United States Department of Agriculture

Food Safety and Inspection Service

CLG-AMG2.09

Confirmation of Aminoglycosides by UHPLC-MS/MS

This method is the laboratory procedure to confirm 9 aminoglycoside residues in muscle, kidney, and liver tissues from cattle, swine, and other species.

Executive Summary

This is a multi-residue confirmation method for analysis of aminoglycoside residues. The method's key performance characteristics include:

- Simultaneous mass spectrometric confirmation of 9 aminoglycosides residues in:
  - Bovine, caprine, ovine, porcine, and poultry kidney
  - Bovine, caprine, ovine, porcine, equine, and poultry muscle
  - Bovine and porcine liver

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. The MLAs for each matrix and individual analytes are found in Tables 19-21.

Analyte	Range of MLAs (ppm)		
	Kidney	Muscle	Liver
Amikacin	0.05	0.05	0.05 - 0.1
Apramycin	0.05 - 0.1	0.05 - 0.1	0.05 - 0.2
Dihydrostreptomycin	1	0.25	0.25 - 1
Gentamicin	0.1 - 0.2	0.2	0.2
Hygromycin B	0.05	0.05 - 0.2	0.05
Kanamycin	0.05	0.05	0.05
Neomycin	0.45	0.6 – 1.2	1.8
Spectinomycin	0.05 - 0.1	0.125 – 0.5	0.125 – 0.25
Streptomycin	1	0.25	0.25 - 1

Notice of Change

FSIS has revised and reformatted its aminoglycosides method to clarify laboratory workflows and modernize the method format. This method will serve as the FSIS laboratories' primary confirmation method with an updated title: *Confirmation of Aminoglycosides by UHPLC-MS/MS* (CLG-AMG2). FSIS will continue to use *Screening for Aminoglycosides by UHPLC-MS/MS* (CLG-AMG4) as the primary screening analysis for aminoglycosides.

CLG-AMG2 will confirm aminoglycoside residues in muscle, kidney, and liver tissues from cattle, swine, poultry, and other species. The method's confirmation scope, target analytes, procedures, and detection criteria remain unchanged. This method revision only impacts work performed by laboratory analysts and does not affect sample collection or result reporting.

Additionally, CLG-AMG2 has been reformatted to enhance clarity and improve user accessibility. Revisions include updating instructions and extraction procedures as well as clarifying standard solution preparation.

A flow chart that describes the overall aminoglycoside analysis process is found in CLG-AMG2 Appendix 1. The flow chart provides an overall sample analysis timeline indicating the best-case scenarios from screening with CLG-AMG4 to confirmation with CLG-AMG2.

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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 22. Follow all applicable federal, state, and local regulations regarding the disposal of chemicals listed in this method.

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Introduction

Aminoglycosides are a class of potent, broad-spectrum 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. 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 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-AMG4 is FSIS' method for screening aminoglycoside residues in meat, poultry, and equine muscle and kidney tissues. When screening results from CLG-AMG4 indicate that an aminoglycoside is found to be "presumptive positive", CLG-AMG2 is used for confirmation. CLG-AMG2 is applicable to confirm 9 aminoglycoside residues (amikacin, apramycin, dihydrostreptomycin, gentamicin, streptomycin, kanamycin, neomycin, spectinomycin, and hygromycin) in kidney tissues from meat and poultry, equine muscle tissue, and bovine and porcine liver tissue. Confirmation analysis is conducted on the presumptive positive sample to confirm the presence of the residue and if the result is a presumptive violation.

KEY DEFINITIONS

Presumptive Positive: Samples that have been found to have screening results that exceed the minimum level of applicability (MLA).

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.

Presumptive Violation: Samples that have been found to confirm but require quantitation analysis for comparison to the tolerance level.

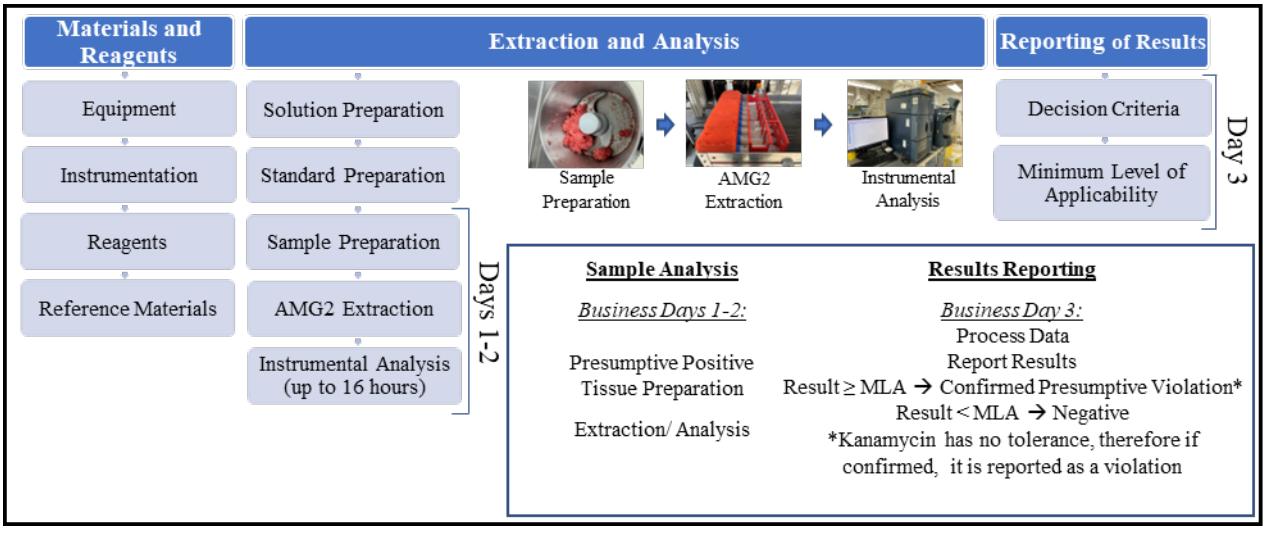

Figure 1: Overview and timeframe for Confirmation of Aminoglycosides by UHPLC-MS/MS (CLG-AMG2)
Materials and reagents are obtained and utilized to prepare solutions and standards. On business days 1-2, after screening analysis, presumptive positive tissue are weighed, extracted, and analyzed by UHPLC-MS/MS. 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

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

In brief, aminoglycoside residues are extracted from tissue using an extraction solution composed of ammonium acetate / trichloroacetic acid and protein precipitation. Weak-cation dispersive solid-phase extraction 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

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.

