US EPA ARCHIVE DOCUMENT

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Analytical Method for the Determination of GS-23199 in Crops by HPLC

Analytical Method No. AG-647

Project Number: 344004

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I. INTRODUCTION/SUMMARY

A. Scope

This method is a modification of REM 154.05[1], "Determination of Residues of Metabolite GS-23199 by Single Column High Performance Liquid Chromatography."

This method is used in the determination of residues of GS-23199, a metabolite of CGA-215944, in crops. This method is not intended for the setting or enforcement of tolerances, but for the gathering of data for dietary risk assessment. The structure and chemical name of the analyte is given in Figure 1.

The limit of detection (smallest standard amount injected during the chromatographic run) for GS-23199 is 1.0 ng. The limit of quantitation (the lowest fortification specified by the method which gives adequate recovery according to EPA guidelines) is 0.02 ppm for GS-23199.

B. Principle

A 5 gram sample of substrate is extracted by shaking with a solution of borax buffer (pH 9) in methanol for one hour. The extract is diluted to 50 mL (100 mL for dry samples) and a 10 mL aliquot is carried through the method.

The aliquot is evaporated to dryness by rotary evaporation using diethylene glycol diethyl ether as a keeper, and reconstituted in methanol/water. The extract solution is acidified and the analyte is partitioned into ethyl acetate, which is then evaporated to dryness by rotary evaporation using 95/5 decane/diethylene glycol diethyl ether as a keeper. The solution is evaporated to dryness and reconstituted in mobile phase 1. Final determination is by HPLC analysis with column switching. The preparatory column is a cyanopropyl column with a mobile phase of 90/5/5/1 (v/v/v/v) hexane/methanol/isopropanol/acetonitrile. The analytical column is an aminopropyl with a mobile phase of 70/20/10/1 (v/v/v/v) methanol/acetonitrile/isopropanol/hexane.

II. MATERIALS AND METHODS

A. Apparatus

1.0 Bottles, square amber wide mouth, 8-oz, with lids.

2.0 Column, HPLC, Zorbax CN, 7 µm, 250 mm x 4.6 mm (Phenomenex No. 00G-0089-EO).

3.0 Column, HPLC, Spherisorb 5 NH_2, 5 µm, 250 mm x 4.6 mm (Phenomenex No. 00G-0108-EO).

4.0 Cylinder, graduated, 50-mL, 100-mL (Fisher No. 08-554D, 08-554E) or equivalent.

5.0 Filter paper, Whatman #5, 7 cm (Fisher No. 09830K) or equivalent.

6.0 Flask, round-bottom, 50-mL, 100-mL (Fisher No. 10-067B, 10-067C) or equivalent.

7.0 Flask, side-arm, 250-mL (Fisher No. 10-180D) or equivalent.

8.0 Funnel, Buchner, Coors Porcelain w/fixed perforated plate, plate diameter 83 mm, height 143 mm, (Fisher No. 10-356C) or equivalent.

9.0 Funnel, with coarse fritted disc, 30-mL, (Fisher No. 10-358F) or equivalent.

10.0 Funnel, separatory, 60-mL (Fisher No. 10-437-2A) or equivalent.

11.0 pH paper, pH 0-14, (EM Scientific No. 9590) or equivalent.

12.0 Pipette, Pasteur, Flint Glass, 9 inch, disposable, (Fisher No. 13-678-7C) or equivalent.

13.0 Pipette, volumetric, 0.5, 1.0, 2.0, and 10.0-mL capacities (Fisher Nos. 13-650A, 13-650B, 13-650C, 13-650L) or equivalent.

14.0 Vials, 1.5-mL E-Z Auto Vial, (Wheaton Part No. 225175SP) or equivalent, with 11 mm Teflon-lined caps (Sunbrokers Part no. 200-152) or equivalent.

B. Reagents and Analytical Standards

All reagents are stored at room temperature. Analytical standards and stock solutions are stored frozen and standard solutions are refrigerated.

1.0 Acetonitrile, HPLC grade (Fisher No. A998-4) or equivalent.

2.0 Borax, Sigma, approx. 99% (Sigma No. B-9876) or equivalent.

3.0 0.05 M Borax - Dilute 19.1 g of Borax to 1000 mL with water.

4.0 Decane, Purum (Fluka No. 30550) or equivalent.

5.0 Diethylene glycol diethyl ether, Purum (Fluka No. 32190) or equivalent.

6.0 Ethyl Acetate, Optima (Fisher No. E196-4) or equivalent.

7.0 Hexane, HPLC Grade (Fisher No. H302-4) or equivalent.

8.0 O-Phosphoric Acid, 85% (Fisher No. A242-4) or equivalent.

9.0 Isopropanol, HPLC Grade (Fisher No. A464-4) or equivalent.

10.0 Methanol, HPLC grade (Fisher No. A452-4) or equivalent.

11.0 Mobile Phase 1 - 90/5/5/1 (v/v/v/v) hexane/methanol/isopropanol/acetonitrile

12.0 Mobile Phase 2 - 70/20/10/1 (v/v/v/v) methanol/acetonitrile/isopropanol/hexane.

13.0 Sodium Sulfate - Certified ACS grade (Fisher No. S421-3) or equivalent.

14.0 Water, deionized and purified in-house using a HYDRO™ purification system, or HPLC grade equivalent.

15.0 GS-23199 analytical standard, Ciba-Geigy Corporation, 410 Swing Road, Greensboro, NC.

C. Analytical Procedure

A flow diagram for the procedure is presented in Figure 2.

1.0 Preparation of sample

Prepare samples according to Ciba SOP 7.21. Store samples frozen at -20°C until just before analysis.

Weigh 5.0 g of plant material into an 8-oz. wide mouth jar. Weigh the samples in a semi-frozen state to avoid losing sample moisture. Allow the samples to thaw fully before extraction.

Prepare procedural fortifications at this point according to section II.J.2.0.

2.0 Extraction

General Procedure

2.1 Add 4 mL of 0.05 M sodium borate (pH 9) to the sample and mix carefully by shaking.

2.2 Add 36 mL of methanol to the sample.

Dry samples (e.g. tomato dry pomace, cured tobacco)

2.1 Add 8 mL of 0.05 M sodium borate (pH 9) to the sample and mix carefully by shaking.

2.2 Add 80 mL of methanol to the sample.

For all samples

2.3 Seal the wide mouth jar tightly and shake at approximately 250 oscillations per minute for 1 hour using a laboratory shaker.

2.4 Vacuum filter the extract using Whatman #5 filter paper. Wash the filter cake with 5 mL of methanol, collecting the washing.

2.5 Adjust the volume of the extract solution to 50.0 mL (100 mL for dry samples) using a volumetric flask.

3.0 GS-23199 Cleanup

3.1 Transfer into aqueous solution

Transfer a 10-mL aliquot of the extract solution into a 50-mL round-bottom flask and add 2 drops of diethylene glycol diethyl ether. Evaporate to aqueous by rotary evaporation (water bath ≤30°C.) Add 0.5 mL of methanol and sonicate the flask for 30 seconds. Add 6 mL of water.

3.2 Liquid/liquid partition

Transfer the solution to a 60-mL separatory funnel, rinsing the round-bottom flask with 1.5 mL of water. Adjust the pH of the solution to ~4.5 with 0.5 M phosphoric acid (about 0.05 to 0.15 mL are needed.) Partition the aqueous solution with 20 mL of ethyl acetate, shaking the funnel for 30 seconds. Place the aqueous phase back into the original 50-mL round-bottom flask and filter the ethyl acetate phase through ~10 g of anhydrous sodium sulfate in a fritted glass funnel into a 100 mL round-bottom flask. Repeat the partition with 2 portions of 15 mL of ethyl acetate, adding the ethyl acetate phase to the previous ethyl acetate solution through the sodium sulfate. Rinse the sodium sulfate with 5 mL of ethyl acetate and collect in the same flask. Add 2 drops of 95/5 (v/v) decane/diethylene glycol diethyl ether

and evaporate to dryness by rotary evaporation (water bath ≤30°C.)

3.3 Final solution

Reconstitute the residue from step 3.2 in 90/5/5/1 (v/v/v/v) hexane/methanol/isopropanol/ acetonitrile for HPLC analysis. Sonicate the solution for ~30 seconds while swirling the flask, being sure to contact all inside surfaces. Transfer to a vial for HPLC analysis.

D. Instrumentation

1.0 Description and Operating Conditions

See Table I for a description of the system components and chromatographic conditions.

2.0 Calibration and Standardization

The instrument is calibrated by construction of a calibration curve from detector response (chromatographic peak height or area and the amount of analyte standard injected), encompassing the range of 1.0 ng to 20 ng. The response curve can be constructed manually, with a calculator, or using a computer. Typical standardization data of analytical standards are presented in Table II and typical standard chromatograms and calibration plots are presented in Figures 3 and 4.

E. Interferences

There are no known interferences resulting from the chemical reagents used in the procedure.

F. Confirmatory Techniques

Analytical method REM 154.05[1] can be used to confirm residues of GS-23199.

G. Time Required for Analysis

1.0 Twelve samples can be taken through the extraction and cleanup procedure in twelve hours.

2.0 Samples can be analyzed overnight if an autosampler is available.

H. Modifications and Potential Problems

1.0 The same extract may be used for analysis for GS-23199 by this method and for CGA-215944 by Analytical Method AG-643[2].

2.0 GS-23199 has a tendency to adhere to glass after solvent evaporation. It is important to put the analyte into solution soon after evaporation.

3.0 Allow the phases to fully separate during the liquid/liquid partition. Place the aqueous phases from each sample into its 50-mL round-bottom flask, keeping the ethyl acetate in the separatory funnel. After the aqueous phases from all of the flasks have been removed, go back to the first separatory funnel. A small amount of water will have separated from the ethyl acetate. Add this to the 50-mL round-bottom and filter the ethyl acetate through the sodium sulfate.

I. Preparation of Standards

1.0 GS-23199 Stock Solution -

GS-23199 is not highly soluble in organic solvents. It is necessary to sonicate the stock solutions for some time in order to achieve complete dissolution.

Weigh 20 mg of GS-23199 into a 100-mL volumetric flask. Add a few milliliters of methanol to the flask and shake and sonicate to dissolve the GS-23199. Dilute to the mark with methanol. The concentration of this stock is 200 mg/mL. Store frozen and in the dark. Stock solutions expire six months after preparation.

2.0 Fortification Standards -

Prepare standards by serial dilution of the stock in methanol. Prepare standards of GS-23199 such that no more than 1 mL of the standard is required to fortify recovery samples. Fortification standards are stable for six months.

3.0 Analytical Standards -

Prepare standards by serial dilution of the stock in 90/5/5/1 (v/v/v/v) hexane/methanol/isopropanol/acetonitrile plus 1 mL of decane/diethylene glycol diethyl ether (95/5 v/v). Prepare at least four standards of GS-23199 in the range of 0.01 to 0.2 µg/mL.

J. Methods of Calculation

1.0 Determination of Sample Residues

Inject 100-µL aliquots of sample final fractions from Sections II.C.3.3 into the HPLC under the same conditions as for the standards. Make appropriate dilutions of the stock to bring the sample response within the range of the standard curve. Compare the response of the unknown samples to the standard curve or enter the value of the response into a least squares program to determine the nanograms of GS-23199 in the injected aliquot. Typical chromatograms for control and fortified control samples are presented in Figures 5 through 12.

To calculate the residue results, the milligrams injected must first be calculated as follows;

(1) mg sample injected =

V_f

mg = milligrams of sample used
V_i = injection volume (mL)
V_f = total volume of final injection solution (mL)

To determine ppm GS-23199 found in the samples use equation 2:

$$(2)\ \text{ppm analyte} = \frac{}{(\text{mg sample injected})\ (R)}$$

R = recovery factor expressed as a fraction (e.g. 100% = 1.0) and is calculated from equation 4.

To convert ppm GS-23199 to CGA-215944 equivalents, multiply the result from equation 2 by 1.89.

2.0 Fortification Experiments

This method is validated for each set of samples analyzed by including an untreated control sample and one or more control samples fortified immediately prior to extraction with GS-23199.

2.1 Add an appropriate amount of fortification standard (from section II.I.2.0) to a sample of control crop sample prior to extraction. The total volume of the added fortification solutions should not exceed 1 mL.

2.2 Proceed to step II.C.2.0. Typical recovery data for control and fortified control samples are given in Tables III.

2.3 Calculate the analyte concentration using equation 3:

$$(3)\ \text{ppm analyte found} = \frac{(\text{ng analyte})}{(\text{mg sample injected})}$$

Determine the recovery factor by first subtracting the background detector response, if any, in the control sample from the analyte response in the recovery sample; then calculate the recovery factor as a percentage (R) by the equation:

$$(4)\ R = \frac{\text{ppm analyte found}}{\text{ppm analyte added}}$$

III. RESULTS AND DISCUSSION

This method has been validated under Ciba-Geigy Residue Chemistry Protocol 628-95 and Amendment 1[3]. The objective of Protocol 628-95 was to validate Analytical Method AG-647 for the determination of GS-23199 in crops. Results from validation are reported herein. The limit of quantitation of AG-647 is 0.02 ppm. The limit of detection of AG-647 is 1.0 ng.

This method is not intended for the setting or enforcement of tolerances, but for the gathering of data for dietary risk assessment.

The average recovery of GS-23199 achieved in validation using the analytical method was 85% (sd=22%, CV=26%, n=56). The average recovery of GS-23199 achieved for substrates fortified at the limit of quantitation (0.02 ppm) was 89% (sd=27%, CV=30%, n=14).

The average recovery for each of the substrates analyzed was between the acceptable limits of 70-120% except for cured tobacco. The average recovery for cured tobacco was 63% with a standard deviation of 6.7%. Because this is not a regulatory method and the standard deviation for the set is low, these results were accepted.

No residues were detected in any of the control samples or reagent blanks.

Recovery results can be found in Table III. Chromatograms from the analysis of control and fortified control samples are presented in Figures 5 through 12.

At the time that this validation was performed, samples containing radiolabeled (^{14}C) GS-23199 were not available. When samples which have been treated with ^{14}C-CGA-215944 become available, they will be analyzed according to this method and the results report in a separate final report.

