I. SUMMARY

MK-0244 (4"-deoxy-4"epi-methylamino avermectin B1 benzoate salt) is proposed as an insecticide to be applied at low levels to various crops, including leafy vegetables and cole crops. It is a mixture of two homologs, which differ by a methylene group, defined as not less than 90% B1a and not more than 10% B1b. To monitor the environmental fate and effects of MK-0244, it is necessary to run a field study measuring the accumulation and dissipation of soil residues. Consequently, a sensitive method to monitor the residues of MK-0244 and its possible photodegradates is required. The validation of such a method is the subject of this study.

Analytical Research Method 244-93-3, "HPLC-Fluorescence Method to Determine the Residues of MK-0244 and its 8,9-Z Isomer in/on Soil," was validated according to protocol no. 3906. The method measures the residue of both the B1a and B1b components as well as a possible photodegradation product, the 8,9-Z isomer of MK-0244. No degradation product of the residue has been identified that is 10% of the application rate or above 0.01 ppm so the method only measures the parent MK-0244.

The method also was tested for ruggedness and interference. The method was determined to be rugged and tolerated variations in supplies and conditioning solvents. Abamectin, a related avermectin, does not interfere in the method either as a detectable peak or by reduced recovery of MK-0244.

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II. PROCEDURES

A. Sample Handling

The soil samples used for this method validation were collected from the fields in which the soil residue trials (protocol 3601; 001-92-6011R, 001-92-6010R and 001-93-3003R) were to be conducted. The samples were designated as methods development soil samples since they were not part of the soil sampling study scheme and were handled separately from the residue trial samples. The soil samples from each location were processed at Three Bridges Farm on August 26, 1993. The processing was varied to determine the best way of handling the soil samples, such as grinding and sieving, etc. For samples from the location of trials 001-92-6010R and 001-93-3003R, the soil was processed by grinding for 1 minute in a Cuisinart followed by sieving through 4, 12, 10 and 16 mesh sieves. The sample from the location of trial 001-92-6011R, the soil was processed by sieving through 4, 12 and 14 mesh sieves. An unprocessed sample of soil from the location 001-92-6010R was used for the ruggedness assay. After processing, the soil from the samples were transferred into individual labelled Nalgene bottles and frozen. The processed samples were shipped and frozen at Rahway until used in the validation.

R. Method

(1) Method Summary

A 10 gram sample was extracted three times with 10 mL of 1% ammonium acetate in methanol using a homogenizer. The combined extracts were evaporated to 1 mL and water and ethyl acetate were added, to partition the residue into ethyl acetate. The ethyl acetate extraction of the aqueous phase was performed twice and then the combined ethyl acetate extracts were loaded onto a prepared propylsulfonic acid (PRS) solid phase extraction column. The PRS column was eluted with 1% ammonium acetate in methanol to extract the MK-0244 and its 8,9-Z isomer (i.e., the avermectins of interest). The eluant was divided into two equal portions (splits), with one portion retained for possible reassay. The other half was evaporated under nitrogen to 1 mL. Water and ethyl acetate were added and the avermectin residue was extracted into the ethyl acetate. The salt (ammonium acetate) was left in the aqueous phase. The ethyl acetate was evaporated again and acetonitrile was added. The samples and corresponding standards (for L'649, MK-0244 B1a and/or 8,9-Z) were derivatized with trifluoroacetic anhydride in the presence of N-methylimidazole and were injected on a reverse phase HPLC with fluorescence detection. Quantitation was based on comparison with external standards. The method had a limit of detection (LOD) of 0.2 ng/g for each analyte. The lower limit of quantification (LOQ) is 0.4 ng/g for each analyte.

This method is able to distinguish as separate residues the B1a component as well as the minor B1b component of MK-0244. The 8,9-Z isomer makes the same fluorescent derivative as the parent MK-0244 and is part of the residue values for MK-0244 B1. The 8,9-Z isomer of the B1a component has been prepared and this relationship established. The 8,9-Z isomer of the B1b component can be considered to behave in an analogous manner.

(2) Fortification Procedures and Standards

The standards were prepared by measuring a known amount of the reference standard or authentic substance powder or solution and diluting with acetonitrile (ACN), as indicated in Table II.

In general, standards for quantitation or fortification were prepared from the reference standard or authentic substances cited below. The material was weighed using an analytical balance and dissolved in acetonitrile to make a concentrated stock standard solution in a volumetric flask. Working standards were prepared by diluting aliquots of the stock standard solution with additional acetonitrile in a volumetric flask. The materials were originally characterized under the USFDA Good Manufacturing Practices (21CFR211) regulations but were later characterized following the USEPA FIFRA Good Laboratory Practices regulations (40CFR160). Copies of the certificates of analyses are included in the appendix.

MK-0244 B1a (from reference standard L-656,748-052S003 containing both B1a and B1b, 93.6% and 3.8%, respectively) was used as the standard for comparison for the B1a component as well as for the B1b component. It was also used for their corresponding delta 8,9-Z isomers since the delta 8,9-Z isomer yields the same derivative as the parent MK-0244. The delta 8,9-Z isomer of the B1a component (authentic substance L-695,638-00iC001, also designated as 8,9-Z, 8,9 Z or just Z) was prepared and this relationship was established by chromatographic comparison of the derivatized authentic samples. The delta 8,9-Z isomer of the B1b component is considered to behave in an analogous manner. Consequently, the residue quantitated at the retention time of the parent derivative represents the sum of the parent plus its delta 8,9-Z isomer. The B1b and B1a components were resolved. Solutions of abamectin (L-676,863-038A003, 0.893% B1a and 0.044% B1b) were prepared for use in ruggedness testing.

