I. INTRODUCTION/SUMMARY

A. Scope

This method is used for the determination of NOA-422601 (Avermectin B1a), NOA-421704 (Avermectin B1b), and NOA-427011 (8,9-Z Avermectin B1a) in soil. The analytes are separated by high performance liquid chromatography (HPLC) with triple quadrupole mass spectrometry detection (LC-MS/MS). A pneumatically- and heat-assisted electrospray interface is used to introduce the HPLC effluent into the mass spectrometer. The analytes are detected in the Multiple Reaction Monitoring (MRM) mode by passing the positive molecular ion through quadrupole 1 (Q1), inducing fragmentation in the collision cell (Q2), and then monitoring a characteristic product ion fragment with quadrupole 3 (Q3). The chemical structures, chemical names, and Chemical Abstracts Registry numbers of the analytes are presented in Figure 1.

The analytical method was validated with a Limit of Quantification (LOQ) of 0.5 ppb for all analytes in soil. The Limit of Detection (LOD), defined as the lowest standard concentration analyzed, was 0.1 ng/mL.

B. Principle

A 10-gram subsample of soil is extracted twice with 70% (v/v) acetonitrile/water at room temperature using mechanical agitation. The sample is centrifuged and filtered with extracts combined. The organic content is removed via rotary evaporation. Acetonitrile is added to the aqueous extract and ammonium hydroxide is added to make the sample basic, and the sample is then passed through a Waters HLB SPE column. The analytes are eluted using methylene chloride. Methylene chloride is removed via rotary evaporation. The final residue is dissolved in acetonitrile/water and analyzed by LC-MS/MS. A flow diagram for the soil method is presented in Figure 2.

II. MATERIALS AND METHODS

A. Apparatus

1.0 Balance, analytical (Mettler BB2400) or equivalent.

2.0 Beaker, glass, 150-mL (Fisher cat. #02-539J) or equivalent.

3.0 125 mL Bottle, wide mouth (Fisher cat. #02-896-2C) or equivalent, appropriate size for extraction/centrifugation of the soil samples.

4.0 Centrifuge, Sorvall Superspeed RC5 (DuPont Instruments cat. #55228-9) or equivalent, with 6-place GSA rotor head (DuPont, Sorvall GSA cat. #08136) or equivalent.

5.0 Cylinder, graduated, 100-mL (Fisher cat. #08-551D) or equivalent.

6.0 Extraction column reservoir and adapter, (J. T. Baker #7120-03 and #7122-00, respectively) or equivalent.

7.0 Filter paper, 9-cm circles, Whatman GF/A (Cat. #1820 090) or equivalent.

8.0 Flasks, round bottom, 500-mL (Fisher cat. #09-552C) or equivalent.

9.0 Buchner Funnel, 100-mm (Fisher cat. #10-356D) or equivalent.

10.0 Mechanical shaker, orbital (Fisher cat. #15-456-6) or equivalent.

11.0 Mixer, Vortex-Genie 2 (Fisher cat. #12-812) or equivalent.

12.0 Pasteur pipet (Fisher cat. #13-678-7C) or equivalent.

13.0 N-Evap Evaporator, Organomation Model 112 or equivalent

14.0 Pipets, glass, class A certified, assorted volumes. These pipets are used when an exact addition of liquid is required (i.e., final addition of solvent to samples).

15.0 Pipetters, Oxford BenchMate adjustable, 40-200 µL volume range (Fisher cat. #21-231), 200-1000 µL volume range (Fisher cat. #21-229) or equivalent. (Note: These adjustable pipetters may only be used for addition of liquid where an exact volume added is not critical, i.e., addition of acid or base.)