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

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.

Decision Criteria

CLG-AMG2 is a mass spectrometric confirmation method and therefore, results are determined based on the method's decision criteria. A sample is considered confirmed negative by CLG-AMG2 if sample does not meet the methods' decision criteria.

As previously discussed in FSIS Laboratory System Introduction, Method Performance Expectations, and Sample Handling for Chemistry (CLG 1), residues with tolerances require both confirmation and quantitation. Apramycin, amikacin, hygromycin, spectinomycin, streptomycin, dihydrostreptomycin, gentamicin, and neomycin have established tolerances, and therefore, if the sample is confirmed as positive based on the method's decision criteria, the sample would be considered a "Presumptive Violation." Presumptive violations will require further analysis through additional methods to quantitate the residue in the sample.

Kanamycin does not have an established residue tolerance, therefore, if the results are confirmed as positive based on the method's decision criteria, the sample would be considered a violation.

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 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-AMG2

Equipment	Supplier and Part Number	Purpose
Centrifuge	General lab supplier	Separates the solid sample material from the extraction solution. Capable of ~ 4000 RPM
Cutting board and knives	General lab supplier	Preparation of sample
Vortex Mixer	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
Top Loading Balance	General lab supplier	Record weight of quality controls and samples. Minimum readability ±0.01 g.
Analytical Balance	General lab supplier	Record weight of standard reagent. Minimum readability ±0.0001 g.
DPX Lever Arm Extractor	DPX Labs, 24 position	Performs SPE cleanup
Centrifuge tubes, Polypropylene (PP) 50 mL, 15 mL	General lab supplier	Contain sample material and extraction vessel
Whatman Mini-Uniprep syringeless Polyvinylidene Difluoride (PVDF) filter vials, 0.2 µm	VWR, 12000-524	Filter final extracts
Cryogenic tubes, 1.2 mL	General lab supplier	Store standard solutions
Fluorinated ethylene propylene (FEP) bottle, 30 mL	General lab supplier	Store standard solutions
Membrane disc filters, 47 mm i.d., 0.2 µm	VWR, 28147-978	Filter mobile phase solutions
Magnetic stirrer	General lab supplier	Prepare extraction solution
Repeating pipettes and tips, 25 µL, 100 µL, 200 µL, 2.5 mL	General lab supplier	Dispense standards and reagents

Shaker	General lab supplier	Facilitates extraction of residue from the sample
Glassware, Class A	General lab supplier	Measuring standards and reagents
Food Processor	Robot Coupe USA Inc.	Homogenize sample
Freezer, -10 °C	General lab supplier	Storage of standards and reagents
PVDF filter disk, 0.2 µm	Xpertek, 9474051	Filter final extracts
Syringe with Luer-Lok tip, 10mL	Becton Dickenson, 309604	Filter final extracts
Fluted filter paper, 12.5cm	General lab supplier	Filter extracts
WCX-DPX tips	General lab supplier	SPE cleanup

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, 1.7 µm	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)-LC Grade	General lab supplier
Water (H_2O), Resistivity of > 18 MΩ-cm	House system
Heptafluorobutyric acid (HFBA)	General lab supplier
1 N Hydrochloric acid (HCl)	General lab supplier
Trichloroacetic acid (TCA)	General lab supplier
Ethylenediaminetetraacetic acid, disodium salt, dihydrate ($Na_2EDTA•2H_2O$), 99+%	General lab supplier

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	PHR1860
Apramycin HCl	Millipore Sigma	A-2024
Dihydrostreptomycin sulfate	USP	1203008
Gentamicin sulfate	Millipore Sigma	G-3632
Streptomycin sulfate	USP	1623003
Kanamycin sulfate	USP	1355006
Neomycin B sulfate	USP	1458009
Spectinomycin HCl	USP	1618003
Hygromycin B	Millipore Sigma	H-7772
Tobramycin	USP	1667508

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

Account for purity and counterions 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 a 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 a1 L volumetric flask. 2) Measure 50 mL of 20 mM HFBA in ACN (Mobile Phase B) and add to the 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 a pH 4 with 1 N HCL or 1N 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 the 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

Mixed AMG standard solutions are prepared from different stock and intermediate stock standard solutions found in Table 6.

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 FEP storage 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, and gentamicin, into a FEP storage bottle. 2) Add 8.75 mL of water. 3) Mix well. 4) Store in freezer at <-10 °C. *Standard expires 3 months after preparation.*
Intermediate individual standard solutions (50 µg/mL):	1) Pipet 250 µL each of amikacin, apramycin, hygromycin B, kanamycin, gentamicin, and spectinomycin into separate FEP storage bottles. 2) Add 9.75 mL of water. 3) Mix well. 4) Store in freezer at <-10 °C. *Standard expires 3 months after preparation.*

Standard Solution

Standard solutions are matrix dependent. Prepare standard solutions based on the matrix of interest. Standard solutions are used to prepare the matrix match standards, as described in Table 16 of the extraction procedure.

Kidney Mixed AMG Standard Solution

Refer to Table 7 to prepare a 4X Kidney Mixed AMG Standard Solution. The 1/2X, 1X and 2X standard solution will need to be prepared as well.

