	Acetonitrile Mix	Acetonitrile	1000
Prednisone	Acetonitrile Mix	Methanol	1000
Oxyphenylbutazone	Acetonitrile Mix	Acetonitrile	1000
Beta/Dexamethasone	Acetonitrile Mix	Acetonitrile	1000
Melengestrol Acetate	Acetonitrile Mix	Acetonitrile	1000
Zeranol (B-Zearalanol)	Acetonitrile Mix	Methanol	1000
Amoxicillin	Beta Lactam Mix	Water	350
Ampicillin	Beta Lactam Mix	Water	250
DCCD	Beta Lactam Mix	Water	300
Cefazolin	Beta Lactam Mix	Water	400
Cloxacillin	Beta Lactam Mix	Water	200
Dicloxacillin	Beta Lactam Mix	Water	200
Naficillin	Beta Lactam Mix	Water	300

Standard Analyte	Category	Solvent used	Stock Standard Concentration (ng/µL)
Oxacillin	Beta Lactam Mix	Water	200
Penicillin G	Beta Lactam Mix	Water	250
Flunixin-d3	Internal Standard Mix	Methanol	1000
13C6 Sulfamethazine Phenyl	Internal Standard Mix	Acetonitrile	1000
d7 Penicillin G	Internal Standard Mix	Water	500

"Acetonitrile Mix" stock standards will expire in 6 months when stored at ≤ -10 ºC or at the time of the earliest expiring component. "Beta Lactam Mix" stock standards will expire in 2 months when stored at ≤ -10 ºC or at the time of the earliest expiring component. "Internal Standard Mix" stock standards will expire in 2 months for d7 Penicillin G, or 6 months for Flunixin-d3 and 13C6 Sulfamethazine Phenyl when stored at ≤ -10 ºC.

Note: Internal standards are optional in this method and can be used to monitor injection sequence performance within a set. For issues observed, such as inconsistent internal standard area counts, samples may be reinjected or reanalyzed as needed. If internal standards are not used, appropriate volumes and chemicals must be adjusted as outlined in appropriate method steps below.

b. Intermediate standard solutions

Prepare individual intermediate standard solutions as described for the analytes below in 10 mL volumetric flasks. Intermediate stock standards will expire in 6 months when stored at ≤ -10 ºC or at the time of the earliest expiring component.

Table 3 – Intermediate standard solutions

Analyte	Stock Standard Concentration (ng/µL)	Solvent used	Volume Stock Standard (µL)	Intermediate Standard Concentration (ng/µL)
Cimaterol	1000	Acetonitrile	500	50
Ractopamine	1000	Acetonitrile	500	50
Salbutamol	1000	Acetonitrile	500	50
Chloramphenicol	1000	Acetonitrile	500	50
Flunixin	1000	Acetonitrile	1000	100
Zeranol (B-Zearalanol)	1000	Acetonitrile	500	50
Melengesterol Acetate	1000	Acetonitrile	500	50

c. Antibiotic drug composite working (spiking) and internal standard (spiking) solutions

   i. Prepare the composite “Acetonitrile Mix” working solution(s) for the veterinary drugs contained in the acetonitrile spiking solutions using the stock and intermediate standard solutions above and the volumes listed in the tables below.

      (a) Calculate or use the volume of stock or intermediate stock solution required to give the concentration listed for each standard type in the table below.

      (b) Pipet the calculated volume of stock into a 10 mL volumetric flask.

      (c) Dilute to 10 mL volume with acetonitrile.

      (d) Cap flask and mix.

      (e) Transfer solution into amber glass LC vials with screw cap lids.

      (f) Composite working solutions will expire in 6 months when stored at $\leq$ -10 ºC or at the time of the earliest expiring component.

Table 4 – "Acetonitrile Mix" kidney working solution preparation

Standard Analyte	Stock or Intermediate Standard Concentration (ng/µL)	Bovine Screening Volume (µL)	Bovine Confirmation Volume (µL)	Porcine Screening Volume (µL)	Porcine Confirmation Volume (µL)	Poultry Screening Volume (µL)	Poultry Confirmation Volume (µL)
2-Quinoxaline Carboxylic Acid (QCA)	15					1000	1000
Beta/Dexamethasone	1000	25	25	25	25	25	25
Chloramphenicol	50	60		60		30	
Chlortetracycline	500	1000	1000	1000	1000	1000	1000
Cimaterol	50	60		60		60	
Ciprofloxacin	1000	12.5	12.5	12.5	12.5	12.5	50
Clindamycin	1000	25	25	25	25	25	25
Danofloxacin	1000	12.5	12.5	12.5	12.5	12.5	12.5
Desethylene Ciprofloxacin	300	41.7		41.7		41.7	
Difloxacin	500	25	25	25	25	25	25
Enrofloxacin	500	25	25	25	25	25	
Erythromycin A	1000	25	25	25	25	25	25
Florfenicol	1000	50	50	50	100	50	50
Flunixin	100	62.5	62.5	62.5	62.5	62.5	62.5
Gamithromycin	500	50	50	50	50	50	50
Lincomycin	500	50	50	50	50	50	50
Melengestrol Acetate	50					200	200
Norfloxacin	1000	12.5	12.5	12.5	12.5	12.5	12.5
Oxyphenylbutazone	1000	50	100				
Oxytetracline	1000	500	500	500	500	250	250
Pirlimycin	1000	125	125	125	125	125	125
Prednisone	1000	25	25	25	25	50	50
Ractopamine	50	30		30		60	60
Salbutamol	50	30	30	30	30	30	30
Sarafloxacin	1000	12.5	12.5	12.5	12.5	12.5	12.5
Sulfachloropyridazine	1000	25	25	25	25	25	25
Sulfadiazine	1000	25	25	25	25	25	25
Sulfadimethoxine	1000	25	25	25	25	25	25
Sulfadoxine	1000	25	25	25	25	25	25
Sulfaethoxypyridazine	1000	25	25	25	25	25	25
Sulfamerazine	1000	25	25	25	25	25	25
Sulfamethazine	1000	25	25	25	25	25	25
Sulfamethizole	1000	25	25	25	25	25	25
Sulfamethoxazole	1000	25	25	25	25	25	25
Sulfamethoxypyridazine	1000	25	25	25	25	25	25
Sulfanitran	500	50	50	50	50	200	200
Sulfapyridine	1000	25	25	25	25	25	25
Sulfaquinoxaline	500	50	50	50	50	50	50
Sulfathiazole	1000	25	25	25	25	25	25
Tetracyline	500	500	500	500	500	500	500
Tilmicosin	1000	30	30	30	30	30	30
Tulathromycin A	1000	500	500			500	
Tylosin	1000	50	50	50	50	50	50
Zeranol (B-Zearalanol)	50			120	120		

