United States Department of Agriculture

Food Safety and Inspection Service

CLG-AVR2.00

Confirmation of Avermectins by UHPLC-MS/MS

This method is the laboratory procedure for confirming Avermectin veterinary drug residues in bovine, porcine, ovine, caprine, and *Siluriformes* (catfish) muscle tissues and processed products.

Executive Summary

This is a multi-residue confirmation method for Avermectins, a class of veterinary drugs. The method is used for mass spectrometric confirmation analysis of 7 avermectins (abamectin, doramectin, emamectin, eprinomectin, ivermectin, moxidectin, and selamectin) in muscle from bovine, porcine, ovine, caprine, and Siluriformes, and processed products. 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 listed in Table 8, where 7.5 ng/g is the MLA for most matrices and avermectin residues.

Notice of Change

This is an improved multi-residue confirmation method for avermectin confirmation analysis and is the original version. The method replaces a previously used muscle confirmation method that utilized liquid chromatography and atmospheric pressure chemical ionization mass spectrometric confirmation analysis, which allows for lower limit detection limits that align better with established drug tolerances. A flow chart, which shows the basic steps of analysis from sample receipt to results reporting, is found in CLG-AVR2 Appendix 1.

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

Introduction

Avermectins are widely used in agriculture to combat both endoparasites and ectoparasites.[1] Despite the useful qualities of these drugs, inadvertent exposure has been associated with possible human health effects.[2,3] The amount and/or presence of avermectins is regulated in livestock animals through regulations that are established and set by the Food and Drug Administration (FDA) through the Federal Food, Drug, and Cosmetic Act.

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 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 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-AVR2 is used to confirm seven avermectin residues (abamectin, doramectin, emamectin, eprinomectin, ivermectin, moxidectin, and selamectin) in muscle tissue of bovine, swine, ovine, caprine, and *Siluriformes* and also in processed products.

Presumptive violations are samples that are presumptive positive and have quantitative results exceeding the specified avermectin tolerance. As shown in Figure 1, CLG-AVR2 is used as the confirmation method for avermectin residues in muscle tissue samples that are a presumptive violation and is the final step for confirmation of a violative result.

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.

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

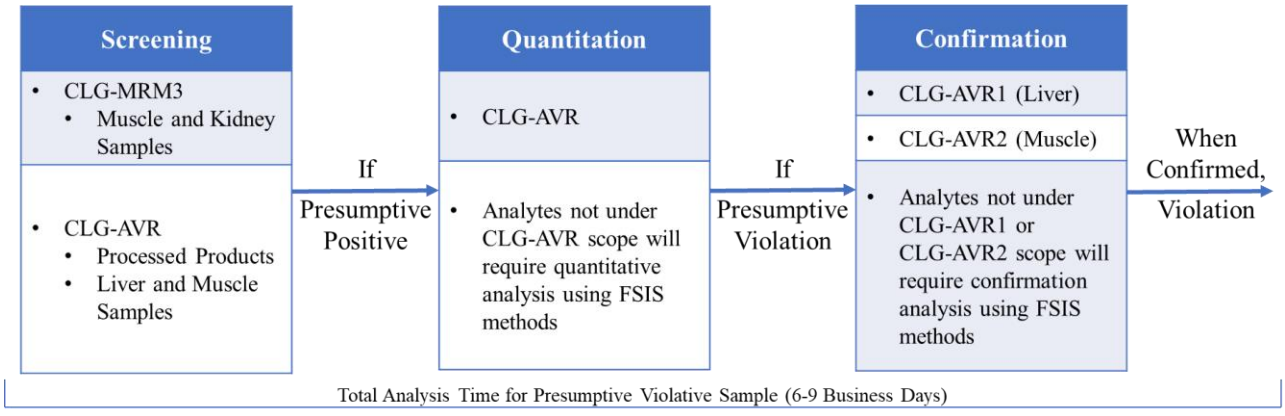

Figure 1: Overview of avermectin residue analysis. This figure represents the best-case scenarios, but analyses may take longer.

This method utilizes the muscle tissue or processed product that were previously prepared during the screening and quantitation analysis. The method employs a protein precipitation extraction under acidic conditions followed by clean up with QuEChERS salts. The resulting

KEY DEFINITIONS

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe): A solid phase extraction method for detection of chemical residues in food.

