United States Department of Agriculture

Food Safety and Inspection Service

CLG-AVR.05

Quantitation of Doramectin, Ivermectin, and Moxidectin by UHPLC

This method is the laboratory procedure for screening and quantitating Avermectin veterinary drug residues in bovine, caprine, equine, ovine, and porcine liver and muscle tissues and processed products.

Executive Summary

This is a screening and quantitation method for avermectin veterinary drug residues in bovine, caprine, equine, ovine, and porcine liver and muscle tissues and processed products. The method's key performance characteristics include:

- Simultaneous screening and quantitation of doramectin, ivermectin, and moxidectin

The minimum level of applicability (MLA) or lowest level at which an FSIS method has been successfully validated for a residue in each matrix for this method is 7.5 ng/g for all matrices and residues.

Notice of Change

The method was reformatted with no substantive changes. A flow chart, which shows the basic steps of analysis from sample receipt to results reporting, is found in CLG-AVR 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 8. 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, and 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-AVR is used as the screening method and is also a quantitation method for samples that have been screened as presumptive positive for avermectin residues (doramectin, ivermectin, and moxidectin) in bovine, caprine, equine, ovine, and porcine liver and muscle tissues and processed products. Presumptive positives require quantitative analysis to determine if the quantitative value for the residue exceed the specified avermectin tolerance and determine if the sample is a presumptive violation. If found to be a presumptive violation, samples require further confirmation analysis. Figure 1 shows an overview of avermectin residue analysis.

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.

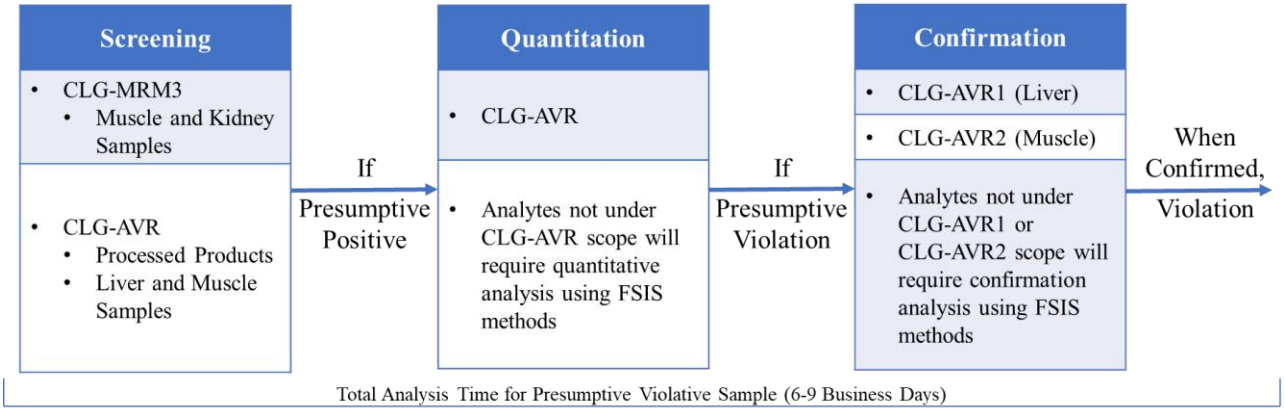

Figure 1: Overview of avermectin residue analysis. This figure represents the best-case scenarios, but analyses may take longer.

Processed products, muscle, and liver tissues, samples are prepared and CLG-AVR is used to screen and determine a quantitative value. For presumptive positive samples from other residue screening analyses, quantitation is required to be conducted on the target tissue of interest. Therefore, the target tissue will need to be prepared prior to quantitation analysis. Doramectin,

[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.

ivermectin, and moxidectin residues are first extracted from tissue or processed product through protein precipitation with acetonitrile. Further cleanup of the extract is conducted with alumina.