Table 5 – "Acetonitrile Mix" muscle working solution preparation

Standard Analyte	Stock or Intermediate Standard Concentration (ng/µL)	Bovine Screen Volume (µL)	Bovine Confirm Volume (µL)	Porcine Screen Volume (µL)	Porcine Confirm Volume (µL)	Poultry Screen Volume (µL)	Poultry Confirm Volume (µL)	Equine Screen Volume (µL)	Equine Confirm Volume (µL)
2-Quinoxaline Carboxylic Acid (QCA)	15					1000	1000	2000	2000
Beta/Dexamethasone	1000	25	25	25	25	25	25	25	25
Chloramphenicol	50	60		60		30		30	
Chlortetracycline	500	1000	1000	1000	1000	1000	1000	1000	1000
Cimaterol	50	30		30		60		60	
Ciprofloxacin	1000	12.5	12.5	12.5	12.5	12.5	25	12.5	50
Clindamycin	1000	25	25	25	25	25	25	25	25
Danofloxacin	1000	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Desethylene Ciprofloxacin	300	41.7		41.7		41.7		41.7	
Difloxacin	500	25	25	25	25	25	25	25	25
Enrofloxacin	500	25	25	25	25	25	25	25	25
Erythromycin A	1000	25	25	25	25	25	25	25	25
Florfenicol	1000	50	50	50	50	50	50	50	50
Flunixin	100	62.5	62.5	62.5	62.5	62.5	62.5	62.5	62.5
Gamithromycin	500	50	50	50	200	50	50	50	50
Lincomycin	500	50	50	50	50	50	50	50	50
Melengestrol Acetate	50					200	200	200	200
Norfloxacin	1000	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Oxyphenylbutazone	1000	25		50	100				
Oxytetracline	1000	250	250	250	250	250	250	500	500
Phenylbutazone	1000			50					
Pirlimycin	1000	125	125	125	125	125	125	125	125
Prednisone	1000	25	25	25	25	25	25	25	25
Ractopamine	50	30	30	30	30	30	30	30	30
Salbutamol	50	30	30	30	30	30	30	30	30
Sarafloxacin	1000	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Sulfachloropyridazine	1000	25	25	25	25	25	100	25	50
Sulfadiazine	1000	25	25	25	25	25	25	25	25
Sulfadimethoxine	1000	25	25	25	25	25	25	25	25
Sulfadoxine	1000	25	25	25	25	25	25	25	25
Sulfaethoxypyridazine	1000	25	25	25	25	25	25	25	25
Sulfamerazine	1000	25	25	25	25	25	25	25	25
Sulfamethazine	1000	25	25	25	25	25	25	25	25
Sulfamethizole	1000	25	25	25	25	25	100	25	100
Sulfamethoxazole	1000	25	25	25	25	25	25	25	25
Sulfamethoxypyridazine	1000	25	25	25	25	25	25	25	25
Sulfanitran	500	50	50	50	50	50	100	50	100
Sulfapyridine	1000	25	25	25	25	25	25	25	25
Sulfaquinoxaline	500	50	50	50	50	50	50	50	50
Sulfathiazole	1000	25	25	25	25	25	25	25	25
Tetracyline	500	500	500	500	500	500		500	1000
Tilmicosin	1000	30	30	30	30	30	30	30	30
Tulathromycin A	1000	500	500	500	500	500	500	500	500
Tylosin	1000	50	50	50	50	50	50	50	50

Using these volumes yield the following concentrations as found in the tables below:

Table 6 – Acetonitrile Mix kidney working standard levels

Standard Analyte	Bovine Screening Level (ng/µL)	Bovine Confirmation Level (ng/µL)	Porcine Screening Level (ng/µL)	Porcine Confirmation Level (ng/µL)	Poultry Screening Level (ng/µL)	Poultry Confirmation Level (ng/µL)
2-Quinoxaline Carboxylic Acid (QCA)					1.5	1.5
Beta/Dexamethasone	2.5	2.5	2.5	2.5	2.5	2.5
Chloramphenicol	0.3		0.3		0.15	
Chlortetracycline	50	50	50	50	50	50
Cimaterol	0.3		0.3		0.3	
Ciprofloxacin	1.25	1.25	1.25	1.25	1.25	5
Clindamycin	2.5	2.5	2.5	2.5	2.5	2.5
Danofloxacin	1.25	1.25	1.25	1.25	1.25	1.25
Desethylene Ciprofloxacin	1.25		1.25		1.25	
Difloxacin	1.25	1.25	1.25	1.25	1.25	1.25
Enrofloxacin	1.25	1.25	1.25	1.25	1.25	
Erythromycin A	2.5	2.5	2.5	2.5	2.5	2.5
Florfenicol	5	5	5	10	5	5
Flunixin	0.625	0.625	0.625	0.625	0.625	0.625
Gamithromycin	2.5	2.5	2.5	2.5	2.5	2.5
Lincomycin	2.5	2.5	2.5	2.5	2.5	2.5
Melengestrol Acetate					1	1
Norfloxacin	1.25	1.25	1.25	1.25	1.25	1.25
Oxyphenylbutazone	5	10				
Oxytetracline	50	50	50	50	25	25
Pirlimycin	12.5	12.5	12.5	12.5	12.5	12.5
Prednisone	2.5	2.5	2.5	2.5	5	5
Ractopamine	0.15		0.15		0.3	0.3
Salbutamol	0.15	0.15	0.15	0.15	0.15	0.15
Sarafloxacin	1.25	1.25	1.25	1.25	1.25	1.25
Sulfachloropyridazine	2.5	2.5	2.5	2.5	2.5	2.5
Sulfadiazine	2.5	2.5	2.5	2.5	2.5	2.5
Sulfadimethoxine	2.5	2.5	2.5	2.5	2.5	2.5
Sulfadoxine	2.5	2.5	2.5	2.5	2.5	2.5
Sulfaethoxypyridazine	2.5	2.5	2.5	2.5	2.5	2.5
Sulfamerazine	2.5	2.5	2.5	2.5	2.5	2.5
Sulfamethazine	2.5	2.5	2.5	2.5	2.5	2.5
Sulfamethizole	2.5	2.5	2.5	2.5	2.5	2.5
Sulfamethoxazole	2.5	2.5	2.5	2.5	2.5	2.5
Sulfamethoxypyridazine	2.5	2.5	2.5	2.5	2.5	2.5
Sulfanitran	2.5	2.5	2.5	2.5	10	10
Sulfapyridine	2.5	2.5	2.5	2.5	2.5	2.5
Sulfaquinoxaline	2.5	2.5	2.5	2.5	2.5	2.5
Sulfathiazole	2.5	2.5	2.5	2.5	2.5	2.5
Tetracyline	25	25	25	25	25	25
Tilmicosin	3	3	3	3	3	3
Tulathromycin A	50	50			50	
Tylosin	5	5	5	5	5	5
Zeranol (B-Zearalanol)			0.6	0.6		