1) Prepare the appropriate 4X standard solution for the tissue and species desired in a volume sufficient to allow for serial dilution.

Table 7: Preparation of Kidney Mixed AMG Standard Solution

AMG	4X (mL)
Neomycin Stock	2.88
Spectinomycin Hydrate Intermediate	1.6
Streptomycin Stock	0.8
Dihydrostreptomycin Stock	0.8
Intermediate standard mixture	1.6
Water Added	2.32

2) Dilute the 4X 1:1 in water and mix well to produce the 2X standard solution.
3) Dilute the 2X 1:1 in water and mix well to produce the 1X standard solution.
4) Dilute the 1X 1:1 in water and mix well to produce the 1/2X standard solution.
5) Store solution in freezer at <-10 °C.

Solution expires 3 months after preparation.

Refer to Tables 23-24 in the Appendix for mixed standard concentrations.

Muscle Mixed AMG Standard Solution

Refer to Table 8-9 to prepare a 4X Muscle Mixed AMG Standard Solution. Table 8 is used to prepare 4X standard solutions for all muscle matrices, except for ovine, which is prepared according to Table 9. The 1/2X, 1X and 2X standard solutions will need to be prepared as well.

All Muscle Except for Ovine

1) Prepare the appropriate 4X standard solution for the tissue and species desired in a volume sufficient to allow for serial dilution.

Table 8: Preparation of Muscle Mixed AMG Standard with Exception of Ovine

AMG	4X (mL)
Neomycin stock	0.48
Spectinomycin Hydrate stock	0.1
Streptomycin stock	0.2
Dihydrostreptomycin stock	0.2
Intermediate standard mixture	1.6
Water added	7.42

2) Dilute the 4X 1:1 in water and mix well to produce the 2X standard solution.
3) Dilute the 2X 1:1 in water and mix well to produce the 1X standard solution.
4) Dilute the 1X 1:1 in water and mix well to produce the 1/2X standard solution.
5) Store solution in freezer at <-10 °C

Solution expires 3 months after preparation.

Refer to Tables 25-26 in the Appendix for mixed standard concentrations.

Ovine Muscle

1) Prepare the appropriate 4X standard solution for the tissue and species desired in a volume sufficient to allow for serial dilution.

Table 9: Preparation of Ovine Muscle Mixed AMG Standard

AMG	4X (mL)
Neomycin stock	0.96
Spectinomycin Hydrate stock	0.1
Streptomycin stock	0.2
Dihydrostreptomycin stock	0.2
Intermediate standard mixture	1.6
Water added	6.94

2) Dilute the 4X 1:1 in water and mix well to produce the 2X standard solution.
3) Dilute the 2X 1:1 in water and mix well to produce the 1X standard solution.
4) Dilute the 1X 1:1 in water and mix well to produce the 1/2X standard solution.
5) Store solution in freezer at <-10 °C.

Solution expires 3 months after preparation.

Refer to Tables 25-26 in the Appendix for mixed standard concentrations.

Liver Mixed AMG Standard Solution

Refer to Table 10 to prepare a 4X Liver Mixed AMG Standard Solution. The 1/2X, 1X and 2X standard solutions will need to be prepared as well.

1) Prepare the appropriate 4X standard solution for the tissue and species desired in a volume sufficient to allow for serial dilution.

Table 10: Preparation of Liver Mixed AMG Standard Solution

AMG	4X (mL)
Neomycin stock	1.44
Spectinomycin Hydrate stock	0.1
Streptomycin stock	0.2
Dihydrostreptomycin stock	0.2
Intermediate standard mixture	1.6
Water added	6.46

2) Dilute the 4X 1:1 in water and mix well to produce the 2X standard solution.
3) Dilute the 2X 1:1 in water and mix well to produce the 1X standard solution.
4) Dilute the 1X 1:1 in water and mix well to produce the 1/2X standard solution.
5) Store solution in freezer at <-10 °C.

Solution expires 3 months after preparation

Refer to Tables 27-28 in the Appendix for mixed standard concentrations.

Spiking Solutions

Spiking solutions are matrix dependent and used during the preparation of positive controls.

- For Tables 11-13, the volumes listed for Amikacin, Apramycin, Gentamicin, Hygromycin B, Kanamycin and Spectinomycin use the 50 µg/mL intermediate individual standard solutions in Table 6. The volumes listed for Streptomycin, Dihydrostreptomycin and Neomycin use the 2000 µg/mL stock solution.
- Portion the spiking solution into 15mL centrifuge tubes in small volume quantities to minimize losses due to thawing and refreezing.

Table 11: Preparation of Kidney AMG Spiking Standard Solution

AMG	Bovine Kidney (mL)	Porcine Kidney (mL)	Poultry Kidney (mL)	Ovine Kidney (mL)	Caprine Kidney (mL)
Amikacin	0.2	0.2	0.2	0.2	0.2
Apramycin	0.2	0.2	0.2	0.4	0.2
Dihydrostreptomycin	0.1	0.1	0.1	0.1	0.1
Gentamicin	0.4	0.4	0.8	0.8	0.4
Hygromycin B	0.2	0.2	0.2	0.2	0.2
Kanamycin	0.2	0.2	0.2	0.2	0.2
Neomycin	0.36	0.36	0.36	0.045	0.36
Spectinomycin	0.2	0.2	0.4	0.8	0.2
Streptomycin	0.1	0.1	0.1	0.1	0.1
Water (mL)	8.04	8.04	7.44	7.155	8.04
Total Volume (mL)	10	10	10	10	10

Store solution in freezer at <-10 °C. *Solution expires 3 months after preparation.*

Refer to Tables 29-30 in the Appendix for spiking standard concentrations.

