HPLC-FLUORESCENCE DETERMINATION FOR AVERMECTIN B_1 AND ITS DELTA 8,9 ISOMER IN CITRUS FRUIT

Method No. 1009

Revision No.2

Merck Sharp and Dohme Research Laboratories
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July 31, 1987

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TABLE OF CONTENTS

HPLC-Fluorescence Determination For Avermectin B1 and its Delta 8,9 Isomer in Citrus Fruit

Merck Sharp & Dohme Research Laboratories
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Method No. 1009R02
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I. SUMMARY

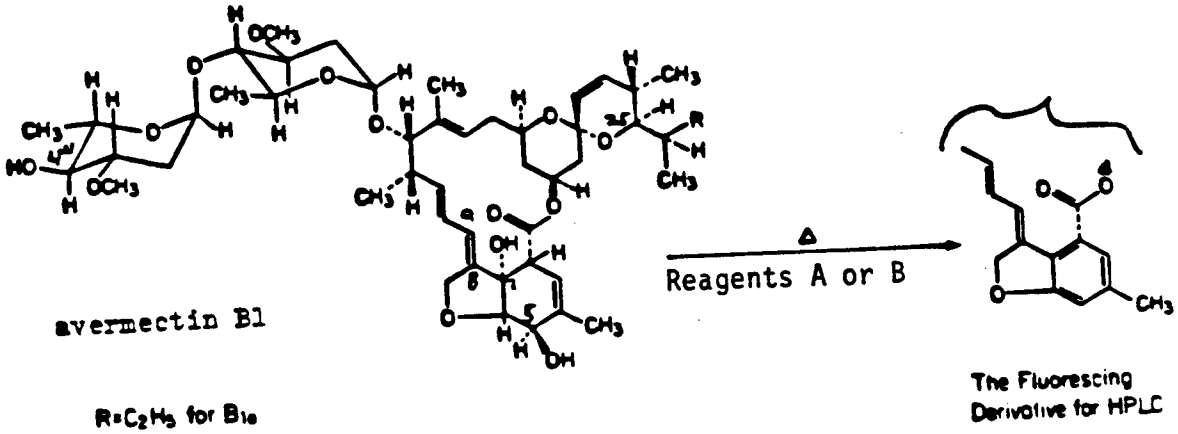
Avermectin B1 (Abamectin) is a mixture of two homologs containing not less than 80% avermectin B1a and not greater than 20% avermectin B1b. These components differ by only one methylene unit (-CH2-) at the 25-carbon position, wherein avermectin B1a contains a sec-butyl group and avermectin B1b contains an isopropyl group.

The first analytical methods developed were for the companion animal health drug ivermectin (22,23-dihydro avermectin B1). As it was known that the avermectins were not amenable to gas chromatography, and the available liquid chromatography methods employed UV detection which lacked the required sensitivity and selectivity, an HPLC method employing fluorescence detection was developed by Tolan et al. (1980) for the determination of ivermectin in plasma. Tway et al. (1981) modified the Tolan method to apply it to tissue. The major result was an improved derivatization procedure which was shorter and more reproducible. Incubation of avermectin B1 with acetic anhydride/1-methylimidazole/DMF for 1 hour at 95°C results in acylation at the 5, 7, and 4" positions, followed by dehydration at the 5 and 7 positions, and ring conjugation. The fluorescent derivative which is formed (see Figure 1) is readily chromatographed using HPLC and gives the sensitivity and selectivity desired for trace residue analysis. This derivatization reaction has since been employed in the determination of avermectin B1 in citrus (Jenkins and Cobin, 1984).

Following more complete characterization of the citrus total toxic residue, it was determined that an analytical method would also be required for the delta 8,9 isomer of avermectin B1. Initially it was thought that the derivatization reaction described above could be used for the determination of both the parent avermectin B1 and its delta 8,9 isomer. Subsequently, it was determined that reaction of the delta 8,9 isomer under these conditions gives an unacceptable result, i.e., apparent low reaction yield of the fluoroderivative which is photolytically unstable. Because stereochemistry suggests that the 7-OH of the delta 8,9 isomer may be hindered, a more reactive acylating reagent, trifluoroacetic anhydride, was substituted for acetic anhydride. Incubation at 30°C for 1 hour yields the same fluorescent derivative as shown in Figure 1 except the 4" position is trifluoroacetylated rather than acetylated (not shown). As this linkage is unstable, the trifluoroacetyl group at the 4" position is cleaved with methanolic ammonium hydroxide to give the stable 4"-OH fluoroderivative. This two step reaction gives a single peak for the delta 8,9 isomer with the same retention time as the parent avermectin B1 fluorescent derivative. Therefore using this derivative, the residue

quantitated represents the sum of avermectin B1a and its delta 8,9 isomer. Residues of avermectin B1a + delta 8,9 isomer below 2 ng/g are non-detectable (reported as ND). Residues between 2-5 ng/g are identified but not quantitated (reported as NQ) and residues above 5 ng/g are identified and quantitated from an avermectin B1a standard curve. Since avermectin B1b is at most 20% (normally less than 10 %) of the active ingredient, residues are generally present at levels less than the limit of quantitation (5.0 ng/g). At levels above 5 ng/g, avermectin B1b/ delta 8,9 isomer residues are quantitated in the same manner as avermectin B1a/delta 8,9 isomer.

Figure 1. Structures of avermectin B1 and derivatization reaction



A. Acetic anhydride/DMF/1-methylimidazole

B. (1) Trifluoroacetic anhydride/DMF/1-methylimidazole

(2) Methanolic ammonium hydroxide

II. METHOD VALIDATION

Table 1 gives the results of the validation of Merck Method No. 1009. Orange and grapefruit homogenates were fortified at 5, 25, and 50 ng/g with avermectin B1a and its delta 8,9 isomer. Orange homogenate was fortified with avermectin B1b at 5 ng/g. Five replicates were prepared for each compound at each fortification level by adding 1 ml of 50 ng/ml, 250 ng/ml, or 500 ng/ml of the working standards to 10 grams of the whole fruit homogenate just prior to extraction with the Polytron blender (step 3). As avermectin B1b is present in the avermectin B1 standard at approximately 10% of the avermectin B1a, 1 ml of the 500 ng/ml avermectin B1 standard (containing 500 ng/ml avermectin B1a and approximately 50 ng/ml avermectin B1b) was used for the avermectin B1b fortifications. Controls were fortified with 1 ml of the acetonitrile used to prepare the standards. For both the parent avermectin B1a and its delta 8,9 isomer, recoveries are similar between fruit types. For avermectin B1a, recoveries averaged 100% (n=10, range 91-113%) for samples fortified at 5 ng/g, 91% (n=10, range 73-108%) for samples fortified at 25 ng/g, and 83% (n=10, range 75-96%) for samples fortified at 50 ng/g. For avermectin B1b recoveries averaged 108% (n=5, range 104-113%) for samples fortified at 5 ng/g.

Recoveries for the delta 8,9 isomer are determined by spiking samples with the delta 8,9 isomer and quantitating versus the avermectin B1a derivative. Subsequent to completion of the validation work for citrus, a more completely characterized delta 8,9 standard of higher purity was obtained (L-652,280-000N005). The purity of the new delta 8,9 standard demonstrates that the purity of the older standard (L-652,280-000N003) was only approximately 70%. Consequently, the recoveries for the delta 8,9 isomer in the past were significantly lower than those determined for the parent avermectin B1a. However, good precision among replicates was still obtained. Using the old standard for the delta 8,9 isomer, recoveries averaged 73% (n=9, range 68-81%) for samples fortified at 5 ng/g, 62% (n=10, range 48-72%) for samples fortified at 25 ng/g, and 58% (n=10, range 53-61%) for samples fortified at 50 ng/g.

More recently, additional data on the recovery of the avermectin delta 8,9 isomer were obtained. Using the new avermectin delta 8,9 standard (L652,280-000N005), recoveries from fortified control oranges at the 5 ng/g level averaged 84% (n=9, range 74-87%) and, at the 25 ng/g level, 81% (n=9, range 73-86%). See Table 1B for detailed data on recoveries. The solid delta 8,9 standard (L-652,280-000N005) has been prepared in glycerol formal and this glycerol formal solution standard (L-652,280-002T001) should be used to prepare all spiking solutions and standards.

Shown in Figures 2 and 3 are example chromatograms for an avermectin B1a 6 ng/ml standard, orange and grapefruit controls, and orange and grapefruit samples fortified with avermectin B1a and the delta 8,9 isomer at 5 ng/g.

Table 1

Recovery of Avermectin B1a, Avermectin B1b, and the Delta 8,9 Isomer from Orange and Grapefruit Whole Fruit Homogenates

Compound	Fortification Level (ng/g)	Percent Recovery	
		Grapefruit	Orange
avermectin B1a	5	113	95
	5	111	101
	5	105	85
	5	106	91
	5	95	97
		AV=106	AV=94
avermectin B1b	5		105
	5		111
	5		113
	5		108
	5		104
			AV=108
delta 8,9 isomer	5	81	
	5	80	68
	5	68	69
	5	78	70
	5	71	75
		AV=76	AV=71
avermectin B1a	25	90	100
	25	84	99
	25	78	108
	25	73	106
	25	74	104
		AV=80	AV=103
delta 8,9 isomer	25	72	65
	25	62	58
	25	71	59
	25	59	55
	25	65	48
		AV=66	AV=57

Table 1 (cont.)