16.0 Rotary evaporator, Buchi (Fisher cat. #09-548-105F) or equivalent.

17.0 Solid phase extraction (SPE) columns: Waters Oasis HLB, 0.2 gram/6 mL capacity/volume (Waters cat. #WAT106202).

18.0 Tube, Vacuum Connecting Adapter, Adrich (cat. no. Z12,240-8) or equivalent

19.0 Vacuum manifold, J. T. Baker #Spe-12G column processor or equivalent.

20.0 Vials, clear or amber, 1.5-mL with screw top caps (National Scientific Company cat. #C4013-15A) or equivalent.

21.0 Vials, clear, 40.7-mL (Fisher cat. #3 338 L) or equivalent.

B. Reagents and Analytical Standards

All reagents and polypropylene glycols are stored at room temperature. Solid analytical standards are stored in a freezer (temperature <-10°C).

1.0 Acetonitrile, HPLC grade (Fisher cat. #A998-4) or equivalent.

2.0 Ammonium formate, certified ACS grade (Fisher cat. #A666-500) or equivalent.

3.0 Ammonium hydroxide, certified ACS grade (Fisher cat. #A669S-500) or equivalent.

4.0 Extraction solvent : 70% Acetonitrile in water. Mix 300 mL of purified water and 700 mL of acetonitrile.

5.0 Methanol, HPLC grade (Fisher cat. #A452-4) or equivalent.

6.0 Methylene chloride, HPLC grade (Fisher cat. #D150SK-1) or equivalent.

7.0 Polypropylene glycol, M.W. 425 (Aldrich cat. #20,230-4).

8.0 Polypropylene glycol, M.W. 1000 (Aldrich cat. #20,232-0).

9.0 Polypropylene glycol, M.W. 2000 (Aldrich cat. #20,233-9).

10.0 PPG tuning solution (for mass calibration of the LC/MS system). Dissolve 0.0014 grams of PPG 425, 0.0100 grams of PPG 1000, 0.0400 grams of PPG 2000, and 0.0126 grams of ammonium formate in 50 mL of methanol, 50 mL water, and 0.1 mL of acetonitrile. Mix well. Store refrigerated in an amber bottle.

11.0 Sample diluent: 50% acetonitrile in water. Mix 500 mL of acetonitrile with 500 mL of purified water.

12.0 SPE rinse solution: 25% acetonitrile in water. Mix 250 mL of acetonitrile with 750 mL of purified water.

13.0 SPE basic rinse solution: 25% acetonitrile in water, 0.1% in ammonium hydroxide. Mix 250 mL of acetonitrile, 750 mL of purified water, and 1 mL of ammonium hydroxide.

14.0 Test analytes tuning solution, 1 ng/µL. Mix one volume of a 10 ng/µL mixed solution of analytes in acetonitrile with nine volumes of 70% methanol/water. Store at refrigerated or frozen temperature.

15.0 Water, HPLC grade, purified in-house with a HYDRO™ purification system or equivalent.

16.0 70% methanol in water. Mix 700 mL of methanol with 300 mL of purified water.

17.0 NOA-422601, NOA-421704, and NOA-427011, Syngenta Crop Protection, Inc., P. O. Box 18300, Greensboro, NC 27419-8300.

C. Safety and Health

Whereas most of the chemicals used and analyzed for in this method have not been completely characterized, general laboratory safety is advised (e.g., safety glasses, gloves, etc. should be used). Ammonium hydroxide is an irritant and should be used in a well-ventilated area (i.e., a fume hood).

D. Analytical Procedure

Note: All glassware, including the bottles for extraction, should be thoroughly cleaned and followed with a rinse of acetonitrile or methanol prior to use. The analysis system is very sensitive and may detect contamination from previous samples if all glassware and extraction bottles are not properly cleaned prior to each use.

1.0 Soil

(Note: Samples must be homogenized prior to analysis using suitable sample preparation techniques.)

1.1 Weigh and record 10.0 ± 0.1 g of soil sample into a plastic extraction bottle.

1.2 Sample fortification, if required for this particular sample, is to be done at this time (refer to Section II.K.2.0).