Table 12: Preparation of Muscle AMG Spiking Standard Solution

AMG	Bovine Muscle (mL)	Porcine Muscle (mL)	Poultry Muscle (mL)	Equine Muscle (mL)	Ovine Muscle (mL)	Caprine Muscle (mL
Amikacin	0.2	0.2	0.2	0.2	0.2	0.2
Apramycin	0.4	0.2	0.2	0.2	0.2	0.2
Dihydrostreptomycin	0.025	0.025	0.025	0.025	0.025	0.025
Gentamicin	0.8	0.8	0.8	0.8	0.8	0.8
Hygromycin B	0.8	0.2	0.2	0.2	0.2	0.2
Kanamycin	0.2	0.2	0.2	0.2	0.2	0.2
Neomycin	0.12	0.06	0.06	0.06	0.12	0.12
Spectinomycin	0.5	1	0.5	2	0.5	0.5
Streptomycin	0.025	0.025	0.025	0.025	0.025	0.025
Water (mL)	6.93	7.29	7.79	6.29	7.73	7.73
Total Volume (mL)	10	10	10	10	10	10

Store solution in freezer at <-10 °C. *Solution expires 3 months after preparation.*

Refer to Tables 31-32 in the Appendix for spiking standard concentrations.

Table 13: Preparation of Liver AMG Spiking Standard Solution

AMG	Bovine Liver (mL)	Porcine Liver (mL)
Amikacin	0.4	0.2
Apramycin	0.8	0.2
Dihydrostreptomycin	0.1	0.025
Gentamicin	0.8	0.8
Hygromycin B	0.2	0.2
Kanamycin	0.2	0.2
Neomycin	0.18	0.18
Spectinomycin	1	0.5
Streptomycin	0.1	0.025
Water (mL)	6.22	7.67
Total Volume (mL)	10	10

Store solution in freezer at <-10 °C. *Solution expires 3 months after preparation.*

Refer to Tables 33-34 in the Appendix for spiking standard concentrations.

Internal Standard Solution

Table 14: 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) Mix well. 4) Store in freezer at <-10 °C *Solution expires 3 months after preparation.*

External Standard Solution

Table 15: Preparation of External Standard for System Suitability

Solution	Procedure
AMG external standard in formic acid	1) Add 25 µL of appropriate kidney, muscle or liver standard (any level is acceptable) to the bottom portion of Whatman Mini-Uniprep filter vial. 2) Add 25 µL of the 40 µg/mL IS solution to the same vial. 3) Add 450 µL 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.*

Sample Preparation

Samples are to be kept cold before and during shipping to the laboratory. Once received at the laboratory, samples are to 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.

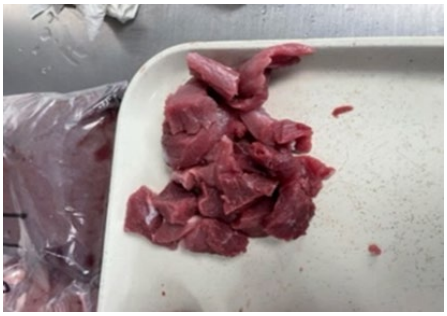

Figure 2: Prepared lean muscle sample with connective tissue removed.
Photo courtesy of Hue Quach, USDA FSIS

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

Aminoglycoside Extraction

Samples

Weigh 2.0 ± 0.1 g of homogenized muscle, kidney or liver sample into labeled 50 mL polypropylene (PP) centrifuge tubes.

KEY DEFINITIONS

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

Matrix matched standard: A sample prepared with the addition of analytes to have a specified concentration level.

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

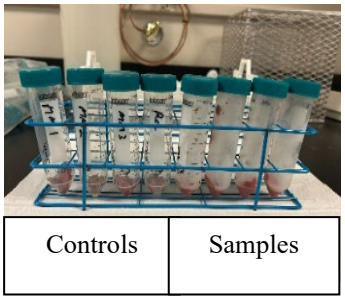

Figure 4: Weighed controls and samples
Photo courtesy of Ivan Lenov, USDA-FSIS

QUALITY CONTROL

Confirmation

1) Prepare a set of controls for each species to be analyzed.
2) Weigh four 2.0 ± 0.1 g portions of blank tissue into 50 mL polypropylene centrifuge tubes. One will be used for the recovery, while the remaining three will be used for the blank and the 1/2X-4X matrix match standard.
   a. The recovery is prepared by spiking 100 µL of the respective matrix AMG Spiking Standard solution at the level of interest.
   b. The blank extracts will be divided into 5 portions (blank and 4 matrix matched standards). The matrix matched standards are to be spiked with the respective matrix AMG Mixed Standard during Step 16 of the extraction procedure.
   c. Weigh one additional portion for an intra-laboratory check, if necessary.

Extraction

1) Add 20 mL of NH_4OAc/EDTA/NaCl/TCA buffer to each tube.
2) Add 100 µL of the 40 µg/mL tobramycin IS to all tubes except blank and matrix matched standards. This amount of tobramycin IS yields 2 µg/g in the tissue.
3) Shake for 10 minutes (min), as shown in Figure 5.
4) Centrifuge at a minimum of ~3700 RCF (4000 RPM) for 3 min.
   a. If floating material is observed, remove it with a spatula.
5) Decant >10 mL supernatant into another 50 mL polypropylene tube.
6) Using a calibrated pH meter or auto-titrator, adjust pH of the sample extracts to 6.50 ± 0.05 with a few drops of 30% NaOH followed by 1 N NaOH and/or 1 N HCl for fine adjustment.

