Attachment 1

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Study Title

HPLC-Fluorescence Assay for Avermectin B_1a, B_1b And The Avermectin B_1a Delta-8,9-Isomer In Bovine Tissues and Milk

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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality under FIFRA §10(d) (1) (A), (B), or (C)

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No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA §10 (d) (1) (A), (B), or (C).

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GLP Statement of Compliance

There are no current EPA requirements for GLP's associated with enforcement methods.

HPLC-FLUORESCENCE ASSAY FOR AVERMECTIN B_{1a}, B_{1b} AND THE AVERMECTIN B_{1a} DELTA 8, 9 ISOMER IN BOVINE TISSUES AND MILK

Method No. 32A

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I. Summary

Abamectin (avermectin B_1) is a combination of about 80% avermectin B_{1a} (B_{1a}) and 20% avermectin B_{1b} (B_{1b}) (see Figure 1). This combination is a very useful pesticide for a variety of crops. Among the crops involved are a fair number that could enter into the diet of the bovine species. Because of this possibility, assay of these avermectins in the various tissues and milk of cattle has been investigated and, as such, is the subject of this report.

Assay of B_{1a} by GLC has been known from the beginning to be a difficult or impossible task. Tolan, Eskola, Fink, Mrozik, and Zimmerman [J. Chromatogr. 190, 367-376 (1980)] devised a fluorescence-HPLC method for assay of a similar species (the dihydrogenated material, ivermectin) in plasma. More recently some of the same people have published an HPLC method with UV detection. Tway modified the Tolan HPLC - fluorescence method for the determination of Ivermectin in a variety of animal tissues [J. Agric. Food Chem. 29, 1059-1063 (1981)]. More recently this method has been adapted to assay Abamectin in tissues.

Following more complete characterization of the total toxic residue in citrus, tomatoes and other agricultural products, it was determined that an analytical method would also be required for the delta 8, 9 isomer of avermectin B_1. It was determined that reaction of the delta 8, 9-isomer using the acetic anhydride derivatization gave an apparently low reaction yield for the fluorescent derivative, which was

itself photolytically unstable. Because stereochemistry suggests that the 7-OH of the delta 8, 9 isomer may be hindered during acylation, 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 the linkage is unstable, the trifluoroacetyl group at 4" position is cleaved by hydrolysis using, methanolic ammonium hydroxide, to give the 4"-OH fluorescent derivative. This two step reaction gives a single peak for the delta 8, 9 isomer and the B_{1a}. Therefore, the quantitated derivatized residue represents the sum of avermectin B_{1a} and its delta 8, 9 isomer.

When the modified derivatization (TFAA) method was applied to bovine tissues (liver, kidney, fat) cleaned up by the standard procedure, fluorescent interferences were seen at retention times similar to those for the avermectin B_{1a} and B_{1b} derivatives. To further clean up these samples before derivatization, an amino propyl (NH_2) Bond Elut column was incorporated into the method. The cleanup for bovine muscle samples obtained from the original method was sufficient even with the new derivatization procedure, so that the aminopropyl column was not necessary for those samples. Details of the modified cleanup method for the assay of avermectin B_{1a} and avermectin B_{1a} delta 8, 9 isomer are given in this report.

The limit of reliable quantitation for avermectin B_{1a}, B_{1b} and the delta 8, 9 isomer is 5 ng/g in muscle and kidney, and 10 ng/g in liver and fat. The detection limit for these compounds in bovine tissues is 2 ng/g. Example chromatograms of standards, control and fortified tissues are depicted in Figure 2.

The TFAA method described in this report has been used successfully to assay for avermectin B_{1a} and avermectin B_{1a} delta 8, 9 isomer at 5 ng/g in bovine muscles and kidneys samples, and at 10 ng/g in bovine fat and livers samples. Method recoveries of avermectin B_{1a} and avermectin B_{1a} delta 8,9 isomer from the fortified bovine samples are given in Tables I, II, III and IV. Recoveries for avermectin B_{1a} and the delta 8, 9 isomer averaged 92%, 93%, 93% and 86% for muscle, kidney, liver and fat, respectively. All of the control kidney and muscle samples had no detectable interferences. A small B_{1a} interference, less than the detection limit of 2 ng/g, was observed in some bovine control fat and liver samples. Only one fat control sample had a B_{1a} interference equal to approximately 4 ng/g.

The original tissue method for Ivermectin published in 1981 (J. Agric. Food Chem 29, 1059-1063 (1981) was modified to determine the concentration of abamectin in raw whole milk. These modifications are detailed in the report "HPLC - Fluorescence Assay for Abamectin B_1 in Bovine Tissues and Milk"

issued March 24, 1986. However, because the original method does not adequately quantitate the delta 8, 9 isomer, the trifluoroacetic anhydride derivatization procedure was applied for the milk assay. It was not necessary to modify the milk method sample cleanup procedure described in the March 24th, 1986 memo to use with the trifluoroacetic anhydride derivatization procedure. This method has a limit of detection for avermectin B_{1a}, B_{1b} and delta 8, 9 isomer residues at 0.5 ng/ml, and a limit at reliable quantitation of 1 ng/ml in raw whole milk. Example chromatograms of standards, control and fortified milk are depicted in Figure 3.

Recovery data for avermectin B_{1a}, B_{1b} and delta 8, 9 isomer from fortified raw whole milk samples are presented in Table V. Recoveries for avermectin B_{1a} and B_{1b} ranged from 87-110% at fortification levels from 1 to 5 ng/ml B_{1a}. Recoveries for the delta 8,9 isomer ranged from 71-100% at levels from 1-50 ng/ml. All of the control milk samples contained no interferences above the limit of detection.

Table I: Summary Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer Recoveries From Fortified Bovine Muscle.

Fortification Compound	Set Number	Fortification Level (Ng/g)	Ng/g Found	% Recovery
Avermectin B1a	RVM3	5.2	5.0	96
	RVM3	5.2	5.1	98
Avermectin B1a	RVM3	10.1	9.9	97
	RVM3	10.1	9.8	97
Avermectin B1a	RVM3	25.3	24.2	96
	RVM3	25.3	24.2	96
				Mean = 97
B1a Delta 8,9	RVM3	5.0	4.3	86
	RVK1	5.0	4.7	94
	RVK1	5.0	4.8	98
B1a Delta 8,9	RVM3	10.0	9.2	92
	RVM3	10.0	8.5	85
B1a Delta 8,9	RVM3	27.4	23.8	87
	RVM3	27.4	23.8	87
				Mean = 90

Three bovine muscle control samples were assayed and each sample yielded a ND result (< 2 Ng/g).

Table II: Summary Of Avermectin Bla And Avermectin Bla Delta 8,9 Isomer Recoveries From Fortified Bovine Kidney.

Fortification Compound	Set Number	Fortification Level (Ng/g)	Ng/g Found	% Recovery
Avermectin Bla	RVDEVK3	5.2	5.1	98
	RVDEVK3	5.2	5.1	98
	RVDEVK3	5.2	5.0	96
	RVDEVK4	5.2	5.2	100
	RVDEVK4	5.2	5.1	98
	RVDEVK4	5.2	5.2	100
	RVDEVK4	5.2	4.8	92
	RVDEVK4	5.2	4.7	90
Avermectin Bla	RVDEVK6	10.1	9.3	92
	RVDEVK6	10.1	8.9	88
	RVDEVK6	10.1	9.9	98
Avermectin Bla	RVDEVK6	25.3	24.0	95
	RVDEVK6	25.3	23.8	94
	RVDEVK6	25.3	25.0	99
				Mean = 96
Bla Delta 8,9	RVDEVK3	5.0	4.7	94
	RVDEVK3	5.0	4.6	92
	RVDEVK4	5.0	4.8	96
	RVDEVK4	5.0	4.6	92
	RVDEVK4	5.0	4.7	94
	RVDEVK4	5.0	4.5	90
	RVDEVK5	5.0	4.4	88
	RVDEVK5	5.0	4.3	86
Bla Delta 8,9	RVDEVK6	10.0	9.0	90
	RVDEVK6	10.0	9.1	91
	RVDEVK6	10.0	9.0	90
Bla Delta 8,9	RVDEVK6	22.8	19.9	87
	RVDEVK6	22.8	21.0	92
	RVDEVK6	22.8	20.1	88
				Mean = 91

Six bovine kidney control samples were assayed and each sample yielded a ND result (< 2 Ng/g).

Table III: Summary Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer Recoveries From Fortified Bovine Liver.

Fortification Compound	Set Number	Fortification Level (Ng/g)	Ng/g Found	% Recovery
Avermectin B1a	RVDEVL1	10.1	9.7	96
	RVDEVL1	10.1	9.5	94
	RVDEVL1	10.1	10.0	99
Avermectin B1a	RVDEVL2	20.2	20.2	100
	RVDEVL2	20.2	18.6	92
	RVDEVL2	20.2	20.1	100
				Mean = 97
B1a Delta 8,9	RVDEVL1	10.0	9.2	92
	RVDEVL1	10.0	9.5	95
	RVDEVL1	10.0	9.2	92
B1a Delta 8,9	RVDEVL2	18.2	17.2	95
	RVDEVL2	18.2	13.0	71
	RVDEVL2	18.2	17.0	88
	RVDEVF1	18.2	16.1	93*
				Mean = 89

* This result was obtained from a rederivatization of the 18.2 Ng/g B1a Delta 8,9 fortified bovine liver sample that had previously yielded a 71 % recovery.

Six bovine liver control samples were assayed and each sample yielded a ND result (< 2 Ng/g).

Table IV: Summary Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer Recoveries From Fortified Bovine Ft.