Fortifications were completed by aliquoting onto the 10 grams of soil an aliquot of the standard solution(s), as indicated in the method. The aliquots were 1.00, 0.4 or 0.2 mL. The fortifications ranged from 120 ng/g of B1a to 0.4 ng/g of B1a and/or 10 ng/g to 0.4 ng/g of the 8,9-Z isomer.

Standards used to quantitate the HPLC results were prepared by aliquoting 0.10, 0.20, 0.50, 0.80 or 1.00 mL of individual standard solutions of MK-0244 B1a (#524) or 0.05, 0.30, 0.50 and 0.80 mL of the delta 8,9-Z isomer of the B1a component (#520). The resultant concentrations of the HPLC standards, following derivatization, ranged from approximately 0.25 ng/mL to 2.5 ng/mL for MK-0244 B1a and approximately 0.25 ng/mL to 4.0 ng/mL for 8,9 Z. The comparison of the quantitation of the 8,9-Z results calculated from the MK-0244 B1a curve versus the 8,9-Z curve is included in this report.

Table II Summary of Standard Solutions (Study No. 93906)

Compound	Date	Aliquot	Final Volume	Concentration	ID#
MK-0244	3/17/93	0.00531 g L-656,748-052S003	50 mL ACN	99.4 mcg/mL B1a 4.0 mcg/mL B1b	480
MK-0244	7/27/93	1.00 mL #480	200 mL ACN	497 ng/mL B1a 20 ng/mL B1b	517
MK-0244	8/10/93	2.00 mL #517	100 mL ACN	9.94 ng/mL B1a (<0.5 ng/mL B1b)	524
MK-0244	9/1/93	0.60 mL #480	50 mL ACN	1190 ng/mL B1a 48 ng/mL B1b	530
8,9-Z Isomer of MK-0244 B1a	6/25/93	0.00543 g L-695,638-001C001	50 mL ACN	101 mcg/mL 8,9-Z B1a	508
8,9-Z Isomer	7/27/93	2.00 mL #508	200 mL ACN	505 ng/mL 8,9-Z B1a	519
8,9-Z Isomer	7/27/93	4.00 mL #519	100 mL ACN	20.2 ng/mL 8,9-Z B1a	520
Abamectin	1/21/93	0.8278 g L-676,863-038A003	25 mL ACN	296 mcg/mL avermectin B1a and 15 mcg/mL avermectin B1b	468
Abamectin	1/21/93	6.76 mL #468	50 mL	40.0 mcg/mL B1a and 2.03 mcg/mL B1b	469
Abamectin	1/21/93	2.50 mL #469	50 mL	2000 ng/mL B1a and 102 ng/mL B1b	470
Abamectin	6/9/93	1.25 mL #470	50 mL ACN	50 ng/mL B1a and 2.6 ng/mL B1b	505

ACN = acetonitrile

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(3) Conditions

The following are the HPLC instruments and conditions used for this validation.

Pump:	Shimadzu LC-10AD
Solvent Selector:	FCV-11AL
Column Heater:	Jones Chromatography 7950, Set at 40 degC.
Autoinjector:	Shimadzu SIL-10A
Detector:	Shimadzu RF551 fluorescent; excitation set at 365 nm and emission set at 470 nm; Xenon lamp.
Controller:	Shimadzu SCL-10A System Controller
Integrator:	Spectra-Physics Chromjet Model 4400 Chart speed at 0.25 cm/min
Column:	ES Industries Chromegabond C-18 (150 x 3 mm; 3 micron) with guard column
Mobile phase:	93:7 Methanol:water at 0.35 mL/min

(4) Data Handling and Calculations

The samples were calculated versus the external standard curves, as mentioned above, using the peak areas at the appropriate retention time(s). A linear regression fit to the standard curve data (plotting peak area versus concentration) generated an equation which was then used to calculate the concentration of the samples. The procedure used for calculating the results was described in a memo Wehner to Egan, dated July 27, 1992 and entitled "Validation of RS/1 Procedure to Calculate Residue Results." Table III summarizes the standard curve information for each set.

The recovery of the delta 8,9 isomer was calculated versus a standard curve of its authentic substance as well as versus a standard curve of the MK-0244 B1a standard. The recovery of MK-0244 B1b was determined from a MK-0244 B1a standard curve. This was to demonstrate the use of a more limited number of standard curves for the determination of incurred residue which could contain an unknown combination of MK-0244 + delta 8,9 isomer.

(5) Deviations to the Method and Protocol

There were no deviations to Analytical Research Method 244-93-3, as described in the September 3, 1993 version, since this validation was intended to demonstrate what procedures worked and the appropriate stopping places. A finalized method description is being prepared concurrently with this report, to reflect the experiences during the validation in this study and during a similar validation conducted by an independent contract laboratory, Analytical Development Corporation, Colorado Springs, Colorado. There were no deviations to the protocol. The method and protocol are in Appendix A.