Table 7 – Acetonitrile Mix muscle working standard levels

Standard Analyte	Bovine Screen Level (ng/µL)	Bovine Confirm Level (ng/µL)	Porcine Screen Level (ng/µL)	Porcine Confirm Level (ng/µL)	Poultry Screen Level (ng/µL)	Poultry Confirm Level (ng/µL)	Equine Screen Level (ng/µL)	Equine Confirm Level (ng/µL)
2-Quinoxaline Carboxylic Acid (QCA)					1.5	1.5	3	3
Beta/Dexamethasone	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Chloramphenicol	0.3		0.3		0.15		0.15	
Chlortetracycline	50	50	50	50	50	50	50	50
Cimaterol	0.15		0.15		0.3		0.3	
Ciprofloxacin	1.25	1.25	1.25	1.25	1.25	2.5	1.25	5
Clindamycin	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Danofloxacin	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Desethylene Ciprofloxacin	1.25		1.25		1.25		1.25	
Difloxacin	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Enrofloxacin	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Erythromycin A	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Florfenicol	5	5	5	5	5	5	5	5
Flunixin	0.625	0.625	0.625	0.625	0.625	0.625	0.625	0.625
Gamithromycin	2.5	2.5	2.5	10	2.5	2.5	2.5	2.5
Lincomycin	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Melengestrol Acetate					1	1	1	1
Norfloxacin	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Oxyphenylbutazone	2.5		5	10				
Oxytetracline	25	25	25	25	25	25	50	50
Phenylbutazone			5					
Pirlimycin	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Prednisone	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Ractopamine	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Salbutamol	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15
Sarafloxacin	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Sulfachloropyridazine	2.5	2.5	2.5	2.5	2.5	10	2.5	5
Sulfadiazine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfadimethoxine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfadoxine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfaethoxypyridazine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfamerazine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfamethazine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfamethizole	2.5	2.5	2.5	2.5	2.5	10	2.5	10
Sulfamethoxazole	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfamethoxypyridazine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfanitran	2.5	2.5	2.5	2.5	2.5	5	2.5	5
Sulfapyridine	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfaquinoxaline	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Sulfathiazole	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Tetracyline	25	25	25	25	25		25	50
Tilmicosin	3	3	3	3	3	3	3	3
Tulathromycin A	50	50	50	50	50	50	50	50
Tylosin	5	5	5	5	5	5	5	5

ii. Prepare the composite working solution(s) for the veterinary drugs contained in the "Beta Lactam mix" spiking solutions using the stock standard solutions above and the volumes listed in the table below.

(a) Calculate or use the volume of stock solution required to give the concentrations listed for each standard type in the table below.

(b) Pipet the calculated volume of stock into a 10 mL volumeteric flask.

(c) Dilute to 10 mL volume with water

(d) Cap flask and mix

(e) Transfer solution into plastic screw cap vials.

(f) Composite working solutions will expire in 2 months when stored at ≤ -10 ºC or at the time of the earliest expiring component.

Table 8 – Beta-lactam kidney working solutions preparation volumes

Standard Analyte	Stock or Intermediate Standard Concentration (ng/µL)	Bovine Screening Volume (µL)	Bovine Confirmation Volume (µL)	Porcine Screening Volume (µL)	Porcine Confirmation Volume (µL)	Poultry Screening Volume (µL)	Poultry Confirmation Volume (µL)
Amoxicillin	350					28.6	
Ampicillin	250	20	20	20	20	20	20
Cefazolin	400	62.5	125	62.5	125	62.5	62.5
Cloxacillin	200	12.5	12.5	12.5	25	12.5	12.5
DCCD	300	333.3	166.7	166.7	166.7	83.3	83.3
Dicloxacillin	200	125	125	125	125	125	125
Naficillin	300	83.3	83.3	83.3	83.3	83.3	83.3
Oxacillin	200	125	125	125	125	125	125
Penicillin G	250	50	50	50	50	50	50

Table 9 – Beta-lactam muscle working solutions preparation volumes

Standard Analyte	Stock or Intermediate Standard Concentration (ng/µL)	Bovine Screen Volume (µL)	Bovine Confirm Volume (µL)	Porcine Screen Volume (µL)	Porcine Confirm Volume (µL)	Poultry Screen Volume (µL)	Poultry Confirm Volume (µL)	Equine Screen Volume (µL)	Equine Confirm Volume (µL)
Amoxicillin	350	114.3		114.3					
Ampicillin	250	20	20	20	20	10	10	20	20
Cefazolin	400	62.5	62.5	62.5	125	62.5	125	125	125
Cloxacillin	200	25	25	25	25	12.5	25	12.5	25
DCCD	300	166.7	166.7	333.3	333.3	166.7	333.3	83.3	166.7
Dicloxacillin	200	125	125	125	125	125	125	125	125
Naficillin	300	83.3	83.3	83.3	83.3	83.3	83.3	83.3	83.3
Oxacillin	200	125	125	125	125	125	125	125	125
Penicillin G	250	50	100	50	200	100	100	100	100

Using these volumes yield the following concentrations as found in the tables below:

Table 10 – Beta-lactam kidney working solution levels

Standard Analyte	Bovine Screening Level (ng/µL)	Bovine Confirmation Level (ng/µL)	Porcine Screening Level (ng/µL)	Porcine Confirmation Level (ng/µL)	Poultry Screening Level (ng/µL)	Poultry Confirmation Level (ng/µL)
Amoxicillin					1	
Ampicillin	0.5	0.5	0.5	0.5	0.5	0.5
Cefazolin	2.5	5	2.5	5	2.5	2.5
Cloxacillin	0.25	0.25	0.25	0.5	0.25	0.25
DCCD	10	5	5	5	2.5	2.5
Dicloxacillin	2.5	2.5	2.5	2.5	2.5	2.5
Naficillin	2.5	2.5	2.5	2.5	2.5	2.5
Oxacillin	2.5	2.5	2.5	2.5	2.5	2.5
Penicillin G	1.25	1.25	1.25	1.25	1.25	1.25

Table 11 – Beta-lactam muscle working solution levels

Standard Analyte	Bovine Screen Level (ng/µL)	Bovine Confirmat Level (ng/µL)	Porcine Screen Level (ng/µL)	Porcine Confirm Level (ng/µL)	Poultry Screen Level (ng/µL)	Poultry Confirm Level (ng/µL)	Equine Screen Level (ng/µL)	Equine Confirm Level (ng/µL)
Amoxicillin	4		4					
Ampicillin	0.5	0.5	0.5	0.5	0.25	0.25	0.5	0.5
Cefazolin	2.5	2.5	2.5	5	2.5	5	5	5
Cloxacillin	0.5	0.5	0.5	0.5	0.25	0.5	0.25	0.5
DCCD	5	5	10	10	5	10	2.5	5
Dicloxacillin	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Naficillin	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Oxacillin	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Penicillin G	1.25	2.5	1.25	5	2.5	2.5	2.5	2.5

iii. Prepare the composite (spiking) solution for the isotopically-labeled veterinary drugs used for internal standards at 20 ppm if internal standards will be used. This solution can contain any number of the internal standards listed in the method.

(a) Calculate the volume of stock solution required to give the 20 ppm level (see the following table).

(b) Pipet the calculated volume of stock into a 5 mL volumeteric flask.

(c) Dilute to 5 mL with acetonitrile.

(d) Cap flask and mix.

(e) Transfer 1.25 mL of solution into 4 amber glass LC vials.

(f) Store at < -10 ºC. The stability of the solution is dependent on which internal standards are present. If the solution contains D7 Penicillin G, then the solution is stable for 2 months. Otherwise, the solution is stable for 6 months.

Table 12 – IS spiking solution

Standard Analyte	Stock or Intermediate Standard Concentration (ng/µL)	Volume (µL)	Final Volume (mL)	Working Standard Concentration (ng/µL)
13C6 Sulfamethazine Phenyl	1000	100	5	20
d7 Penicillin G	500	200	5	20
Flunixin-d3	1000	100	5	20

3. Preparation of External Calibration Curve (Optional)

Use the following table to prepare external standards.

Table 13 – Preparation of external standards

Target Conc.	Volume Acetonitrile Standard Mix (µL)	Volume Beta Lactam Standard Mix (µL)	Volume Internal Standard Mix (µL)	Volume Acetonitrile (µL)	Volume of 0.1% Formic Acid in water
0 X	0	0	20	120	860
1/4 X	10	10	20	110	850
1/2 X	20	20	20	100	840
1 X	40	40	20	80	820
2 X	80	80	20	40	780
3 X	120	120	20	0	740

Note: The volume of 0.1% Formic Acid in water will be increased by 20 µL to maintain a total volume of 1 mL if no internal standard solution is added.

E. SAMPLE PREPARATION

Samples collected fresh must be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples must be frozen (< -10 °C) prior to grinding if they cannot be prepared on the day of receipt. Once frozen, the sample should be allowed to thaw, while keeping it as cold as possible. Dissect away fat and connective tissue. Grind tissue in blender or vertical cutter-mixer until homogeneous. Store samples frozen (< -10 °C) prior to analysis.

F. ANALYTICAL PROCEDURE

1. Preparation of Controls and Samples

   a. Weigh 2 ± 0.1 g of homogenized samples into labeled 50 mL polypropylene centrifuge tubes.

      Note: Use corresponding blank tissue for controls for each specific species and tissue sample being analyzed.

      i. Screening - Weigh one portion each for a blank (negative control), a 1/2 X recovery (positive control), a matrix matched standard, and a check sample, if necessary.

      ii. Confirmation - Weigh six 2 g portions of blank tissue into 50 ml polypropylene centrifuge tubes. One for 1/2 X recovery (positive control), one for the blank (negative control) and four for the matrix matched standards (1/2X, 1X, 2X, and 3X). Weigh one additional portion for a check sample, if necessary.

      iii. Prepare recoveries, check samples, blank, matrix matched standard(s), and samples using the solutions and volumes in the table below:

Table 14 – Preparation of controls and samples

Sample Type	Acetonitrile Standard Mix (µL)	Beta Lactam Standard Mix (µL)	Internal Standard Mix (µL)	Acetonitrile (µL)	Water (µL)
Samples and Negative Controls			40	160	160
Matrix Matched Standards				200	160
½ X Recovery	40	40	40	120	120
1X Recovery	80	80	40	80	80
2X Recovery	160	160	40		

Note: If no internal standard mix is used, the volume of acetonitrile added to each tube must be increased by 40 µL.

Note: The “1/2 X Recovery” sample type represents the minimum level of applicability (refer to Section J.3).

2. Extraction Procedure

   a. Vortex all uncapped tubes 10 seconds each to mix chemicals with matrix and allow to stand 5 minutes.

Note: Press down to start swirling action slowly and then increase speed to mix without splashing.

b. Add 9.7 mL of 4/1 (v/v) acetonitrile/water to all tubes using a calibrated solvent dispenser. Cap tubes well.

c. Place the racks of tubes in platform shaker on high for 5 minutes.

d. Centrifuge the tubes at >3000 rcf for 5 minutes.

e. Decant each extract into a pre-labeled 50 mL polypropylene centrifuge tubes containing 0.50 g of C18.

f. Add 10 mL of hexane (saturated with acetonitrile) using a calibrated dispenser to all tubes. Cap all tubes well.

g. Shake all tubes in the platform shaker on high for 1 minute.

h. Centrifuge all tubes at approximately 3000 rcf for 5 minutes.

i. Aspirate hexane to waste using a Pasteur pipette on a hose connected to a side-arm Erlenmeyer flask.

j. Pipet a 5 mL aliquot of the extract into a pre-labeled 15 mL polypropylene centrifuge tubes.

k. Evaporate extract to ≤ 0.5 mL in TurboVap at 45 ± 2°C at 15 psi setting, changing to 20 psi after 10 minutes. Total time to evaporate to ≤ 0.5 mL is 45 to 60 minutes.

l. Per the table below, add the volumes of the standard mixes and solutions to the matrix matched standards, add 140 µL acetonitrile to all other tubes, and dilute to 1.0 mL with 0.1% formic acid in water. Vortex all tubes for 5 seconds.

