HPLC-FLUORESCENCE DETERMINATION FOR AVERMECTIN B_1 AND 8,9-Z-AVERMECTIN B_1 IN PEARS AND APPLES

Method No. 8000, Rev. 4

Merck Research Laboratories
Division of Merck & Co., Inc.
P.O. Box 2000
Rahway, NJ 07065-0900

December 14, 1992

Prepared by: Michael B Hicks
Michael B. Hicks
Staff Chemist
Analytical Research

Audited by: Helene Rosenthal
Helene Rosenthal
Auditor
Quality Assurance

Approved by: Patricia C. Tway
Patricia C. Tway, Ph.D.
Executive Director
Analytical Research

Collaborators:

Teresa A. Wehner, Ph.D.
Richard S. Egan, Ph.D.
Janice Cobin
Sunil V. Prabhu, Ph.D.

TABLE OF CONTENTS

HPLC-Fluorescence Determination For Avermectin B_1 and 8,9-Z-Avermectin B_1 in Pears and Apples

Merck Research Laboratories
Division of Merck & Co., Inc.
P.O. Box 2000, Rahway, NJ 07065-0900

I SUMMARY

Avermectin B_1 (Abamectin) is a mixture of two homologs containing not less than 80% avermectin B_{1a} and not more than 20% avermectin B_{1b}. These components differ by only one methylene unit (-CH2-) at the 25-carbon position, wherein avermectin B_{1a} contains a sec-butyl group and avermectin B_{1b} contains an isopropyl group.

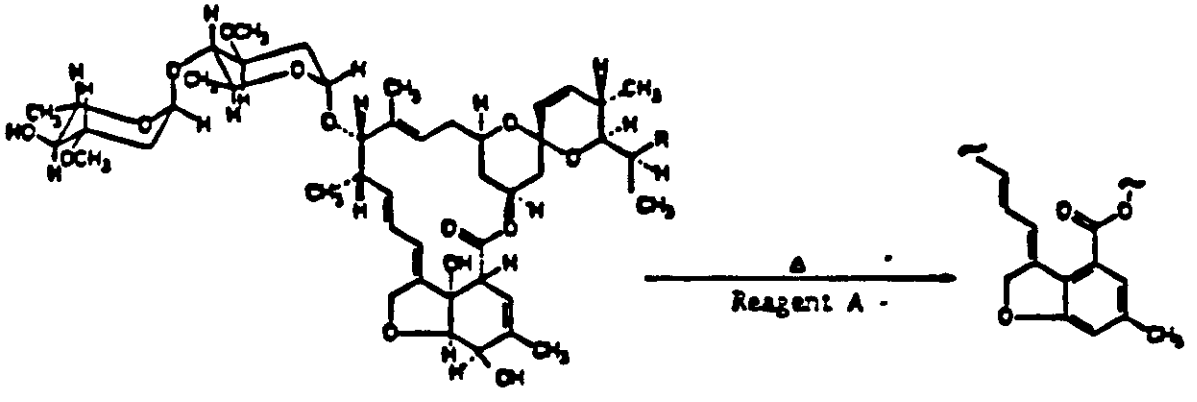
The first analytical methods developed were for the animal health drug ivermectin (22,23-dihydroavermectin B_1). The decomposition of the avermectins during gas chromatographic analysis and the low sensitivity of the liquid chromatography assays employing UV detection, made the detection of the avermectins at trace levels an analytical problem. In 1980 Tolan et al. (1) developed an HPLC method employing the fluorescence detection of ivermectin in plasma. Then in 1981 Tway et al. (2) 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 B_1 with acetic anhydride, 1-methylimidazole and 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 by Jenkins and Cobin (3) in the determination of avermectin B_1 in citrus.

Following more complete characterization of the total toxic residue, it was determined that an analytical method would also be required for 8,9-Z-avermectin B_1. Initially it was thought that the derivatization reaction described above could be used for the determination of both the parent avermectin B_1 and 8,9-Z-avermectin B_1. Subsequently, it was determined that reaction of 8,9-Z-avermectin B_1 under these conditions gives an unacceptable result, i.e., apparent low reaction yield of the fluorescent derivative which is photolytically unstable. Because stereochemistry suggests that the 7-OH of 8,9-Z-avermectin B_1 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 8,9-Z-avermectin B_1 isomer with the same retention time as the parent avermectin B_1 fluorescent derivative. Therefore using this derivative, the residue quantitated represents the sum of avermectin B_{1a} and 8,9-Z-avermectin B_{1a}.

For apples and pears, residues of avermectin B_{1a} + 8,9-Z-avermectin B_{1a} below 1 ng/g are reported as non-detectable (reported as ND). Residues between 1 - 2 ng/g are identified but not quantified (reported as NQ) and residues above 2 ng/g are identified and quantitated from avermectin B_{1a} standard curve. Since avermectin B_{1b} is at most 20% (normally less than 10%) of the active ingredient, residues are generally present at levels less than the limit of quantitation for pears and for apples (2 ng/g). At levels above the quantitation limit, avermectin B_{1b} residues are quantitated in the same manner as avermectin B_{1a}/8,9-Z-avermectin B_{1a}.

Figure 1

Structures of Avermectin B_1 and
Derivatization Reaction



$R=C_2H_5$ for B_{1a}
$R=CH_3$ for B_{1b}

The Fluorescing
Derivative for HPLC

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

(2) Methanolic ammonium hydroxide

II METHOD VALIDATION

Method 8000, Rev. 2 has been validated for both pears and apples. In each experiment, a 10 g sample was fortified with avermectin B_1 or 8,9-Z-avermectin B_1. Recoveries for the avermectin B_1 (L-676,863-038A003) and 8,9-Z-avermectin B_{1a} (L-652,280-002T001) were determined by spiking samples with avermectin B_{1a} or with the 8,9-Z-avermectin B_{1a}, and they were quantitated versus the B_{1a} component from avermectin B_1 standards. Pear and apple samples used in method development and validation were purchased at local supermarkets and were obtained from Merck field Trial #001-87-5007R, 001-90-6028R and 001-90-6029R (pears). The control pear and apple samples had no peaks which would interfere with the avermectin B_{1a}/8,9-Z-avermectin B_{1a} or B_{1b} derivative peaks.

Table 1 gives the results of the validation of the method for pear matrices. Recoveries were good and ranged from 81 to 126% with precision that ranged from 3.4 to 12% RSD. Table II gives the results for method validation for the apple matrix. Recoveries were good and ranged from 71 to 108% with an RSD that ranged from 5.6 to 14%.

Typical chromatograms Avermectin B_1 standard, control sample, control samples fortified with avermectin B_{1a} and 8,9-Z-avermectin B_{1a} and avermectin B_{1b} are shown in Figures 2, 3, and 4.

Table 1

Recovery of Avermectin B_{1a}, Avermectin B_{1b}, and 8,9-Z-Avermectin B_{1a} from Pear Homogenates

Sample No.	Pear Type	B_{1a} Conc. ng/g calc.	Fort. Level ng/g	%Rec
C-1	Bartlett	0.0	0.0	NA
C-2	Bartlett	0.0	0.0	NA
C-3	Bartlett	0.0	0.0	NA
C-4	Bartlett	0.0	0.0	NA
C-5	Bartlett*	0.0	0.0	NA
C-6	Bartlett*	0.0	0.0	NA
C-7	Bartlett*	0.0	0.0	NA
C-8	Bartlett*	0.0	0.0	NA
CB-1	Bosc	0.0	0.0	NA
CB-2	Bosc	0.0	0.0	NA
CB-3	Bosc	0.0	0.0	NA
16701-70-1	Bartlett	N.D.	0.0	NA
16701-71-1	Bartlett	N.D.	0.0	NA
16701-72-1	Bartlett	N.D.	0.0	NA
16701-70-2	Bartlett	2.2	2.1	104
16701-70-3	Bartlett	2.2	2.1	105
16701-70-4	Bartlett	2.4	2.1	116
16701-71-2	Bartlett	1.8	2.1	87
16701-71-3	Bartlett	1.7	2.1	81
16701-72-2	Bartlett	2.1	2.1	100
16701-72-3	Bartlett	2.2	2.1	105
			Mean Recovery = $\overline{100}$ rsd 12%	
5-B_{1a}-1	Bartlett	4.8	5.1	94
5-B_{1a}-2	Bartlett	4.9	5.1	96
5-B_{1a}-3	Bartlett	4.9	5.1	96
5-B_{1a}-4	Bartlett	4.7	5.1	92
5-B_{1a}-5	Bartlett*	4.9	5.1	96
5-B_{1a}-6	Bartlett*	5.6	5.1	110
5-B_{1a}-7	Bartlett*	5.3	5.1	104
5B-B_{1a}-1	Bosc	4.9	5.1	96
5B-B_{1a}-2	Bosc	4.8	5.1	94
5B-B_{1a}-3	Bosc	5.2	5.1	102
			Mean Recovery = $\overline{98}$ rsd 5.7%	