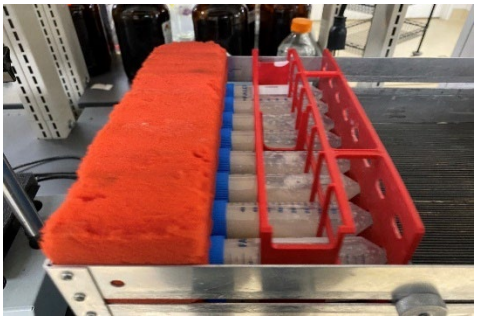

Figure 5: Samples undergoing shaking for extraction.
Photo courtesy of Ryan Matsuda, USDA-FSIS

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 ~3700 RCF (4000 RPM) for 3 min.
8) Pipet 10 mL of extract into labeled 15 mL PP tube (this is equivalent to 1 g of sample when using the IS).
   a. If floating material is observed, remove by filtering the extract through a 0.2 µm PVDF filter disk with a 10 mL syringe.
9) For DPX cleanup place the WCX-DPX tips in the lever arm apparatus (as shown in Figure 6), including the rubber O-ring to ensure gas tight seal. Position the lever arm to give 3 mL of air in the plastic syringes and then attach the manifold cover. Slowly raise and lower the arm to fill and empty the tips.
10) Using the setup described in Figure 7, place properly labeled 15 mL PP tubes in the collection rack. This setup is for 12 samples. This method requires 6 tubes for each sample.

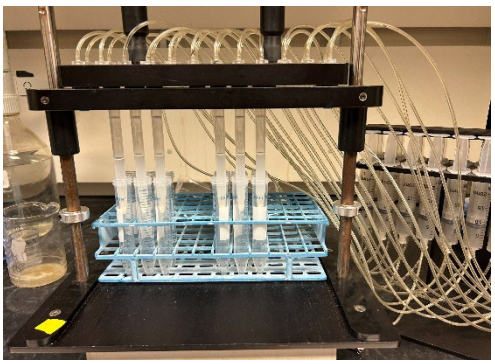

Figure 7: Samples undergoing SPE for extraction.
Photo courtesy of Darren Montgomery, USDA-FSIS

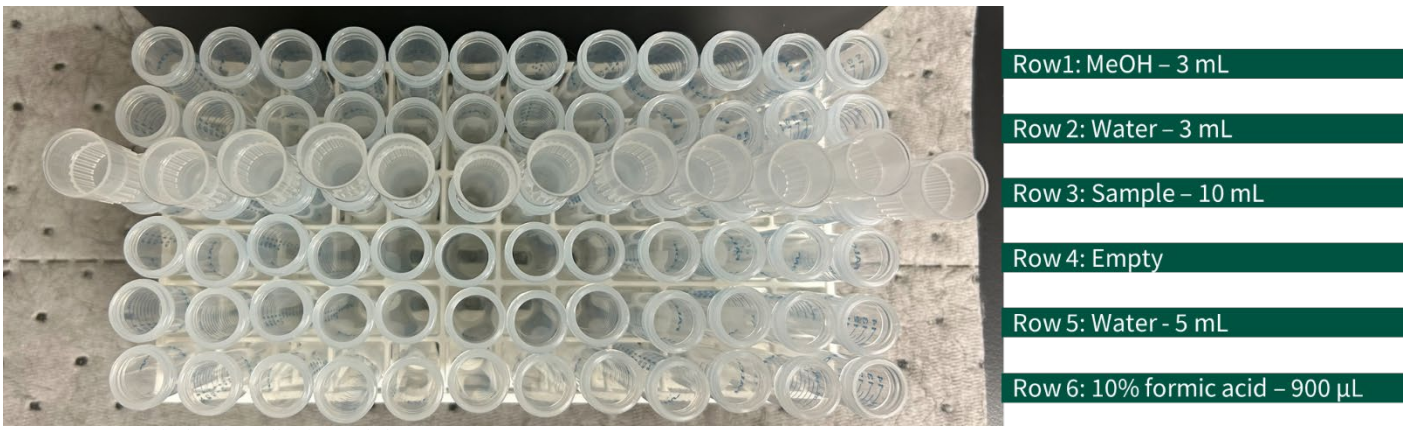

Figure 6: Setup of tubes for collection rack for SPE.
Photo courtesy of Ivan Lenov, USDA-FSIS and Darren Montgomery, USDA-FSIS

11) Condition the WCX-DPX tip (Shown in Figure 8) with 3 mL of Methanol and elute to waste. Then rinse the tip with 3mls of water 2 times and elute to waste.

12) Load all 10 mL of each sample extract onto sorbent tip in 4 x 2.5 mL portions.

    a. Make sure that the sorbent is interacting with the extract for at least 30 seconds each time

    b. Draw the first 2.5 mL slowly into the WCX-DPX tip. Return that portion to the sample extract tube and repeat a second time before discarding the extract into the waste tube.

    c. Draw the three remaining 2.5 mL portions of sample through the WCX-DPX tip one portion at a time.

Figure 8: WCX-DPX tip. Photo courtesy of Ivan Lenov, USDA-FSIS

13) Slowly draw 5 mL of water into each tip to rinse away possible contaminants.

    After 30 seconds, empty the water to waste.

14) Elute the sorbent with 900 µL of 10% formic acid in water as follows.

    Slowly draw the 900 µL into the tip. After at least 30 seconds, slowly empty the solution back into the original tube. Repeat this process 4 additional times to ensure that the analytes are eluted from the sorbent.

15) Add 100 µL of water to each cleaned extract and mix well, except for the blank extracts used to prepare the matrix matched standards.

16) For the matrix blanks, combine the three matrix blanks into one of the 15 mL tubes (2.7 mL total volume). 0.45 mL is used for the negative control (blank) and the four matrix-matched standards. Spike according to Table 16.

    a. For Kidney use the AMG Mixed standards from Table 7.
    b. For Muscle use the AMG Mixed standards from Table 8 and Table 9 (Ovine Muscle).
    c. For Liver use the AMG Mixed standards from Table 10.

