United States Department of Agriculture

Food Safety and Inspection Service

CLG-AMG4.05

Screening for Aminoglycosides by UHPLC-MS/MS

This method is the laboratory procedure for screening aminoglycoside residues in bovine, porcine, poultry, ovine, and caprine kidney and muscle tissues.

Executive Summary

This multi-residue screening method is used to screen for 9 aminoglycoside residues in kidney and muscle tissues from 6 animal species. The method's key performance characteristic includes:

- Simultaneous screening of highly polar antibiotics, aminoglycosides through Ultra-High Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry (UHPLC-MS/MS).

The minimum levels of applicability (MLA) or lowest levels at which an FSIS method has been successfully validated for a residue in each matrix for this method are found in the table below and also in Table 13 and Table 14.

AMG	Bovine (µg/g)		Porcine (µg/g)		Poultry (µg/g)		Equine (µg/g)		Ovine (µg/g)		Caprine (µg/g)	
	K	M	K	M	K	M	K	M	K	M	K	M
Amikacin	0.05	0.05	0.05	0.05	0.05	0.05	N/A	0.05	0.05	0.05	0.05	0.05
Apramycin	0.05	0.05	0.05	0.05	0.05	0.05	N/A	0.05	0.05	0.05	0.05	0.05
Dihydrostreptomycin	1	0.25	1	0.25	1	0.25	N/A	0.25	1	0.25	1	0.25
Gentamicin	0.05	0.05	0.05	0.05	0.05	0.05	N/A	0.05	0.05	0.05	0.05	0.05
Hygromycin B	0.05	0.05	0.05	0.05	0.05	0.05	N/A	0.05	0.05	0.05	0.05	0.05
Kanamycin	0.05	0.05	0.05	0.05	0.05	0.05	N/A	0.05	0.05	0.05	0.05	0.05
Neomycin	3.6	0.6	3.6	0.6	3.6	0.6	N/A	0.6	3.6	0.6	3.6	0.6
Spectinomycin	0.05	0.05	0.05	0.05	0.05	0.05	N/A	0.05	0.05	0.05	0.05	0.05
Streptomycin	1	0.25	1	0.25	1	0.25	N/A	0.25	1	0.25	1	0.25
K = Kidney M = Muscle N/A = Not Applicable												

Notice of Change

The method was updated to include expiration dates for the prepared solutions. In addition to these method changes, an executive summary and a safety hazards section has been added to method. No changes were made to flow chart in CLG-AMG4 Appendix 1.

Table of Contents

Safety Precautions

The personnel performing the analysis must read the Safety Data Sheets (SDS) for the standards and reagents used in this method. The hazards and recommended safe procedures for use are listed in Table 15. Follow applicable federal, state, and local regulations regarding the disposal of chemicals listed in this method.

Introduction

Aminoglycosides are a class of broad-spectrum potent antibiotics used to treat bacterial infections.[1] The use of aminoglycosides in the livestock industry has declined due to antibiotic resistance and health problems associated with toxicity when present at high levels. 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 such as aminoglycosides. The FDA establishes and publishes regulations setting tolerances for residues of animal drugs. Some aminoglycosides are not approved for use in livestock animals and therefore have a zero-tolerance policy, while other aminoglycosides are approved and have a tolerance or maximum allowable level.

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, and egg products for veterinary drugs and pesticides to verify that these products are below tolerances and are 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 veterinary 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

Due to their highly polar chemical structures, aminoglycosides are unable to be retained using standard reversed-phase HPLC conditions. Therefore, CLG-MRM3, FSIS's method for analysis of several animal drugs, cannot be utilized for analysis of aminoglycosides. In order to analyze aminoglycosides, ion-pairing additives are added to the mobile phase to improve chromatographic separation and retention.

CLG-AMG4 is used for screening of 9 aminoglycoside drugs (amikacin, apramycin, dihydrostreptomycin, gentamicin, streptomycin, kanamycin, neomycin, spectinomycin, and hygromycin). The method is applicable for analysis of the aminoglycoside residues in bovine, caprine, ovine, porcine, equine, and poultry muscle and kidney tissues.

