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A. INTRODUCTION

1. Theory

The acetonitrile extract obtained from the determinative method (CLG-AVR) prior to derivatization for LC analysis is further purified by using a C8 solid phase extraction (SPE) cartridge and an alumina-B SPE cartridge. The purified extract is analyzed by injecting into an LC/APCI mass spectrometer. The identity of the compound is confirmed by comparison of its retention time and relative intensity with those of a standard or a positive control.

2. Applicability

This method is applicable for confirmation of Ivermectin, Doramectin and Moxidectin in liver and muscle of bovine, ovine, porcine, caprine and equine species as well as their processed products at $\geq 25$ ppb.

Note: Occasionally, some additives may interfere with confirmation of low level findings.

B. EQUIPMENT

Note: Equivalent apparatus or instrumentation may be substituted for any of the following.

1. Apparatus
   a. Amber ABC screw-top vials - 2 mL Cat. No. 27331, Supelco.
   b. EDP Plus Micropipettes - 250 $\mu$L capacity, Rainin Instrument Inc.
   c. Mechanical shaker - Eberbach model 610 equipped with shaker box model 6040, Thomas Scientific.
   e. 50-mL polypropylene centrifuge tubes - Cat. No. 2098, Becton Dickerson Labware.
   f. 16-port Vacuum manifold - Altech Associates, Inc.
   g. 15 mL disposable glass conical centrifuge tubes with snap caps, Cat No. 21020, VWR.
   h. N-Evap - Model 112, Organomation Assoc. Inc.
i. C8 Bond Elut cartridge - Part No. 1210-2002, Varian.

j. Sep-Pak Plus Alumina B Cartridge - Part No. WAT 020505, Waters Corporation.

2. Instrumentation

a. LC/APCI MS - Waters 2690 LC with Waters/Micromass QA MS
   LC column - Waters Nova-Pak 15 cm x 2 mm id. C-18, 4 \( \mu \)m particles with an Optigard 1mm. C-18 guard column. LC column temperature at approximately 23 \(^\circ\)C.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted for any of the following.

1. Reagents

a. Acetonitrile (ACN) - HPLC grade.

b. Methanol - HPLC grade.

c. Water - HPLC grade.

d. Hexane - HPLC grade.

e. Triethylamine - HPLC grade.

f. Methylene chloride - HPLC grade.

g. Ethyl acetate - HPLC Grade.

2. Solutions

a. SPE wash solution - ACN + water + triethylamine (30 mL + 70 mL + 0.1 mL). Make fresh immediately prior to use.

b. 50 mM Ammonium Acetate Buffer - Use the following formula to calculate the amount of ammonium acetate needed.

\[
\text{mg of ammonium acetate} = 50 \times \text{Liter of buffer needed} \times 77.08.
\]

The ammonium acetate is dissolved in deionized water and adjusted with acetic acid to pH 4.

c. LC Mobile Phase - 70% ACN + 25% water + 5% Buffer (v/v).

Note: It is possible to shorten the retention times of the compound of interest for cleaner samples by increasing the flow rate or decreasing the % water in the mobile phase. With dirtier sample extracts, it may be necessary to change the
retention time by replacing some of the water with methanol, reducing the acetonitrile level or increasing the % of water.

d. Methylene Chloride/Ethyl Acetate (3:1):
   Mix one volume of ethyl acetate to three volumes of methylene chloride.

D. STANDARDS

1. Source
   a. Ivermectin standard catalog no. L-640,471-076P004
      Merck, Sharpe and Dhome
      Rahway, NJ 07065
   b. Abamectin standard catalog no. L-676-863-038A003
      Merck Sharpe and Dhome
      Rahway, NJ 07065
   c. Doramectin standard
      Pfizer
      Lee-Summit, MO 64081-2998
   d. Moxidectin standard-catalog no. 301423
      Fort Dodge Animal Health
      Division of Wyeth
      P.O. Box 5366
      Princeton, NJ 08543-5366

2. Preparation of Standard Solutions
   a. Stock standard solution (125 μg/mL):
      Dissolve an appropriate amount of moxidectin, doramectin, and ivermectin separately in acetonitrile to make a concentration of 125 μg/mL.
   b. Working standard solution (0.5 μg/mL):
      Dilute 1 mL of each stock solution (a) to 250 mL with acetonitrile in a 250 mL volumetric flask. Use this working solution as the fortification solution for positive control and standard.
3. Storage conditions:
   a. Store stock solution in freezer.
   b. Working standard solutions may be stored at room temperature.

4. Shelf Life Stability
   a. Stock: 1 year.
   b. Working solution: 90 days.

E. SAMPLE PREPARATION

Weigh, extract, and clean up the sample as described in Determinative Method (CLG-AVR), section F.1.a. through F.1.f.

Note: Fortify the positive control with the Avermectin(s) of interest or the multi-avermectin working standard at a concentration equivalent to that of the compound of interest.