Table 1

Recovery of Avermectin B_{1a}, Avermectin B_{1b}, and 8,9-Z-Avermectin B_{1a} from Pear Homogenates

Sample No.	Pear Type	B_{1a} Conc. ng/g calc.	Fort. Level ng/g	%Rec
30-B_{1a}-1	Bartlett	27.9	31.0	90
30-B_{1a}-2	Bartlett	28.8	31.0	93
30-B_{1a}-3	Bartlett	27.0	31.0	87
30-B_{1a}-4	Bartlett	28.8	31.0	93
30-B_{1a}-5	Bartlett	29.4	31.0	95
			Mean Recovery = $\overline{92}$ rsd 3.4%	
50-B_{1a}-1	Bartlett	45.6	51.0	89
50-B_{1a}-2	Bartlett	43.8	51.0	86
50-B_{1a}-3	Bartlett	50.4	51.0	99
50-B_{1a}-4	Bartlett	50.4	51.0	99
50-B_{1a}-5	Bartlett	46.2	51.0	91
			Mean Recovery = $\overline{93}$ rsd 6.4 %	

Table 1 (con't)

Recovery of Avermectin B_{1a}, Avermectin B_{1b}, and 8,9-Z-Avermectin B_{1a} from Pear Homogenates

Sample No.	Pear Type	8,9-Z-avermectin B_{1a} Conc. ng/g calc.	Fort. Level ng/g	%Rec
5-8,9-1	Bartlett	4.3	4.6	93
5-8,9-2	Bartlett	4.6	4.6	100
5-8,9-3	Bartlett	4.1	4.6	89
5-8,9-4	Bartlett	4.8	4.6	104
5-8,9-5	Bartlett*	3.9	4.6	85
5-8,9-6	Bartlett*	5.0	4.6	109
5-8,9-7	Bartlett*	4.9	4.6	109
5-8,9-8	Bartlett*	4.9	4.6	107
5B-8,9-1	Bosc	4.9	4.6	107
5B-8,9-2	Bosc	4.7	4.6	102
5B-8,9-3	Bosc	4.3	4.6	93
			Mean Recovery =	$\overline{100}$ rsd 8.3%
25-8,9-1	Bartlett	22.5	23.0	98
25-8,9-2	Bartlett	21.9	23.0	95
25-8,9-3	Bartlett	22.8	23.0	99
25-8,9-4	Bartlett	21.3	23.0	93
25-8,9-5	Bartlett	21.0	23.0	91
			Mean Recovery =	$\overline{95}$ rsd 4.3%
50-8,9-1	Bartlett	39.0	46.0	85
50-8,9-2	Bartlett	44.4	46.0	97
50-8,9-3	Bartlett	41.4	46.0	90
50-8,9-4	Bartlett	40.2	46.0	87
50-8,9-5	Bartlett	40.8	46.0	89
			Mean Recovery =	$\overline{90}$ rsd 5.1%

*Control Bartlett Pears from Field Trial 001-87-5007R
NA - Not applicable.

Table 1 (con't)

Recovery of Avermectin B_{1a}, Avermectin B_{1b}, and 8,9-Z-Avermectin B_{1a} from Pear Homogenates

Sample No.	Pear Type	B_{1b} Conc. ng/g calc.	Fort. Level ng/g	%Rec
4-B_{1b}-1	Bartlett	4.1	3.8	108
4-B_{1b}-2	Bartlett	4.8	3.8	126
4-B_{1b}-3	Bartlett	4.3	3.8	113
4-B_{1b}-4	Bartlett	3.8	3.8	100
4-B_{1b}-5	Bartlett	3.7	3.8	97
			Mean Recovery =	$\overline{109}$ rsd 11%

*Control Bartlett Pears from Field Trial 001-87-5007R
NA - Not applicable

Note: All control samples were free from any detectable avermectin residues.

Table 2

Recovery of Avermectin B_{1a}, Avermectin B_{1b}, and 8,9-Z-Avermectin B_{1a} from Apple[a] Homogenates

Sample Name	Set No.	Apple Type	B_{1a} Conc. ng/g Cal.	Fort. Level ng/g	% Rec.
CONT·APPLE	APPSET01	Red Delicious	0	0	NA
CONT·APPLE	APPSET02	Red Delicious	0	0	NA
CONT·APPLE	APPSET03	Red Delicious	0	0	NA
CONT·APPLE	APPSET04	Golden Delicious	0	0	NA
FORT APPLE1	APPSET03	Red Delicious	1.5	1.9	79
FORT APPLE1'	APPSET03	Red Delicious	1.6	1.9	84
FORT APPLE1"	APPSET03	Red Delicious	1.6	1.9	84
5FORT APP	APPSET04	Golden Delicious	1.9	1.9	100
					Mean = 87
					RSD 11%
FORT APPLE2	APPSET03	Red Delicious	4.1	4.8	85
FORT APPLE2'	APPSET03	Red Delicious	4.4	4.8	92
FORT APPLE2"	APPSET03	Red Delicious	4.4	4.8	92
6FORT APP	APPSET04	Golden Delicious	3.7	4.8	77
					Mean = 87
					RSD 8.3%
FORT APPLE1	APPSET01	Red Delicious	43	40	108
FORT APPLE1'	APPSET01	Red Delicious	38	40	95
FORT APPLE1"	APPSET01	Red Delicious	38	40	95
7FORT APP	APPSET04	Golden Delicious	35	40	88
					Mean = 97
					RSD 8.6%
FORT APPLE2	APPSET01	Red Delicious	80	79	101
FORT APPLE2'	APPSET01	Red Delicious	81	79	103
FORT APPLE2"	APPSET01	Red Delicious	72	79	91
10FORT APP	APPSET04	Golden Delicious	58	79	73
11FORT APP	APPSET04	Golden Delicious	65	79	82
					Mean = 90
					RSD 14%

Table 2 (cont'd)
Recovery of Avermectin B_{1a}, Avermectin B_{1b}, and
8,9-Z-Avermectin B_{1a} from Apple Homogenate

Sample Name	Set No.	Apple Type	8,9-Z-avermectin B_{1a} Conc. ng/g Cal.	Fort. Level ng/g	% Rec.
FORT APPLE1	APPSET02	Red Delicious	4.9	5.3	92
FORT APPLE1'	APPSET02	Red Delicious	4.1	5.3	77
FORT APPLE1"	APPSET02	Red Delicious	4.6	5.3	87
1FORT APP	APPSET04	Golden Delicious	4.7	5.3	89
					Mean = 86
					RSD 7.5%
FORT APPLE2	APPSET02	Red Delicious	24	26	92
FORT APPLE2'	APPSET02	Red Delicious	25	26	96
FORT APPLE2"	APPSET02	Red Delicious	25	26	96
2FORT APP	APPSET04	Golden Delicious	22	26	85
					Mean = 92
					RSD 5.6%
FORT APPLE3	APPSET02	Red Delicious	64	70	91
FORT APPLE3'	APPSET02	Red Delicious	62	70	89
FORT APPLE3"	APPSET02	Red Delicious	68	70	97
3FORT APP	APPSET04	Golden Delicious	50	70	71
					Mean = 87
					RSD 13%

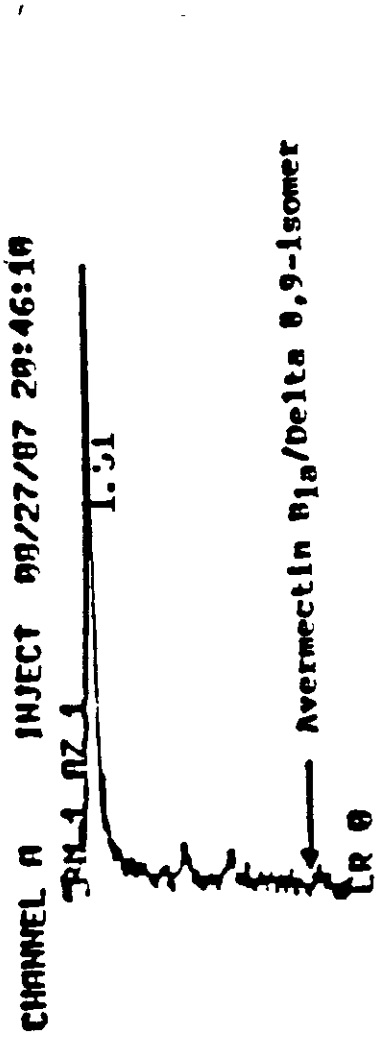
Table 2 (cont'd)
Recovery of Avermectin B_{1a}, Avermectin B_{1b}, and
8,9-Z-Avermectin B_{1a} from Apple Homogenate