In brief, aminoglycoside residues are initially extracted from tissue using an extraction solution composed of ammonium acetate / trichloroacetic acid and protein precipitation. Weak-cation dispersive solid-phase extraction (SPE) media is used for further extraction and sample clean up. The extracted samples are then analyzed through ion paring Ultra-High Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry (UHPLC-MS/MS).

KEY DEFINITIONS

Reversed-phase HPLC: A type of chromatography that uses a non-polar stationary phase.

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.

Solid-Phase Extraction (SPE): An extraction technique that utilizes a solid support that contains an adsorbing surface or chemical coating that can interact with analyte.

UHPLC-MS/MS: An analytical technique where there is a physical separation of target compounds followed by their mass-based detection.

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

[1] KM Kause, AW Serio, TR Kane, LE Connolly, "Aminoglycosides: An Overview", Cold Spring Harb. Perspect. Med. 6 (2016) doi: 10.1101/cshperspect.a027029

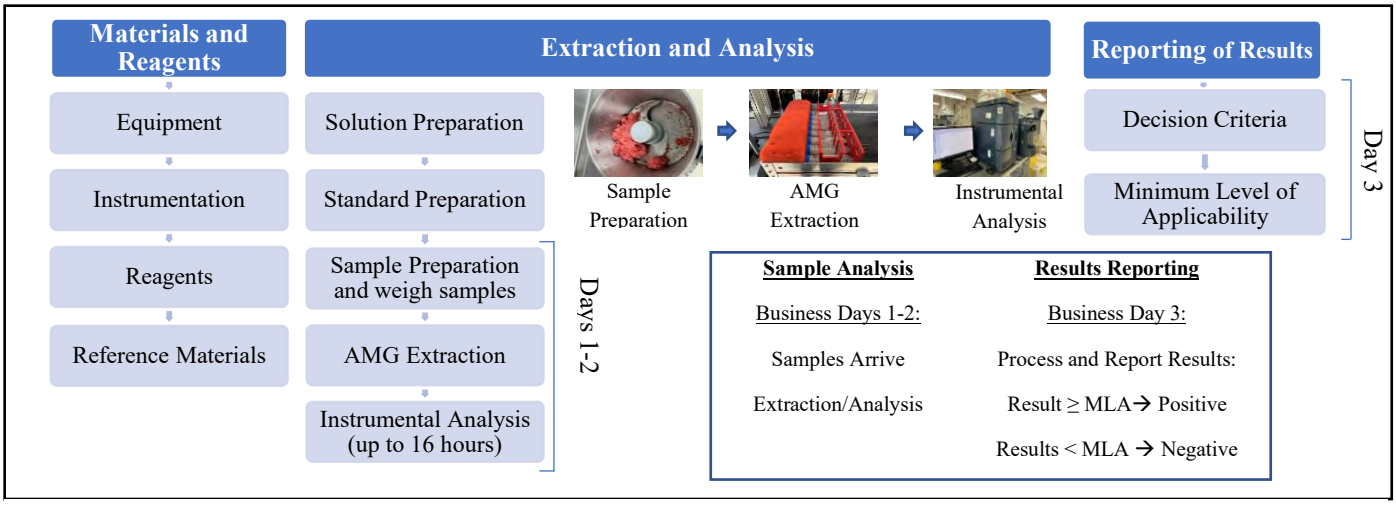

Figure 1: Overview and timeframe of Aminoglycosides (AMG4). Materials and reagents are obtained and utilized to prepare solutions and standards. The samples arrive at laboratory, are prepared into a homogenized mixture, weighed, extracted and analyzed by UHPLC-MS/MS on business days 1-2. Results are reported on business day 3. This figure represents the best-case scenarios, but analyses may take longer. Photos courtesy of Hue Quach, USDA-FSIS and Ryan Matsuda, USDA-FSIS.

Decision Criteria

A sample is considered negative if the results are less than the Minimum Level of Applicability (MLA). A sample is considered a presumptive positive if the results are greater than or equal to the MLA. Screened positive results will require further analysis through confirmation or quantitative methods.

