United States Department of Agriculture

Food Safety and Inspection Service

CLG-MRQ1.01

Quantitation and Confirmation of Animal Drug Residues by High-Resolution Accurate-Mass Liquid Chromatography Mass Spectrometry (HRAM LC-MS)

This method is the laboratory procedure to quantify and confirm veterinary drugs from 10 drug classes (analgesics/anti-inflammatory, avermectins, beta-agonists, beta-lactam/cephalosporins, benzimidazole, fluoroquinolines, general drugs, macrolides, sulfonamides, and tetracyclines) in bovine muscle, kidney, and liver.

Executive Summary

This multi-residue method is used to quantify and confirm veterinary drugs from 10 drug classes (analgesics/anti-inflammatories, avermectins, beta-agonists, beta-lactams/cephalosporins, benzimidazoles, fluoroquinolones, general drugs, macrolides, sulfonamides, and tetracyclines) in bovine muscle, kidney, and liver. The applicable drugs are listed in the table below. The method's benefits include:

- Quantitate and confirm multiple veterinary drug residues simultaneously in a single analysis with high mass accuracy
- Archive several single analyte or specific drug class quantitation methods and a traditional residue bioassay that uses older technology
- Decrease time to result for presumptive positive residues

Applicable Veterinary Drugs		
Muscle	**Kidney**	**Liver**
2-Aminosulfone albendazole	Ampicillin	2-Aminosulfone albendazole
Ampicillin	Chlortetracycline	Albendazole
Chlortetracycline	Cloxacillin	Chlortetracycline
DCCD	DCCD	Danofloxacin
Erythromycin A	Desacetyl cephapirin	Emamectin
Flunixin	Erythromycin A	Enrofloxacin
Levamisole	Oxytetracycline	Fenbendazole
Oxytetracycline	Penicillin G	Flunixin
Penicillin G	Tetracycline	Levamisole
Sulfachloropyridazine	Tylosin	Oxytetracycline
Sulfadimethoxine		Pirlimycin
Sulfaethoxypyridazine		Ractopamine
Sulfamethazine		Sulfachloropyridazine
Sulfaquinoxaline		Sulfadimethoxine
Tetracycline		Sulfaethoxypyridazine
Tilmicosin		Sulfamethazine
Tylosin		Sulfaquinoxaline
		Tetracycline
		Thiabendazole
		Tilmicosin

The minimum levels of applicability (MLA) or lowest levels at which an FSIS method has been successfully validated for a residue in each matrix are found in the Tables 21, 23, and 25 for bovine muscle, kidney, and liver, respectively. The range acceptable recovery values are listed in Tables 22, 24, and 26 for bovine muscle, kidney, and liver, respectively

Notice of Change

This revision includes method updates that expand the applicability of the method to other matrices. The first version of CLG-MRQ1 (CLG-MRQ1.00) only included veterinary drug quantitation in bovine muscle, however, bovine kidney and liver are also target tissues for some chemical residue tolerances. Therefore, in addition to bovine muscle, bovine kidney and liver quantitation analysis were added to scope of the method. Several additional chemical residues were also added for quantitation in bovine kidney and liver to further expand the scope of the method. Additionally, mass spectrometric confirmation criteria were added to the method to simultaneously confirm and quantitate the analytes of interest in bovine muscle, kidney, and liver. Overall, these additions expand the capability of CLG-MRQ to analyze veterinary drugs with established tolerances in edible tissues.

The flow chart found in CLG-MRQ1 Appendix 1 was updated to include bovine kidney and liver and confirmation to the workflow.

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Safety Precautions

The personnel performing the analysis are to read the Safety Data Sheets for the standards and reagents used in this method. The hazards and recommended safe procedures for use are listed in Table 27. Follow all applicable federal, state, and local regulations regarding the disposal of chemicals listed in this method.

Introduction

Veterinary drugs and antibiotics are used to aid growth promotion and feed efficiency, while also being used to prevent or treat diseases in livestock and food producing animals. The Food and Drug Administration (FDA) through the Federal Food, Drug, and Cosmetic Act has the authority to approve and regulate the use of animal drugs. The FDA establishes and publishes regulations by setting tolerance levels for residues of animal drugs in edible tissues.

The National Residue Program (NRP) is an interagency program that is designed to identify, prioritize, and analyze residues in meat, poultry, and egg products. The Food Safety and Inspection Service (FSIS) administers the NRP by collecting and testing samples of domestic and imported meat (including *Siluriformes* fish products), poultry, egg products for veterinary drugs to verify that these products are below tolerances and safe, wholesome, and accurately labeled. FSIS publishes an Annual Sampling Plan to provide information on the process of sampling meat, poultry, and egg products for animal drugs of public health concern. The NRP is monitored and modified annually to set priorities based on data analyses that identify trends in detected residues.

Method Overview

KEY DEFINITIONS
Presumptive Positive: Samples that have been found to have screening results that exceed the MLA.

Presumptive positives, with residue tolerances, require quantitative analysis to determine if the quantitative residue value exceeds the specific veterinary drug tolerance level. If the amount found exceeds the tolerance, the sample is a presumptive violation and would require further confirmatory analysis. Presumptive positives without tolerances only require confirmatory analysis. CLG-MRQ1 is used to simultaneously quantitate and confirm veterinary drugs from 10 drug classes (analgesics/anti-inflammatory, avermectins, beta-agonists, beta-lactam/cephalosporins, benzimidazole, fluoroquinolones, general drugs, macrolides, sulfonamides, and tetracyclines) in bovine muscle, kidney, and liver. Figure 1 shows an overview of the presumptive positive sample quantitative and confirmation analysis through CLG-MRQ1. Presumptive positive analytes not found under the scope of CLG-MRQ1 will require further analysis through additional methods.