Sample Name	Set No.	Apple Type	B_{1b} Conc. ng/g Cal.	Fort. Level ng/g	% Rec.
FORT APPLE3	APPSET03	Red Delicious	5.8	5.9	98
FORT APPLE3'	APPSET03	Red Delicious	5.9	5.9	100
FORT APPLE3"	APPSET03	Red Delicious	5.2	5.9	88
4FORT APP	APPSET04	Golden Delicious	4.5	5.9	76
8FORT APP	APPSET04	Golden Delicious	5.7	5.9	97
9FORT APP	APPSET04	Golden Delicious	6.0	5.9	102
					Mean = 94
					RSD 11%

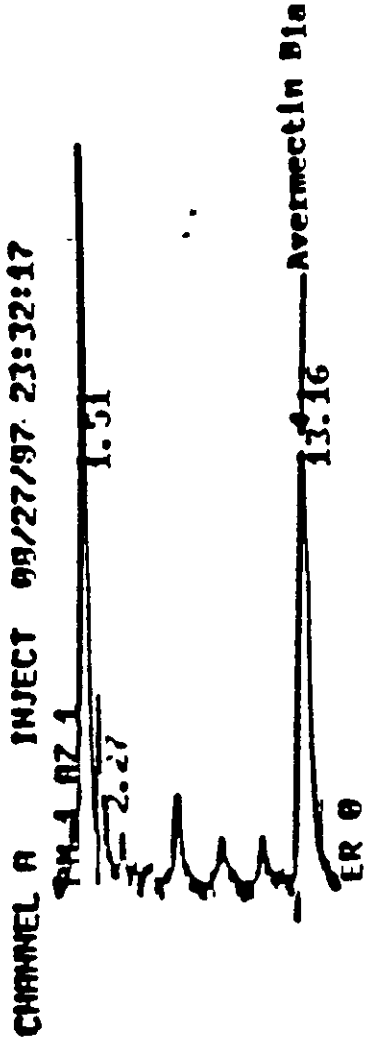
[a]Apple samples were obtained from a grocery store.
NA - Not applicable.

Figure 2

Typical Chromatograms of Control Pears
and Avermectin B_1 in Bartlett Pears



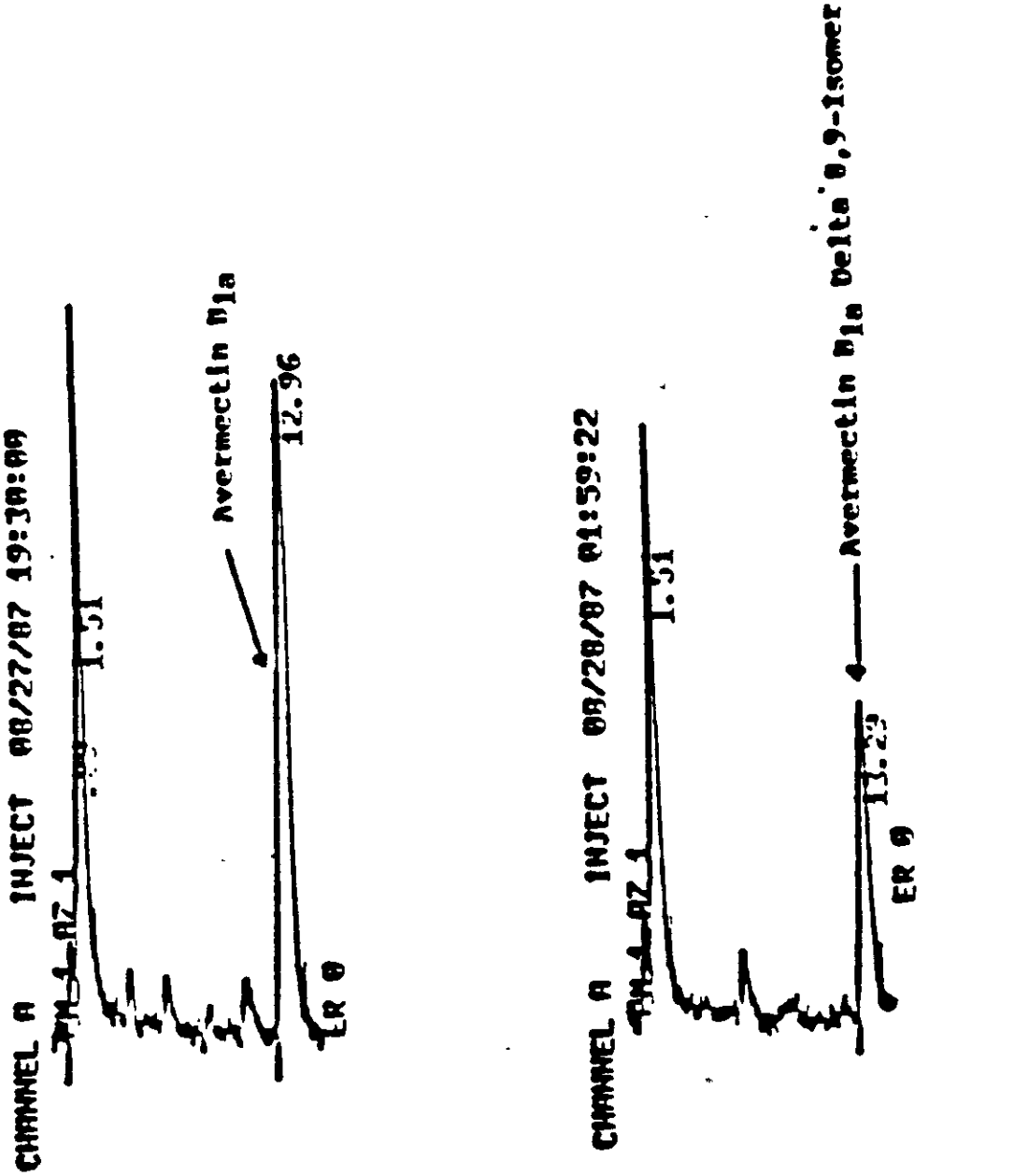
CONTROL BARTLETT
PEAR SAMPLE



CONTROL BARTLETT
PEAR SAMPLE FORTIFIED
WITH 5.1 ng/g AVERMECTIN B1a

Figure 3

Typical Chromatograms of Avermectin B_{1a} Standard
and 8,9-Z-Avermectin B_{1a} in Bartlett Pears