1.3 Add 100 mL of extraction solvent (70% acetonitrile/water) to the sample.

1.4 Place the sample on an mechanical shaker and agitate the sample at high speed for thirty minutes at room temperature.

1.5 Centrifuge the sample at approximately 8500 RPM for 7 minutes, or at an alternate speed and time if the results are considered satisfactory.

1.6 Pass the extract through a piece of filter paper in a buchner funnel under vacuum with the aid of a vacuum connecting adapter tube into a roundbottom flask. When all of the extract has passed through the filter paper, rinse the filter paper with approximately 10 mL of methanol or acetonitrile, using a squirt bottle.

1.7 Repeat the extraction adding 100 mL of the extraction solvent (70% acetonitrile/water) to the sample and repeat Steps 1.4 through 1.6, adding the second extract to the first contained in roundbottom flask, from Step 1.6.

1.8 Remove the organic content from the sample until only aqueous remains via rotary evaporation with a water bath temperature of approximately 35-40°C. The volume of aqueous remaining should be approximately 50 mL.

1.9 Add 17 mL of acetonitrile to the sample to dissolve any residues adsorbed to the glass surface of the round bottom flask. Swirl to mix the contents.

1.10 Basify the sample by adding 0.1 mL of ammonium hydroxide.

1.11 Attach the SPE columns (Waters Oasis HLB, 0.2 g/6mL) to an appropriate SPE vacuum manifold. Condition the SPE columns by first passing one column volume (approximately 6 mL) of methanol through the column followed by one column volume of 25% acetonitrile in water. Do not permit the column to dry out before adding the sample.

1.12 Load the samples onto the SPE columns. (It is recommended to attach an appropriate-sized reservoir to the SPE columns during the loading step.) Adjust vacuum so that a fast drip rate is obtained. (An appropriate rate is about 1 drop per second.) Discard the eluate.

1.13 Rinse the sample container with approximately 5 mL of 25% acetonitrile/water, 1% in ammonium hydroxide. When all of the sample from Step 1.12 has passed through the SPE column, pass this rinsate through the column. Discard the eluate.

1.14 When the rinsate from Step 1.13 has passed through the column, rinse the column with one column volume of 25% acetonitrile in water. Discard the eluate.

1.15 Dry the column by increasing the vacuum to maximum and pulling air through the SPE column for approximately five minutes.

1.16 Elute analytes from SPE by passing one column volume (approximately 6 mL) of methylene chloride through the column into a 40.7 mL vial.

1.17 Remove methylene chloride from the sample using a N-evap Evaporator until dry with a water bath temperature of approximately 35-40°C.

1.18 Pipette 5.0 mL of acetonitrile into the vial. Place on a vortex mixer for approximately thirty seconds to dissolve the residue.

1.19 Pipette 5.0 ml of water into the vial. Place on a vortex mixer for approximately thirty seconds to dissolve the residue.

1.20 Additional dilution, if necessary, may be made using 50% acetonitrile in water. The sample should be stored refrigerated (<5°C) until the time of analysis. Samples should be stored frozen for long term storage (> 2 weeks).

1.21 Analyze the sample using LC-MS/MS.

E. Instrumentation

1.0 Description and Operating Conditions: HPLC

See Table 1 for a description of the reversed phase HPLC system and chromatographic conditions used for the analysis.

2.0 Description and Operating Conditions: LC-MS/MS

NOA-422601, NOA-421704, and NOA-427011 are monitored as positive ions. Triple quadrupole analysis (MS/MS) of the unique precursor/ product ion pair is suggested to achieve the low method LOQ. See Table 2 for a description of the mass spectrometer instrumentation and operating conditions.

3.0 Description and Operating Conditions: LC-MS/MS Turbo Ionspray Interface

The optimized values for the turboion interface may vary with time and may need to be periodically re-optimized. With the Sciex API-3000 LC-MS/MS system, typical electrospray operating conditions are described in Table 2.