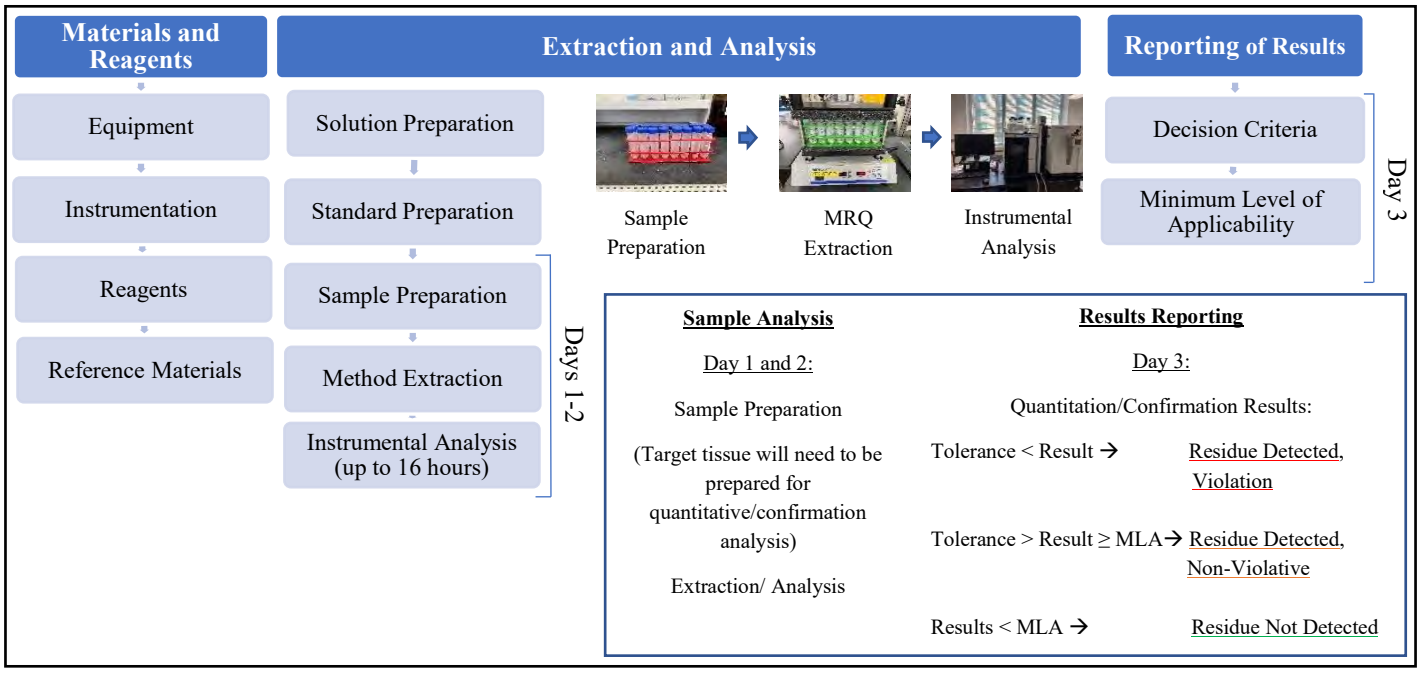

Figure 1: Overview and timeframe of presumptive positive analysis through CLG-MRQ1. Materials and reagents are obtained and utilized to prepare solutions and standards. On business days 1-2, after screening analysis, target tissue from presumptive positive samples are prepared or presumptive positives are weighed, extracted, and analyzed by HRAM LC-MS. Results are reported on business day 3. This figure represents the best-case scenarios, but analyses may take longer. Photos courtesy of Ryan Matsuda, USDA-FSIS.

Quantitation and confirmation analysis is required to be conducted on the target tissue. The target tissue (i.e., kidney, muscle, or liver) is defined in Title 21 of the Code of Federal Regulations, which establishes the edible tissues to monitor specific veterinary drug tolerance levels. Therefore, depending on the tissue or veterinary drug, the target tissue will need to be prepared prior to quantitation and confirmation analysis.

In brief, chemical residues from veterinary drugs are extracted from tissue through a protein precipitation extraction using a solution of acetonitrile and water. The extracted residues are examined and quantitated with high-resolution accurate-mass liquid chromatography mass spectrometry (HRAM LC-MS).

The minimum levels of applicability, recovery limits, and quantitation range for analytes listed under the scope of CLG-MRQ1 for bovine muscle, kidney, and liver are listed in Tables 21-26, respectively.

This method is to be performed using the standards/solutions for the respective analyte(s) of interest. Only applicable standards/solutions are necessary for reporting results.

KEY DEFINITIONS

Protein precipitation: An extraction technique resulting in solid material being left at the bottom of an extraction vessel with the extract or liquid layer containing the analyte. The liquid layer can be separated out for further analysis.

HRAM LC-MS: an analytical technique where there is a physical separation of target compounds followed by their accurate mass-based detection.

Decision Criteria

CLG-MRQ1 is only validated for quantitation and confirmation analysis in bovine muscle, kidney, and liver. The quantitative results from CLG-MRQ1 are compared to established tolerances and the method's minimum level of applicability (MLA) for bovine muscle, kidney, or liver. The confirmation results are determined based on the method's decision criteria for confirmation. If the results are greater than the tolerance and confirm, the sample is considered detected and is a violation. If the results are greater than or equal to the MLA but less than the tolerance and confirm, the sample is considered detected and non-violative. A sample is considered negative if the results are less the than the minimum level of applicability (MLA) or does not confirm.

Key Definitions

MLA: Lowest level at which an FSIS method has been successfully validated for a residue in each matrix. Full definition is on the CLG website.

Disclosure Statement

FSIS does not specifically endorse any test products listed in this method. FSIS acknowledges that equivalent equipment, reagents, or solutions may be suitable for laboratory use. The FSIS laboratory system utilizes the method performance requirements when evaluating the equivalence of an alternative equipment, reagent, or solution for a given analyte and sample matrix pair. Significant equivalence changes would require FSIS laboratory leadership approval.

Materials and Reagents

Equipment

Table 1: Equipment Required to Perform CLG-MRQ1

Equipment	Supplier and Part Number	Purpose
Cutting board and knives	General lab supplier	Preparation of sample.
Food Processor	Robot Coupe USA Inc.	Homogenize sample.
Glassware: Class A	General lab supplier	Measure standards and reagents.
Repeating pipettes and tips, 2 µL to 20 µL, 20 µL to 1000 µL, 500 µL to 2500 µL	General lab supplier	Dispense standards and reagents.
Bottle-Top Dispensers, 1 mL to 5 mL, 2 mL, and 10 mL	General lab supplier	Dispense solutions.
Freezer, -10 °C	General lab supplier	Store standards and reagents.
Pulsating vortex platform shaker	General lab supplier	Facilitates extraction of residue from the sample.
Centrifuge capable of ~ 4700 RCF	General lab supplier	Separates the solid sample material from the extraction solution.
Analytical Balance	General lab supplier	Record weight of standard reagent. Minimum accuracy ±0.0001 g.
Top Loading Balance	General lab supplier	Record weight of quality controls and samples. Minimum accuracy ±0.01g.
Centrifuge tubes Polypropylene (PP), 50 mL	General lab supplier	Contain sample material and extraction vessel.
Autosampler Vials - Amber screw top vials with PTFE/Silicone septa, 2 mL	General lab Supplier	Storage of extracts.
Plastic screw cap vials - Polypropylene, 4 mL	General lab supplier	Store standard solutions.

