Title:

Determination of Ivermectin in Animal Feed by LC-MS/MS

1. Purpose

This method is applicable for the analysis of animal feed for ivermectin. Feed samples are subjected to liquid extraction using methanol followed by dilution. An aliquot of the clarified extract is filtered and transferred for LC-MS/MS analysis. Ivermectin is confirmed by retention time and the presence of its characteristic parent/daughter ion transitions. Quantitation is performed using peak areas relative to a calibration curve constructed with matrix-matched standards and an isotopically labeled internal standard (ivermectin-d_2).

2. Scope / Field of Application

Using the appropriate dilution factor, this method will accurately quantify ivermectin from 0.5 µg/g to 625 µg/g in animal feed when analyzed on a Thermo TSQ Quantis Plus LC-MS/MS system.

3. Definitions and Acronyms

Following terms and acronyms found in this protocol are defined as follows:

LC	Liquid Chromatography
HPLC	High Performance Liquid Chromatography
UPLC	Ultra-high Performance Liquid Chromatography
MS	Mass Spectrometry
MS/MS	Tandem Mass Spectrometry
SOP	Standard operating procedure (or protocol)
IVM	Ivermectin
IVM-d_2	Ivermectin-d_2
ACN	Acetonitrile
PVDF	Polyvinlidene Fluoride
WS	Work Solution
QC	Quality Control

4. Warning and Safety Precautions

Handle ACN, methanol, and formic acid in a fume hood. Follow laboratory PPE requirements. Dispose of organic waste according to chemical waste rules. All liquid waste is collected in 4 L bottles for disposal.

5. Reagents and Chemicals

All reagents are “reagent grade or better” or “HPLC grade” unless otherwise noted. Unopened solvents may be extended past their manufacturer expiration date for up to 1 year when solvent purity will not affect extraction efficiency, LC performance, or target analyte concentration. All solvents and mobile phase components are HPLC grade, unless otherwise noted. For hazards, refer to the appropriate SDS. Current manufacturers and product and lot numbers are listed in the ICN database

1. Acetonitrile.
2. Ammonium Acetate.
3. Formic acid.
4. Isopropanol.
5. Methanol.
6. Water.
7. Ivermectin, Millipore Sigma, Part # I8898-1G. CAS# 70288-86-7.
8. Ivermectin-d_2, Cayman Chemical, Part # 35345.

6. Equipment and Apparatus

1. Amber vial, 4 and 20 mL.
2. Balance, Analytical Balance – readability to 0.0001 g.
3. Balance, Top load Balance – readability to 0.01 g.
4. Beakers, assorted sizes.
5. Centrifuge tubes, 2, 15 and 50 mL.
6. DisQuE tubes (optional), 2 mL, Waters Part# 186004832.
7. Graduated cylinders, 100 mL, 1 and 2 L.
8. HPLC vials, amber screw neck, 12x32 mm with bonded pre-slit PTFE silicone septa (Waters or equivalent).
9. HPLC vial racks, capable of holding 50 of 12x32 mm vial.
10. HPLC C18 Column, reverse phase, 100 x 2.1 mm (L x I.D.) and 1.7 µm particle size (Waters Acquity UPLC BEH C18, part # 186002352 or equivalent).
11. Lighting: the room in which sample preparation is performed should be equipped with special UV filtered lighting to prevent degradation of Ivermectin.
12. LC-MS/MS System, Thermo TSQ Quantis Plus or equivalent.
13. Pipettes with respective tips: 200 µL, 1000 µL and 10 mL adjustable volume models (Eppendorf or equivalent).
14. Shaker, a reciprocating/wrist-action shaker, Burrell Wrist-Action® Shaker Model 95 or equivalent.
15. Syringes, 5 mL disposable;
16. Syringe filters, 13 mm 0.2 µm PVDF filters or finer may be substituted as long as it does not compromise filtration, Whatman #6779-1304 or equivalent (Do not use nylon filters).
17. Vortexer.

