United States Department of Agriculture

Food Safety and Inspection Service

# CLG-MRQ1.00

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

This method describes the laboratory procedure to quantify 17 veterinary drugs from 7 drug classes (analgesics/anti-inflammatory, β-lactam/cephalosporins, benzimidazole, general drugs, macrolides, sulfonamides, and tetracyclines) in bovine muscle.

# Notice of Change

The method is a new multi-residue quantitation method for veterinary drugs and is the original version with no changes. The method is used to quantitate 17 veterinary drugs from 7 drug classes (analgesics/anti-inflammatory, β-lactam/cephalosporins, benzimidazole, general drugs, macrolides, sulfonamides, and tetracyclines) in bovine muscle. The method's benefits include:

- Simultaneous measurement of multiple veterinary drug residue amounts in a single analysis with high mass accuracy.
- Quantification of several veterinary drugs that were previously difficult to measure.
- Archival of several single analyte or specific drug class quantitation methods that use older technology (CLG-CEF2, CLG-FLX4, CLG-PENG1, CLG-SUL4, and CLG-TIL1).
- Transition from using a traditional residue bioassay for several analytes to a modernized MS instrument platform.
- Lower detection limits to align better with established drug residue tolerances.
- Decreased time to result for presumptive positive residues.

# Table of Contents



**Safety Precautions**

The personnel performing the analysis are to read the Safety Data Sheets (SDS) for the standards and reagents used in this method. Follow 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

CLG-MRM3 is FSIS's method for screening of several veterinary drugs in meat, poultry, and egg products. If screening results from CLG-MRM3 indicate that veterinary drug residues are found to be "presumptive positive" for an analyte(s) in bovine tissue, further quantitation analysis is required. CLG-MRQ1 is used to quantitate 17 veterinary drugs from 7 drug classes (analgesics/anti-inflammatory, β-lactam/cephalosporins, benzimidazole, general drugs, macrolides, sulfonamides, and tetracyclines) in bovine muscle. Figure 1 shows an overview of the presumptive positive sample quantitative analysis through CLG-MRQ1. Presumptive positive analytes not found under the scope of CLG-MRQ1 will require further analysis through additional methods.

CLG-MRM3 is conducted in both muscle and kidney tissues, therefore if screened in kidney, muscle samples will need to be prepared for quantitative analysis with CLG-MRQ1. If previously screened in muscle tissue, prepared muscle tissue will be used for quantitative analysis, without any need to prepare additional tissue samples.