[1] De Souza, RB, Guimaraes, JR. 2022. Effects of avermectins on the environment based on its toxicity to plants and soil invertebrates - a review. Water, Air, and Soil Pollution 233:259.
[2] El-Saber Batiha, G, Alqahtani, A, Ilesanmi, OB, Saati, AA, El-Mleeh, A, Hetta, HF, Magdy Beshbishy, A. 2020. Avermectin derivatives, pharmacokinetics, therapeutic and toxic dosages, mechanism of action, and their biological effects. Pharmaceuticals 13:196.
[3] Lin, D, Tang, N, Zhang, C, Cheng, J, Zhang, Z, Wang, S, Wu, C, Zhang, L, Tao, L, Li, Z, Zhang, Y. 2021. Avermectin induced DNA damage to the apoptosis and autophagy in human lung epithelial A549 cells. Ecotoxicology and Environmental Safety 215:112129.

extract is further cleaned up with reverse phased dispersive solid phase extraction (SPE) media. The final extract is then solvent exchanged and reconstituted with the mobile phase used for analysis. The final extracted residues are examined using reversed-phased chromatography using Ultra-High Performance Liquid Chromatography Coupled with Tandem Mass Spectrometry (UHPLC-MS/MS). Figure 2 shows an overview of the sample analysis through CLG-AVR2.

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.

Solid Phase Extraction (SPE): An extraction technique that utilizes a solid support that contains an adsorbing surface or chemical coating that can interact with the 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/solutions for the presumptive avermectin analyte(s). Only applicable standards/solutions are necessary for reporting results.