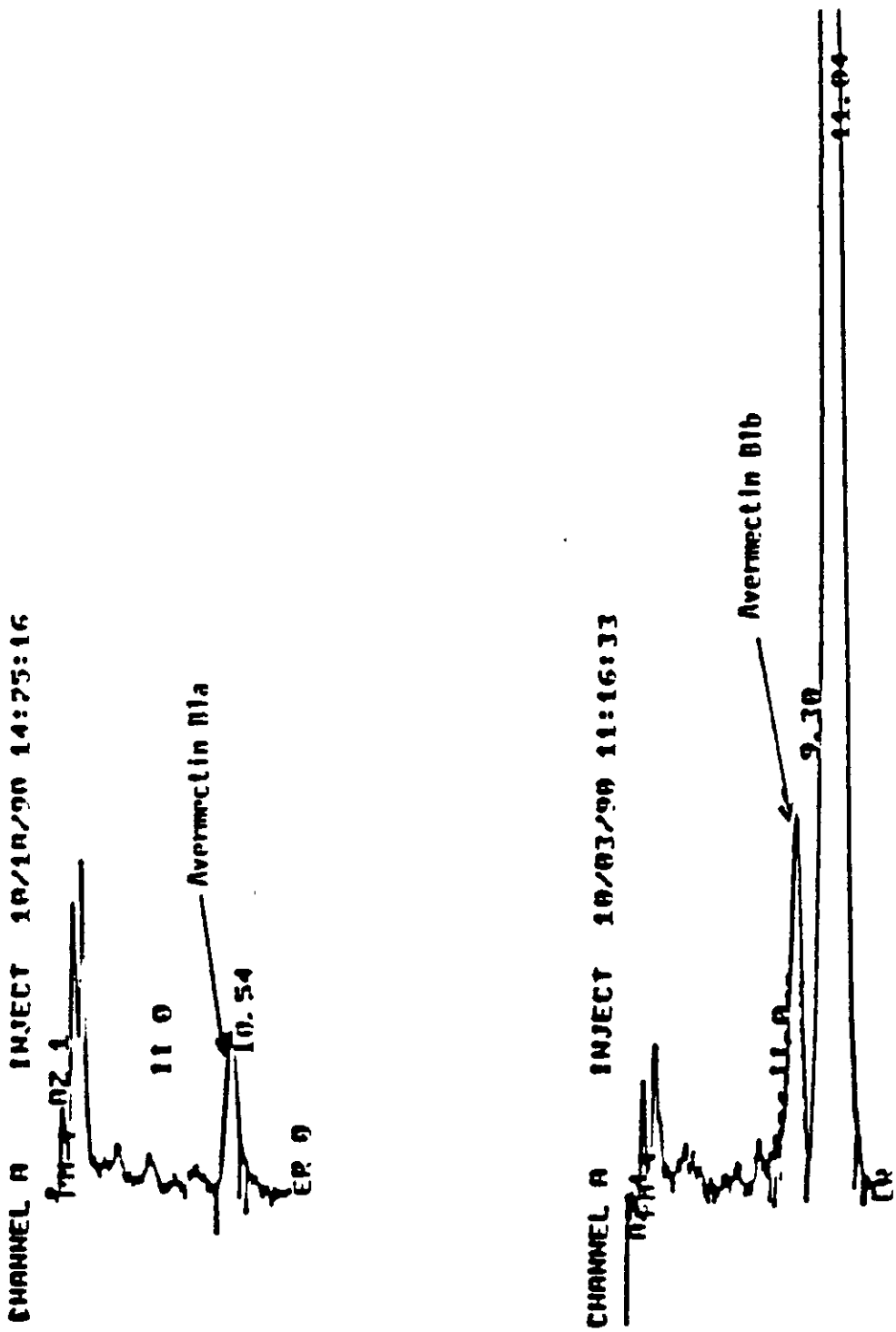


Avermectin B_{1a}
Standard 8.2 ng/mL

Control Bartlett
Pear Sample Fortified
with 4.6 ng/g
8,9-Z-Avermectin B_{1a}

Figure 4

Typical Chromatograms of Avermectin B_{1a} and B_{1b} in Red Delicious Apples



Control Red Delicious Apple Sample Fortified with 1.9 ng/g Avermectin B_{1a}

Control Red Delicious Apple Sample Fortified with 5.9 ng/g Avermectin B_{1b}

III PRINCIPLE

Ten gram pear or apple samples are treated with pectinase. The pectinase hydrolyzes the pectin (Polygalacturonic acid, MW = 30 - 300K) making the sample soluble in the extraction solvent.

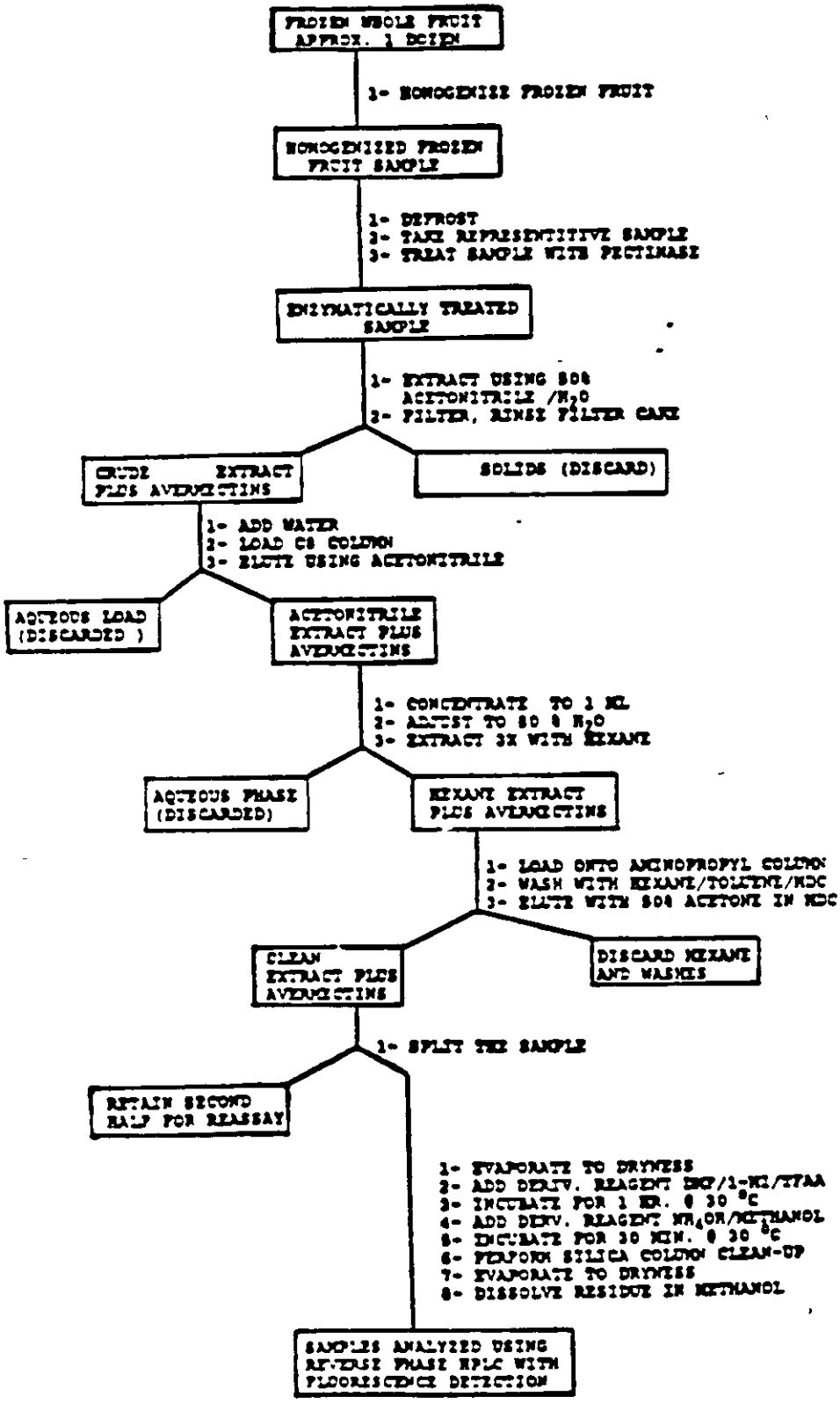
Residues of avermectin B_1 and 8,9-Z-avermectin B_1 are extracted from the hydrolyzed apple or pear homogenate by blending with 50:50 acetonitrile/water. The extract is filtered and made more aqueous by the addition of water. The aqueous solution is passed through a C-8 column, loading the avermectins onto the column. The eluant is discarded and the avermectins are eluted with a small volume of acetonitrile. The acetonitrile is concentrated and water is added to the acetonitrile. The aqueous acetonitrile is extracted with hexane three times, removing the avermectins from the aqueous acetonitrile. The hexane extracts are combined and loaded onto an aminopropyl column, which retains the avermectins. The hexane eluant is discarded. The column is washed with hexane, toluene, and methylene chloride and the avermectins are eluted with 50:50 acetone/methylene chloride. This eluant is evaporated to dryness and the residue is dissolved and diluted to 10 mL using methylene chloride. The sample is split, evaporated to dryness and a fluorescent derivative is formed by reaction with a mixture of N,N-dimethylformamide, trifluoroacetic anhydride and 1-methylimidazole (Reagent A-1) for 1 hour at 30°C, followed by reaction with methanolic ammonium hydroxide (Reagent A-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 the 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 8,9-Z-avermectin B_1 produces the same derivative as avermectin B_1, the derivatized residue quantitated represents the sum of avermectin B_1 and 8,9-Z-avermectin B_1, as shown below:

Parent	**heat**	**single**
Avermectin B_1 + 8,9-Z-Avermectin B_1	**------->**	**fluorescent peak**
	Reagent A	

A flow chart illustrating the analytical methodology used for this assay is given in Figure 5.