Fortification Compound	Set Number	Fortification Level (Ng/g)	Ng/g Found	% Recovery
Avermectin B1a	KTC	8	7	88
	KTC	8	7	88
	KTC	8	7	88
Avermectin B1a	KTC	19	16	84
	KTC	19	15	79
	KTC	19	17	89
Avermectin B1a	RVDEVF1	40.5	40.6	100
	RVDEVF1	40.5	40.0	99
Avermectin B1a	KTC	53	44	83
Avermectin B1a	KTC	60	48	80
Avermectin B1a	KTC	106	98	92
	KTC	106	100	94
				Mean = 89
B1a Delta 8,9	RVDEVF1	10.0	8.0	80
	RVDEVF1	10.0	8.4	84
	RVDEVF1	10.0	8.0	80
B1a Delta 8,9	RVDEVF1	18.2	14.7	81
	RVDEVF1	18.2	14.2	78
	RVDEVF1	18.2	15.4	89
B1a Delta 8,9	RVDEVF1	41.0	36.4	86
	RVDEVF1	41.0	35.3	86
	RVDEVF1	41.0	34.3	84
				Mean = 83

Nine bovine fat control samples were assayed. One control sample yielded a NQ result (< 5 Ng/g) and eight control samples yielded a ND result (< 2 Ng/g).

Table V: Summary of Avermectin B1a, B1b and Avermectin B1a Delta 8,9 Isomer Recoveries From Fortified Control Raw/Whole Milk

Fortification Compound	Set Number	Fortification Level (ng/ml)	ng/g Found	Percent Recovery
Avermectin B1a	MK936-305	1.0	0.97	97.0
	MK936-305	1.1	0.96	87.0
	MK936-305	1.0	1.1	110.0
	MK936-307	1.0	1.0	100.0
	MK936-307	1.0	1.1	110.0
Avermectin B1a	MK936-303	5.1	4.7	92.0
	MK936_303	5.1	5.2	102.0
Avermectin B1a	MK936_305R	9.4	8.9	95.0
	MK936_305R	9.4	8.9	95.0
Avermectin B1a	MK936_306	14.1	14.0	99.0
	MK936_306	14.1	15.0	106.0
Avermectin B1a	MK936-305R	51.0	51.0	100.0
	MK936_305R	51.0	51.0	100.0
	MK936_305R	51.0	53.0	104.0
		Mean % Rec.	B1a	100.0
B1a Delta 8,9	MK936_306	1.0	0.74	74.0
	MK936_306	1.0	0.73	73.0
	MK936_306	1.0	0.92	92.0
B1a Delta 8,9	MK936_307	5.1	3.6	71.0
	MK936_307	5.1	3.8	75.0
B1a Delta 8,9	MK936_306	10.0	8.2	82.0
	MK936_306	10.0	9.3	93.0
	MK936_306	10.0	8.9	89.0
B1a Delta 8,9	MK936_306	50.0	43.0	86.0
	MK936_306	50.0	46.0	94.0
	MK936_306	50.0	49.0	100.0
		Mean % Rec.	delta 8,9	84.0
Avermectin B1b	MK936_306	0.7	0.74	106.0
	MK936_306	0.7	0.71	101.0
	MK936_306	0.7	0.74	106.0
Avermectin B1b	MK936_306	1.1	0.98	89.0
	MK936_306	1.1	1.2	109.0
		1.1	1.2	109.0
Avermectin B1b	MK936_307	1.3	1.4	108.0
	MK936_307	1.3	1.4	108.0
Avermectin B1b	MK936_305R	3.8	4.0	105.0
	MK936_305R	3.8	3.8	100.0
		3.9	4.1	105.0
		Mean % Rec.	B1b	104.0

Five control raw/whole milk samples were assayed and each sample yielded a non-detectable residue (ND < 0.5 ng/ml).

The milk used for controls and fortified controls in this study was raw/whole milk obtained from Brown Swiss Dairy Cows on March 18, 1987. The milk was purchased from Ken Moser Farms in Stewardsville, New Jersey.

II. Method of Analysis for Tissues

A. Summary of the Determinative Residue Method

The tissue is homogenized with acetone-water and the avermectin B_1 and the delta 8, 9 isomer are extracted from the tissue with isooctane. The isooctane is evaporated, and solvent-solvent distributions into acetonitrile out of hexane, and into hexane out of acetonitrile-water are performed. A) Solvent is removed and fluorescence produced by B). After adding chloroform, the reaction mixture is washed through a silica gel Sep-Pak, the solvent is removed, and HPLC analysis (fluorescence detection) on Zorbax C18 is performed on the residue dissolved in methanol. A flow diagram of the assay is shown in Figure 4.

A) An aminopropyl solid phase extraction is performed on the hexane extract for bovine fat, kidney and liver samples.

B) For 1 hour at 30°C with n-methylimidazole/dimethyformamide/trifluoroacetic anhydride derivatization reagent and then for 30 minutes at 30°C with methanolic ammonium hydroxide.

II. Method of Analysis for Tissues (continued)

B. Reagents

Acetone - Mallinckrodt, Nanograde.

Acetonitrile Burdick & Jackson, distilled in glass.

Ammonium Hydroxide - Mallinckrodt, Analytical reagent (28-30%).

Chloroform - Burdick & Jackson, distilled in glass.

Dimethyl Formamide (DMF) - Baker, reagent grade.

Hexane - Burdick & Jackson, distilled in glass.

Methanol - Burdick & Jackson, distilled in glass.

Methylene Chloride - Burdick & Jackson, distilled in glass.

1-Methylimidazole - 99%, Aldrich Chemical Company.

Nitrogen - the equivalent of Matheson extra dry compressed gas.

Sodium Chloride - Mallinckrodt, Analytical Reagent

Sylon-CT - Supelco, Inc.

Trifluoroacetic Anhydride - 99+% Pure, Pierce Chemical Company.

Toluene - Mallinckrodt, Analytical Reagent.

2,2,4-Trimethylpentane - Mallinckrodt, Nanograde.

Water, Filtered Millipore - Distilled water is treated with a Milli-Q system and then filtered through a Millipore Type HA 0.45 μm disc.

II. Method of Analysis for Tissues (continued)

C. Solutions

Trifluoroacetic Anhydride Derivatizing Reagent.

Add 3.6 ml dimethylformamide to 0.4 ml 1-methylimidazole in a 15 ml centrifuge tube and mix. Place the tube in an ice bath and slowly add 0.6 ml trifluoroacetic anhydride. Vortex to mix.

Methanolic Ammonium Hydroxide Derivatizing Reagent.

Add 0.2 ml of ammonium hydroxide reagent (28-30% ammonia) to 3 ml methanol. Vortex to mix.

Acetone/Water - 50% (v/v).

Acetone:Methylene Chloride - 1:1

90% Methanol/Water - Dilute 200 ml of filtered Millipore water to 2 liters with methanol. Deaerate by slowly bubbling nitrogen through for 10 minutes.

D. Apparatus

Balance - analytical, capable of weighing 1 mg accurately.

Balance - capable of weighing 5 g accurately into an approximately 50 g tube.

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

Constant temperature water bath capable of holding the temperature at 30°C ± 0.5°C.

Bond Elut-aminopropyl (NH_2) 500 mg. 3 ml column (Cat No.611303,) Analytichem International.

II. Method of Analysis for Tissues

Apparatus (continued)

Centrifuge, IEC Model HN-S-II, with six place rotor IEC #958 and 15 and 50 ml cups. The centrifuge is run at 2000 to 2500 rpm. The centrifuge used gives ca. 700 to 750 X g maximum centrifugal force (RCF).

Centrifuge tubes, glass, 15 and 50 ml with polyethylene stoppers to fit.

Centrifuge tubes, 50 ml polypropylene, Corning #25331. (Used only for storing standard solutions.)

Centrifuge tubes, 15 ml, silylated approximately once every two months. (Used only for the derivatization reaction.) Pick 15 ml tubes that the stoppers fit tightly. Fill each tube to the top with Sylon-CT. Let stand 20 minutes. Immediately and quickly rinse, first thoroughly with toluene and then with methanol. Fill with methanol. Let stand 20 minutes, rinse thoroughly with acetone, and dry. All glassware used should be completely free of all acidic and alkaline residues. (These tubes should be cleaned by hand by first soaking in methylene chloride immediately after use and then in detergent for at least several hours, followed by thorough rinsing with hot water, distilled water, and acetone before thorough drying. Variations in the washing regimen are not recommended, since some analysts have had poor results when the standard washing method was not followed.)

II. Method of Analysis for Tissues

D. Apparatus (continued)

Dispensing pipettors, 10, 15, and 20 ml.

Freezer - capable of reaching temperatures of -20°C.

Gloves - disposable PVC from Fisher Chemical.

Graduate - 25 ml, 100 ml.

Graduate - 2000 ml with ground glass stopper.

Homogenizer - Polytron - Brinkmann Instruments, #27-11-200-5 with PTA 10S generator #27-11-330-3.

Pipets, disposable, Pasteur.

Pipets, graduated 1.0 ml, and 2.0 ml and 5.0 ml

Pipets, volumetric 0.5 ml, 1 ml, 2 ml, 3 ml, 4 ml, and 5 ml.

Reciprocating shaker, variable speed - Eberbach, J. T. Baker Catalog #8287-E30 or equivalent.

Repipet, 5 to 10 ml volume.

Sep-Pak, silica cartridge, Part No. 51900 - Waters Associates.

Sep-Pak, sample cartridge rack, modified to hold 15 ml centrifuge tubes, waters & associates.

Spatula - stainless steel.

Syringe - 50 mcl, 250 mcl, and 5 ml and 10 ml.

Tape - #13 2956, one-half inch from Ace Scientific.

Ultrasonic bath - Sonogen Automatic Cleaner - Branson Model 520 or equivalent.

II. Method of Analysis for Tissues

D. Apparatus (continued)

Vacuum Manifold - Applied separations Spe-ed Mate-30 System or equivalent with a modified rack to hold 15 ml centrifuge tubes.

Other reagents or apparatus may be substituted if equivalent performance can be demonstrated.

Vortex mixer.