Instrumentation

Table 2: Instrumentation

Instrument	Supplier and Model Number	Purpose
Thermo Scientific Q Exactive mass spectrometer with Vanquish UHPLC system	Thermo Scientific	Extract analysis.
Phenomenex Kinetex core-shell 1.7 µm XB-C18 100 Å column (2.1 x 150 mm)	Phenomenex 00F-4498-AN	Extract analysis.
UltraShield UHPLC Pre-Column Filter	Macmod Analytical MMUS-1505	Extract analysis.

Reagents

Table 3: Reagents

Reagent	Supplier and Part Number
Acetonitrile (ACN), LC-MS Grade	General lab supplier
Formic acid, LC-MS Grade	General lab supplier
Water - Resistivity of > 18 MΩ-cm	House System
Methanol (MeOH)	General lab supplier
Dimethyl sulfoxide (DMSO)	General lab supplier

Reference Materials

Table 4: Reference Materials

Standard	Supplier	Catalog Number
2-Aminosulfone albendazole	LGC Standards	DRE-C10065200
Ampicillin	LGC Standards	DRE-C10243080
Chlortetracycline	MilliporeSigma	PHR1520
Cloxacillin	MilliporeSigma	PHR1922
Danofloxacin	LGC Standards	DRE-C11960400
DCCD	LGC Standards	TRC-D289905
Desacetyl cephapirin	LGC Standards	TRC-D288970
Erythromycin A	MilliporeSigma	PHR1039
Fenbendazole	MilliporeSigma	PHR1832
Flunixin	LGC Standards	DRE-C13726900
Flunixin - d_3	LGC Standards	DRE-C13727010
Levamisole	LGC Standards	DRE-C14629700

Oxytetracycline	LGC Standards	DRE-C15820000
Penicillin G	LGC Standards	DRE-C15935000
Penicillin G - d_7	LGC Standards	TRC-B288600
Pirlimycin	LGC Standards	TRC-P509300
Ractopamine	LGC Standards	DRE-C16805000
Sulfachloropyridazine	LGC Standards	DRE-C16990100
Sulfadimethoxine	LGC Standards	DRE-C16990550
Sulfaethoxypyridazine	LGC Standards	DRE-C16990650
Sulfamethazine	LGC Standards	DRE-C16996500
Sulfamethazine phenyl - $^{13}C_6$	LGC Standards	DRE-C16996502
Sulfaquinoxaline	LGC Standards	DRE-C16990000
Tetracycline	LGC Standards	DRE-C17396150
Thiabendazole	LGC Standards	DRE-C17450000
Tilmicosin	LGC Standards	DRE-C17582000
Tylosin	LGC Standards	PHR2652

When possible, reference materials are to be purchased from manufacturers accredited to ISO Standard 17034.

Purity and counterions are to be considered when calculating standard concentrations. In-house prepared standards are to be assigned an expiration date that is no later than the stability stated in the method.

Extraction and Analysis

Solution Preparation

Table 5: Preparation of Solutions

Solution	Procedure
80:20 (v/v) **Acetonitrile:Water**	1) Measure 800 mL of acetonitrile using a graduated cylinder and transfer to a container (at least 1 L size). 2) Measure 200 mL of water using a graduated cylinder and add to the container holding the acetonitrile. 3) Mix solution well and transfer to a dispenser bottle. 4) Store at room temperature. *Solution expires 1 year after preparation.*
50:50 (v/v) **Acetonitrile:Methanol**	1) Measure 50 mL of acetonitrile using a graduated cylinder and transfer to a container. 2) Measure 50 mL of methanol using a graduated cylinder and add to the container holding the acetonitrile. 3) Mix solution well. 4) Store at room temperature. *Solution expires 1 year after preparation.*
0.03 M Sodium Hydroxide	1) Add 0.12 g of NaOH to a 100 ml volumetric flask containing 80 mL of water. 2) Mix and allow solution to cool. 3) Adjust to final volume using water. 4) Store in a plastic container. 5) Store at room temperature. *Solution expires 1 year after preparation.*
12.5% DMSO in Methanol	1) Measure 12.5 mL of DMSO and transfer to a 100 mL volumetric flask. 2) Dilute to volume with methanol. 3) Mix solution well and transfer to a storage bottle. 4) Store at room temperature. *Solution expires 1 year after preparation.*
Mobile Phase A (0.1% Formic Acid in Water)	1) Add 1.0 mL of formic acid to a 1 L volumetric flask. 2) Dilute to volume with water. 3) Mix well and transfer to the aqueous reservoir of the LC. 4) Store at room temperature. *Solution expires 1 year after preparation.*

Mobile Phase B (0.1% Formic Acid in Acetonitrile)	1) Add 1.0 mL of formic acid into a 1 L volumetric flask. 2) Bring to volume using acetonitrile. 3) Mix well and transfer to the organic reservoir of the LC. 4) Store at room temperature. *Solution expires 1 year after preparation.*

Standard Preparation

Table 6: Single Stock Standard Solutions

Solution	Procedure
Single-analyte Stock Standard Solutions	1) For each stock solution, calculate the amount of base material needed (ex. accounting for purity and/or water and counterion content) to prepare at the concentration listed in Table 7 using the appropriate solvent listed. 2) Other concentrations are to be used based on two criteria: a) Solubility of the drug in the solvent b) Cost and availability of the drug 3) Store in freezer at <-10 °C.