7. Solutions

A. Ivermectin Standard Solutions

1. Stock Solution, 10 mg/mL - Weigh 0.500 g ± 0.0001 g of ivermectin and quantitatively transfer to a 50 mL amber volumetric flask. Fill to volume with methanol and mix well. Expiry is 1 month from the date of preparation. Store in freezer at -20°C ±10°C.
2. Intermediate Solution, 1 mg/mL - Pipette 1000 µL of 10 mg/mL of stock solution into a 10 mL amber volumetric flask. Fill to volume with methanol and mix well. Expiry is one month from the date of preparation. Store in freezer at -20°C ±10°C.
3. Intermediate Solution, 10 µg/mL - Pipette 100 µL of 1 mg/mL of stock solution into a 10 mL amber volumetric flask. Fill to volume with methanol and mix well. Expiry is one week from the date of preparation. Store in freezer at -20°C ±10°C.
4. Work Solution A, 100 ng/mL - Pipette 1000 µL of 10 µg/mL of work solution A into a 10 mL amber volumetric flask. Fill to volume with methanol and mix well. Freshly prepared.
5. Work Solution B, 10 ng/mL - Pipette 1000 µL of 10 µg/mL of work solution A into a 10 mL amber volumetric flask. Fill to volume with methanol and mix well. Freshly prepared.

B. Internal Standard Solutions

1. Stock Solution, 100 µg/mL –1 mg of ivermectin-d_2 was transferred to a 10 mL amber volumetric flask. Fill to volume with methanol and mix well. Expiry is 1 month from the date of preparation. Store in freezer at -20°C ±10°C.
2. Work Solution, 100 ng/mL - Pipette 20 µL of 100 µg/mL of stock solution into a 20 mL amber volumetric flask. Fill to volume with methanol and mix well. Expiry is one week from the date of preparation. Store in freezer at -20°C ±10°C.

C. Dilution Solutions

Dilution solution, 80% acetonitrile in water is prepared as follows: mix 800 mL of acetonitrile with 200 mL water. Expiry is 1 year from date of preparation.

D. Calibration Standard Solutions

Pipette the corresponding volumes (Table 1) of ivermectin work solutions (Section 7. A. 3 &4) into dilution solvent. Then add 50 µL of the ivermectin-d_2 work solutions.

Table 1. Preparation of Calibration Curve

Calibration Standards	STD1	STD2	STD3	STD4	STD5	STD6
IVM Intermediate Solution used	B, 10 ng/mL			A, 100 ng/mL		
Volume of Intermediate spiked (µL)	50	100	250	50	100	250
Volume of solvent added (µL)	950	900	750	950	900	750
IVM in sample (µg/g)	0.5	1	2.5	5	10	25

E. Other Solutions

1. LC-MS/MS Mobile Phase solution A, 5 mM ammonium acetate/0.1% formic acid in water is prepared as follows:

   Add 1 mL of formic acid and 315.3 mg of ammonium acetate to 1000 mL LCMS grade water. Mix thoroughly and filter the solution through a 0.2 µm nylon filter. This solution may be prepared in multiples as needed. Expiry is 1 year from date of preparation or reagent expiry, whichever is sooner.

2. LC-MS/MS Mobile Phase solution B, 0.1% formic acid in 95:5 acetonitrile:water is prepared as follows:

   Add 1 mL of formic acid to a mixture of 950 mL of ACN and 50 mL of water. Mix thoroughly and filter the solution through a 0.2 µm nylon filter. This solution may be

   prepared in multiples as needed. Expiry is 1 year from date of preparation or reagent expiry, whichever is sooner.

3. LC-MS/MS Sample Manager/Purge Wash, 25% Methanol, 25% Isopropanol, 25% Acetonitrile, 25% Water is prepared as follows:

   Combine 500 mL of water, 500 mL of methanol, 500 mL of acetonitrile, and 500 mL of isopropanol. Expiry is 1 year from date of preparation.