Liver tissue samples require additional clean up with solid phase extraction. After cleanup, all extracts undergo derivatization to fluorescently label the avermectin residues. Following derivatization, extracts are analyzed by ultra-high performance liquid chromatography (UHPLC) with fluorescence detection. Figure 2 shows an overview of the sample analysis through CLG-AVR.

KEY DEFINITIONS

Derivatization: The process of transforming a chemical compound to a related compound of similar structure.

Fluorescence: The emission of light by a molecule after the molecule has become electronically excited by the absorption of a photon.

UHPLC: An analytical technique involving physical separation of target compounds.

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.

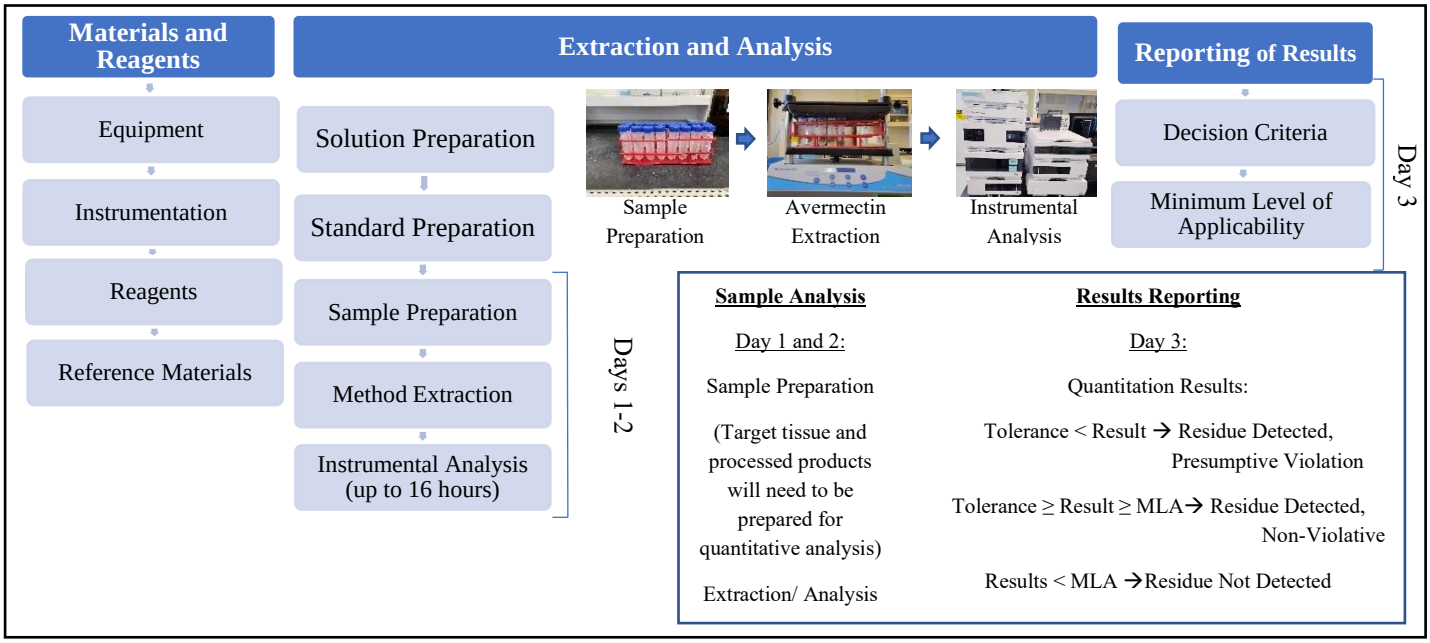

Figure 2: Overview and timeframe of initial analysis of processed products, presumptive positive analysis with CLG-AVR. Materials and reagents are obtained and utilized to prepare solutions and standards. On business days 1-2, after screening analysis, target tissues from presumptive positive samples are prepared or presumptive positives are weighed, extracted, and analyzed by HPLC. Results are reported on business day 3. This figure represents the best-case scenarios, but analyses may take longer. Photo courtesy of Eric Flynn and Ralph DiCosty, USDA-FSIS.