Compound	Fortification Level (ng/g)	Percent Recovery	
		Grapefruit	Orange
avermectin B1a	50	84	83
	50	84	78
	50	96	82
	50	85	84
	50	75	80
		AV=85	AV=81
delta 8,9 isomer	50	58	57
	50	59	59
	50	61	61
	50	53	55
	50	61	59
		AV=58	AV=58

TABLE 1 B

Recoveries for the New Avermectin Delta 8,9 Standard (L 652,280-000N005) From Fortified Control Oranges

Compound	Fortification Level (ng/g)	Percent Recovery
delta 8,9 isomer	4.6	83
	4.6	83
	4.6	76
	4.7	87
	4.7	77
	4.7	83
	4.7	74
	4.7	74
	4.7	81
		AV= 84
delta 8,9 isomer	22.8	82
	22.8	86
	22.8	74
	23.3	77
	23.3	84
	23.3	73
	23.3	81
	23.3	85
	23.3	85
		AV= 81

Figure 2. Typical Chromatograms of Avermectin B1 and its Delta 8,9 Isomer in Orange.

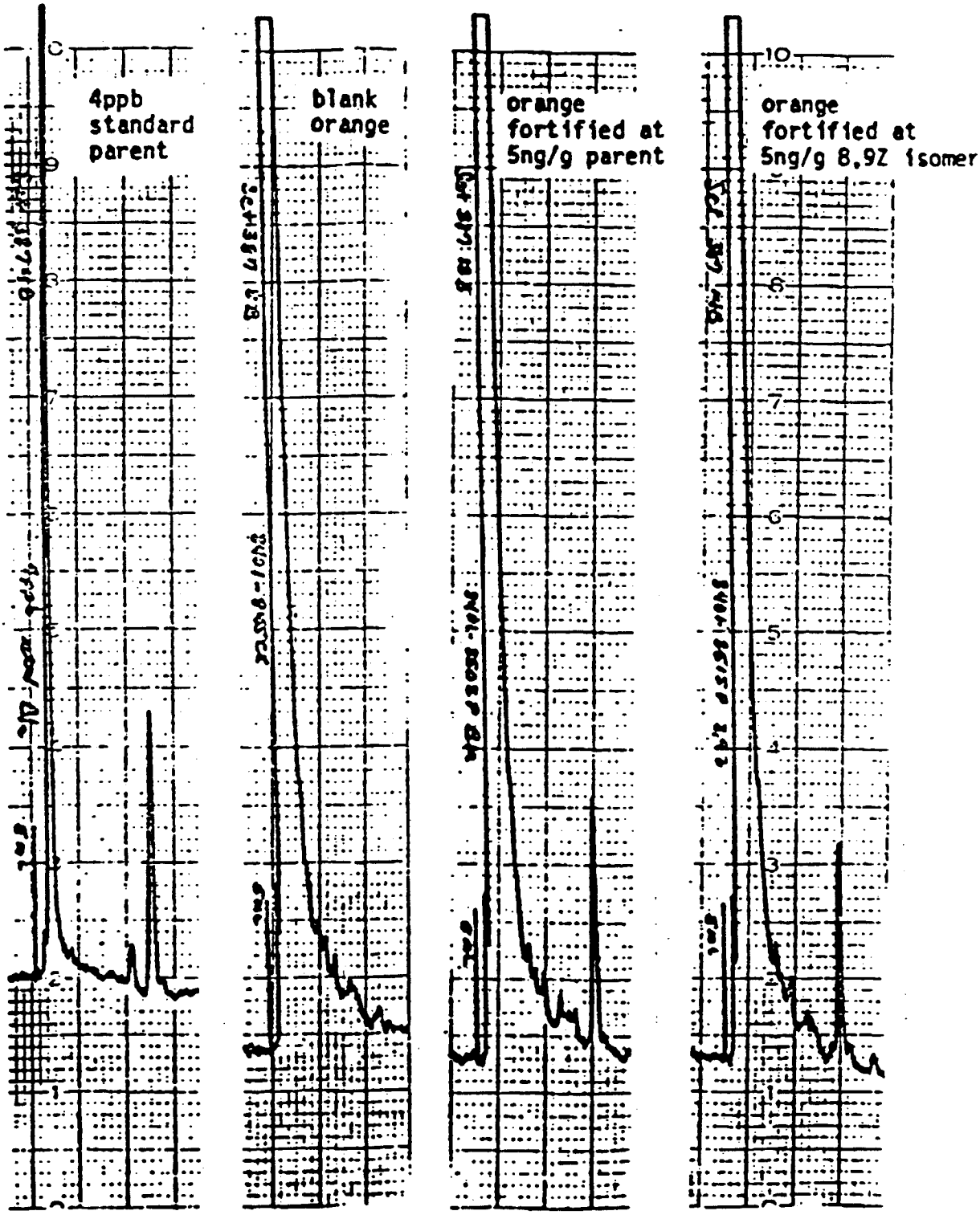
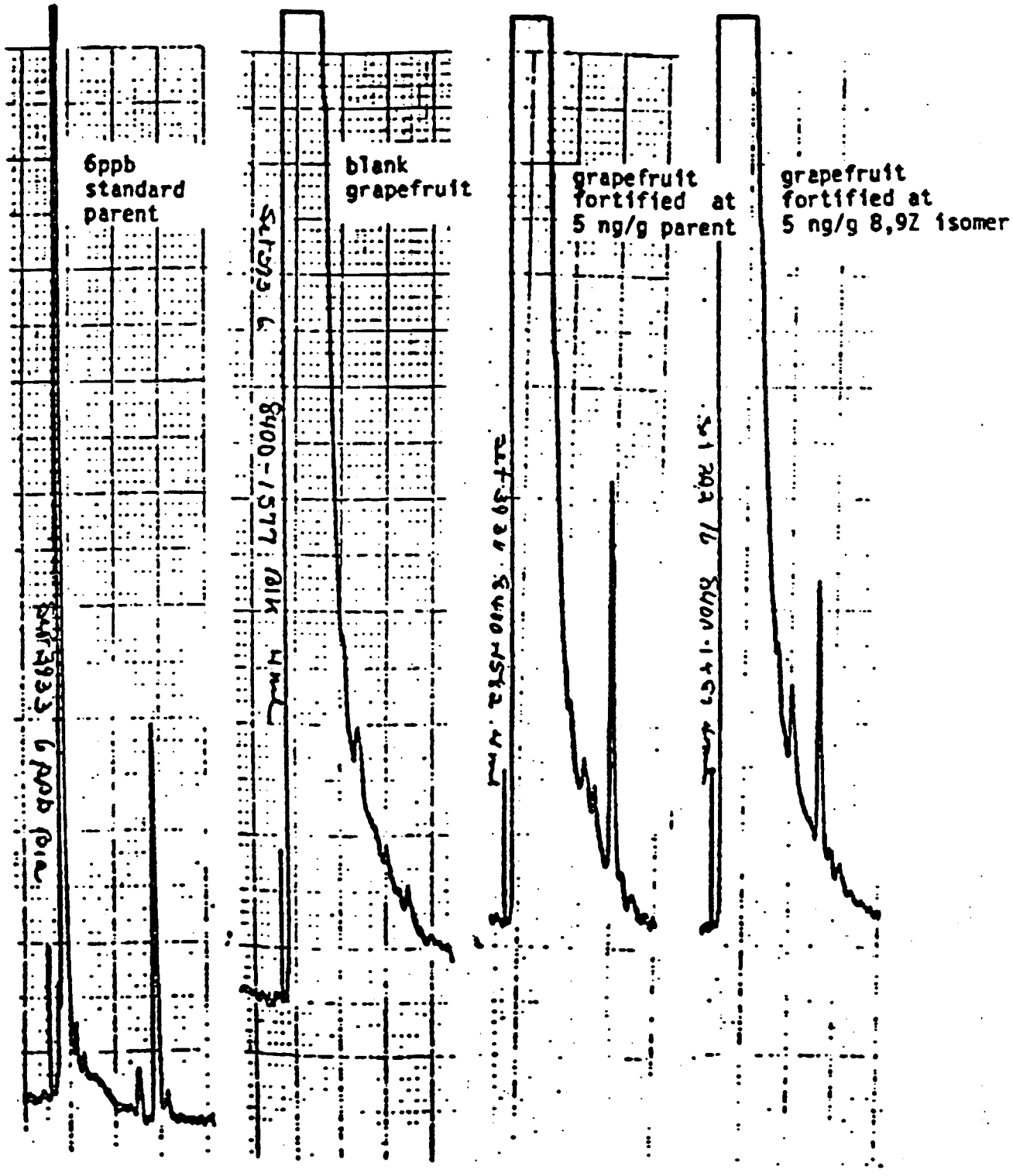


Figure 3. Typical Chromatograms of Avermectin B1 and its Delta 8,9 Isomer in Grapefruit.



III. PRINCIPLE

Residues of avermectin B1 and its delta 8,9 isomer are extracted from citrus fruit homogenate by blending with methanol. The filtrate is extracted twice with isooctane and the isooctane discarded. 10% NaCl solution is added to the methanol extract and this mixture is extracted 2 times with 0.01% t-butanol in methylene chloride. The combined organic extracts are concentrated. Acidic alumina is used for column clean-up of the concentrate. The eluant is evaporated to dryness and a fluorescent derivative is formed by reaction with N,N-dimethylformamide/trifluoroacetic anhydride/1-methylimidazole reagent (Reagent B-1) for 1 hour at 30°C, followed by reaction with methanolic ammonium hydroxide (Reagent B-2) for 1/2 hour at 30°C. The reaction mixture is dissolved in chloroform and passed through a silica gel column for separation of the derivatized residue from derivatization reagents. The eluant is taken to dryness and dissolved in methanol. The derivatized residue is determined by reversed-phase liquid chromatography with fluorescence detection. As derivatization of the delta 8,9 isomer results in a peak with the same retention time as the parent avermectin, the derivatized residue quantitated represents the sum of avermectin and its delta 8,9 isomer, as shown below:

Parent Avermectin	+	delta 8,9 isomer	heat ---------> Reagent B	single fluorescent peak

NOTE TO THE ANALYST

Avermectin B1 has a very low water solubility, approximately 7.8 ppb. In addition, there are indications that this compound will form a monolayer at phase boundaries [liquid-air, liquid-liquid, or liquid-solid (glass or plastic) interface]. Because of these properties, loss of avermectin B1 due to adsorption to glassware may be critical when working in the low ppb range. Care should be taken to not take samples to dryness whenever possible. When samples are taken to dryness, sonication upon dissolution is crucial.