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-AMG4

Equipment	Supplier and Part Number	Purpose
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 samples, standards, and reagents.
Repeating pipettes and tips, **2 µL - 20 µL,** **20 µL - 1000 µL,** **500 µL - 2500 µL**	General lab supplier	Dispense standards and reagents.
Bottle-Top dispensers, **1 mL - 5 mL,** **2 mL and 10 mL**	General lab supplier	Dispense reagents and solutions.
Glassware, Class A	General lab supplier	Measuring standards and reagents
Centrifuge tubes Polypropylene (PP), 15, 50 mL	General lab supplier	Contain sample material and extraction vessel.
Analytical Balance	General lab supplier	Record weight of standard reagent. Minimum readability ±0.0001 g.
Top Loading Balance	General lab supplier	Record weight of quality controls and samples. Minimum readability ±0.01 g.
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.
Shaker	General lab supplier	Facilitates extraction of residue from the sample
pH meter	General lab supplier	Ensure pH of reagents and extracts during extraction
Auto-titrator	General lab supplier	Titrate sample extracts
Nalgene fluorinated ethylene propylene (FEP) bottle, 30 mL	General lab supplier	Store standard solutions

Cryogenic tubes, 1.2 mL	**General lab supplier**	**Store standard solutions**
Fluted filter paper, Grade 313, 15 cm	VWR, 28333-043	Filter extracts
CUCCX1 (carboxylic acid) Sorbent	Sorbent Selectra Bulk Sorbents, CUCCXOOK	Dispersive solid phase extraction
Magnetic stirrer	General lab supplier	Prepare extraction solution
Membrane disc filters, 47 mm i.d., 0.2 µm	VWR, 28147-978	Filter mobile phase solutions
Whatman Mini-Uniprep syringeless PVDF filter vials, 0.2 µm	VWR, 12000-524	Filter final extracts
PVDF filter disk, 0.2 µm	Xpertek, 9474051	Filter final extracts
Syringe, 3 mL	Becton Dickenson, 309657	Filter final extracts
Screw top, amber glass, autosampler vials, PTFE septa, 2 mL	General lab supplier	Store extract.

Instrumentation

Table 2: Instrumentation

Instrument	Supplier and Model Number	Purpose
Waters UPLC-MS/MS System	Waters Xevo I-Class LC, Waters Xevo TQD Mass Spectrometer	Sample extract analysis
Waters UPLC BEH C18, 2.1 × 50 mm, 1.7µm	Waters, 186002350	Sample extract analysis
Waters VanGuard Pre-column UHPLC BEH C18, 2.1 × 5.0 mm, 1.7 µm	Waters, 186003975	Sample extract analysis

Reagents

Table 3: Reagents

Reagent	Supplier and Part Number
Acetonitrile (ACN) - LC-MS Grade	General lab supplier
Methanol (MeOH)	General lab supplier
Water – Resistivity of > 18 MΩ-cm	House system
Heptafluorobutyric acid (HFBA)	Millipore Sigma, 77249
1 N Hydrochloric acid (HCl)	General lab supplier
Trichloroacetic acid (TCA)	Millipore Sigma, T6399
Ethylenediaminetetraacetic acid, disodium salt, dihydrate ($Na_2EDTA•2H_2O$), 99+%	Millipore Sigma, E5134
Ammonium acetate (NH_4OAc)	General lab supplier
1 N Sodium hydroxide (NaOH) solution	General lab supplier
Sodium chloride (NaCl)	General lab supplier
Formic acid	General lab supplier
0.5 N NaOH	General lab supplier
30% w/v NaOH	General lab supplier

Reference Materials

Table 4: Reference Materials

Standard	Supplier	Catalog Number
Amikacin	Millipore Sigma	A-1774
Apramycin HCl	Millipore Sigma	A-2024
Dihydrostreptomycin sulfate	USP	1203008
Gentamicin sulfate	Millipore Sigma	G-3632
Streptomycin sulfate	USP	1623003
Kanamycin sulfate	Millipore Sigma	K-1876
Neomycin B sulfate	Millipore Sigma	N-1876
Spectinomycin HCl	USP	1618003
Hygromycin B	Millipore Sigma	H-7772
Tobramycin	Millipore Sigma	T-4014