Decision Criteria

CLG-AVR is validated for screening and quantitation. The results from CLG-AVR are compared to established tolerances and the method's MLA for the species and matrix. If the results are greater than the tolerance, the sample is considered detected and is a presumptive violation. The sample would then need to undergo further confirmation analysis through CLG-AVR1 or CLG-AVR2 to confirm the violative result. If the results are greater than or equal to the MLA but less than or equal to the tolerance, the sample is considered detected and non-violative. A sample is considered negative if the results are less than the MLA.

Disclosure Statement

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

Materials and Reagents

Equipment

Table 1: Equipment Required to Perform CLG-AVR

Equipment	Supplier and Part Number	Purpose
Centrifuge	General lab supplier	Separates the solid sample material from the extraction solution. Capable of 450 RCF (~1500 RPM).
Mini Vortex Mixer	General lab supplier	Facilitates extraction of residue from the sample.
Platform Vortex Mixer	General lab supplier	Facilitates extraction of residue from the sample.
Analytical Balance	General lab supplier	Record weight of sample and reagent. Minimum accuracy ±0.01 g.
Nitrogen Evaporator Apparatus with Heated Water Bath	General lab supplier	Reduces extraction solution down to desired volume
Centrifuge tubes, Polypropylene (PP), 50 mL	General lab supplier	Contain sample material and extraction vessel.
HPLC Vials - Screw top vials with PTFE/Silicone septa, 2 mL	General lab Supplier	Storage of extracts.
Pipettes and tips, for volumes ranging from 37.5 µL to 2 mL	General lab supplier	Dispense standards and reagents.
Repeating pipettes and tips, for volumes of 150 and 200 µL	General lab supplier	Dispense standards and reagents.
Bottle-Top Dispensers, for volumes ranging from 0.5 to 8 mL	General lab supplier	Add reagents.
Glassware, Class A	General lab supplier	Measuring standards and reagents.
Cutting board and knives	General lab supplier	Preparation of sample.
Food Processor	Robot Coupe USA Inc.	Homogenize sample.
Glass culture tubes, 20 x 150 mm and 13 x 100 mm	General lab supplier	Processing of extracts.

SPE column, with frit, unpacked	General lab supplier	Retain deactivated alumina.
SPE cartridge, C18, 100 mg, 3 mL	Agilent 12102099	Filtration of liver extracts.

Instrumentation

Table 2: Instrumentation

Instrument	Supplier and Model Number	Purpose
Agilent LC System with Fluorescence Detector	Agilent Infinity II LC System with UHPLC capability	Extract analysis
Analytical Column	Agilent, 699975-302	Extract analysis
Guard Column	Agilent, 823750-911	Extract analysis

Reagents

Table 3: Reagents

Reagent	Supplier and Part Number
Acetonitrile (ACN), HPLC grade	General lab supplier
Aluminum oxide (Al_2O_3)	Sigma Aldrich 199974 (activated, neutral, Brockmann I)
1-methylimidazole ($CH_3C_3H_3N_2$) **Store in desiccator.**	Sigma Aldrich M50834
Trifluoroacetic anhydride ($C_4F_6O_3$) **Store in desiccator.**	Sigma Aldrich 106232
Methanol (MeOH), HPLC grade	General lab supplier
Water- Resistivity of > 18 MΩ-cm	House System

Reference Materials

Table 4: Reference Materials

Standard	Supplier	Catalog Number
Abamectin, 100 µg/mL in acetonitrile, 1 mL ampoule (Internal Standard)	LGC	DRE-XA10001000AL
Doramectin, 100 µg/mL in acetonitrile, 1 mL ampoule	LGC	DRE-A13083000AL-100
Ivermectin, 100 µg/mL in acetonitrile, 1 mL ampoule	AccuStandard	P-1084S-CN
Moxidectin, 100 µg/mL in acetonitrile, 1 mL ampoule	LGC	DRE-A15335000AL-100