Suggested assay stopping points are after steps 7, 20 and 28 for storage of samples overnight or over the weekend at 4°C or below.

IV. METHOD

A. Sample Preparation

1. The entire sample must be processed using a Hobart food processor or equivalent. Grind the sample until a homogeneous blend is attained. Processing is more efficient if the sample is broken into small pieces that are more manageable by the food processor.

2. Store approximately 500 grams of each treated sample and approximately 1000 grams of the control in square 500 ml Nalgene bottles in a freezer until analysis.

NOTE: Samples must remain frozen throughout the sample preparation steps. While processing the sample, dry ice may be added if sample begins to thaw.

B. Extraction From Crop

3. Weigh out exactly 10 grams of the sample homogenate and place it in an 8 oz French square bottle or equivalent glass container. Add 50 ml methanol.

4. Position the probe of the Polytron blender near the bottom of the bottle and grind the mixture using setting 7 for 1 minute.

5. Pour the methanol and any accompanying macerate through a 63 mm porcelain funnel fitted with Whatman #50 filter paper which has been prerinsed with 3ml methanol. Collect the filtrate in a 250 ml filter flask. Remove filtrate under vacuum.

6. Add 50 ml methanol to the French square bottle. Run the Polytron at a setting of 7 for 1 minute to rinse any residual avermectin off of the probe. Add methanol rinse to porcelain funnel. Rinse the probe between grinding samples with methanol by running the probe in a container of clean methanol.

7. Wash the filter cake with a small amount of methanol (5-10ml). The volume of combined extracts should be less than 125 ml. Remove the vacuum and begin the solvent partition.

C. Coextractive Removal By Solvent Partition

8. Transfer the combined methanol extracts to a 500 ml separatory funnel. Rinse the filter flask with three 2-3 ml portions of methanol and add to the separatory funnel.

9. Add 100 ml isooctane to the separatory funnel. Stopper, shake for 30 seconds, allow the layers to separate.

10. Transfer the methanol extract (bottom layer) to a 250 ml separatory funnel. Discard the isooctane.

11. Add a second 100 ml portion of isooctane to the 250 ml separatory funnel containing the methanol extract. Stopper and shake for 30 seconds.

12. Allow the layers to separate and transfer the methanol extract back into the 500 ml separatory funnel. Discard the isooctane.

13. Add 250 ml of 10% NaCl solution to the 500 ml separatory funnel, then add 60 ml of 0.01% t-butanol in methylene chloride to the separatory funnel, stopper, shake for 30 seconds, allow the layers to separate.

14. Transfer the 0.01% t-butanol in methylene chloride lower layer to a 250 ml round-bottomed flask by filtering the extract through 7 grams of anhydrous sodium sulfate in a small glass funnel (sodium sulfate held in place with a small amount of silanized glass wool).

15. Repeat the extraction with a second 60 ml portion of 0.01% t-butanol in methylene chloride. After the second 60 ml extract has been dried over the sodium sulfate, rinse the sodium sulfate with 5 ml of methylene chloride adding the rinse to the combined extracts.

16. Reduce the combined extracts to about 2-3 ml by vacuum rotary evaporation at a water bath temperature not exceeding 40^{o}C.

17. Transfer the concentrate to a 15 ml graduated centrifuge tube using a disposable Pasteur pipet. Rinse the flask with three 2-3 ml portions of methylene chloride and add to the centrifuge tube. Use the same disposable pipet to make all transfers.

18. If necessary, reduce total volume to less than 10 ml with nitrogen in a 50°C water bath, being careful not to take to dryness.

19. Adjust the volume to 10 ml with methylene chloride using the graduations on the tube. Cap and vortex briefly.

20. Transfer 5 ml of the sample to a 15 ml graduated centrifuge tube, using the graduations on the centrifuge tube containing the sample and a Pasteur pipet. Cap the remainder of the sample and store it at -10°C. (Polypropylene centrifuge tubes may be used for storage).

NOTE: One half of the sample is saved in case a repeat analysis is necessary.

21. Reduce the 5 ml taken from the sample to less than 1ml with nitrogen in a 50°C water bath. Do not let the sample go to dryness. Dilute the sample to 1 ml using methylene chloride and the graduations on the centrifuge tubes. Vortex the sample briefly before addition to the alumina column.

D. Column Clean-up Chromatography

22. Prepare the acidic alumina column by dry packing 0.7 grams of heat-treated acidic alumina into an Analytichem International brand 1 ml column fitted with a 20 micron frit. Tap to settle. Place another frit on top of the column. Place the packed column on the Baker 10 SPE System and apply low vacuum (10 in. Hg). Place a 25 ml reservoir, fitted with an adapter on top of the packed column.

NOTE: Remove the alumina from the oven just prior to packing columns. Packed columns may be stored in a desiccator for a period not to exceed 12 hrs.

NOTE: Apply low vacuum (10 in. Hg) to the Baker 10 SPE System during elution in steps 23 to 28, interrupting the vacuum after each step.

23. Wash the column with 5 ml methylene chloride.

24. Place 1 ml matrix concentrate on the column.

25. Rinse the tube with two 1 ml portions of 1% isopropanol in methylene chloride using the vortex mixer. Place each rinse on the column using a Pasteur pipet. The volume of the 1% isopropanol in methylene chloride should not exceed 1 ml per rinse. The graduation marks on the centrifuge tubes may be used as a measure. Discard the eluate collected. Prepare fresh daily the 1% isopropanol in methylene chloride.

26. Place a 25 ml vial (70 mm x 25 mm, Arthur Thomas Co., No.9710L17) under the column. Elute column with 20 ml of 15% isopropanol in methylene chloride. Collect the eluant in vial.

27. Reduce the volume to 2-3 ml with nitrogen in a water bath at 50°C. Do not take the sample to dryness.

28. Quantitatively transfer with three 3 ml portions of methylene chloride to a 15 ml silylated centrifuge tube using a Pasteur pipet.

NOTE: Silylate tubes as described in Section VI. G. (Tubes only need to be silyated once every two months).

E. Derivatization

29. Take the sample to dryness with nitrogen in a 50°C water bath.

NOTE: Care must be taken to insure that no moisture is present in the tubes prior to the addition of the derivatization reagent.

30. Add 0.2 ml of the trifluoroacylation derivatization reagent to the tube, stopper, vortex and sonicate to dissolve the residue.

NOTE: Standards should be derivatized with unknowns.

31. Centrifuge briefly, and put unknowns and standards together in a 30°C water bath for one hour.

32. Remove the tubes and add 0.1 ml of the methanolic ammonium hydroxide reagent. Vortex and return stoppered tubes to the 30°C water bath for 30 minutes.

33. Remove the tubes, add 4 ml chloroform to each tube and vortex. The sample should appear white and cloudy after the addition of the chloroform.

34. Wash a silica SEP-PAK cartridge with 5 ml of chloroform using vacuum of approximately 10 in. Hg to pull the chloroform from a reservoir into a waste collection tube.

35. After removal of the vacuum, place a 15 ml graduated centrifuge tube under the SEP-PAK cartridge and add the sample in the 4 ml of chloroform to the cartridge reservoir using a Pasteur pipet. Apply the vacuum and collect the eluant in a 15 ml graduated centrifuge tube. A SEP-PAK sample cartridge rack or equivalent may be used to clean-up several samples in parallel.

36. Remove the vacuum. Wash the silylated centrifuge tube two times with 2-3 ml chloroform. Use the same pipet that was used to transfer the sample into the cartridge reservoir to transfer the chloroform wash into the cartridge reservoir. Apply the vacuum between washes only.

37. Elute the column with an additional 5-7 ml of chloroform to give a final eluant volume of approximately 13 ml.

38. Take to dryness with nitrogen in a 70^{o}C bath. Samples should be reduced to an oily residue, less than 0.1 ml.

39. Pipet exactly 5.0 ml methanol into the tube, vortex and sonicate to completely dissolve the residue.

NOTE: The amount of methanol used as the final volume may vary depending on the expected residue levels.

40. Centrifuge briefly prior to injection of the supernatant on HPLC.

F. Confirmation of the presence of avermectin B1

The following derivatization procedure forms the 4" acetyl fluorescent derivative of avermectin B1. Using the HPLC conditions described, the presence of avermectin B1 above 5 ng/g is confirmed by a peak with a relative retention time of 1.7, relative to the 4" hydroxy fluorescent derivative formed by the derivatization procedure in the above assay.

1. Perform steps 1-29 of the primary assay.

2. Step 30- Add 0.1 ml of the acetyl derivatization reagent to the tube, stopper, vortex and sonicate to dissolve the residue.

