1 0 INTRODUCTION

1.1 Scope

The analytical method presented is used for the determination of parent milbemectin ($M.A_3$ and $M.A_4$), and its potential degradation products including the photoproducts ($8,9Z\text{-}M.A_3$ and $8,9Z\text{-}M.A_4$), hydroxy metabolites ($27\text{-hydroxy-}M.A_3$ and $27\text{-hydroxy-}M.A_4$) and keto metabolites ($27\text{-keto-}M.A_3$ and $27\text{-keto-}M.A_4$)

The method may be used for the simultaneous determination of the combined residue of $M.A_3$ and $8,9Z\ M.A_3$ and the combined residue of $M.A_4$, and $8,9Z\text{-}M.A_4$. This is a direct result of the conversion of $M.A_3$ and $8,9Z\text{-}M.A_3$ to the same anhydro-$M.A_3$ compound resulting from the derivatization procedure employed to generate compounds that can be detected and quantitated. Likewise, the combined residue of $M.A_4$, and $8,9Z\text{-}M.A_4$ form the same anhyro-$M.A_4$ derivative via this derivatization procedure and, thus, can also be quantitated as a single combined residue.

Alternatively, $M.A_3$, $M.A_4$, $8,9Z\text{-}M.A_3$ and $8,9Z\text{-}M.A_4$ may be determined as individual components by incorporation of a fractionation step prior to derivatization of a composite extract. The resulting individual fractions would then be derivatized and analyzed separately for quantitation of the respective anhydro derivatives.

The procedure used for extraction of $M.A_3$, $M.A_4$, $8,9Z\text{-}M.A_3$ and $8,9Z\text{-}M.A_4$ from soil concurrently extracts the hydroxy metabolites ($27\text{-hydroxy-}M.A_3$ and $27\text{-hydroxy-}M.A_4$) and keto metabolites ($27\text{-keto-}M.A_3$ and $27\text{-keto-}M.A_4$). Methodology for the analysis of $27\text{-keto-}M.A_3$ and $27\text{-keto-}M.A_4$ consists of a direct chromatographic method since the keto metabolites do not form anhydro derivatives using the derivatization procedure presented in this method. Analysis of the $27\text{-hydroxy-}M.A_3$ and $27\text{-hydroxy-}M.A_4$ follow a similar methodology scheme to that of the parent compounds and their photoproducts, i.e., the same initial extraction, solid-phase clean up, and derivatization. The hydroxy anhydro derivatives are analyzed using chromatographic conditions used for either parent and/or photoproduct anhydro derivatives.

Limits of quantitation (LOQ) achievable using the method presented below are summarized as follows

1.00 µg a.i./Kg for $M.A_3$ and $M.A_4$
1.12 µg a.i./Kg for $8,9Z\text{-}M.A_3$ and $8,9Z\text{-}M.A_4$
1.00 µg a.i./Kg for $27\text{-hydroxy-}M.A_3$ and $27\text{-hydroxy-}M.A_4$
2.00 µg a.i./Kg for $27\text{-keto-}M.A_3$ and $27\text{-keto-}M.A_4$

1.2 Principle

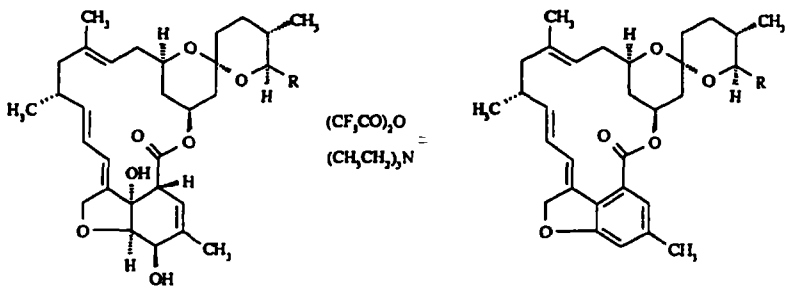
Milbemectin, a mixture of $M.A_3$ and $M.A_4$; the photoproducts of milbemectin, $8,9Z\text{-}M.A_3$ and $8,9Z\text{-}M.A_4$; and the potential degradation products $27\text{-hydroxy-}M.A_3$, $27\text{-hydroxy-}M.A_4$, $27\text{-keto-}M.A_3$ and $27\text{-keto-}M.A_4$ are extracted from soil using methanol. The extracts are cleaned up using a C_{18} solid-phase extraction column. Residues are eluted from the column, the eluant evaporated to dryness and the residue reconstituted in

hexane/isopropanol. At this point, an aliquot of reconstituted eluant can be fractionated using high performance liquid chromatography (HPLC) to separate M.A_3/M.A_4 from 8,9Z-M.A_3/8,9Z-M A_4 Fractionation provides individual fractions, which can be dried and derivatized, thus allowing quantitation of the individual components.

For analysis of total[1] M.A_3 (including any 8,9Z-M.A_3 present), total[1] M.A_4 (including any 8,9Z-M A_4 present), 27-hydroxy-M.A_3, 27-hydroxy-M.A_4, 27-keto-M.A_3, and 27-keto-M.A_4, a volumetric aliquot of the reconstituted eluant from the C_{18} SPE column is transferred to a centrifuge tube and the residue subsequently derivatized to the fluorescence-active anhydro-M.A_3, anhydro-M.A_4, anhydro-27-hydroxy-M.A_3 and anhydro-27-hydroxy-M.A_4 derivatives. An aliquot of the remaining column eluant is evaporated to dryness, redissolved in hexane/isopropanol and analyzed directly for 27-keto-M.A_3 and 27-keto-M.A_4 using gradient-elution, normal phase HPLC with ultraviolet absorption (UV) detection. As noted above, this procedure generates results for total M.A_3 that includes M.A_3 and its corresponding photoproduct, 8,9Z-M.A_3, since they form the same anhydro-M.A_3 derivative. Likewise, the above procedure provides results for total M A_4 that includes its corresponding photoproduct, 8,9Z-M.A_4, since they form the same anhydro-M A_4 derivative. Also, as noted above, the method may be altered to obtain separate values for parent and photoproduct components. To determine separate concentrations for these components, an aliquot of the solid-phase column eluant must be fractionated prior to performing the derivatization with collection of separate fractions for the parent and photoproduct moieties.