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

Purity and counterions are to be taken into account 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
20 mM HFBA in ACN (Mobile Phase B):	1) Measure 2.6 mL of HFBA and add to 1 L volumetric flask. 2) Dilute to volume with ACN and mix well. 3) Filter through a 0.2 µm filter disc if necessary. 4) Transfer to a glass storage container for use. 5) Store at room temperature. *Solution expires 1 year after preparation.*
20 mM HFBA in 95:5 Water:ACN (Mobile Phase A):	1) Measure 2.47 mL of HFBA and add to 1 L volumetric flask. 2) Measure 50 mL of 20 mM HFBA in ACN (Mobile Phase B) and add to same flask. 3) Dilute to volume with water and mix well. 4) Filter through a 0.2 µm filter disc if necessary. 5) Transfer to a glass storage container for use. 6) Store at room temperature. *Solution expires 3 months after preparation.*
Extraction solvent mixture (10 mM NH_4OAc, 0.4 mM EDTA, 0.5% NaCl and 2% TCA in water):	1) Measure 1.54 g of NH_4OAc and add to a 2 L glass container. 2) Add 1.95 L of water. 3) Measure and adjust pH to pH 4 with 1 N HCl or 1 N NaOH. 4) Measure 0.3 g of $Na_2EDTA•2H_2O$ and add to the same container. 5) Measure 10 g of NaCl and add to the same container. 6) Measure 40 g of TCA and add to the same container. 7) Mix to ensure salts are dissolved. 8) Dilute to a 2 L volume with water. 9) Mix well and transfer to a glass storage container for use. 10) Store at room temperature. *Solution expires 1 year after preparation.*
10% Formic Acid in Water:	1) Add 80 mL of water to a 100 mL volumetric flask. 2) Measure and add 10 mL of formic acid to same flask. 3) Dilute to volume with water. 4) Mix well and transfer to a glass storage container for use. 5) Store at room temperature. *Solution expires 1 year after preparation.*

Standard Preparation

Table 6: Stock and Intermediate Stock Standard Solutions

Solution	Procedure
Individual AMG stock solutions (2000 µg/mL in water):	1) For each stock solution, calculate the amount of material that contains 20 mg AMG base, accounting for purity and/or water and sulfate content. Weigh this amount to the nearest 0.1 mg. 2) Transfer to a 30 mL Nalgene FEP bottle and add by weight (1 g/mL density for water) the exact amount of water (≈10 mL) to yield 2000 µg/mL concentration of the pure drug. 3) Mix well. 4) Store in freezer at <-10 °C. *Solution expires 3 months after preparation.*
Intermediate standard mixture of AMGs in water (50 µg/mL)	1) Pipet 250 µL each of amikacin, apramycin, hygromycin B, kanamycin, gentamicin, and spectinomycin into a 30 mL FEP bottle. 2) Add 8.50 mL of water. 3) Store in freezer at <-10 °C. *Solution expires 3 months after preparation.*

Table 7: Mixed AMG spiking solution in water:

Following Table 7, combine the amounts of 2,000 µg/mL AMG stock solution for streptomycin, dihydrostreptomycin, neomycin and for Intermediate standard mixture of AMGs in water (50 µg/mL) to prepare the mixed working standards in a 30 mL FEP bottle for kidney, or muscle (use given volumes depending on matrix):

AMG	Standard	Concentration (µg/mL)	Volume for Kidney, Fortification Standard, (mL)	Kidney Fortification Standard (µg/mL)	Volume for Muscle Fortification Standard, (mL)	Muscle Fortification Standard (µg/mL)
Neomycin	Stock	2000	0.72	144	0.12	24
Streptomycin	Stock	2000	0.2	40	0.05	10
Dihydrostreptomycin	Stock	2000	0.2	40	0.05	10
Hygromycin B	AMG Intermediate Standard Mix	50	0.4	2	0.4	2
Amikacin						
Kanamycin						
Apramycin						
Gentamicin						
Spectinomycin						
Water	N/A	N/A	8.48	N/A	9.38	N/A

Mix well. Store in freezer at <-10 °C. *Solution expires 3 months after preparation.*
The spiking solution is used to fortify samples (recoveries and check samples). The spiking solution should be portioned into polypropylene centrifuge tubes in quantities such that the volume in each tube is consumed on a sample set thus minimizing losses due to thawing and refreezing.