3. Step 31- Centrifuge briefly, put unknowns and standards together in a 95°C oil bath for 1 hour.

4. Omit step 32.

5. Step 33- add 1 ml of chloroform instead of 4 ml. No white cloudiness is observed.

6. Perform steps 34-40.

G. Preparation of the Standards and Quantitation

Analytical Standards-

Avermectin B1 (L-676,863-038A002)
Avermectin B1a delta 8,9 isomer (L-652,280-002T001)

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

Preparation

1. To prepare 500 ng/ml stock solutions of avermectin B1a, B1b and the B1a delta 8,9 isomer, weigh out enough of the standard glycerol formal solution to produce 500 ml of a 500 ng/ml solution for each standard. The standards should be prepared separately and diluted with acetonitrile. The avermectin B1 glycerol formal solution contains 0.956% avermectin B1a and 0.071% avermectin B1b w/w. The avermectin B1a delta 8,9 standard contains 0.38% w/w of the avermectin B1a delta 8,9 isomer. The exact concentration of all standards used should be reported and used throughout all calculations.

2. To prepare 50 ng/ml intermediate stock solutions of the above standards pipet 10 ml of the 500 ng/ml stock standard into separate 100 ml volumetric flasks. Make to volume with acetonitrile. Store standards at 0°C or lower. Label all glassware and standard storage containers with exact concentrations, notebook reference and the date of preparation.

3. To prepare 2.0, 4.0, 6.0, 8.0 and 10.0 ng/ml working standards, transfer to separate silylated 15 ml tubes 0.2, 0.4, 0.6, 0.8 and 1.0 ml, respectively, of the 50 ng/ml avermectin B1a intermediate stock solution.

4. Derivatize and perform all subsequent operations for injection on the HPLC, as described above (steps 29-40).

V. REAGENTS

A. Solvents

Acetonitrile, methanol, 2,2,4-trimethylpentane (isooctane), methylene chloride, chloroform, isopropanol - EM Science (Pesticide Grade) or Burdick & Jackson (Distilled in Glass solvents); t-butanol - Fisher Scientific; Sylon-CT - Supelco Inc.; ultrapure water from Milli-Q Purification System.

B. HPLC Solvent

Prepare HPLC solvent by diluting 200 ml of Milli-Q purified water to 2.0 liters using HPLC grade methanol. Vacuum filter mixed HPLC solvent through Rainin 47 mm nylon 66 filter, 0.45 micron pore size. Equivalent filtering systems may be used.

C. Inorganics

Aluminum Oxide, acid washed (heat treated over night at 110-120°C) - J.T. Baker Chemical Co.; Anhydrous sodium sulfate (heat treated over night at 120°C) - J.T. Baker Chemical Co. or EM Science; Sodium chloride - Fisher Scientific; Silica-Gel SEP-PAKs - Waters Associates, Inc.

10% (w/v) NaCl solution should be prepared by weighing out 400 grams of NaCl and diluting to 4 liters in an erlenmeyer flask. NaCl may be dissolved readily by using a large teflon spin bar and a stirring plate.

NOTE: When the alumina and sodium sulfate are removed from the oven they must be stored in a desiccator when not being actively used. This storage period is not to exceed 12 hours.

Aluminum oxide from manufacturers other than J.T. Baker or new lots of aluminum oxide may be tested for possible interferences and abamectin recoveries by the method described in Procedure I. If unacceptable results are obtained, the alumina may be washed by the method described in Procedure II.

PROCEDURE I FOR ALUMINA TESTING

Validation of a New Lot or Manufacturer of Alumina

1) Weigh out five 10 gram samples of processed control citrus as described in steps 1-3 of Method 1009 revision No. 2.

2) Carry these five separate 10 gram samples through steps 3-19 of Method 1009 revision No. 2. (This step produces five 10 ml control extracts.)

3) Combine the five 10 ml control extracts of methylene chloride and mix thoroughly. Pipet out eight 5 ml aliquots of the combined extracts. (Each 5 ml aliquot represents the extract from 5 grams of control matrix.)

4) Fortify each of the extracts at the following levels:

2 - with no fortification
2 - with 5 ng/g avermectin B1a *
2 - with 50 ng/g avermectin B1a
2 - with 5 ng/g avermectin delta 8,9 isomer

The fortification of each of the samples must be performed by pipetting the appropriate quantity of avermectin in acetonitrile into the centrifuge tubes before the addition of the 5 ml of control extract. The acetonitrile should be taken to dryness at 50°C using a gentle stream of dry nitrogen. The residue is then redissolved in a 5 ml aliquot of the control extract, vortexed and ultrasonicated for 15 seconds.

* The fortification at 50 ng/g of avermectin B1a can be used to determine B1b recoveries.

5) These fortified 5 ml control extracts should then be carried through steps 21-40 of Method 1009 revision 2. Recoveries for each of the compounds tested should be calculated and control samples should be examined and shown to be free from detectable interferences.

Acceptable recoveries range from 80 to 110%.

PROCEDURE II FOR ALUMINA WASHING

Alumina Column Washing Procedure to Remove Interference

1. Pack the column as described in Method 1009, revision No. 2, step 22.

2. Wash the column with 30 ml of 15% isopropanol in methylene chloride.

3. After the wash solvent has completely eluted through the column, wash the column with 30 ml of methylene chloride.

4. Continue drawing a low vacuum (less than 10 in. Hg) for 20 minutes to air dry the column and continue the procedure with step 23.

D. Derivatization Reagents

Trifluoroacetic anhydride - Pierce; N,N-dimethylformamide - J. T. Baker; acetic anhydride, 1-methylimidazole - Aldrich; ammonium hydroxide (28-30%) - Fisher Scientific or Mallinckrodt.

To prepare the trifluoroacetyl derivatization reagent, add 3.6 ml dimethylformamide to 0.4 ml 1-methylimidazole in a 15 ml centrifuge tube, mix. Place the tube in an ice bath, allow the solution to chill for a minute, then slowly add 0.6 ml trifluoroacetic anhydride. Vortex until the solution is thoroughly mixed. Use this reagent immediately after preparation.

To prepare the acetyl derivatization reagent, add 0.6 ml of acetic anhydride to 1.8 ml N,N-dimethylformamide in a centrifuge tube. To this mixture add 0.4 ml of 1-methylimidazole. Vortex to mix.

To prepare the methanolic ammonium hydroxide reagent, add 0.2 ml of ammonium hydroxide reagent (28-30% ammonia) to 3 ml methanol. Vortex to mix. This volume should be added using a 1 ml graduated pipet. Volumetric pipets should not be used for the preparation of this reagent because the high viscosity causes errors in the volume retained in the tip of the pipet.

These reagents must be prepared just prior to derivatization. This procedure provides enough of each reagent to derivatize 15 samples and 5 standards.

VI. GENERAL APPARATUS

A. Crop Processor- Hobart model 81486 food processor with an 18 inch bowl, or equivalent food processor that is capable of producing a homogeneous sample from the frozen samples.

B. Homogenizer- Brinkmann Instruments Polytron blender model PT-35 with probe generator model PT20 or equivalent.

C. Vacuum Rotary Evaporator- Buchi Model R, Brinkmann Instruments or equivalent.

D. Centrifuge- IEC Tabletop Model HN-S II or equivalent.

E. Ultrasonicator- L&R Transistor/Ultrasonic Model T-21 or equivalent ultrasonicator with the same wattage.

F. Vacuum Manifold- Baker 10 SPE system with vacuum gauge or equivalent.

G. 15 ml Graduated Centrifuge Tubes-Fisher Scientific. Graduations are required.

Prepare silylated 15 ml graduated centrifuge tubes for the derivatization reaction, using the following procedure: Fill each clean tube to the top with Sylon-CT. Let stand 20 minutes. Rinse three times with 15 ml of toluene followed by three times with 15 ml of methanol. Fill with methanol and let stand 20 minutes. Rinse three times with 15 ml acetone and dry. As the derivatization reaction requires anhydrous conditions, tubes should be absolutely free of moisture prior to use. Moisture free conditions are insured by storing tubes in a 120^{o}C oven when not in use. Tubes will need to be re-silylated approximately every two months.

After each use, silylated tubes should be cleaned by first soaking in methylene chloride and then in detergent for at least several hours each, followed by thorough rinsing with hot water, distilled water, and acetone.

VII. HPLC APPARATUS

A. Mobile Phase Delivery System- Beckman Model 112 pump or equivalent. The pump must be able to operate at 1.5 ml/min, under the given conditions.

B. Injector- Waters WISP Model 710B or equivalent.

C. Pre-Column- Whatman Inc. 70 mm x 21 mm column containing 25-37 micron Co-Pell ODS packing or equivalent.

D. In-Line Filter- SSI high pressure 0.5 micron column prefilter or equivalent.

E. Analytical Column- ES Industries CHROMEGABOND MC-18, 150 x 4.6 mm I.D., 3 micron particle size. Other columns producing adequate chromatographic results will be accepted.

F. Fluorescence Detector- Kratos / Schoeffel Instruments Model FS 950. Other detector models should be tested for adequate senstivity before work begins.

G. 10 mv strip chart recorder or a data acquisition system which can provide a signal to noise ratio of greater than 5 for the lowest standard.

VIII. HPLC OPERATING CONDITIONS

Chromatographic Conditions:

Mobile Phase- 10% water in methanol (v/v);
Flow Rate- 1.5 ml/min Injection Volume- 50 microliters
Column Temperature- 30°C.

Detector Parameters:

Lamp- Kratos FSA 110, Blue phosphor coated Hg vapor lamp.
Excitation filter- Kratos FSA 403, 365 nm bandpass filter.
Emission filter- Kratos FSA 426, 418 nm cut off filter.
Sensitivity (pmt voltage)= 570v to 750v.
Range- 0.02 microamps. Time constant- 6 secs.

The recorder should be set at 10 mV, and the detector output should be set at 100 mV. All data acquisition systems must be able to produce a signal to noise ratio greater than or equal to 5 for the 2 ng/ml avermectin B1a standard.