All anhydro derivatives, to include anhydro-M.A_3 (arising from either M.A_3 or 8,9Z-M.A_3), anhydro-M.A_4 (arising from either M.A_4 or 8,9Z- M.A_4), anhydro-27-hydroxy-M.A_3 and anhydro-27-hydroxy-M.A_4, are analyzed chromatographically using gradient-elution, reversed-phase HPLC with fluorescence detection.

The derivatization reaction is illustrated below using milbemectin components M.A_3 and M.A_4 as examples. The analogous reaction occurs for the photoproducts 8,9Z- M.A_3 and 8,9Z- M.A_4, and the hydroxy metabolites, 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4.



M.A_3: R = CH_3
M.A_4: R = CH_3CH_2

Anhydrous M.A_3: R = CH_3
Anhydrous M.A_4: R = CH_3CH_2

1 The reference to "total M.A_3" or "total M A_4" within this report refers to the combined residue of M.A_3 and its photoproduct 8,9Z-M.A_3 or M.A_4 and its photoproduct 8,9Z-M.A_4, respectively

It is apparent from the above discussion that the methodology presented offers several options for analysis depending upon the moieties for which quantitative information is desired. To aid the analyst, the method is illustrated diagrammatically in Figure 3 with reference to the appropriate analytical sections.

2.0 APPARATUS

Note: Apparatus listed may be replaced with equivalent apparatus or other apparatus serving the same function if verification supports such substitutions.

2.1 Syringes, assorted sizes

2.2 Volumetric flasks, 100 and 200 mL, Class A, Kimax

2.3 Beakers, Kimax, 150 - 250 mL

2.4 Volumetric pipets, 10 and 20 mL, Class A, Kimax

2.5 Centrifuge tubes, 15 mL, graduated to 0.10 mL, Kimax

2.6 Vacuum manifold, Vac Master 10, Catalog No. 12H016, Jones Chromatography

2.7 Centrifuge bottles, Pyrex, 150 mL, Catalog No. 1265-150, Corning

2.8 Sonic Dismembrator, Model 300 operated at 60% power, Fisher

2.9 Centrifuge, Centra-8R, IEC

2.10 Filtering apparatus, filter funnel, 140 mL, 60-mm disk diameter, EC with 24/40 male adapter, WDLF-0002, At-Mar Glass Company

3.0 REAGENTS

Note Reagents may be replaced with equivalent reagents from alternative vendors if verification supports such substitutions.

3.1 Methanol, HPLC grade, (203 nm UV cutoff), Burdick and Jackson,

3.2 Acetonitrile, HPLC grade (188 nm UV cutoff), Burdick, and Jackson

3.3 Water, Barnstead NANOpure®, ASTM type II, 17.8 MΩ-cm

3.4 2-Propanol, HPLC grade, Fisher

3.5 n-Hexane, HPLC grade, Burdick & Jackson

3.6 Benzene, HPLC grade, Sigma Aldrich

3.7 Triethylamine (TEA), analytical reagent grade, Aldrich

3.8 Trifluoroacetic anhydride (TFAA), analytical reagent grade, Aldrich

3.9 Phosphoric acid, reagent grade, Mallickrodt

3.10 C_{18}-reversed phase solid-phase extraction columns, 1000 mg, Bakerbond

3.11 Glass fiber filters, GF/A, Whatman

3.12 Celite 545, Catalog No. 3371-05, J.T. Baker

4 0 REFERENCE STANDARDS IDENTIFICATION

M.A_3: (10E,14E,16E,22Z)-(1R,4S,5'S,6R,6'R,8R,13R,20R,21R,24S)-21,24-dihydroxy-5',6',11,13,22-pentamethyl-3,7,19-trioxa-cyclo[15.6.1.$1^{4,8}$.$O^{20,24}$]-pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one.

M.A_4: (10E,14E,16E,22Z)-(1R,4S,5'S,6R,6'R,8R,13R,20R,21R,24S)-6'-ethyl-21,24-dihydroxy-5',11,13,22-tetramethyl-3,7,19-trioxacyclo[15.6.1.$1^{4,8}$.$O^{20,24}$]-pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one.

8,9Z-M A_3: (10E,14E,16Z,22Z)-(1R,4S,5'S,6R,6'R,8R,13R,20R,21R,24S)-21,24-dihydroxy-5',6',11,13,22-pentamethyl-3,7,19-trioxacyclo[15.6.1.$1^{4,8}$.$O^{20,24}$]-pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one.

8,9Z-M.A_4: (10E,14E,16Z,22Z)-(1R,4S,5'S,6R,6'R,8R,13R,20R,21R,24S)-6'-ethyl-21,24-dihydroxy-5',11,13,22-tetramethyl-3,7,19-trioxacyclo[15.6.1.$1^{4,8}$.$O^{20,24}$]-pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one.

27-hydroxy- M.A_3: 27-hydroxy-(10E,14E,16E,22Z)-(1R,4S,5'S,6R,6'R,8R,13R,20R,21R,24S)-21,24-dihydroxy-5',6',11,13,22-pentamethyl-3,7,19-trioxa-cyclo [15.6.1.$1^{4,8}$.$O^{20,24}$]-pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one

27-hydroxy- M.A_4: 27-hydroxy-(10E,14E,16E,22Z)-(1R,4S,5'S,6R,6'R,8R,13R,20R,21R,24S)-6'-ethyl-21,24-dihydroxy-5',11,13,22-tetramethyl-3,7,19-trioxacyclo [15.6.1.$1^{4,8}$.$O^{20,24}$]-pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one.

27-keto- M.A_3: 27-keto-(10E,14E,16E,22Z)-(1R,4S,5'S,6R,6'R,8R,13R,20R,21R,24S)-21,24-dihydroxy-5',6',11,13,22-pentamethyl-3,7,19-trioxa-cyclo[15.6.1.$1^{4,8}$.$O^{20,24}$]-pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one.

27-keto- M A_4 27-keto-(10E,14E,16E,22Z)-(1R,4S,5'S,6R,6'R,8R,13R,20R,21R,24S)-6'-ethyl-21,24-dihydroxy-5',11,13,22-tetramethyl-3,7,19-trioxacyclo[15.6.1.$1^{4,8}$.$O^{20,24}$]-pentacosa-10,14,16,22-tetraene-6-spiro-2'-tetrahydropyran-2-one.