Note: After dilution to 1.0 mL with 0.1% formic acid in water, the extracts will contain ≈14% acetonitrile by volume.

Table 15 – Preparation of matrix matched standards

Sample Type	Acetonitrile Standard Mix (µL)	Beta Lactam Standard Mix (µL)	Internal Standard Mix (µL)	Acetonitrile (µL)
Samples, Recoveries, and Negative Controls				140
1/2X Matrix Matched Standard	20	20	20	100
1X Matrix Matched Standard	40	40	20	80
2X Matrix Matched Standard	80	80	20	40
3X Matrix Matched Standard	120	120	20	0

Note: If no internal standard mix is used, the volume of acetonitrile added to each tube must be increased by 20 µL.

m. Pipet 500 µL of all final extracts and calibration standards into bottom portions of pre-labeled Whatman Mini UniPrep Syringless Filter Vials, PVDF, 0.2 micron.

n. Pressed filter/caps onto the vials and placed in autosampler tray for UHPLC-MS/MS analysis.

3. Instrumental Settings

Note: The instrument parameters may be optimized to ensure system suitability.

a. Instrument Operating Parameters - UHPLC system

i. Mobile phase for Residue analysis:

Mobile Phase A - 95% water / 5% ACN / 0.1% Formic Acid
Mobile Phase B - 100% ACN / 0.1% Formic Acid

Flush column with 1:1 A/B at a flow rate of 0.5 mL/min for three minutes. Change the mobile phase initial conditions to 100% A. Allow column to equilibrate until the “delta” value on the pressure reading is < 20.

ii. UHPLC gradient program:

Flow rate: 0.5 mL/min
Pressure Limits: 200 psi minimum; 15,000 psi maximum
Run time: 12.9 minutes

Table 16 – UHPLC gradient program

Time (min)	*% Mobile Phase A*	*% Mobile Phase B*	*Gradient*
0.00	100	0	none
0.10	80	20	linear
8.00	80	20	linear
9.50	60	40	linear
9.60	10	90	linear
12.90	10	90	linear

b. Autosampler program:

i. Run time: 12.90 minutes

ii. Injection loop: 20 µL

iii. Loop option: Full Loop

iv. Injection volume: 20 µL

v. Weak wash solvent: 10/90 Acetonitrile/Water

vi. Weak wash volume: 1200 µL

vii. Strong wash solvent: 50/50 Acetonitrile/Water

viii. Strong wash volume: 400 µL

ix. Sample temperature: 10 °C

c. Column manager

i. Column valve position: To match column location.

ii. Column manager temperature: 40 °C

iii. Use divert valve to divert eluant to waste 0.25 minutes prior to first peak and 0.25 minutes after last analyte peak.

d. Instrument Operating Parameters - Mass Spectrometer

Mass Spectrometer calibration and resolution are to be done according to the manufacturer's specification using the manufacturer's supplied calibration solution.

i. Type: MS/MS

ii. Electrospray Source Parameters:

(a) Capillary (kV): 3.0

(b) Cone (V): Variable - analyte dependent

(c) Extractor (V): 3.0

(d) RF (V): 0.10

(e) Source Temperature (°C): 150

(f) Desolvation Temperature (°C): 450

(g) Cone Gas Flow (L/hr): 20

(h) Desolvation Gas Flow (L/hr): 900

(i) Collision Gas Flow (mL/min): 0.10

iii. Analyzer Parameters:

(a) LM 1 Resolution: 10.6

(b) HM 1 Resolution: 14.8

(c) MSMS Mode Entrance: 1

(d) MSMS Mode Collision Energy: Variable – analyte dependent

(e) MSMS Mode Exit: 0.5

(f) LM 2 Resolution: 9.5

(g) HM 2 Resolution: 15.8

iv. MS Method Parameters:

(a) Type: MRM

(b) Ion Mode: ES+

(c) Dwell (s): 0.005

(d) Start time (min): 0.4

(e) End time (min): 6.1

v. Instrumental Settings - Scan Parameters

Table 17 – Instrument settings

Analyte	RT (min)	window (min)	Dwell Times (ms)	Precursor Ion(m/z)	Product Ions (m/z)	Collision Energy (V)	Cone (V)
2-Quinoxaline Carboxylic Acid	2.34	2.22-2.60	5	174.95	**129.00** **131.00** **102.00**	16 16 30	22
Sulfanilamide	1.06	0.42 - 2.00	5	172.87	**92.88** 75.90 65.90	36 22 20	44
Amoxicillin	1.43	1.25 – 1.90	5	366.14	**114.00** 349.34 208.09	22 10 14	20
Salbutamol	1.43	1.27 - 1.67	5	240.23	**148.18** 222.30 166.15	20 10 15	20
Cimaterol	1.47	1.32 - 1.72	5	220.04	**142.97** 115.88 88.91	24 16 10	20
DCCD	1.66	1.55 - 2.10	5	549.10	**182.96** 241.12 125.91	30 20 76	40
Lincomycin	1.82	1.69 - 2.09	5	407.31	**126.10** 359.22 389.21	35 20 15	40
Sulfadiazine	1.91	1.79 - 2.19	5	251.10	**156.08** 108.02 158.08	15 20 15	30
Ampicillin	1.95	1.82 - 2.35	5	350.14	**106.07** 114.00 160.07	24 30 24	26
Desethylene Ciprofloxacin	1.98	1.88 - 2.30	5	306.20	**288.19** 245.18 289.28	20 20 10	35
Sulfathiazole	2.00	1.90 - 2.30	5	256.07	**156.08** 108.04 101.03	15 25 25	25
Sulfapyridine	2.09	1.92 - 2.37	5	250.09	**156.08** 92.00 108.11	18 26 28	32
Norfloxacin	2.07	1.95 - 2.43	5	320.17	**276.19** 233.10 219.03	20 30 20	35
Tulathromycin A	2.08	1.93 - 2.38	5	806.75	**72.01** 577.47 116.04	35 25 30	40