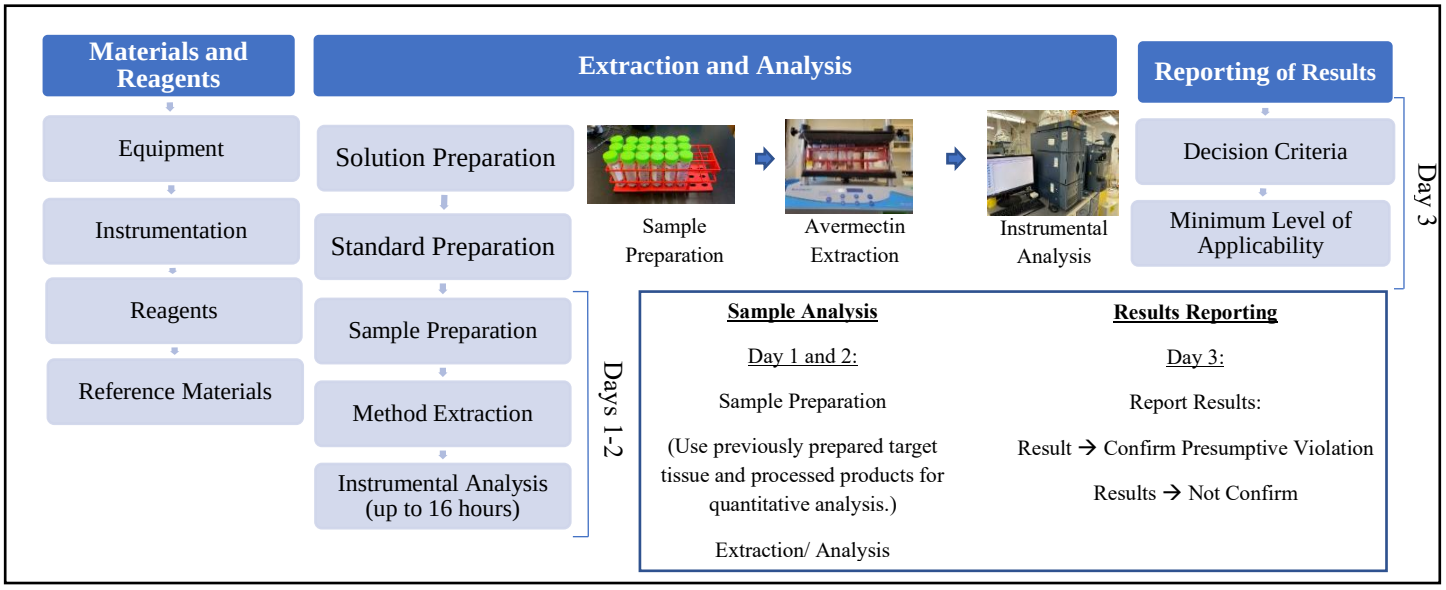

Figure 2: Overview and timeframe of "Confirmation of Avermectins" (CLG-AVR2). Materials and reagents are obtained and utilized to prepare solutions and standards. On business days 1-2, after screening and quantitation analysis, target tissues from presumptive positive samples are prepared or presumptive positives 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 Bruno Giri, USDA-FSIS

Decision Criteria

CLG-AVR2 is only validated for confirmation. A sample is considered confirmed negative by CLG-AVR2 if the sample does meet the method's decision criteria. A sample is considered confirmed positive if the results are confirmed based on the method's decision criteria. Residues with tolerances (i.e., abamectin, doramectin, emamectin, eprinomectin, ivermectin, and moxidectin) have been reported to have a quantitative value that exceeds the tolerance and are found to be confirmed positive are reported as violative. Residues that have no established tolerances (i.e., selamectin) that are found to be confirmed positive are reported as violative.

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-AVR2

Equipment	Supplier and Part Number	Purpose
Glassware, Class A	General lab supplier	Measuring standards and reagents.
Repeating pipettes and tips, 2 µL - 20 µL, 20 µL to 1000 µL, 500 µL to 2500 µL	General lab supplier	Dispense standards and reagents.
Bottle-Top Dispensers, 1 mL to 5 mL, 2 mL and 10 mL	General lab supplier	Adds solutions.
Analytical Balance	General lab supplier	Record weight of standard reagent. Minimum accuracy ±0.0001 g.
Top Loading Balance	General lab supplier	Record weight of standard reagent. Minimum accuracy Minimum accuracy ±0.01 g.
Centrifuge	General lab supplier	Separates the solid sample material from the extraction solution. Capable of 3155 RCF.
Centrifuge tubes, Polypropylene (PP), 50 mL	General lab supplier	Contain sample material and extraction vessel.
Centrifuge tubes, Polypropylene (PP), 15 mL	General lab supplier	Contain sample material and extraction vessel.
Multi-Tube Vortexer	General lab supplier	Facilitates extraction of residue from the sample.
Vortexer	General lab supplier	Facilitates extraction of residue from the sample.
Ultra Sonic Bath	General lab supplier	Facilitates extraction of residue from the sample.
Nitrogen Evaporator Apparatus with Heated Water Bath	General lab supplier	Reduces extraction solution down to desired volume.
PTFE Syringe Filter, 0.2 µm, 13 mm	Pall Corporation, 4423T	Filter final extracts.

Syringe, Plastic, 3 mL	**Becton Dickenson, 309657**	**Filter final extracts**
HPLC Vials- Amber screw top vials with PTFE/Silicone septa, 2 mL	General lab Supplier	Storage of extracts.
Freezer, -10 °C	General lab supplier	Storage of standards, reagents, and samples

Instrumentation

Table 2: Instrumentation

Instrument	Supplier and Model Number	Purpose
LC-MS/MS	Agilent, 6490	Extract analysis
Waters UPLC BEH C18, 2.1 x 50 mm, 1.7µm	Waters, 186002350	Extract analysis
Waters VanGuard Pre-column UHPLC BEH C18, 2.1 x 5.0 mm, 1.7 µm	Waters, 186003975	Extract analysis

Reagents

Table 3: Reagents

Reagent	Supplier and Part Number
QuEChERS Salts Packets (4 g Magnesium Sulfate ($MgSO_4$) & 1 g Sodium Chloride (NaCl))	Agilent Technologies, Cat. No. 5982-6550
QuEChERS Salts Tubes (250 mg MgSO4 and 250 mg CEC18)	UCT, Cat. No. ECQUUS1050CT
Acetic Acid	Millipore Sigma, Cat. No. A6283
Methanol HPLC Grade	Millipore Sigma, Cat. No. MMX0475P6
Acetonitrile HPLC Grade	Fisher Scientific, Cat. No. A998-4
Ammonium Formate	Millipore Sigma, Cat. No. 516961-100G
Formic Acid	Millipore Sigma, Cat. No. F0507-1L
Water- Resistivity of > 18 MΩ-cm	House System

