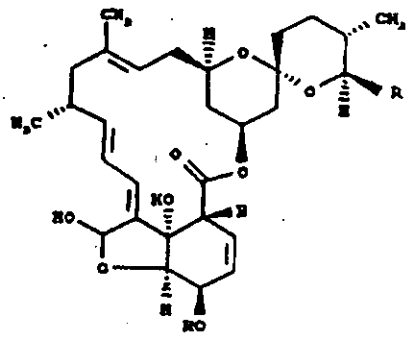
1.0 STRUCTURE



R = CH_3 :M.A_3
R = C_2H_5 :M.A_4
M.A_3:M.A_4 = 3:7
ISO Name: milbemectin

2.0 INTRODUCTION

2.1 Scope

This analytical method is used for the determination of milbemectin in freshwater and saltwater. A limit of quantitation (LOQ) for freshwater of 0.005 µg a.i./L was achieved; the LOQ for saltwater was 0.0134 µg a.i./L. Method validation results are presented in Tables 1 and 2. A flowchart of the analytical procedure is presented in Figure 1. A sample linear regression analysis is shown in Figure 2. Representative chromatograms have been supplied in Figures 3-16.

2.2 Principle

Residues of milbemectin are extracted from aqueous solutions by partitioning twice with dichloromethane. The organic extracts are evaporated to 2-4 mL and quantitatively transferred to centrifuge tubes. The extracts are evaporated to dryness under a stream of nitrogen. The residues are converted to their respective anhydride derivatives by addition of triethylamine in benzene and trifluoroacetic anhydride to each of the tubes. The solutions are heated in a water bath after which the reaction is completed by addition of additional triethylamine. The solutions are evaporated to approximately 200 µL under nitrogen and the residues are dissolved in acetonitrile/water, 95/5 (v:v). Quantitation is effected by isocratic, reverse-phase, high performance liquid chromatography (HPLC) with fluorescence detection.

3.0 APPARATUS

NOTE: All apparatus listed may be replaced by equivalent apparatus from alternative sources if experimental verification supports such substitutions.

3.1 Separatory funnels, 125-250 mL with glass-ground fittings

3.2 Graduated cylinders, 10-25 mL

3.3 Volumetric pipets, 1 mL, 10 mL and 15 mL, class A

3.4 Pear-shaped evaporation flasks, 50 mL, Pyrex or Kimax

3.5 Graduated centrifuge tubes, 15 mL

3.6 Disposable Pasteur pipettes, Baxter, 9"

3.7 Rotary evaporators, Büchi RE 111

3.8 Nitrogen evaporator, Organomation Multivap Model 111

3.9 Vortex, Fisher Genie 2

3.10 Volumetric flasks, 100 mL, for preparing analytical stocks and standards

3.11 Analytical balance capable of five decimal accuracy, Mettler AE 240

3.12 HPLC vials, 2 mL, with crimp caps, Hewlett-Packard

4.0 REAGENTS

NOTE: All reagents listed may be replaced with equivalent reagents from alternative sources if experimental verification supports such substitutions.

4.1 Methanol, Burdick and Jackson, HPLC grade

4.2 Acetonitrile, Burdick and Jackson, HPLC grade

4.3 Water, Barnstead NanoPure ®, HPLC grade

4.4 Dichloromethane, Burdick and Jackson, pesticide grade

4.5 Benzene, Sigma-Aldrich, HPLC grade

4.6 Triethylamine, Fisher, HPLC reagent grade

4.7 Trifluoroacetic anhydride, Aldrich, 99+%

5.0 PREPARATION OF ANALYTICAL STANDARDS

NOTE: Store all standard solutions in a refrigerator maintained at approximately 4°C. Standard solutions are presumed stable for at least six months unless standard comparison data indicates otherwise.