Analyte	RT (min)	window (min)	Dwell Times (ms)	Precursor Ion(m/z)	Product Ions (m/z)	Collision Energy (V)	Cone (V)
Oxytetracycline	2.13	1.94 - 2.40	5	461.23	**426.35** 443.39 201.24	20 15 25	25
Ciprofloxacin	2.13	2.00 - 2.45	5	332.23	**245.18** 288.39 203.21	25 20 40	35
Ractopamine	2.19	2.08 - 2.48	5	302.20	**164.03** 106.98 120.98	15 30 30	20
Sulfamerazine	2.21	2.10 - 2.49	5	265.04	**91.88** 155.92 107.91	30 15 15	35
Danofloxacin	2.22	2.12 - 2.52	5	358.12	**96.03** 314.18 283.14	30 15 25	30
Tetracycline	2.27	2.16 - 2.56	5	445.23	**154.13** 410.22 427.32	30 20 15	30
Enrofloxacin	2.30	2.20 - 2.60	5	360.24	**316.40** 245.25 203.16	20 25 40	35
Sulfamethizole	2.44	2.30 - 2.70	5	271.06	**156.08** 91.97 107.98	16 30 26	28
Sulfamethazine	2.46	2.26 - 2.65	5	279.14	**186.14** 156.08 108.12	20 20 25	35
Sulfamethazine-6C13	2.46	2.24 - 2.73	5	285.17	**186.06** 98.02 124.05	18 32 26	32
Cefazolin	2.44	2.32 - 2.80	5	455.13	**156.03** 323.15 112.08	16 12 35	20
Sulfamethoxy pyridazine	2.48	2.34 - 2.72	5	281.12	**156.09** 126.17 108.06	20 20 25	30
Difloxacin	2.53	2.27 - 2.75	5	400.25	**356.43** 299.23 285.28	20 30 40	35
Sarafloxacin	2.48	2.35 - 2.75	5	386.09	**342.18** 299.17 270.20	20 25 45	40
Pirlimycin	2.67	2.44 - 2.84	5	411.29	**112.20** 363.33 110.34	40 20 45	30

Analyte	RT (min)	window (min)	Dwell Times (ms)	Precursor Ion(m/z)	Product Ions (m/z)	Collision Energy (V)	Cone (V)
Chlortetracycline	2.76	2.63 - 3.03	5	479.19	**154.08** 444.26 462.20	30 20 20	30
Clindamycin	2.81	2.68 - 3.08	5	425.30	**126.22** 377.40 124.32	40 20 45	45
Gamithromycin	2.85	2.70 - 3.10	5	777.79	**83.02** 115.97 158.10	48 36 46	66
Sulfachloropyridazine	2.84	2.29 - 3.00	5	285.00	**156.08** 107.98 91.97	16 26 30	28
Tilmicosin	3.00	2.83 - 3.25	5	869.79	**174.16** 132.23 696.63	35 35 35	45
Sulfadoxine	3.00	2.85 - 3.25	5	311.15	**156.08** 108.14 140.09	20 30 30	35
Sulfamethoxazole	3.00	2.89 - 3.29	5	254.02	**92.06** 155.97 107.89	25 20 20	30
Sulfaethoxypyridazine	3.03	2.91 - 3.31	5	295.13	**156.09** 140.18 108.09	20 20 25	30
Florfenicol	3.05	2.93 - 3.33	5	358.10	**241.01** 206.00 130.36	18 22 60	20
Chloramphenicol	3.25	3.00 - 3.60	5	323.11	**274.97** 164.99 118.78	15 30 40	15
Erythromycin A	3.45	3.28 - 3.68	5	734.75	**158.15** 115.89 576.48	30 40 20	30
Sulfadimethoxine	3.46	3.33 - 3.75	5	311.14	**156.10** 108.04 245.22	20 30 20	35
Sulfaquinoxaline	3.46	3.35 - 3.75	5	301.10	**156.13** 107.98 91.97	18 28 36	34
Prednisone	3.56	3.37 - 3.85	5	359.15	**341.11** 146.94 267.28	10 26 15	22
Tylosin	3.63	3.46 - 3.86	5	916.76	**174.20** 101.08 145.15	35 35 35	45

Analyte	RT (min)	window (min)	Dwell Times (ms)	Precursor Ion(m/z)	Product Ions (m/z)	Collision Energy (V)	Cone (V)
Pen G-d7	3.74	3.63 - 4.20	5	342.13	**183.10** 160.10 98.10	26 24 54	46
Penicillin G	3.77	3.61 - 4.03	5	335.13	**176.02** 160.10 114.01	16 18 30	18
Beta/Dexamethasone	4.01	3.89 - 4.29	5	393.22	**373.24** 147.07 355.29	10 28 14	20
Sulfanitran	4.05	3.93 - 4.33	5	336.15	**156.08** 134.13 92.71	14 28 38	30
Zeranol (B-Zearalanol)	4.26	4.13 - 4.53	5	323.21	**305.20** 189.09 149.02	10 24 30	16
Oxacillin	4.30	4.17 - 4.70	5	402.14	**160.00** 243.08 144.06	20 18 34	22
Cloxacillin	4.56	4.43 - 5.05	5	436.16	**160.06** 277.14 114.07	12 16 44	22
Nafcillin	4.69	4.55 - 5.10	5	415.22	**199.17** 171.07 115.11	16 42 70	20
Oxyphenylbutazone	4.74	4.59 - 4.99	5	325.18	**120.06** 148.16 204.12	24 30 16	26
Flunixin	4.82	4.61 - 5.03	5	297.05	**279.05** 109.00 264.04	22 50 32	42
Flunixin-d3	4.81	4.63 - 5.03	5	300.05	**282.12** 112.03 264.04	24 54 36	40
Dicloxacillin	4.94	4.80 - 5.35	5	470.19	**160.06** 311.11 114.07	14 16 48	22
Phenylbutazone	5.86	5.69 - 6.09	5	309.12	**119.95** 76.90 91.80	22 24 18	32
Melengesterol acetate	6.20	6.04 - 6.44	5	397.35	**279.33** 337.46 221.28	20 15 40	30

Note: Product ions are listed with the expected screening ion in bold (top) followed by diagnostic ions 1 and 2 (middle and bottom).