   NOTE: Solution is needed to rinse LC parts to prevent carry-over/cross- contamination but is not critical to the sample results unless the reservoir runs out causing system components to become contaminated with analyte.

8. Preparing Samples for Analysis

NOTE: ***These steps must be performed with the UV filtered lamps switched on, and normal lights turned off. Sun and normal light sources degrade the ivermectin and ivermectin-d_2.***

Pull samples, ivermectin work solutions (Sections 7.C and 7.D) and ivermectin-d_2 work solutions (Sections 7.B.2) from freezer; allow all materials to thaw completely at room temperature.

A. Preparation of Quality Controls.

Prepare quality controls at 2, 10 and 20 µg/g of ivermectin as follow:

Pipette the corresponding volumes (Table 2) of ivermectin intermediate solutions (Section 7.A.2) into 10 gram of drug-free animal feed.

Table 2. Preparation of Quality Control

Quality Controls	QC-LS	QC-MS	QC-HS
IVM Intermediate Solution used	1 mg/mL		
Volume of Intermediate added (µL)	20	100	200
Corresponding IVM in sample (µg/g)	2	10	20

B. Liquid Extraction

1) Add 100 ± 0.5 mL methanol into 10 ± 0.05 grams of samples.
2) Shake on a reciprocating / wrist-action shaker for 1 hour at high speed.
3) Filter the extract to collect at least 10 mL of clear solution.
4) Dilute the extract by mixing appropriate volume (**Aliquot 1, Table 3)** of the extract with methanol to make the final volume of the solution to 10 mL.

Table 3. Dilution table

Ivermectin Guarantee (µg/g)	Aliquot 1 (mL)	Volume of Methanol (mL)
0.5 - 24.99	1	9
25 – 99.99	0.25	9.75
100 – 249.99	0.1	9.9
250 – 625	0.04	9.96

C. Dilution

1) Transfer 100 µL of the extract into 900 µL of 80% acetonitrle in water.
2) Add 50 µL of the ivermectin-d_2 work solution to each tube. Mix well by vortex.

3) Filter the solution with 0.2 µm filtration to an amble HPLC vial.

When significant MS signal suppression is observed due to matrix interferences, the samples should be further purified using dSPE tubes.

D. Optional: Dispersive cleanup (dSPE)

4) Transfer the diluted extract into a 2 mL DisQue tube.
5) Cap and vortex 15 s vigorously (ensure complete contact with sorbents).
6) Centrifuge for 5 min at 10,000 rpm.
7) Filter the solution with 0.2 µm filtration to an amble HPLC vial.

9. Instrument Analysis of Samples

1. Instrument set-up varies based on instrument:

   a. In TraceFinder, for Thermo Quantis Plus LCMS, select the current ivermectin template.

   b. Verify the UPLC mobile phase gradient and MS/MS conditions are correct using sections 2-4 below.

   c. Load the appropriate template. Set up the data file with sample list. Start the sequence with several injections of solution containing analyte, mid-level standards and/or controls, injected to help stabilize the LC-MS/MS system. Quality controls should be run following the calibration curve/before samples, approximately every 10 samples, and at the end of the sequence. Confirm the samples, controls, blank, and reference standards are in the rack positions designated in the sample list.

2. UPLC conditions:

   Load the appropriate run template, inlet method, and tune method. UPLC instrument parameters should be as follows:
   a. Column: UPLC BEH C_{18} 2.1x 100 mm, 1.7 µm (or equivalent).
   b. Column temperature: 50°C; Sampler temperature: 10°C.
   c. Run time: 10 min.
   d. Injection volume: 10 µL.