Standard	*Expiration*
Beta-Lactam mix stock standard	*2 months*
Penicillin G - d_7 stock standard	*2 months*
Acetonitrile mix stock standards	*6 months*
Flunixin - d_3 stock standard	*6 months*
Sulfamethazine Phenyl – $^{13}C_6$ stock standards	*6 months*

Table 7: Stock Standard Concentrations

Standard Analyte	Category	Solvent used	Stock Standard Solution Concentration (ng/µL)
2-Aminosulfone albendazole	Acetonitrile Mix	12.5% DMSO in Methanol	1000
Ampicillin	Beta Lactam Mix	Water	250
Chlortetracycline	Acetonitrile Mix	Methanol	5000
Cloxacillin	Beta Lactam Mix	Water	200
Danofloxacin	Acetonitrile Mix	0.03 M NaOH	1000
*DCCD	Beta Lactam Mix	Water	300

Standard Analyte	Category	Solvent used	Stock Standard Solution Concentration (ng/µL)
Desacetyl cephapirin	Beta Lactam Mix	Water	250
Erythromycin A	Acetonitrile Mix	Acetonitrile	1000
Fenbendazole	Acetonitrile Mix	12.5% DMSO in Methanol	1000
Flunixin	Acetonitrile Mix	Methanol	1000
Flunixin - d_3	Internal Standard Mix	Methanol	1000
Levamisole	Acetonitrile Mix	12.5% DMSO in Methanol	1000
Oxytetracycline	Acetonitrile Mix	Methanol	5000
Penicillin G	Beta Lactam Mix	Water	250
Penicillin G - d_7	Internal Standard Mix	Water	500
Pirlimycin	Acetonitrile Mix	50:50 Acetonitrile:Methanol	1000
Ractopamine	Acetonitrile Mix	Water	1000
Sulfachloropyridazine	Acetonitrile Mix	Acetonitrile	1000
Sulfadimethoxine	Acetonitrile Mix	Acetonitrile	1000
Sulfaethoxypyridazine	Acetonitrile Mix	Acetonitrile	1000
Sulfamethazine	Acetonitrile Mix	Acetonitrile	1000
Sulfamethazine phenyl -$^{13}C_6$	Internal Standard Mix	Acetonitrile	1000
* Sulfaquinoxaline	Acetonitrile Mix	Acetonitrile	500
Tetracycline	Acetonitrile Mix	Methanol	5000
Thiabendazole	Acetonitrile Mix	Methanol	1000
Tilmicosin	Acetonitrile Mix	Acetonitrile	2000
Tylosin	Acetonitrile Mix	Acetonitrile	1000

The stock standard solutions identified with an asterisk (*) symbol may require gentle heating at the time of preparation and before preparation of mixed working standards to aid in the dissolution of material.

Composite working (spiking) and internal standard working (spiking) mix preparation

Table 8: Acetonitrile Mix Working Solution

Working Solution	Procedure
Acetonitrile Mix Working Solution	1) Calculate the volume of stock or intermediate stock solution required to give the working standard concentration listed for each analyte in Tables 9-11. 2) Pipet the calculated volume of stock into a 25 mL volumetric flask. 3) Dilute to 25 mL volume with acetonitrile. 4) Cap the flask and mix. 5) Transfer into 4 mL amber glass LC vials with screw cap lids. 6) Store in freezer at <-10 °C. *Solution expires 6 months after preparation.*

Table 9: Acetonitrile Mix Working Standard Preparation for Muscle

Standard Analyte	Stock Standard Solution Concentration (ng/µL)	Stock Standard Solution Volume (µL)	Acetonitrile Mix Working Standard Solution Concentration (ng/µL)
2-Aminosulfone albendazole	1000	62.5	2.5
Chlortetracycline	5000	500	100
Erythromycin A	1000	125	5
Flunixin	1000	31.25	1.25
Levamisole	1000	125	5
Oxytetracycline	5000	500	100
Sulfachloropyridazine	1000	125	5
Sulfadimethoxine	1000	125	5
Sulfaethoxypyridazine	1000	125	5
Sulfamethazine	1000	125	5
Sulfaquinoxaline	500	250	5
Tetracycline	5000	500	100
Tilmicosin	2000	62.5	5
Tylosin	1000	250	10

Table 10: Acetonitrile Mix Working Standard Preparation for Kidney

Standard Analyte	Stock Standard Solution Concentration (ng/µL)	Stock Standard Solution Volume (µL)	Acetonitrile Mix Working Standard Solution Concentration (ng/µL)
Chlortetracycline	5000	3000	600
Erythromycin A	1000	125	5
Oxytetracycline	5000	3000	600
Tetracycline	5000	3000	600
Tylosin	1000	250	10

Table 11: Acetonitrile Mix Working Standard Preparation for Liver

Standard Analyte	Stock Standard Solution Concentration (ng/µL)	Stock Standard Solution Volume (µL)	Acetonitrile Mix Working Standard Solution Concentration (ng/µL)
2-Aminosulfone albendazole	1000	250	10
Albendazole	1000	250	10
Chlortetracycline	5000	1500	300
Danofloxacin	1000	250	10
Emamectin	1000	62.5	2.5
Enrofloxacin	500	250	5
Fenbendazole	1000	1000	40
Flunixin	1000	156.25	6.25
Levamisole	1000	125	5
Oxytetracycline	5000	1500	300
Pirlimycin	1000	625	25
Ractopamine	1000	112.5	4.5
Sulfachloropyridazine	1000	125	5
Sulfadimethoxine	1000	125	5
Sulfaethoxypyridazine	1000	125	5
Sulfamethazine	1000	125	5
Sulfaquinoxaline	1000	125	5
Tetracycline	5000	1500	300
Thiabendazole	1000	125	5
Tilmicosin	2000	750	60

Table 12 Beta-Lactam Mix Working Solution Preparation

Working Solution	Procedure
"Beta-Lactam Mix" working solution	1) Calculate the volume of stock solution required to give the working standard concentration listed for each analyte in Tables 13-14. 2) Pipet the calculated volume of stock into a 25 mL volumetric flask. 3) Dilute to 25 mL volume with water. 4) Cap flask and mix. 5) Transfer into 4 mL polypropylene vials with screw cap lids. 6) Store in freezer at <-10 °C. *Solution expires 2 months after preparation.*

Table 13: Beta-Lactam Mix Working Solution Preparation for Muscle

Standard Analyte	Stock Standard Solution Concentration (ng/µL)	Stock Standard Solution Volume (µL)	Beta-Lactam Mix Working Standard Solution Concentration (ng/µL)
Ampicillin	250	50	0.5
DCCD	300	4167	50
Penicillin G	250	250	2.5

Table 14: Beta-Lactam Mix Working Solution Preparation for Kidney

Standard Analyte	Stock Standard Solution Concentration (ng/µL)	Stock Standard Solution Volume (µL)	Beta-Lactam Mix Working Standard Solution Concentration (ng/µL)
Ampicillin	250	50	0.5
Cloxacillin	200	62.5	0.5
DCCD	300	1667	20
Desacetyl cephapirin	250	500	5
Penicillin G	250	250	2.5

There are no Beta-Lactam compounds for liver analysis.