II. Method of Analysis for Tissue (continued)

E. Chromatographic Apparatus

A Beckman-Altex Model 110A liquid chromatographic pump complete with Waters Wisp Autosampler and Kratos-Schoeffel Instruments Model FS950 fluorescence detector with a 1 millivolt recorder was used. A 5 μm, 4.6 mm ID, C18 standard Brownlee Labs guard column (Spheri-5 RP-18 OD-GU obtained from Rainin Instrument Co., Inc.) was used before the analytical column. This guard column is replaced monthly unless the pressure reaches 2000 psi, in which case it is replaced immediately.

Conditions: 15 cm length x 4.6 mm ID Zorbax ODS-C18 column 5 μm.

Mobile phase - 90% methanol in water (v/v).

Column temperature - 30°C.

Flow - 1.8 ml/minute (usual pressure 1000 psi, 500 to 2000 psi acceptable).

Detector Settings:

Excitation lamp - FSA 110, standard 365 nm.

Standard Kratos Flowcell - FSA 210.

Excitation filter - FSA 403, 365 nm band pass filter.

Emission filter - FSA 426, 418 nm. cutoff filter

Sensitivity range - 0.05 microamp.

Time constant - about 6 sec.

II. Method of Analysis for Tissues

E. Chromatographic Apparatus (continued)

The avermectin B_{1a}/avermectin B_{1a} delta 8, 9 isomer derivative has a retention time of about 17 minutes when the indicated conditions are used.

F. Standards for the Determinative Assay

For the most sensitive scale, accurate aliquots of 0.2, 0.4, 0.8, 1.2, 1.6 and 2.0 ml of ca. 50 ng/ml standard solution of avermectin B_{1a} in acetonitrile are added to silylated 15 ml centrifuge tubes and the samples blown to dryness at 60°C with nitrogen purge. After reaction, Sep-Pak treatment, etc., these samples are redissolved in one ml of methanol to give 5, 10, 20, 30, 40, and 50 ng/ml standard solution equivalents for use on the 0.05 scale.

Starting with a solution of about 0.956% (w/w) avermectin B_{1a} in glycerol formal, stock solutions of about 100 micrograms/ml can be prepared by weighing accurately about 0.5 gram of the solution into a 50-ml volumetric flask. Acetonitrile is used to dilute to the mark, and the solution is thoroughly mixed. Storage of this stock solution and all dilutions of same should be in polypropylene tubes or teflon lined capped amber borosilicate vials (40 ml) at -20°C.

II. Method of Analysis for Tissues

G. Procedure for the Determinative Assay of Tissues

Homogenization

All Tissues:

1. Weigh accurately 5.0 grams of sample into a 50-ml glass centrifuge tube.

Liver, Muscle, Kidney

2. Add 15 ml of 50% acetone/water to the tube from a dispensing pipettor.
3. Homogenize for 15 to 20 seconds or shorter time with a Polytron setting of 7. Homogenize until a homogeneous looking mixture results.
4. Put a clean 50-ml tube in place containing 15 ml of isooctane from a dispensing pipettor.
5. Homogenize 5 to 10 seconds and pour the isooctane into the 50-ml tube with the homogenate. Clean the homogenizer probe between samples with isooctane, distilled water, and acetone.

Fat Only:

2. Add 15 ml of the acetone/water and 10 ml of isooctane to the tube and blend until a homogeneous mixture results.
3. Rinse probe with 10 ml isooctane in a clean 50-ml tube by homogenizing for 5 to 10 seconds.
4. Pour isooctane into the 50-ml tube with the homogenate.

G. Procedure for the Determinative Assay of Tissues

Fat Only: (continued)

5. Add 1 gram of solid sodium chloride to the tube containing the homogenate.

Extractions - All Tissues

6. Stopper the tube, shake well for one minute, and centrifuge for 10 minutes.
7. Transfer the upper or isooctane layer to a second 50-ml centrifuge tube with a disposable pipet. Completely avoid the lower layer.
8. Break up the plug by using a vortex mixer and/or shaking; add 15 ml of fresh isooctane, repeating the extraction and combining isooctane layers.
9. Evaporate the combined isooctane layers down to a small volume (or ca. 5 ml for fat) using an 80°C bath and nitrogen purge.
10. Repeat the extractions with two more passes of 15 ml each of isooctane adding the isooctane in each case to the same tube which had the previous two isooctane extracts.
11. Again evaporate down as far as possible using the 80°C bath and purge.
12. Add 6 ml of methanol to all samples except fat and dissolve or resuspend completely with an ultrasonic bath and/or vortex mixer.

G. Procedure for the Determinative Assay of Tissues

Extractions - All Tissues (continued)

13. Place all samples except fat in a refrigerator or freezer until thoroughly cooled (at this point the sample is best left overnight in the refrigerator or freezer).

Fat Only:

14. Either add 2 ml of hexane and 5 ml of acetonitrile to the still hot and molten fat or freeze overnight in a freezer at -20°C. For samples frozen overnight, remelt in a 80°C bath and add the hexane and acetonitrile the next day.
15. Shake the still warm mixture thoroughly for ca. 1 minute and immediately centrifuge.
16. Cool in ice water until the lower or fat layer congeals and the upper or acetonitrile layer can be decanted completely into a clean 15-ml centrifuge tube.
17. Repeat the melting, extraction with 5 ml of acetonitrile, and transfer into the same 15 ml tube.
18. Add 2 ml of hexane to the 15-ml tube, shake thoroughly, centrifuge, and remove the upper, hexane layer to waste by disposable pipet or by aspiration. Go to Step 28.

G. Procedure for the Determinative Assay of Tissues

Extractions (continued)

Liver, Muscle and Kidney:

19. Centrifuge the cold 50 ml tube for 5 minutes, and decant off the clear supernatant to a clean 15-ml centrifuge tube.
20. Wash the residue in the 50-ml tube with 1 ml of methanol using a vortex mixer, centrifuge, and decant off into the same 15-ml tube. (If solids are decanted off in either step, recentrifuge the 15-ml tube and decant off into a new 15 ml tube to get a clear solution to continue the assay with.)
21. For muscles only, perform a 2-ml hexane extraction of the residue solids and add to the combined methanol.
22. Using a 60°C bath and nitrogen purge, evaporate off all the methanol.
23. By repipet add 3 ml of hexane to the tube previously containing the methanol and use an ultrasonic bath to remove all material from the walls of the centrifuge tube.
24. Add 4 ml by repipet of acetonitrile and repeat the ultrasonic treatment briefly.
25. Shake thoroughly, centrifuge 5 minutes, and move the lower or acetonitrile layer to a clean 15-ml tube.

G. Procedure for the Determinative Assay of Tissues

Extractions

Liver, Muscle and Kidney: (continued)

26. Re-extract the hexane layer with a second 4 ml of acetonitrile and combine acetonitrile layers.
27. Extract the combined acetonitrile layers with 1 ml of hexane, centrifuge to clear the layers, and move the upper, hexane layer to waste by disposable pipet or by aspiration.

All Tissues:

28. Evaporate the acetonitrile solution to 1 ml using a ca. 60°C bath and nitrogen flush.
29. If the acetonitrile is <1 ml, make up to 1 ml with fresh acetonitrile.
30. Use an ultrasonic bath to get a homogeneous mix if necessary.
31. Add 4 ml of distilled water (2 ml for kidney samples) and 5 ml of hexane; shake ca. 1 minute and centrifuge for 5 minutes.
32. Move the upper, hexane layer to a clean 15-ml centrifuge tube using a disposable pipet. Avoid the lower layer.
33. Repeat with 5 ml and, then, 4 ml hexane extractions combining all three hexane extracts.

G. Procedure for the Determinative Assay of Tissues

Extractions - All Tissues (continued)

34. Attach a 500 mg 3 ml aminopropyl column fitted with an adaptor and a 25 ml reservoir to the Spe-ed Mate-30 system. Condition the column with 6 ml of hexane.

35. 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 centrifuge tube under the column and elute the column with 5 ml acetone:methylene chloride 1:1.

36. Reduce the acetone/methylene chloride extract to dryness.

Muscle Only:

37. Evaporate to dryness (or as near dryness as possible) using a 40°C bath and nitrogen purge. (To get completely down, more heat may need to be applied at the end of the evaporation. The bath may go up to ca. 80°C at this point.)

38. Redissolve the residue in exactly 1.0 ml of methanol using a vortex mixer and ultrasonic bath.

39. Mix thoroughly and centrifuge for 5 minutes.

40. Pipet exactly 0.5 ml of the solution from Step 39 into the bottom of a silylated 15 ml centrifuge tube. Use a 0.5 ml volumetric pipet.

41. Completely evaporate the solvent carefully using a 60°C bath and nitrogen purge. Avoid spattering.

G. Procedure for the Determinative Assay of Tissues

All Tissues: (continued)

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

NOTE: Standards should be derivatized with unknowns.

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

44. 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.

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

46. Wash a silica Sep-Pak cartridge with 5 ml of chloroform using vacuum of approximately 125 mm Hg to pull the chloroform from a reservoir into a waste collection tube. A SEP-PAK sample cartridge rack or equivalent may be used to clean up several samples in parallel.

47. 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.

G. Procedure for the Determinative Assay of Tissues

Derivatization - All Tissues (continued)

48. Release the vacuum. Wash the silylated centrifuge tube two times with 2 to 3 ml chloroform. Use the same pipet that was used to transfer the sample to transfer the chloroform washes to the SEP-PAK reservoir. Apply the vacuum to elute the washes, releasing after each wash.

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

50. Take the sample to dryness with nitrogen in a 70°C water bath. Samples should be reduced to an oily residue less than 0.05 ml.

51. Pipet exactly 1.0 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.

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

53. Generate a standard curve by performing a linear regression analysis of response (peak height) versus concentration (ng/ml). The curve should be linear with a regression coefficient (r^2) of 0.97 or better. Use the regression equation to determine the concentration of unknowns with an observed peak height.