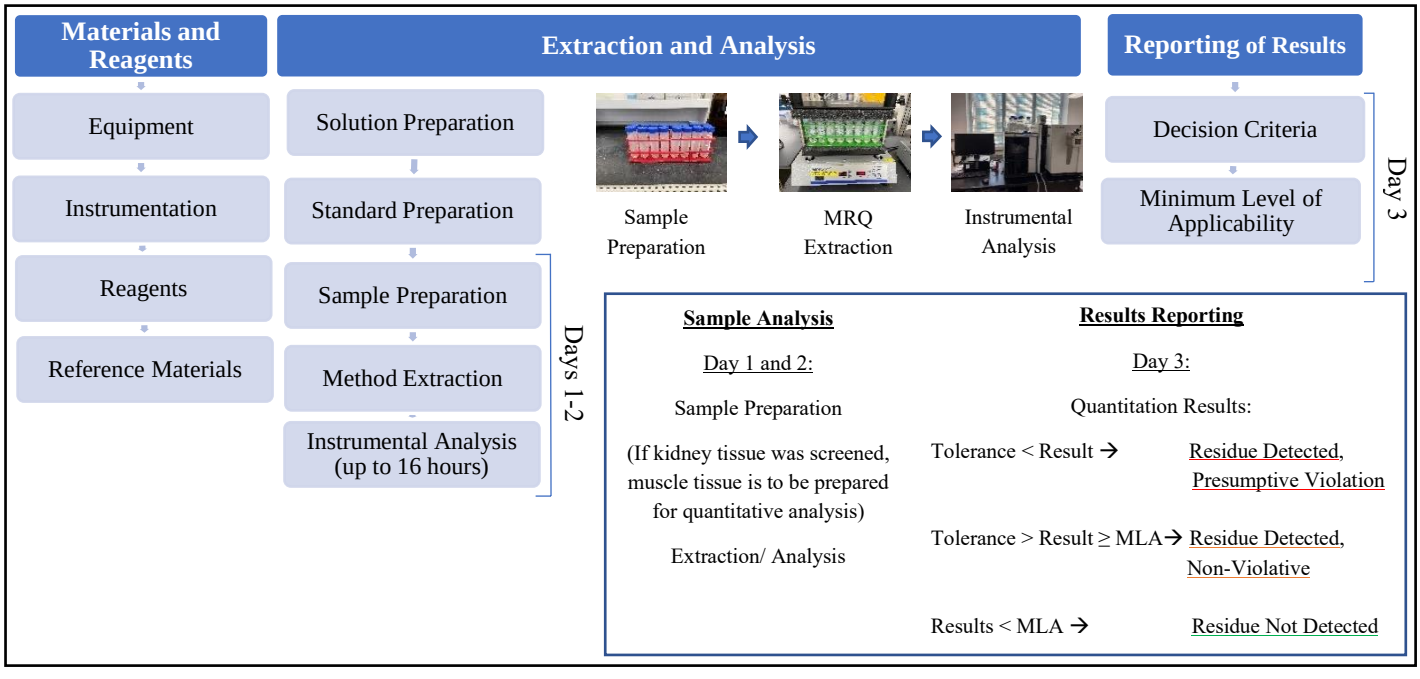


**Figure 1:** Overview and timeframe of presumptive positive analysis through CLG-MRQ1. Materials and reagents are obtained and utilized to prepare solutions and standards. On business days 1-2, after screening analysis, target tissue from presumptive positive samples are prepared or presumptive positives 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.

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 are listed in Tables 17-18. This method may be performed using standards or solutions that contain fewer analytes than the method applicability. When that occurs, the excluded analytes would not be included in the reported results.

**KEY DEFINITIONS**

**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

**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 here.

For presumptive positive results, residues with tolerances undergo both confirmation and quantitation analysis. CLG-MRQ1 is only validated for quantitation analysis in bovine muscle. The results from CLG-MRQ1 are compared to established tolerances and the method's minimum level of applicability (MLA) for bovine muscle and quantitated based on the method's decision criteria. If the results are greater than the tolerance, the sample is considered detected and is a presumptive violation. The sample would then need to undergo further confirmation analysis to confirm the violative result. If the results are greater than or equal to the MLA but less than the tolerance, 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).

## 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 |
|---|---|---|
| **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. |
| **Polypropylene Centrifuge Tubes: 50 mL** | General lab supplier | Hold sample material and extraction container. |
| **Top Loading Balance** | General lab supplier | Record weight of quality controls and samples. Minimum accuracy ±0.01g. |
| **Analytical balance** | General lab supplier | Record weight of standard reagent. Minimum accuracy ±0.0001g. |
| **Plastic screw cap vials - Polypropylene, 4 mL** | General lab supplier | Store standard solutions. |
| **Autosampler Vials - Amber screw top vials with PTFE/Silicone septa, 2 mL** | General lab Supplier | Storage of extracts. |
| **Repeating pipettes and tips: 2 µL - 20 µL, 20 µL to 100 µL, 100 µL to 1000 µL, 500 µL to 5000 µL** | General lab supplier | Dispense standards and reagents. |
| **Bottle-Top Dispensers: 1 mL to 10 mL** | General lab supplier | Dispense solutions. |
| **Glassware: Class A** | General lab supplier | Measure standards and reagents. |
| **Cutting board and knives** | General lab supplier | Preparation of sample. |
| **Food Processor** | Robot Coupe USA Inc. | Homogenize sample. |
| **Freezer, -10 °C** | General lab supplier | Store standards and reagents. |
| **LC vials - Amber glass screw top with lids, 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 | TRC-A580950 |
| **Ampicillin** | Sigma Aldrich | A1593 |
| **Chlortetracycline** | US Pharmacopeia | 1129007 |
| **DCCD** | LGC Standards | TRC-D289905 |
| **Erythromycin A** | Sigma Aldrich | E0774 |
| **Flunixin** | US Pharmacopeia | 1274607 |
| **Flunixin - $d_3$** | Sigma Aldrich | 34083 |
| **Levamisole** | Sigma Aldrich | 31742 |
| **Oxytetracycline** | US Pharmacopeia | 1491004 |
| **Penicillin G** | US Pharmacopeia | 1502508 |