Table III HPLC Injection Set Parameters for MK-0244 Soil Method Validation 244-93-3 (Study 93906)

TABLE ID*	HPLC DATE	SLOPE	INTER-CEPT	R^2
VPS1	8SEPT 93	466300	-33310	0.9903
VPS2	10SEPT93	450700	-25740	0.9991
VPS2R	13SEPT93	429000	-18100	0.9979
VPS3	14SEPT93	423600	-7567	0.9985
VPS3S	15SEPT93	408300	3406	0.9986
VPS3SZ	15SEPT93	347100	22980	0.9976
VPS3SR	16SEPT93	400100	3446	0.9972
VPS4	23SEPT93	429200	-5188	0.9918
VPS4R	24SEPT93	406600	4162	0.9997
VPS4S	24SEPT93	406500	-9940	0.9906

*Table ID: VPS = Validation Payne Soil set number;
R = Reinjection or dilution; S = Split; Z = 8,9-Z standard curve.

HPLC Sets 4 (4R, 4S) used for interference and ruggedness testing.

R^2 = coefficient of determination for linear regression fit of line

Derived from #soilvalhplc.

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C. Assay Timing and Storage

The method validation a'so demonstrated those points in the procedure during which the samples could be stored frozen over some period of time (generally overnight or over a weekend) and maintain acceptable recovery. The data reported here reflect samples which were stored at the recommended places or not stored at all. The method description recommends the appropriate stopping points. The method description dated September 3, 1993 contained a typographical error which has been corrected in the finalized description. An appropriate stopping place is at steps 7 or 8 and not step 5, as indicated in the earlier version of the method.

In general, the validation has demonstrated that the method can be conducted on a number of samples up to 12, simultaneously. The number of samples to be assayed in a set depends on the purpose of the assay. While conducting the assays of 12 samples, including untreated control and method recovery samples, an analyst can complete the assay procedure in one day, with overnight automatic injections on the HPLC.

The stability of the MK-0244 formulation under the conditions of use is the subject of a separate study. The stability of the MK-0244 and degradate standards in solution was investigated before this method validation and there was no problem with the standard stability for the time periods over which the standard solutions were used. The freezer storage stability of the residues in soil is the subject of a separate study, still ongoing (Study number 93601), part of the field dissipation residue study.

Raw data, including chromatograms and standard curves, are presented in Appendix B by analysis set. The original analytical raw data, including chromatograms and study specific notebooks, will have been archived before this study is signed by the study director. The validation data as well as the method validation final report will be archived in the Animal Science Communication Center, presently located at Merck & Co., Inc, Metropolitan Corporate Plaza, 485 Route 1 South, Building C, Iselin, New Jersey 08830, WBC-125. This study was initiated on September 8, 1993 when the study director signed the protocol. Some preparatory work, such as preparation of the standard/authentic substance solutions and the laboratory notebook was begun before study initiation date. The study completion date will be recorded when the final report is signed by the study director.

I. INTRODUCTION

A. Background

The avermectins are a family of macrocyclic lactones produced by the soil microorganism, Streptomyces avermitilis. The major fermentation product is avermectin B1 (abamectin, MK-0936) which is a potent broad spectrum acaricide/insecticide registered for use worldwide (Campbell, 1989). Ivermectin, the 22,23-dihydro-derivative of abamectin, is chemically modified from abamectin and has found wide use as an antiparasitic agent in animals (Campbell et al., 1983). A semisynthetic second generation avermectin, 4"-deoxy-4"-*epi*-methylamino-avermectin B1 benzoate salt, or MK-0244, is being developed for its different spectrum of activity against lepidopteran larvae (Dybas et al., 1989).

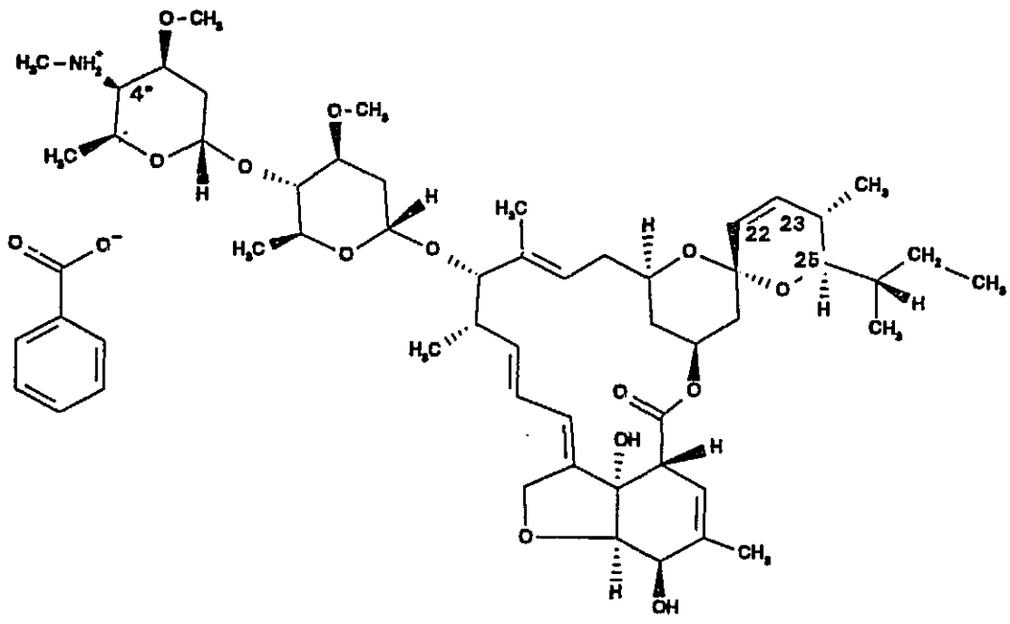
Similar to abamectin, MK-0244 is a mixture of two homologs designated B1a and B1b (Figure 1). These homologs differ by one methylene unit (CH_2) at the 25 position, where B1a contains a sec-butyl group and B1b contains an isopropyl group. The ratio of these homologs in MK-0244 is specified as being at minimum 90% B1a and at maximum 10% B1b. MK-0244 differs from abamectin in that the 4"-hydroxy group of abamectin is replaced by a 4"-epimethylamino and is isolated as the benzoate salt. The designation MK-0243 is applied to the hydrochloride salt.