G. Procedure for the Determinative Assay of Tissues

Derivatization - All Tissues (continued)

54. Calculate the sample concentration as follows:

$$C\ (ng/ml) = (PK\ HT - I)/S$$

where:

PK HT = Peak height of the fluorescent derivative

S = Slope of the regression line

I = y-intercept of the regression line

The B_{1a}/delta 8,9 isomer residue (ng/g) in a sample is calculated by the equation:

$$\text{Residue (ng/g)} = \frac{C\ (ng/ml)\ X\ FV}{W\ X\ Frac}$$

where:

FV = Volume of HPLC solution (ml)

W = Sample weight taken in Step 1 of the method (gm).

Frac = Sample fraction taken for the derivatization in Step 40 of the method.

II. Method of Analysis for Tissues (continued)

H. Suitable Stopping Places for Tissue Assays

The following stopping places allow storage for a maximum of four days.

1. In a refrigerator at 4°C.
   a. Only in methanol after Step 12.
2. In a freezer at -20°C.
   a. After Step 11 for fat only.
   b. After Step 38, dissolution of residue in methanol.
   c. After Step 51, dissolution of the residue in methanol.

If stopping places other than the above are needed, assay stability at said stopping place should first be demonstrated.

I. Assay Timing

For almost any analysis, the schedule adhered to depends greatly on the objectives of the analyst and the equipment available to carry out the procedure. For the abamectin residue method, our own desires were to maximize accuracy and minimize time per assay, allowing time for other duties in an eight-hour day. Achieving single results in minimum time was not a high priority. However, assays can be completed in a single day if needed to meet other objectives.

I. Assay Timing (continued)

To best meet our stated objectives with the available equipment, we devised the schedule shown in Figure 5 as follows:

Day 1 - Grind 11 samples plus one control fortified with an appropriate level of standard. Complete isooctane extractions, blow downs, and dissolution in methanol. Place in freezer to cool. (Steps 1-13, Point A in the Figure 4 flow diagram.)

Day 2 - Do the acetonitrile, hexane, water extractions, aminopropyl cleanup, and evaporations. Make derivative, SEP-PAK and dissolve for LC. (Steps 14-51. Using a WISP, the HPLC analysis can be accomplished over the second night.)

Day 3 - Same as day 1 plus run LC of sample from day 1 and calculate results.

The overall composite effect of this schedule is that one analyst completes 11 samples and one recovery per each two working days. If no fortified sample is included, the analyst does six samples/day. A number of analysts have been able to easily keep to this schedule for months at a time using the ivermectin assay, almost identical to the abamectin assay except for solid phase extraction and the HPLC mobile phase composition.

I. Assay Timing (continued)

An alternative schedule has also been devised to complete analyses in a shorter time frame from initiation of assay to result. This procedure calls for completing the day 1 schedule before lunch on four to six samples, cooling at -20°C over lunch, and doing the day 2 schedule post lunch. Such a schedule can and has in our hands been completed on six samples in one 8-hour day. A more reasonable approach for routine analysis is to attempt only four samples/day. If one combines this second schedule with automatic injection, four to six samples can be completed in a day not including the calculations. With a proper computational unit attached directly to the apparatus, one could achieve four to six assays in a single day (with the HPLC run overnight). We regard the second schedule as more hectic and hence, less desirable than the first.

III. Method of Analysis for Milk

A. Summary of the Method

Ammonium hydroxide and ethanol are added to milk and the avermectin B_{1a}, B_{1b}, and delta 8,9 isomer are extracted with ethyl acetate and isooctane. The organic solvents are removed from the extract by evaporation and the residue is distributed between hexane and acetonitrile. After dissolution in acetonitrile to 10 ml, the sample is split in half. Half of the sample is evaporated to dryness and is reacted with trifluoroacetic anhydride and 1-methylimidazole in N,N-dimethylformamide, followed by reaction with methanolic ammonium hydroxide. The reaction mixture is cleaned up by SEP-PAK/chloroform treatment and the chloroform is removed by evaporation. A methanolic solution of the derivatized residue is chromatographed through a C-18 column at 30°C with 7% water in methanol as the mobile phase, and with fluorescence detection. Peak height comparisons with standards are made to determine ng/ml concentrations of avermectin B_{1a}, B_{1a} delta 8,9 isomer, and avermectin B_{1b}. Linear regression analysis between peak height and the concentration of avermectin B_{1a} standards in conjunction with mathematical interpolation is used to determine the concentration of the residue.

IV. Method of Analysis for Milk (continued)

B. Solvents and Reagents

Trifluoroacetic Anhydride - Pierce Chem. Co., 99+% purity.

Acetone - any reagent grade.

Acetonitrile - Burdick & Jackson, distilled in glass.

Ammonium Hydroxide - Mallinckrodt, Analytical Reagent (28 to 30%).

Chloroform - Burdick & Jackson, distilled in glass.

N,N-Dimethylformamide (DMF) - Baker, Baker Analyzed Reagent.

Ethanol - U.S. Industrial Chemicals Co., ethyl alcohol U.S.P. 190 proof punctilious.

Ethyl Acetate - Fisher, HPLC Grade.

Hexane - Burdick & Jackson, high purity.

Isooctane - Mallinckrodt, 2,2,4-trimethylpentane, Nanograde.

Methanol - Burdick & Jackson, high purity.

Methylene Chloride - any reagent grade.

1-Methylimidazole - Aldrich, 99%.

Nitrogen - The equivalent of Matheson extra dry compressed gas.

Sylon-CT - Supelco.

Water - Filtered, Millipore treated, distilled water.

IV. Method of Analysis for Milk (continued)

C. Solutions

Trifluoroacetic Anhydride Derivatizing Reagent - Add 3.6 ml dimethylformamide to 0.4 ml 1-methylimidazole in a 15 ml centrifuge tube and mix. Place the tube in an ice bath and slowly add 0.6 ml trifluoroacetic anhydride. Vortex to mix.

Methanolic Ammonium Hydroxide Derivatizing Reagent - Add 0.2 ml of ammonium hydroxide reagent (28 to 30% ammonia) to 3 ml methanol. Vortex to mix.

Mobile phase - In a 2000 ml graduated cylinder dilute 140 ml of water to 2000 ml with methanol. Mix and bubble nitrogen through this solution slowly for 10 minutes.

D. Apparatus

Balance - Analytical, capable of weighing 1 mg accurately.

Bath - water with temperature set at 30°C.

Bath - water with temperature variable 50 to 80°C.

Centrifuge - IEC Model HN-S-II, with six place rotor IEC #958 and 15 and 50 ml cups. The centrifuge is run at 2000 to 2500 rpm.

Centrifuge tubes - Glass, 15 and 50 ml with polyethylene stoppers to fit.

Centrifuge tubes - Glass, 15 ml, silylated approximately every two months (used only for derivatization reaction). Fill each tube with Sylon-CT and allow to stand 20 minutes. Rinse thoroughly with toluene and then with methanol. Fill with methanol and let stand

IV. Method of Analysis for Milk

D. Apparatus

Centrifuge tubes (continued)

20 minutes, rinse thoroughly with acetone and allow to dry. All glassware used should be completely free of all acidic and alkaline residues. (These tubes should be cleaned by hand by first soaking in methylene chloride immediately after use and then in detergent for at least several hours each, followed by thorough rinsing with hot tap water, distilled water, and acetone before thorough drying. Variations in the washing regimen are not recommended, since some analysts have had poor results when the standard washing method was not followed. After silylation this washing should be carried out two or three times to avoid interferences.)

Dispensers - Labindustries, Repipet, 0 to 10 ml.

Dispensing pipettors (pouring) - 5 ml fixed.

Gloves - Disposable PVC from Fisher.

Freezer - Capable of reaching -20°C.

Pipets, disposable Pasteur.

Pipets, graduated - 1.0 ml, 10 ml.

Pipets, volumetric - 0.5 ml, 1 ml, 5 ml, 10 ml.

Pipets, blowout - 0.2 ml.

Sep-Pak, silica cartridge - Waters Associates Part No. 51900.

IV. Method of Analysis for Milk

D. Apparatus (continued)

Stoppers, polyethylene to fit centrifuge tubes.

Ultrasonic bath - Sonogen Automatic Cleaner, Branson Model 520 or equivalent.

Vortex mixer - Scientific Industries Model K-550-G.

E. Chromatographic System

A Beckman-Altex Model 110A pump was used to supply the mobile phase (7% water in methanol v/v) through a Waters WISP Autosampler, a 4.6 mm I.D. standard Brownlee Labs guard column containing 5 µm C-18 (Spheri-5 RP-18 OD-GU obtained from Rainin Instrument Co., Inc.), a 15 cm x 4.6 mm I.D. Zorbax ODS C-18 column and a Kratos-Schoeffel Instruments Model FS950 fluorescence detector connected to a 1 mv recorder. The jacketed column was kept at 30°C by a circulating water bath (Haake, Model FE2). The flow rate was 1.5 ml/minute at a usual pressure of 1400 psig (500 to 2500 psig is acceptable).

The detector used a standard 365 nm lamp (FSA 110), and standard Kratos flow cells (FSA 210). The filters were a 365 nm band pass filter (FSA 403) for excitation and a 418 nm cutoff filter for emission. The range was set at 0.02 µA, the sensitivity at 730 V and the time constant at 6 seconds.

IV. Method of Analysis for Milk

E. Chromatographic System (continued)

Under these conditions the retention time of the derivative of avermectin B_{1a}/B_{1a} delta 8,9 isomer was about 8 minutes. The retention time for the avermectin B_{1b} derivative was about 6.3 minutes.

F. Standards

Starting with a solution of about 0.956% (w/w) avermectin B_{1a} in glycerol formal (L-676,873-038A002), stock solutions of about 100 mcg/ml may be prepared by accurately weighing about 0.5 gram of the solution into a 50 ml volumetric flask, diluting to the mark with acetonitrile and thoroughly mixing. Storage of this stock solution and all dilutions of it should be in polypropylene tubes or Teflon lined capped amber borosilicate vials (40 ml) at -20°C.