5.1 Primary Stock Solution

Using an analytical balance, weigh 100 mg (as active ingredient) of milbemectin on a weigh paper and transfer to a 100-mL volumetric flask. Rinse the weigh paper with methanol into the volumetric flask. Dilute to volume with methanol. This solution reflects a concentration of 1000 µg a.i./mL.

5.2 Fortification Standards

Serially dilute the primary stock solution (10 mL to 100 mL) with methanol to produce a 100-µg a.i./mL secondary stock. Continue serially dilutions, using methanol, to produce 10.0, 1.00, 0.100 and 0.0100 µg a.i./mL fortification stock solutions. The 1.00, 0.100 and 0.0100 µg a.i./mL solutions will be used in the preparation of recovery samples.

5.3 HPLC Calibration Standards

Prepare calibration standards by fortifying graduated centrifuge tubes with milbemectin stock solutions, evaporating the solutions to dryness and derivatizing as described in section 6.2: Derivatization of Extracted Residues. Typical calibration standards may be prepared as follows (Note 1):

Stock Concentration (µg a.i./L)	Fortification Volume (µL)	Final Volume After Derivatization (mL)	Standard Concentration (µg a.i./L)
10.0	50.0	2.00	0.250
100	10.0	2.00	0.500
100	20.0	2.00	1.00
100	30.0	2.00	1.50
100	40.0	2.00	2.00
100	50.0	2.00	2.50

6.0 ANALYTICAL METHOD

6.1 Extraction and Evaporation

6.1.1 Measure the requisite volume of freshwater or saltwater into a 125-mL or 250-mL separatory funnel (Note 2). Volumes used in the method validation trials ranged from 1.00-100 mL, depending on the nominal concentration of milbemectin. When using an initial volume of 1.00 mL, 10-20 mL of matrix should be added to the sample following fortification (Note 3).

6.1.2 Fortify validation samples with the standard solutions prepared in Sections 5.2 or 5.3 (as appropriate). Swirl each sample to ensure homogeneity.

6.1.3 For sample concentrations requiring less than 100 mL, add 10 mL of dichloromethane to the separatory funnel. For sample concentrations requiring 100 mL or more, add 15 mL of dichloromethane to the separatory funnel. See Tables 1 and 2 for volumes used in the method validation trials (Note 3).

6.1.4 Shake the separatory funnel (with venting) for approximately one minute. Allow the phases to separate.

6.1.5 Drain the organic phase (lower) into a 50-mL pear-shaped evaporation flask.

6.1.6 Repeat the partition one additional time (Steps 6.1.3-6.1.5). Combine the extracts in the 50 mL pear-shaped flask.

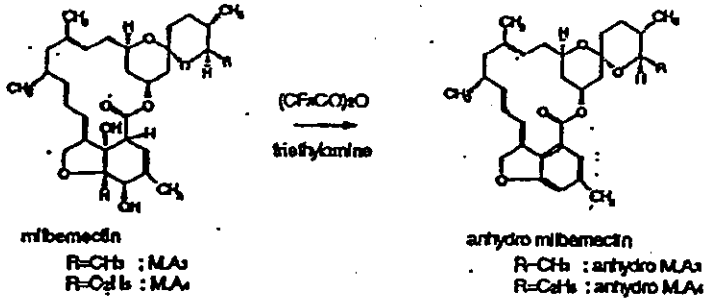
6.1.7 Rotary evaporate the sample to 2-4 mL using a waterbath maintained at 40°C. Do not rotary evaporate the sample to dryness (Note 4).

6.1.8 Using a disposable pipet, quantitatively transfer the extract to a 15-mL graduated centrifuge tube. Add approximately 2 mL of dichloromethane to the flask and swirl well. Transfer this rinsate to the centrifuge tube. Add an additional 2 mL of dichloromethane to the flask. Swirl the solution and transfer to the centrifuge tube (Note 5).

6.1.9 Observe the solution in the centrifuge tube. If any water is present on the surface of the dichloromethane, remove this water with a disposable pipet (Note 6).