Sample identification and test and reference substance information can be found in Residue Test Report RI-MV-016-95.[4] The raw data, Residue Test Report, and final report are stored in the Ciba Agricultural Group Archives in Greensboro, NC. No samples are archived.

There were no deviations from the protocol, nor any circumstances affecting the quality or integrity of the data.

IV. CONCLUSION

Analytical Method AG-647 is a valid and accurate method for the determination GS-23199 in crops.

V. CERTIFICATION

The report and experimental results included in this study, laboratory Project I. D. AG-647 are certified to be authentic accounts of the experiments.

2/20/96
Date

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CERTIFICATION OF GOOD LABORATORY PRACTICES

The analytical work reported in AG-647 was performed in accordance with Good Laboratory Practice Standards, 40 CFR Part 160.

2/20/96
Date

Timothy Joseph
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2-20-96
Date

W. T. Beidler
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VI. QUALITY ASSURANCE STATEMENT

Ciba-Geigy Corporation
Ciba Crop Protection
Quality Assurance Unit

QUALITY ASSURANCE STATEMENT

Report Title: Analytical Method for the Determination of GS-23199 in Cucurbits, Tobacco, and Fruiting Vegetables, Including Tomato Processed Fractions, by HPLC

Study Director: T. A. Joseph, Ciba

Study Coordinator: T. G. Thorn, Ciba

Ciba Study No.: Protocol 628-95 with Amendment(s)

Project No.: 344004

Pursuant to Good Laboratory Practice Standards, this statement verifies that this study was inspected and/or audited and the findings reported to the study director and management by the Ciba Crop Protection Quality Assurance Unit on the dates listed below.

INSPECTION/AUDIT TYPE	INSPECTION/AUDIT DATES	REPORTING DATES
Protocol Audit	11/3/95	11/3/95
In-Progress Inspection	12/6/95	12/6/95
Final Report Audit	1/12, 15-16/96	1/17/96

Prepared by: Roger A. Edwards Date: 1/17/96
Roger A. Edwards
Senior Quality Assurance Auditor

VII. TABLES AND FIGURES

TABLE I. HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC CONDITIONS

Instrumentation:

Pump 1	- Applied Biosystems LC-250
Pump 2	- Applied Biosystems LC-250
Autoinjector	- Alcott 738A
Detector	- Applied Biosystems 785A
Column 1	- Zorbax CN 7 µm, 250 mm x 4.6 mm
Column 2	- Spherisorb 5 NH_2 5 µm, 250 mm x 4.6 mm

Conditions:

Mobile Phase 1	-	90/5/5/1 (v/v/v/v) hexane/methanol/isopropanol/acetonitrile
Mobile Phase 2	-	70/20/10/1 (v/v/v/v) methanol/acetonitrile/isopropanol/hexane
Flow Rate	-	1 mL/min.
Injection Volume	-	100 µL
Detection	-	260 nm
Approximate cut time	-	9.9 - 10.9 min.
Approximate retention time	-	32 min.

TABLE II. TYPICAL STANDARDIZATION DATA FOR GS-23199

ng of GS-23199 injected	Peak Area
1.0	6619
1.0	4576
5.0	26938
10.0	56185
10.0	56633
20.0	113899

Correlation Coefficient	0.99978
Slope	5707.9
Intercept	-570.2

Data is from the analysis of green tobacco.

TABLE III. RECOVERY RESULTS FOR GS-23199 FORTIFIED IN CONTROL SUBSTRATES

Sample Code	Substrate	Fortification Level (ppm)	% Recovery GS-23199
CC.CA	Cucumbers	Control	<0.02 ppm
CC.CB	Cucumbers	Control	<0.02 ppm
CC.02A	Cucumbers	0.02	96
CC.02B	Cucumbers	0.02	74
CC.05A	Cucumbers	0.05	80
CC.05B	Cucumbers	0.05	66
CC.10A	Cucumbers	0.10	75
CC.10B	Cucumbers	0.10	75
CC.20A	Cucumbers	0.20	72
CC.20B	Cucumbers	0.20	80
Average Recovery			77
Standard Deviation			8.9
CN.C	Cantaloupes	Control	<0.02 ppm
CN.02A	Cantaloupes	0.02	[a]
CN.02B	Cantaloupes	0.02	171
CN.05A	Cantaloupes	0.05	118
CN.05B	Cantaloupes	0.05	101
CN.10A	Cantaloupes	0.10	107
CN.10B	Cantaloupes	0.10	100
CN.20A	Cantaloupes	0.20	103
CN.RB	Cantaloupes	reagent blank	<0.02 ppm
Average Recovery			117
Standard Deviation			28
TM.CA	Tomatoes	Control	<0.02 ppm
TM.CB	Tomatoes	Control	<0.02 ppm
TM.02A	Tomatoes	0.02	94
TM.02B	Tomatoes	0.02	95
TM.05A	Tomatoes	0.05	119
TM.05B	Tomatoes	0.05	134
TM.10A	Tomatoes	0.10	84
TM.10B	Tomatoes	0.10	121
TM.20A	Tomatoes	0.20	129
TM.20B	Tomatoes	0.20	109
Average Recovery			111
Standard Deviation			18
TW.CA	Tomato wet pomace	Control	<0.02 ppm
TW.02A	Tomato wet pomace	0.02	[b]
TW.02B	Tomato wet pomace	0.02	92
TW.05A	Tomato wet pomace	0.05	68
TW.05B	Tomato wet pomace	0.05	79
TW.10A	Tomato wet pomace	0.10	85
TW.10B	Tomato wet pomace	0.10	65
TW.20A	Tomato wet pomace	0.20	68
Average Recovery			76
Standard Deviation			11

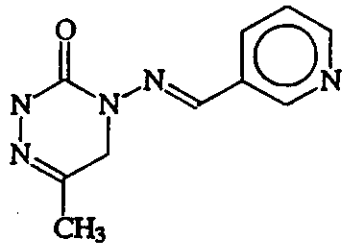
[a] Due to reinjections, not enough remained for analysis.
[b] An aqueous layer appeared in the final fraction, biasing the result.

TABLE III. RECOVERY RESULTS FOR GS-23199 FORTIFIED IN CONTROL SUBSTRATES (Continued)

Sample Code	Substrate	Fortification Level (ppm)	% Recovery GS-23199
TD.CA	Tomato dry pomace	Control	<0.02 ppm
TD.02A	Tomato dry pomace	0.02	93
TD.02B	Tomato dry pomace	0.02	80
TD.05A	Tomato dry pomace	0.05	85
TD.05B	Tomato dry pomace	0.05	[c]
TD.10A	Tomato dry pomace	0.10	77
TD.10B	Tomato dry pomace	0.10	81
TD.20A	Tomato dry pomace	0.20	79
Average Recovery			82
Standard Deviation			6.0
PP.CA	Peppers	Control	<0.02 ppm
PP.02A	Peppers	0.02	92
PP.02B	Peppers	0.02	89
PP.05A	Peppers	0.05	84
PP.05B	Peppers	0.05	88
PP.10A	Peppers	0.10	81
PP.10B	Peppers	0.10	84
PP.20A	Peppers	0.20	74
Average Recovery			84
Standard Deviation			5.9
TG.CA	Tobacco, green	Control	<0.02 ppm
TG.02A	Tobacco, green	0.02	72
TG.02B	Tobacco, green	0.02	63
TG.05A	Tobacco, green	0.05	71
TG.05B	Tobacco, green	0.05	84
TG.10A	Tobacco, green	0.10	62
TG.10B	Tobacco, green	0.10	74
TG.20A	Tobacco, green	0.20	72
TG.RB	Tobacco, green	Reagent Blank	<0.02 ppm
Average Recovery			71
Standard Deviation			7.4
TC.CA	Tobacco, cured	Control	<0.02 ppm
TC.CB	Tobacco, cured	Control	<0.02 ppm
TC.02A	Tobacco, cured	0.02	72
TC.02B	Tobacco, cured	0.02	61
TC.05A	Tobacco, cured	0.05	60
TC.05B	Tobacco, cured	0.05	65
TC.10A	Tobacco, cured	0.10	59
TC.10B	Tobacco, cured	0.10	72
TC.20A	Tobacco, cured	0.20	52
TC.20B	Tobacco, cured	0.20	62
TG.RB	Tobacco, cured	Reagent Blank	<0.02 ppm
Average Recovery			63
Standard Deviation			6.7

[c] Due to reinjections, not enough remained for analysis..

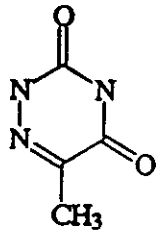
FIGURE 1. CHEMICAL NAMES AND STRUCTURES



CGA-215944

4,5-Dihydro-6-methyl-4-[(3-pyridinylmethylene)-amino]-1,2,4-triazin-3(2H)-one

CAS # 123312-89-0



GS-23199

6-Methyl-1,2,4-triazine-3,5(2H,4H)-dione

FIGURE 2. FLOW DIAGRAM FOR THE DETERMINATION OF GS-23199 IN CROPS

Extraction

5-gram sample

↓

General Procedure	Dry Crops
↓	↓
Add 4 mL of Borax + 36 mL of methanol	Add 8 mL of Borax + 80 mL of methanol
↓	↓
Shake for 1 hour	Shake for 1 hour
↓	↓
Filter	Filter
↓	↓
Wash filter cake with 5 mL of methanol	Wash filter cake with 5 mL of methanol
↓	↓
Adjust volume to 50 mL with methanol	Adjust volume to 100 mL with methanol
↓	↓

↓

Take a 10-mL aliquot and add 1 drop of diethylene glycol diethyl ether (DGDE)