Note: Store reference standards frozen

5 0 ANALYTICAL STANDARD SOLUTION PREPARATION

5.1 Stock Solution Preparation - M.A_3 and M A_4

Stock solutions of milbemectin are prepared by accurately weighing 100 mg (corrected for purity of the analytical standard) of each of the standards, M.A_3 and M.A_4, onto weigh paper, transferring to separate clean 100-mL class A volumetric flasks and adjusting to final volume using methanol. These stock solutions contain 1.00 mg a.i./mL of M.A_3 and M.A_4, respectively. The solutions are then volumetrically diluted 10 mL to 100 mL using methanol to achieve 100-mg a.i./L stock solutions Serial dilutions of 10 mL to 100 mL are successively made in methanol to prepare stock solutions containing 10.0 and 1.00 mg a i./L of M.A_3 and M.A_4. Combined stocks, containing both M.A_3 and M.A_4 may also be prepared. These solutions are used for the preparation of standards and for fortification of recovery samples.

5.2 Stock Solution Preparation - 8,9Z-M.A_3 and 8,9Z-M.A_4

Stock solutions of the photoproducts of milbemectin are prepared by accurately weighing 100 mg (corrected for purity of the analytical standards) of each of the standards, 8,9Z-M.A_3 and 8,9Z-M.A_4, onto weigh paper, transferring to clean separate 100-mL class A volumetric flasks and adjusting to final volume using methanol These stock solutions contain 1.00 mg a.i./mL of 8,9Z-M.A_3 and 8,9Z-M.A_4, respectively. The solutions are then volumetrically diluted 10 mL to 100 mL using methanol to achieve 100 mg a.i./L stock solutions. Serial dilutions of 10 mL to 100 mL are successively made in methanol to prepare stock solutions containing 10.0 and 1.00 mg a.i./L of 8,9Z-M.A_3 and 8,9Z-M.A_4. Combined stocks, containing both 8,9Z-M.A_3 and 8,9Z-M.A_4 may also be prepared These solutions are used for fortification of the recovery samples for the photoproduct method validation trials.

5.3 Stock Solution Preparation - 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4

Stock solutions of the 27-hydroxy metabolites of milbemectin are prepared by accurately weighing 25 mg (corrected for purity of the analytical standards) of each of the standards, 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4, onto weigh paper, transferring to separate clean 50-mL class A volumetric flasks and adjusting to final volume using acetonitrile. These stock solutions contain 0.500 mg a.i./mL of 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4, respectively. The solutions are then volumetrically diluted 20 mL to 100 mL using acetonitrile to achieve 100-mg a.i./L stock solutions. Serial dilutions of 10 mL to 100 mL are successively made in acetonitrile to prepare stock solutions containing 10 0 and 1.00 mg a.i./L of 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4. Combined stocks, containing both 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4 may also be prepared. These solutions are used for fortification of the recovery samples for the 27-hydroxy metabolite method validation trials.

5.4 Stock Solution Preparation - 27-keto-M.A_3 and 27-keto-M.A_4

Stock solutions of the 27-keto metabolites of milbemectin are prepared by accurately weighing 25 mg (corrected for purity of the analytical standards) of each of the standards,

27-keto-M.A_3 and 27-keto-M.A_4, onto weigh paper, transferring to separate clean 50-mL class A volumetric flasks and adjusting to final volume using acetonitrile. These stock solutions contain 0.500 mg a.i./mL of 27-keto-M.A_3 and 27-keto-M.A_4, respectively. The solutions are then volumetrically diluted 20 mL to 100 mL using acetonitrile to achieve 100-mg a.i./L stock solutions. Serial dilutions of 10 mL to 100 mL are successively made in acetonitrile to prepare stock solutions containing 10.0 and 1.00 mg a.i./L of 27-keto-M.A_3 and 27-keto-M.A_4. Combined stocks, containing both 27-keto-M.A_3 and 27-keto-M.A_4 may also be prepared. These solutions are used for fortification of the recovery samples for the 27-keto metabolite method validation trials.

5.5 Combined Stock Solution - M.A_3, M.A_4, 8,9Z-M.A_3 and 8,9Z-M.A_4

Stock solutions containing the four components, M.A_3, M.A_4, 8,9Z-M.A_3 and 8,9Z-M.A_4, are prepared for the total residue and fractionation portions of the method. Ten milliliters each of the 100 mg a.i./L methanolic stock solutions of M.A_3, M.A_4, 8,9Z-M.A_3 and 8,9Z-M.A_4 are volumetrically combined in a 100-mL class A volumetric flask. The final volume is adjusted to 100 mL with methanol, resulting in a solution containing 10.0 mg a.i./L of each of the four components. From this solution, 10.0 mL is volumetrically transferred to a 100-mL volumetric flask partially filled with methanol and then brought to volume with methanol. This solution contains 1.00 mg a.i./L of each component.

5.6 Calibration Standard Solutions of Anhydro-M.A_3 and Anhydro-M.A_4

Since M.A_3 and M.A_4, and the respective 8,9Z-M.A_3/8,9Z-M.A_4 photoproducts form the same anhydro-M.A_3 and anhydro-M.A_4 derivatives, M.A_3 and M.A_4 are derivatized and used to quantitate both parent milbemectin and its photoproducts. Calibration standards are prepared by addition of aliquots of the M.A_3 and M.A_4 stock solutions prepared in Sections 5.1 and 5.2 to clean, dry, 15-mL, centrifuge tubes using a gas-tight syringe. The stock solutions are subsequently evaporated to dryness and derivatized as described in the Section 6.5, Derivatization.

Stock Concentration (mg a.i./L)[1]	Fortification Volume (µL)	Final Volume (mL)	Standard Concentration (µg a.i./L)[2]
0.100	35.0	10.0	0.350
0.100	100	10.0	1.00
0.100	250	10.0	2.50
0.100	350	10.0	3.50
0.100	500	10.0	5.00

[1] The stock concentration contains 0.100 mg a.i./L of M.A_3 and 0.100 mg a.i./L of M.A_4.
[2] Solutions contain M.A_3 and M.A_4, each at the concentration presented.