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
Deactivated alumina	1) Weigh 88 g aluminum oxide and combine with 12 g of water. 2) Cap, and shake until clumps disappear. 3) Store at room temperature. *Solution expires 1 week after preparation.*
1:1 1-methylimidazole : acetonitrile (v/v)	1) Combine equal volumes of 1-methylimidazole and acetonitrile. 2) Vortex to mix. *Solution expires on the day of preparation.*
1:1 trifluoroacetic anhydride : acetonitrile (v/v)	1) Combine equal volumes of trifluoroacetic anhydride and acetonitrile. 2) Vortex to mix. *Solution expires on the day of preparation.*
Mobile phase, 3% water in methanol	1) Add 60 mL of water to a 2 L volumetric flask. 2) Dilute to volume with methanol. 3) Mix well and transfer to glass storage container for use. 4) Store at room temperature. *Solution expires 1 year after preparation.*

Standard Preparation

Table 6: Fortification Standards

Solution	Procedure
Abamectin Internal Fortification Standard in Acetonitrile (0.50 µg/mL)	1) Calculate the amount of abamectin standard reference solution needed to make 200 mL of a solution with a final abamectin concentration of 0.50 µg/mL. 2) Add the calculated amount of standard reference solution to a 200 mL volumetric flask. Bring to volume with acetonitrile. Mix well. 3) Store at room temperature. *Solution expires 3 months after preparation.*

Multi-Avermectin Fortification Standard in Acetonitrile (0.50 µg/mL)	1) Calculate the amounts of doramectin, ivermectin, and moxidectin standard reference solutions needed to make 200 mL of a solution with final analyte concentrations of 0.50 µg/mL. 2) Add the calculated amounts of standard reference solutions to a 200 mL volumetric flask. Bring to volume with acetonitrile. Mix well. 3) Store at room temperature. *Solution expires 3 months after preparation.*

Sample Preparation

All samples are stored frozen (≤ -10 °C). Processed products and muscle tissue will need to be prepared for screening and quantitative analysis. If there is a presumptive positive, prepare the target tissue of interest for quantitative 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 or product in blender or vertical cutter-mixer until homogeneous (Figure 4). Store homogenized samples frozen (≤ -10 °C) prior to analysis.

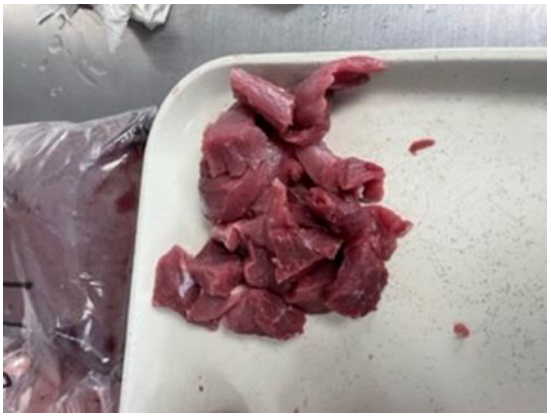

Figure 3: Prepared lean muscle sample with connective tissue removed. Photo courtesy of Hue Quach, USDA-FSIS.

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

Avermectins Extraction

Samples

Weigh 2.5 ± 0.2 g of sample into a 50 mL polypropylene centrifuge tube.

QUALITY CONTROL

Weigh two 2.5 ± 0.2 g portions of blank tissue into 50 mL polypropylene centrifuge tubes (Figure 5).

1) One for the blank (negative control)
2) One for the recovery (positive control).

Weigh one additional portion for an intra-laboratory check sample, if necessary.

Figure 5: Weighed controls and samples. Photo courtesy of Eric Flynn, USDA-FSIS.

External Calibration Curve

Prepare external calibration curve in glass culture tubes as per Table 7.