↓

Evaporate to dryness

↓

Reconstitute in 0.5 mL of methanol, sonicate for 30 seconds

↓

Add 6 mL of water

↓

Transfer to 60-mL separatory funnel with 1.5 mL water rinse

↓

Adjust pH to 4.5 with 0.5 M phosphoric acid

↓

Partition with 1 x 20 mL + 2 x 15 mL of ethyl acetate, discard aqueous

↓

Filter partitions through 10 g of sodium sulfate

↓

Wash sodium sulfate with 5 mL of ethyl acetate

↓

Add 2 drop of 95/5 (v/v) decane/DGDE and evaporate to dryness

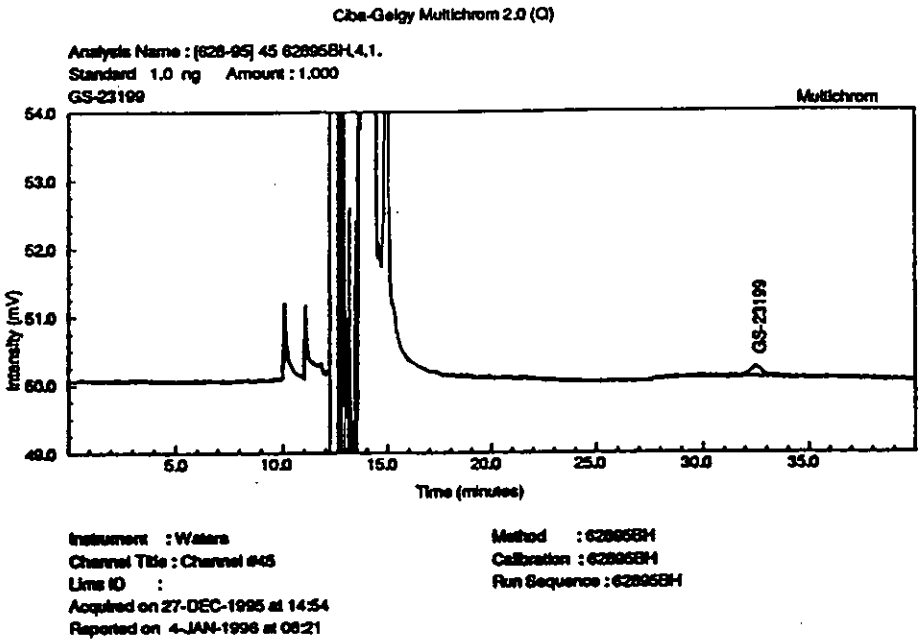
↓

Reconstitute in 90/5/5/1 (v/v/v/v) hexane/methanol/isopropanol/acetonitrile

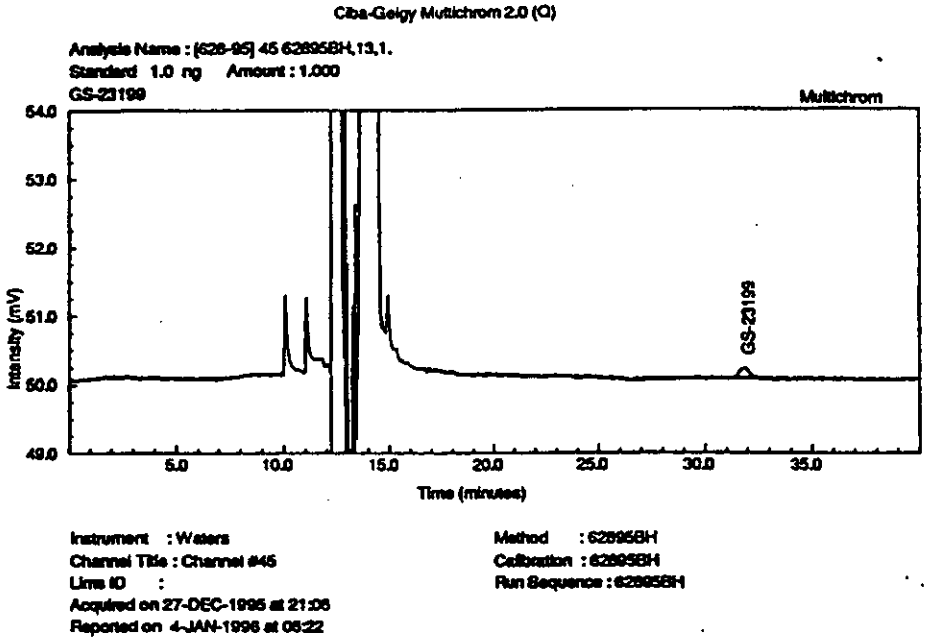
↓

Analyze by HPLC

FIGURE 3. TYPICAL CHROMATOGRAMS OF GS-23199 STANDARD SOLUTIONS

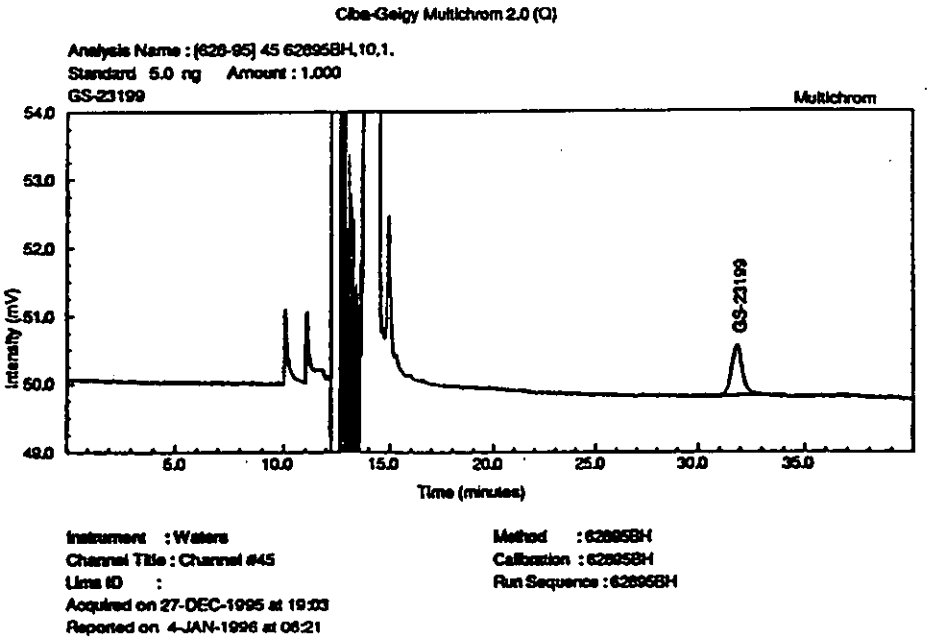


1. 0.01 ng/µL, 100 µL injected

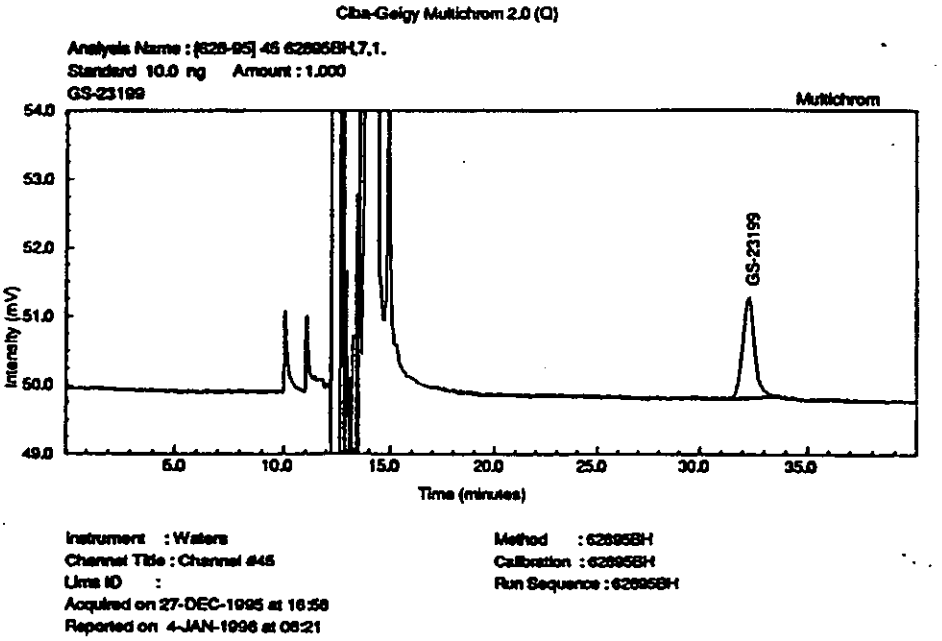


2. 0.01 ng/µL, 100 µL injected

FIGURE 3. TYPICAL CHROMATOGRAMS OF GS-23199 STANDARD SOLUTIONS (Continued)

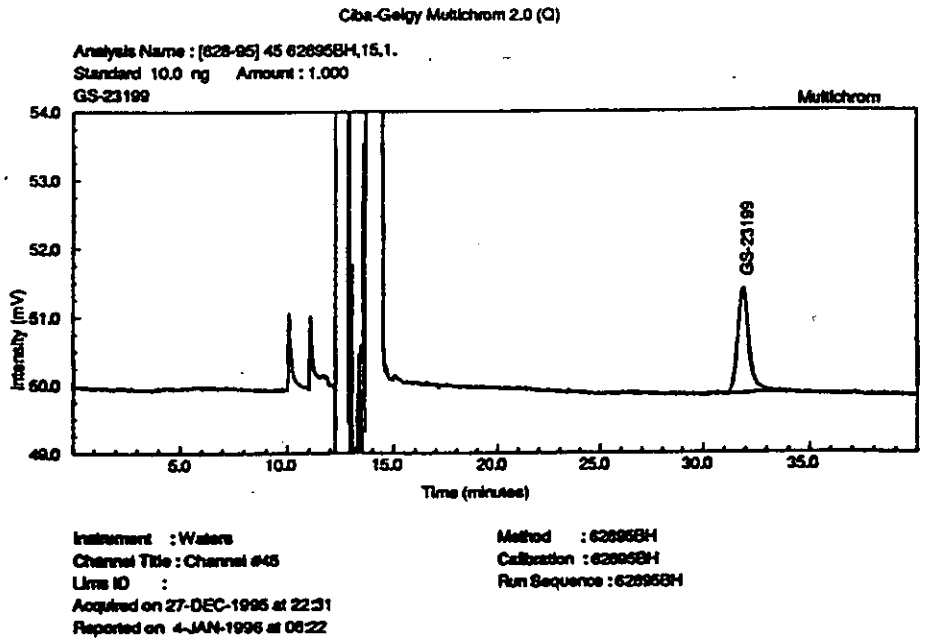


3. 0.05 ng/µL, 100 µL injected

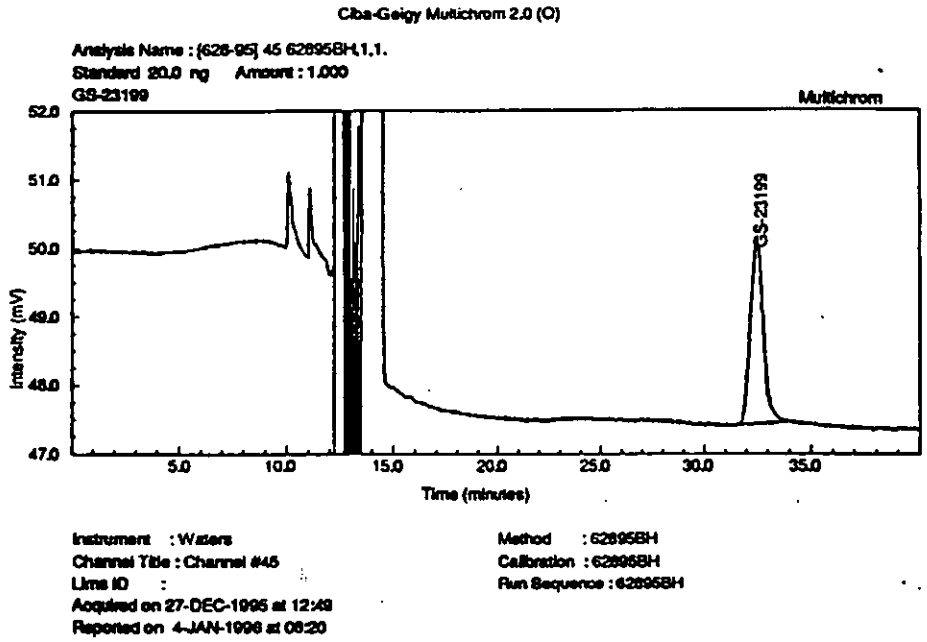


4. 0.1 ng/µL, 100 µL injected

FIGURE 3. TYPICAL CHROMATOGRAMS OF GS-23199 STANDARD SOLUTIONS (Continued)

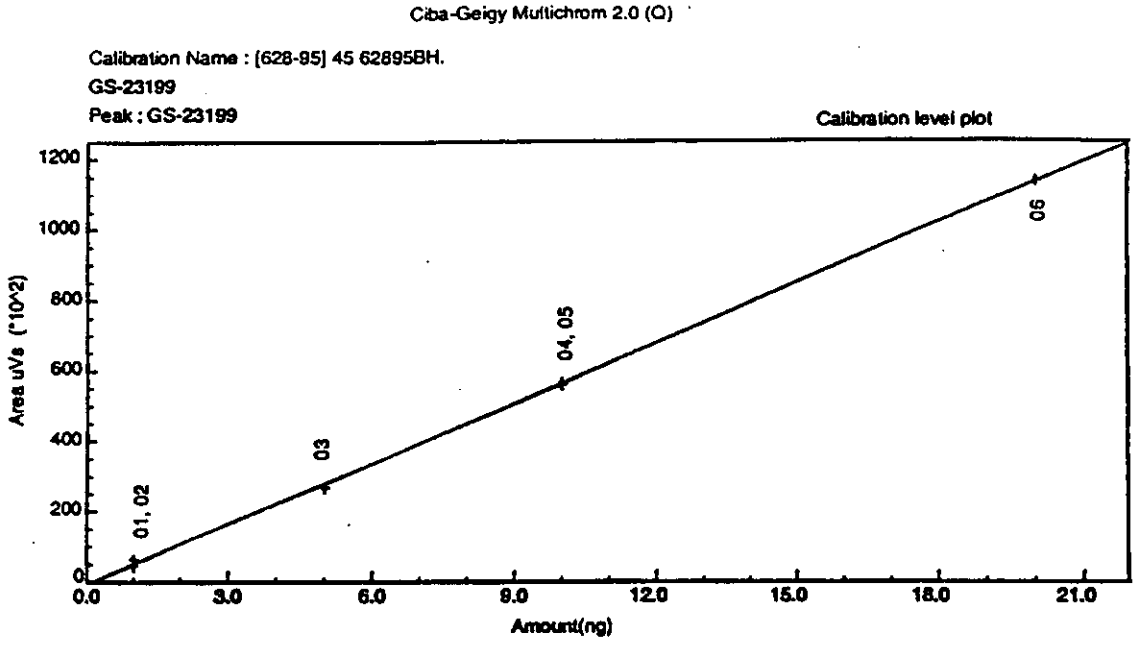


5. 0.1 ng/µL, 100 µL injected



6. 0.2 ng/µL, 100 µL injected
Chromatograms from the analysis of green tobacco

FIGURE 4. TYPICAL CALIBRATION PLOT OF GS-23199 STANDARD SOLUTIONS



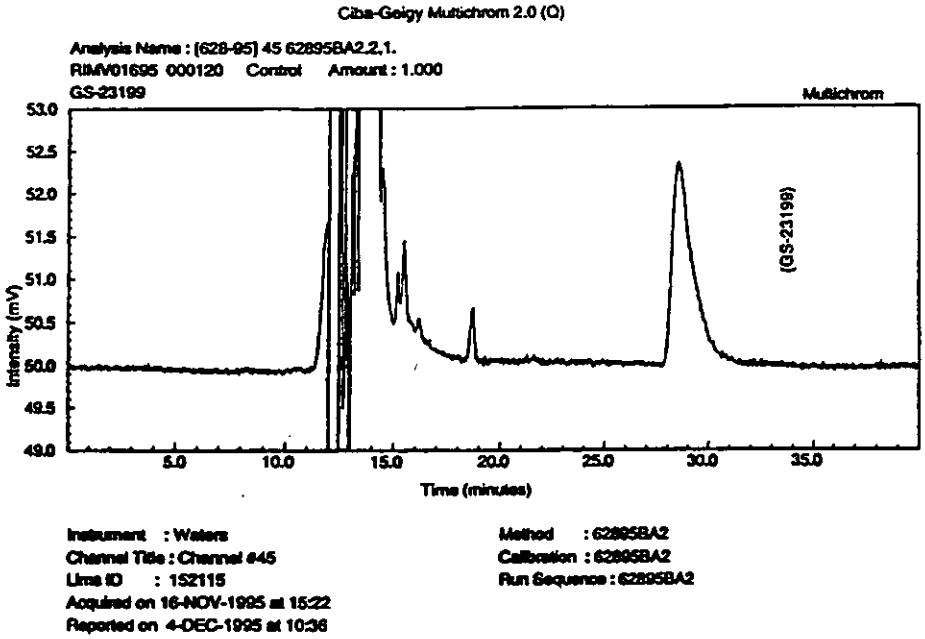
Constant : -5.702146E+2
1st degree : 5.70789E+3

Curve fit : Linear
Correlation coefficient : 0.99978
Standard error : 9.73335E+2

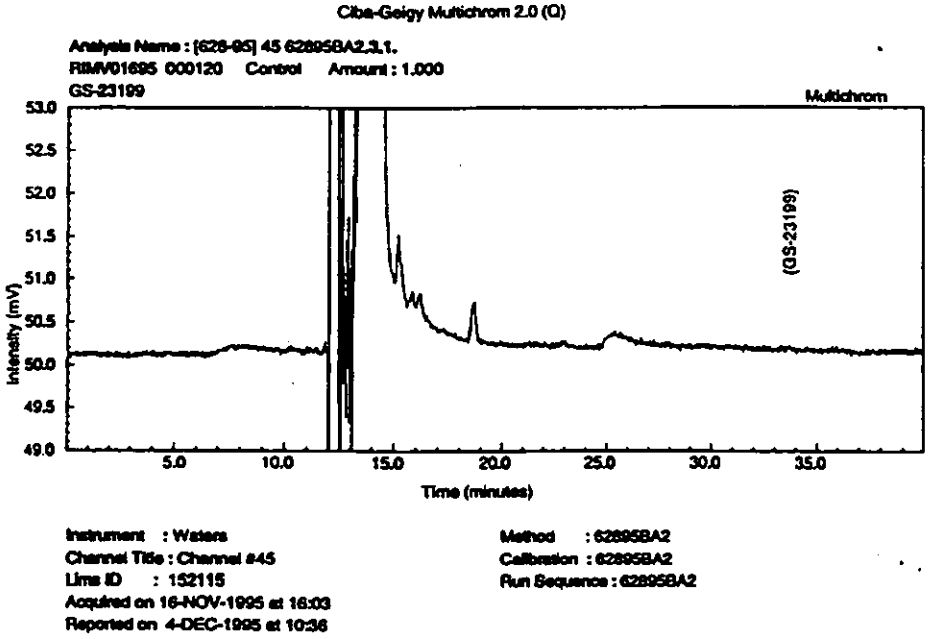
Reported on 3-JAN-1996 at 15:56

Curve from the analysis of green tobacco

FIGURE 5. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CUCUMBERS FOR GS-23199