5.7 Calibration Standard Solutions of Anhydro-27-Hydroxy-M.A_3 and Anhydro-27-Hydroxy-M.A_4

Since 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4 form distinctly different anhydro-derivatives from M.A_3 and M.A_4, namely anhydro-27-hydroxy-M.A_3 and anhydro-27-hydroxy-M.A_4, a separate set of standards may be derivatized and used to quantitate 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4. Alternatively, calibration standards may be prepared by fortifying centrifuge tubes with the four components, M.A_3, M.A_4, 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4, derivatizing and diluting the mixture as a singular combined standard. This approach is preferable since standards containing the four anhydro-derivatives are simultaneously chromatographed and the use of a combined standard minimizes the number of injections, thereby lessening the total run time. All combined standards are fortified, evaporated, derivatized and diluted using the methodology described in Section 5 6.

5.8 Calibration Standard Solutions of 27-Keto-M.A_3 and 27-Keto-M.A_4

Using the derivatization procedures previously described, the 27-keto-moieties of milbemectin, 27-keto-M.A_3 and 27-keto-M.A_4, do not form the corresponding anhydro-27-keto-M.A_3 and anhydro-27-keto-M.A_3. Therefore, a separate set of calibration standards containing 27-keto-M.A_3 and 27-keto-M.A_4 must be prepared as follows:

Stock Concentration (mg a.i./L)[1]	Fortification Volume (µL)	Final Volume (mL)	Standard Concentration (µg a.i./L)[2]
10.0	40.0	100	4.00
10.0	100	100	10.0
10.0	150	100	15.0
10.0	200	100	20.0
10.0	250	100	25.0

[1] The stock concentration contains 10.0 mg a.i./L of 27-keto-M.A_3 and 10.0 mg a.i./L of 27-keto-M.A_4.

[2] Solutions contain 27-keto-M A_3 and 27-keto-M A_4, each at the concentration presented.

6.0 ANALYTICAL METHOD

6.1 Sample Preparation

6 1.1 Using an appropriate technique, mix soil in order to ensure a homogenous sample.

6 1.2 Weigh 25 grams of soil into a tared, pre-labeled, 150-mL, glass centrifuge bottle Record the weight.

6.2 Laboratory Fortification

6.2.1 For the preparation of procedural recovery samples, soil is fortified as appropriate using stock solutions prepared in Sections 5.1 through 5.5.

6 2.2 For fractionation of $M.A_3$ and $M.A_4$ from 8,9Z-$M.A_3$ and 8,9Z-$M.A_4$, fortify the soil samples with combined stock solutions of the respective components. Typical fortification levels for the fractionation method (parent/photoproduct) are 2.00, 10.0, 50 0 and 100 µg a.i./Kg

6.2.3 For analysis of $M.A_3$, $M.A_4$, 27-hydroxy-$M.A_3$, 27-hydroxy-$M.A_4$, 27-keto-$M.A_3$ and 27-keto-$M.A_4$, fortify the soil samples with combined stock solutions of the six components. Typical fortification levels for the combined method are 2 00, 10.0 and 100 µg a.i./Kg for $M.A_3$, $M.A_4$, 27-hydroxy-$M.A_3$, 27-hydroxy-$M.A_4$ and 5.00, 10.0 and 100 µg a i./Kg for 27-keto-$M.A_3$ and 27-keto-$M.A_4$.

6.3 Sample Extraction

6.3.1 To the centrifuge bottle containing the sample, add 50 mL of methanol using a graduated cylinder or tiltapet.

6.3.2 Using a sonic disruptor set at 60% power, disrupt the sample for approximately five minutes. The sample at this stage should be converted to fine particles If another sample needs to be immediately extracted, rinse the disruptor horn with a small quantity of methanol and collect the rinsate in the centrifuge bottle containing the sample.

6.3.3 Cap the centrifuge bottle and centrifuge the bottle at a setting of 2500 rpm for five minutes.

6.3 4 Prepare a filtering aid by adding 10 grams of Celite 545 to a glass beaker and adding sufficient reagent grade water to make the mixture flowable. Using vacuum, pour this mixture into a filtering apparatus with sintered glass frit (or Buchner funnel) fitted with a glass fiber filter. Ensure that the filtering aid forms an even, homogenous bed with a depth of approximately 5 to 10 mm of Celite.

6.3.5 Gently remove the sample from the centrifuge, ensuring that the solids are not disturbed. Vacuum filter the supernatant through the filtering aid and collect the filtrate in a labeled, 200-mL, glass, Class A, volumetric flask.

6 3.6 Add 50 mL of methanol to the centrifuge bottle and return to the sonic disruptor. Repeat steps 6.3.2, 6.3.3 and 6.3.5 combining the filtrate with the initial filtrate in the volumetric flask. The same filter aid prepared and used in step 6.3.4 for filtering the initial extract should also be used for the second extract.

6.3 7 Rinse the probe with 5 to 10 mL of methanol and collect the rinsate in the centrifuge bottle. Filter the rinsate through the Celite and collect in the same volumetric flask.

Note: The total volume of methanol in the flask must not exceed 135 mL or breakthrough during the subsequent solid-phase extraction step may occur.

6.3.8 To the volumetric flask, add 65 mL of reagent grade water. Adjust the final volume to a final volume of 200-mL with methanol. Invert several times to ensure homogeneity. This solution will consist of 70% or less methanol and approximately 30% or greater water.

6.4 Solid-Phase Extraction

6 4 1 For each sample, prepare a Bakerbond C_{18} SPE (1000 mg) column by rinsing the column with 2 to 3 column volumes of methanol followed by 2 to 3 column volumes of reagent-grade water. Ensure that the column does not go to dryness until step 6.4.4.

6.4.2 Using a Class A volumetric pipet, transfer a portion of the extract (from step 6.4 1) to the column In order to achieve the specified LOQs, a 20-mL aliquot of the extract is required.

6.4 3 Using a low vacuum, allow the sample to pass through the C_{18} column. When the sample just reaches the top of the C_{18} packing, add 5 mL of CH_3OH/H_2O, 70:30 (v:v) and continue elution. Successively add two additional 5 mL rinses of CH_3OH/H_2O, 70:30 (v:v). Discard all eluants.

6 4.4 Allow the column to elute to dryness and leave under vacuum for approximately 15 minutes in order to remove residual water.

Note: Increase vacuum as necessary. It is important that the column be allowed to dry completely under vacuum since residual water will slow the subsequent evaporation process and if not removed, will inhibit the formation of the anhydro-derivatives.

6 4 5 Volumetrically add 5 mL of 100% methanol to the column and elute into a 15-mL, graduated, centrifuge tube.