These conditions are for the HPLC equipment and columns described above. With other equipment or columns minor changes in operating conditions may be required to obtain equivalent performance.

IX. DETERMINATION

Retention times for the fluorescent derivatives of avermectin B1a/delta 8,9 isomer and avermectin B1b/delta 8,9 isomer are approximately 10 minutes and 8.5 minutes, respectively. Residues of avermectin B1a/delta 8,9 isomer below 2 ng/g are non-detectable (reported as ND). The peaks representing avermectin B1a/delta 8,9 residues between 2-5 ng/g are identified but not quantitated (reported as NQ) and the peaks for residues above 5 ng/g are identified and quantitated. Since avermectin B1b is at most 20% (usually less than 10%) of the active ingredient, residue levels are generally less than the quantitation limit (5 ng/g) or the detection limit (2 ng/g). The peak representing avermectin B1b/delta 8,9 isomer is identified but not quantitated when the residue level is between 2-5 ng/g. Residues of avermectin B1b/delta 8,9 isomer above 5 ng/g are identified and quantitated in the same manner as the avermectin B1a/delta 8,9 isomer, using the avermectin B1a standard curve for quantitation.

An analysis set is comprised of no less than 5 standards and no more than 12 samples. The standards are run before and after the samples to ensure the stability of the HPLC system, the standards and the samples. For each analysis set, the slope and intercept are determined from the linear regression of the standards' peak height vs. concentration in nanograms per milliliter. Occasionally it has been observed that the peak height 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. The concentration of avermectin B1a/delta 8,9 isomer in a residue sample is determined as follows:

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

Where:

C=concentration of avermectin B1a/avermectin delta 8,9 isomer in ng/ml in the final volume used for HPLC analysis, PK HT= peak height of avermectin B1a/delta 8,9 isomer derivative, I= intercept, S= slope, FV= final volume used for HPLC analysis, SW= sample weight, UNK= concentration of avermectin B1a/delta 8,9 isomer in ng/g in the unknown residue sample. Frac= Fraction of the sample used for the assay (usually 0.5). Avermectin B1b residues are calculated in the same manner.

X. BIBLIOGRAPHY

Tolan, J.W., P. Escola, D. W. Fink, H. Mrozik, L. A. Zimmerman. 1980. "Determination of Avermectins in Plasma at Nanogram Levels Using High-Performance Liquid Chromatography with Fluorescence Detection". J. Chromatography, Vol. 190, pp. 367-376.

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Jenkins, J. J. and J. Cobin. "Determination of Avermectin in Citrus by Reversed Phase Liquid Chromatography with Fluorescence Detection". Abstract. American Chemical Society, Division of Pesticide Chemistry, Philadelphia, Pennsylvania, 8/26/84-8/31/84.

Analytical Research Residue Method Revision

Revision 1 01/13/87 page 1 of 3

Method 1009 was modified as follows:

A) Section II Method Validation.

Documentation indicating the purity of the new delta 8,9 Z Bla standard (L-652,280-000N005) versus the old delta 8,9 Z Bla standard (L-652,280-000N003) was added.

Justification: See memo from Morgan to Tway forthcoming.

B) Section IV A Method, steps 1 and 2.

Hobart food processors and sample processing steps were revised.

Justification: Samples could not be processed frozen using the Cuisinart food processor. The Cuisinart sample capacity is too small to process the whole sample.

C) Section IV C Method, steps 13 and 14.

Steps 13 and 14 were combined. The methanol extract and salt solution does not need to be mixed before the addition of the 0.01 % t-butanol in methylene chloride.

Justification: This step was carried over from method development work at Three Bridges. Acceptable validation work was performed at Three Bridges using this alteration in the method.

D) Section IV D Method, steps 19 and 20.

Steps 19 and 20 were changed providing a more detailed method for splitting the samples prior to derivatizations. The storage temperature for the samples was changed to - 10°C.

Justification: Description of sample splitting procedure was not accurate. The storage temperature of -10°C was validated in Rahway.

E) Section IV D Method, step 25.

Addition of 1 ml of the 1% isopropanol to the alumina column was changed to a quantitative 1 ml volume.

Justification: Low recoveries were obtained when any volume greater than 1 ml was used to wash the sample.

Method 1009 was modified as follows:

F) Section IV E method, step 33.

The volume of chloroform added to the sample after derivatization was changed from 1 ml to 4 ml.

Justification: The increased amount of chloroform decreases the polarity of the solvent thereby decreasing the solubility of ammonium trifluoroacetate. This aids in its removal during silica SEP-PAK ™ clean up. The solution turns cloudy after the addition of the 4 ml of chloroform producing a good visual indication that the reaction was completed successfully. When there is a lack of cloudiness at this point the derivatization was not performed properly.

G) Section IV E Method, step 34-37.

The SEP-PAK ™ cartridge rack has been approved for the silica clean-up step. This method may be used in addition to the older syringe method.

Justification: This device allows more samples to be processed and provides similar results when compared to the syringe method. See analyst validation reports for method 1009 (Analysts: JMM, KTC).

H) Section IV G Method.

New analytical standard preparations have been obtained. (L-676,863-038A002 Avermectin B1 and L-652,280-002T001 Delta 8,9 Z Avermectin B1a). Details on standard preparation were clarified .

I) Section V Reagents.

The lot number for the alumina was changed to 619464, and the preparation of the 10 % NaCl solution was scaled up.

Justification: Improper lot number was listed. The preparation of larger quantities of the 10 % NaCl solution was necessary.

Method 1009 was modified as follows:

J) Section VI General Apparatus.

In certain cases an equivalent instrument or apparatus may be substituted. When this type of substitution is allowable it was noted in the method.

Justification: In many cases these substitutions are not critical to the method. ~~However~~ All substitutions should be included in report writing.

K) Section VIII HPLC Operating Conditions.

These conditions were reformatted and clarified. A requirement was added to the detector specifications. The detector used must be able to produce a signal to noise ratio greater than or equal to 5 for the lowest standard (2 ng/ml).

Justification: This is required for validation see protocol A3-F1 for details.

L) Section IX Determination.

Quantitation of residues for avermectin B1b may be performed using the B1a standard curve.

Justification: See memo from Morgan to Tway forthcoming.

M) Sections I through X, The entire method.

Typographical errors, spelling errors and format were changed. No alterations in content were made.

Justification: To clarify document.

Prepared by: _____ January 19, 1987
John M. Morgan Date

Approved by: Patricia C. Tway January 19, 1987
Patricia C. Tway Date

Analytical Research Residue Method Revision

Method 1009 revision 1 was modified as follows:

a) Section V C, Reagents

Procedures I and II for alumina testing and prewashing were added to the method.

Justification: See memos from Morgan to Tway, and Wehner to Tway issued 4/21/87.

b) Section IV G., Preparation of Standards

Step 3 was revised and step 4 was omitted. The remaining steps were renumbered.

Justification: Use of the delta 8,9 or B1b standards is not necessary in performing routine analysis.

c) Revisions, page 3, section L

A memo was issued from Morgan to Tway 3/16/87.

d) Section I and IX, description of determination

The description of the determination or quantitation of avermectin B1a/delta 8,9 isomer was expanded and clarified.

Justification: To clarify the way avermectin B1a/delta 8,9 is quantitated and reported.

e) Section II, Method Validation

Table 1B was added.

Justification: Additional data with the new delta 8,9 isomer standard was available.

Method 1009 revison 1 was modified as follows:

f) Sections I through X, The entire method.

Typographical errors, spelling errors and format were changed. No alterations in content were made.

Justification: To clarify document.

Prepared by: John M. Morgan 8-5-87 Date

Approved by: Patricia C. Tway 8/5/87 Date
Patricia C. Tway

SUGGESTIONS FOR THE ANALYST PERFORMING MERCK RESIDUE METHOD NO. 1009, REV. 2

HPLC-FLUORESCENCE DETERMINATION FOR AVERMECTIN B1 AND ITS DELTA 8,9 Z ISOMER IN CITRUS FRUIT

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July 31, 1987

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TABLE OF CONTENTS

Citrus Method (1009R02, 7/31/87)
Suggestions for the Analyst - July 31, 1987

INTRODUCTION

Background

Avermectin B1 (abamectin) is a very effective acaricide and insecticide. [1,2] Its utility has been investigated in citrus such as oranges, grapefruit, lemons and tangelos. Because it is effective at such low application rates (typically 0.025 lbs ai/acre), the resultant residues are quite low and present a significant analytical challenge. Avermectin B1 is a mixture of at least 80% avermectin B1a and not more than 20% avermectin B1b (Figure 1). An environmental photodegradate is formed from the isomerization of the 8,9 double bond to the Z form. It has been determined from metabolism and toxicity studies that it is necessary to quantitate the residues on exposed citrus of avermectin B1a, avermectin B1b and the 8,9 isomer of avermectin B1a.

Method chemistry

It is evident from the structure that avermectin B1 has very little volatility and none of the heteroatoms (e.g., Cl, N or P) which have enhanced detection capabilities. Conventional gas chromatographic pesticide methods will not work to chromatograph or detect avermectin B1. High pressure liquid chromatography (HPLC) assays have been developed using either ultraviolet [3] or fluorescent detection for the related compound, ivermectin (dihydroavermectin B1), in animal plasma [4] or tissue [5]. To achieve the sensitivity and selectivity required for citrus, fluorescent detection is necessary [6]. Because the compound itself is not sufficiently fluorescent, various derivatives have been investigated. Fortunately, it is not necessary to add a fluorescent tag to the structure but only to aromatize a ring (as shown in Figure 1).