3. Mobile phase and gradient conditions:

   a. Mobile Phase A: 5 mM ammonium acetate/0.1% formic acid in water.

   b. Mobile Phase B: 0.1% formic acid in 95:5 Acetonitrile:Water.

   c. Gradient times and flow rates are as noted on the table below:

Table 3. UPLC Gradient Times and Flow Rates

Time (min)	Flow Rate (mL min-1)	A (%)	B (%)	Curve
Initial	0.3	20	80	6
0.10	0.3	20	80	6
7.00	0.3	20	80	6
7.10	0.4	2	98	6
8.50	0.4	2	98	6
8.51	0.3	20	80	6
10.00	0.3	20	80	6

Retention times on Thermo Quantis Plus:

Ivermectin: ~ 6.0 min
Ivermectin-d_2: ~ 6.0 min

These times may vary with slight fluctuations in mobile phase and UPLC conditions. Column pressure should be checked, gradient conditions should be verified, and column temperature should be confirmed if retention times are not consistent with previous run. Refer to system readiness section 12. If all conditions have been confirmed, the retention times may be adjusted as necessary.

4. MS/MS conditions - Set up the mass spectrometer by selecting the appropriate, tune file and ion mode, according to the following parameters:

NOTE: Global parameters not listed here may vary based on current mass resolution and calibration data.

MRM transitions:

Compound	Retention Time (min)	RT Window (min)	Precursor (m/z)	Product (m/z)	Collision Energy (V)	RF Lens (V)	Min Dwell Time (ms)
IVM	6	4	892.7	307.1	24	167	748.245
IVM	6	4	892.7	569.1	15	167	748.245
IVM-d2	6	4	894.7	309.1	26	167	748.245
IVM-d2	6	4	894.7	571.1	15	167	748.245

Ion Source Settings:

Ion Source Type: **H-ESI**
Spray Voltage: **Static**
Positive Ion (V): **3200**
Negative Ion (V): **2500**
Current LC Flow (µL/min): **0**
Sheath Gas (Arb): **50**
Aux Gas (Arb): **10**
Sweep Gas (Arb): **1**
Ion Transfer Tube Temp (°C): **325**
Vaporizer Temp (°C): **350**

Instrument Notes:

i. All chromatographic and mass-spectrometric settings used in this method were optimized to meet internal performance requirements and were verified during routine preventative maintenance and instrument calibration.
ii. The retention time windows, collision energies, and the selected precursor/product ion transitions were established during method development and validation.
iii. Retention time windows may be adjusted as needed to compensate for column aging or to improve chromatographic resolution, provided that all target analyte and internal-standard peaks remain clearly defined.
iv. Collision energies may be refined to enhance ionization efficiency or signal response.
v. Precursor and product ion target masses may be optimized within ± 0.9 m/z of the exact mass, without exceeding the next whole-number value (e.g., for an exact mass of 892.7, acceptable target masses fall between 892.0 and 892.9).
vi. Any adjustments made to improve chromatographic or detection performance must comply with the acceptance criteria outlined in the method's Quality Assurance Plan.
vii. Modifications that would significantly alter method performance require equivalency evaluation and prior approval from laboratory management.

10. Sample Set

The injection sequence outlined below may be adjusted as necessary; however, all required controls must be included. System suitability must be established prior to initiating the sample batch.

1) Solvent Blank
2) Matrix Blank (negative control)
3) Quality control (positive control)
4) Samples up to a maximum of 20
5) Reinjection of quality control

11. Calculation

The injection sequence outlined below may be adjusted as necessary; however, all required controls must be included. System suitability must be established prior to initiating the sample batch.

$$CIVM = CInstrument \times DF \times \frac{10\ ml}{V\ \text{aliquot1}} \times \frac{VExtraction}{WSample} \times 0.001$$

Where:
C_{IVM} = Ivermectin content in the original animal feed in µg/g (ppm).
$C_{Instrument}$ = Instrument reading amount based on peak area ratio of IVM vs IVM-d_2 in ng/ml.
$V_{Aliquot\ 1}$ = Volume of Aliquot 1 in ml (**Table 3**)
DF = Dilution factor (Step 8.C), 10.
$V_{Extraction}$ = Volume of Extraction, 100 mL.
W_{Sample} = Weight of Sample, 10 g.
0.001 = coverts ng/g to µg/g.