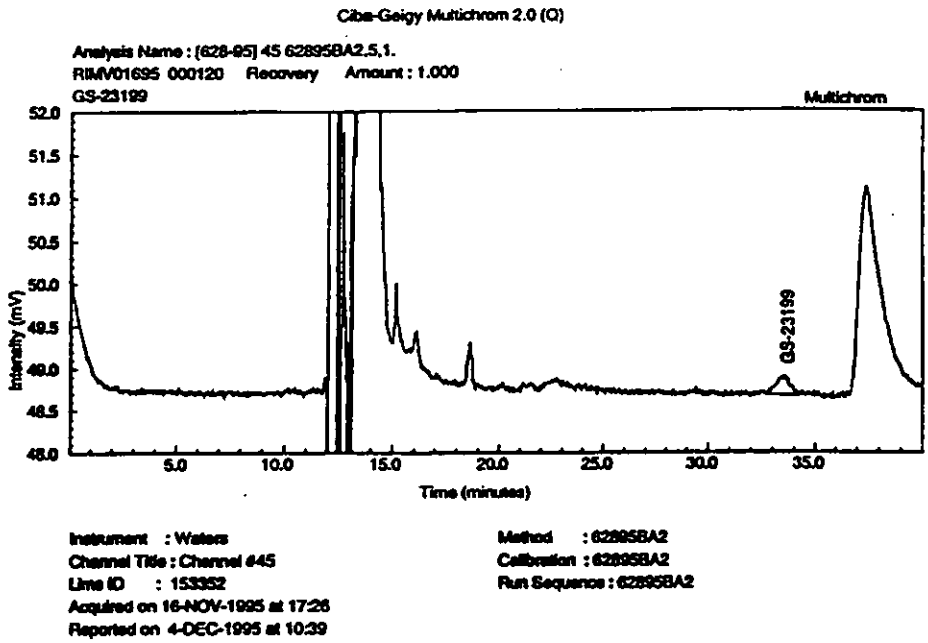


1. CC.CA, control, 100 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

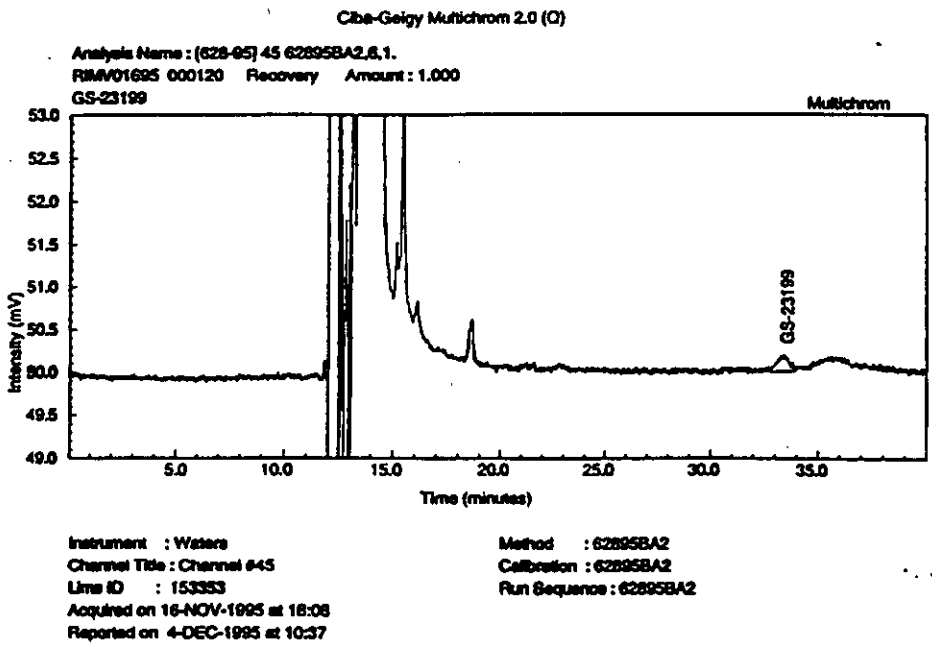


2. CC.CB, control, 100 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

FIGURE 5. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CUCUMBERS FOR GS-23199 (Continued)

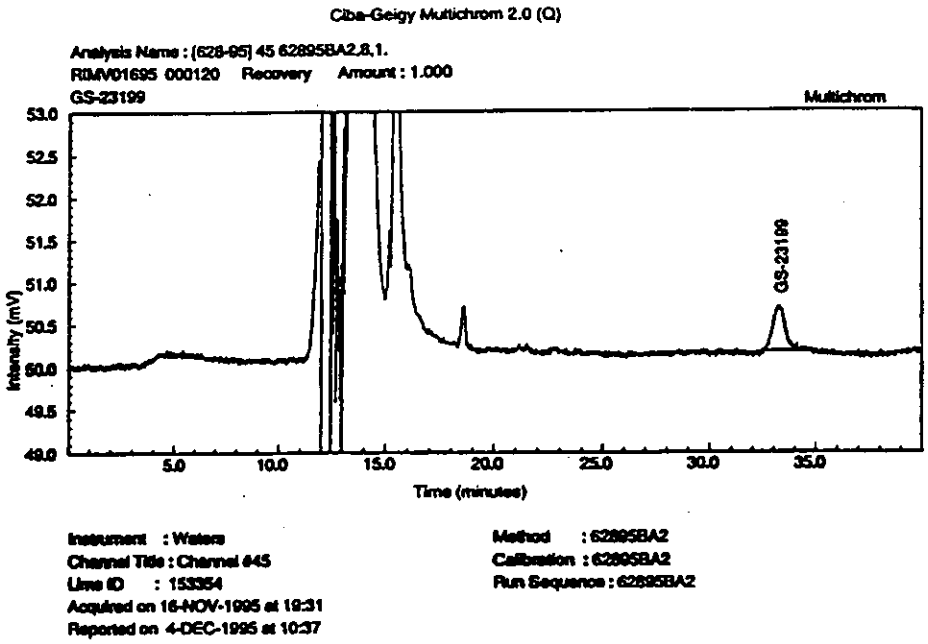


3. CC.02A, 0.02 ppm fortification, 100 mg injected, 1.92 ng GS-23199 found, 0.019 ppm GS-23199 found, 96% recovery

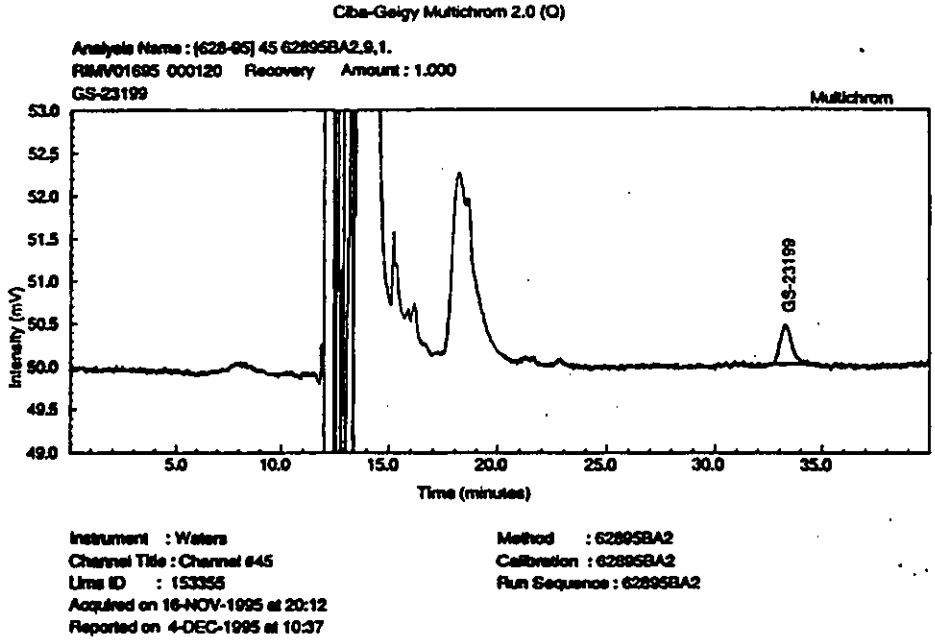


4. CC.02B, 0.02 ppm fortification, 100 mg injected, 1.47 ng GS-23199 found, 0.015 ppm GS-23199 found, 74% recovery

FIGURE 5. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CUCUMBERS FOR GS-23199 (Continued)

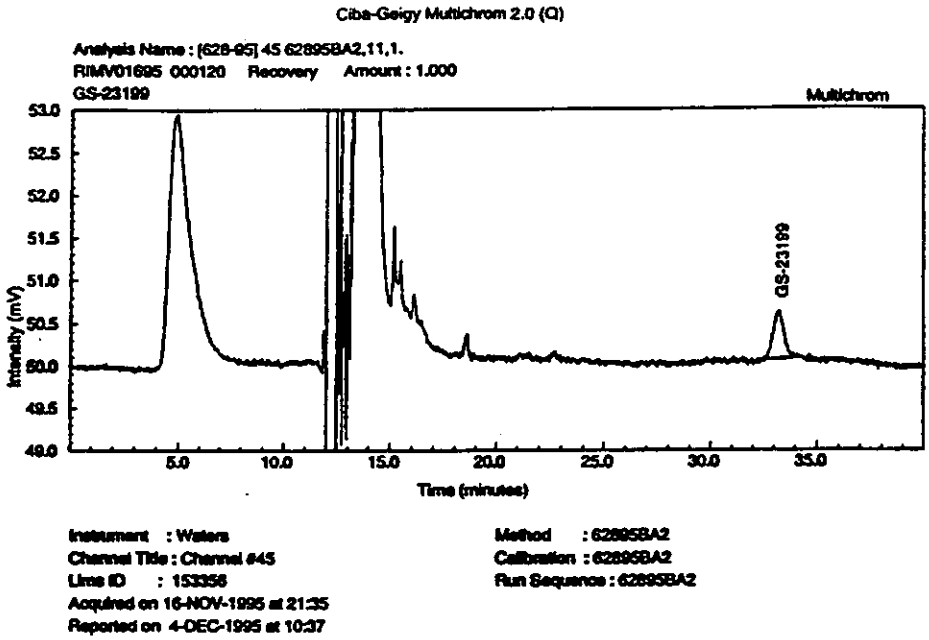


5. CC.05A, 0.05 ppm fortification, 99 mg injected, 3.99 ng GS-23199 found, 0.040 ppm GS-23199 found, 80% recovery

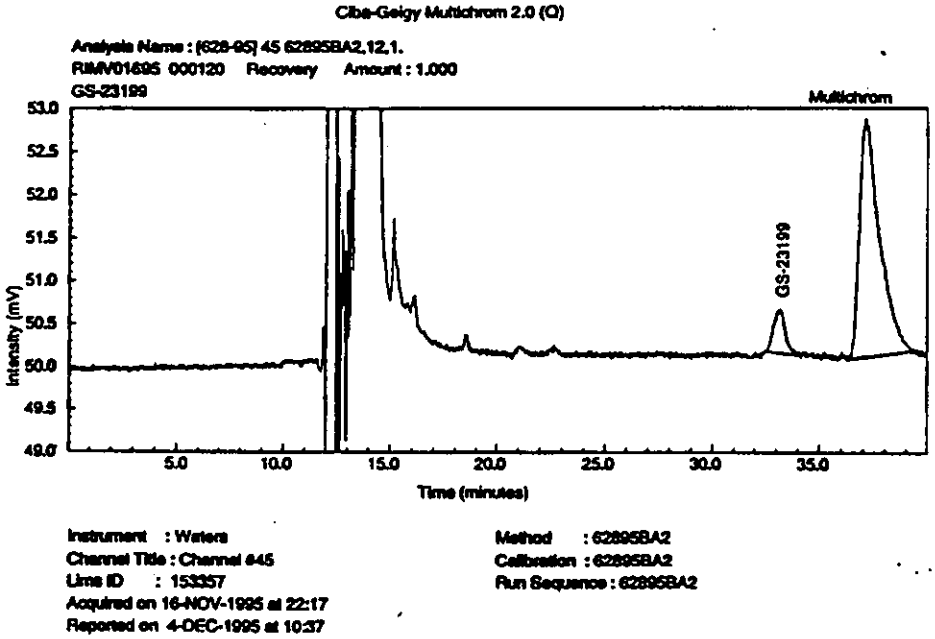


6. CC.05B, 0.05 ppm fortification, 101 mg injected, 3.30 ng GS-23199 found, 0.033 ppm GS-23199 found, 66% recovery

FIGURE 5. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CUCUMBERS FOR GS-23199 (Continued)