The most useful technique to quantitate avermectin B1a, avermectin B1b and the photodegradate delta 8,9 isomer employs trifluoroacetic anhydride and 1-methylimidazole in dimethylformamide. Incubation with these reagents at 30°C for 60 minutes yields the fluorescent derivative shown with the 4" position trifluoroacetylated on the saccharide (oleandrosyl) group (not shown). To stabilize the derivative, the trifluoroacetyl group is cleaved with methanolic ammonium hydroxide to leave a hydroxyl at the 4" position. This two-step process yields a single HPLC peak for both the avermectin B1a and the delta 8,9 isomer. Under the given chromatographic conditions, the fluorescent derivative of avermectin B1b is resolved from and elutes before the B1a or delta 8,9 peak. Thus, two component

residues can be quantitated, (1) avermectin B1a + delta 8,9 isomer and (2) avermectin B1b. Since avermectin B1b is present in much smaller quantities in the formulation and the resultant residues, there is seldom enough to quantitate. Both components of the residue are quantitated in levels at or above 5 ng/g and are identified only when present at levels from 2 to 5 ng/g. They are not detectable below 2 ng/g.

OVERVIEW OF ASSAY

Principle

The ground whole citrus fruit is homogenized and extracted with methanol to solubilize the residues of avermectin B1 and its delta 8,9 isomer. The macerate is filtered and the methanol filtrate is washed twice with isooctane. An aqueous salt solution is added to the methanol extract and the avermectin residue is extracted from the mixture into methylene chloride containing 0.01% t-butanol. The methylene chloride phase is concentrated by evaporation and loaded onto an acidic alumina column for additional purification. The eluant (15% isopropanol in methylene chloride) is evaporated to dryness and reconstituted in the derivatization reagent mixture (reagent A in Figure 1) of 1-methylimidazole and trifluoroacetic anhydride in dimethyl-formamide. The fluorescent derivative is formed by reaction at 30°C for 60 minutes followed by addition of methanolic ammonium hydroxide (reagent B) and reaction for 30 minutes at 30°C. Chloroform is then added to the reaction mixture and the combination is washed through a silica gel column to purify the derivative. The eluant is evaporated to dryness and reconstituted in methanol for high pressure liquid chromatographic analysis with fluorescence detection. A flow diagram detailing the assay method is shown in Figure 2.

Assay timing

For almost any analysis, the assay scheduling depends greatly on the objectives of the analyst and the equipment available to complete the procedure. This avermectin B1 assay method has been designed to maximize accuracy and minimize time per assay. Achieving a single result in minimum time was not our highest priority. Thus, we routinely assay 10 samples plus a control and a fortified control in two eight hour days with the final quantitative assay step performed overnight using an automatic HPLC injector. It is possible to assay a more limited number of samples within a 24 hour period, including the HPLC quantitation since there are no prolonged steps (e.g. no overnight reactions). This approach is only desirable if there are a limited number of samples or for enforcement purposes because it is not as efficient. Notations will be

made throughout this description of the opportunities for efficient scheduling or handling.

Solubility properties

Avermectin B1 (including the delta 8,9 isomer) has very low water solubility (in the ppb range). This provides some advantages for sample cleanup since the compound of interest can be separated from aqueous co-extractives quite easily. The solubility properties also provide some disadvantages because there is a tendency for losses on surfaces, such as on glass. It is important to not take the samples to dryness except as directed in the method. Avermectin B1 has high solubility in alcohols, such as methanol, isopropanol or t-butanol. For example, the addition of t-butanol to the methylene chloride extraction solvent helps keep the avermectin in solution during the solvent evaporation step after the extractions.

Motives behind assay

The steps in the assay have been shown to provide the necessary cleanup of samples while maintaining sufficient recovery. The methanol extraction does a thorough job of extracting the avermectin B1 residue (including 8,9 isomer) from the matrix, but it also extracts extraneous material. The liquid-liquid partitions plus the alumina column cleanup are needed to remove the undesirable co-extractives, taking advantage of the solubility properties of avermectin. Formation of the fluorescent derivative provides the sensitivity and selectivity to quantitate the low residues incurred with normal usage.

EQUIPMENT

The equipment listed in the method has been used to assay avermectin B1 (including 8,9 isomer) in citrus. Substitutions of equivalent equipment may be suitable if the performance is equivalent or superior to the specified equipment. A well-equipped residue laboratory should have all of the items listed in the method or at least have suitable substitute equipment, with the possible exception of the HPLC fluorescence detector. In general, different types of glassware may be used without problems, except that the derivatization tubes always should be silanized. Equipment such as centrifuges and evaporators which do not directly contact the sample only need to meet the performance specifications since there is no opportunity for contamination. Equipment such as the Polytron homogenizer or HPLC injector which does contact the sample should be tested for equivalent performance with the procedure as specified.

PREPARATION OF STANDARDS

Solid avermectin B1a, avermectin B1b or 8,9 isomer are not available in easily handled forms so the analytical standards are dilute solutions in glycerol formal. The standards and all dilutions should be stored in the dark in a freezer capable of maintaining -10°C. They should be weighed out after coming to room temperature using an analytical balance capable of measuring below 1 mg accurately. Each standard should be weighed onto a glass or platinum weigh boat. The small boat may be rinsed thoroughly by placing in a glass funnel with a stem too narrow to let the boat pass through but wide enough to allow copious amounts of solvent through. The funnel is placed in the opening of the volumetric flask in which the standard will be diluted during the rinsings of the weigh boat. Dilute solutions of the standards should be prepared in acetonitrile where stability over several months has been demonstrated in a dark freezer. Because the available avermectin standard is a mixture of B1a and B1b, it is important to know the exact purity of the standard to determine the concentration of B1a and B1b in solution. The purity is generally given in weight percent. An avermectin standard that is 0.9% B1a has 9 mg of avermectin B1a in every 1000 mg of standard.

ASSAY PROCEDURE

Sample preparation

A field sample usually consists of several fruit that have had the same treatment. These fruit must be crushed and blended together to form a homogeneous sample. A sample is most efficiently ground using the Hobart food processor after breaking up the whole frozen fruit into smaller pieces. The whole fruit can be broken into pieces by placing the strong plastic-impregnated cloth bag which contains the frozen fruit on a clean hard surface (such as a lab counter) and striking the fruit inside the bag with a large hammer or mallet until all of the individual fruit is broken into pieces that the food processor can grind. To avoid overloading the food processor, the sample pieces should be added slowly to the grinding vessel. As some pieces are ground, more pieces can be added until there are no discernible chunks of whole fruit. After the whole sample is in the food processor, grinding is continued until the mixture is homogeneous. This usually takes a minimum of 5 minutes. To keep the sample primarily frozen during the grinding process, dry ice is added to the processor. The sample mixes best when it is a slush, not completely solid. It is important to not allow it to completely thaw, however, since the solids and liquid will tend to separate. Other food processors such as a Cuisinart may be used to grind

samples. The Cuisinart has capacity for only smaller samples but it tends to grind the samples into finer pieces.

After the grinding has been completed, portions of the sample are carefully transferred into a properly-labelled square Nalgene polypropylene bottle. A square polypropylene bottle is used for more efficient storage in an unbreakable container but the shape does not affect the samples. The bottle should not be filled to the top to allow space for expansion of the sample during the freezing and thawing process and for thorough mixing prior to analysis. The dry ice used during the grinding process should be allowed to dissipate before sealing the bottles. To avoid cross contamination between samples during processing, the removable parts on the food processor are cleaned by washing them with hot soapy water and then rinsing with distilled water and methanol. Control samples are generally processed before treated samples. Samples suspected to contain the highest residues are processed last.

Extraction from crop

To ensure a homogeneous and representative sample, the frozen ground matrix should be thawed before weighing the 10.0 gram portion. The sample can be thawed by either gently warming the sample container in a water bath or by leaving the sample overnight in a refrigerator or on a counter. For assays with quick turnaround, the sample will not have been stored frozen after grinding so the sample merely has to be thawed more completely to allow mixing. After the sample has thawed, it should be shaken thoroughly and mixed with a spatula to avoid separation of solids and liquid. Continuous mixing during the removal of the 10 gram subsample may be necessary to assure a representative sample.

The ten gram sample is weighed into the homogenization vessel. We have found that the French square bottle is convenient and helps the homogenization process in methanol. The bottle is tall and narrow, preventing loss of the sample during the blending process. The bottle can stand upright by itself since it has a flat bottom. The square corners of the bottle encourage turbulent flow during homogenization by the Polytron which enhances mixing. After the sample is weighed out, the solvent is added and then homogenized with methanol for at least 1 minute or as long as necessary to assure a homogeneous sample extract. If the sample is a fortified control, the small volume (1 ml or less) of fortification standard in acetonitrile is added and the solvent is allowed to evaporate before the methanol is added. If a Polytron homogenizer is not available, the samples could be homogenized in a blender (such as from Eberbach). The same solvent volumes should be used but

additional homogenization time may be necessary to completely solubilize the avermectin residue.

The methanol and macerate are poured into a porcelain funnel containing filter paper wetted with methanol and the filtrate is collected in a filter flask by drawing vacuum through the filter. The Polytron probe is rinsed by homogenization in the bottle with additional methanol which also is used to rinse the filter and macerate. For the most efficient use of time and energy, the filtration apparatus including the vacuum line should be set up near the homogenizer. To avoid sample loss during filtration, it is essential that the end of the porcelain funnel be below or pointing away from the vacuum side arm of the filtering flask. The filter cake is washed with an additional small amount of methanol (5-10 ml).