Using a 1 ml graduated pipet, aliquots of a 50.0 ng/ml and a 5.0 ng/ml standard solution of B_{1a} in acetonitrile are added to silylated 15 ml centrifuge tubes, and the acetonitrile is evaporated off at 60° in a nitrogen stream. After reaction, SEP-PAK treatment, etc., these samples are redissolved in 2 ml of methanol to give final concentrations of 1.3, 2.5, 5, 10, and 15 ng/ml.

IV. Method of Analysis for Milk (continued)

G. Procedure

1. Shake raw whole milk to make homogeneous.
2. With a volumetric pipet, transfer 10 ml milk to a 50 ml centrifuge tube.
3. Using a 10 ml graduated pipet, add 1.5 ml concentrated ammonium hydroxide and agitate with a vortex mixer (vortex) briefly.
4. Add 10 ml ethanol by volumetric pipet and shake for 30 seconds.
5. Add 10 ml ethyl acetate by volumetric pipet and shake for 20 seconds.
6. Add 10 ml isooctane from a dispensing pipettor and shake for 20 seconds.
7. Centrifuge 4 minutes.
8. Using a disposable Pasteur pipet, transfer the upper layer, avoiding the lower layer completely, to a fresh 50 ml centrifuge tube.
9. Repeat 5, 6, and 7 once on the raffinate.
10. Combine the upper layer with that from 8 above.
11. Evaporate off the solvents (to an oil that no longer reduces volume) at 70°C in a stream of nitrogen.
12. With a dispenser add 3 ml hexane, vortex, sonicate, and shake.

IV. Method of Analysis for Milk

G. Procedure (continued)

13. With a dispenser add 4 ml acetonitrile, shake for 1 minute, and centrifuge 5 minutes.
14. Using a disposable Pasteur pipet, transfer the lower layer to a 15 ml centrifuge tube.
15. Repeat 13 once on the hexane raffinate.
16. Combine the lower layer with that from 14 above.
17. Using a dispenser add 1 ml hexane to the combined acetonitrile layers, shake 1 minute, and centrifuge 4 minutes.
18. Using a disposable Pasteur pipet transfer or aspirate the upper layer to waste.
19. At 70° evaporate the sample to less than 10 ml with a stream of nitrogen. Dilute the sample to 10 ml with acetonitrile and split the sample by transferring 5 ml to a silylated centrifuge tube using a disposable Pasteur pipet and the graduation marks on the centrifuge tube.
20. Prepare standards as described in Section F.
21. Run one set of standards and one set of samples.
22. Completely evaporate the solvent carefully using a 60°C bath and nitrogen purge. Avoid spattering.

IV. Method of Analysis for Milk

G. Procedure (continued)

23. 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.

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

25. 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.

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

27. Wash a silica SEP-PAK cartridge with 5 ml of chloroform using vacuum of approximately 125 mm Hg to pull the chloroform from a reservoir into a waste collection tube. A SEP-PAK sample cartridge rack or equivalent may be used to clean up several samples in parallel.

28. 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.

IV. Method of Analysis for Milk

G. Procedure (continued)

29. Release the vacuum. Wash the silylated centrifuge tube three times with 2 to 3 ml chloroform. Use the same pipet that was used to transfer the sample, to transfer the chloroform washes to the Sep-Pak reservoir. Apply the vacuum to elute the washes, releasing after each wash.

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

31. Take the sample to dryness with nitrogen in a 70°C water bath. Samples should be reduced to an oily residue less than 0.05 ml.

32. Pipet exactly 2.0 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.

33. Centrffuge briefly prior to injection of the supernatant on HPLC.

IV. Method of Analysis for Milk

G. Procedure (continued)

34. Inject two sets of standards with one set of samples. Dilute samples if necessary to keep within the range of the standards. Generate a standard curve by performing a linear regression analysis of response (peak height) versus concentration (ng/ml). The curve should be linear with a correlation coefficient (r^2) of 0.97 or better. Use the regression equation to determine the concentration of unknowns with an observed peak height.

35. Calculate ng/ml (milk) by the following equation:

$$\text{ng/ml (s)} = \text{ng/ml (c)} \times \frac{V_1 \times D}{V_2 \quad V_3}$$

where: V_1 = ml sample dissolved in before LC (2.0 ml)

V_2 = fraction of sample used (0.5 ml)

V_3 = ml milk originally taken (10 ml).

D = dilution of LC sample to stay in range of standard curve or 1 if no dilution made.

ng/ml (c) = ng/ml in final solution injected into liquid chromatograph. (Determined from regression analysis).

ng/ml (s) = ng/ml in the original sample.

and the usual equation is:

$$\text{ng/ml (s)} = \text{ng/ml (c)} \times \frac{D}{2.5}$$

IV. Method of Analysis for Milk

H. Suitable Stopping Places for Milk Assays

The samples may be held refrigerated (4°C) overnight after step 3, the addition of ammonium hydroxide and agitation.

Samples may also be held overnight in the freezer at -20°C after completion of the first series of extractions with ethyl acetate, ethanol, and isooctane.

The following stopping places allow storage for a maximum of four days in a freezer at -20°C.

1. After Step 19 - Acetonitrile solution after division.
2. After Step 32 - Derivatization.

I. Assay Timing

For almost any analysis, the schedule adhered to depends greatly on the objectives of the analyst and the equipment available to carry out the procedure. For the abamectin residue method, our own desires were to maximize accuracy and minimize time per assay, allowing time for other duties in an eight-hour day. Achieving single results in minimum time was not a high priority. However, assays can be completed in a single day if needed to meet other objectives. The next several paragraphs describe the timing for the milk assay.

IV. Method of Analysis for Milk

I. Assay Timing (continued)

On day one 12 samples are carried through all of the partition steps and split into silanized tubes.

On day two 12 samples, plus the analytical standards may be derivatized and chromatographed.

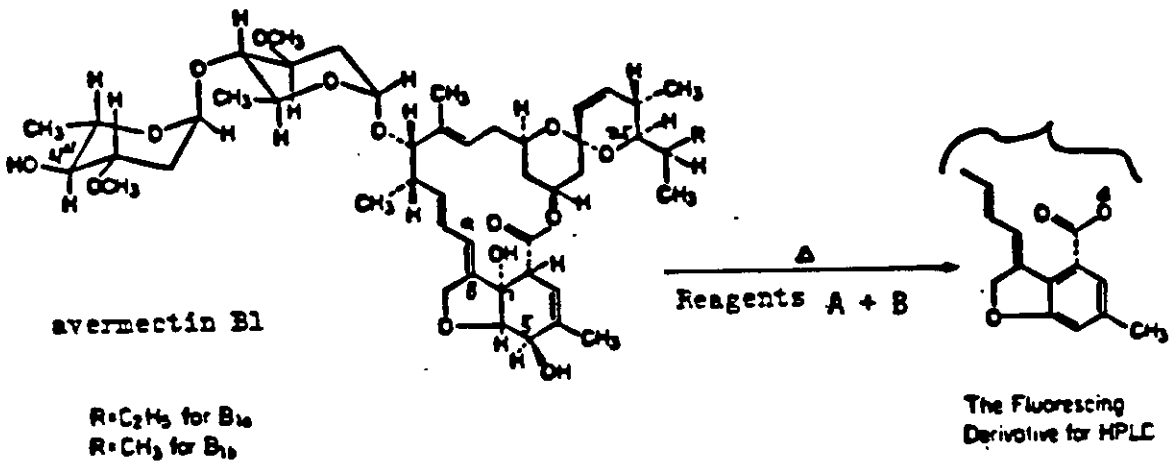
Six samples may be completed analyzed in a single day.

A P P E N D I X A.

Figures

Figure 1.

Structures of Avermectin B_1 and Derivatization Reaction

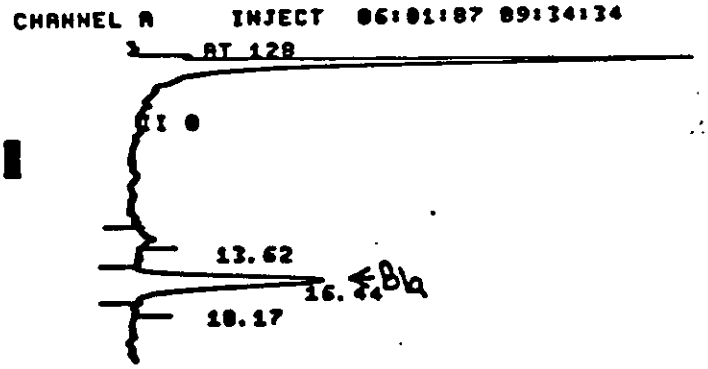


A. Trifluoroacetic anhydride/DMF/1-methylimidazole

B. Methanolic Ammonium Hydroxide

Figure 2: Example Chromatograms Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer In Bovine Tissue

10.3 ng/ml Avermectin B1a Standard Equivalent To 4 ng/g



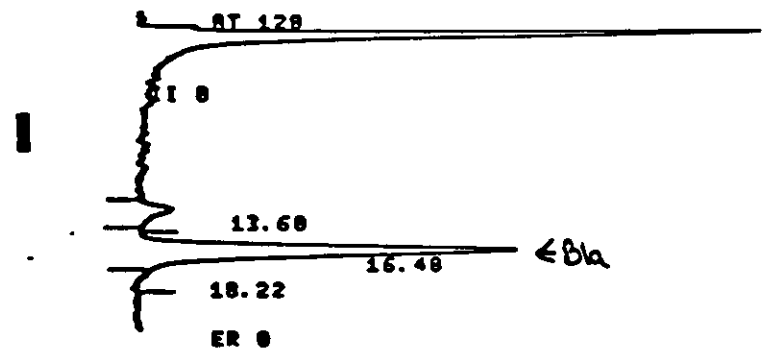
AVERMECTIN B1A IN CATTLE 06:01:87 09:34:3

FILE 2. METHOD 0. RUN 267 INDEX

PEAK#	HT%	RT	PK HT BC	
1	6.955	13.62	1721 01	
2	92.866	16.44	22975 02	←B1a
3	0.179	18.17	44 03	

TOTAL 100. 24741

20.6 ng/ml Avermectin B1a Standard Equivalent To 8.2 ng/g



AVERMECTIN B1A IN CATTLE 06:01:87 09:18:31

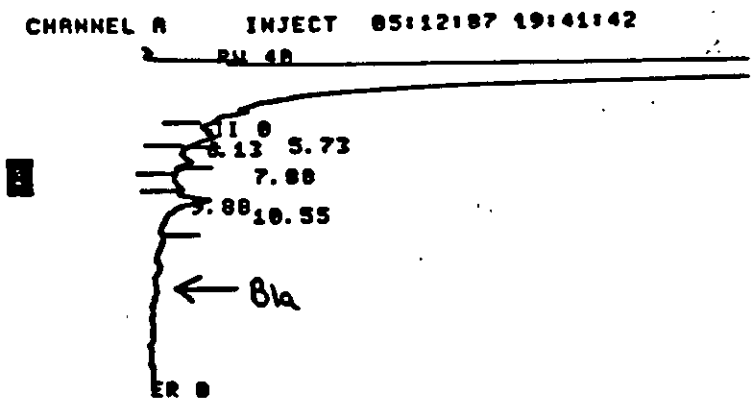
FILE 2. METHOD 0. RUN 266 INDEX 26

PEAK#	HT%	RT	PK HT BC	
1	7.36	13.6	3679 01	
2	92.64	16.48	46315 03	←B1a

TOTAL 100. 49994

Figure 2: Example Chromatograms Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer In Bovine Tissue (Continued).