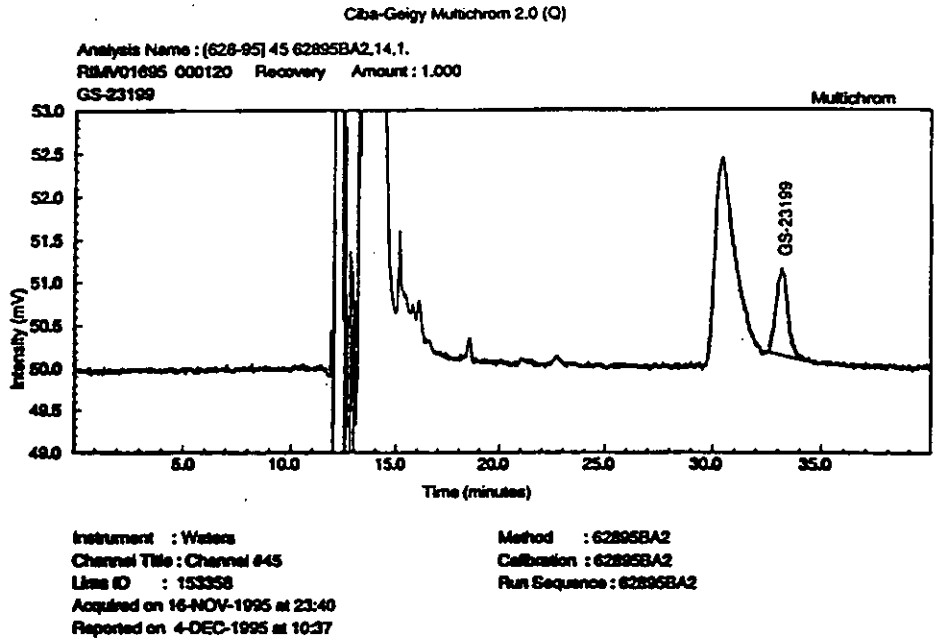


7. CC.10A, 0.10 ppm fortification, 50 mg injected, 3.72 ng GS-23199 found, 0.075 ppm GS-23199 found, 75% recovery

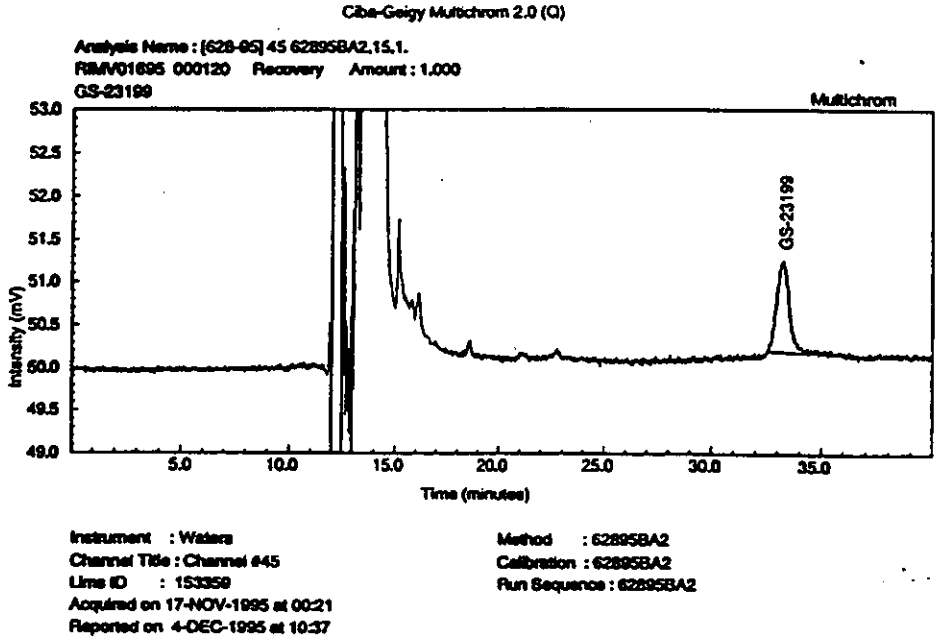


8. CC.10B, 0.10 ppm fortification, 50 mg injected, 3.76 ng GS-23199 found, 0.075 ppm GS-23199 found, 75% recovery

FIGURE 5. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CUCUMBERS FOR GS-23199 (Continued)

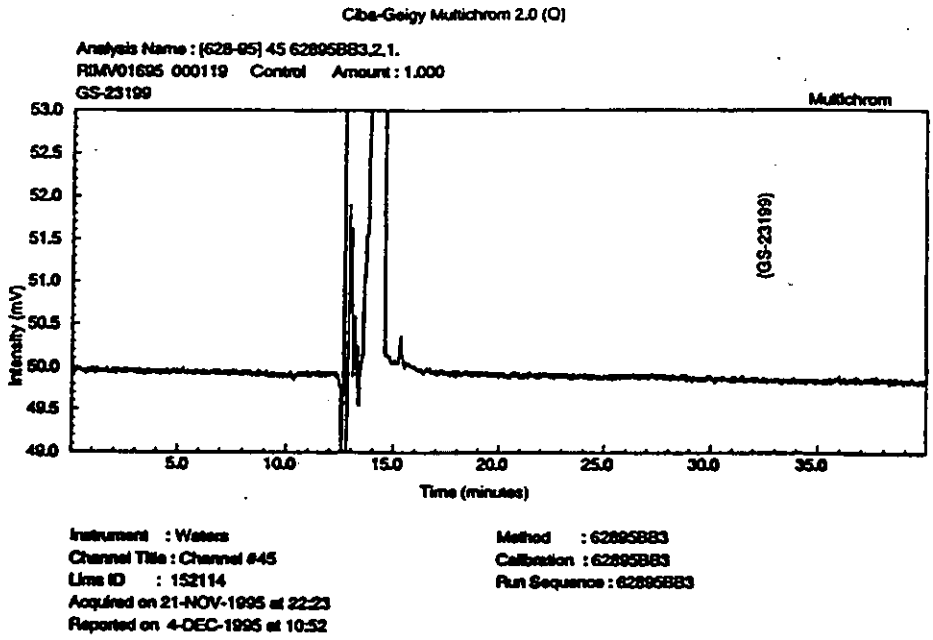


9. CC.20A, 0.20 ppm fortification, 50 mg injected, 7.26 ng GS-23199 found, 0.14 ppm GS-23199 found, 72% recovery

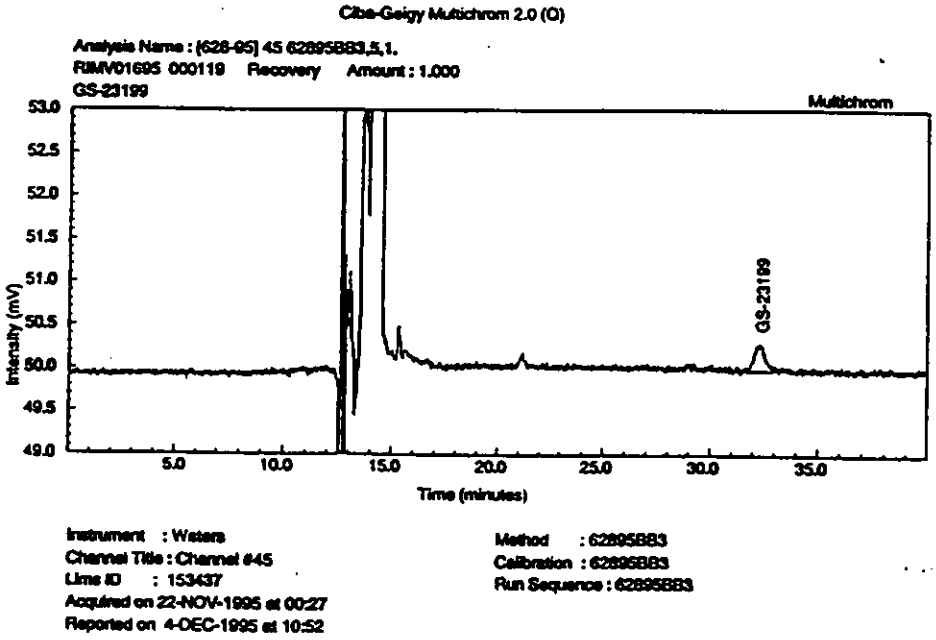


10. CC.20B, 0.20 ppm fortification, 50 mg injected, 8.06 ng GS-23199 found, 0.16 ppm GS-23199 found, 80% recovery

FIGURE 6. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CANTALOUPES FOR GS-23199

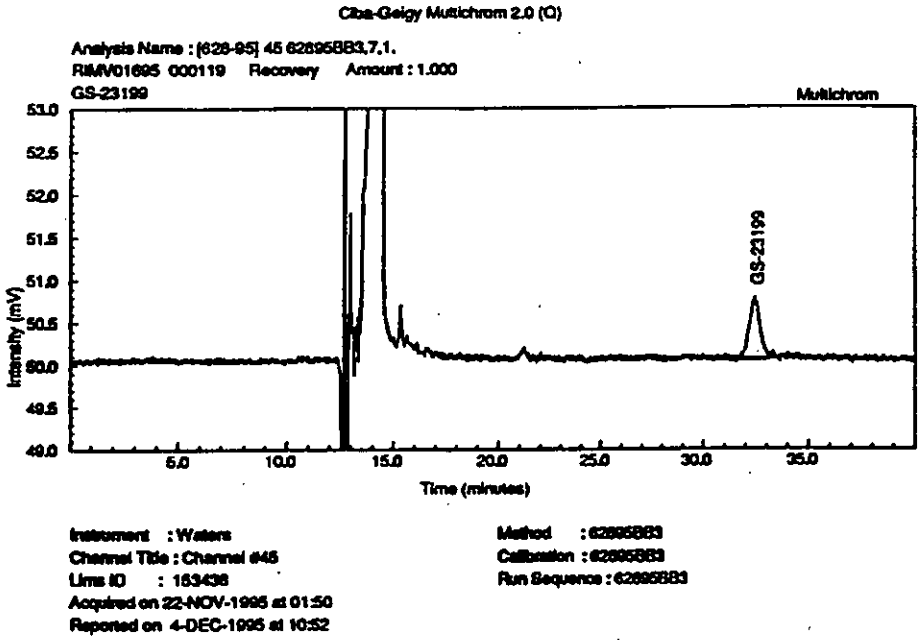


1. CN.C, control, 100 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

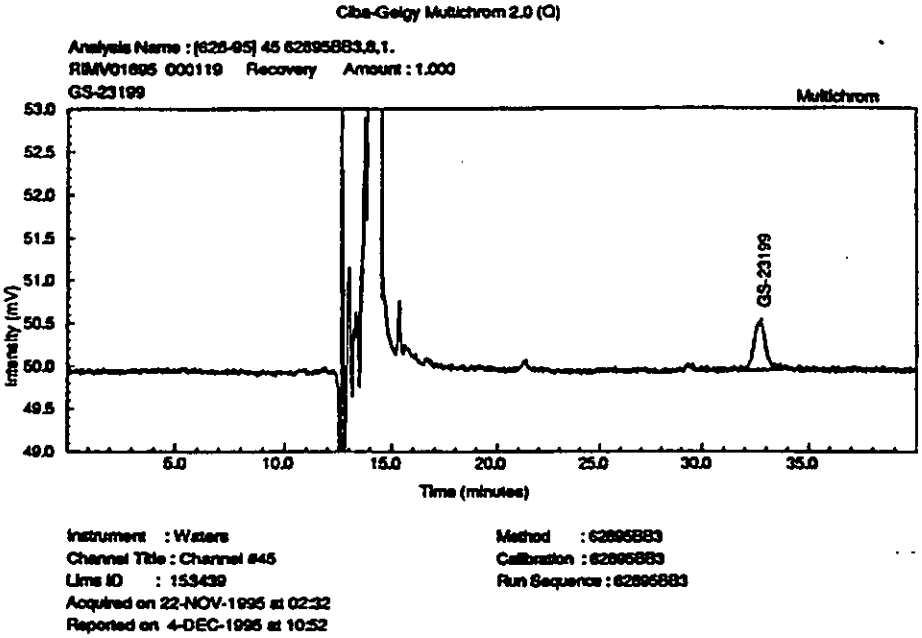


2. CN.02B, 0.02 ppm fortification, 101 mg injected, 3.45 ng GS-23199 found, 0.034 ppm GS-23199 found, 171% recovery

FIGURE 6. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CANTALOUPES FOR GS-23199 (Continued)

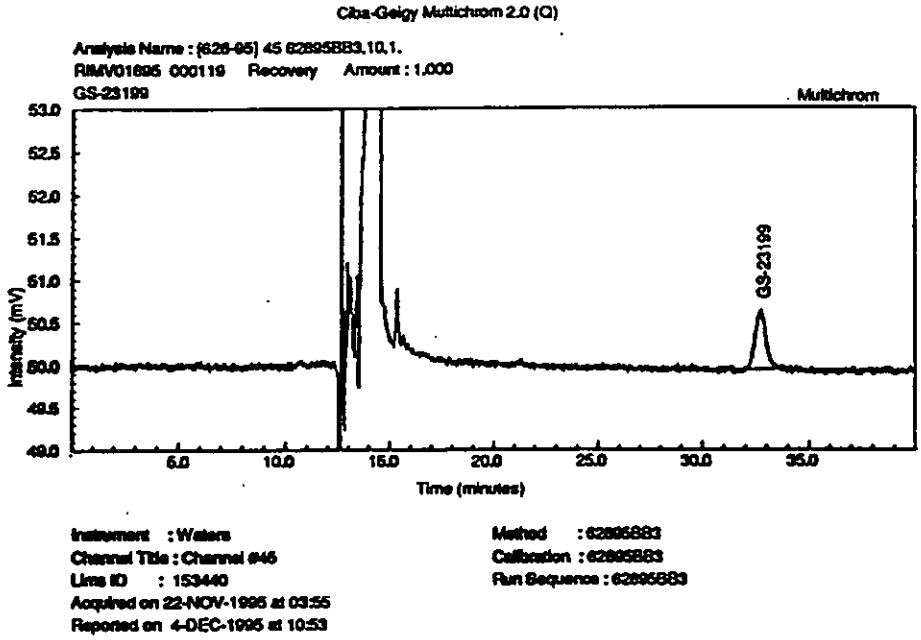


3. CN.05A, 0.05 ppm fortification, 100 mg injected, 5.90 ng GS-23199 found, 0.059 ppm GS-23199 found, 118% recovery

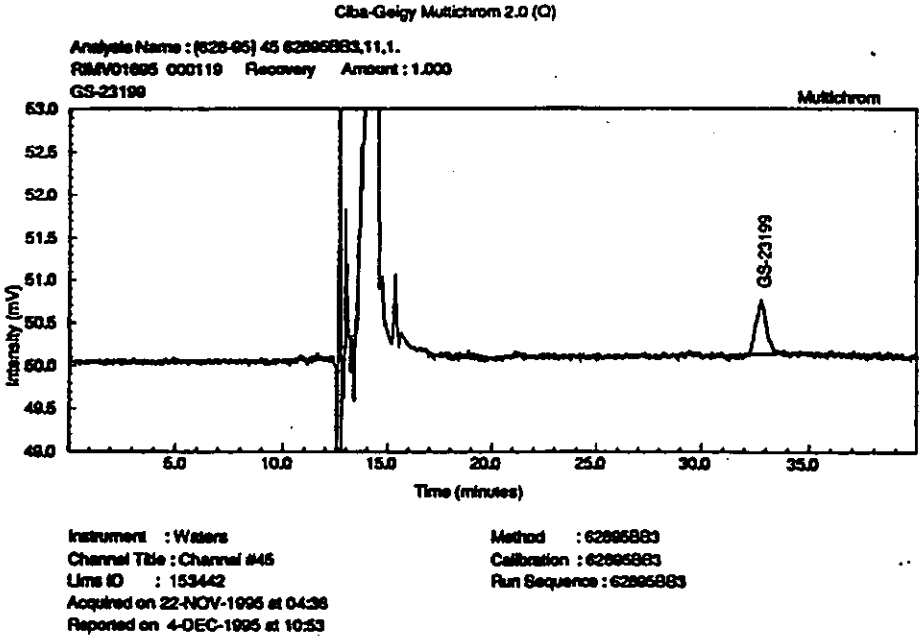


4. CN.05B, 0.05 ppm fortification, 100 mg injected, 5.02 ng GS-23199 found, 0.050 ppm GS-23199 found, 101% recovery

FIGURE 6. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CANTALOUPES FOR GS-23199 (Continued)