Figure 5

Analytical Methodology for the
Analysis of Avermectins in Pears and Apples



NOTES TO THE ANALYST

Avermectin B_1 has a very low water solubility, approximately 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 B_1 due on adsorption 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, and at no point should samples be left to dry for a prolonged time.

Solvent composition is critical to the solid phase extraction columns. The solvent volumes used throughout this method should be accurately measured.

Suggested assay stopping points are after steps 9, 14, 15, 19, and 20. These stopping points are 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.

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 homogenized sample into a 4 oz French square bottle and add 13 mL of the buffered pectinase solution (made fresh daily see section V). Incubate in a water bath at 37 °C (± 1 °C) for 1 hour.

   Note: When assaying the fortified samples for recovery, fortification of control samples is performed before the addition of the pectinase solution.

4. Add 13 mL of acetonitrile. Position the Polytron probe near the bottom of the bottle and homogenize on a setting of seven for 30 seconds, or until homogeneous (but not less than 30 sec).

5. Apply a reduced vacuum (-5 in. Hg) to a 70 mm porcelain funnel fitted with a Whatman #4 filter paper and a 250 mL sidearm flask. Wet the filter paper by prerinsing it with 2 mL of acetonitrile.

   NOTE: Use of a smaller diameter funnel will result in prolonged filtration times and a possible reduction in recoveries.

6. Pour the aqueous acetonitrile and any accompanying macerate through the filtration unit.

7. While the first extract is filtering, add 25 mL of a 50% acetonitrile/H_2O solution to the French square bottle. Position the probe near the bottom of the flask and run on a setting of 7 for 15 seconds, in order to rinse the blades. Swirl the flask while homogenizing so that the walls of the bottle are rinsed with the extracting solution.

8. Pour this extract into the Buchner funnel with the first sample extract and increase the vacuum to -20 in. of Hg.

9. Rinse the French square bottle with 4 mL of acetonitrile (2 X 2 mL using a disposable pipet and a 2 mL bulb), pour the rinse into the buchner funnel and allow the vacuum to dry the filter cake. Wash the filter cake with three 2 mL volumes of acetonitrile and allow the vacuum to dry the filter cake again.

10. Remove the funnel and add 25 mL of Milli-Q water to the filtration flask. Swirl the flask to mix the solvents.

C. <u>C-8 Column Clean-Up</u>

11. Attach a 1000 mg/6 mL C-8 Bond-Elut column fitted with an adapter and a 75 mL reservoir to the Spe-ed Mate-30 System. Condition the column by rinsing it with a 5 mL volume of acetonitrile, followed by a 5 mL volume of Milli-Q water.

12. Transfer most of the aqueous acetonitrile extract from the 250 mL filter flask to the 75 mL Analytichem reservoir fitted with the C-8 Bond-Elut cartridge. Use the Spe-ed Mate-30 and a 2 liter vacuum trap to load the sample. Maintain a vacuum of 10 in. of Hg and load the remainder of the sample. Using this setup, 12 to 15 samples can be loaded in parallel. Discard the eluant.

    (Note: The composition of the aqueous acetonitrile extract to be loaded on the C-8 column should not have more than 40% acetonitrile.)

13. Remove the vacuum. Place a 15 mL centrifuge tube under the column. Add 12 mL of acetonitrile to the 250 mL filter flask, swirl to rinse. Pour the 12 mL of acetonitrile from the flask into the C-8 column reservoir.

14. Apply a vacuum (-10 in. Hg) and collect the eluant in the centrifuge tube.

15. Reduce the acetonitrile eluant to 1 mL with nitrogen at 70°C using heat lamps or a water bath. Avoid reducing the volume to below 1 mL. If the volume is reduced to below 1 mL, dilute to 1 mL using acetonitrile and the graduations on the centrifuge tube.

D. Hexane/Water-Acetonitrile Partition

16. To the 1 mL acetonitrile eluant in the 15 mL centrifuge tube, add 4 mL distilled water. Stopper and mix well. Add 5 mL hexane. Stopper and shake for 1 minute. Centrifuge for 5 minutes. Remove the upper hexane layer to a new 15 mL centrifuge tube. Repeat the extraction with 5 mL and then 4 mL hexane combining hexane extracts. Total hexane volume should be 14 mL. When removing top hexane layer, be careful not to remove any of the lower acetonitrile layer.

E. Aminopropyl Column Cleanup

17. Attach a 500 mg/3 mL aminopropyl Bond-Elut column fitted with an adapter and a 25 mL reservoir to the Speed mate 30 system. Condition the column with 2 column volumes (2 X 3 mL) of hexane.

18. Place the 14 mL hexane sample on the column. Wash the column with 4 mL hexane followed by 3 mL toluene and 15 mL methylene chloride. Discard the eluant. Place a 15 mL silylated centrifuge tube under the column and elute the column with 5 mL of 50% acetone in methylene chloride (v/v). Use a vacuum of -10 in. of Hg to perform these steps.

NOTE: When rinsing the aminopropyl column with hexane/toluene and when eluting the avermectins using 50% acetone in methylene chloride, these solvents should be added to the appropriate sample tubes before addition to the corresponding column reservoir. While the solvents are in the sample tube, the tube should be vortexed before addition to the reservoir. This ensures a quantitative transfer of avermectins to the aminopropyl column.

19. Dilute the samples to 10 mL using methylene chloride and the graduations on the centrifuge tubes.

20. Sonicate for 15 seconds, vortex, and transfer half (5 mL) of the sample into a polypropylene or glass centrifuge tube. This split may be performed using a disposable pipet and the graduations on the centrifuge tube. Store the split at -10°C.

NOTE: If the second half of the samples are to be stored for more than 48 hours, the second half should be taken to dryness and redilued in 5.0 mL of acetonitrile before storage.

F. Derivatization

21. Take the half of the sample in the silylated centrifuge tube to dryness with nitrogen in at 50 to 70 °C using heat lamps or a 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. Standards should be derivatized with unknowns.

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

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

24. 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.

25. 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.

26. Wash a silica Bond Elut cartridge with 5 mL of chloroform using vacuum of approximately 10 in. of Hg to pull the chloroform from a reservoir into a waste collection tube.

27. After removal of the vacuum, place a 15 mL graduated centrifuge tube under the Bond Elut 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 Bond Elut sample cartridge rack or equivalent may be used to clean-up several samples in parallel.

28. Remove the vacuum. Wash the silylated centrifuge tube two times with 2 mL of 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.

29. Elute the column with an additional 5 mL of chloroform to give a final eluant volume of approximately 13 mL.

30. Evaporate the chloroform with nitrogen in a 70°C water bath. Samples should be reduced to an oily residue, less than 0.1 mL.

31. 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.

32. Centrifuge briefly prior to injection of the supernatant on HPLC. Reserve derivatized solutions by storing in a freezer until quantitation is completed.

G. Preparation of the Standards and Quantitation

Analytical Standards -

Avermectin B_1 (current standard is L-676,863-O38A003)
8,9-Z-Avermectin B_1 (current standard is L-652,280-002T001)

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

The most current standard should be used.

Preparation

1. To prepare 25 µg/mL stock solutions of avermectin B_{1a}, and 8,9-Z-avermectin B_{1a}, weigh out enough of the standard glycerol formal solutions to produce 50 mL of a 25 µg/mL solution for avermectin B_{1a} (~140 mg), and 25 mL of a 25 µg/mL solution for 8,9-Z-avermectin B_{1a} (~165 mg). The standards should be prepared separately and diluted with acetonitrile. The current avermectin B_1 glycerol formal solution contains 0.893% avermectin B_{1a} and 0.044% avermectin B_{1b} w/w. The current 8,9-Z-avermectin B_{1a} standard contains 0.38% w/w of 8,9-Z-avermectin B_{1a}. The exact concentration of all standards used should be reported and used throughout all calculations. The 25 µg/mL stock solution of avermectin B_{1a} can be used to prepare a 50 ng/mL avermectin B_{1b} standard solution.

2. To prepare 500 ng/mL intermediate stock solutions of the above standards, pipet 2 mL of the 25 µg/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. The 50 ng/mL standards can be prepared by diluting the 500 ng/mL intermediate stock solutions 1 to 10.

3. To prepare 2.0, 4.0, 6.0, 8.0 and 10.0 ng/mL working standards for the derivatization, 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 B_{1a} standard solution.