Environmental fate and metabolism work using radiolabelled MK-0244 applied to soil indicates that MK-0244 is the major compound present under both anaerobic and aerobic conditions (Study 93257, "Aerobic Soil Metabolism of [^{14}C]4"-Epimethylamino-4"-Deoxyavermectin B1a Benzoate").

The avermectins are not volatile enough for gas chromatographic determinations so the chromatographic separation of choice is high pressure liquid chromatography. Methods using ultraviolet detection have not been sensitive nor selective enough to detect the low levels of the avermectins in complex matrices (Vuik, 1991; Pivnichny et al., 1983; Fox and Fink, 1985). Fluorescence derivatization, taking advantage of the inherent structure of the avermectins, has been the most successful technique (Tolan et al., 1980; Tway et al. 1981; deMontigny et al. 1990; Prabhu et al. 1991; Prabhu et al. 1992; Wehner et al. 1993) for analyzing ivermectin and abamectin.

The present method (244-93-3) is partially based on the MK-0244 total toxic residue method for vegetables (244-92-3). Its objective is to determine the residues of MK-0244 and its 8,9-Z isomer in/on soil down to sub-ppb levels.

Figure 1
Structure of MK-0244 B1a:
4"-deoxy-4"-*epi*-methylamino-avermectin B1a



B. Method Summary

A flow diagram of the method is presented in Figure 2.

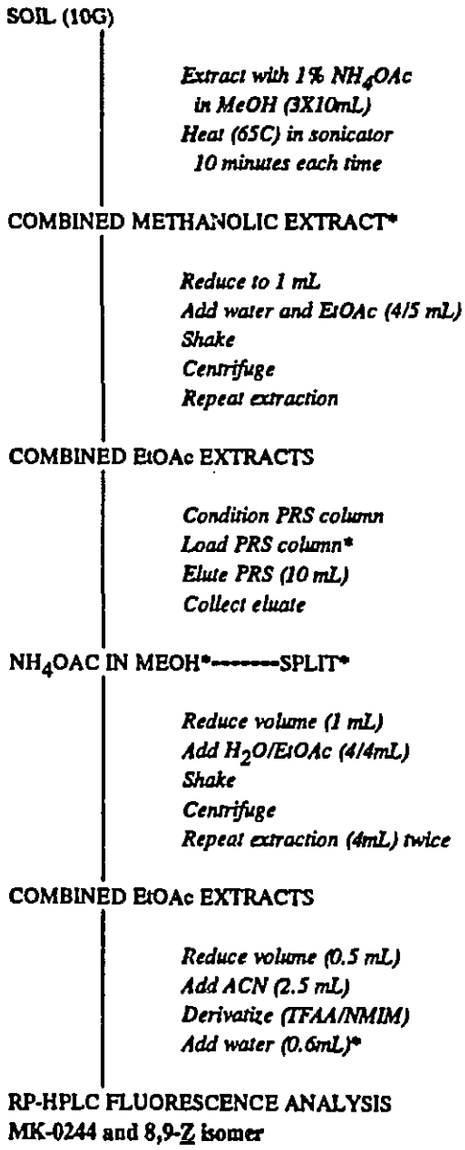
A 10 gram sample of soil is extracted three times with 10 mL of a 1% ammonium acetate in methanol solution in a heated sonicator. The combined extracts are evaporated down to 1 mL and water is added. The aqueous solution is extracted twice with ethyl acetate and the ethyl acetate is loaded onto a prepared propylsulfonic acid (PRS) solid phase extraction column.

The PRS column is eluted with 1% ammonium acetate in methanol to remove MK-0244 and its 8,9-Z isomer. The methanolic eluant is split in half. One half is retained for possible reassay and the other half is evaporated to 1 mL. Water and ethyl acetate are added and the avermectin residue is extracted into the ethyl acetate phase, leaving the ammonium acetate in the aqueous phase. The ethyl acetate is evaporated to 0.5 mL and acetonitrile is added. The samples and corresponding standards are derivatized with trifluoroacetic anhydride in the presence of N-methylimidazole (Figure 3). The derivatized samples and standards are injected on a reverse phase HPLC with fluorescence detection. Quantification is based on comparison with external standards.

This method is able to distinguish as separate residues the B1a component as well as the minor B1b component of MK-0244. As was experienced for abamectin (Arison and Flynn, 1987), the 8,9-Z isomer makes the same fluorescent derivative as the parent MK-0244 and is part of the residue values for MK-0244 B1. The 8,9-Z isomer of the B1a component has been prepared and this relationship established. The 8,9-Z isomer of the B1b component can be considered to behave in an analogous manner.

The method has a limit of detection (LOD) of 0.2 ng/g for each analyte. The lower limit of quantification (LOQ) is 0.4 ng/g for each analyte.