4.0 Calibration and Standardization: LC-MS/MS

4.1 Calibrate and tune the mass spectrometer prior to analyzing samples. Check the calibration and tune by infusing a standard solution of polypropylene glycols (PPG), or another suitable mass calibration solution, into the mass spectrometer using the turbo ionspray interface while monitoring positive ions. The study director, or system analyst, will determine how frequently mass calibration needs to be performed.

4.2 Determine the specific ion to monitor for each analyte by infusion of an analyte test solution (approx. 1 ng/µL in 70% methanol/water) or flow injection analysis (FIA) while scanning the Q1 quadrupole mass analyzer to find the optimum ion. For the avermectin analytes, the predominant ion is the sodium ion adduct of the molecular ion. Determine the specific product ion fragment to monitor for each analyte in the MS/MS mode by passing the characteristic precursor ion through Q1, fragmenting the ion in Q2, and scanning the resulting ion fragments in Q3. The selected product ion

chosen to monitor will depend on the intensity of the ion fragment along with the possibility that an interference also has the same fragment ion. Table 3 lists the precursor/product ion pairs that are monitored ions for each analyte. Typical MS/MS mass fragmentation spectra will be presented for each analyte in the final report.

4.3 Determine the retention time of the analytes by injecting a standard solution into the HPLC. During a series of analyses, the analyte retention time should vary no more than 2% from its mean value, on a daily basis.

4.4 Calibrate the instrument by constructing a calibration curve from detector response (chromatographic peak height or area) and the amount of analyte injected. Typical calibration data and chromatograms of calibration standards will be presented in the final report.

F. Interferences

1.0 There are no known interferences originating from the sample cleanup procedure. However, interferences can originate from impure chemicals, solvents, contaminated glassware, and the HPLC water supply.

G. Confirmatory Techniques

1.0 No confirmatory analysis procedure is included in this method. This method employs highly specific LC-MS/MS for the detection mode, coupled with the characteristic retention time observed for the analyte on the appropriate HPLC column.

H. Time Required

1.0 The sample extraction and cleanup procedure can be completed for a set of twenty soil samples in an eight-hour work day.

2.0 Each HPLC analysis requires approximately 11 minutes.

I. Modifications and Potential Problems

1.0 Contaminants from chemicals, solvents, glassware, and the HPLC water supply can interfere with the analysis. It is recommended that a reagent blank be run with an analysis set to verify that no

interferences are originating from the chemicals and reagents used in this procedure. MS techniques are very sensitive. All glassware should be solvent-rinsed before use to prevent inadvertent contamination of control or low level samples.

2.0 Analytical Method 116-00 was validated only for the soil type listed in the final method. Samples from different locations may exhibit binding or interference problems which were not observed with this sample.

3.0 "Bumping" is sometimes observed for samples during the solvent removal steps via rotary evaporation. Periodic venting of the vacuum and the use of solvent traps helps minimize inadvertent losses during these steps.

4.0 Suppression of the ion signal due to soil matrix has been seen while using LC-MS/MS detection. This effect is minimized by: (1) the SPE cleanup procedure which removes the majority of the interfering matrix, (2) the use of a relatively slow linear gradient elution, and (3) injection of a small volume of sample. Signal suppression as great as 50% has been observed for some analytes fortified into control samples when the above three steps were not taken. If matrix suppression of the analyte signal is observed, it can frequently be minimized by injecting smaller volumes of the sample into the system.

5.0 No analyte stability or solubility problems have been observed when standard solutions have been prepared and stored as detailed in Section II.J.

6.0 Long-term optimization of the LC/MS signal by infusion of a test mixture of analytes into the system will result in lingering high backgrounds for the molecular ions. While the background signals will decrease with time or cleaning of the orifice plate, it may be severe enough to affect the ability to achieve desired signal to noise ratios for lowest standards. For this reason it is highly recommended that optimizing/calibrating with analytical standards be done with dilute solutions and the optimizing/calibrating time be minimized. It is also recommended after calibrating/optimizing with test analytes, to turn the power off to the electronics, remove the ionspray interface, and thoroughly wipe clean the orifice plate using a lint-free tissue wetted with methanol. Repeat several times.