Control Muscle



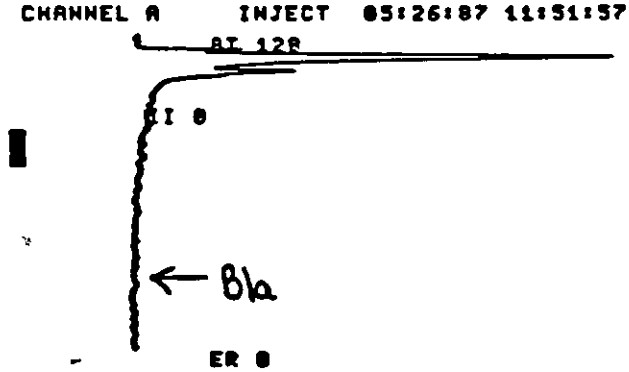
AVERMECTIN B1A IN MUSCLE 05:12:87 19:41:42 CH= "A" PS= 1.

FILE 2. METHOD 0. RUN 70 INDEX 70

PEAK#	HT%	RT	PK HT BC
1	11.931	5.73	1519 82
2	22.436	6.13	2856 83
3	13.061	7.88	1663 81
4	9.356	9.88	1191 82
5	43.217	10.55	5501 83
TOTAL	100.		12730

No B1a

Control Kidney

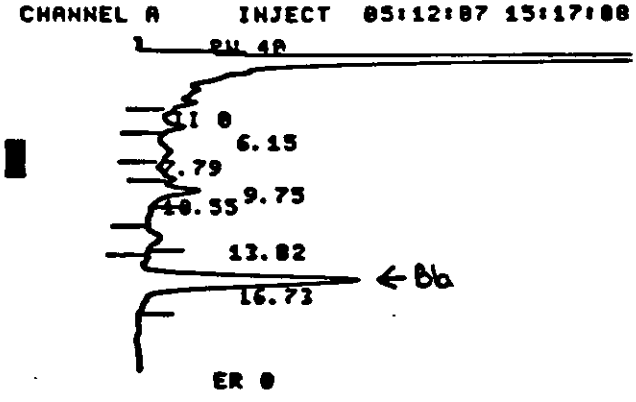


NO DATA, CHANNEL A

No B1a

Figure 2: Example Chromatograms Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer In Bovine Tissue (Continued).

Control Muscle Fortified At 5.2 ng/g Avermectin B1a (98% Recovery)

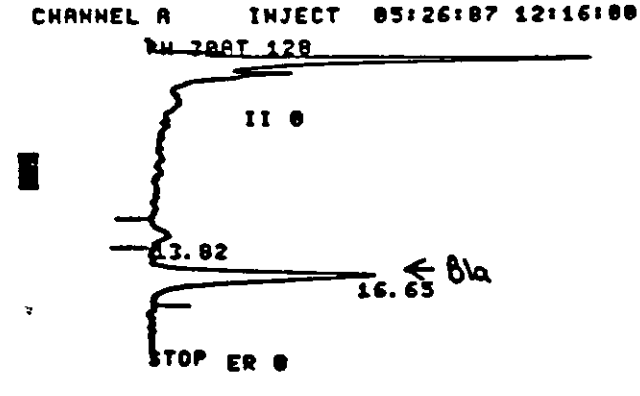


AVERMECTIN B1A IN MUSCLE 05:12:87 15:17:88

FILE 2. METHOD 0. RUN 59 INDEX 59

PEAK#	HT%	RT	PK HT BC	
1	6.906	6.15	2870 02	
2	4.349	7.79	1807 02	
3	6.091	9.75	2531 02	
4	14.249	10.55	5921 03	
5	4.217	13.82	1752 01	
6	64.188	16.73	26675 01	← B1a
TOTAL	100.		41557	

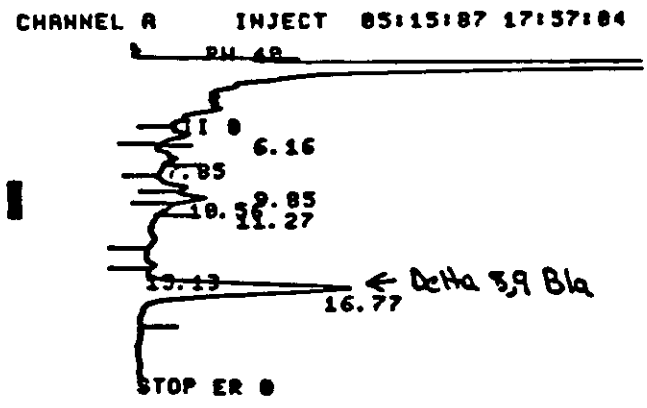
Control Kidney Fortified At 5.2 ng/g Avermectin B1a (100% Recovery)



AVERMECTIN B1A IN KIDNEYS 05:26:87 1

FILE 2. METHOD 0. RUN 199 INDEX 199

PEAK#	HT%	RT	PK HT BC	
1	8.318	13.82	2504 02	
2	91.682	16.65	27598 03	← B1a
TOTAL	100.		30101	

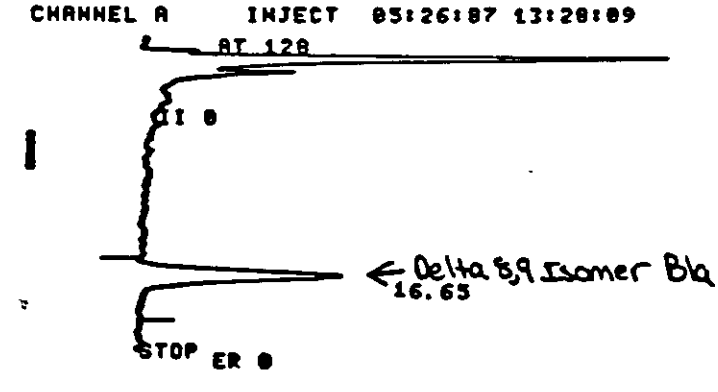


Control Muscle Fortified At
5.0 ng/g Avermectin Bla
Delta 8,9 Isomer
(96% Recovery)

AVERMECTIN B1A IN KIDNEYS 05:15:87 17:57:84 1.

FILE 2. METHOD 0. RUN 99 INDEX 99

PEAK#	HT%	RT	PK HT	BC	
1	7.382	6.16	3114	01	
2	5.13	7.85	2188	01	
3	7.855	9.85	3358	02	
4	13.919	10.56	5936	02	
5	3.21	11.27	1369	03	
6	2.858	15.13	1219	02	
7	59.727	16.77	25471	03	← Delta 8,9 Isomer
TOTAL	100.		42645		



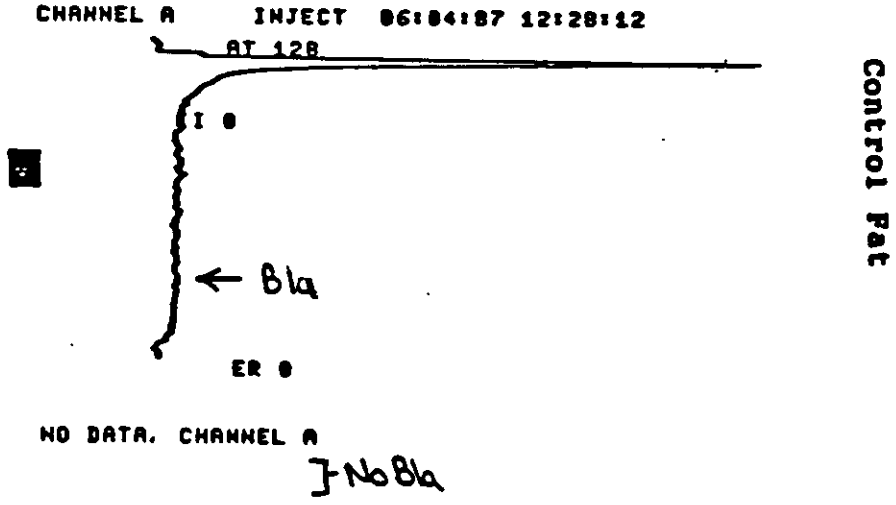
Control Kidney Fortified At
5.0 ng/g Avermectin Bla
Delta 8,9 Isomer
(96% Recovery)

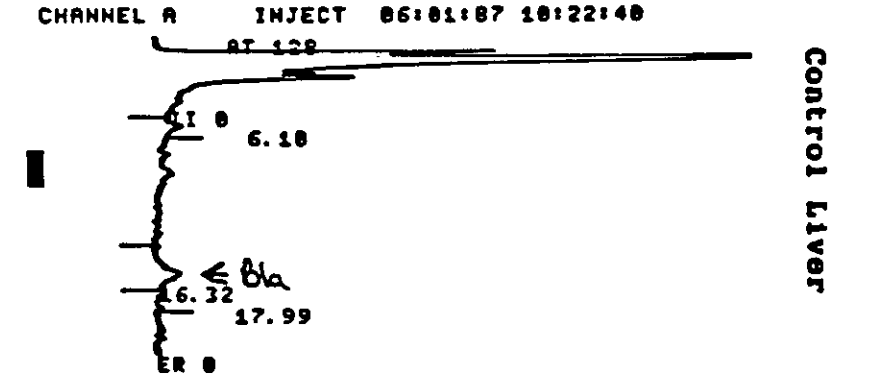
AVERMECTIN B1A IN KIDNEYS 05:26:87 13:28:89 CH= "A" PS= 1.