6.4 6 Place the centrifuge tube in a waterbath maintained at approximately 40°C and evaporate the extract to dryness under a gentle stream of nitrogen

Note. Ensure that no water is present in the centrifuge tube prior to derivatization. If a small quantity of water remains following evaporation, it may be azeotroped by addition of absolute ethanol followed by evaporation under nitrogen.

6.5 Separation of Milbemectin and its Potential Degradation Products

6 5 1 For analysis of total M A_3 and M.A_4 residues, proceed to section 6.6.

6.5.2 For fractionation of M.A_3/M.A_4 from 8,9Z-M.A_3/8.9Z-M.A_4, proceed to section 6 7

6.5 2 For analysis of M.A_3, M.A_4, 27-hydroxy-M.A_3, 27-hydroxy-M.A_4, 27-keto-M.A_3 and 27-keto-M.A_4, proceed to section 6.8.

6.6 Derivatization

6 6.1 To the centrifuge tube, add 1.00 mL of 0.5M triethylamine (TEA) in benzene and 100 µL of trifluoroacetic anhydride Vortex the tube for a few seconds to ensure mixing Place the uncapped tube in a water bath maintained at approximately 40°C and allow the reaction to proceed for thirty minutes.

6.6.2 Remove the tube from the water bath and add 50 µL of 100% triethylamine. Vortex the solution for a few seconds.

6.6.3 Return the tube to the 40°C water bath and evaporate the solution under a gentle stream of nitrogen.

Note: The solution will not evaporate to dryness due to the presence of triethylamine. The final volume should be approximately 0.2 mL.

6.6.4 Adjust the final volume of each sample (using the graduations on the centrifuge tube) with CH_3CN:H_2O, 95:5 (v:v) Vortex the solution for a few seconds and transfer a portion to an autosampler vial for analysis.

Note: The final volume will be dictated by the residue level present. In order to achieve an LOQ of 1.00 µg a.i./Kg, a final volume of 5.00 mL is required when the low standard is 0.500µg a.i./L.

6 7 Separation of M.A_3/M.A_4 and 8,9Z-M.A_3/8,9Z-M.A_4

An aliquot of the reconstituted residue resulting following drying of the eluant of the solid-phase column clean up may be fractionated to separate parent milbemectin and its photoproducts using the following procedure.

6.7.1 Dissolve the residue (from step 6.4.6) in a centrifuge tube by volumetrically adding 1.00 mL of a solution of n-hexane:2-propanol, 99:1, (v:v). Vortex the centrifuge tube for approximately 30 seconds.

6.7.2 If determination of the total milbemectin residue is desired, an aliquot of the sample may be taken for subsequent derivatization/analysis with an additional aliquot retained for determination of the individual components For analysis of total residues, volumetrically transfer 250 µL of the solution to a 15-mL graduated centrifuge tube, evaporate to dryness under a gentle stream of nitrogen and derivatize by following steps 6 6.1 through 6.6.4.

6.7.3 To separate M A_3/M.A_4 from the photoproducts 8,9Z-M.A_3/8,9Z-M.A_4, transfer an aliquot of the reconstituted dried eluant to an HPLC autosampler vial for injection and fractionation using normal phase HPLC

6.7.4 Ensure that the HPLC column is equilibrated and that the instrument is fully operational. Chromatographic conditions for the separation of M.A_3/M.A_4 from 8,9Z-M.A_3/8,9Z-M.A_4 are presented in Section 7 of this method.

6.7.5 Prepare a reference standard in n-hexane:2-propanol, 99:1, (v:v) that contains M.A_3, M.A_4, 8,9Z-M.A_3 and 8,9Z-M.A_4 at such a level that the components can be detected by UV using the supplied operating conditions Inject this standard to ensure retention time stability.

6.7 6 Identify the retention times of the four components. Using the chromatographic conditions summarized in Section 7 of this method., the retention time of the photoproducts should be in the range of 8 to 12 minutes; the parent components in the range of 12 to 16 minutes. Retention ranges must be confirmed daily.

6.7.7 Inject samples and collect fractions as follows. Define the time range for the photoproducts and parent components such that the column eluant is collected well before and after the peaks of interest. Samples may be collected manually or with the use of an automated fraction collector.

6.7.8 Inject the reference standard after chromatographing the sample set to ensure retention time stability was maintained.

6.7.9 Evaporate the eluant fractions to dryness under a gentle stream of nitrogen. Proceed with derivatization, as presented in Sections 6.6.1 through 6.6.4.

6.8 Fractionation of M.A_3/M.A_4, 27-hydroxy-M.A_3/27-hydroxy-M.A_4 and 27-keto-M.A_3/27-keto-M.A_4

An aliquot of the reconstituted residue resulting following drying of the eluant of the solid-phase column clean up may be fractionated to separate parent milbemectin, the 27-hydroxy metabolites and the 27-keto metabolites using the following procedure.

6 8.1 Dissolve the residue (from step 6.4.6) in the centrifuge tube by volumetrically adding 5.00 mL of a solution of n-hexane:2-propanol, 99:1, (v:v). Vortex the centrifuge tube for approximately 30 seconds.

6.8.2 For analysis of M.A_3, M.A_4, 27-hydroxy-M.A_3 and 27-hydroxy-M.A_4, volumetrically transfer 1000 µL of the reconstituted solution to a second graduated centrifuge tube.

6.8.3 Evaporate the 1000 µL of solution to dryness under a gentle stream of nitrogen. Proceed with derivatization, as presented in Sections 6.6.1 through 6.6.4.

6 8.4 For analysis of 27-keto-M.A_3 and 27-keto-M.A_4, evaporate the remaining 4 mL of extract to dryness under a gentle stream of nitrogen. Reconstitute to volume with n-hexane:2-propanol, 99.1 (v:v) and submit samples to analysis

by normal-phase HPLC. Chromatographic conditions are presented in section 7.3.

6.9 HPLC Determination

Concentrations of residues that have been converted via derivatization to their anhydro derivatives are determined using HPLC with fluorescence detection. Concentrations of the keto metabolites are determined using HPLC with UV detection. Complete chromatographic conditions are presented in Section 7.0.