7.0 LC-MS/MS is required to achieve the method LOQ for all analytes.

8.0 Reversed phase columns from other manufacturers may be substituted for the column used in this study provided that the analyst demonstrates acceptable peak shape and sensitivity with the substituted column. The mobile phase gradient may need to be altered if a different column is used.

9.0 To achieve the soil method LOQ of 0.5 ppb, the author extracted and processed 10 g of soil, used a final sample volume of 10 mL, and injected 10 µL of sample onto the HPLC column. Matrix suppression of analyte signal may prevent injecting more sample mass to improve sensitivity.

10.0 Several injections (3-4) of samples or standards should be made into the LC/MS prior to initiation of the sample run sequence. This is necessary to stabilize analyte retention times and to stabilize the MS response to the analytes.

11.0 The product insecticide Abamectin consists of NOA-422601 (≥ 80%) and NOA-421704 (≤ 20%).

J. Preparation of Standard Solutions

All stock solutions are stored in amber bottles in a freezer (< -10°C) when not in use. Mixed standards may be stored in a freezer or refrigerated (< 5°C). No analyte stability or solubility problems have been observed in the standard solutions used in this study. The mixed standards are used for fortifications and as HPLC standards.

1.0 Prepare individual 100 ng/µL stock solutions for each analyte. Weigh approximately 10.0 mg of analyte. Determine the appropriate volume of acetonitrile to add using the equation presented below. The concentration of the analytical standard is corrected for its chemical purity.

$$V\,(mL) = \frac{w\,(mg) \times P}{C\,(ng/uL)} \times 10^3$$

Where "V" is the volume of acetonitrile needed; "w" is the weight, in mg, of the solid analytical standard; "P" is the purity, in decimal form, of the analytical standard; "C" is the desired concentration of the final solution, in ng/µL; and 10^3 is a conversion factor.

For example:

The volume required to dilute 9.9 mg of an analyte, of 98.0% purity, to a final concentration of 100 ng/µL is:

$$V\,(mL) = \frac{9.9\ mg \times 0.98}{100\ ng/uL} \times 10^3 = 97.0\ mL$$

2.0 A 1.0 ng/µL mixed standard is prepared by combination of the analyte stock solutions and dilution with acetonitrile. Pipette 1.0 mL of each analyte 100 ng/µL stock solution into a 100-mL volumetric flask and then dilute to the calibration mark with acetonitrile.

3.0 Fortification and calibration standards are prepared by subsequent dilutions of the 1.0 ng/µL solution with 50% acetonitrile/water. Fortification standards should be prepared such that no more than 1.0 mL of the fortification solution is added to a sample. (Example: For a 10-g soil sample, the addition of 0.5 mL of a 0.01 ng/µL fortification solution will result in a fortification level of 0.5 ppb.)

K. Methods of Calculation

1.0 Determination of Residues in Samples

1.1 Inject the sample solution from Step II.D.1.21 into the analysis system. The sample solution may be diluted if the analyte response exceeds the range of the calibration curve. The amount of analyte injected (ng) is determined by entering the value of the chromatographic peak height, or area, in the calibration response curve (Step II.E.4.4) and calculating the corresponding value of nanograms injected. Typical chromatograms for control and fortified control soil are presented in Figures 3-10.

2.0 Determination of Residues in Fortified Samples

Validate the method for each set of samples analyzed by including a control sample and one or more control samples fortified prior to the extraction procedure with 0.05 ppb or more of each analyte in soil.

2.1 Add an appropriate volume of a fortification solution to the sample prior to any of the cleanup steps. The total volume of the added fortification solution should not exceed 1.0 mL.