Table 8: Preparation of Internal Standard

Solution	Procedure
Tobramycin internal standard (IS) in water (40 µg/mL):	1) Measure 200 µL of 2000 µg/mL tobramycin stock solution and add to a 15 mL polypropylene centrifuge tube. 2) Add 9.8 mL of water to the same tube. 3) Store in freezer at <-10 °C. *Solution expires 3 months after preparation.*

Table 9: Preparation of External Standard for system suitability

Solution	Procedure
AMG external standard in formic acid:	1) Add 0.050 mL of appropriate kidney or muscle calibration/spiking solutions to bottom portion of Whatman Mini-Uniprep filter vial. 2) Add 0.050 mL of the 40 µg/mL IS solution to the same vial. 3) Add 0.400 mL of 10% formic acid to the same vial. 4) Mix well. 5) Place filter cap on top of vial and push down to filter. 6) Store in refrigerator at 2 - 8 °C. *Solution expires 5 days after preparation..*

Table 10: AMG Concentration in fortified tissue with 100 µL of appropriate standard

AMG	Kidney (µg/g)	Muscle (µg/g)
Neomycin	3.6	0.6
Spectinomycin	0.05	0.05
Streptomycin	1	0.25
Dihydrostreptomycin	1	0.25
Gentamicin	0.05	0.05
Hygromycin B	0.05	0.05
Amikacin	0.05	0.05
Kanamycin	0.05	0.05
Apramycin	0.05	0.05

Sample Preparation

Samples 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, temper (partially thaw) the sample while keeping it as cold as possible. As shown in Figure 2, trim away fat and connective tissue from the sample. As shown in Figure 3, grind sample in blender or vertical cutter-mixer until homogeneous. Store homogenized samples frozen (≤-10 °C) prior to analysis.

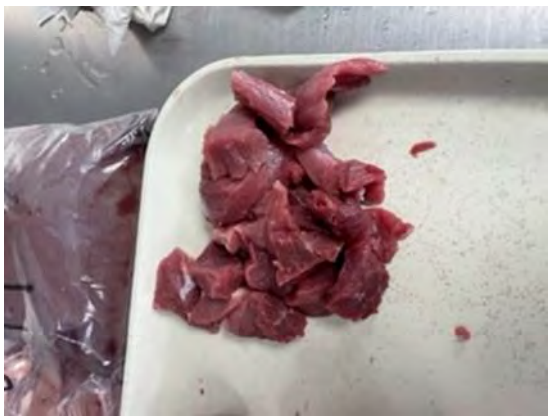

Figure 2: Prepared lean muscle sample with connective tissue removed. Photo courtesy of Hue Quach, USDA FSIS.

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

Aminoglycoside Extraction

Samples

Weigh 4.0 ± 0.1 g of homogenized muscle or kidney sample into labeled 50 mL polypropylene centrifuge tubes.

QUALITY CONTROL

1. Weigh three 4.0 ± 0.1 g portions of blank tissue into 50 mL polypropylene centrifuge tubes (see Figure 4). One for the blank (negative control), one for the decision level control, and one for the Recovery (positive control). Weigh one additional portion for a check sample, if applicable.
2. Prepare decision level and recovery controls by fortifying the sample with 100 µL of the appropriate fortification standard.

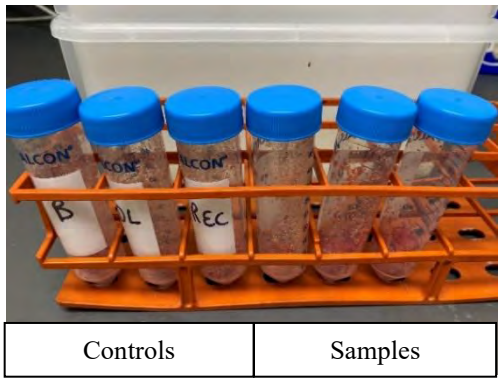

Figure 4: Weighed controls and samples. Photo courtesy of Ryan Matsuda, USDA-FSIS

KEY DEFINITIONS

Blank (negative control): A quality control sample that is negative for all analytes of interest.

Decision level control: Sample is prepared with addition of analytes that have a concentration level comparable to MLA. Negative and positive controls are compared to "Decision level control."