7.0 CHROMATOGRAPHIC CONDITIONS

7.1 HPLC Conditions for the analysis of Anhydro-M.A_3, Anhydro-M.A_4, Hydroxy M.A_3 and Hydroxy M.A_4 Derivatives

Instrument:	Hewlett Packard Model 1090 High Performance Liquid Chromatograph
Detector:	Jasco Model FP-920 Fluorescence Detector
Analytical Column:	YMC-Pack ODS-AM 250 mm x 4.6 mm, 3-µm particle size
Flow Rate:	1.0 mL/min
Column Temperature:	40°C
Mobile Phase Composition:	Solvent A: 20% CH_3CN: 80% H_2O: 0.1% H_3PO_4 Solvent B: 98%CH_3CN: 2% H_2O: 0.1% H_3PO_4

Time (min)	%A	%B
0.01	45	55
1.00	45	55
15.00	0	100
25.00	0	100
25.10	45	55
30.00	45	55

Injection Volume:	100 µL
Retention Times of Anhydro-M.A_3	Approximately 20.8 minutes
Anhydro M.A_4	Approximately 22.5 minutes
27-Hydroxy M.A_3	Approximately 15.5 minutes
27-Hydroxy M.A_4	Approximately 16.8 minutes
Excitation/Emission Wavelength:	360/460 nm

7.2 Normal Phase HPLC Conditions for the Fractionation of 8,9Z-M.A_3, 8,9Z-M.A_4, M.A_3 and M.A_4

Instrument:	Hewlett-Packard Model 1090 High Performance Liquid Chromatograph
Detector:	Diode Array Detector
Analytical Column:	Phenomenex LUNA Silica 250 mm x 4.6 mm, 5-µm particle size

Flow Rate:	2.0 mL/min
Column Temperature:	40°C
Mobile Phase Composition.	99:1, Hexane : 2-Propanol (v:v)
Injection Volume:	250 µL
Retention Times of $M.A_3$	Approximately 20.7 minutes
$M.A_4$	Approximately 18.3 minutes
8,9Z-$M.A_3$	Approximately 13.6 minutes
8,9Z-$M.A_4$	Approximately 13.0 minutes
Primary Analytical Wavelength:	244 nm

7.3 Isocratic HPLC Operational Parameters For The Analysis of Anhydro-$M.A_3$ and Anhydro-$M.A_4$

Instrument:	Hewlett Packard Model 1090 High Performance Liquid Chromatograph
Detector:	Jasco Model FP-920 Fluorescence Detector
Analytical Column:	HP ODS Hypersil 200 x 4.6 mm, 5 µm
Flow Rate:	1.0 mL/min
Column Temperature:	40°C
Mobile Phase Composition:	95:5 Acetonitrile : Water (v:v)
Injection Volume:	100 µL
Retention Times of Anhydro-$M.A_3$	Approximately 7.4 minutes
Anhydro-$M.A_4$	Approximately 8.6 minutes
Excitation/Emission Wavelength:	360/460 nm

7.4 Normal Phase HPLC Conditions for the Analysis of 27Keto-$M.A_3$ and 27Keto-$M.A_4$

Instrument:	Waters Model 1090 High Performance Liquid Chromatograph
Detector:	Waters Model 486 Detector
Analytical Column:	YMC-Silica 250 mm x 2.0 mm, 5-µm particle size
Flow Rate:	1.0 mL/min
Column Temperature.	40°C
Mobile Phase Composition:	Solvent A: 99% Hexane 1% IPA Solvent B: 95% Hexane 5% IPA

Time (min)	%A	%B
0.01	100	0
1.00	100	0
14.00	0	100
14 10	100	0
18.00	100	0

Injection Volume:	200 µL
Retention Times of 27Keto-$M.A_3$	Approximately 10.0 minutes
27Keto-$M.A_4$	Approximately 9.5 minutes
Primary Analytical Wavelength:	280 nm

8.0 CALIBRATION

Calibration standards containing M.A_3, M A_4, 27-hydroxy-M A_3 and 27-hydroxy-M A_4 were prepared by fortifying dry 15 mL graduated centrifuge, evaporating the solvent to dryness under a gentle stream of nitrogen and derivatizing according to sections 6.6.1-6 6 4 These standards yielded concentrations ranging from 0.350-5.00 µg a.i./L. Linear regression equations were generated using the respective concentrations of the calibration standards versus peak area responses of the anhydro-M.A_3, anhydro-M.A_4, anhydro-27-hydroxy-M.A_3, and anhydro-27-hydroxy-M.A_4 derivatives, as follows

$$\text{Concentration (µg a.i./Kg)} = \frac{\text{(Peak area - y-intercept) x dilution factor}}{\text{Slope}}$$

Four representative calibration curves for the parent and the 27-hydroxy components for a standard range of 0.350-5.00 µg a i./L are presented in Figure 4, 5, 6 and 7

Calibration standards containing 27-keto-M A_3 and 27-keto-M.A_4 at concentrations ranging from 4.00-25 0 µg a.i./L were fortified directly into 100 mL volumetric flasks containing 99.1 hexane IPA (v v). Two representative calibration curves for the 27-keto derivatives are presented in figures 8 and 9

9.0 CALCULATIONS

The concentration of M.A_3, M.A_4, 8,9Z-M A_3, 8,9Z-M A_4, 27-hydroxy-M.A_3, 27-hydroxy-M.A_4, 27-keto-M.A_3 and 27-keto-M.A_4 (milbemectin component) in samples is determined by substitution of the peak area response into the regression equation obtained from calibration standards

Milbemectin component(µg a.i /Kg) = [(Peak Area - Y-Intercept)/Slope]*Dilution Factor

where the Dilution Factor adjusts for the dilution and/or concentration of sample by the method

$$\text{Dilution Factor} = \frac{V_{f2}}{V_{i2}} \times \frac{V_{f1}}{M_i}$$

Where:

M_i= Initial mass of soil extracted
V_{f1}= Final extracted volume
V_{i2}= Volume of extract on the c_{18} column
V_{f2} = Final volume

Recoveries for fortified samples are calculated as follows.

$$\text{Percent Recovery} = \frac{\text{Measured milbemectin component (µg a i./Kg)}}{\text{Nominal milbemectin component (µg a.i /Kg)}} \times 100$$

An example calculation using a sample fortified at 10.0 µg a.i./Kg with $M.A_3$ follows:

where Peak Area = 517 44684
Y-Intercept = 7.44679
Slope = 221.6711
Dilution factor = 4.00

$$\text{Dilution Factor} = \frac{5.00}{10.0} \times \frac{200}{25.0} = 4\ 00$$

$$\text{Milbemectin in sample (µg a.i./Kg)} = \frac{(517.44684 - 7.44679)}{221.6711} \times 4.00$$

$$= 9.20\ \text{µg a.i./Kg}$$

$$\text{Percent Recovery} = \frac{9.20\ \text{µg a.i./Kg milbemectin}}{10.0\ \text{µg a.i./Kg milbemectin}} \times 100$$

Percent Recovery = 92.0%

Representative chromatograms of low and high-level calibration standards of anhydro-$M.A_3$ and anhydro-$M.A_4$ are shown in figures 12 and 13 respectively.