| | | |
|---|---|---|
| **Penicillin G - $d_7$** | LGC Standards | TRC-B288600 |
| **Sulfachloropyridazine** | Sigma Aldrich | 46778 |
| **Sulfadimethoxine** | Sigma Aldrich | 46794 |
| **Sulfaethoxypyridazine** | Sigma Aldrich | 02743 |
| **Sulfamethazine** | Sigma Aldrich | S6256 |
| **Sulfamethazine Phenyl - $^{13}C_6$** | Sigma Aldrich | 32519 |
| **Sulfaquinoxaline** | Sigma Aldrich | 45662 |
| **Tetracycline** | US Pharmacopeia | 1651009 |
| **Tilmicosin** | Sigma Aldrich | 33864 |
| **Tylosin** | Sigma Aldrich | T6134 |

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).<br>2) Measure 200 mL of water using a graduated cylinder and add to the container holding the acetonitrile.<br>3) Mix solution well and transfer to a dispenser bottle. |
| **50:50 (v/v) Acetonitrile:Methanol** | 1) Measure 500 mL of acetonitrile using a graduated cylinder and transfer to a container (at least 1 L size).<br>2) Measure 500 mL of water using a graduated cylinder and add to the container holding the acetonitrile.<br>3) Mix solution well and transfer to a dispenser bottle. |
| **Mobile Phase A (0.1% Formic Acid in Water)** | 1) Add 1.0 mL of formic acid to a 1 L volumetric flask.<br>2) Dilute to volume with water.<br>3) Mix well and transfer to the aqueous reservoir of the LC. |
| **Mobile Phase B (0.1% Formic Acid in Acetonitrile)** | 1) Add 1.0 mL of formic acid into a 1 L volumetric flask.<br>2) Bring to volume using acetonitrile.<br>3) Mix well and transfer to the organic reservoir of the LC. |

## Standard Preparation

**Table 6: Single Stock Standard Solutions**

| | |
|---|---|
| **Single-analyte Stock Standard Solutions**<br><br>**Beta-Lactam Stock Standard Solutions expire after 2 months.**<br><br>**Acetonitrile mix stock standards expire after 6 months.**<br><br>**Internal Standard Mix stock standards expire after 2 months for Penicillin G - $d_7$, or 6 months for Flunixin - $d_3$ and Sulfamethazine Phenyl - $^{13}C_6$.**<br><br>**Store at ≤ -10 °C.** | 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.<br>2) Other concentrations are to be used based on two criteria:<br>a) Solubility of the drug in the solvent.<br>b) Cost and availability of the drug.<br>3) When DMSO is referenced in Table 7, Solvent used, dissolve the weighed standard with an appropriate volume of DMSO and dilute to volume with appropriate solvent. |

**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 |
| *DCCD | Beta Lactam Mix | Water | 300 |
| Erythromycin A | Acetonitrile Mix | Acetonitrile | 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 |
| 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 |
| Tilmicosin | Acetonitrile Mix | Acetonitrile | 2000 |
| Tylosin | Acetonitrile Mix | Acetonitrile | 1000 |

**The stock standard solutions identified with an asterisk (*) symbol in Table 7 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**

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

**Table 9: Acetonitrile Mix Working Standard Preparation**

| Standard Analyte | Stock standard Con. (ng/µL) | Stock standard volume (µL) | Acetonitrile Mix Con. (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 Beta-Lactam Mix Working Solution Preparation**

| | |
|---|---|
| Beta-Lactam Mix working solution<br><br>**Expires after 2 months.**<br>**Solutions will be stored at ≤ -10 °C.** | 1) Calculate the volume of stock solution required to give the working standard concentration listed for each analyte in Table 11.<br>2) Pipet the calculated volume of stock into a 25 mL volumetric flask.<br>3) Dilute to 25 mL volume with water.<br>4) Cap flask and mix.<br>5) Transfer into 4 mL polypropylene vials with screw cap lids. |