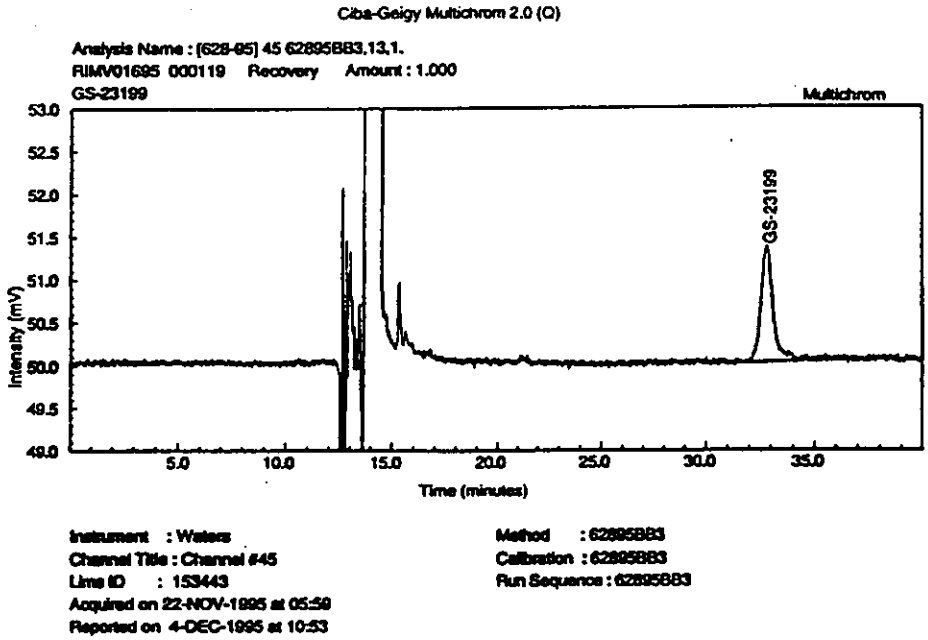


5. CN.10A, 0.10 ppm fortification, 50 mg injected, 5.38 ng GS-23199 found, 0.11 ppm GS-23199 found, 107% recovery

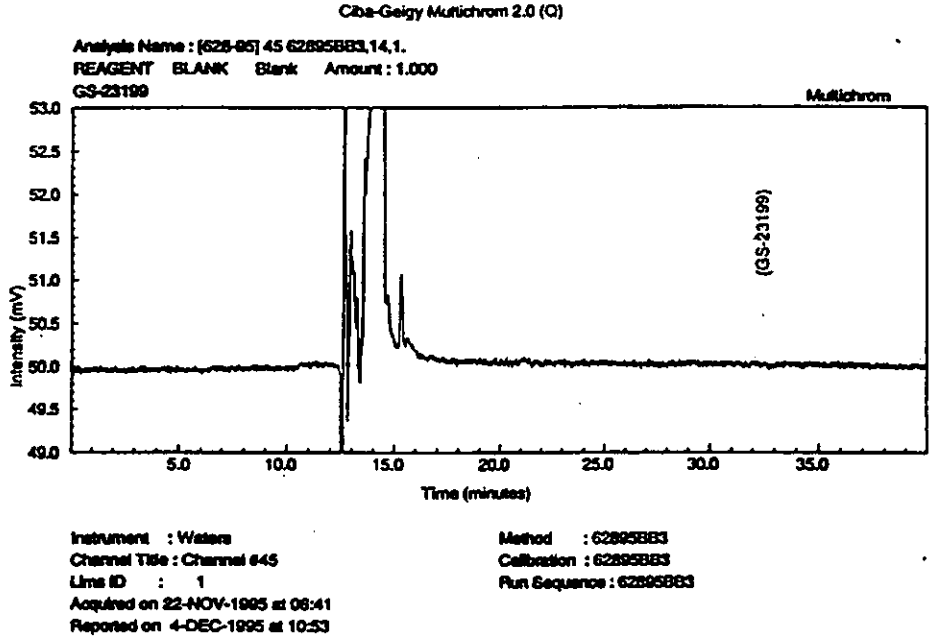


6. CN.10B, 0.10 ppm fortification, 50 mg injected, 4.97 ng GS-23199 found, 0.10 ppm GS-23199 found, 100% recovery

FIGURE 6. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CANTALOUPES FOR GS-23199 (Continued)

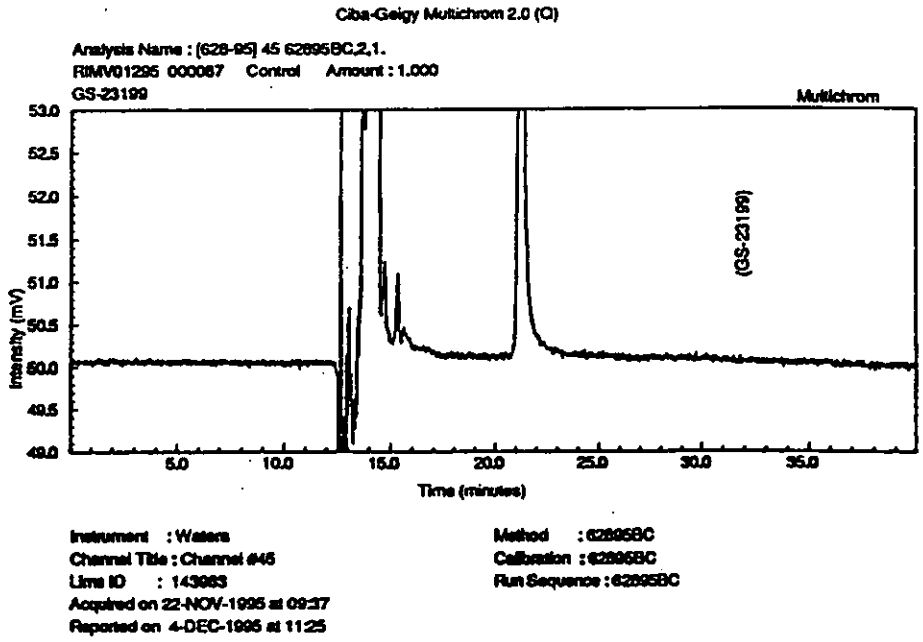


7. CN.20A, 0.20 ppm fortification, 50 mg injected, 10.3 ng GS-23199 found, 0.21 ppm GS-23199 found, 103% recovery

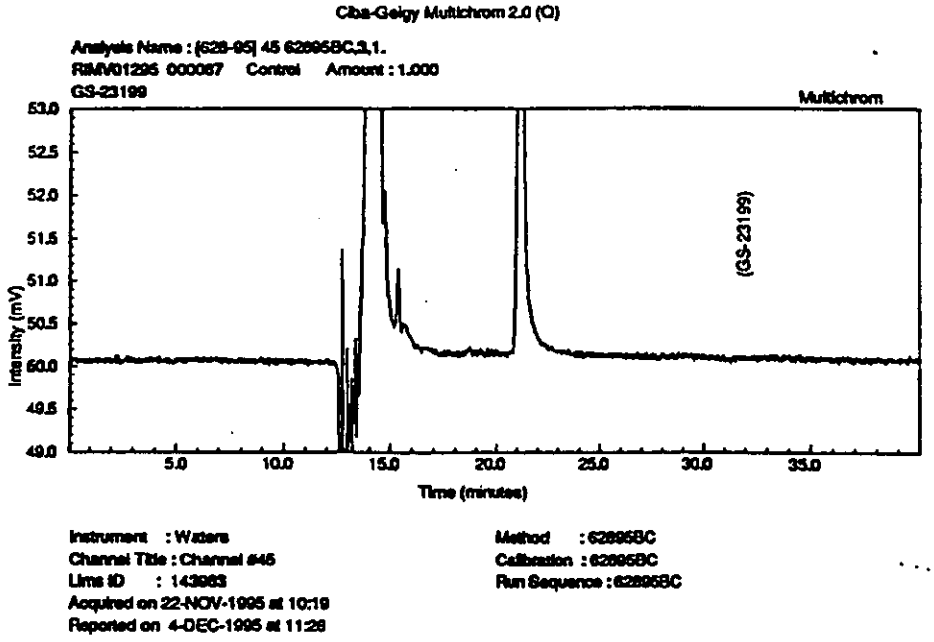


8. CN.RB, reagent blank, 100 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

FIGURE 7. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATOES FOR GS-23199

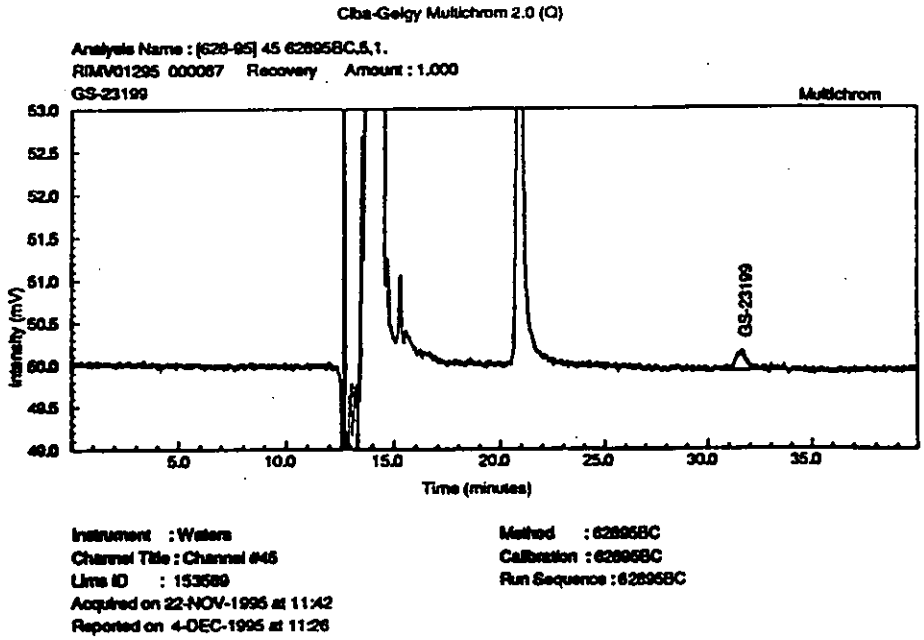


1. TM.CA, control, 101 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

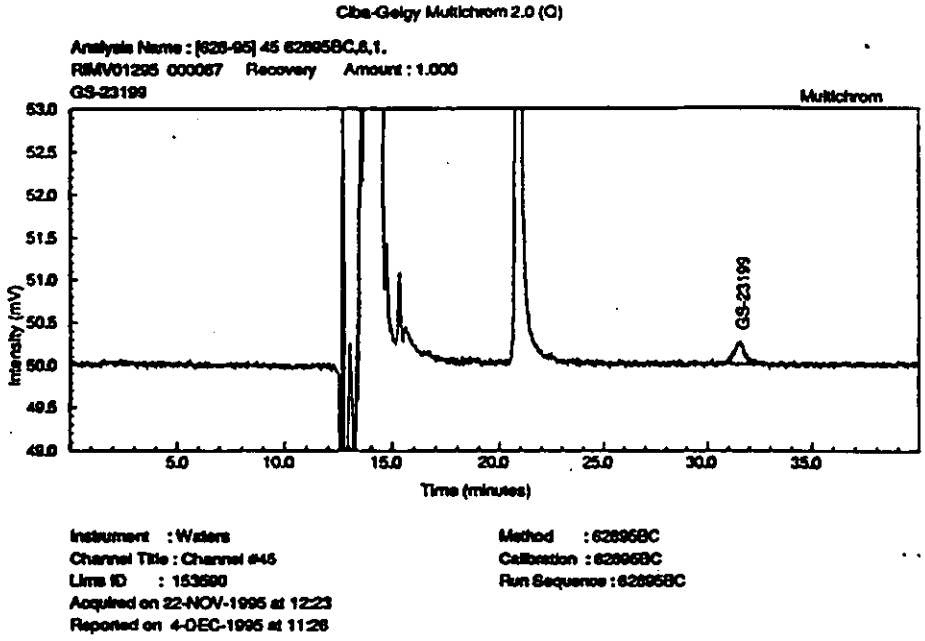


2. TM.CB, control, 99 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

FIGURE 7. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATOES FOR GS-23199 (Continued)