12. Data Acceptability Criteria

Analytical data generated under this method must meet the acceptance criteria listed below before results can be reported. Failure of any required criterion must be evaluated, documented, and resolved prior to data release. Re-injection, re-extraction, or instrument troubleshooting may be required.

1. System Suitability

   System suitability must be demonstrated prior to analysis of samples.

   a) System pressure: Within ± 4400 psi of historical operating pressure.
   b) Stability injections: Initial injections of mid-level standards or controls must show stable retention times and peak shapes for both ivermectin and ivermectin-d_2.
   c) Blank injections: Solvent and matrix blanks must show no detectable ivermectin above 1/10 of the response of the lowest calibration standard.

2. Calibration Curve

a) The calibration curve must contain all required levels and must be constructed using matrix-matched standards spiked with internal standard.
b) Correlation coefficient (R^2): $\geqslant$ 0.995 (instrument software and MS Excel calculation).
c) Each calibration point must back-calculate to 80-110% of its nominal concentration, except the lowest standard which may be ± 25%.
d) Internal standard response must be present and stable across the curve; IVM-d_2 response factor > 1000.

3. Quality Control Samples

Quality controls (low, mid, and high) must be included at the beginning of the run, approximately every 10 samples, and at the end of the run.
a) QC results must fall within 80–110% of their nominal concentrations.
b) Mean QC bias across all QC levels must remain within 80–110%.
c) QC failure requires evaluation; affected sample results cannot be reported until QC performance is acceptable.

4. Sample Data Quality

Retention times for ivermectin and ivermectin-d_2 must be within the established windows for the instrument platform used.

a) Peak shape must be symmetrical and well-resolved from background noise.
b) Internal standard response must be present and within the range seen for standards and QCs.
c) Samples requiring dilution must have final values corrected by the dilution factor (Section 8).

5. Re-Analysis and Corrective Action

a) Re-analysis is required when any of the following occur:
b) Calibration curve or QC criteria are not met.
c) Internal standard response is absent or significantly suppressed.
d) Chromatographic peak abnormalities (poor shape, split peaks, co-elution).
e) Retention time shifts outside established acceptance windows.
f) Blank contamination exceeds allowable limits.

Corrective actions may include system maintenance, column flushing or replacement, re-injection, or full sample re-extraction.

13. References

1. **USDA Food Safety and Inspection Service (FSIS).** *CLG-AVR2.00: Ivermectin and Abamectin Residue Analysis by LC-MS/MS.* Office of Public Health Science, Chemistry Laboratory Guidebook. (2024).
2. Croubels, S., De Baere, S., Cherlet, M., & De Backer, P. *Determination of ivermectin B1a in animal plasma by liquid chromatography combined with electrospray ionization mass spectrometry.* **Journal of Mass Spectrometry**, 37, 840–847 (2002).
3. De Sousa, F. G., et al. *LC–MS/MS Determination of Macrocyclic Lactones in Biological Matrices.* **Molecules**, 27(3), 998 (2022).
4. Pérez, R., Godoy, C., Palma, C., Cabezas, I., Muñoz, L., Rubilar, L., Arboix, M., & Alvinerie, M. *Plasma Profiles of Ivermectin in Horses following Oral or Intramuscular Administration.* **Journal of Veterinary Medicine, Series A**, 50, 297–302 (2003).