6.1.10 Evaporate the extract to dryness in a waterbath maintained at 40°C under a gentle stream of nitrogen. Ensure that no water is present (Note 7). If a small amount of water remains, a small volume of absolute ethanol may be added to the centrifuge tube and the evaporation repeated.

6.2 Derivatization of Extracted Residues



6.2.1 Add 1 mL of a 0.5N triethylamine solution (prepared in benzene) and 100 μL of trifluoroacetic anhydride to the graduated centrifuge tube. Vortex the tube for a few seconds (Note 8).

6.2.2 Place the uncovered tube in a waterbath maintained at 40°C and allow the derivatization to proceed for 30 minutes.

6.2.3 Remove the tube from the waterbath and add 50 μL of 100% triethylamine. Vortex the solution for a few seconds.

6.2.4 Return the centrifuge tube to the waterbath and evaporate the solution under a gentle stream of nitrogen. Note: The solution will not evaporate to dryness due to the presence of triethylamine. Evaporate the solution to approximately the 0.200 mL graduation.

6.2.5 Remove the centrifuge tube from the waterbath. Add a solution of 95/5, CH_3CN/H_2O (v:v) to the tube and adjust the final volume using the gradations on the centrifuge. Vortex the solution for a few seconds. Note: The minimum final volume should be no less than 2 mL. Final volumes used in the method validation trials are presented in Tables 1 and 2.

6.2.6 Transfer a portion of the sample to an autosampler vial and submit for analysis by high performance liquid chromatography.

6.3 High Performance Liquid Chromatographic Determination

Use a Hewlett-Packard ODS Hypersil, 200 mm x 4.6 mm, 5μ column to achieve separation. Use a Hewlett-Packard Model 1090 HPLC (or equivalent) and a Jasco Model 821 FP fluorescence detector (or equivalent) to provide adequate selectivity and sensitivity (Note 9).Chromatographic and detection conditions are presented in Section 7.0.

6.4 Safety Precautions

Use normal safety precautions, which include wearing gloves, labcoats and safety glasses. The use of a fume hood is necessary to minimize exposure to the analyte, solvents and reagents used in this procedure. Special precautions should be taken when using dichloromethane, benzene and trifluoroacetic anhydride. Dichloromethane and benzene are suspected carcinogens and trifluoroacetic anhydride is extremely corrosive. It is suggested that use of these materials be limited only to a fumehood.

6.5 Limit of Quantitation

The limit of quantitation (LOQ) for the analysis of milbemectin in freshwater and saltwater was defined as the product of the lowest standard concentration and the dilution factor of the matrix blanks. The LOQ in freshwater was calculated to be 0.005 µg a.i./L; the LOQ for saltwater was calculated to be 0.0134 µg a.i./L. Adjust the detector gain, calibration standard concentration, initial sample volume and final extract volume to allow detection of milbemectin at 50-75% of the LOQ.

6.6 Time Required for Analysis

An experienced analyst can process a set of approximately 15 samples and prepare them for injection on the HPLC in approximately one 8-hour day.

6.7 Interferences and Potential Problems

Fluorescence detection conditions have been optimized to provide significant selectivity and sensitivity of the two components of milbemectin. No interferences have been observed in any analysis of milbemectin in freshwater or saltwater. Several areas in this methodology are critical to the quantitative recovery of milbemectin from aqueous matrices. Please pay special attention to Section 10: NOTES.

7.0 HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS

7.1 Description and Typical Operating Conditions

Instrument: Hewlett-Packard Model 1090 high performance liquid chromatograph equipped with a Jasco Model 821 FP fluorescence detector. Data was collected and processed with HP Chem Station software.