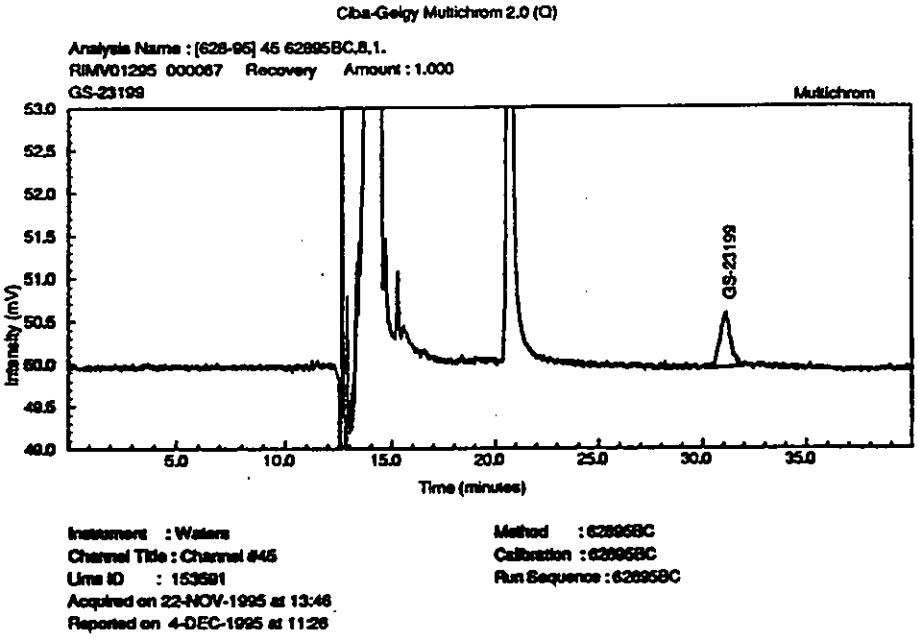


3. TM.02A, 0.02 ppm fortification, 99 mg injected, 1.87 ng GS-23199 found, 0.019 ppm GS-23199 found, 94% recovery

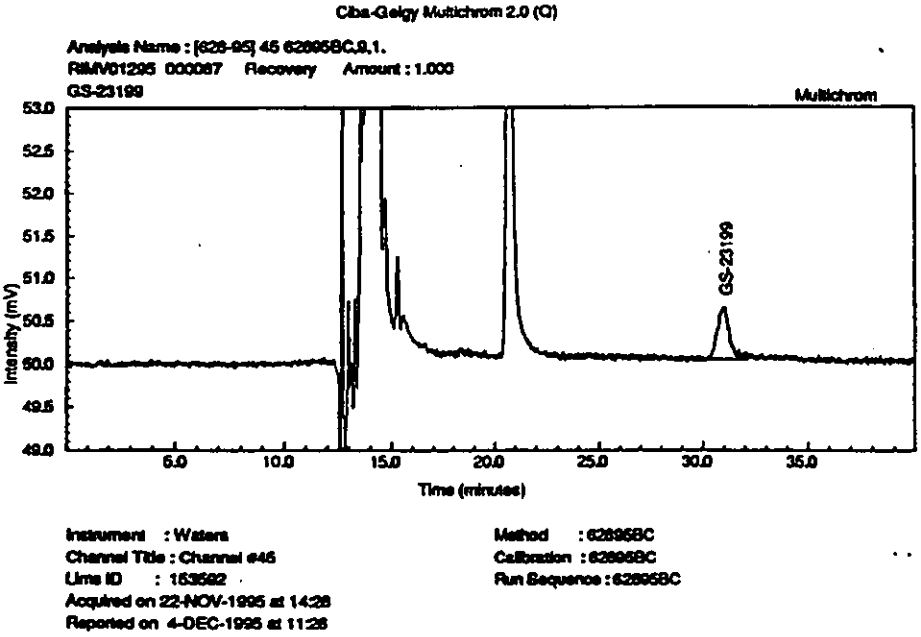


4. TM.02B, 0.02 ppm fortification, 100 mg injected, 1.91 ng GS-23199 found, 0.019 ppm GS-23199 found, 95% recovery

FIGURE 7. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATOES FOR GS-23199 (Continued)

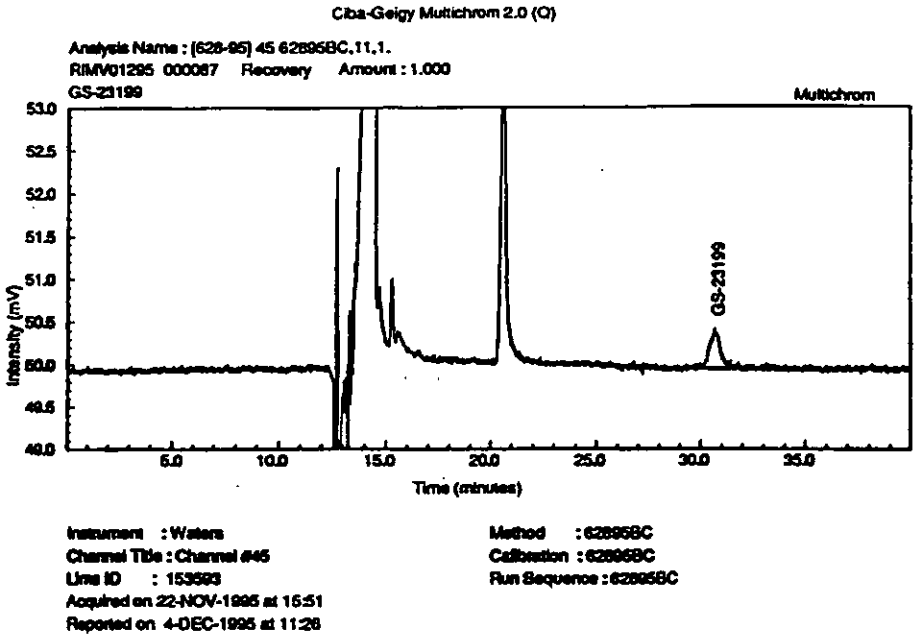


5. TM.05A, 0.05 ppm fortification, 100 mg injected, 5.95 ng GS-23199 found, 0.059 ppm GS-23199 found, 119% recovery

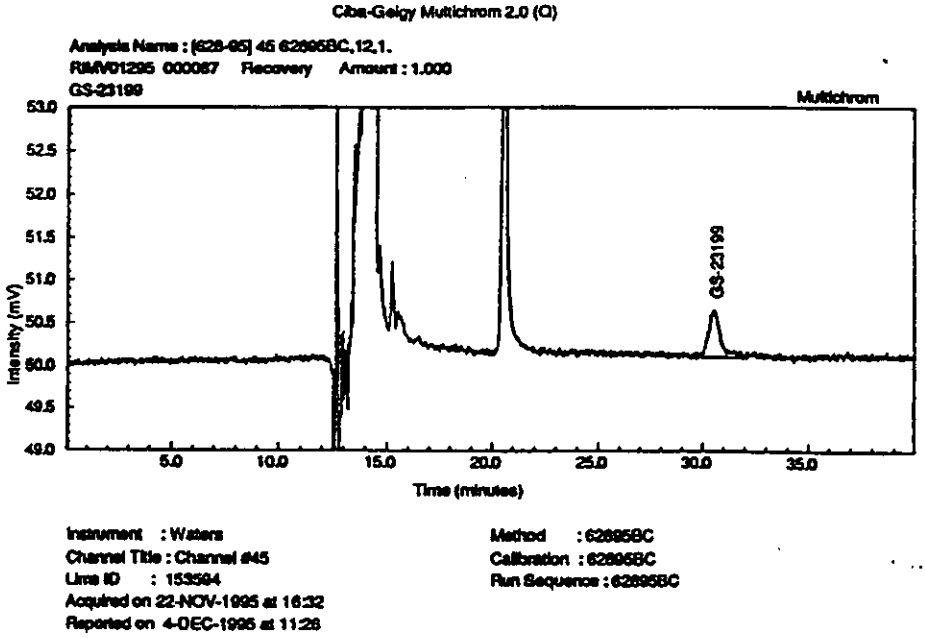


6. TM.05B, 0.05 ppm fortification, 100 mg injected, 6.75 ng GS-23199 found, 0.067 ppm GS-23199 found, 134% recovery

FIGURE 7. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATOES FOR GS-23199 (Continued)

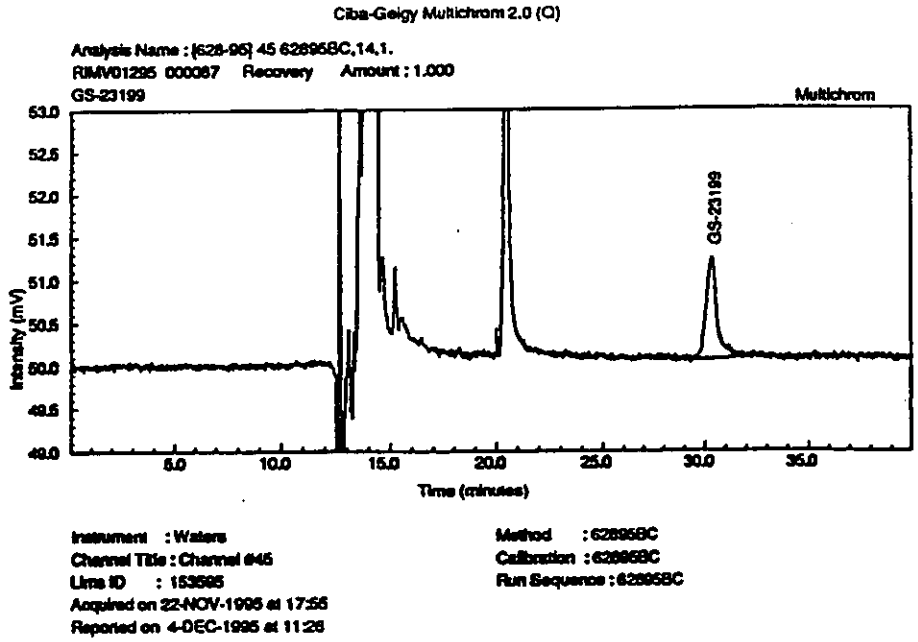


7. TM.10A, 0.10 ppm fortification, 50 mg injected, 4.21 ng GS-23199 found, 0.084 ppm GS-23199 found, 84% recovery

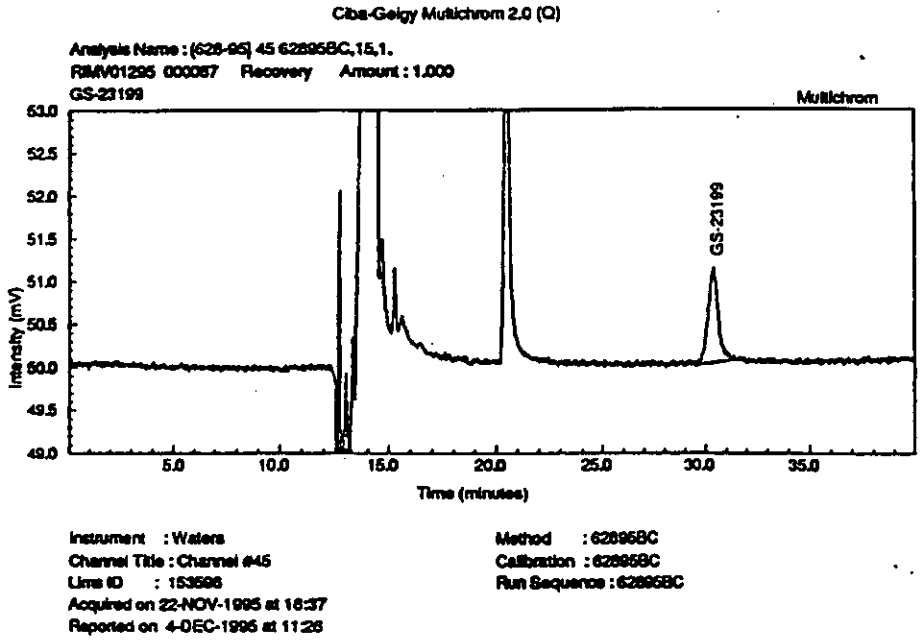


8. TM.10B, 0.10 ppm fortification, 51 mg injected, 6.13 ng GS-23199 found, 0.12 ppm GS-23199 found, 121% recovery

FIGURE 7. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATOES FOR GS-23199 (Continued)

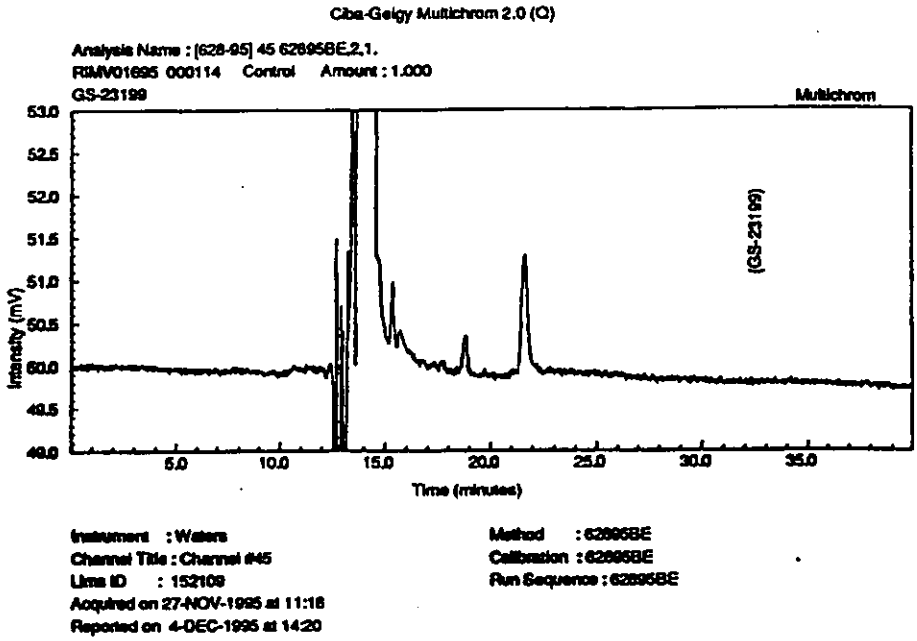


9. TM.20A, 0.20 ppm fortification, 50 mg injected, 13.0 ng GS-23199 found, 0.26 ppm GS-23199 found, 129% recovery

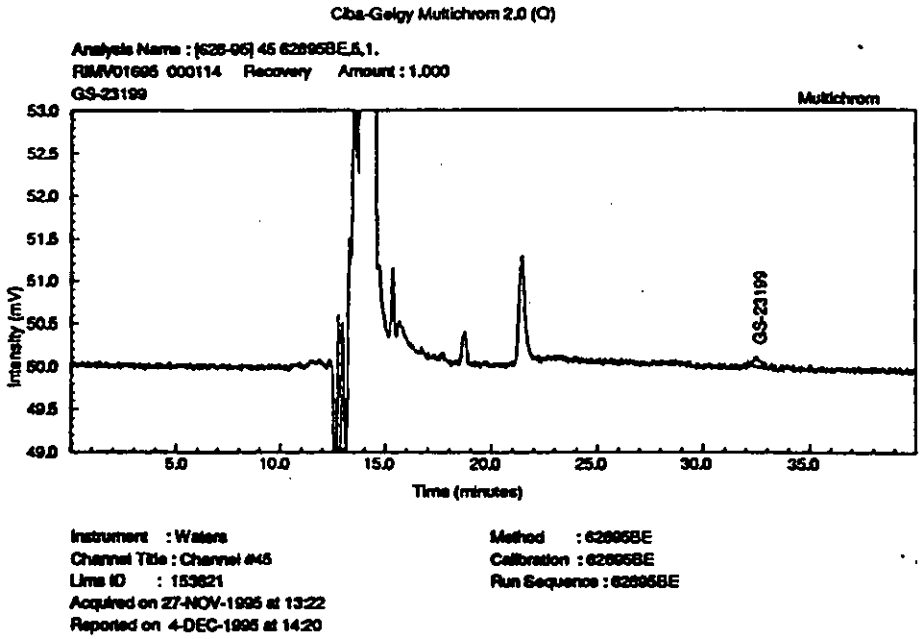


10. TM.20B, 0.20 ppm fortification, 50 mg injected, 10.9 ng GS-23199 found, 0.22 ppm GS-23199 found, 109% recovery

FIGURE 8. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATO WET POMACE FOR GS-23199