Table 16: Preparation of Matrix Matched Standards

Sample Type	AMG Mixed Std (µL)	Internal Standard Mix Volume (µL)	Water (µL)	Blank Final Extracts (mL)
0X Matrix Blank	-	25	25	0.45
1/2X Matrix Matched Standard	25	25	-	0.45
1X Matrix Matched Standard	25	25	-	0.45
2X Matrix Matched Standard	25	25	-	0.45
4X Matrix Matched Standard	25	25	-	0.45

17) For the 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.

Instrumental Analysis

Chromatographic Parameters

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

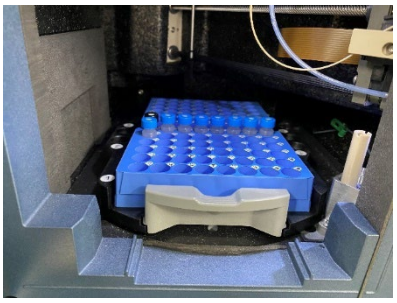

Figure 9: Prepared samples in UPLC-MS/MS instrument.
Photo courtesy of Ryan Matsuda, USDA-FSIS

Table 17: LC Gradient Program

Time (min)	% Mobile Phase A	% Mobile Phase B	Gradient
Initial	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 (min): 3.0
    b) Injection loop (µL): 20
    c) Sample injection mode: Partial loop needle overfill
    d) Injection Volume (µL): 15
    e) Weak wash solvent: Mobile Phase A
    f) Weak wash volume (µL): 500
    g) Strong wash solvent: Mobile Phase B
    h) Strong wash volume(µL): 500
    i) Sample temperature: (°C): 7
6) Column manager
    a) Column valve position: To match column location
    b) Column manager temperature (°C): 40
    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+
    c) MRM Transitions

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

Table 18: MRM Transitions

Compound	Start–End Time (min)	Dwell Time (ms)	Precursor ion *(m/z)*	Product ions *(m/z)*	Collision Energy (V)	Cone (V)
Spectinomycin Hydrate	0.9-1.2	22	351.24	**98.00** 333.33 140.10	30 20 25	40
Hygromycin B	1.1-1.3	22	528.20	**177.05** 352.03 257.00	30 24 30	44
Streptomycin	1.2-1.4	22	582.17	**263.09** 246.05 176.00	32 40 40	70
Dihydrostreptomycin	1.2-1.4	52	584.17	**263.09** 246.05 176.00	30 38 40	70
Amikacin	1.5-1.7	50	586.43	**163.21** 247.37 101.98	35 45 40	35
Kanamycin A	1.6-1.8	50	485.36	**163.22** 324.33 102.14	20 15 45	30
Apramycin	1.9-2.1	11	540.41	**217.20** 378.31 199.35	25 15 35	35
Gentamicin c1a	2.0-2.2	11	450.39	**160.16** 322.37 112.17	25 15 30	35
Gentamicin c2+c2a	2.0-2.2	11	464.42	**160.23** 322.39 163.14	25 15 20	35
Gentamicin c1	2.0-2.3	11	478.42	**157.25** 160.16 322.42	30 25 15	40
Neomycin B	2.1-2.3	11	615.30	**163.38** 293.03 160.53	35 24 33	52
Tobramycin (IS)	2.0-2.1	11	468.36	**163.19** 145.10	25 30	25

Most abundant product ion is in bold.

Instrumental 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 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) Matrix matched standards
2) Recovery (positive control)
3) Intra-Laboratory Check Sample (If applicable)
4) Blank (Negative Control)
5) Up to 20 Samples
6) Re-injection of the Recovery (positive control) or the matrix matched standards (for system suitability).

INTRA-LABORATORY

CHECK SAMPLE

Defined on the CLG website.

Reporting Results

Decision Criteria

Confirmation

A sample is confirmed positive for an analyte if the following criteria are met:

1) The recovery retention times must match the retention time of the matrix matched standard within 5%.
2) The retention time for the samples must match the retention time of the positive control or the matrix matched standard within 5%.
3) All product ions specified for ratio matching are present with a signal-to-noise ratio ≥ 3. This will be verified by visual inspection. Visual inspection for detection will also include assessment of peak shape or drift in relation to standard peaks.
4) One of the following ion ratio matching conditions is met:
   i. If two product ions are assessed, one sample ion ratio should match the calculated average ratio of the matrix-matched standards within a ± 10% absolute difference.
   ii. If three product ions are monitored, the presence of two sample ion ratios should match the calculated average ratio of the matrix-matched standards within a ± 20% absolute difference.

Key Facts:

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

5) The recovery fortified at the minimum level of applicability (Tables 19-21) of the analyte must exceed 10% of the matrix matched standard at the corresponding level.
6) The blank (negative control) response must be less than 10% of the matrix-matched standard at the MLA.
7) The sample response equals or exceeds the recovery fortified at the MLA.

QUALITY CONTROL

Quality Control Procedures for Confirmation

1) For set acceptance, eight of the nine analytes must meet confirmation criteria. For sample reporting purposes, the analytes of interest in the fortified recovery (positive control) must meet confirmation criteria.
2) The blank (negative control) must be negative using the confirmation criteria for the analytes of interest.