For twelve samples, it takes 1-2 hours to weigh, homogenize, extract and filter from the matrix. The methanol extracts may be retained in covered flasks overnight or over the weekend in the freezer.

The methanol extract and rinses are transferred from the flask into a 500 ml separatory funnel. If the extract was stored in the freezer, it should be allowed to warm to room temperature and mixed by swirling and sonication before being transferred into the separatory funnel. The filter flask is rinsed with three small portions (2-3 ml) of methanol and the rinsings are added to the separatory funnel. The total volume of the methanol extract and rinses should be below 125 mls.

Solvent partition

Isooctane is added to the separatory funnel to wash nonpolar co-extractives from the methanol extract. Much of the color from the methanol extract goes into the isooctane phase, which sometimes makes it difficult to see the solvent interface. As the methanol is drained from the separatory funnel, the interface becomes more obvious in the narrower parts of the funnel. The methanol can be drained into a 250 ml separatory funnel or back into the filter flask. If it is put into the filter flask, then the methanol is returned to the 500 ml separatory funnel after the isooctane is discarded, and washed again with another aliquot of isooctane. If the 250 ml separatory funnel is used, then the next step is to repeat the isooctane wash and the methanol is returned to the 500 ml funnel after the second isooctane phase is discarded.

The sodium chloride solution is added to the methanol to assist the removal of the polar co-extractives and force the avermectin residue into the methylene chloride phase containing 0.01% t-butanol. The salt helps to minimize the

formation of emulsions during the partition. Because of the volatility of the methylene chloride, it is essential to vent the separatory funnel regularly during shaking. Venting needs to be done about every 5-10 seconds during the first methylene chloride extraction and about every 15 seconds during the second methylene chloride extraction.

The methylene chloride (with 0.01% t-butanol) extracts are filtered through anhydrous sodium sulfate (pre-wetted with methylene chloride) in a funnel to remove excess water from the extract. The drying funnels are prepared manually and certain precautions should be observed. The silanized glass wool should be packed just tight enough to avoid the loss of the solid phase during the liquid movement. Glass wool which is packed too tightly inhibits flow through the bed of sodium sulfate and slows analysis. If it is packed too loosely, the sodium sulfate may fall into the round bottom flask collecting the filtrate. Experience will demonstrate the appropriate packing procedure. The seven grams of sodium sulfate can be rapidly measured into the funnels by measuring the volume of 7 grams in a graduated cylinder and then using the cylinder to measure and pour the sodium sulfate into each funnel. If only a few samples are to be assayed, this time-saving is not necessary.

The combined methylene chloride (containing 0.01% t-butanol) extract is evaporated to 2-3 ml using a vacuum rotary evaporator. When performing the evaporation, changes in vacuum pressure should be made slowly to avoid "bumping" or drawing some of the liquid into the vacuum adapter, condensor or trap. When the sample reaches approximately 3 ml, the vacuum should be slowly changed to ambient pressure. Experience will help in judging the 3 ml volume left in the flask. If the sample goes to dryness during the rotary evaporation step, it should be noted and then 3 ml of 0.01% t-butanol in methylene chloride should be added to the flask, swirled and sonicated to completely redissolve the residue before transfer.

The sample is transferred to a 15 ml centrifuge tube and brought to a volume of 10 ml (measured by the tube graduations) with methylene chloride. Half of the sample (5 ml) is quantitatively transferred to a polypropylene centrifuge tube for storage. The remaining half of the sample may be stored in a freezer overnight or over the weekend until the next step is performed.

To handle a sample set of 10 samples plus control and fortification, it may be most efficient to divide the set in half. The first half of samples are partitioned with solvents up to the rotary evaporation step. The second half of samples in the set are then partitioned with solvents while the first half is being evaporated. This requires diligence to assure that the assay is performed properly

with each sample but saves time when handling multiple samples. If many rotary evaporators are available, then this approach may not be necessary. The solvent partition and rotary evaporation steps take approximately 3 hours for 12 samples (including the control and fortification).

Column cleanup

Preparing the alumina column

The alumina column is prepared by transferring 0.70 to 0.72g of heat-treated acidic alumina into a 1 ml column reservoir with a 20 micron polyethylene frit. If the empty reservoir comes with two frits already inserted, it is necessary to use a small probe such as a piece of wire or 1/16" tubing to push them out and only 1 frit is used at the base of the column. Prepacked columns have not been used because of the expense to obtain custom-packed columns and because of the need to verify the performance of every lot of alumina used for the method. The alumina, either in the packed column or before packing, must not be left exposed to humid lab air for prolonged periods of time. The alumina should be heated or stored in an oven at 120°C at least overnight before being packed in the columns. This is normal good practice in using alumina, which tends to adsorb lab vapors.

The packed column is washed with methylene chloride just before use to condition the column. If the lot of alumina or reservoirs have displayed some potential interferences, particularly for avermectin B1b, the packed column with the reservoir in place can be washed with 30 ml of 15% isopropanol in methylene chloride before use. After the wash solvent has completely eluted, the column should be conditioned by washing with 30 ml of 100% methylene chloride. The column can then be air-dried and used normally.

Different lots or manufacturers of alumina can be validated by conducting an experiment as described in Procedure I (memo Morgan to Tway, 4/21/87).

Using the vacuum manifold

We have used the Baker 10 SPE or the Analytichem Vac-Elut vacuum manifold to perform the alumina column cleanup. Twenty ml of 15% isopropanol in methylene chloride measured from a fixed volume dispensing pipet are used to elute the avermectin from the column. These vacuum manifolds are not deep enough to hold 20 or even 15 ml test tubes so we use 25 or 40 ml flat-bottomed vials, as mentioned in the methods. Neither manufacturer sells a rack specifically for these vials so some ingenuity is required. There are at least three alternative ways to use the vacuum manifold to collect larger sample volumes.

For the first alternative, there are ten positions available on the manifold with a stainless steel cannula at each position directing the eluant from the column into the receptacle. The taller 40 ml vials can be left free-standing in the box and the cannulas are lined up and inserted into the corresponding vials. The ten vials fill the box tightly enough that none of the vials fall over. This does require care to ensure that each cannula gets inside a vial so none of the eluant is lost. The free-standing system only works if all ten positions have vials to fill the box. This means that "dummy" vials are needed if not all 10 positions are required. The dummy vials are reused.

The second alternative uses commercially available racks which can be purchased from either manufacturer for use in the vacuum manifold. Both systems have racks that can hold volumetric flasks. The 25 ml vials are placed in the flask rack and held in place with a rubber band wrapped horizontally around the rack and vials. The vials are held rigidly in place and the rack helps to align the vials with the cannulas. The rack also supports the vials above the bottom of the box and keeps the vacuum orifice unencumbered. The rubber band does not last for more than a few uses since the solvent vapors or lab atmosphere tend to cause disintegration, but it is an inexpensive item to replace

The third alternative is to use different, deeper manifolds which are now available, from manufacturers such as Applied Separations (Bethlehem, PA), Analytichem International (Harbor City, CA) or Applied Science (Deerfield, IL).

The 5 ml of remaining sample are concentrated to 1 ml and loaded onto the alumina column. The tube is rinsed with two 1 ml portions of 1%isopropanol (IPA) in methylene chloride and the rinsings are loaded onto the column. Loading the column with more than 2-3 ml of 1%IPA tends to wash the avermectin off the column prematurely. The column is eluted with 20 ml of 15% IPA in methylene chloride. The eluant is evaporated to 2-3 ml and quantitatively transferred to a 15 ml silanized centrifuge tube to use in the derivatization reaction. Three 3 ml portions of methylene chloride are used to rinse the collection vessel (vial) and transferred to the silanized tube.

The samples may be stored overnight in a freezer at this point. The alumina column steps (manipulating the sample from the rotary evaporation flask to the dried residue ready for derivatization) take approximately 2 hours to complete for 12 samples (including control and fortification).

Derivatization

The solvent is removed from the silanized derivatization tube by evaporation under a stream of nitrogen. It is important to try to concentrate the residue as much as possible in the tip of the centrifuge tube. Evaporation that is too rapid will splash sample up the sides of the tube and reduce the derivatization yield. Some additional rinsing of the tube walls with methylene chloride may be necessary to ensure that all of the sample is in the bottom and will thus be reconstituted in the derivatization reagents.

The derivatization reagents are measured into a centrifuge tube using a graduated 1.0 or 5.0 ml pipet and mixed by vortexing. When vortexing the DMF and 1-methylimidazole mixture, it is important to make sure that the reagents are mixed well before proceeding. Several short interrupted pulses on the vortex will help the mixing. The mixture should be visually inspected to see if the agitation was adequate. The differences in viscosities or refractive index make it apparent when the reagents are inadequately mixed. The trifluoroacetic anhydride (TFAA) is volatile and generates heat when mixed with the dimethylformamide (DMF) and 1-methylimidazole mixture so the reagent tube containing the mixture should be chilled for about a minute prior to the addition of the TFAA. Addition of the TFAA causes a white cloud to form above the reagent mixture which disappears when well-mixed.

The derivatization reagent mixture is aliquoted to each sample or standard tube using a 1.00 ml graduated pipet (1/100 ml calibration). The 0.2 ml aliquot for each tube is dispensed and then thoroughly mixed. Aliquoting for five tubes can be done consecutively using the 1.00 ml pipet before refilling. During sonication of the tubes, those tubes containing sample matrix should be monitored to ensure that all of the residue goes into solution. All of the tubes, both standard and sample, are added to the 30°C water bath simultaneously. Timing of the reaction starts after all the tubes are in the bath. It is important that the bath be maintained at 30°C $\pm$ 1°C. After the 60 minute reaction time has passed, the methanolic ammonium hydroxide is added, which is also done using a 1.00 ml graduated pipet to dispense the 0.1 ml of reagent to ten tubes consecutively. During the derivatization, the samples will change colors, which vary with the matrix and range from yellow to cherry-red. The standards do not change colors.