F. ANALYTICAL PROCEDURE

1. Extraction Procedure
   a. Immediately following step F.1.f. of the Determinative Method (CLG-AVR) add 5 mL of hexane and vortex for 30 sec.
   b. Centrifuge for 3 min. at 2500 rpm or allow to stand until layers separate (approximately 5 min.). Remove and discard hexane top layer.
   c. Evaporate acetonitrile under a gentle stream of dry nitrogen or dry air at approximately 65 ± 5 °C.
   d. Reconstitute the dried sample using 5.0 mL ± 50 µL acetonitrile. Vortex to mix.
   e. Add 10 mL HPLC grade water and 50 µL triethylamine. Vortex for 1 min. or shake vigorously for 5 min.
   f. Precondition a C8 Bond-Elut SPE cartridge by passing 4 mL ACN followed by 3 mL SPE wash solution.
   g. Pass extract in aliquots through the preconditioned C8 Bond Elut SPE cartridge with 10 mL reservoir until all extracts have been added at a flow rate of one drop per sec. Use a vacuum manifold to control the flow rate.
   h. Rinse empty sample tube with 3 mL SPE wash and add onto the SPE cartridge; discard eluates.
i. Dry SPE cartridge using a vacuum manifold for 5 minutes under vacuum (< 5 in. Hg).

j. Elute Avermectin residues with 6 mL of acetonitrile into a clean centrifuge tube.

k. Discard the C8 cartridges.

Note: Irreversible adsorption of the analytes to the C8 cartridge may occur and may result in decreased recovery of the analyte. The use of a different solvent to elute the Avermectin residues off the column may be necessary. For example: 3:1 methylene chloride/ethyl acetate or another solvent may be substituted for the acetonitrile as needed.

l. Evaporate the eluate to dryness on a nitrogen evaporator at 60 ± 5 °C and reconstitute with 6 mL of 3:1 methylene chloride/ethyl acetate solution. Add onto a pre-washed Alumina-B cartridge [Pre-washed with 4 mL methylene chloride and dried for 1 min. under vacuum (< 5 in. Hg)]. Discard eluates.

m. Draw air through Alumina-B cartridge for 30 sec.

n. Use a clean 15 mL centrifuge tube to collect eluates.

o. Attach a 10 mL Luer tip syringe to the cartridge. Pass 1.0 mL acetone through the Alumina B cartridge using gravity flow while collecting the eluate.

p. Allow cartridge to drain and continue elution with 4 mL of methanol using gravity flow.

q. Evaporate the eluate to dryness on a nitrogen evaporator at 60 ± 5 °C and reconstitute with 200 µL of Mobile phase (C.2.c).

r. Confirm residues by injecting into a LC/APCI mass spectrometer.

2. LC/APCI Mass Spectrometer operating conditions:

Note: It may be necessary to optimize the parameters when different mass spectrometers are used. The key parameters include the vaporizer and capillary temperature, the voltage of the capillary and the tube lens, the resolution of the mass spectrometer, the way of tuning, and the flow rate and composition of the LC mobile phase.

a. Flow rate - 0.5 mL/min.

b. APCI probe temperature - 500 °C

c. Source temperature - 140 °C

d. Ionization mode - Positive ion APCI

e. Corona discharge current - 5 µA
f. Desolvation gas flow rate - 450 L/hr.
g. Cone gas flow rate - 50 L/hr.
h. Tune and calibration compounds - Avermectin of interest or compounds with known spectra to cover the mass range of the ions monitored.
i. Electron multiplier voltage - 800 volts
j. Peak width at 50% peak height - 0.5 µm or larger.
k. Window width of each ion - 0.3 to 0.6 amu.
l. Dwell time of each ion - 250 ms.
m. Scanning mode - Selected ion monitoring.

3. LC/MS confirmation criteria:
   a. At least three characteristic ions, including the molecular weight ion, should be present.
   b. Two or more relative abundances are ± 20% of the fortified sample (or the standard) run on the same day under the same conditions.
   c. The retention time is ± 5% of the average of the standard or fortified sample.
   d. The tissue blank has no confirmable target compound.
   e. The signal to noise (S/N) ratio for the ions ratioed is > 3.
Table 1 shows the summary of fragment ions useful for confirmation of ivermectin, doramectin, and moxidectin.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mode</th>
<th>Ion 1 m/z</th>
<th>Ion 2 m/z</th>
<th>Ion 3 m/z</th>
<th>Ion 4 m/z</th>
<th>Ion 5 m/z</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ivermectin</td>
<td>+ APCI</td>
<td>892.5</td>
<td>567.3</td>
<td>551.3</td>
<td>307.2</td>
<td>569.3</td>
</tr>
<tr>
<td></td>
<td>- APCI</td>
<td>873.5</td>
<td>855.5</td>
<td>567.3</td>
<td>837.5</td>
<td></td>
</tr>
<tr>
<td>Doramectin</td>
<td>+ APCI</td>
<td>916.5</td>
<td>899.5</td>
<td>593.3</td>
<td>575.3</td>
<td>331.2</td>
</tr>
<tr>
<td>Moxidectin</td>
<td>+ APCI</td>
<td>640.4</td>
<td>622.4</td>
<td>498.3</td>
<td>496.3</td>
<td>528.3</td>
</tr>
<tr>
<td>Other ions</td>
<td>+ APCI</td>
<td>510.3</td>
<td>604.4</td>
<td>590.4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>may include</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4. Sample Chromatograms
   See APPENDIX K.1 (a-c) for chromatograms.