Note: The following parameters will be used if screening for Chloramphenicol in negative electrospray ionization mode:

Analyte	RT	Window	Dwell Time	Precursor	Product	Collision	Cone
ES (-)	(min)	(min)	(ms)	Ion (m/z)	Ions (m/z)	Energy (V)	(V)
Chloramphenicol	3.25	3.00-3.60	0.008	321.09	152.03	15	35

4. Sample Set
   a. Screening Set
      i. External Standard(s) (optional)
      ii. Matrix matched standard
      iii. Recovery(ies) (positive controls)
      iv. Check sample (if necessary)
      v. Blank (negative control)
      vi. Up to 27 Samples
      vii. External standard, matrix matched standard, or recovery
   b. Confirmation Set
      i. External Standard(s) (optional)
      ii. Matrix matched standards
      iii. Recovery(ies) (positive control)
      iv. Check sample (if necessary)
      v. Matrix Matched Blank (negative control)
      vi. Up to 24 Samples
      vii. External standard, matrix matched standard, or recovery

Note: Placing solvent blanks in the sample injection sequence is prudent in case a high finding leads to carry-over. Additionally, one may want to include an additional external standard, matrix matched standard, or recovery within the sample injection sequence to verify retention time and instrument response stability.

G. CALCULATIONS / IDENTIFICATION

1. Screening
   a. The screening ion for a given analyte must be present. The required ion for each compound is listed in Table 17.
   b. The retention times for the screening ion in the fortified recoveries must match

the retention time of the screening ion in the matrix-matched standard within 5%. Retention time for the screening ions in the samples must match the retention time of the screening ions in a fortified recovery or the matrix matched standard within 5%.

c. The screening ion must have a signal-to-noise ratio ≥ 3. This may be verified by visual inspection.

d. A sample is screened positive for an analyte if the following criteria are met:

i. The fortified recovery of the analyte must exceed 10% of the 1/2 X matrix matched standard level.

ii. The sample response equals or exceeds the 1/2 X (or level of interest) fortified recovery level.

e. The level of the screening ion in the blank (negative control) must be less than 10% of the 1/2 X level for the matrix-matched standard.

Note: If a sample shows a positive response for a compound which did not meet screening criteria in the associated QC samples, then further testing of that sample is warranted.

2. Confirmation

a. Monitored ions for each analyte will be assessed as follows:

i. Recovery retention times must match the retention time of the matrix matched standard within 5%. Retention time for the samples must match the retention time of the positive control or the matrix matched standard within 5%.

ii. All product ions specified for ratio matching are present with a signal-to-noise ratio ≥ 3. This may be verified by visual inspection.

iii. One of the following ion ratio matching conditions is met:

Note: Ratios are calculated by dividing the area count of each diagnostic ion by the area count of the base ion. Ion ratios should be less than 1. If the ratio is not less than 1 for a sample set, the inverse of this ratio may be used.

(a) If two product ions are assessed, one sample ion ratio should match the calculated average ratio of the matrix-matched standards within a ± 10% absolute difference.

(b) If three product ions are monitored, the presence of two sample ion ratios should match the calculated average ratio of the matrix-matched standards within a ± 20% absolute difference.

iv. The fortified recovery of the analyte must exceed 10% of the 1/2 X matrix matched standard level.

v. The blank (negative control) must be less than 10% of the 1/2 X level for the matrix matched standard.

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment -Safety eyewear, protective gloves, and lab coat.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Antibiotic standards	Some individuals may have allergic reactions to certain β-lactams, sulfa, or other drugs.	Wear appropriate personal protective equipment to avoid dermal contact.
Acetonitrile, Methanol	Flammable	Keep in well-closed containers away from ignition sources. Avoid contact or prolonged exposure to vapors. Work in fume hood. Keep away from flame or heat.
Formic acid	Corrosive, Caustic	Wear personal protective equipment, avoid skin contact.

3. Disposal Procedures

Follow local, state and federal guidelines for disposal.

I. QUALITY ASSURANCE PLAN

1. Performance Standard

a. Screening Criteria

i. For set acceptance, 90% of the monitored analytes in the fortified recovery (positive control) must meet screening criteria. For sample reporting purposes, the analytes of interest in the fortified recovery (positive control) must meet screening criteria.

ii. The blank (negative control) must be negative using the criteria in Section G.

b. Confirmation Criteria

i. For set acceptance, nine of the following ten analytes must meet confirmation criteria (Beta-Dexamethasone, Sarafloxacin, Erythromycin A, Florfenicol, Flunixin, Oxytetracycline, Penicillin G, Prednisone, Sulfadimethoxine, and Sulfamethazine). For sample reporting purposes, the analytes of interest in the fortified recovery (positive control) must meet confirmation criteria.

ii. The blank (negative control) must be negative using the criteria in Section G for the analytes of interest.

2. Critical Control Points and Specifications

Record	*Acceptable Control*
none known at this time	

3. Intralaboratory Check Samples

a. System, minimum contents.

i. Frequency: One per week per analyst when samples analyzed.

ii. Records are to be maintained.

b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Investigate following established procedures.

ii. Take corrective action as warranted.

4. Sample Condition upon Receipt

Cool or frozen

J. APPENDIX

1. References

[Reserved]

2. Chromatograms/spectra

[Reserved]