Figure 2
Flow Diagram of the Analytical Method 244-93-3
to Determine the Residues of MK-0244 and its 8,9-Z Isomer in/on Soil



* stopping point

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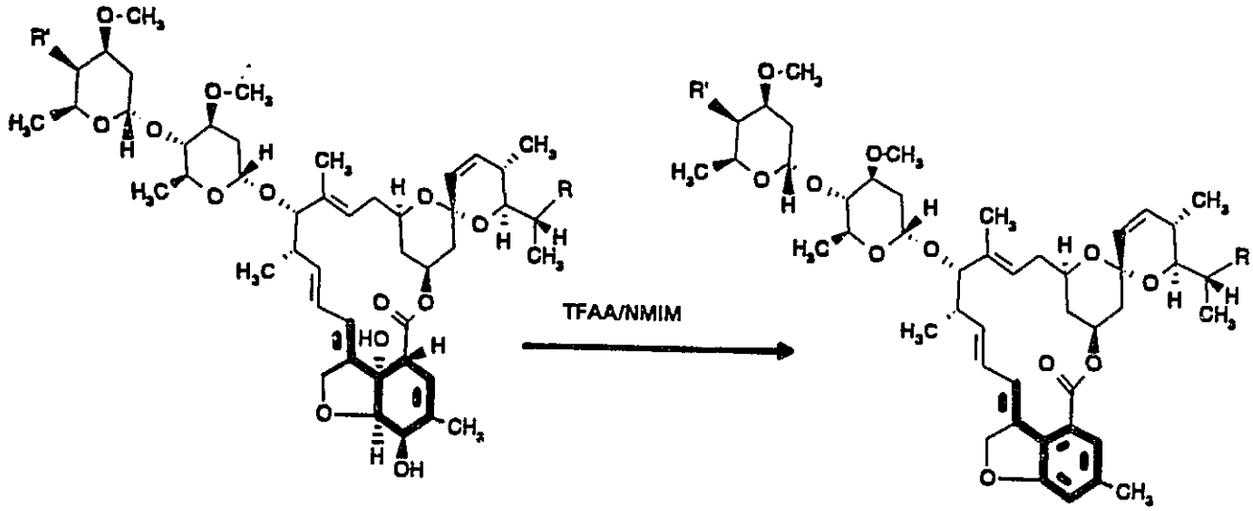
Figure 3
Reaction to Form Fluorescent Derivative



NON-FLUORESCENT

FLUORESCENT

618-244-93906

III. EQUIPMENT AND SUPPLIES

A. REAGENTS AND SOLVENTS

Acetonitrile (ACN) - Burdick and Jackson, HPLC grade, or equivalent.

Ammonium Acetate (NH_4OH) - Sigma, ACS Reagent grade, or equivalent.

Ethyl Acetate (EtOAc) - Burdick and Jackson, HPLC grade, or equivalent.

Helium or Nitrogen - the equivalent of Matheson extra dry compressed gas, used to deaerate mobile phase or evaporate solvents.

Methanol (MeOH) - Burdick and Jackson, HPLC grade or Mallinckrodt, Nanograde, or equivalent.

1-Methylimidazole (N-Methylimidazole, NMIM) - Aldrich Chemical Company, 99% pure, or equivalent.

Phosphoric Acid - EM Science, 85% solution, reagent grade, or equivalent.

Trifluoroacetic Anhydride (TFAA) - Pierce, reagent grade, or equivalent.

Water, Filtered Millipore - Distilled water is treated with a Milli-Q system including a Millipore Type HA 0.45 m disc, or equivalent.

B. SOLUTIONS

1% Ammonium Acetate in Methanol (W/V) - Weigh 40 g (± 0.1g) of ammonium acetate and quantitatively transfer to a 4 L bottle of methanol or equivalent. Cap and shake to completely dissolve the ammonium acetate. Sonicate if necessary.

Derivatization Reagent (1:2 (V:V) TFAA:ACN) - This reagent has to be prepared fresh just before use. The total volume of the reagent prepared depends on the number of samples to be assayed. For a typical set of 12 samples and five standards, mix 5.0 mL of acetonitrile and 2.5 mL trifluoroacetic anhydride in a 15 mL centrifuge tube. For each sample or standard tube, 0.3 mL of derivatization reagent is used. (Note: Extra care to avoid skin contact should be taken while handling TFAA reagent. TFAA should be handled in a fume hood.)

Mobile Phase - 7% Water in Methanol - Dilute 140 mL (± 1mL) of Millipore water to two liters with methanol. Deaerate as needed by slowly bubbling with helium or nitrogen, or equivalent. This solution can be used to dilute samples as well.

1% Phosphoric Acid in Methanol (W/V) - Pipet 6 mL (± 0.1mL) (10 g) phosphoric acid into a 1 L volumetric flask and dilute to the mark with methanol. Mix well.

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C. GENERAL EQUIPMENT

1,3,6 mL Adapter - Varian (Analytichem International), or equivalent.

Balance - analytical, capable of accurately weighing 1.00 mg.

Balance - analytical, capable of accurately weighing 150 mg.

Bath, ice-water - at 0°C or Kryorack - Streck Laboratories, Inc., model #40-190, or equivalent (see note below).

Bath, water - variable temperature 40 to 80°C.

Bottles - Glass, 1 L, with screw cap, for storage of solutions.

Cartridge - Propylsulfonic Acid (PRS), Varian (Bond Elut, Analytichem International #1210-2039), 500 mg, 2.8 mL (or ~3mL), or equivalent.