Recovery (positive control): Sample is prepared with addition of analytes that have a concentration level comparable to MLA. Samples are compared to recovery.

Extraction

1) Add 20 mL of NH_4OAc/EDTA/NaCl/TCA buffer to each tube.
2) To each tube add 200 µL of the 40 µg/mL tobramycin IS to yield 2 µg/g in the tissue.
3) As shown in Figure 5, shake for 10 minutes (min).
4) Centrifuge at a minimum of ~3500 RCF (4000 RPM) for 5 min.
5) Decant supernatant into a 50 mL polypropylene tube or container used for titration
    a. If floating material is observed, remove it with a spatula or filter by using a fluted filter paper (Filtering set up illustrated in Figure 6)
6) Using a calibrated pH meter or auto-titrator, adjust pH of the sample extracts to 7.50 ± 0.25 with a few drops of 30% NaOH followed by 1 N NaOH and/or 1 N HCl for fine adjustment. (Auto-titration system set up shown in Figure 6.)

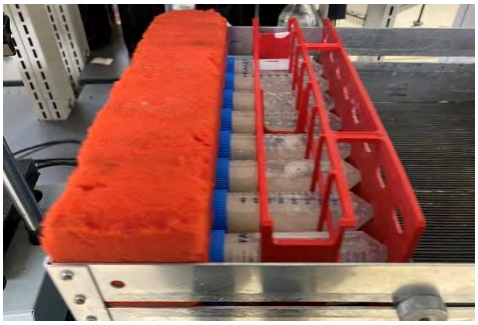

Figure 5: Samples undergoing shaking for extraction. Photo courtesy of Ryan Matsuda, USDA-FSIS

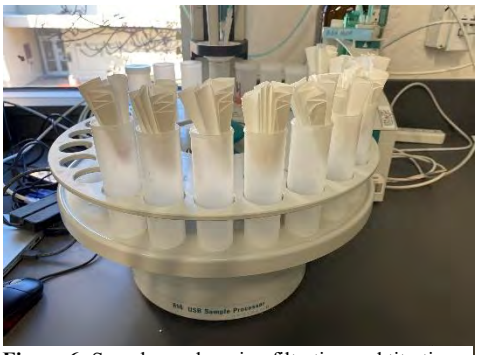

Figure 6: Samples undergoing filtration and titration. Photo courtesy of Ryan Matsuda, USDA-FSIS

> **Technical Option:**
> Using more dilute concentrations of NaOH and HCl, such as 0.5 N, is allowable for fine adjustment of the pH.

7) Centrifuge at a minimum of ~3500 RCF (4000 RPM) for 3 min.
8) Decant each extract into a pre-labeled 50 mL polypropylene centrifuge tubes containing approximately 0.50 g of weak-cation exchange (carboxylic acid) sorbent.
9) As shown in Figure 7, cap tubes and vortex on a platform vortex for 3 min.
10) Centrifuge tubes at a minimum of ~3500 RCF (4000 RPM) for 3 min.

Figure 7: Clean up through dispersion SPE. Photo courtesy of Ryan Matsuda, USDA-FSIS

11) Aspirate and discard supernatant, leaving centrifuged sorbent at bottom of tube.
12) Add 2 mL 10% Formic Acid to each tube containing sorbent, cap and vortex on a platform vortex for 3 min.
13) Centrifuge tubes at a minimum of ~3500 RCF (4000 RPM) for 3 min.
14) For both samples and controls place 500 µL of each final extract into bottom piece of Mini Uni-Prep PVDF syringeless filter vial. Then insert top filter vial and press together. (See Figure 8)

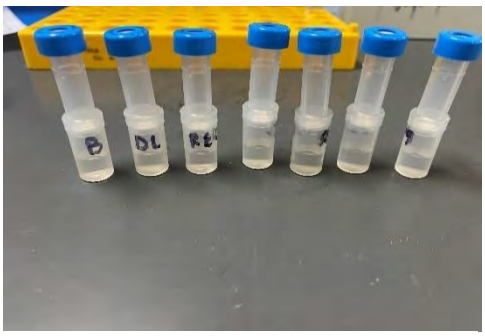

Figure 8: Final extracts before being flited. Photo courtesy of Ryan Matsuda, USDA-FSIS

Instrumental Analysis

An example of a sample tray for an LC-MS/MS system and an example of an LC-MS/MS instrument are shown in Figure 9 and Figure 10, respectively.