Table 15: Internal Standard Mix Working Standard Solution Preparation

Working Solution	Procedure
Internal Standard Mix Working Standard Solution	1) Calculate the volume of stock solution required to give the working standard concentration listed for each analyte in Table 16. 2) Pipet the calculated volume of stock into a 25 mL volumetric flask. 3) Dilute to 25 mL with acetonitrile. 4) Cap flask and mix. 5) Transfer into 4 mL amber glass LC vials with screw cap lids. 6) Store in freezer at <-10 °C. *Solution expires 2 months after preparation.*

Table 16: Internal Standard Mix Working Standard Solution

Standard Analyte	Stock Standard Solution Concentration (ng/µL)	Stock Standard Solution Volume (µL)	Working Standard Solution Concentration (ng/µL)
Flunixin - d_3	1000	500	20
Penicillin G - d_7	500	1000	20
Sulfamethazine phenyl - $^{13}C_6$	1000	500	20

Sample Preparation

All samples are stored frozen (≤ -10 °C). Prepare presumptive positive samples for quantitative and confirmation analysis. Partially thaw all tissue samples, while keeping them as cold as possible. Trim away fat and connective tissue (Figure 2). Grind tissue in blender or vertical cutter-mixer until homogeneous (Figure 3). Store homogenized samples frozen (≤ -10 °C) prior to analysis.

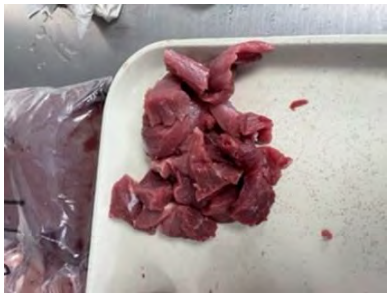

Figure 2: Prepared lean muscle sample with connective tissue removed. Photo courtesy of Hue Quach, USDA FSIS.

Figure 3: Homogenized sample. Photo courtesy of Hue Quach, USDA FSIS

Extraction

Samples

Weigh 2.00 ± 0.10 g of sample into a 50 mL polypropylene centrifuge tube.

KEY DEFINITIONS

Negative control (Blank): A sample that is negative of all analytes.
Recovery (positive control): A sample is prepared by the addition of analytes prior to extraction that have a concentration level comparable to MLA.

QUALITY CONTROL

1. Weigh five 2.00 ± 0.10 g portions of blank tissue into 50 mL polypropylene centrifuge tubes. One for the blank (negative control), one for the recovery (positive control), and at least 3 fortified calibration points ranging from ½ X to 4 X.
2. Weigh one additional portion for a check sample, if necessary.
3. Prepare recovery, fortified calibration curve, check sample, and samples using the tissue specific solutions and volumes in Table 17. Ensure that the calibration curve is within the acceptable analytical range for the analyte.

Table 17: Example Preparation of Controls and Samples

Levels	Acetonitrile Standard Mix Volume (µL)	Beta-Lactam Standard Mix Volume (µL) (Muscle and Kidney Only)
½ X	20	20
1 X	40	40
2 X	80	80
4 X	160	160

Extraction

1) Add 30 µL of Internal Standard Mix to all tubes.
2) Vortex all tubes 10 seconds each to mix chemicals with the matrix and allow sample tubes to stand for 5 minutes (min).
3) Add 10 mL of 80:20 (v/v) acetonitrile/water to all tubes. Cap tubes well.
4) Vortex the tubes using the pulsating vortex platform shaker for 5 min, as shown in Figure 4. Visually verify that the tissue and solvent are effectively mixed (Figure 5).

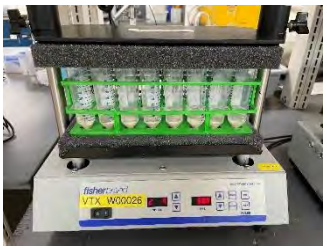

Figure 4: Samples being vortexed. Photo courtesy of Ryan Matsuda, USDA-FSIS

Figure 5: Vortexed sample. Photo courtesy of Stephen Kubota, USDA-FSIS.

5) Centrifuge the tubes at 4708 RCF (~4600 RPM) for 10 min.
6) Pipette a minimum of 500 µL of samples extract into a labeled amber glass autosampler vials.

Instrumental Analysis

An example of a HRAM LC-MS instrument and a sample tray for an HRAM LC-MS instrument are shown in Figure 6 and Figure 7, respectively.

Chromatographic Parameters

1) Mobile phases for MRQ1 analysis
   a) Mobile Phase A – 0.1% Formic Acid in Water
   b) Mobile Phase B – 0.1% Formic Acid in Acetonitrile
2) Run time: 12.9 min
3) Gradient Program

Figure 6: Example of HRAM LC-MS instrument. Photo courtesy of Abdulahi Duale, USDA-FSIS

Table 18: UHPLC Gradient Program

Time (min)	Flow (mL/min)	% Mobile Phase B	Gradient
0.000			
0.500	0.300	0.0	5
2.000	0.300	15.0	8
3.000	0.300	30.0	3
5.000	0.300	35.0	5
7.000	0.300	100.0	6
7.300	0.400	100.0	5
8.800	0.400	100.0	5
10.00	0.300	0.0	3
12.90		Stop Run	

4) Autosampler Program:
    a) Run time: 12.90 min
    b) Injection loop: 25 µL
    c) Injection Volume: 7 µL
    d) Sample temperature: 7 °C (refrigerated)
5) Column manager
    a) Column valve position: To match column location
    b) Column manager temperature: 30 °C