7.1 Description and Typical Operating Conditions (continued)

Column: Hewlett-Packard ODS Hypersil, 200 mm x 4.6 mm, 5μ particle size

Column Temperature: 40°C

Mobile Phase: 95% acetonitrile : 5% water (v:v)

Flowrate: 1.00 mL/minute

Injection Volume: 100 μL

Excitation Wavelength: 360 nm

Emission Wavelength: 460 nm

Typical Retention Times: $M.A_3$ = 7.7 minutes
$M.A_4$ = 9.1 minutes

7.2 Calibration

The HPLC is calibrated using the calibration standards prepared in Section 5.3, in concentrations typically ranging from 0.250 μg a.i./L to 2.50 μg a.i./L (Note 1). Inject a minimum of five standards at the beginning of the run, one standard after every five recovery samples throughout the run and a minimum of five standards at the end of the run. A linear regression equation is generated using the sum of the resulting peak areas of the components of milbemectin, $M.A_3$ and $M.A_4$, versus the concentration of the standards. The coefficient of determination should be equal to or greater than 0.985. The sample concentration is determined by interpolation of the sum of the resulting peak areas of the components of milbemectin, $M.A_3$ and $M.A_4$, from the appropriate standard curve linear regression equation.

7.3 Representative Chromatograms

A representative method flowchart is provided in Figure 1. A typical calibration curve is presented in Figure 2. Typical chromatograms illustrating low and high HPLC calibration standards are presented in Figures 3 and 4. Chromatograms reflecting a reagent blank, matrix blank and 0.0050-20.0 μg a.i./L recoveries from freshwater are presented in Figures 5-12, respectively. Chromatograms reflecting a matrix blank and 0.0267-20.0 μg a.i./L recoveries from saltwater are presented in Figures 13-16, respectively.

8.0 CALCULATIONS

8.1 The sample extract concentration is calculated by interpolation of the sum of the resulting peak areas of the components of milbemectin, $M.A_3$ and $M.A_4$ from the standard curve linear regression equation as follows:

Concentration of Extract (μg a.i./L) = (Sum of $M.A_3/M.A_4$ areas - $Y_{intercept}$)/Slope

8.2 The concentration of the original aqueous sample is calculated as the product of the extract concentration and the dilution factor as follows:

Conc. of Milbemectin (μg a.i./L) = Conc. of Extract (μg a.i./L) x (V_{final} / $V_{initial}$)
Where: V_{final} = final extract volume (mL)
$V_{initial}$ = aqueous sample volume (mL)

8.3 The recovery of milbemectin from the aqueous sample is calculated as follows:

$$\text{Percent recovery} = \frac{\text{Measured Milbemectin concentration } (\mu g \text{ a.i./L})}{\text{Nominal Milbemectin concentration } (\mu g \text{ a.i./L})} \times 100$$

8.4 An example calculation for a typical saltwater recovery sample follows:

Wildlife International, Ltd. I.D. Number.: 420C-101-VMAS-10
Sequence Number: SAN3
Run number: SAN3A29A
See Figure Number 14

Where:

Nominal concentration: 0.0267 μg a.i./L
Aqueous sample volume: 75.0 mL
Final extract volume: 2.00 mL
Dilution factor: 0.0267
Sum of peak areas ($M.A_3/M.A_4$): 436.12
Y-intercept: 25.21408
Slope: 413.42

Concentration of Milbemectin Extract (μg a.i./L) = (436.12 - 25.21408)/413.42
Concentration of Milbemectin Extract (μg a.i./L) = 0.99392

Concentration of Milbemectin (μg a.i./L) = 0.99392 μg a.i./L x 0.0267
Concentration of Milbemectin (μg a.i./L) = 0.0265

$$\text{Percent recovery} = \frac{0.0265 \ \mu g \text{ a.i./L}}{0.0267 \ \mu g \text{ a.i./L}} \times 100 = 99.3\%$$

10. NOTES

10.1 Method validation results were obtained from three validation trials. Calibration standard concentrations were varied according to the nominal concentrations of the recovery samples.

10.2 The volume of the recovery samples may be varied depending on the selected calibration standard range and the sensitivity of the detector. The smallest volume used for recovery samples was 1.00 mL; the largest volume was 100 mL.