Chromatographic Parameters

1) Mobile phases for AMG analysis
   a) Mobile Phase A – 95% water / 5% ACN / 20 mM HFBA
   b) Mobile Phase B – 100% ACN / 20 mM HFBA
2) Flow rate: 0.5 mL/min
3) Run time: 3.00 min
4) Gradient Program

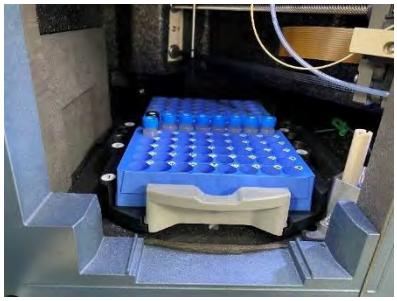

Figure 9: Prepared samples in LC-MS-MS instrument. Photo courtesy of Ryan Matsuda, USDA-FSIS

Table 11: LC Gradient Program

Time (min)	% Mobile Phase A	% Mobile Phase B	Gradient
0.00	100	0	none
0.50	80	20	linear
1.00	80	20	none
2.00	60	40	linear
2.05	10	90	linear
2.50	10	90	none
2.55	100	0	linear
3.00	100	0	none

5) Autosampler program
   a) Run time: 3.0 min
   b) Injection loop: 20 µL
   c) Sample injection mode: Partial loop needle overfill
   d) Injection Volume: 15 µL
   e) Weak wash solvent: Mobile Phase A
   f) Weak wash volume: 500 µL
   g) Strong wash solvent: Mobile Phase B
   h) Strong wash volume: 500 µL
   i) Sample temperature: 7° C
6) Column manager
   a) Column valve position: To match column location
   b) Column manager temperature: 40 °C
   c) Use divert valve to divert eluant to waste 0.25 min prior to first peak and 0.25 min after last analyte peak.

Mass Spectrometry Parameters

1) Type: MS/MS
2) 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): 0.20
3) Analyzer Parameters
   a) LM1 Resolution: 12.50
   b) HM 1 Resolution: 12.50
   c) MSMS Mode Entrance: -5
   d) MSMS Mode Collision Energy: Variable – analyte dependent
   e) MSMS Mode Exit: 1
   f) LM 2 Resolution: 12.50
   g) HM 2 Resolution: 12.50
4) MS Method Parameters:
   a) Type: MRM
   b) Ion Mode: ES+

Figure 10: LC-MS-MS instrument.
Photo courtesy of Ryan Matsuda, USDA-FSIS

c) MRM Transitions:

Table 12: MRM Transitions

Start–End Time (min)	Dwell Time (ms)	Compound	Precursor ion *(m/z)*	Product ion *(m/z)*	Cone (V)	Collision Energy (V)
0.9-1.2	66	Spectinomycin Hydrate	351.24	333.33	40	20
1.1-1.3	66	Hygromycin B	528.20	177.05	44	30
1.2-1.4	44	Streptomycin	582.17	263.09	70	32
1.2-1.4	52	Dihydrostreptomycin	584.17	263.09	70	30
1.5-1.7	150	Amikacin	586.43	163.21	30	35
1.6-1.8	150	Kanamycin A	485.36	163.22	30	20
1.9-2.1	33	Apramycin	540.41	217.20	35	25
2.0-2.1	22	Tobramycin (IS)	468.36	163.19	25	25
2.0-2.2	33	Gentamicin c1a	450.39	160.16	35	25
2.0-2.2	33	Gentamicin c2+c2a	464.42	160.23	35	25
2.0-2.3	33	Gentamicin c1	478.42	157.25	40	30
2.1-2.3	22	Neomycin B	615.30	163.38	52	35

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 time windows may be adjusted to account for aging of UHPLC columns, GC 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.
  - Target masses for precursor and product ions can be optimized to a *m/z* value that falls within the unit mass resolution of the exact mass, but not to exceed the next integer value (e.g., if the exact mass is 787.5, an allowable target mass range includes 787.0-787.9).
- 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 all controls.