Centrifuge - IEC model HN-S-II, with six place rotor IEC #958 and 15 and 50 mL cups, or equivalent. The centrifuge is run at 2000 to 2500 RPM. The centrifuge used gives ca. 700 to 750 x g maximum centrifugal force (RCF).

Centrifuge tubes - glass, graduated, 15 mL, with polyethylene stoppers to fit, Pyrex, or equivalent.

Centrifuge tubes - Blue Max™, polypropylene with screw cap, 50 mL, Becton Dickinson Labware (Cat. No. 2098), or equivalent.

Dispensers - Labindustries, Repipet, 0-50 mL, or equivalent.

Flasks - Volumetric, 100, 1000, and 2000 mL with ground glass stoppers.

Freezer - Capable of reaching temperatures of -10°C or below.

Gloves - disposable PVC from Fisher Chemical, or equivalent.

Nitrogen evaporation manifold.

Pipets - disposable, Pasteur.

Pipets - graduated, 0.5, 1, 2, 5, 10, and 25 mL.

Pipets - volumetric, 0.5, 1, 2, 4, and 5 mL.

Reservoir - 25 mL capacity, Varian, or equivalent.

Spatula - stainless steel.

Stopcocks - for use between cartridge and vacuum manifold, Worldwide Monitoring, or equivalent.

Ultrasonic Bath - Sonogen Automatic Cleaner, Branson Model 7000, or equivalent.

Vacuum manifold - Worldwide Monitoring or Varian Vac Elut SPS24 or equivalent, capable of holding 15 mL tubes.

Vortex Mixer - Fisher Scientific, or equivalent.

Note: Use of an ice bath during the derivatization reaction is recommended, but is not required. Apparatus or reagents demonstrated to yield equivalent recoveries may be substituted for the items listed above.

D. HPLC APPARATUS AND CHROMATOGRAPHIC CONDITIONS

Several chromatographic systems with interchangeable components may be used. Other equivalent equipment may be substituted if it can be shown to provide comparable performance. Selection of mobile phase composition depends on the instruments.

Liquid chromatography pump - Shimadzu Model 10AD, or equivalent.

Autosampler - Shimadzu SIL-10A, or an equivalent.

Guard column - C-18 standard Brownlee Labs, Newguard, 7 μm, 15 mm x 3.2 mm, from Rainin Instrument Co., Inc, or equivalent.

Analytical column - Chromegabond C-18, 15 cm x 3.0 mm ID, 3 μm particles, ES Industries, or equivalent.

Column heater - Jones Chromatography 7950, or equivalent, or no heater.

Fluorescence detector - Shimadzu Model RF-551, or equivalent.

Integrator - Spectra-Physics Model 4400 Chromjet, or equivalent.

Chromatographic conditions:

Mobile Phase - 7% water in methanol, isocratic.

Column Temperature - 40°C (recommended) or ambient .

Flow Rate - 0.35 mL/min for 3.0 mm ID columns (pressure 800 to 1500 psi is acceptable).

Detector Settings:

Xenon lamp - Excitation 365 nm, Emission 470 nm. Time constant, range and sensitivity should be set appropriate to the needs of the method and the operation of the specific instrument, usually on the most sensitive settings in order to achieve the low limits of quantitation and detection specified by the method.

The MK-0244 B1a derivative has a retention time of approximately 12-14 minutes, as determined by the analytical standards injected with each set. The B1b derivative should be baseline-resolved and elutes 1 to 2 minutes before the MK-0244 B1a. The retention time windows for calculations are dependent on the exact conditions used.

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E. PREPARATION OF THE STANDARDS

Analytical Standard:

MK-0244 (L-656,748-052S007) or most recent designated reference standard.

Authentic Substance:

MK-0244 B1a 8,9-Z isomer (L-695,638-001C001) or most recent authentic substance.

Chemical Data
Merck Research Laboratories
P.O. Box 2000
Rahway, NJ 07065

Preparation:

1. The MK-0244 standard (listed above) contains 91.9% B1a and of 4.7% B1b (W/W). To prepare an approximately 100 mcg/mL MK-0244 B1a stock solution accurately weigh 5 mg of L-656,748-052S007 into a 50 mL volumetric flask, and dilute to the mark with acetonitrile. The 10 ng/mL standard can be prepared by diluting the 500 ng/mL intermediate stock solution, 2 mL to 100 mL, with acetonitrile. All standards should be transferred to capped polypropylene tubes, or other appropriate container, for freezer storage at or below -10°C.

2. To prepare approximately 0.25, 0.5, 1.25, 2, and 2.5 ng/mL calibration curve standards for the derivatization, transfer to separate 15 mL tubes 0.1, 0.2, 0.5, 0.8, and 1.0 mL, respectively, of the 10 ng/mL MK-0244 B1a standard solution. Proceed as described in **IV, Procedures, Section E.**

3. The authentic sample listed below has the assigned purity as follows:

   (L-695,638-001C001) 92.9% B1a

   The procedure for the preparation of the approximately 100 mcg/mL MK-0244 B1a stock solution may be used to prepare approximately 100 mcg/mL stock solutions of the above authentic sample. More dilute solutions of individual compounds or of several compounds in one solution may be prepared from the approximately 100 mcg/mL stock solutions.

4. Derivatize and perform all subsequent operations for injection on the HPLC. Derivatized standards and samples are stable over 96 hours when stored at or below -10°C in the freezer.

5. The exact concentration of all standards used should be reported and used throughout all calculations. The standards are stable several months when stored in the freezer below -10°C.