14. Revision History

The first version 12/11/2025

15. Appendix

Representative chromatograms for ivermectin and ivermectin-d_2 are shown below

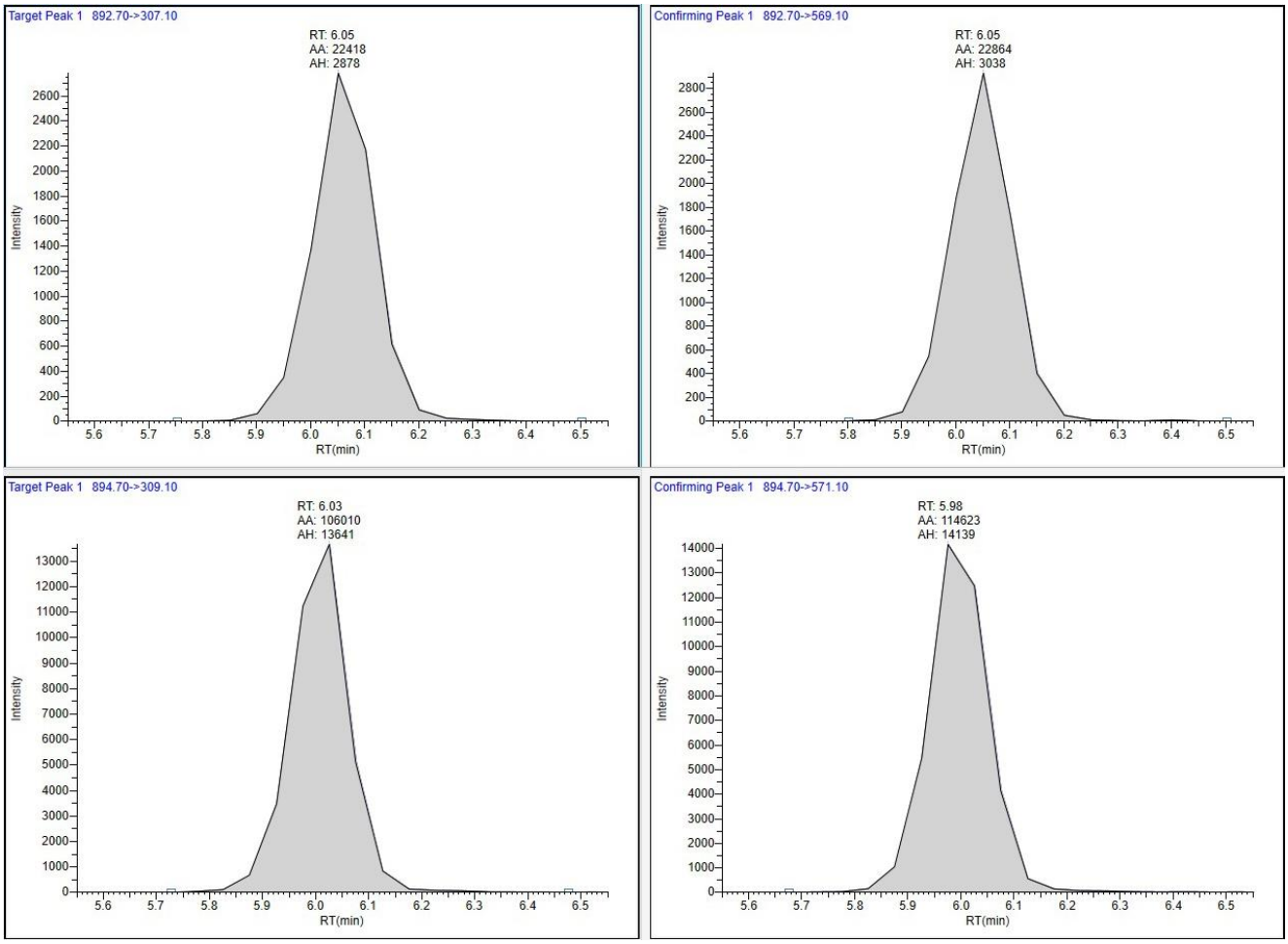

Figure 1 Chromatogram of IVM an IVM-d_2 in Calibration Standard on Thermo Quantis Plus