2.2 Proceed with the sample cleanup procedure.

3.0 Calculations

Calculations may be performed by computer program or manually as follows (all concentrations are based on wet weight):

3.1 Calculate the analyte concentration (in ppb) from equation (1):

$$(1)\ \text{ppb analyte} = \frac{\text{ng analyte found}}{\text{g sample injected}}$$

The mass of sample injected is calculated from equations (2) and (3), respectively.

The grams of sample injected is calculated from equation (2).

$$(2)\ \text{grams of soil injected} = g \times \frac{Vi}{Vf}$$

where, g is the grams of soil (wet weight) extracted, Vi is the volume (mL) injected onto the HPLC column, and Vf is the final volume (mL) of the cleaned-up sample (from Step II.D.1.21).

The amount (ppb) of analyte found is calculated from equation (3).

$$(3)\ \text{ppb analyte found} = \frac{\text{ng analyte found}}{\text{g sample injected}}$$

3.2 The accuracy of the method is determined by the average recovery of the analytes fortified into the test substrate. The precision is estimated by the relative standard deviation of the determined concentration.

V. TABLES

TABLE 1. HPLC SYSTEM AND OPERATING CONDITIONS

Instrumentation:

Shimadzu LC-10Atvp HPLC System

Operating Conditions:

Injection Volume: 10 µL
Mobile Phase Flow Rate: 0.3 mL/min
Column: Phenomenex Luna 5µ phenyl-hexyl 150 x 2 mm.
Mobile Phase A: Methanol
Mobile Phase B: Water

Mobile Phase Gradient Program:

Time (min.)	% A	% B
0.0	70	30
0.2	70	30
2.0	95	5
6.4	95	5
6.5	70	30
9.0	Stop	

Total Analysis Time: 11 min.

Analyte Retention Times:

NOA-421704	7.10 min
NOA-422601	7.30 min
NOA-427011	7.60 min

TABLE 2. MASS SPECTROMETRY SYSTEM AND OPERATING CONDITIONS

Instrumentation:

PE Sciex API 3000 LC-MS/MS

Software:

MacQuan Version 1.7.1

Instrument Parameters

(IS) Ion Source:	5000	(IQ2) Inter Quad 2 Lens:	-58
(TEM) Temperature:	500°C	(R02) Quad 2 Rod Offset:	-63
(OR) Orifice Plate:	90	(ST3) Stubbies:	-78
(RNG) Focusing Ring:	350	(R03) Quad 3 Rod Offset:	-68
(Q0) Quad 0 Rod Offset:	-10	(DF) Deflector:	-400
(IQ1) Inter Quad 1 Lens:	-10.9	(CEM) Channel Electron Multiplier:	2400
(ST) Stubbies (Prefilter):	-17	(NEB) Nebulizer Gas (N_2)	12
(R01) Quad 1 Rod Offset:	-12	(CUR) Curtain Gas (N_2)	8
(CAD) Collisionally Activated Dissociation Gas (N_2)			8

*Note: State file values will vary slightly from instrument to instrument. The values often will be changed slightly during instrument optimization procedures.

Data Collection

Time 0 → 4.0 minutes, pause, no data collected
Time 4.0 → 10.0 minutes, collect data

Mode: MRM on positive detection

Dwell Time: 0.30 sec per ion transition

Ion Transitions Monitored

895.5 > 751.5 NOA-422601
881.5 > 737.5 NOA-421704
895.5 > 751.5 NOA-427011

TABLE 3. TYPICAL ANALYTE MONITORING IONS: LC-MS/MS

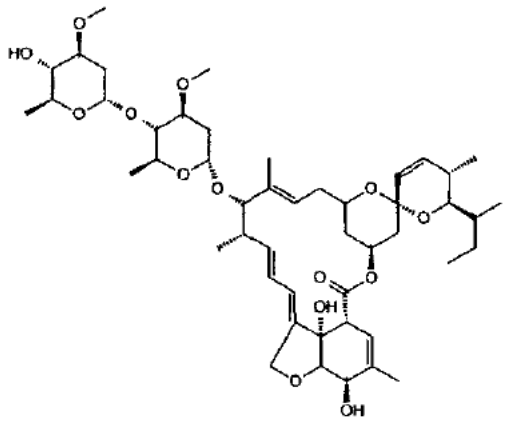
Analyte	Exact Molecular Weight	Q1 Molecular Ion*	Q3 Product Ion
NOA-422601	872.5	895.5	751.5
NOA-421704	858.5	881.5	737.5
NOA-427011	872.5	895.5	751.5