IV. PROCEDURE

A. EXTRACTION OF SOIL

1. Weigh 10.0g (± 0.05g) into 50 mL plastic centrifuge tubes, or equivalent. Fortify samples, if required, with up to 1 mL of standard. Place in hood for approximately 30 minutes to allow most of the fortification solvent, usually acetonitrile, to evaporate.

2. Dispense 10-15 mL of the 1% NH_4OAc in MeOH solution onto the soil. Cap and vortex. A pre-extraction with 20 mL of methanol can be performed before the extraction with 1% NH_4OAc in MeOH.

3. Place the centrifuge tubes containing the soil in rack in a sonicator heated to 65-75C (higher temperatures can be used at higher altitudes). Sonicate approximately 10 minutes. Remove tubes from the sonicator and shake tubes for approximately 1 minute. Return tubes to sonicator and continue to sonicate for approximately 1 to 2 minutes more.

4. Remove the tubes from the sonicator and centrifuge at full speed (approximately 700g) for approximately 5 minutes.

5. Decant the methanolic supernatant from the 50 mL centrifuge tubes into 15 mL glass centrifuge tubes.

6. Evaporate the methanolic extract in the 15 mL tubes to a small volume using a nitrogen manifold and a water bath at approximately 65-75C, so that the next extract can be added to the 15 mL tube. Alternatively, the extracts can be blown down in a 50 mL tube and then quantitatively transferred to a 15 mL tube. Do not evaporate to dryness. This can be done at the same time as Step 7.

7. Repeat Steps 2 through 6 twice for a total of three 1% NH_4OAc in MeOH extractions and a total volume of 30 to 45 mL. If the 20 mL methanol extraction was performed as well, then the total volume may be 65 mL.

B. SOLVENT TRANSFER

8. Evaporate the combined methanolic extracts to approximately 1 mL using a nitrogen manifold and a water bath at approximately 65-75C. If the volume is evaporated to below 1 mL, dilute to the 1 mL mark on the tube with methanol. Add 4 mL of water and vortex.

9. Repipet 5 mL of ethyl acetate (EtOAc) into the tubes. Cap. Shake moderately by inversion of the tubes, or vortex, for ~1 minute. Centrifuge at full speed (~700g) for ~5 minutes.

10. Remove the top EtOAc layer from the tubes to a reservoir of a prepared PRS solid phase extraction column (See Section C following).

11. Repeat steps 9 and 10 once for a total EtOAc volume of 10 mL.

C. SOLID PHASE CATION EXCHANGE

12. Prepare one 500 mg/3 mL PRS (cation exchange) column cartridge for each sample by conditioning with approximately 5 mL rinses of each of the following solutions in order: 1% NH_4OAc in MeOH, 1% (w/v) phosphoric acid in MeOH, water, MeOH, and EtOAc. A clean 25 mL, or equivalent, reservoir should be fitted to the column with an adapter and the column fitted to a vacuum manifold with a stopcock. The stopcock is used to prevent sucking the column to dryness when several are treated concurrently on a multiple-outlet vacuum manifold.

13. Transfer the upper, ethyl acetate phase from Step 10 (See **Section B** above) to the reservoir above a prepared PRS cartridge. The transfer must be completed by carefully using a pipet.

14. Load the second EtOAc fractions into the reservoir.

15. Begin the vacuum loading of the sample after the audition of the second extract. (Some of the extract may load by gravity before the use of vacuum.) Pass the EtOAc extract at approximately 1 to 2 drops per second through the PRS column by drawing a low vacuum. The ethyl acetate that passes through the column is waste.

16. Rinse the inside of the reservoir with 3 mL ethyl acetate.

D. SECOND ETHYL ACETATE PARTITION

17. Elute the PRS column into a 15 mL centrifuge tube using 10-12 mL of 1% NH_4OAc in MeOH. Use low vacuum similar to the rate used in Step 15 above, until the eluant flow stops and then increase vacuum to collect the last solvent from the column. Stopper the tube and mix well. Divide (split) the eluant equally between two 15 mL centrifuge tubes using a disposable pipet and graduations on the tube. Stopper one tube and store in the freezer for future use, if necessary. If sensitivity is an issue, the samples do not have to be split.

    NOTE: Adequate mixing before splitting is essential.

18. Evaporate the eluant in the remaining tube to 1 mL by carefully using a nitrogen manifold and a water bath at approximately 65-75°C. If the volume is evaporated to below 1 mL, dilute to the 1 mL mark on the centrifuge tube with methanol. It is recommended to avoid evaporation to dryness at this step.

19. Add 4 mL water and 4 mL ethyl acetate to the tube containing the methanolic solution. Stopper the tube.

20. Shake moderately by inversion, or vortex, for 1 min and centrifuge at full speed (approximately 700g) for approximately 5 minutes.

21. Remove the ethyl acetate phase to a second 15 mL centrifuge tube and add another 4 mL ethyl acetate to the tube containing the aqueous solution. Stopper the tube and repeat Step 20.

22. Repeat Step 21 with a third aliquot of 4 mL EtOAc.

23. Combine the third EtOAc extract with the first and second extracts and carefully evaporate to 0.5 mL using a nitrogen evaporation manifold and a water bath at approximately 60-70C. Again avoid evaporation to dryness. Dilute to the 1.0 mL mark on the centrifuge tube with acetonitrile. Add an additional 2.0 mL acetonitrile to each sample.