Intralaboratory Check Samples (If applicable)

1) Acceptability criteria.
   a. Eight of the nine analytes must meet confirmation criteria in a fortified Intra-Laboratory Check.
   b. All of the monitored 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 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 19: Minimum Level of Applicability (MLA) for Kidney Confirmation

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	0.1	0.05
Dihydrostreptomycin	1	1	1	N/A	1	1
Gentamicin	0.1	N/A	0.2	N/A	0.2	0.1
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	0.45	0.45	0.45	N/A	0.45	0.45
Spectinomycin	0.05	0.05	0.1	N/A	N/A	0.05
Streptomycin	1	1	1	N/A	1	1

N/A = Not Applicable

Table 20: Minimum Level of Applicability (MLA) for Muscle Confirmation

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	0.1	0.05	0.05	0.05	0.05	0.05
Dihydrostreptomycin	0.25	0.25	0.25	0.25	0.25	0.25
Gentamicin	N/A	0.2	0.2	0.2	N/A	0.2
Hygromycin B	0.2	0.05	0.05	0.05	0.05	0.05
Kanamycin	0.05	0.05	0.05	0.05	0.05	0.05
Neomycin	1.2	0.6	0.6	0.6	1.2	1.2
Spectinomycin	0.125	0.25	0.125	0.5	0.125	0.125
Streptomycin	0.25	0.25	0.25	0.25	0.25	0.25

N/A = Not Applicable

Table 21: Minimum Level of Applicability (MLA) for Liver Confirmation

AMG	Bovine (µg/g)	Porcine (µg/g)
Amikacin	0.1	0.05
Apramycin	0.2	0.05
Dihydrostreptomycin	1	0.25
Gentamicin	N/A	0.2
Hygromycin B	N/A	0.05
Kanamycin	N/A	0.05
Neomycin	N/A	1.8
Spectinomycin	0.25	0.125
Streptomycin	1	0.25

N/A = Not Applicable

Safety Hazards

Table 22: 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. The National Residue Program Roles Functions and Responsibilities | Food Safety and Inspection Service (usda.gov)

Contact Information and Inquiries

Inquiries about methods can be submitted through the FSIS website via the AskFSIS or please contact:

Chemistry Section
Laboratory Quality Assurance, Response, and Coordination Staff
USDA/FSIS/OPHS
950 College Station Road
Athens, GA 30605
OPHS.LQAD@usda.gov

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

William K. Shaw, Jr., PhD
Executive Associate for Laboratory Services

Appendix

The standards solutions listed in Table 23 are used for preparation of standards (matrix matched, and external standards, if needed)

Table 23: Kidney AMG Mixed Standard Solution Concentration

AMG	1/2X (µg/mL)	1X (µg/mL)	2X (µg/mL)	4X (µg/mL)
Amikacin	1	2	4	8
Apramycin	1	2	4	8
Dihydrostreptomycin	20	40	80	160
Gentamicin	1	2	4	8
Hygromycin B	1	2	4	8
Kanamycin	1	2	4	8
Neomycin	72	144	288	576
Spectinomycin	1	2	4	8
Streptomycin	20	40	80	160

Table 24 gives the AMG concentrations in kidney for recovery samples when 100 µL is spiked into 2 g samples and 25 µL is added to 0.5 g equivalent final extracts.

Table 24: Kidney AMG Mixed Standard Solution Concentration in Matrix

AMG	1/2X (µg/g)	1X (µg/g)	2X (µg/g)	4X (µg/g)
Amikacin	0.05	0.1	0.2	0.4
Apramycin	0.05	0.1	0.2	0.4
Dihydrostreptomycin	1	2	4	8
Gentamicin	0.05	0.1	0.2	0.4
Hygromycin B	0.05	0.1	0.2	0.4
Kanamycin	0.05	0.1	0.2	0.4
Neomycin	3.6	7.2	14.4	28.8
Spectinomycin	0.05	0.1	0.2	0.4
Streptomycin	1	2	4	8

The standard solutions listed in Table 25 are used for the preparation of standards (matrix matched, and external standards, if needed)

Table 25: Muscle AMG Mixed Standard Solution Concentration

AMG	Matrix	1/2X (µg/mL)	1X (µg/mL)	2X (µg/mL)	4X (µg/mL)
Amikacin	Muscle	1	2	4	8
Apramycin	Muscle	1	2	4	8
Dihydrostreptomycin	Muscle	5	10	20	40
Gentamicin	Muscle	1	2	4	8
Hygromycin B	Muscle	1	2	4	8
Kanamycin	Muscle	1	2	4	8
Neomycin	Muscle/ Ovine Muscle	12/24	24/48	48/96	96/192
Spectinomycin	Muscle	2.5	5	10	20
Streptomycin	Muscle	5	10	20	40

Table 26 gives the AMG concentrations in muscle for recovery samples when 100 µL is spiked into 2 g samples and 25 µL is added to 0.5 g equivalent final extracts.

Table 26: Muscle AMG Mixed Standard Solution Concentration in Matrix

AMG	Matrix	1/2X (µg/g)	1X (µg/g)	2X (µg/g)	4X (µg/g)
Amikacin	Muscle	0.05	0.1	0.2	0.4
Apramycin	Muscle	0.05	0.1	0.2	0.4
Dihydrostreptomycin	Muscle	0.25	0.5	1	2
Gentamicin	Muscle	0.05	0.1	0.2	0.4
Hygromycin B	Muscle	0.05	0.1	0.2	0.4
Kanamycin	Muscle	0.05	0.1	0.2	0.4
Neomycin	Muscle/ Ovine Muscle	0.6/1.2	1.2/2.4	2.4/4.8	4.8/9.6
Spectinomycin	Muscle	0.125	0.25	0.5	1
Streptomycin	Muscle	0.25	0.5	1	2

The standard solution listed in Table 27 are used for preparation of standards (matrix matched, and external standards, if needed)

Table 27: Liver AMG Mixed Standard Solution Concentration

AMG	1/2X (µg/mL)	1X (µg/mL)	2X (µg/mL)	4X (µg/mL)
Amikacin	1	2	4	8
Apramycin	1	2	4	8
Dihydrostreptomycin	5	10	20	40
Gentamicin	1	2	4	8
Hygromycin B	1	2	4	8
Kanamycin	1	2	4	8
Neomycin	36	72	144	288
Spectinomycin	2.5	5	10	20
Streptomycin	5	10	20	40

Table 28 gives the AMG concentrations liver for recovery samples when 100 µL is spiked into 2 g samples and 25 µL is added to 0.5 g equivalent final extracts.