4. Derivatize and perform all subsequent operations for injection on the HPLC, as described above (steps 21 - 32).

V Reagents

Solvents - acetone, acetonitrile, methylene chloride (MDC), chloroform, hexane, toluene, methanol - EM Science (Pesticide Grade or Distilled in Glass); Sylon CT - Supelco Inc.; Ultrapure water - Milli-Q water purification system.

Reagents for Enzymatic Digestion - Pectinase (polygalacturonase; Poly-[1,4-alpha-D-galacturonide] glycanohydrase; EC 3.2.1.15) from Asperigillus niger, a solution in 40% glycerol - Sigma Chemical Co., Cat No. P 5146; Buffer salt, dry pH 4.01 (Potassium acid phthalate buffer) - Fisher Scientific, Cat No. B-79.

To prepare the phthalate buffer solution dissolve one package of the dry buffer salt (Fisher #B-79) in 1 liter of Milli-Q water using a volumetric flask. This produces a 50 mM pH 4 phthalate buffer. Make fresh weekly, store at room temperature.

To prepare the pectinase solution used to digest the samples, pipette 2300 units of the enzyme into a graduated cylinder and dilute to 200 mL using the phthalate buffer (50 mM, pH 4). Make fresh daily.

The number of enzyme units of pectinase per milliliter of glycerol can be calculated by multiplying the concentration of protein in mg/mL by the enzyme activity in units/mg. Both of these numbers should be provided by the manufacturer. Once the number of units of enzyme per milliliter have been calculated, the volume of the solution to be pipetted can be determined. This volume is calculated by dividing 2300 units by the number of enzyme units per milliliter. This volume is then rounded up to the nearest mL. Below is an example of this calculation.

Sample Calculation

Enzyme Activity (provided by the manufacturer) = 9.1 units/mg
Protein Conc. (provided by the manufacturer) = 12.7 mg/mL

Unit Conc. = 9.1 units/mg X 12.7 mg/mL = 116 units/ mL

2300 units (required)/116 units/mL = 19.8 mL

19.8 mL is then rounded up to the nearest milliter (20 mL)

Therefore in order to prepare the pectinase solution to be used for sample digestion, 20 mL of the pectinase in glycerol (containing 2300 units of the enzyme) is diluted to 200 mL with the Phthalate buffer.

Extraction solvent - Add 1 liter of acetonitrile to 1 liter of Milli-Q water and mix thoroughly. Place this extraction solvent in a 4 liter bottle fitted with a 25 or 50 mL repipet.

Clean-up columns- Octyl (C-8) 1000 mg/6 mL, cat. no. 606406, Analytichem International; Aminopropyl (NH2) 500 mg/3 mL, cat no. 611303, Analytichem International; Sep-Pak silica cartridges-Waters Associates or Bond Elut, 1000 mg, 6 ml, part No. 601406, Analytichem International.

NOTE: Recovery of avermectin B_1 may need to be determined for each new lot of bonded phase columns.

HPLC Solvents - 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 or degassing systems may be used.

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

Filter Paper - Whatman No. 4 - Fisher Scientific.

To prepare the trifluoroacetyl derivatization reagent, add 0.4 mL 1-methylimidazole to 3.6 mL dimethylformamide 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 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 of the solution 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

Homogenizer - Brinkmann Instruments Polytron Blender model PT-35 with probe generator model PT10ST or equivalent.

Crop Processor - Hobart Food Processor or Cuisinart food processor model DLC-X.

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

Repipet - Dispensor and Diluters, 10, 25, and 50 mL.

Sonicator - L&R Transistor/Ultrasonic model T-21 or equivalent.

Vacuum manifolds - Applied Separations Spe-ed Mate-30, Waters Associates Sep-Pak Cartridge Rack, or equivalent.

15 mL Graduated Centrifuge tubes - Fisher Scientific or equivalent.

To prepare silylated 15 mL graduated centrifuge tubes used for derivatization reaction, silylate approximately once every two months using the following procedure: Fill each tube to the top with Sylon-CT. Let stand 20 minutes. Rinse thoroughly with toluene followed by methanol. Fill with methanol. Let stand 20 minutes, rinse thoroughly with acetone and dry. Following use, tubes should be cleaned by hand 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 before reuse. As the derivatization reaction requires anhydrous conditions, tubes should be absolutely free of moisture prior to use. Oven drying ensures this condition.

VII HPLC Apparatus

Pump - Beckman model 110A, or equivalent.

Injector - Waters Wisp Model 710B, or equivalent.

Analytical column - ES Industries, Chromegabond MC18 column, 15 cm X 4.6 mm, 3 micron particle size, or equivalent.

Guard Column - Rainin Instrument, Spheri-5 OD-GU, RP-18, 30 x 4.6 mm column containing 5 mcm packing, or equivalent.

Fluorescence Detector - Kratos Instruments FS950, or equivalent.

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.

Column Heater - Fiatron CH-30 column heater with TC-55 controller or equivalent system.

VIII HPLC Operating Conditions

Mobile phase - 10% ultrapure water in methanol (v/v); flow rate 1.5 mL/min; injection volume - 50 microliters; detector parameters: sensitivity (PMT gain)- 570 to 780 V; Range - 0.02 microamps; time constant - 6; column temperature - 30° C. (These conditions are for the ES Industries Chromegabond MC18 columns). Columns such as the Dupont Zorbax ODS (150 mm x 4.6 mm 5 micron particle size) may be substituted. Retention times for the fluorescent derivatives of avermectin B_{1a}/8,9-Z-avermectin B_{1a} and avermectin B1b are approximately 11 to 13 minutes and 8 to 10 minutes, respectively, using the Chromegabond column and the above conditions. Using the Zorbax column, retention times are slightly different.

Note: 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

For apples and pears, residues of avermectin B_{1a} + 8,9-Z-avermectin B_{1a} below 1 ng/g are reported as non-detectable (reported as ND). Residue between (1 - 2) ng/g are identified but not quantified (reported as NQ) and residues above 2 ng/g are identified and quantitated from avermectin B1a standard curve. Since avermectin B_{1b} is at most 20% (usually less than 10%) of the active ingredient, B_{1b} residue levels are generally less than the quantitation limit for pears and for apples (2 ng/g). The peak representing avermectin B_{1b} is identified and quantitated in the same manner as the avermectin B_{1a}/8,9-Z-avermectin B_{1a}, using the avermectin B_{1a} standard curve.

An analysis set is comprised of no less than 5 standards and no more than 15 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 both injections of a single standard is much lower than expected. Because it is known that this observation can be attributed to low derivatization reaction yield, the data for this standard may be discarded in determining the regression coefficients. An acceptable standard curve will have a coefficient of determination $r^2 = 0.97$ or better. The concentration of avermectin B_{1a}/8,9-Z-avermectin B_{1a} in a residue sample is determined as follows:

$$C = (PK\ HT\text{-}I)/S \quad UNK = (CxFV)/(SW\ X\ FRAC)$$

Where:

C = concentration of avermectin B_{1a}/8,9-Z-avermectin B_{1a} in ng/mL in the final volume used for HPLC analysis, PK HT = peak height of avermectin B_{1a}/8,9-Z-avermectin B_{1a} isomer derivative, I = intercept, S = slope, FV = final volume used for HPLC analysis, SW = sample weight, UNK = concentration of avermectin B_{1a}/8,9-Z-avermectin B_{1a} in ng/g in the unknown residue sample. Frac = Fraction of the sample used for the assay (usually 0.5). Avermectin B_{1b} residues are calculated in the same manner.

Samples which have peak heights above the standard curve should be diluted and reinjected with the standards again to be more accurately quantitated.

X SUGGESTIONS FOR THE ANALYST PERFORMING MERCK RESIDUE METHOD NO. 8000

A. Introduction

i. Background

Avermectin B_1 (abamectin) is a very effective acaricide and insecticide. [4,5] Its utility has been investigated in citrus, cotton, celery, tomatoes and pears as well as other crops. Because it is effective at such low application rates (typically in the realm of 0.025 lbs ai/acre), the resultant residues are quite low and present a significant analytical challenge. Avermectin B_1 is a mixture of at least 80% avermectin B1a and not more than 20% avermectin B_{1b} (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 of avermectin B_{1a}, 8,9-Z-avermectin B_{1a}, and avermectin B_{1b} on exposed fruit.

ii. Method chemistry

It is evident from the structure that avermectin B_1 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 B_1. High pressure liquid chromatography (HPLC) assays have been developed using either ultraviolet [6] or fluorescent detection for the related compound, ivermectin (dihydroavermectin B_1), in animal plasma [1] or tissue [2]. To achieve the sensitivity and selectivity required for the detection of avermectins in pears, fluorescent detection is necessary [3]. 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 B_{1a}, avermectin B_{1b} and the photodegradate 8,9-Z-avermectin B_{1a} 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 B_{1a} and 8,9-Z-avermectin B_{1a}. Under the given chromatographic conditions, the fluorescent derivative of avermectin B_{1b} is resolved from and elutes before the B_{1a} or 8,9-Z-avermectin B_{1a}. Thus, two component residues can be quantitated, (1) avermectin B_{1a} + 8,9-Z-avermectin B_{1a} and (2) avermectin B_{1b}. Since avermectin B_{1b} is present in much smaller quantities in the formulation and the resultant residues in pears or apples, there is seldom enough to quantitate. Both components of the residue are quantitated in levels at or above 2 ng/g and are identified only when present at levels from 1 to 2 ng/g. Components are not detectable below 1 ng/g.