3. Minimum Level of Applicability

Table 18 - Minimum Level of Applicability for kidney

Standard Analyte	Bovine Screening Level (ng/g)	Bovine Confirmation Level (ng/g)	Porcine Screening Level (ng/g)	Porcine Confirmation Level (ng/g)	Poultry Screening Level (ng/g)	Poultry Confirmation Level (ng/g)
2-Quinoxaline Carboxylic Acid (QCA)					30	30
Amoxicillin					20	
Ampicillin	10	10	10	10	10	10
Beta/Dexamethasone	50	50	50	50	50	50
Cefazolin	50	100	50	100	50	50
Chloramphenicol	6		6		3	
Chlortetracycline	1000	1000	1000	1000	1000	1000
Cimaterol	6		6		6	
Ciprofloxacin	25	25	25	25	25	100
Clindamycin	50	50	50	50	50	50
Cloxacillin	5	5	5	10	5	5
Danofloxacin	25	25	25	25	25	25
DCCD	200	100	100	100	50	50
Desethylene Ciprofloxacin	25		25		25	
Dicloxacillin	50	50	50	50	50	50
Difloxacin	25	25	25	25	25	25
Enrofloxacin	25	25	25	25	25	
Erythromycin A	50	50	50	50	50	50
Florfenicol	100	100	100	200	100	100
Flunixin	12.5	12.5	12.5	12.5	12.5	12.5
Gamithromycin	50	50	50	50	50	50
Lincomycin	50	50	50	50	50	50
Melengestrol Acetate					20	20
Naficillin	50	50	50	50	50	50
Norfloxacin	25	25	25	25	25	25
Oxacillin	50	50	50	50	50	50
Oxyphenylbutazone	100	200				
Oxytetracline	1000	1000	1000	1000	500	500
Penicillin G	25	25	25	25	25	25
Pirlimycin	250	250	250	250	250	250
Prednisone	50	50	50	50	100	100
Ractopamine	3		3		6	6
Salbutamol	3	3	3	3	3	3
Sarafloxacin	25	25	25	25	25	25
Sulfachloropyridazine	50	50	50	50	50	50
Sulfadiazine	50	50	50	50	50	50
Sulfadimethoxine	50	50	50	50	50	50
Sulfadoxine	50	50	50	50	50	50
Sulfaethoxypyridazine	50	50	50	50	50	50
Sulfamerazine	50	50	50	50	50	50
Sulfamethazine	50	50	50	50	50	50
Sulfamethizole	50	50	50	50	50	50
Sulfamethoxazole	50	50	50	50	50	50

Standard Analyte	Bovine Screening Level (ng/g)	Bovine Confirmation Level (ng/g)	Porcine Screening Level (ng/g)	Porcine Confirmation Level (ng/g)	Poultry Screening Level (ng/g)	Poultry Confirmation Level (ng/g)
Sulfamethoxypyridazine	50	50	50	50	50	50
Sulfanitran	50	50	50	50	200	200
Sulfapyridine	50	50	50	50	50	50
Sulfaquinoxaline	50	50	50	50	50	50
Sulfathiazole	50	50	50	50	50	50
Tetracyline	500	500	500	500	500	500
Tilmicosin	60	60	60	60	60	60
Tulathromycin A	1000	1000			1000	
Tylosin	100	100	100	100	100	100
Zeranol (B-Zearalanol)			12	12		

Table 19 - Minimum Level of Applicability for muscle

Standard Analyte	Bovine Screen Level (ng/g)	Bovine Confirm Level (ng/g)	Porcine Screen Level (ng/g)	Porcine Confirm Level (ng/g)	Poultry Screen Level (ng/g)	Poultry Confirm Level (ng/g)	Equine Screen Level (ng/g)	Equine Confirm Level (ng/g)
2-Quinoxaline Carboxylic Acid (QCA)					30	30	60	60
Amoxicillin	80		80					
Ampicillin	10	10	10	10	5	5	10	10
Beta/Dexamethasone	50	50	50	50	50	50	50	50
Cefazolin	50	50	50	100	50	100	100	100
Chloramphenicol	6		6		3		3	
Chlortetracycline	1000	1000	1000	1000	1000	1000	1000	1000
Cimaterol	3		3		6		6	
Ciprofloxacin	25	25	25	25	25	50	25	100
Clindamycin	50	50	50	50	50	50	50	50
Cloxacillin	10	10	10	10	5	10	5	10
Danofloxacin	25	25	25	25	25	25	25	25
DCCD	100	100	200	200	100	200	50	100
Desethylene Ciprofloxacin	25		25		25		25	
Dicloxacillin	50	50	50	50	50	50	50	50
Difloxacin	25	25	25	25	25	25	25	25
Enrofloxacin	25	25	25	25	25	25	25	25
Erythromycin A	50	50	50	50	50	50	50	50
Florfenicol	100	100	100	100	100	100	100	100
Flunixin	12.5	12.5	12.5	12.5	12.5	12.5	12.5	12.5
Gamithromycin	50	50	50	200	50	50	50	50
Lincomycin	50	50	50	50	50	50	50	50
Melengestrol Acetate					20	20	20	20
Naficillin	50	50	50	50	50	50	50	50
Norfloxacin	25	25	25	25	25	25	25	25
Oxacillin	50	50	50	50	50	50	50	50
Oxyphenylbutazone	50		100	200				
Oxytetracline	500	500	500	500	500	500	1000	1000
Penicillin G	25	50	25	100	50	50	50	50
Phenylbutazone			100					
Pirlimycin	250	250	250	250	250	250	250	250

Standard Analyte	Bovine Screen Level (ng/g)	Bovine Confirm Level (ng/g)	Porcine Screen Level (ng/g)	Porcine Confirm Level (ng/g)	Poultry Screen Level (ng/g)	Poultry Confirm Level (ng/g)	Equine Screen Level (ng/g)	Equine Confirm Level (ng/g)
Prednisone	50	50	50	50	50	50	50	50
Ractopamine	3	3	3	3	3	3	3	3
Salbutamol	3	3	3	3	3	3	3	3
Sarafloxacin	25	25	25	25	25	25	25	25
Sulfachloropyridazine	50	50	50	50	50	200	50	100
Sulfadiazine	50	50	50	50	50	50	50	50
Sulfadimethoxine	50	50	50	50	50	50	50	50
Sulfadoxine	50	50	50	50	50	50	50	50
Sulfaethoxypyridazine	50	50	50	50	50	50	50	50
Sulfamerazine	50	50	50	50	50	50	50	50
Sulfamethazine	50	50	50	50	50	50	50	50
Sulfamethizole	50	50	50	50	50	200	50	200
Sulfamethoxazole	50	50	50	50	50	50	50	50
Sulfamethoxypyridazine	50	50	50	50	50	50	50	50
Sulfanitran	50	50	50	50	50	100	50	100
Sulfapyridine	50	50	50	50	50	50	50	50
Sulfaquinoxaline	50	50	50	50	50	50	50	50
Sulfathiazole	50	50	50	50	50	50	50	50
Tetracyline	500	500	500	500	500		500	1000
Tilmicosin	60	60	60	60	60	60	60	60
Tulathromycin A	1000	1000	1000	1000	1000	1000	1000	1000
Tylosin	100	100	100	100	100	100	100	100

K. APPROVALS AND AUTHORITIES

1. Approvals on file.

2. Issuing Authority: Director, Laboratory Quality Assurance Division.