24. Prepare a standard series in 15 mL centrifuge tubes containing 0.5 mL ethyl acetate and a total of 2.5 mL acetonitrile including the volume of standard solution (in acetonitrile) used (**Section III.E**). Derivatize samples and standards, as described in **Section IV.E.**

E. DERIVATIZATION AND HPLC ANALYSIS

25. A standard series is prepared from an acetonitrile solution of MK-0244, approximately 10 ng/mL, by placing 0.1, 0.2, 0.5, 0.8 and 1.0 mL in separate tubes and diluting to 1.0 mL with acetonitrile. The concentration of this series is approximately 0.25, 0.5, 1.25, 2, and 2.5 ng/mL when diluted to a final volume after derivatization of 4 mL.

26. To the standard series in 0.5 mL ethyl acetate and 2.5 mL total acetonitrile, add 0.1 mL methylimidazole. Vortex, and then chill approximately 5 to 10 minutes in a Kryorack or wet ice bath.

27. Prepare a 1:2 trifluoroacetic anhydride:acetonitrile reagent solution (**Section III.B**) and chill with the standards and samples in Step 26.

28. To chilled standards and samples, add 0.3 mL chilled trifluoroacetic anhydride:acetonitrile reagent. Vortex. Allow the samples and standards to come to room temperature for approximately 5 to 10 minutes.

29. Add 0.6 mL of water to both standards and samples and vortex. The final volume is 4 mL before dilutions.

30. Centrifuge briefly, if necessary, and inject on HPLC with fluorescence detection.

F. STOPPING POINTS AND NOTES TO THE ANALYST

After the assay has started, the following stopping places allow for storage in a freezer at or below -10°C for several days.

1. In 1% NH_4OAc in MeOH, after Step 7 or 8, before the addition of water.

2. On the PRS column, wrapped in plastic, after Step 16.

3. In 1% NH_4OAc in MeOH, after Step 17.

4. After derivatization, after Step 30.

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The following are additional notes to the analyst.

1. Samples stored in the freezer in EtOAc (Step 11 or 23) will not derivatize, therefore do not use EtOAc as a stopping point. Other steps, in addition to those listed above, can be used as stopping points as long as they have been demonstrated to give adequate recoveries.

2. Samples may be diluted with mobile phase (for example, 93% methanol/7% water) after derivatization, to be within the range of the standard curve. This dilution may be made up to 96 hours after the original sample injection provided that the dilution is injected with reinjections of standards from the corresponding original derivatization set.

3. The method requires approximately 6 to 7 hours for sample preparation prior to HPLC analysis. The initial extraction step is the most tedious, however, other steps or procedures can be set up, or results calculated, while the samples are being sonicated.

V. QUANTIFICATION AND SYSTEM SUITABILITY

The fluorescent derivatives of MK-0244 B1a and its 8,9-Z isomer elute as a distinct peak between approximately 12-14 minutes. The fluorescent derivatives of MK-0244 B1b and its 8,9-Z isomer should elute as a smaller distinct peak approximately 1 to 2 minutes before the B1a counterparts. MK-0244 B1a should be baseline-resolved from B1b and any other peaks that may be matrix-related. A set of at least five external standards are injected before and after the sample as a check of the system suitability as well as for quantification. Coefficients of determination of the external standards should be 0.97 or greater and method recoveries of fortified samples should have recoveries values of 70-120%.

For a 10 gram soil sample, residues of MK-0244 B1a/8,9 Z, below 0.2 ng/g are non-detectable (reported as ND). The peak representing MK-0244 B1a/8,9-Z residues between 0.2-0.4 ng/g are identified but not quantified (reported as NQ) and the peaks for residues above or equal to 0.4 ng/g are identified and quantified. Since MK-0244 B1b is at most 10% MK-0244 of the B1a component, residue levels of B1b are generally less than the limit of quantification (0.4 ng/g). The peak representing MK-0244 B1b/8,9-Z isomer is identified at residue levels between 0.2-0.4 ng/g, and quantified in the same manner as MK-0244 B1a/8,9-Z isomer, using the B1a standard curve. Residues below 0.2 ng/g are reported as ND.

An analysis set is comprised of no less than 5 standards and no more than 15 samples between each set of standard injections. The standards are run before and after the samples to ensure the stability of the HPLC system, the standards and the samples. Smaller sets may be used for dilutions or reinjections. For each analysis set, the slope and intercept are determined from the linear regression of the standards' peak area or height vs. concentration in nanograms per milliliter. It is recommended that peak area be used, but peak height can be used if it is shown that adequate recoveries are obtained when peak height is used. Occasionally it has been observed that the peak response for one standard is much lower than expected. Because it is known that this observation can be attributed to low derivatization reaction yield, a single errant standard may be

discarded in determining the regression coefficients and linear regression equation (see below).

The concentration of each group of analytes (see above) in a residue sample is determined as follows:

$$C=(PK\ AR-I)/S \quad UNK=(CxFV)/(SW\ X\ FRAC)$$

Where:

C= concentration of the analytes of interest in ng/mL in the final volume used for HPLC analysis, PK AR= peak area or height of the analytes of interest, I= intercept, S= slope, FV= final volume used for HPLC analysis, SW= sample weight (usually 10g), UNK= concentration of the analytes of interest in ng/g in the unknown residue sample. FRAC= fraction of the sample used for the HPLC-fluorescence assay (usually 0.5). MK-0244 B1b/8,9-Z residues are calculated from the MK-0244 B1a standard curve.

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