NOTES (continued)

10.3 Sample volumes should be selected such that the mass of milbemectin added to the sample is relatively constant and less than or equal to 20 ng. Sample recoveries are optimized in the 1-20 ng range. Do not prepare samples in volumetric flasks with the intention of subsampling. Fortify all samples directly in separatory funnels. Milbemectin adsorbs to glassware surfaces and recoveries will be low if secondary sampling is effected.

10.4 Do not rotary evaporate to dryness. Our laboratory observed low recoveries when samples were inadvertently rotary evaporated to dryness.

10.5 The transfer of the dichloromethane extract from the pear-shaped flask to the graduated centrifuge tube appears to be one of the more critical steps. Ensure that sufficient rinses with dichloromethane are carried out and that all portions of the organic extract are quantitatively transferred.

10.6 The simplest method to remove residual water on the surface of the dichloromethane extract is to use a disposable pipet. Pipet all the water followed by one-half of a pipet of the organic extract. Allow the phases to separate in the pipet and return the dichloromethane to the centrifuge tube.

10.7 Prior to derivatization, the centrifuge tube must be completely dry; i.e. no water may be present. The derivatization effects dehydration of $M.A_3$ and $M.A_4$ to form the corresponding aromatic, anhydride moieties. The presence of any water will inhibit the dehydration process resulting in low or no recoveries.

10.8 Prepare the 0.5N triethylamine in benzene daily. A convenient way to prepare the reagent is to pipet 3.5 mL of triethylamine into approximately 25 mL of benzene contained in a 50 mL graduated cylinder. Adjust the final volume to 50 mL with benzene.

10.9 The method validation trials were carried out using a Jasco Model 821 FP fluorescence detector. Our laboratory is currently using a Jasco Model FP-920 which affords approximately three times more sensitivity.

FLOW CHART FOR THE ANALYSIS OF MILBEMECTIN IN FRESHWATER AND SALTWATER

Rinse all glassware with acetone followed by dichloromethane.

↓

Prepare recovery samples in the appropriate aqueous matrix directly in separatory funnels. Fortify samples with the appropriate Milbemectin stock solution. Leave the matrix blank unfortified.

↓

Extract each sample twice with 10-15 mL of dichloromethane. Combine the two extracts in a 50-mL pear-shaped flask. Rotary evaporate each sample, using a waterbath of approximately 4-°C, to a 2-4 mL volume. Do not rotary evaporate to dryness.

↓

Transfer each extract to a prelabelled, 15-mL graduated centrifuge tube. Rinse the flask with 2-4 mL of dichloromethane and transfer the rinsate to the centrifuge tube.

↓

Evaporate the extract to dryness in a waterbath maintained at ~40°C under a gentle stream of nitrogen.

↓

To each centrifuge tube, add 1 mL of a 0.5 N triethylamine solution (prepared in benzene) and 100 μL of trifluoroacetic anhydride. Vortex each tube for few seconds.

↓

Place each tube in a waterbath maintained at ~40°C and allow the derivatization to proceed for 30 minutes.

↓

Following this period, add 50 μL of triethylamine to each of the tubes. Vortex each solution for a few seconds.

↓

Evaporate the resulting solutions to approximately 200 μL in a ~40°C waterbath using a gentle stream of nitrogen.

↓

To the samples, add a solution of 95% CH_3CN : 5% H_2O; adjust the final volume using the gradations on the centrifuge tube.

↓

Transfer a portion of the final extracts to HPLC autosampler vials for analysis.

↓

Prepare calibration standards by fortifying requisite volumes of the appropriate analytical stock solution into dry, graduated centrifuge tubes. Evaporate the standards to dryness using a gentle stream of nitrogen. Follow steps 7-12 to complete standard preparation.

Figure 1. Analytical method flow chart for the analyses of Milbemectin in freshwater and saltwater.