Reference Materials

Table 4: Reference Materials

Standard	Supplier	Catalog Number
Abamectin (ABA)	Millipore Sigma	31732
Doramectin (DORA)	Millipore Sigma	33993
Emamectin Benzoate (EMA)	Millipore Sigma	31733
Eprinomectin (EPRINO)	Millipore Sigma	32526
Ivermectin B1a (IVER)	Millipore Sigma	I8898
Moxidectin (MOXI)	Millipore Sigma	33746
Selamectin (SEL)	Millipore Sigma	32746

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
Extraction Solvent (Acetonitrile with 2% Acetic Acid (v/v))	1) Add 20 mL of acetic acid to a 1 L volumetric flask. 2) Dilute to volume with acetonitrile. 3) Mix well and transfer to a glass storage container for use. 4) Store at room temperature. *Solution expires 1 year after preparation.*
Mobile Phase A (5 mM ammonium acetate/0.1% formic acid in water by volume)	1) Measure 0.771 g ammonium acetate. 2) Dissolve and transfer the ammonium acetate to a 2 L graduated cylinder or volumetric flask with water. 3) Measure and add 2 mL of formic acid to same cylinder or volumetric flask. 4) Dilute to volume with water. 5) Mix well and transfer to a glass storage container for use. 6) Store at room temperature. *Solution expires 1 year after preparation.*
Mobile Phase B (0.1% formic acid in 95:5 Acetonitrile:Water)	1) Measure 1 mL of formic acid into a 1 L volumetric flask. 2) Measure 50 mL of water and add to same volumetric flask. 3) Dilute to volume with acetonitrile. 4) Mix well and transfer to a glass storage container for use. 5) Store at room temperature. *Solution expires 1 year after preparation.*
Isocratic Mobile Phase (20:80 Mobile Phase A: Mobile Phase B)	1) Measure 20 mL of Mobile Phase A and add to a glass storage container. 2) Measure 80 mL of Mobile Phase B and add to same container. 3) Mix well for use. 4) Store at room temperature. *Solution expires 1 year after preparation.*

Standard Preparation

Table 6: Standard Solutions

Standard Solution	Procedure
Individual Avermectin stock solutions **(1 mg/mL in acetonitrile)**	1) For each stock solution, weigh approximately 10 mg to the nearest 0.1 mg. 2) Add to a 10 mL volumetric flask. 3) Dilute to volume with acetonitrile. 4) Mix well and transfer to a glass storage container for storage and use. 5) Store in freezer at <-10 °C *Solution expires 1 year after preparation.*
Intermediate Mixture, 2.5 µg/mL in methanol	1) Pipette ~250 µL of each stock solution (adjusted from the determined actual stock standard concentration) into a 100 mL volumetric flask. 2) Dilute to volume with methanol. 3) Mix well and transfer to a glass storage container for storage and use. 4) Store in freezer at <-10 °C *Solution expires 6 months after preparation.*

Sample Preparation

All samples are stored frozen (≤ -10 °C). Processed products and muscle tissue will need to be prepared for confirmation analysis. If there is a presumptive positive, prepare the muscle of interest for confirmation analysis. All tissue samples should be allowed to partially thaw, while keeping them as cold as possible. Trim away fat and connective tissue (Figure 3). Grind tissue in blender or vertical cutter-mixer until homogeneous (Figure 4). Store homogenized samples frozen (≤ -10 °C) prior to analysis.

Figure 3: Processed meat product. Photo courtesy of Getty Images.

Figure 4: Homogenized sample. Photos courtesy of Hue Quach, USDA-FSIS

Avermectin Extraction

Samples

Weigh 5 ± 0.1 g of sample into labeled 50 mL polypropylene centrifuge tubes, as shown in Figure 5.