Table 1

Typical Gradient HPLC Conditions For The Analysis of $M.A_3$, $M.A_4$, 27-Hydroxy $M.A_3$ and 27-Hydroxy $M A_4$

INSTRUMENT:	Waters Model 2690 High Performance Liquid Chromatograph with a Jasco FP-920 Detector
DATA ACQUISITION:	Millenium version 2.15
ANALYTICAL COLUMN.	YMC-Pack ODS-AM Column (250 mm x 4.6 mm, 3 µm particle size)
OVEN TEMPERATURE:	40°C
FLOW RATE.	1.0 mL/minute
MOBILE PHASE:	Solvent A: 20% Acetonitrile : 80% Water : 0.1% Phosphoric Acid Solvent B: 98% Acetonitrile : 2% Water : 0.1% Phosphoric Acid

Time (min)	%A	%B
0.01	45	55
1.00	45	55
15.00	0	100
25 00	0	100
25.10	45	55
30.00	45	55

INJECTION VOLUME:	100 µL
RETENTION TIME:	$M.A_3$: ~ 20.8 $M A_4$: ~ 22.5 27-Hydroxy $M.A_3$: ~15 5 27-Hydroxy $M.A_4$: ~16.8
FLUORESCENCE WAVELENGTH:	Excitation λ. 360 nm Emission λ: 460 nm

Table 2

Typical Normal Phase HPLC Conditions For The Fractionation of 8,9Z-M.A_3, 8,9Z-M.A_4, M A_3 and M.A_4

INSTRUMENT:	Hewlett-Packard Model 1090 High Performance Liquid Chromatograph with a Diode Array Detector
DATA ACQUISITION	HP Chemstation version 8.04
ANALYTICAL COLUMN:	Phenomenex LUNA Silica (250 mm x 4 6 mm, 5 µm particle size)
OVEN TEMPERATURE.	40°C
FLOW RATE.	2.0 mL/minute
MOBILE PHASE:	99.1, Hexane :2-Propanol (v:v)
INJECTION VOLUME:	250 µL
RETENTION TIME:	M.A_3 : ~20.7 minutes 8,9Z-M.A_3 : ~13.6 minutes M.A_4 ~ 18.3 minutes 8,9Z-M.A_4 : ~13 minutes
PRIMARY ANALYTICAL WAVELENGTH:	244nm

Table 3

Typical Normal Phase HPLC Conditions For The Analysis of 27-Keto M.A_3 and 27-Keto M.A_4

INSTRUMENT	Waters Model 1090 High Performance Liquid Chromatograph with a Waters Model 486 Detector
DATA ACQUISITION.	Millenium version 2.15
ANALYTICAL COLUMN	YMC Silica (250 mm x 2.0 mm, 5 µm particle size)
OVEN TEMPERATURE.	40°C
FLOW RATE	1.0 mL/minute
MOBILE PHASE	Solvent A: 99% Hexane : 1% IPA Solvent B: 95% Hexane : 5% IPA

Time (min)	%A	%B
0.01	100	0
1.00	100	0
14.00	0	100
14.10	100	0
18.00	0	0

INJECTION VOLUME:	200 µL
RETENTION TIME:	27-Keto M.A_3 : ~10.0
	27-Keto M.A_4 . ~9.5
PRIMARY ANALYTICAL WAVELENGTH:	280 nm

Figure 1 Analytical method flow chart for the analysis of Milbemectin and its metabolites in soil.

METHOD OUTLINE FOR THE PROCESSING OF MILBEMECTIN AND ITS METABOLITES IN FIELD SOILS

1. Weigh 25 grams of soil into a tared container or directly into a tared glass centrifuge tube.
2. If necessary, transfer the sample into a centrifuge tube. Fortify QC samples with the appropriate M.A_3/M.A_4, 8,9Z-M.A_3/8,9-ZM.A_4, 27-hydroxy M.A_3/27-hydroxy M.A_4 or 27-keto M.A_3/27-keto M.A_4 stock solutions.
3. To each centrifuge tube, add 50 mL of methanol.
4. Using a sonic disrupter, disrupt for approximately 5 minutes.
5. Centrifuge the mixture at 2500 rpm for 5 minutes.
6. Gently remove the sample from the centrifuge and vacuum filter the supernatant, using a glass fiber filter (containing approximately 10 grams of Celite 545 added as a slurry in water) placed on the sintered glass frit. Collect the filtrate in a 200-mL, class A volumetric flask.
7. To the centrifuge tube, add 50 mL of methanol. Swirl well or vortex briefly to loosen the compacted soil. Repeat steps 4-6, combining the extracts in the volumetric flask
8. Rinse the filter and glassware appropriately with methanol. Add 65.0 mL of NanoPure water to the volumetric flask and adjust the flask to the 200-mL line using methanol.
9. Prepare a Bakerbond 1 gram C_{18} SPE column by washing with 2-3 column volumes of methanol followed by 2-3 column volumes of NanoPure water. Do not allow the column to elute to dryness.
10. Volumetrically transfer the requisite volume of each extract to its respective column.
11. Drain the solvent to just above the surface of the C_{18} packing material Wash the column with three 5 mL portions of 70% CH_3OH . 30% H_2O. Discard the eluate. Allow the column to dry under vacuum for approximately 15 minutes.
12. To the column, add 5 mL of methanol and elute the residues into a 15-mL graduated centrifuge tube
13. Place the centrifuge tube in a water bath maintained at 40°C. Evaporate the solvent to dryness under a gentle stream of nitrogen.

14 Reconstitute the milbemectin residues in the requisite volume of 1% isopropanol (IPA)·99% hexane Vortex each tube for approximately 30 seconds.