Figure 7: HRAM LC-MS tray. Photo courtesy of Ryan Matsuda, USDA-FSIS

Mass Spectrometry Parameters

1) Scan Type: Full MS - AIF
2) Electrospray Source Parameters
    a) Sheath gas flow rate: 50
    b) Auxiliary gas flow rate: 12
    c) Sweep gas Flow rate: 3
    d) Spray voltage (kV): 3.60
    e) Capillary temperature (°C): 250
    f) S-lens RF level: 60.0
    g) Auxiliary gas heater temperature (°C): 350
3) Analyzer Parameters
    a) Use lock masses: Best
    b) Chromatography peak width: 10 s
    c) Method duration: 12.90 min
    d) Properties of Full MS /All Ion Fragmentation (AIF)
        i) Runtime: 0 to 12.9 min
        ii) Polarity: positive
        iii) Full MS
            1) Resolution: 70,000
            2) Automatic Gain Control (AGC) target: $1e^6$
            3) Maximum Orbitrap Injection Time (IT): 50 ms
            4) Scan range: 80 to 1200 *m/z*

Table 19: Chromatographic and Mass Spectrometry Parameters

Compound Name	Formula	Expected Retention Time / Retention Time Windows (min)	Quant Ion (*m/z*)	Internal Standard
2-Aminosulfone albendazole	$C_{10}H_{13}N_3O_2S$	4.59	240.08012	Flunixin-d_3
Albendazole	$C_{12}H_{15}N_3O_2S$	6.41	266.09577	Flunixin-d_3
Ampicillin	$C_{16}H_{19}N_3O_4S$	4.00 - 5.50*	350.11690	Penicillin G-d_7
Chlortetracycline	$C_{22}H_{23}ClN_2O_8$	4.50 - 6.00*	479.12157	Flunixin-d_3
Cloxacillin	$C_{19}H_{18}ClN_3O_5S$	7.78	436.07285	Penicillin G-d_7
Danofloxacin	$C_{19}H_{20}FN_3O_3$	4.27	358.15615	Flunixin-d_3
^DCCD	$C_{17}H_{20}N_6O_7S_4$	4.40 - 5.40*	275.02109	Penicillin G-d_7
Desacetyl cephapirin	$C_{15}H_{15}N_3O_5S_2$	6.64	382.05259	Penicillin G-d_7
Emamectin	$C_{49}H_{75}NO_{13}$	7.68	886.53112	Flunixin-d_3
Enrofloxacin	$C_{19}H_{22}FN_3O_3$	4.30	360.17180	Flunixin-d_3
Erythromycin A	$C_{37}H_{67}NO_{13}$	5.23	734.46852	Flunixin-d_3
Fenbendazole	$C_{15}H_{13}N_3O_2S$	7.51	300.08012	Flunixin-d_3
Flunixin	$C_{14}H_{11}F_3N_2O_2$	7.98	297.08454	Flunixin-d_3
Flunixin-d_3	$C_{14}H_{11}F_3N_2O_2$	7.97	300.10337	Internal Standard
Levamisole	$C_{11}H_{12}N_2S$	4.75	205.07940	Flunixin-d_3
Oxytetracycline	$C_{22}H_{24}N_2O_9$	4.60 - 5.60*	461.15546	Flunixin-d_3
Penicillin G	$C_{16}H_{18}N_2O_4S$	6.75	335.10600	Penicillin G-d_7
Penicillin G-d_7	$C_{16}H_{18}N_2O_4S$	6.70	342.14994	Internal Standard
Pirlimycin	$C_{17}H_{31}ClN_2O_5S$	4.48	411.17150	Flunixin-d_3
Ractopamine	$C_{18}H_{23}NO_3$	4.25	302.17507	Flunixin-d_3
Sulfachloropyridazine	$C_{10}H_9ClN_4O_2S$	5.73	285.02075	Sulfamethazine phenyl-$^{13}C_6$
Sulfadimethoxine	$C_{12}H_{14}N_4O_4S$	6.33	311.08085	Sulfamethazine phenyl-$^{13}C_6$
Sulfaethoxypyridazine	$C_{12}H_{14}N_4O_3S$	5.78	295.08594	Sulfamethazine phenyl-$^{13}C_6$
Sulfamethazine	$C_{12}H_{14}N_4O_2S$	5.35	279.09100	Sulfamethazine phenyl-$^{13}C_6$
Sulfamethazine phenyl-$^{13}C_6$	$C_{12}H_{14}N_4O_2S$	5.35	285.11115	Internal Standard
Sulfaquinoxaline	$C_{14}H_{12}N_4O_2S$	6.29	301.07537	Sulfamethazine phenyl-$^{13}C_6$
Tetracycline	$C_{22}H_{24}N_2O_8$	4.50 - 5.50*	445.16054	Flunixin-d_3
Thiabendazole	$C_{10}H_7N_3S$	4.24	202.04334	Flunixin-d_3
^Tilmicosin	$C_{46}H_{80}N_2O_{13}$	4.89	435.29030	Flunixin-d_3
Tylosin	$C_{46}H_{77}NO_{17}$	5.00 - 6.00*	916.52643	Flunixin-d_3

The analyte identified with a caret (^) symbol in Table 19 is analyzed using the doubly charged ion, $[M+2H]^{2+}$.

*** An area sum of the retention time window will be used when these analytes have multiple peaks in the range that shift ratios. If only a single peak is visible, a single peak integration will be used.**