* All analytes form sodium adducts as the primary molecular ion.

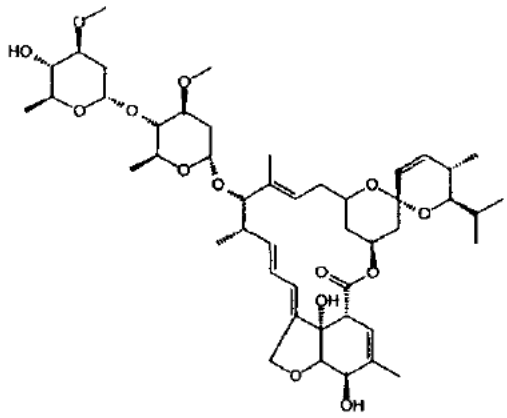
Note: The masses set for Q1 and Q3 in data acquisition should be based on the center of the observed precursor ion mass in Q1 and the center of the observed product ion mass in Q3. These masses may vary as much as 0.1-0.2 amu from the exact molecular mass obtained by manual calculations.

VI. FIGURES

FIGURE 1. CHEMICAL NAMES AND STRUCTURES

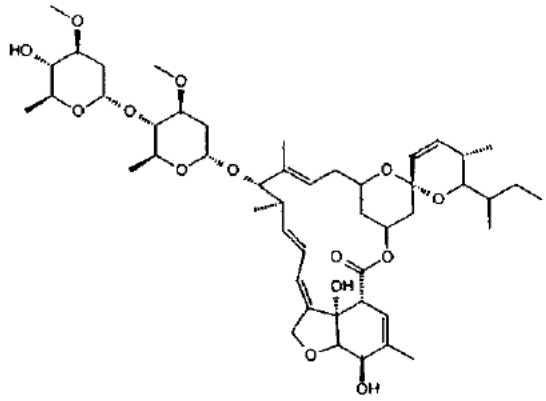


NOA-422601 (Avermectin B1a)
CAS No.: 65195-55-3



NOA-421704 (Avermectin B1b)
CAS No.: 65195-56-4

FIGURE 1. CHEMICAL NAMES AND STRUCTURES (Continued)



NOA-427011 (8,9-Z Avermectin B1a)
CAS No.: 113665-89-7

FIGURE 2. FLOW DIAGRAM FOR SOIL

Weigh 10 g sample of soil.
(Fortify, if necessary)
Extract with 100 mL of 70% acetonitrile/water for 30 minutes at room temperature using mechanical agitation.
Centrifuge/filter.
Repeat extraction with additional 100 mL of 70% acetonitrile/water.
Centrifuge/filter and combine with first extract.

↓

Remove organic content via rotary evaporation.
Add 17 mL of acetonitrile to sample.
Swirl contents to ensure analytes are not adsorbed to the glass surface.
Basify sample by adding ammonium hydroxide.

↓

Isolate the analytes by passing the sample through a Waters HLB SPE column.
Rinse column.
Apply vacuum to dry the column.
Elute the analytes with methylene chloride into a concentration tube.

↓

Remove the methylene chloride via N-Evap Evaporator.
Add 5 mL of acetonitrile to the sample.
Vortex mix to redissolve residue.
Add 5 mL water.
Vortex mix sample.
Perform additional dilution of the sample, if required.

↓

Analyze by LC-MS/MS using reversed phase HPLC.