Table 7: Preparation of External Calibration Curve

Calibration Level (ng/g)	Volume of multi-avermectin standard (µL)	Volume of abamectin internal standard (µL)
0	0	150
7.5	37.5	150
15	75	150
30	150	150
60	300	150

Evaporate, reconstitute, and derivatize the external calibration curve beginning at Extraction Step 10 below.

Extraction

1) Add 8 mL acetonitrile to each tube.
2) Fortify all tubes with 150 µL (30 ng/g) of abamectin fortification internal standard.
3) Fortify recovery with 75 µL (15 ng/g) of multi-avermectin fortification standard.
4) Vortex tubes for at least 30 sec (Figure 6) and centrifuge for 3 minutes at 450 RCF (1500 rpm).
5) Prepare deactivated alumina columns by weighing 2.0 ± 0.2 g into an empty SPE cartridge with frit. Columns must be prepared on the day of analysis.

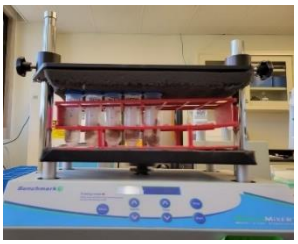

Figure 6: Samples being vortexed. Photo courtesy of Eric Flynn, USDA-FSIS.

6) Pour acetonitrile supernatant through a deactivated alumina column and collect filtrate in a glass culture tube, as shown in Figure 7.

Figure 7: Sample extracts filtering through alumina columns. Photo courtesy of Nicholas Paganella, USDA-FSIS.

Figure 8: Samples in evaporator. Photo courtesy of Eric Flynn, USDA-FSIS.

7) Repeat extraction steps 4 – 5 with an additional 8 mL of acetonitrile. Centrifuge and pour through alumina column as above, combining filtrates.

8) Transfer glass tubes to an evaporator and evaporate acetonitrile to dryness under a gentle stream of nitrogen while maintaining water bath at 65 ± 5°C, as shown in Figure 8.

9) Prepare muscle and liver extracts for derivatization.

Muscle

For muscle tissue and external calibration curve, add 2.5 mL acetonitrile.

Liver

1) For liver tissue, condition the C-18 SPE cartridge by washing with 1 mL acetonitrile. Discard washing. Do not allow cartridge to dry or recoveries will be low.
2) Reconstitute the dry sample with 0.5 mL acetonitrile. Vortex.
3) Load the 0.5 mL acetonitrile onto the C-18 SPE cartridge. Collect eluate in a glass culture tube.
4) Add 2 mL acetonitrile to the sample tube. Vortex. Load the 2 mL onto the same C-18 SPE cartridge, combining eluates

10) Add 200 µL 1-methylimidazole/acetonitrile reagent to the eluate. Vortex.

11) Add 200 µL trifluoroacetic anhydride/acetonitrile reagent to the eluate. Vortex.

12) Place samples in the dark and allow derivatization to proceed for at least 15 min. Transfer samples to HPLC vials, minimizing exposure of derivatized samples to strong light.

13) Analyze by UHPLC with fluorescence detection. See Figures 9 and 10.

Figure 9: Prepared samples in UHPLC instrument. Photo courtesy of Ralph DiCosty, USDA-FSIS.

Figure 10: UHPLC instrument. Photo courtesy of Ralph DiCosty, USDA-FSIS.

Instrumental Analysis

Chromatographic Parameters

1) Mobile phase: 3% water / 97% methanol
2) Sample temperature: 4 °C
3) Injection volume: 5 µL
4) Flow rate: 0.625 mL/min
5) Gradient: None
6) Run time: 4.5 min
7) Column temperature: 55 °C
8) Detection mode: Fluorescence
9) Excitation wavelength (nm): 365 ± 10
10) Emission wavelength (nm): 465 ± 10

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 may be adjusted to account for aging of UHPLC columns or for improved separation to ensure that all chromatographic peaks are present.
- 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.

1) External Standard Curve
2) Negative Control
3) Positive Control
4) Intra-laboratory check sample (if needed)
5) Samples, up to a maximum of 18
6) Re-injection of the positive control (instrument safeguard)

INTRA-LABORATORY CHECK SAMPLE

Defined on the CLG website.