FILE 2. METHOD 0. RUN 202 INDEX 202

PEAK#	HT%	RT	PK HT	BC	
1	100.	16.65	25191	01	← Bla Delta 8,9 Isomer
TOTAL	100.		25191		

Figure 2: Example Chromatograms Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer In Bovine Tissue (Continued).





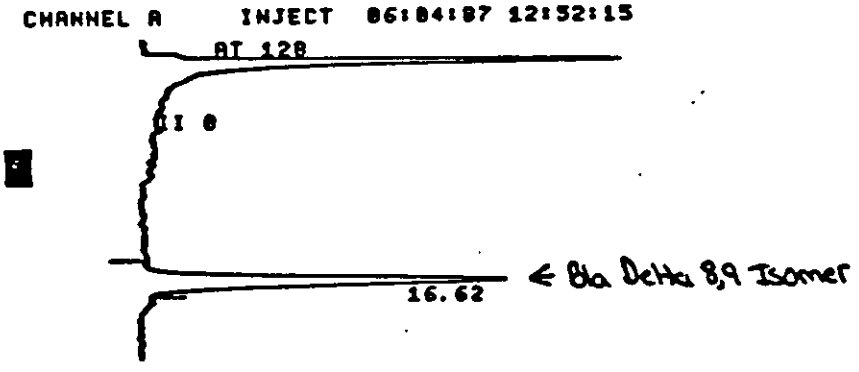
AVERMECTIN B1A IN CATTLE 86:81:87 10:22:40 CH= "A" PS= 1.

FILE 2. METHOD 0. RUN 269 INDEX 269

PEAK#	HT%	RT	PK HT BC	
1	41.182	6.1	2130 01	
2	55.818	16.32	2887 02	← Bla
3	3.	17.99	155 03	
TOTAL	100.		5173	

Figure 2: Example Chromatograms Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer In Bovine Tissue (Continued).

Control Fat Fortified At 10.0 ng/g Avermectin B1a Delta 8,9 Isomer (80% Recovery)

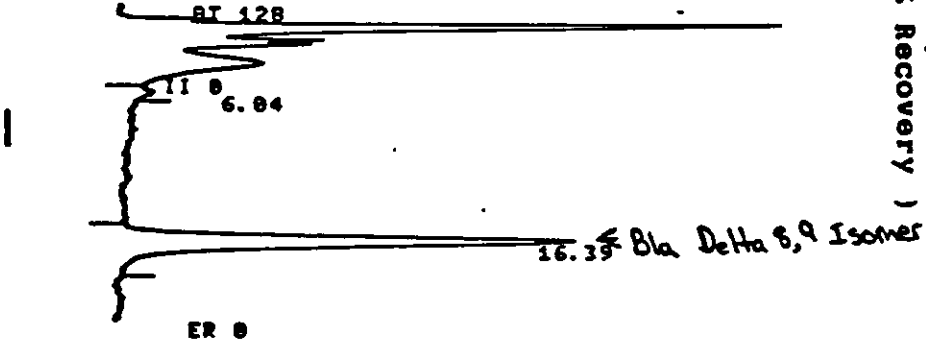


AVERMECTIN B1A IN CATTLE 06:04:87 12:52:15 CH= "A" PS= 1.

FILE 2. METHOD 0. RUN 323 INDEX 323

PEAK#	HT%	RT	PK HT BC	
1	100.	16.62	43547 01	← B1a Delta 8,9 Isomer
TOTAL	100.		43547	

Control Liver Fortified At 10.0 ng/g Avermectin B1a Delta 8,9 Isomer (95% Recovery)



AVERMECTIN B1A IN CATTLE 06:01:87 12:22:56 CH= "A" PS= 1.

FILE 2. METHOD 0. RUN 274 INDEX 274

PEAK#	HT%	RT	PK HT BC	
1	3.448	6.84	1977 01	
2	96.552	16.39	55363 01	← B1a Delta 8,9 Isomer
TOTAL	100.		57340	

Figure 2: Example Chromatograms Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer In Bovine Tissue (Continued).

Figure 2: Example Chromatograms Of Avermectin B1a And Avermectin B1a Delta 8,9 Isomer In Bovine Tissue (Continued).

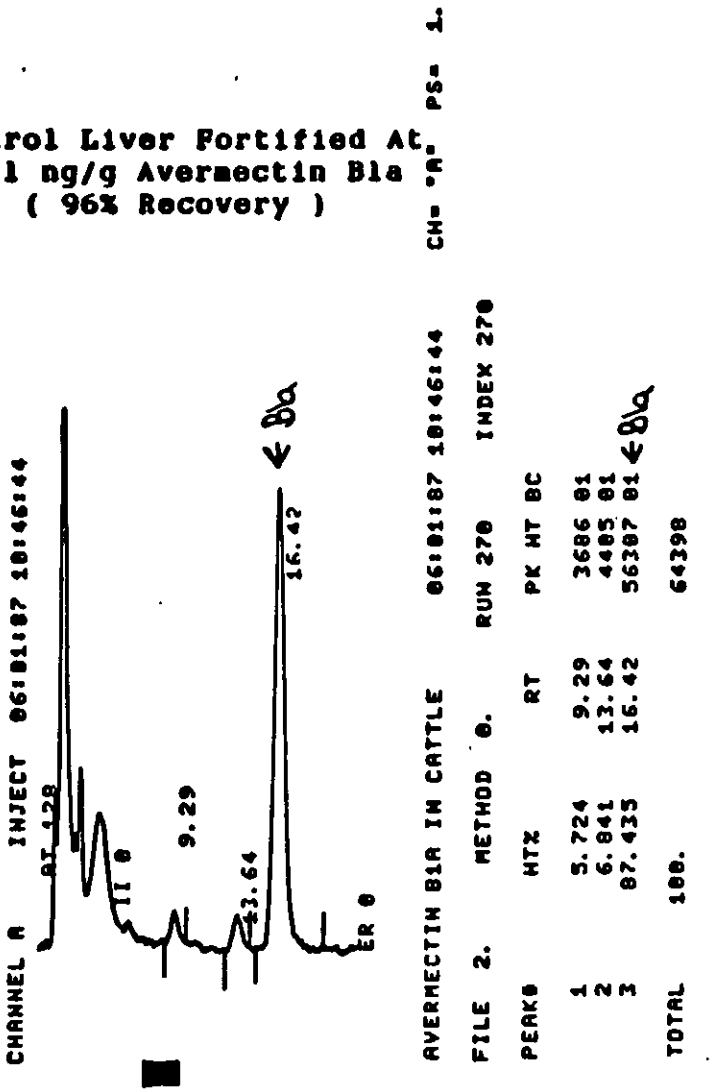


Figure 3.

Typical Chromatograms of Avermectin B_{1a} Standard, Control Raw Whole Milk, Control Raw Whole Milk Fortified with Avermectin B_{1a}, Avermectin B_{1a} Delta 8,9-Isomer and, Avermectin B_{1b}