**Table 11: Beta Lactam Mix Working Solution Preparation**

| Standard Analyte | Stock standard Con. (ng/µL) | Stock standard volume (µL) | B-Lactam Mix Con. (ng/µL) |
|---|---|---|---|
| Ampicillin | 250 | 50 | 0.5 |
| DCCD | 300 | 4167 | 50 |
| Penicillin G | 250 | 250 | 2.5 |

**Table 12: Internal Standard Mix Working Standard Solution Preparation**

| | |
|---|---|
| Internal Standard Mix Working Standard Solution<br><br>**Expires after 2 months.**<br>**Store at ≤ -10 °C.** | 1) Calculate the volume of stock solution required to give the working standard concentration listed for each analyte in Table 13.<br>2) Pipet the calculated volume of stock into a 25 mL volumetric flask.<br>3) Dilute to 25 mL with acetonitrile.<br>4) Cap flask and mix.<br>5) Transfer into 4 mL amber glass LC vials with screw cap lids. |

**Table 13: 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). Presumptive positive muscle samples do not require any additional preparation. If screening was conducted in kidney, muscle tissue samples are to be prepared. Muscle samples should be allowed to partially thaw, while keeping them as cold as possible. Trim away fat and connective tissue. Grind tissue in blender or vertical cutter-mixer until homogeneous. Store homogenized samples frozen (≤ -10 °C) prior to analysis.

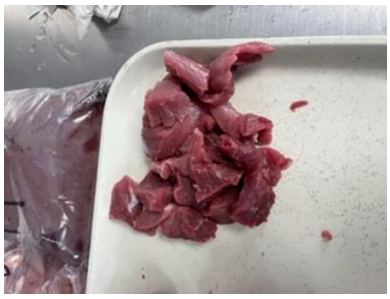

**Figure 2:** Prepared lean muscle sample with connective tissue removed. Photo courtesy of Hue Quach, USDA FSIS.

**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.

**QUALITY CONTROL**

**Bovine Muscle Controls**

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 QC Recovery, and at least 3 fortified calibration points ranging from ½ X to 4 X.

Weigh one additional portion for a check sample, if necessary.

Prepare blank, QC Recovery, fortified calibration curve, check sample, and samples using the solutions and volumes in Table 14.

**Table 14:** Example Preparation of Controls and Samples

| Sample Type | Acetonitrile Standard Mix Volume (µL) | Beta Lactam Standard Mix Volume (µL) | Internal Standard Mix Volume (µL) |
|---|---|---|---|
| Samples and Negative Controls | 0 | 0 | 30 |
| QC Recovery (1x) | 40 | 40 | 30 |
| Fortified Curve ½ X | 20 | 20 | 30 |
| Fortified Curve 1 X | 40 | 40 | 30 |
| Fortified Curve 2 X | 80 | 80 | 30 |
| Fortified Curve 4 X | 160 | 160 | 30 |

### Extraction

1) Vortex all tubes 10 seconds each to mix chemicals with matrix and allow fortified sample tubes to stand for 5 minutes (min).
2) Add 10 mL of 80:20 (v/v) acetonitrile/water to all tubes using a calibrated solvent dispenser. Cap tubes well.
3) 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 mixing effectively.
4) Centrifuge the tubes ~ 4700 RCF for 10 min.
5) Pipette a minimum of 500 µL of samples extract into a labeled amber glass autosampler vials.


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

## Instrumental Analysis

An example of a HRAM LC-MS instrument and a sample tray for an HRAM LC-MS instrument are shown in Figure 5 and Figure 6, 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 5:** Example of HRAM LC-MS instrument with high resolution. Photo courtesy of Abdulahi Duale, USDA-FSIS

**Table 15: UHPLC Gradient Program**

| *Time (min)* | *Flow (ml/min)* | *% Mobile Phase B* | *Curve* |
|---|---|---|---|
| 0.000 | | Run | |
| 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

**Instrumental Note:**

Autosampler program parameters are modified or optimized if needed to ensure that all chromatographic peaks are present.

**Figure 6:** 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

**Instrumental Note:**

Mass spectrometry parameters are optimized and adjusted as needed during annual preventative maintenance and calibration.