5. Tentative fragmentation patterns:
   a. Ivermectin.

\[\text{Figure 4. Tentative fragmentation pattern of ivermectin}\]
b. Doramectin

\[
\begin{align*}
\text{m/z} & \\
916 & [M + NH_4]^+ \\
899 & [M + H]^+ \\
593 & [A]^+ \\
575 & [A - H_2O]^+ \\
331 & [B]^+
\end{align*}
\]
c. Moxidectin

\[
\begin{align*}
\text{m/z} & \quad [M + H]^+ \\
640 & \\
622 & \quad [M + H - H_2O]^+ \\
498 & \quad [M - B - (CH_3CH_2CH_2)^+ + H]^+ \\
496 & \quad [M - A - CH_3OH + H]^+ \\
528 & \quad [M - A + H]^+
\end{align*}
\]

G. CALCULATIONS

[Reserved]

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Safety glasses, appropriate gloves, lab coat.
2. Hazards

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Hazard</th>
<th>Recommended Safe Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile, Methanol, Acetone, Ethyl acetate, Triethylamine, Hexane, Methylene Chloride</td>
<td>Flammable and corrosive, may cause skin or respiratory irritation.</td>
<td>Avoid contact or prolonged exposure to vapors. Work in a fume hood. Keep away from flame or heat.</td>
</tr>
<tr>
<td>Ivermectin</td>
<td>Weak teratogen and possible mutagen.</td>
<td>Handle with extreme caution.</td>
</tr>
<tr>
<td>Abamectin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Doramectin</td>
<td>Severe explosion hazard.</td>
<td>Handle with extreme caution.</td>
</tr>
<tr>
<td>Moxidectin</td>
<td>May cause skin or respiratory irritation. The toxic effects of this material have not been fully evaluated.</td>
<td>Work in a well-ventilated area. Store material in a secure, dry, cool well ventilated room.</td>
</tr>
</tbody>
</table>

3. Disposal Procedures

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Hazard</th>
<th>Recommended Safe Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic solvents</td>
<td>See above</td>
<td>Collect waste in tightly sealed container and store away from non-compatibles in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.</td>
</tr>
</tbody>
</table>

I. QUALITY ASSURANCE PLAN

1. Performance Standard
2. Critical Control Points and Specifications

<table>
<thead>
<tr>
<th>Record</th>
<th>Acceptable Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>a. Sample weight</td>
<td>2.5 g. ± 0.2 g</td>
</tr>
<tr>
<td>b. Final dilution volume</td>
<td>200 μL</td>
</tr>
</tbody>
</table>

3. Readiness To Perform

a. Familiarization

i. Phase I: Standards - Inject standards at the limit of confirmation and determine the ratios of the ions of interest for each component peak of compounds to be studied. Determine instrument sensitivity for each compound of interest.

ii. Phase II: Fortified samples - For each species and tissue to be monitored, clean up and inject:

   (a) A blank tissue extract

   (b) A positive control for all compounds of interest at the tolerance level or at the lowest confirmable level.

   Note: Phase I and Phase II may be performed concurrently.

iii. Phase III: Check samples for analyst accreditation.

   (a) Analyze a total of 8 samples in the tissues and species to be monitored. Samples should include liver, muscle, and processed products fortified at 25 ppb. At least one but no more than two should be blank. The fortification levels of the samples should be unknown to the analyst.

   (b) Authorization from the QAM and Supervisor is required to commence official sample analysis.

b. Acceptability criteria.

   Must meet confirmation criteria in section F.3 and I.1.
4. Intralaboratory Check Samples  
   a. Frequency: Once per week per analyst when samples are analyzed.  
   b. Records are to be maintained  
   c. Acceptability criteria.  
      If unacceptable values are obtained, then:  
      i. Stop all official analyses by that analyst for this method.  
      ii. Take corrective action.

5. Sample Acceptability and Stability  
   a. Matrix: Liver, muscle, and processed product.  

6. Sample storage:  
   a. Time: 6 months.  
   b. Condition: Frozen

7. Sample Set  
   a. Each set should include:  
      i. Tissue blank  
      ii. Positive control fortified at level comparable with the amount found using the determinative method  
      iii. Samples  
      iv. Standard

8. Sensitivity  
   Minimum Level of Applicability (MLA): 25 ppb

J. WORKSHEET  
   [Reserved]
K. APPENDIX

1. Chromatograms
   
   a. Chromatograms of standards
b. Chromatograms of Beef Muscle Positive Control
c. Chromatograms of Beef Liver Positive Control
2. Reference

L. APPROVALS AND AUTHORITIES
   Approvals on file.
   Issuing Authority: Director, Laboratory Quality Assurance Division (LQAD).