B. Overview of assay

i. Principle

Ten gram pear or apple samples are treated with pectinase. The pectinase hydrolyzes the pectin in the pears or apples (Polygalacturonic acid, MW=30-300K) making the sample more soluble in the extraction solvent.

Residues of avermectin B_1 and 8,9-Z-avermectin B_1 are extracted from the hydrolyzed pear or apple homogenate by blending with 50:50 acetonitrile/water. The extract is filtered and made more aqueous by the addition of water. The aqueous solution is passed through a C-8 column, loading the avermectins onto the column. The eluant is discarded and the avermectins are eluted with a small volume of acetonitrile. The acetonitrile is concentrated and water is added to the acetonitrile. The aqueous acetonitrile is extracted with hexane three times, removing the avermectins from the aqueous acetonitrile. The hexane extracts are combined and loaded onto an aminopropyl column, which retains the avermectins. The hexane eluant is discarded. The column is washed with hexane, toluene, and methylene chloride and the avermectins are eluted with 50:50 acetone/methylene chloride. This eluant is evaporated to dryness and the residue is dissolved and diluted to 10 mL using methylene chloride. The sample is split and evaporated to dryness and a fluorescent derivative is formed by reaction with a mixture of N,N-dimethylformamide, trifluoroacetic anhydride and 1-methylimidazole (Reagent A-1) for 1 hour at 30°C, followed by reaction with methanolic ammonium hydroxide (Reagent A-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 8,9-Z-avermectin B_1 produces the same derivative as avermectin B_1, the derivatized residue quantitated represents the sum of avermectin B_1 and 8,9-Z-avermectin B_1, as shown below:

Parent	**heat**	**single**
Avermectin B_1 + 8,9-Z-Avermectin B_1	**------->**	**fluorescent peak**
	Reagent A	

The analytical methodology used for the pear or apple assay is illustrated in Figure 5.

ii. 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 B_1 assay method has been designed to maximize accuracy and minimize the time per assay. Achieving a single result in minimum time was not our highest priority. Thus, we routinely assay 13 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.

iii. Solubility properties

Avermectin B_1 (including 8,9-Z-avermectin B_1) 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 B_1 has high solubility in alcohols, such as methanol.

iv. Motives behind assay

The steps in the assay have been shown to provide the necessary cleanup of samples while maintaining sufficient recovery. The acetonitrile/H_2O extraction efficiently extracts the avermectin B_1 residue (including 8,9-Z-avermectin B_1) from the matrix, but it also extracts extraneous material. The column cleanup steps in conjunction with the liquid-liquid partitions are needed to remove the undesirable co-extractives. These partitions and column clean-ups are efficient because they take advantage of the unique solubility properties of the avermectins. Formation of the fluorescent derivative provides the sensitivity and selectivity to quantitate the low incurred residues found in whole fruit treated with avermectin at the normal application rate.

C. Equipment

The equipment listed in the method has been used to assay avermectin B_1 (including 8,9-Z-avermectin B_1) in pears or apples. 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.

D. Preparation of standards

Solid avermectin B_{1a}, avermectin B_{1b} or the 8,9-Z-avermectin B_{1a} 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 rinsing 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 B_{1a} and B_{1b}, it is important to know the exact purity of the standard to determine the concentration of B_{1a} and B_{1b} in solution. The purity is generally given in weight percent. An avermectin standard that is 0.9% B_{1a} has 9 mg of avermectin B_{1a} in every 1000 mg of standard.

E. Assay Procedure

i. Sample preparation

A field sample consists of several fruit that have had the same treatment. These fruit must be crushed and blended together to form a homogeneous sample. To avoid overloading the food processor, the sample 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. 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. 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 should be processed before treated samples. Samples suspected to contain the highest residues are processed last.

To ensure a homogeneous and representative sample, the frozen ground matrix should be thawed thoroughly before weighing the 10.0 gram portion. The sample can be thawed by either gently warming the sample container in a water bath (approx. 50°C) or by leaving the sample overnight in a refrigerator, or on a counter several hours before treatment with pectinase. For assays with a 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 a tared homogenization vessel. Try to place all of the sample in the bottom of the bottle so that when the extractant and other reagents are added to the bottle the sample will be in complete contact with the solvent and other reagents. Try to avoid touching the sample to the walls near the mouth of the French square bottle, this prevents the sample from coming in contact with the reagents added later. If some sample does remain near the top of the bottle try to push it to the bottom of the bottle using the spatula, or by washing it down when adding reagents later in the method (e.g. the enzyme solution, or the extraction solvent). We have found that the French square bottle is convenient and helps the homogenization process. 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. If the sample is a fortified control, the small volume (1 mL or less) of the fortification standard in acetonitrile is added and the solvent is allowed to evaporate before the enyzmatic treatment with pectinase.

ii. Enzymatic Treatment

After the samples are weighed and the appropriate controls are fortified the sample must be treated with an enzyme known as pectinase; this enzyme treatment helps digest the complex carbohydrate matrix in the pears or apples making the sample more soluble in the extraction solvent. The enyzme is added to the sample in an aqueous phthalate buffer (pH 4). This is the buffer required for maximal enzyme activity.

The buffer is prepared from a dry buffer salt. This buffer salt produces a 50 mM, pH 4.0 phthalate buffer which should be made fresh weekly. The pH of the buffer should be tested after preparation using a pH meter or pH paper. The pH should be 4.0 ± 0.1 pH unit.

When preparing the pectinase in the buffer solution the pectinase may be added to the buffer using a 25 mL graduated pipet. The enzyme solution may be added to the samples using a 20 mL repipet or a graduated cylinder. When adding the pectinase solution to the sample be sure to swirl the flask gently (using a circular motion) in order to suspend the pear solids in the enzyme solution. Do not swirl the sample vigorously, this causes the sample to splash high on the walls of the extraction vessel preventing it from being submersed in the 37°C water bath.

The water bath temperature for the hydrolysis should be 37°C ± 1°C. The sample must be in the water bath for a minimum period of 1 hr. Although it has been shown that an extended period of the enzymatic treatment (2.5 hrs) has no detrimental affect on the recoveries, they should not be left in the water bath for a period longer than 1 hr and 15 minutes.

iii. Extraction from crop

After the enzymatic treatment, acetonitrile is added to the sample producing an extractant which is 50% acetonitrile and 50 % buffer solution. The acetonitrile may be added to the sample using a 25 mL graduated repipet. This solution is then homogenized using a Polytron homogenizer. 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. When using the polytron homogenizer be sure to adjust the space between the bottom of the probe and the bottom of the French square bottle to maximize the turbulence in the homogenizing vessel (approx. 1 cm).

Only the buchner funnel size specified should be used for this assay, substitute funnels are not recommended. This is due to the fact that smaller funnels have shorter stems, which when placed in a 250 mL filter flask end directly in front of the side arm to the vacuum. This allows some of the sample to be aspirated towards the vacuum. This can be easily avoided by using the funnel size listed. The larger surface area of the funnel listed also provides a more rapid filtration for the pear extracts because of the increased surface area of the filter. For the most efficient use of time, two vacuum filtration set-ups should be set up near the homogenizer.