Table 28: Liver AMG Mixed Standard Solution Concentration in Matrix

AMG	1/2X (µg/g)	1X (µg/g)	2X (µg/g)	4X (µg/g)
Amikacin	0.05	0.1	0.2	0.4
Apramycin	0.05	0.1	0.2	0.4
Dihydrostreptomycin	0.25	0.5	1	2
Gentamicin	0.05	0.1	0.2	0.4
Hygromycin B	0.05	0.1	0.2	0.4
Kanamycin	0.05	0.1	0.2	0.4
Neomycin	1.8	3.6	7.2	14.4
Spectinomycin	0.125	0.25	0.5	1
Streptomycin	0.25	0.5	1	2

Table 29 provides AMG concentrations in solution for kidney. These standards are for use as spiking solutions (recoveries and check samples) for confirmation analyses.

Table 29: Kidney AMG Spiking Standard Concentrations in Solution

AMG	Bovine Kidney (µg/mL)	Porcine Kidney (µg/mL)	Poultry Kidney (µg/mL)	Ovine Kidney (µg/mL)	Caprine Kidney (µg/mL)
Amikacin	1	1	1	1	1
Apramycin	1	1	1	2	1
Dihydrostreptomycin	20	20	20	20	20
Gentamicin	2	2	4	4	2
Hygromycin B	1	1	1	1	1
Kanamycin	1	1	1	1	1
Neomycin	72	72	72	9	72
Spectinomycin	1	1	2	4	1
Streptomycin	20	20	20	20	20

Table 30 provides the AMG concentration for kidney in confirmation recovery samples using 100 µL of the respective standard spiked into 2 g of sample.

Table 30: Kidney AMG Spiking Standard Concentrations in Matrix

AMG	Bovine Kidney (µg/g)	Porcine Kidney (µg/g)	Poultry Kidney (µg/g)	Ovine Kidney (µg/g)	Caprine Kidney (µg/g)
Amikacin	0.05	0.05	0.05	0.05	0.05
Apramycin	0.05	0.05	0.05	0.1	0.05
Dihydrostreptomycin	1	1	1	1	1
Gentamicin	0.1	0.1	0.2	0.2	0.1
Hygromycin B	0.05	0.05	0.05	0.05	0.05
Kanamycin	0.05	0.05	0.05	0.05	0.05
Neomycin	3.6	3.6	3.6	0.45	3.6
Spectinomycin	0.05	0.05	0.1	0.2	0.05
Streptomycin	1	1	1	1	1

Table 31 provides AMG concentrations in solution for muscle. These standards are for use as spiking solutions (recoveries and check samples) for confirmation analyses.

Table 31: Muscle AMG Spiking Standard Concentrations in Solution

AMG	Bovine Muscle (µg/mL)	Porcine Muscle (µg/mL)	Poultry Muscle (µg/mL)	Equine Muscle (µg/mL)	Ovine Muscle (µg/mL)	Caprine Muscle (µg/mL)
Amikacin	1	1	1	1	1	1
Apramycin	2	1	1	1	1	1
Dihydrostreptomycin	5	5	5	5	5	5
Gentamicin	4	4	4	4	4	4
Hygromycin B	4	1	1	1	1	1
Kanamycin	1	1	1	1	1	1
Neomycin	24	12	12	12	24	24
Spectinomycin	2.5	5	2.5	10	2.5	2.5
Streptomycin	5	5	5	5	5	5

Table 32 provides the AMG concentration for liver in confirmation recovery samples using 100 µL of the respective standard spiked into 2 g of sample.

Table 32: Muscle AMG Spiking Standard Concentration in Matrix

AMG	Bovine Muscle (µg/g)	Porcine Muscle (µg/g)	Poultry Muscle (µg/g)	Equine Muscle (µg/g)	Ovine Muscle (µg/g)	Caprine Muscle (µg/g)
Amikacin	0.05	0.05	0.05	0.05	0.05	0.05
Apramycin	0.1	0.05	0.05	0.05	0.05	0.05
Dihydrostreptomycin	0.25	0.25	0.25	0.25	0.25	0.25
Gentamicin	0.2	0.2	0.2	0.2	0.2	0.2
Hygromycin B	0.2	0.05	0.05	0.05	0.05	0.05
Kanamycin	0.05	0.05	0.05	0.05	0.05	0.05
Neomycin	1.2	0.6	0.6	0.6	1.2	1.2
Spectinomycin	0.125	0.25	0.125	0.5	0.125	0.125
Streptomycin	0.25	0.25	0.25	0.25	0.25	0.25

Table 33 provides AMG concentrations in solution for liver. These standards are for use as spiking solutions (recoveries and check samples) for confirmation analyses.

Table 33: Liver AMG Spiking Standard Concentrations in Solution

AMG	Bovine Liver (µg/mL)	Porcine Liver (µg/mL)
Amikacin	2	1
Apramycin	4	1
Dihydrostreptomycin	20	5
Gentamicin	4	4
Hygromycin B	1	1
Kanamycin	1	1
Neomycin	36	36
Spectinomycin	5	2.5
Streptomycin	20	5

Table 34 provides the AMG concentration for liver in confirmation recovery samples using 100 µL of the respective standard spiked into 2 g of sample.

Table 34: Liver AMG Spiking Standard Concentration in Matrix

AMG	Bovine Liver (µg/g)	Porcine Liver (µg/g)
Amikacin	0.1	0.05
Apramycin	0.2	0.05
Dihydrostreptomycin	1	0.25
Gentamicin	0.2	0.2
Hygromycin B	0.05	0.05
Kanamycin	0.05	0.05
Neomycin	1.8	1.8
Spectinomycin	0.25	0.125
Streptomycin	1	0.25