Table 20: Confirmation Fragment Ions

Compound Name	Fragment Ion 1 (*m/z*)	Fragment Ion 2 (*m/z*)	Fragment Ion 3 (*m/z*)	Fragment Ion 4 (*m/z*)	Fragment Ion 5 (*m/z*)
2-Aminosulfone albendazole	198.03304	106.05311	133.06345	N/A	N/A
Albendazole	234.0692	159.04254	192.02222	N/A	N/A
Chlortetracycline	154.04965	98.06049	444.08344	462.09384	N/A
Cloxacillin	160.04245	114.03743	178.0052	277.03683	206.03661
Danofloxacin	96.08147	340.14532	314.16613	255.05603	283.1236
DCCD	183.03351	241.03865	126.01223	N/A	N/A
Desacetyl cephapirin	111.01402	112.02196	152.01635	124.02162	226.02239
Emamectin	158.11732	82.06572	126.09131	302.19556	N/A
Enrofloxacin	316.18158	245.10817	72.08162	203.0612	N/A
Erythromycin A	158.11768	83.04994	116.07102	127.07558	576.37354
Fenbendazole	268.05298	159.04231	190.00638	109.01085	131.04764
Flunixin	279.07376	264.05014	109.04523	259.06802	277.07843
Levamisole	91.05486	178.06837	123.02652	N/A	N/A
Oxytetracycline	426.1178	201.05443	337.06979	154.04976	365.06546
Penicillin G	91.05487	114.03761	160.04254	176.07037	N/A
Pirlimycin	112.11233	363.16708	110.06018	N/A	N/A
Ractopamine	107.0496	121.06503	91.05486	164.10693	136.0757
Sulfachloropyridazine	108.04467	156.01111	92.04996	68.05025	N/A
Sulfadimethoxine	108.04476	156.07666	92.05	245.10283	N/A
Sulfaethoxypyridazine	108.04464	156.01112	140.08154	N/A	N/A
Sulfamethazine	124.08695	108.04467	204.04341	156.01106	213.11313
Sulfaquinoxaline	108.04475	156.01114	92.05	146.07117	N/A
Tetracycline	410.1225	154.04976	427.15002	428.13376	N/A
Thiabendazole	131.06062	175.03227	65.03957	N/A	N/A
Tilmicosin	174.11218	696.4657	132.10187	88.07628	116.07083
Tylosin	174.11234	101.06014	145.08578	132.10187	83.04981

N/A = Not Applicable

Instrument Note

All chromatographic and instrument parameters were optimized in accordance with FSIS laboratory system method performance requirements and during annual preventative maintenance and calibration.

- Retention time windows, collision energies, and selected masses for precursor and product ions were set and utilized at time of method validation.
  - Retention times and retention time windows may be adjusted based on the standard or positive control to account for aging of UHPLC columns or for improved separation to ensure that all chromatographic peaks are present.
  - Collision energies may be adjusted and optimized for improved mass spectrometry detection.
- Parameter modifications to improve instrument performance to ensure all chromatographic peaks are present must meet the acceptance criteria listed in the method's Quality Assurance Plan.
- Significant changes that affect method performance require equivalency testing and FSIS laboratory leadership approval.

Sample Set

The injection sequence below can be modified, as needed, but must include the controls.

1) Fortified Calibration Curve Standards
2) Blank (negative control)
3) Recovery (positive control)
4) Intra-laboratory check sample (if needed)
5) Solvent Blank (If analyzing tetracyclines, required before samples)
6) Up to 35 samples
7) Reinjection of a fortified calibration curve standard or recovery (positive control).

INTRA-LABORATORY CHECK SAMPLE

Defined on the CLG website

System suitability is to be demonstrated prior to sample set injection.

Reporting of Results

Decision Criteria

Confirmation

Confirmation criteria are required only for analyte(s) of interest.

Key Definitions
Analyte(s) of Interest: An analyte(s) of interest is the screened positive residue that was found to be a presumptive positive.

1) Monitored ions for each analyte will be assessed as follows:
   a. The quant ions for a given analyte must be present. The required ion for each compound is listed in Table 19.
   b. The quant ion must have a signal-to-noise ratio ≥ 3. This will be verified by visual inspection. Visual inspection for detection also includes assessment of peak shape or drift in relation to standard peaks.
   c. The highest analyte peak in the sample must be ≤ 0.2 min, or within ± 2.5% (not to exceed 0.5 minutes) of the retention time of the fortified recovery (positive control).
   d. The quant ion must exceed 10% of the area of the recovery.
2) At least 1 fragment ion must be present.
   a. The fragment ion must have a mass difference less than 10 ppm from the reference mass found in Table 20 for the respective fragment ion.

QUALITY CONTROL

Quality Control Procedures for Confirmation

1) For set acceptance, the analyte(s) of interest (i.e., analytes to be confirmed) in the fortified recovery (positive control) must meet confirmation criteria.
2) The blank (negative control) must be negative using the confirmation criteria for the analyte(s) of interest.

Intra-Laboratory Check Samples (If applicable)

1) Acceptability criteria.
   a. The analyte(s) of interest in a fortified Intra-laboratory Check must meet confirmation criteria.
   b. The analyte(s) of interest in an unfortified Intra-laboratory Check must not meet confirmation criteria.
   c. FSIS Field Service Laboratories are to refer to internal FSIS Quality Control Procedures when unacceptable values are obtained:
      i. Refer to LW-Q1002, Chemistry Non-Conformance Tables, for how to proceed and whether to take corrections or corrective actions.

Quantitation

1) Ensure that all quant ions for the analyte(s) of interest used for the construction of standard curve are present at a signal-to-noise ratio ≥ 3. This will be verified by visual inspection. Visual inspection for detection also includes assessment of peak shape or drift in relation to standard peaks.
2) The highest analyte peak in the sample must be ≤ 0.2 min, or within ± 2.5% (not to exceed 0.5 minutes) of the retention time of the recovery (positive control).
3) The blank must be less than 10% of the recovery.
4) The internal standard response for Penicillin G-d_7 for the samples is to be within 50-150% of the internal standard response for Penicillin G-d_7 of the recovery.
5) Using a linear regression on the instrument software, calculate the slope, intercept, and the coefficient of determination of a standard curve for the analyte in question. This is constructed by plotting the peak area ratio using the Quant Ion / Internal Standard (as listed in Table 19) versus concentration (µg/g) for the calibration curve standards.
6) Calculate results when the following conditions are met:
   a. The coefficient of determination (r^2) for the standard curve is ≥ 0.990.
   b. The recovery (positive control) falls within the limits specified in the Quality Control Procedures. Control limits are applicable for the validated analytical range.

QUALITY CONTROL

Quality Control Procedures for Quantitation

1) For set acceptance, the analyte(s) of interest in the fortified recovery (positive control) must meet the acceptable recovery range for the analytes listed in Tables 22, 24, and 26.
2) The blank (negative control) must be negative using the quantitation criteria for all analytes of interest.
3) The internal standard response for Penicillin G-d_7 for the Blank (negative control) and Calibration Curve Standards must be within 50-150% of the internal standard response for Penicillin G-d_7 of the recovery (positive control).