After the derivatization reaction is completed, four ml of chloroform are added to each tube and vortexed. The solution should be cloudy and have a white suspension after the chloroform addition. The silica SEP-PAK cleanup can be performed on multiple samples using a cartridge rack or on

individual samples using a Luer-lok syringe as the solvent reservoir. It is important to rinse the derivatization tube thoroughly with the chloroform as well as the silica SEP-PAK, as described in the procedure. The chloroform eluant is evaporated to dryness and reconstituted in methanol. It is important to adequately vortex and sonicate the methanol solution before HPLC injection. It takes about 3 hours to derivatize and prepare 12 samples and 5 standards for HPLC analysis.

HPLC Determination

The high pressure liquid chromatographic (HPLC) system suitability should be determined before any samples are assayed. The performance of the overall system is demonstrated by a steady baseline from the detector and by an appropriate response and retention time for standards. Generally, the fluorescence detector is being run at such sensitive levels that it is necessary to leave it on most of the time, but individual manufacturers' recommendations should be followed. Fresh mobile phase should be pumped through the system long enough to determine the stability of system. Sources of extraneous baseline noise or drift should be eliminated as much as possible before assays begin. The highest standard should be injected to verify that the detector and recorder/data system settings are appropriate with the high standard being at least 75% of full scale deflection (but not offscale). The lowest standard should be at least five times the signal to noise. To achieve sufficient sensitivity with a recorder, it may be necessary to set the recorder input voltage to be lower than the output voltage of the detector. For example, the recorder could be set to 1 mV while the detector is set to 10 mV. In routine practice, when the HPLC system is being used daily to assay avermectin, it will be apparent when the performance has degraded without extensive system checks. Generally, replacing the used guard column with a new one improves chromatographic performance. If this does not work, additional troubleshooting is necessary.

QUANTITATION

For routine analysis of avermectin B1, including its 8,9 isomer, samples are run in sets of twelve, including one control and one fortified sample. The standards, at least five, are derivatized and assayed on the HPLC with the samples. Generally, the five standards are injected before the samples to demonstrate the system performance and then injected again after the samples to demonstrate the system stability. A standard curve using the 10 injections is determined by performing a linear regression on the standards' peak height versus the concentration of the standards in ng/ml. The slope and intercept from the

regression line are used to calculate the concentrations of the sample solutions based on the peak heights observed for the samples. The standard curve occasionally has an aberrant standard, with a peak height too low to fit on the line. A single errant standard can be discarded from the linear regression calculation. If more than one standard appears abnormal, then the derivatization must be repeated with the retained underivatized portions of the samples and fresh standards.

After the concentration of the sample in the final volume is determined from the linear regression curve, then the concentration in ng/g of the original sample may be determined based on the fraction of the sample derivatized (FRAC), the starting sample weight (SW) and the final volume (FV) for HPLC analysis. For example, a sample which calculates to have 10 ng/ml in the HPLC solution and which was treated in the usual manner during the sample preparation will have 10 ng/ml X 5 ml (FV) or 50 ng in the total HPLC solution. This 50 ng came from half of the 10 gram sample or

50 ng / [0.5 (FRAC) X 10 g (SW)] = 10 ng/g

Determination of avermectin B1b from avermectin B1a

Avermectin B1b residues in samples can be determined from the avermectin B1a standard curve as described in the memo from Morgan to Tway (3/16/87). Experimental results have demonstrated that avermectin B1b residues in citrus can be accurately quantitated from the avermectin B1a standard curve without needing to generate a B1b standard curve. This is reasonable since the structure of the fluorophores is so similar for the two compounds and avermectin B1b is rarely observed at quantifiable levels. The retention time of avermectin B1b is established by the highest standards of avermectin B1a which have enough B1b to determine its retention time.

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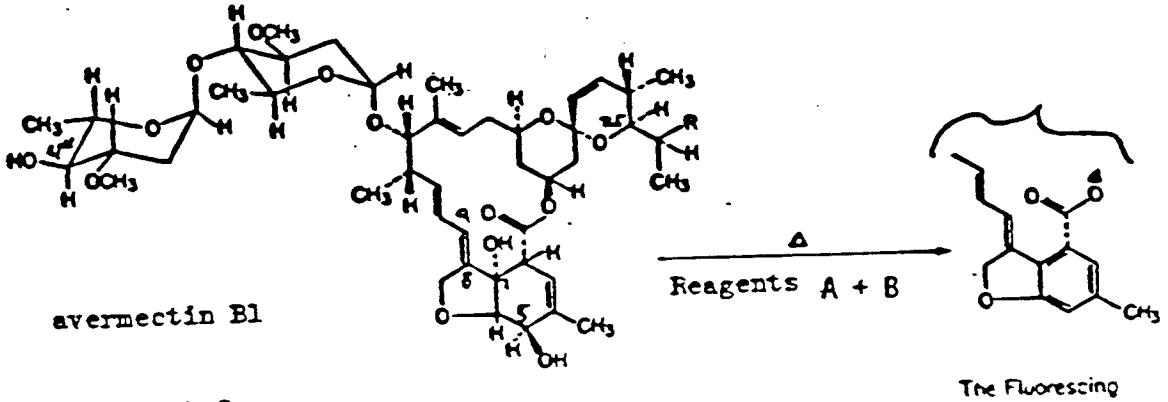
Figure 1
Structure of Avermectin and Fluorescent Derivative

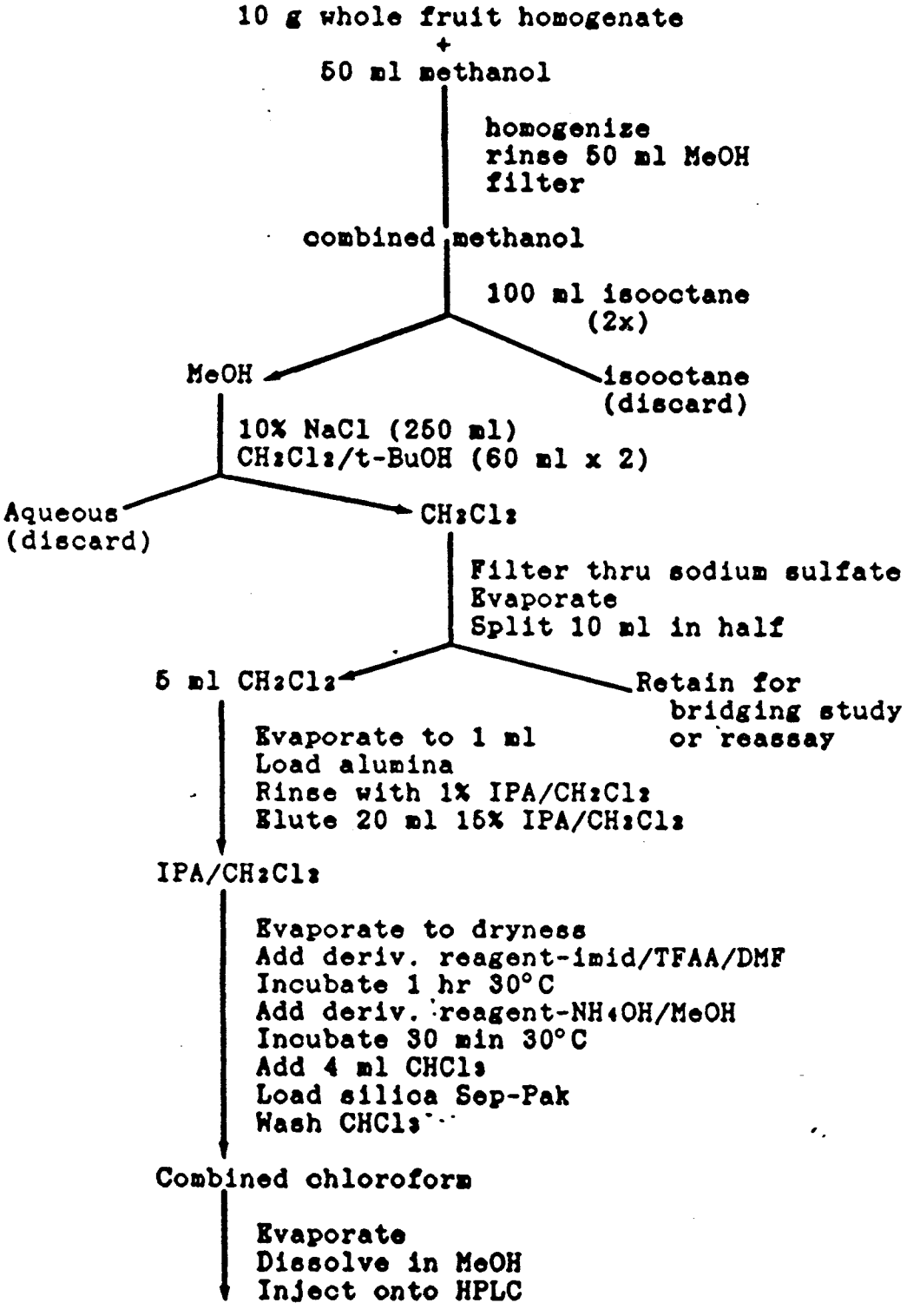


A. Trifluoroacetic anhydride/DMF/1-methylimidazole

B. Methanolic Ammonium Hydroxide

FIGURE 2

Avermectin Citrus Whole Fruit Assay



Reverse phase HPLC with fluorescence detection