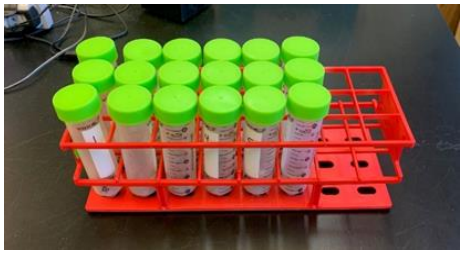

Figure 5: Weighed controls and samples. Photos courtesy of Bruno Giri, USDA-FSIS

KEY DEFINITIONS

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

Matrix matched control: Sample is spiked with analytes at the end after extraction. The analyte concentration is comparable to the MLA. Negative and positive controls are compared to the "Matrix matched control."

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

QUALITY CONTROL

Weigh 5 g portions of previously analyzed tissue or processed product that is blank into 50 mL polypropylene centrifuge tubes for each of the following controls.

1) Blank (negative control).
2) Recovery (positive control) – fortify with 15uL of standard which produces a 7.5 ng/g control.
3) Matrix matched control (reference standard) - fortify with 15uL of standard which produces a 7.5 ng/g control.
4) Intra-laboratory check sample, if necessary.

Extraction

1) Add 10 mL of the 2% acetic acid/acetonitrile solution.
2) Add QuEChERS salts from the pre-weighed QuEChERS salts packets containing 1g of sodium chloride and 4 g of magnesium sulfate anhydrous to the tubes.
3) Vortex tubes for 1 minute, as shown in Figure 6.
4) Further extract the samples by immersing tubes into an ultrasonic bath for 5 minutes, as shown in Figure 7.

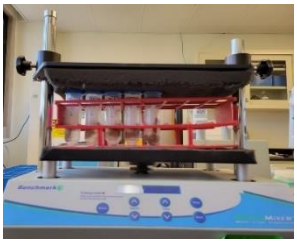

Figure 6: Samples being vortexed. Photo courtesy of Eric Flynn, USDA-FSIS.

Figure 7: Immerse tubes into an ultrasonic bath. Photos courtesy of Bruno Giri. USDA-FSIS

5) Centrifuge samples at 10 °C for 10 minutes in a refrigerated centrifuge at 3155 x g.
6) Transfer the supernatant solution to 50 mL polypropylene QuEChERS salts tubes that contain 250 mg of dispersive C18 and 250 mg of magnesium sulfate. Vortex briefly.
7) Allow samples to rest for 5 minutes.
8) Vortex again briefly to allow the QuEChERS salts to further interact with the sample and centrifuge at 3155 x g at 10 °C for 10 minutes.
9) Transfer a 4 mL aliquot to a 15 mL polypropylene centrifuge tube.
10) Evaporate samples at 47.5 °C ± 2.5 °C until dryness.
11) Reconstitute in 1 mL of Isocratic Mobile Phase, vortex briefly and syringe filter with PTFE filters into an autosampler vial.
12) Fortify matrix matched control with intermediate mixed standard to give a final concentration to the appropriate MLA for the respected product or matrix.

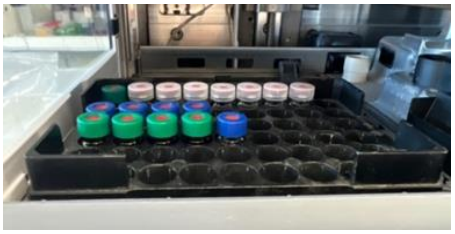

Figure 8: Prepared samples in UHPLC-MS/MS. Photos courtesy of Dagny Morales, USDA-FSIS.

Instrumental Analysis

Chromatographic Parameters

1) Mobile phases for AVR2 analysis
   a) Mobile Phase A – 5 mM ammonium acetate/0.1% formic acid in water by volume
   b) Mobile Phase B – 0.1% formic acid in 95:5 Acetonitrile:Water
2) Flow rate (mL/min): 0.3
3) Run time (min): 6
4) Gradient Program: Isocratic Elution
5) Autosampler program
   a) Run time (min): 6
   b) Injection Volume (µL): 10
   c) Sample temperature (°C): 4
6) Column manager
   a) Column temperature (°C): 35

Figure 9: UHPLC-MS/MS. Photos courtesy of Dagny Morales, USDA-FSIS

Mass Spectrometry Parameters

1) Type: MS/MS
2) Mass Spectrometer Parameters
    a) Gas temperature (°C): 80
    b) Gas flow (L/min): 11
    c) Nebulizer pressure (psi): 30
    d) Sheath gas temperature (°C): 350
    e) Sheath gas flow (mL/min): 7
    f) Capillary voltage (mV): 3500
    g) MRM Transitions