Intra-laboratory Check Samples (If applicable)

1) Acceptability criteria.
   a. The analyte(s) of interest in a fortified Intra-laboratory Check must meet the acceptable recovery range for the analytes listed in Tables 22, 24, and 26.
   b. The analyte(s) of interest in an unfortified Intra-laboratory Check must be negative using the quantitation criteria for all analytes of interest.
   c. FSIS Field Service Laboratories are to refer to internal FSIS Quality Control Procedures when unacceptable values are obtained:
      i. Refer to LW-Q1002, Chemistry Non-Conformance Tables, for how to proceed and whether to take corrections or corrective actions.

Minimum Level of Applicability

Table 21: Minimum Level of Applicability (MLA) for Confirmation in Bovine Muscle

Analyte	MLA (µg/g)
2-Aminosulfone albendazole	0.0250
Ampicillin	NA (Quantitation Only)
Chlortetracycline	1.00
DCCD	0.500
Erythromycin A	0.0500
Flunixin	0.0125
Levamisole	0.100
Oxytetracycline	1.00
Penicillin G	0.0250
Sulfachloropyridazine	0.0500
Sulfadimethoxine	0.0500
Sulfaethoxypyridazine	0.0500
Sulfamethazine	0.0500
Sulfaquinoxaline	0.0500
Tetracycline	1.00
Tilmicosin	0.0500
Tylosin	0.100

Table 22: Analytical Ranges and Acceptable Recoveries for Bovine Muscle

Analyte	Analytical Range (µg/g)	Control Limits (%)
2-Aminosulfone albendazole	0.0250-0.100	61-131
Ampicillin	0.0100-0.0200	45-163
Chlortetracycline	1.00-4.00	63-124
DCCD	0.500-2.00	67-131
Erythromycin A	0.0500-0.200	80-127
Flunixin	0.0125-0.0500	88-108
Levamisole	0.100-0.200	63-142
Oxytetracycline	1.00-4.00	60-139
Penicillin G	0.025-0.100	75-119
Sulfachloropyridazine	0.0500-0.200	75-113
Sulfadimethoxine	0.0500-0.200	85-112
Sulfaethoxypyridazine	0.0500-0.200	90-112
Sulfamethazine	0.0500-0.200	93-105
Sulfaquinoxaline	0.05-0.200	81-108
Tetracycline	1.00-4.00	64-137
Tilmicosin	0.0500-0.200	73-122
Tylosin	0.100-0.400	76-126

Table 23: Minimum Level of Applicability (MLA) for Confirmation in Bovine Kidney

Analyte	MLA (µg/g)
Ampicillin	NA (Quantitation Only)
Chlortetracycline	12
Cloxacillin	0.02
DCCD	0.8
Desacetyl cephapirin	0.05
Erythromycin A	0.05
Oxytetracycline	12
Penicillin G	0.025
Tetracycline	12
Tylosin	0.2

Table 24: Analytical Ranges and Acceptable Recoveries for Bovine Kidney

Analyte	Analytical Range (µg/g)	Control Limits (%)
Ampicillin	0.020-0.040	24-176%
Chlortetracycline	12-24	64-136%
Cloxacillin	0.020-0.040	70-130%
DCCD	0.800-1.60	71-129%
Desacetyl cephapirin	0.050-0.200	87-113%
Erythromycin A	0.050-0.200	88-112%
Oxytetracycline	12-24	76-124%
Penicillin G	0.025-0.100	96-104%
Tetracycline	12-24	77-123%
Tylosin	0.200-0.400	35-154%

Table 25: Minimum Level of Applicability (MLA) for Confirmation in Bovine Liver

Analyte	MLA (µg/g)
2-Aminosulfone albendazole	0.1
Albendazole	0.2
Chlortetracycline	6
Danofloxacin	0.1
Emamectin	0.025
Enrofloxacin	0.05
Fenbendazole	0.4
Flunixin	0.0625
Levamisole	0.05
Oxytetracycline	6
Pirlimycin	0.25
Ractopamine	0.09

Analyte	MLA (µg/g)
Sulfachloropyridazine	0.05
Sulfadimethoxine	0.05
Sulfaethoxypyridazine	0.05
Sulfamethazine	0.05
Sulfaquinoxaline	0.05
Tetracycline	6
Thiabendazole	0.1
Tilmicosin	0.6

Table 26: Analytical Ranges and Acceptable Recoveries for Bovine Liver

Analyte	Analytical Range (µg/g)	Control Limits (%)
2-Aminosulfone albendazole	0.100-0.400	77-123%
Albendazole	0.200-0.400	71-129%
Chlortetracycline	6-12	78-122%
Danofloxacin	0.100-0.400	81-119%
Emamectin	0.025-0.100	63-137%
Enrofloxacin	0.050-0.200	77-123%
Fenbendazole	0.400-1.60	79-121%
Flunixin	0.0625-0.250	97-103%
Levamisole	0.050-0.200	78-122%
Oxytetracycline	6-12	67-133%
Pirlimycin	0.250-1.000	82-118%
Ractopamine	0.090-0.180	67-133%
Sulfachloropyridazine	0.0500-0.200	90-110%
Sulfadimethoxine	0.0500-0.200	91-109%
Sulfaethoxypyridazine	0.0500-0.200	92-109%
Sulfamethazine	0.0500-0.200	97-103%
Sulfaquinoxaline	0.0500-0.200	91-109%
Tetracycline	6-12	71-129%
Thiabendazole	0.100-0.200	70-130%
Tilmicosin	0.600-2.400	83-117%

References

1) 21CFR 556 for tolerance values set by FDA.
2) The National Residue Program sets the number of samples analyzed each year for animal drugs.

Safety Hazards

Table 27: Safety Hazards and Recommended Safe Procedures

Procedure Step	Hazard	Recommended Safe Procedures
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, Sodium hydroxide	Corrosive, Caustic	Wear personal protective equipment, avoid skin contact.
Veterinary Drug Standards	Some individuals may have allergic reactions to veterinary drug compounds, which may cause skin and respiratory irritation. Possible reproductive toxicity.	Wear personal protective equipment, avoid skin contact. Handle with extreme caution. Work in a well-ventilated area.

Contact Information and Inquiries

Inquiries about methods can be submitted through the USDA website via the "Ask USDA" portal at https://ask.usda.gov or please contact:

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This method has been validated, reviewed, approved, and deemed suitable and fit for purpose for use in the USDA FSIS Field Service Laboratories.

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