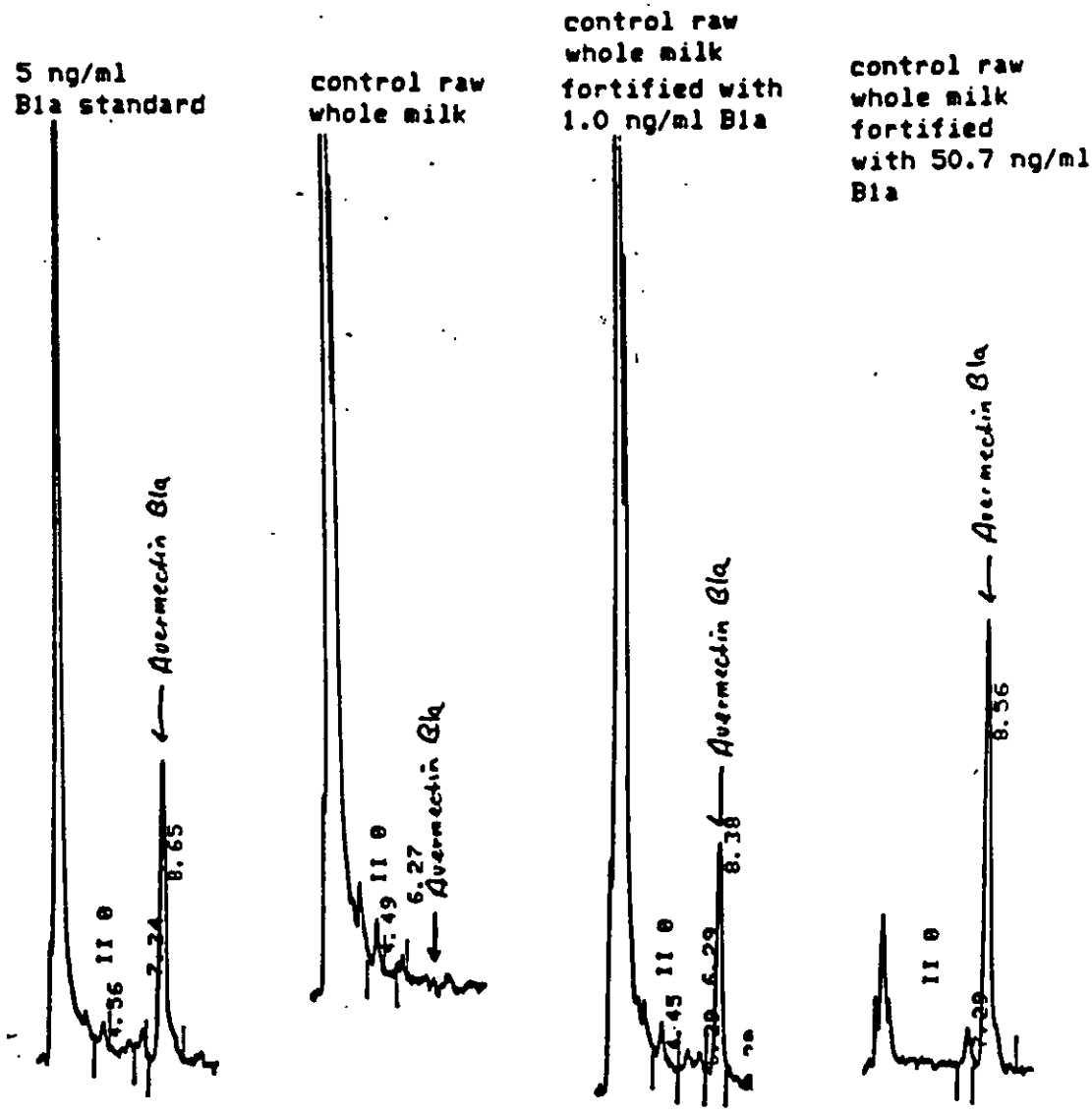


Figure 3. (continued)

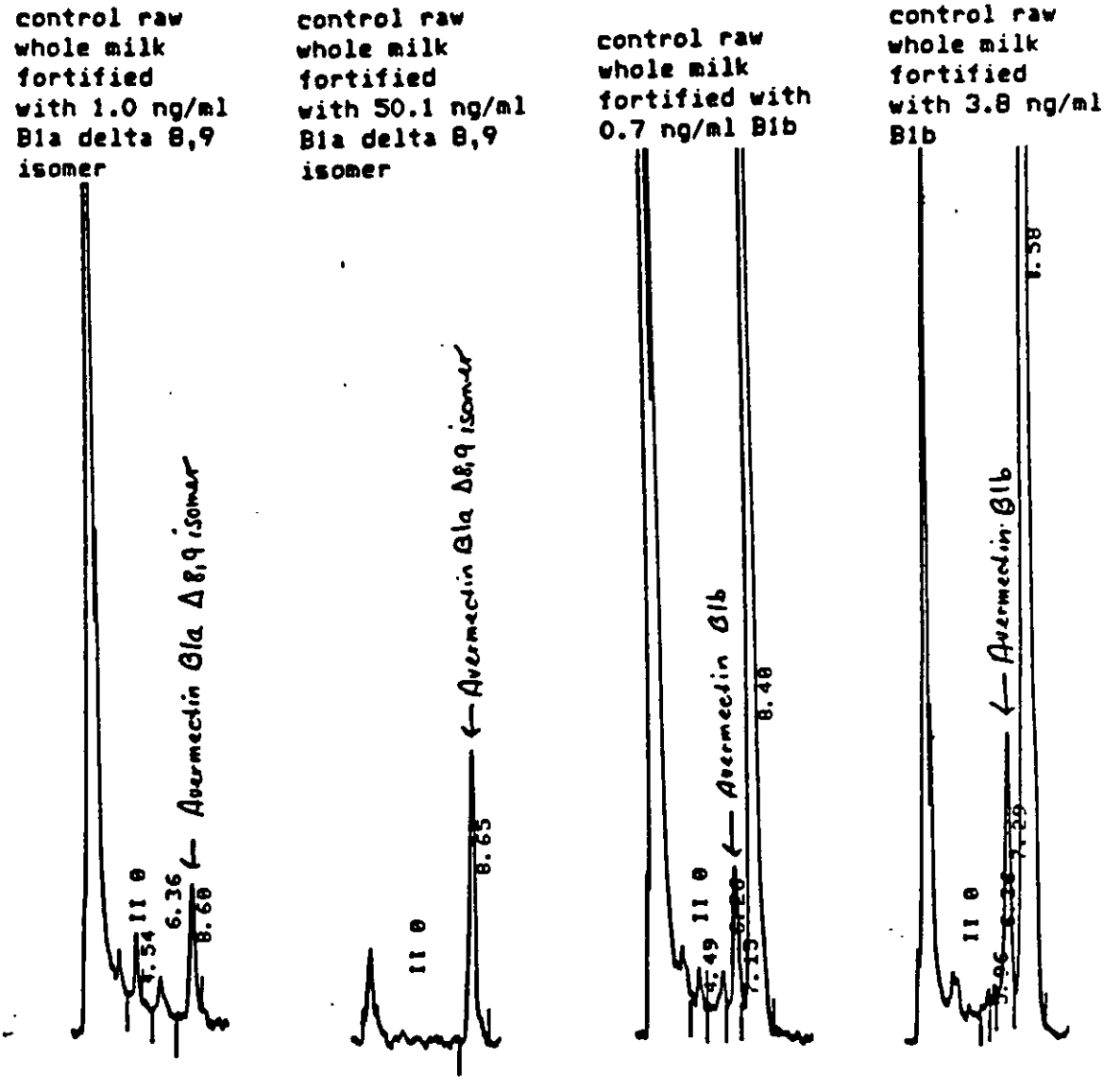


Figure 4.

Flow Diagram of Abamectin Tissue Assay

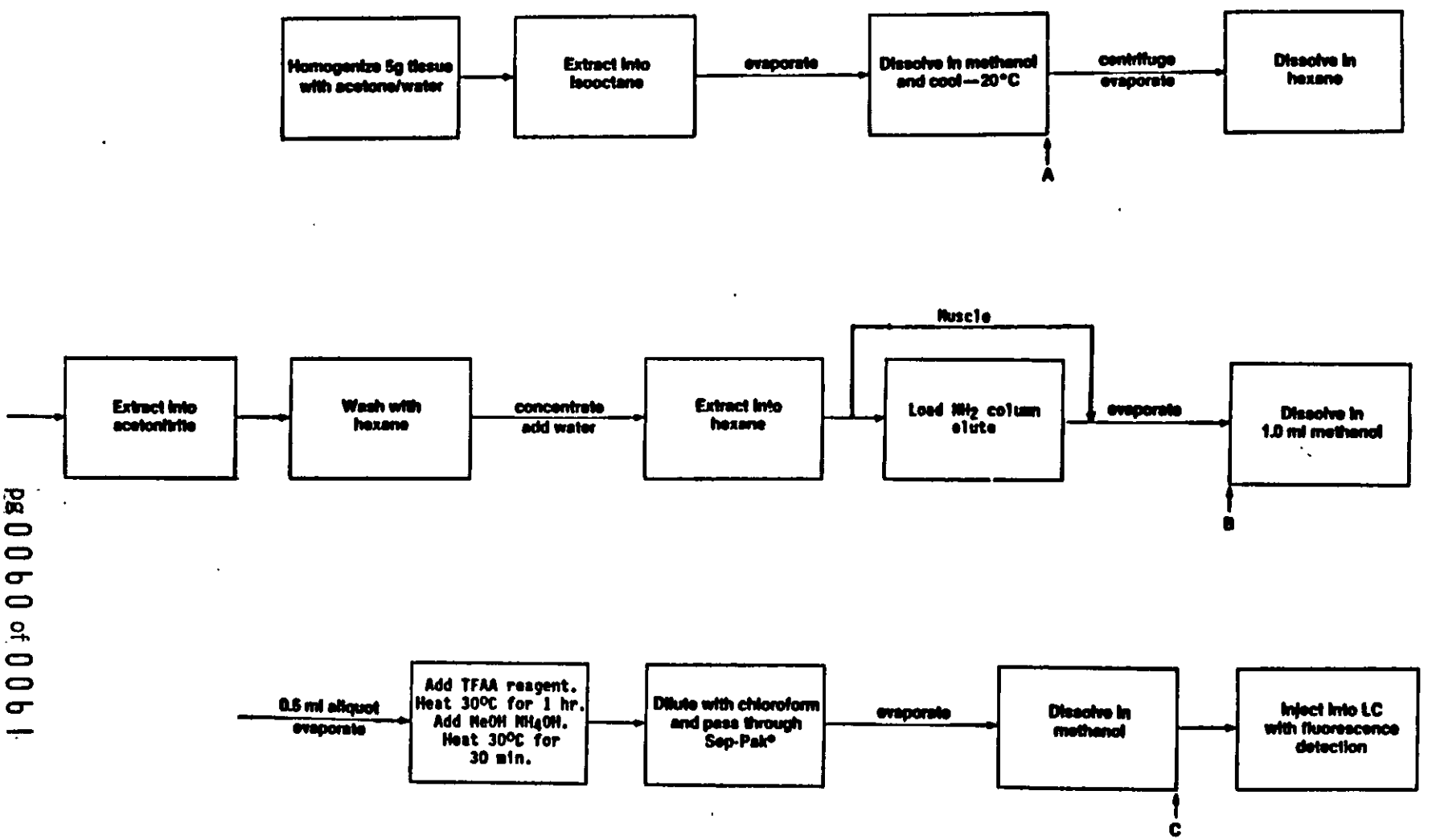


Figure 5.

Assay Timing

Day 1-

Grind 11 samples plus one control fortified with an appropriate level of standard. Complete isooctane extractions, blow downs, and dissolution in methanol. Place in freezer to cool. (Point A in Flow Diagram.)

Day 2-

Do the acetonitrile, hexane, water extractions, aminopropyl cleanup, and evaporations. Make derivative, SEP-PAK, and dissolve for LC. (Point C in Flow Diagram.)

Day 3-

Same as Day 1. Plus run LC of samples from Day 1 and calculate results.