**Table 16: Chromatographic and Mass Spectrometry Parameters**

| Compound Name | Formula | Expected Retention Time / Retention Time Windows | Quant Ion (m/z) |
|---|---|---|---|
| 2-Aminosulfone Albendazole | $C_{10}H_{13}N_3O_2S$ | 4.59 | 240.08012 |
| Ampicillin | $C_{16}H_{19}N_3O_4S$ | 4.00 - 5.50 | 350.11690 |
| ^DCCD | $C_{17}H_{20}N_6O_7S_4$ | 4.40 - 5.40 | 275.02109 |
| Chlortetracycline | $C_{22}H_{23}ClN_2O_8$ | 4.50 - 6.00 | 479.12157 |
| Erythromycin A | $C_{37}H_{67}NO_{13}$ | 5.23 | 734.46852 |
| Flunixin | $C_{14}H_{11}F_3N_2O_2$ | 7.98 | 297.08454 |
| Flunixin-$d_3$ | $C_{14}H_{11}F_3N_2O_2$ | 7.97 | 300.10337 |
| Levamisole | $C_{11}H_{12}N_2S$ | 4.75 | 205.07940 |
| Oxytetracycline | $C_{22}H_{24}N_2O_9$ | 4.60 - 5.60 | 461.15546 |
| Penicillin G | $C_{16}H_{18}N_2O_4S$ | 6.75 | 335.10600 |
| Penicillin G-$d_7$ | $C_{16}H_{18}N_2O_4S$ | 6.70 | 342.14994 |
| Sulfachloropyridazine | $C_{10}H_9ClN_4O_2S$ | 5.73 | 285.02075 |
| Sulfadimethoxine | $C_{12}H_{14}N_4O_4S$ | 6.33 | 311.08085 |
| Sulfaethoxypyridazine | $C_{12}H_{14}N_4O_3S$ | 5.78 | 295.08594 |
| Sulfamethazine | $C_{12}H_{14}N_4O_2S$ | 5.35 | 279.09100 |
| Sulfamethazine Phenyl-$^{13}C_6$ | $C_{12}H_{14}N_4O_2S$ | 5.35 | 285.11115 |
| Sulfaquinoxaline | $C_{14}H_{12}N_4O_2S$ | 6.29 | 301.07537 |
| Tetracycline | $C_{22}H_{24}N_2O_8$ | 4.50 - 5.50 | 445.16054 |
| ^Tilmicosin | $C_{46}H_{80}N_2O_{13}$ | 4.89 | 435.29030 |
| Tylosin | $C_{46}H_{77}NO_{17}$ | 5.00 - 6.00 | 916.52643 |

**(^) The analytes identified with a caret (^) symbol in Table 16 are analyzed using the doubly charged ion, $[M+2H]^{2+}$.**

### Sample Set

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

1) Fortified Calibration Curve Standards
2) QC Tissue Blank (negative control)
3) QC 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) End QC injection of one of the Fortified Curve samples or QC Recovery (for system suitability)

**INTRA-LABORATORY CHECK SAMPLE**

Defined on the CLG website here

**Technical Note:**

A solvent blank or negative control is used to demonstrate that any carryover is less than 10% of the QC recovery.

# Reporting of Results

## Decision Criteria

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.

**Key Definitions**

**Analyte(s) of Interest:** An analyte(s) of interest is the screened positive residue from CLG-MRM3 that was found to be a presumptive positive.

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 fortified QC recovery.
3) The blank must be less than 10% of the QC 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 QC Recovery (positive control).
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 (most abundant ion of the Analyte/Internal Standard) 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 Assurance Plan.

## QUALITY CONTROL

**Quality Control Procedures**

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 Table 14.
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 QC Tissue Blank (negative control) and Calibration Curve Standards must be within 50-150% of the internal standard response for Penicillin G-$d_7$ of the QC 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 Table 14.
   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 17: Minimum Level of Applicability (MLA) for Bovine Muscle

| Analyte | MLA (µg/g) |
|---|---|
| 2-Aminosulfone Albendazole | 0.0250 |
| Ampicillin | 0.0100 |
| 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 18: 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 |

## 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.

## 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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