Instrument system suitability is to be demonstrated prior to sample set injection.

1) Decision Level control
2) Recovery (positive control)
3) Intra-Laboratory Check Sample (If applicable)
4) Blank (negative control)
5) Up to 44 Samples
6) Reinjection of Recovery or Decision Level control (for set suitability)

INTRA-LABORATORY CHECK SAMPLE

Defined on the CLG website.

Reporting of Results

Decision Criteria

Screening

1) The retention time for the recoveries and samples must match the retention time of the decision level control within 5%.
2) Blank must be less than 10% of the decision level control.
3) The screening ion for a given analyte must be present. The required ion for each compound is listed in Table 12.
4) The screening ion must have a signal-to-noise ratio ≥ 3. This may be verified by visual inspection.
5) A sample is screened positive if the following criteria are met:
   a) The fortified recovery of the analyte must exceed 10% of the decision level control.
   b) The sample response equals or exceeds the level of interest recovery (positive control) level.

QUALITY ASSURANCE PLAN

Quality Control Procedures

1) For set acceptance, all analytes in the fortified recovery (positive control) must meet screening criteria.
2) The blank (negative control) must be negative using the screening criteria

Intra-Laboratory Check Samples (If applicable)

1) Acceptability criteria.
   a. All analytes in a fortified Intra-Laboratory Check must meet screening criteria.
   b. All analytes in an unfortified Intra-Laboratory Check must be negative using the screening criteria.
   c. FSIS Field Service Laboratories are to refer to internal FSIS Quality Control Procedures, if 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 13: Minimum Level of Applicability (MLA) for Kidney Screening

AMG	Bovine (µg/g)	Porcine (µg/g)	Poultry (µg/g)	Equine (µg/g)	Ovine (µg/g)	Caprine (µg/g)
Amikacin	0.05	0.05	0.05	N/A	0.05	0.05
Apramycin	0.05	0.05	0.05	N/A	0.05	0.05
Dihydrostreptomycin	1	1	1	N/A	1	1
Gentamicin	0.05	0.05	0.05	N/A	0.05	0.05
Hygromycin B	0.05	0.05	0.05	N/A	0.05	0.05
Kanamycin	0.05	0.05	0.05	N/A	0.05	0.05
Neomycin	3.6	3.6	3.6	N/A	3.6	3.6
Spectinomycin	0.05	0.05	0.05	N/A	0.05	0.05
Streptomycin	1	1	1	N/A	1	1
N/A = Not Applicable						

Table 14: Minimum Level of Applicability (MLA) for Muscle Screening

AMG	Bovine (µg/g)	Porcine (µg/g)	Poultry (µg/g)	Equine (µg/g)	Ovine (µg/g)	Caprine (µg/g)
Amikacin	0.05	0.05	0.05	0.05	0.05	0.05
Apramycin	0.05	0.05	0.05	0.05	0.05	0.05
Dihydrostreptomycin	0.25	0.25	0.25	0.25	0.25	0.25
Gentamicin	0.05	0.05	0.05	0.05	0.05	0.05
Hygromycin B	0.05	0.05	0.05	0.05	0.05	0.05
Kanamycin	0.05	0.05	0.05	0.05	0.05	0.05
Neomycin	0.6	0.6	0.6	0.6	0.6	0.6
Spectinomycin	0.05	0.05	0.05	0.05	0.05	0.05
Streptomycin	0.25	0.25	0.25	0.25	0.25	0.25

Safety Hazards

Table 15: 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, Hydrochloric acid, Sodium Hydroxide, Trichloroacetic Acid, Ethylenediaminetetraacetic acid Heptafluorobutyric Acid	Corrosive, Caustic	Wear personal protective equipment, avoid skin contact.
Aminoglycoside Standards	Some individuals may have allergic reactions to aminoglycosides, which may cause skin and respiratory irritation. Possible reproductive toxicity and ototoxicity	Wear personal protective equipment, avoid skin contact. Handle with extreme caution. Work in a well-ventilated area.

References

1) 21CFR 556 for tolerance values set by FDA.

2) The National Residue Program sets the number of aminoglycosides to be sampled each year.

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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