Reporting of Results

Decision Criteria

1) The retention time of each analyte must match that of the recovery (positive control) or the external standard injected most recently before the sample within 5%.
2) Blank must be less than the MLA.
3) Each analyte must have a signal-to-noise ratio ≥ 3. This may be verified by visual inspection.
4) Positive samples must meet the above criteria and be greater than or equal to the MLA for analyte of interest.

Technical Note:

If a sample concentration is greater than the highest standard used in the calibration curve, the sample should be re-analyzed. To keep the sample concentration in the range of the calibration curve, the sample weight may be reduced to as little as 1.0 g or the calibration curve may be extended such that the highest calibration standard is greater than the calculated concentration of the sample.

QUALITY ASSURANCE PLAN

Quality Control Procedures

1) For set acceptance, all analytes of interest in the recovery (positive control) must meet method criteria and recover in the range of 60-120%.
2) The blank (negative control) must be less than the MLA for analyte of interest.
3) The correlation coefficient of the calibration curve must be greater than 0.995 for analyte of interest.

Intra-laboratory Check Samples (if applicable)

1) All analytes of interest in a fortified intra-laboratory check sample must meet method criteria.
2) All analytes of interest in an unfortified intra-laboratory check sample must be less than the MLA.
3) FSIS Field Service Laboratories are to refer to internal FSIS Quality Control Procedures, if unacceptable values are obtained:
    a. Refer to LW-Q1002, Chemistry Non-Conformance Tables, for how to proceed and whether to take corrections or corrective actions.

Calculations

Concentrations are calculated based on response ratios using peak area as the response:

$$Response\ Ratio = \frac{\text{Doramectin, ivermectin, or moxidectin response}}{\text{Abamectin response}}$$

Alternatively, peak height can be use instead of peak area for the response

Construct a linear regression line using the response ratios and standard concentrations.

The equation is y = mx + b, where

x = doramectin, ivermectin, moxidectin concentration (ng/g)

y = doramectin, ivermectin, or moxidectin response ratio

m = slope

b = y-intercept

Concentrations of doramectin, ivermectin, or moxidectin in tissue are calculated per the regression line.

Minimum Level of Applicability

The minimum level of applicability for doramectin, ivermectin, and moxidectin in bovine, caprine, equine, ovine, and porcine liver, muscle, and processed products is 7.5 ng/g.

Safety Hazards

Table 8: Safety Hazards and Recommended Safe Procedures

Procedure Step	Hazard	Recommended Safe Procedures
Acetonitrile	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.
Trifluoroacetic Anhydride, 1-methylimidazole	Corrosive, may cause skin or respiratory irritation.	Avoid contact or prolonged exposure to vapors. Work in a fume hood.
Ivermectin, Abamectin	Weak teratogen and possible mutagen.	Handle with extreme caution.
Doramectin	Severe explosion hazard if in powdered form.	Handle with extreme caution.
Moxidectin	May cause skin or respiratory irritation. The toxic effects of this material have not been fully evaluated.	Work in a well-ventilated area.

References

1) 21 CFR 556 for tolerance values set by FDA.
2) USDA Food Safety and Inspection Service, National Residue Program.
3) Wang, H, Wang, Z, Liu, SY, Liu, Z. 2009. Rapid method for multi-residue determination of avermectins in bovine liver using high-performance liquid chromatography with fluorescence detection. Bulletin of Environmental Contamination and Toxicology 82:395.

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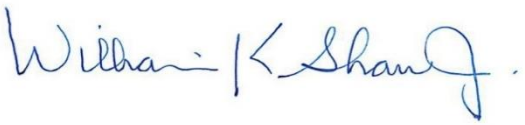
This method has been validated, reviewed, approved, and deemed suitable and fit for purpose for use in the USDA FSIS Field Service Laboratories.



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