15 A. For analysis of 8,9Z M.A_3 8,9Z M.A_4, transfer the reconstituted extract to an autosampler vial and fractionate using normal phase HPLC. Following evaporation (under nitrogen) of the collected fractions, derivatize the residues according to steps 16-18. See Figure 2.

B. For analysis of M.A_3/M.A_4, 27-hydroxy M.A_3/27-hydroxy M.A_4, volumetrically transfer an aliquot of the reconstituted solution (A) to a second centrifuge tube (B). Evaporate the aliquot (B) to dryness and proceed with step 16.

C. For analysis of 27-keto M.A_3 and 27-keto M.A_4, evaporate (under nitrogen) the remaining solution (A) to dryness and reconstitute to volume with 1% isopropanol (IPA)·99% hexane Vortex each tube for approximately 30 seconds. Analyze by normal phase HPLC.

16. To the dry centrifuge tubes (B), add 1 mL of 0.5M triethylamine (TEA) in benzene and 100 µL of trifluoroacetic anhydride Vortex each tube for a few seconds. Place the tubes in a waterbath (maintained at 40°C) for thirty minutes.

17. Remove the tubes from the waterbath and add 50 µL of 100% TEA. Vortex the solution for a few seconds. Return the tubes to the waterbath and evaporate the solutions under a gentle stream of nitrogen. The solutions will not evaporate to dryness; the final volume achieved should be approximately 200 µL

18. To each sample, adjust the final volume (using the graduations on the centrifuge tube an/or transferring to volumetric flasks) with 95% CH_3CN: 5% H_2O. Vortex the solutions and transfer the final extracts to autosampler vials for analysis.

Figure 2 Analytical method flow chart for the fractionation of the parent and photoproducts of milbemectin

METHOD OUTLINE FOR THE FRACTIONATION OF M.A$_3$, M.A$_4$, 8,9Z-M.A$_3$ AND 8,9Z-M.A$_4$ IN FIELD SOILS

1. Follow method outline through #14. (Figure 1)

2. Transfer reconstituted extract to an auto sampler vial and fractionate using normal phase HPLC.

3. Fractionation· set up HPLC. Test inject a standard 2 times to find the correct retention times.
   a. Once the retention times are established, start the sequence.
   b For each sample, collect manually in individual labeled graduated centrifuge tubes (15 mL) the photoproducts (earlier retention time) and the parent material (later retention time). i.e.; If the sample set consists of 10 samples then 20 centrifuge tubes will be needed.

4. Evaporate all fractions under nitrogen and derivatize according to the method outline (Steps 16 through 18, Figure 1.)

Flow Chart for Processing of Milbemectin and Potential Metabolites in Soil

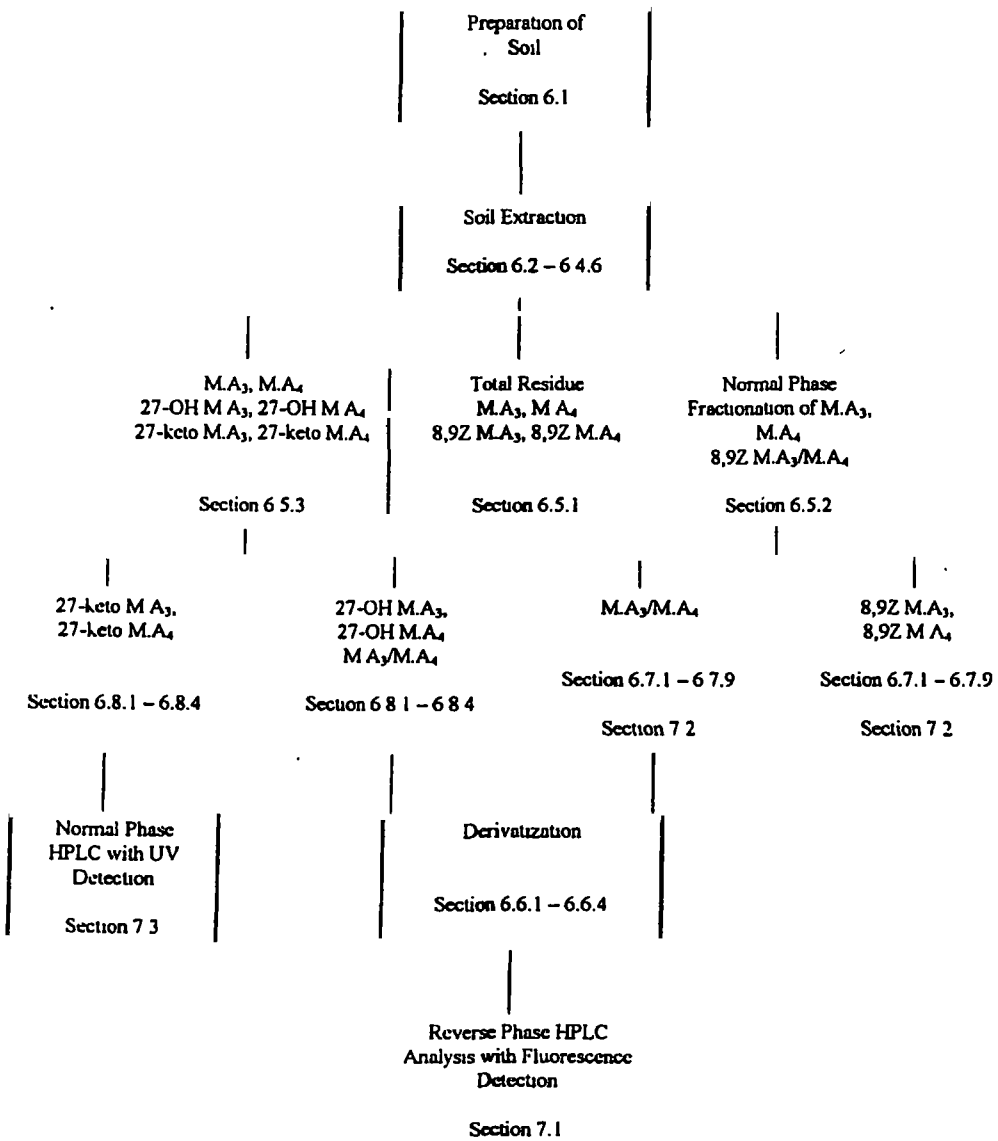


Figure 3 Flow chart for Processing of Milbemectin and Potential Metabolites in Soil