Table 7: MRM Transitions

Compound	Precursor (*m/z*)	Quant Ion (*m/z*)	Qual Ion 1 (*m/z*)	Qual Ion 2 (*m/z*)
ABA	890.5	305	567.3	113
DORA	916.6	331	593.2	219.1
EMA	886.5	158	126	82
EPRINO	914.5	186	154	112
IVER	892.5	307.1	569.2	194.9
MOXI	640.4	199	528.2	498.2
SELA	770.4	203	333	276

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 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 required controls. System Suitability is to be demonstrated prior to sample set injection.

1) Solvent Blank
2) Blank (negative control)
3) Matrix matched control
4) Recovery (positive control)
5) Intra-laboratory check sample (if applicable)
6) Samples up to a maximum of 20
7) Reinjection of standard/positive control

INTRA-LABORATORY

CHECK SAMPLE

Defined on the CLG website.

Reporting of Results

Decision Criteria

Confirmation Criteria

1) The recovery (positive control) of the analyte of interest must exceed 10% of the matrix matched control.

2) Monitored ions for each analyte will be assessed as follows:

   a. Recovery retention times must match the retention times of the matrix matched control within 5%. Retention time for the samples must match the retention time of the recovery within 5%.

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

   c. The two sample ion ratios should match the ratio of the recovery within ± 20% relative difference. For example, if the average product ratio is 0.46, the acceptance range for the samples is 0.37-0.55.

      i. Ratios are calculated by dividing the area count of each qual ion by the area count of the quant ion. Ion ratios should be less than 1. If the ratio is not less than 1 for a sample set, the inverse ratio may be used.

QUALITY CONTROL

Quality Control Procedures

Confirmation Criteria

1) For set acceptance, each compound of interest in the fortified recovery (positive control) must meet the confirmation criteria.

2) The blank (negative control) must be negative using the confirmation criteria for the analytes of interest.

Intra-laboratory Check Samples (If applicable)

1) Acceptability criteria.

   a. All analytes in a fortified Intra-Laboratory Check must meet criteria.

   b. All analytes in an unfortified Intra-Laboratory Check must be negative using the 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 8: Minimum Level of Applicability (MLA) for Confirmation level per species

	Bovine	Porcine	Ovine	Caprine	Siluriformes	Processed Products
Analyte	**MLA (ng/g)**					
Abamectin	7.5	7.5	7.5	7.5	7.5	7.5
Doramectin	7.5	7.5	7.5	7.5	7.5	7.5
Emamectin	7.5	7.5	7.5	7.5	7.5	7.5
Eprinomectin	7.5	7.5	7.5	7.5	7.5	7.5
Ivermectin	7.5	7.5	7.5	7.5	7.5	7.5
Moxidectin	7.5	7.5	7.5	7.5	7.5	7.5
Selamectin	7.5	7.5	7.5	7.5	7.5	15

Safety Hazards

Table 9: Safety Hazards and Recommended Safe Procedures

Procedure Step	Hazard	Recommended Safe Procedures
Ivermectin, Abamectin	Weak teratogen and possible mutagen. May cause skin or respiratory irritation. Possible reproductive toxicity.	Handle with extreme caution.
Doramectin	Severe explosion hazard if in powdered form. May cause skin or respiratory irritation. Possible reproductive toxicity.	Handle with extreme caution.
Emamectin benzoate, Eprinomectin, Moxidectin, Selemectin	May cause skin or respiratory irritation. Possible reproductive toxicity.	Work in a well ventilated area.
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, Acetic acid	Corrosive, Caustic	Wear personal protective equipment, avoid skin contact.

References

1) Da Silva, GR, Lima, JA, de Souza, JA, Santos, FA, Lana, MAG, de Assis, DCS, de Vasconcelos Cancado, S. 2017. Multiresidue method for identification and quantification of avermectins, benzimidazoles and nitroimidazoles residues in bovine muscle tissue by ultra-high performance liquid chromatography tandem mass spectrometry (UHPLC-MS/MS) using a QuEChERS approach. Talanta 171: 307.

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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