In order to prevent the pear or apple solids from going underneath the filter paper the filter paper is prewet with approx. two mL of acetonitrile while drawing a weak vacuum (-5 in. Hg), this ensures a good seal between the funnel and the paper. If a good seal is not made between the filter paper and the funnel, increase the vacuum to -20 in. Hg temporarily until the sample is added. Then decrease the vacuum back to -5 in. Hg and continue with the method. These extra precautions must be taken to prevent the sample from passing under the filter paper and clogging the C—8 column entrance frit. The homogenized sample extract from the French square bottle is then added slowly to the funnel, collecting the filtrate in the filter flask. When adding the second volume of extractant (50% acetonitrile and water) to the French square bottle carefully wash down the walls of the vessel. The 50% acetonitrile/H_2O should be prepared by mixing 500 mL of acetonitrile and 500 mL of Milli-Q® water, not by diluting 500 mL of water to 1 liter. Diluting up to 1 liter using acetonitrile may cause problems in the loading of the C-8 column due to the higher percentage of acetonitrile in the extractant (the higher percentage of acetonitrile is caused by a volume reduction on mixing of the acetonitrile and water, and the addition of more acetonitrile to bring the solution up to 1 liter).

The addition of the 50 % acetonitrile/H_2O was achieved in our laboratory using a repipet and adding 5 X 5 mL, rotating the flask after each 5 mL addition (this ensures a good rinsing of the bottle walls). The second

extraction is then performed and added to the funnel with the first portion of the extract, and the vacuum is increased to - 20 in. Hg. A simple way to create the two different vacuum levels is the insertion of a two way valve in the vacuum line between the vacuum source and the filter flask. For low vacuum the two way valve is open to the atmosphere, the filter flask, and the vacuum source. For high vacuum the valve is closed to the atmosphere, but open to the filter flask and the vacuum source. After rinsing the homogenization vessel and letting the filter cake dry, the filter cake is washed with an additional small volume of acetonitrile (5-7 mL). This is done by adding 3 times 2 mL of acetonitrile to the funnel using a 2 mL rubber bulb and a disposible pipet. The rinse is distributed evenly among the pear solids, concentrating longer on areas were there appears to be more solids.

For fifteen samples, excluding the time for defrosting the samples, it takes 3 hours to weigh, treat the samples with pectinase, homogenize, extract, and filter. The extracts may be retained in covered flasks overnight or over the weekend, in the freezer.

The combined acetonitrile extracts and rinses are made more aqueous by the addition of 25 mL of water. Swirl the flask upon the addition of the water to obtain a homogenous sample. If the extract was stored in the freezer, it should be warmed to room temperature and mixed thoroughly by swirling and sonication before addition of the water.

iv. C-8 Column Clean-up

In a given sample set twelve to fifteen samples may be loaded in parallel if the Spe-ed Mate -30 vacuum manifold is used in conjunction with a 2 liter solvent trap. The solvent trap is placed between the vacuum source and the manifold to collect the waste from the sample load. After pre-conditioning of the columns the samples are added to the sample reservoirs; this leaves approx. 10 to 20 mL in the filtration flasks. A vacuum of 10 in. of Hg is used to load and condition the columns. This vacuum is NOT to be exceeded due to a possible implosion of the Spe-ed Mate -30 cover and the fact that increased flow rates during the loading of the C-8 column may decrease recovery. A safety device to prevent implosion of the cover at stronger vacuums is available from the manufacturer. The vacuum may fluctuate by $\pm$ 2 in. Hg during the load due to liquid flowing through the vacuum lines. The remaining contents of the flasks are added to the C-8 column reservoirs after the initial load is approximately 50 % complete. When the load is complete properly labeled tubes are placed under the positions associated with the C-8 columns (double check these positions). The filter flasks are then rinsed by adding 12 mL of acetonitrile to them and swirling them to rinse the walls. This rinse, usually a white cloudy solution, is added to the 75 mL reservoirs to elute the samples. Once the samples are eluted they may be stored at -10°C overnight or they may be concentrated to 1 mL and stored at -10°C overnight. However, if you begin the hexane/acetonitrile partition you must complete the aminopropyl column cleanup before you will reach the next suitable overnight stopping point in the assay.

v. Hexane/Acetonitrile-water partition

In this partition the compounds of intermediate polarity are concentrated in the aqueous acetonitrile layer and the avermectins are concentrated in the combined hexane layers. The 4 mL of water may be added to the concentrated acetonitrile extract using a repipet. The water should be taken fresh from a Milli-Q water system or equivalent weekly to prevent the chance of microbial contamination.

The hexane used for this partition was also measured using a repipet. Changing the volume of the third and final extract to 4 mL simply prevents the centrifuge tubes from becoming too full. Since you are partitioning 15 samples three times and combining the extracts, concentrate on the sample labels in order to ensure the combination of the proper extracts, it is easy to switch the order of two tubes when transferring samples to and from the centrifuge and sample rack.

After completion of the partition the samples must be carried through the aminopropyl cleanup before a suitable overnight stopping point is reached. It has been shown in our laboratory that stopping in hexane with the pear matrix produces larger deviations in fortified recoveries.

vi. Aminopropyl Column Cleanup

In the aminopropyl column clean-up the combined hexane extract is loaded onto the aminopropyl column and washed with several solvents which elute interfering compounds. The avermectins are then eluted using a solution of 50 % acetone in methylene chloride. The Spe-ed Mate -30 is used to carry 12 to 15 samples through this step in parallel.

The important step in this section of the method is the rinsing of the centrifuge tubes with hexane, toluene and acetone/methylene chloride. When adding these solvents to the aminopropyl column reservoirs, they should be added to the centrifuge tubes that contained the sample in hexane first. The tube should then be vortexed using your fingers or a mechanical vortex before adding the solvent to the appropriate reservoir. The rinses are done in this manner for the hexane and toluene rinses, and for the acetone/methylene chloride eluant. Make certain that each sample tube is rinsed into the appropriate sample reservoir (check labels before each rinse is performed).

The 50% acetone/methylene chloride solution should be prepared daily to prevent changes in composition due to evaporation. The samples should be eluted into silyated centrifuge tubes so that the derivatization can be performed.

After elution using the acetone/methylene chloride solution the samples are diluted to 10 mL using methylene chloride and the graduations on the side of the centrifuge tubes. The sample is then split by transferring 5 mL to another glass or polypropylene centrifuge tube. This half of the sample is stored at -10°C in case of an unsuccessful derivatization. The remaining half of the sample in the silyated tube is then used for the derivatization. If the second half of the samples are to be stored for more than 48 hours, they must be taken to dryness and rediluted with 5.0 mL of acetonitrile before storage.

vii. 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. This reagent should be prepared just prior to derivatization.

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. Centrifuge the tubes after the sonication and vortexing. 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 or 15 samples and 5 standards for HPLC analysis.

The fluorescent derivatives have an accitation maximum at 365nm and an emission maximum at 470nm. The HPLC equipment, especially the detector, will dictate the settings required to detect the derivatives.

viii. 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.

F. Quantitation

For routine analysis of avermectin B_1, including 8,9-Z-avermectin B_1, samples are run in sets of twelve or fifteen, 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 which contains a total of 100 ng or 10 ng/g. The following example illustrates this calculation;

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

If the peak height for a sample is larger than the highest standard, this sample should be diluted and reanalyzed (see general guideline AB-P1).

i. Determination of avermectin B_{1b} from avermectin B_{1a}

Avermectin B_{1b} residues in samples can be determined from the avermectin B_{1a} standard curve as described in the memo from Morgan to Tway (3/16/87). Experimental results have demonstrated that avermectin B1b residues in citrus and pears can be accurately quantitated from the avermectin B_{1a} standard curve without needing to generate a B_{1b} standard curve. This is reasonable since the structure of the fluorescent derivatives is the same for both compounds as determined by NMR spectroscopy. The retention time of avermectin B_{1b} is established by the highest standards of avermectin B_{1a} which have enough B_{1b} to determine its retention time.

XI BIBLIOGRAPHY

1. Tolan, J.W., P. Eskola, 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.

2. Tway, P. C., J. S. Wood, G. V. Downing. 1981. "Determination of Ivermectin in Cattle and Sheep Tissues Using High-Performance Liquid Chromatography with Fluorescence Detection". J. Agri. Food Chem., Vol. 29, pp. 1059 - 1063.

3. 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.

4. I. Putter, J.G. MacConnell, F.A. Preiser, A.A. Haidri, S.S. Ristich and R.A. Dybas; Experentia 37, 963-4 (1981).

5. J.R. Egerton, D.A. Ostlind, L.S. Blair, C.H. Eary, D. Suhayda, S. Cifelli, R.F. Riek and W.C. Campbell; Antimicrob. Agents Chemother. 15, 372-378 (1979).

6. J.V. Pivnichny, J.S.K. Shim and L.A. Zimmerman; J. Pharm. Sci. 72, 1447-1450 (1983).