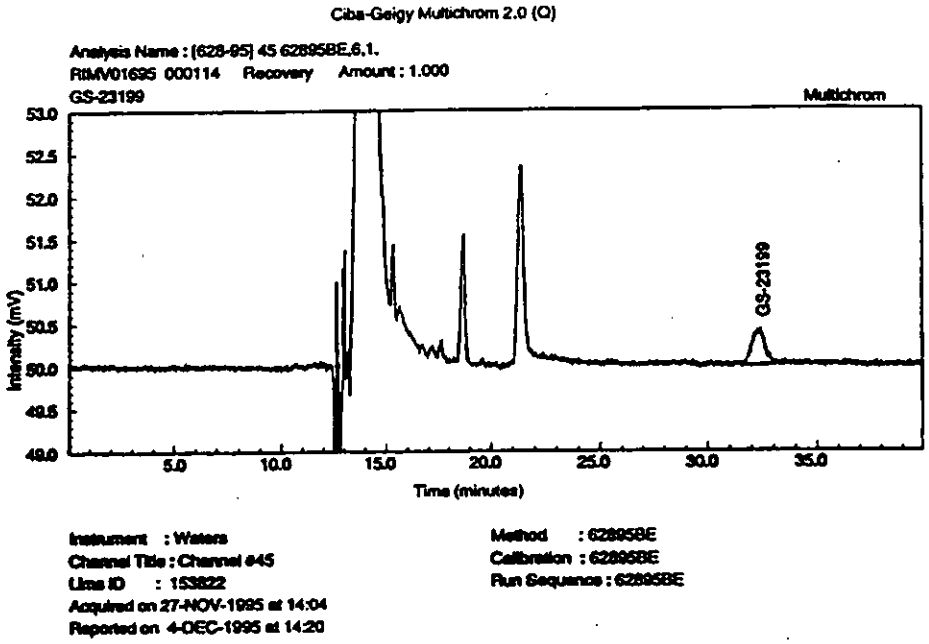


1. TW.CA, control, 99 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

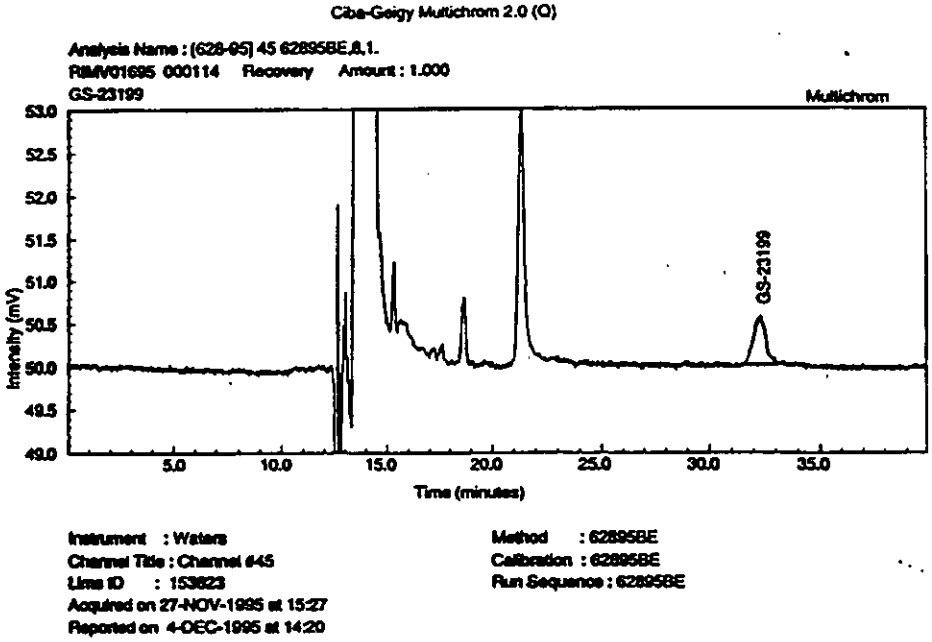


2. TW.02B, 0.02 ppm fortification, 50 mg injected, 0.91 ng GS-23199 found, 0.018 ppm GS-23199 found, 92% recovery

FIGURE 8. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATO WET POMACE FOR GS-23199 (Continued)

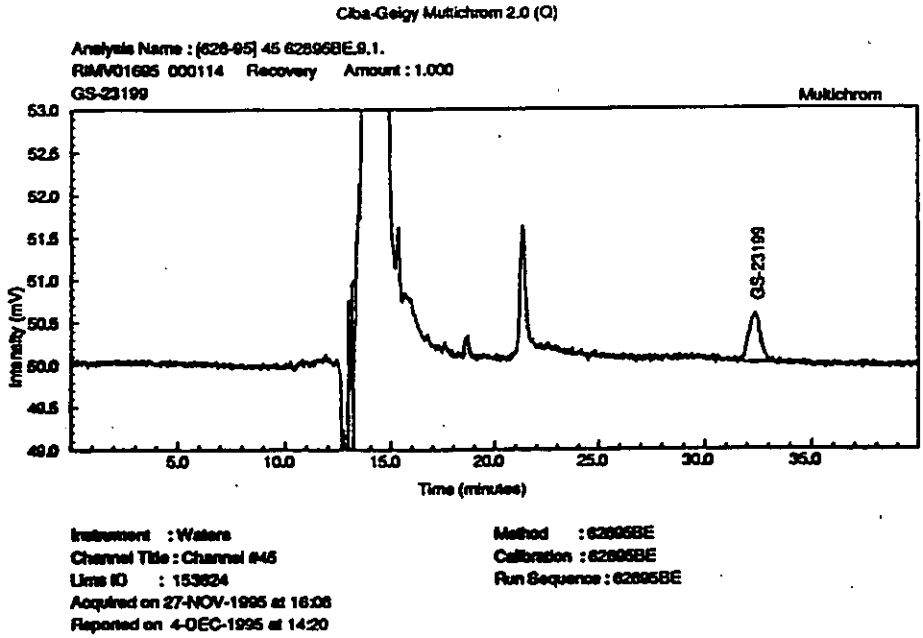


3. TW.05A, 0.05 ppm fortification, 100 mg injected, 3.38 ng GS-23199 found, 0.034 ppm GS-23199 found, 68% recovery

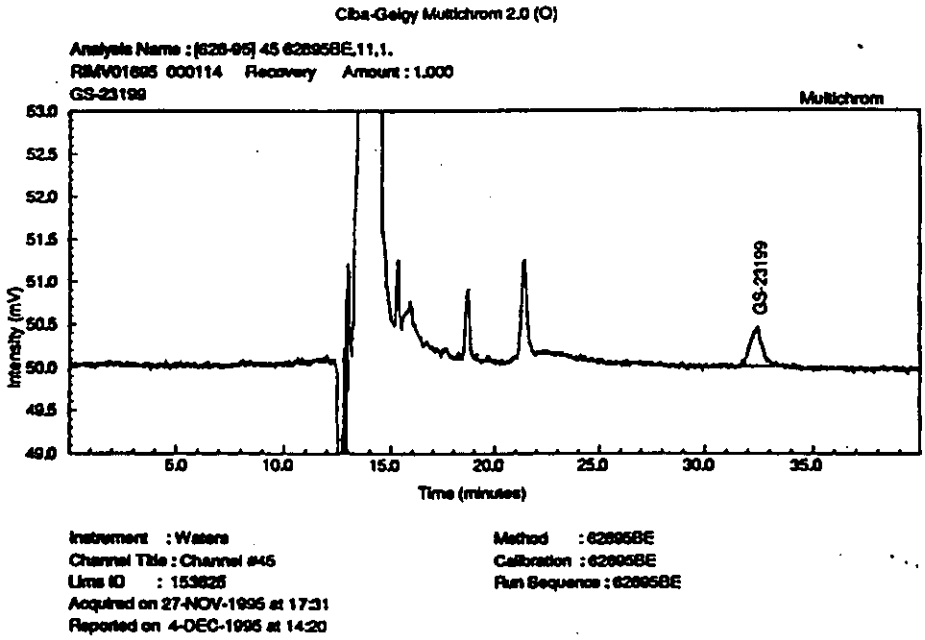


4. TW.05B, 0.05 ppm fortification, 99 mg injected, 3.93 ng GS-23199 found, 0.040 ppm GS-23199 found, 79% recovery

FIGURE 8. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATO WET POMACE FOR GS-23199 (Continued)

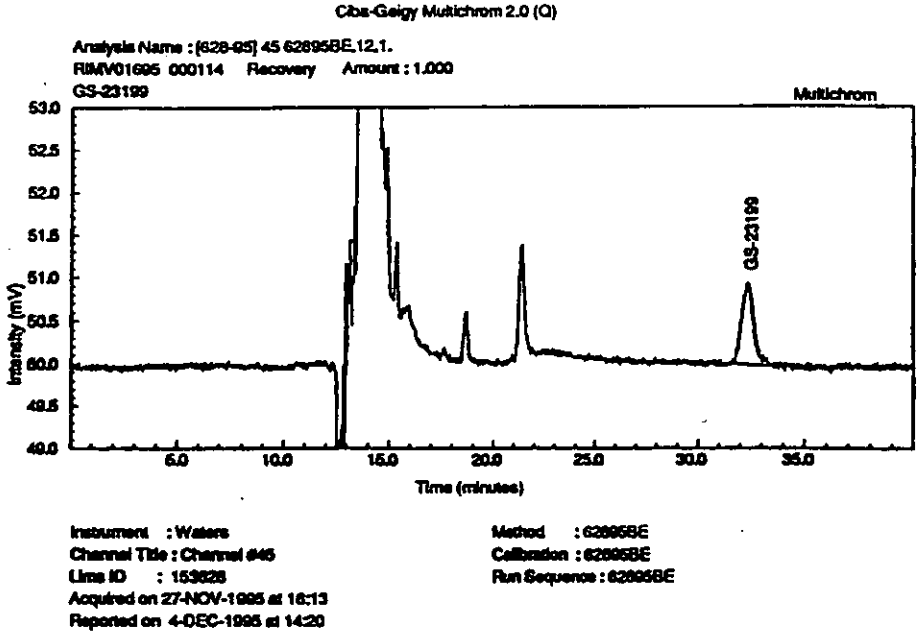


5. TW.10A, 0.10 ppm fortification, 50 mg injected, 4.23 ng GS-23199 found, 0.085 ppm GS-23199 found, 85% recovery



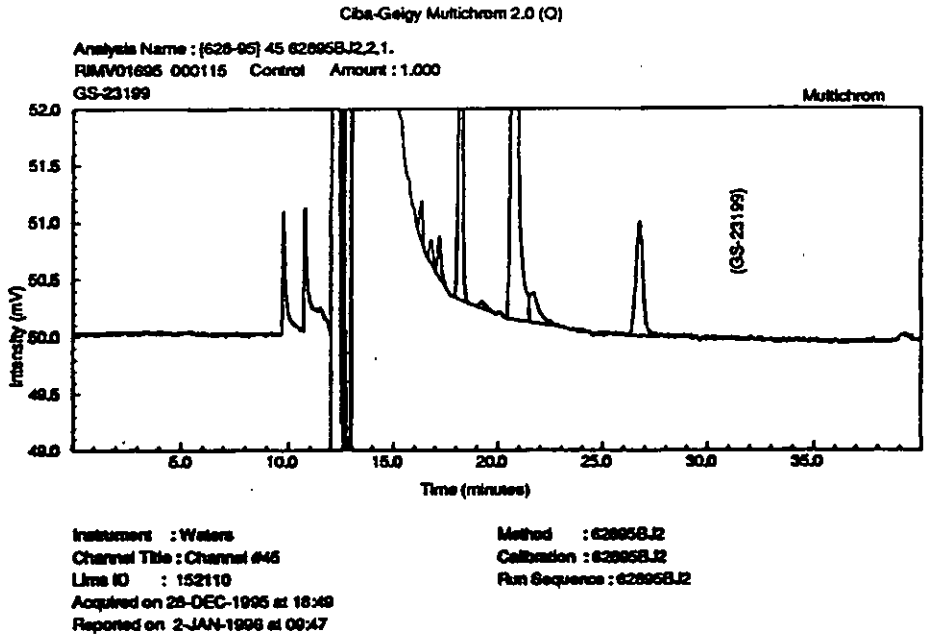
6. TW.10B, 0.10 ppm fortification, 50 mg injected, 3.29 ng GS-23199 found, 0.065 ppm GS-23199 found, 65% recovery

FIGURE 8. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATO WET POMACE FOR GS-23199 (Continued)

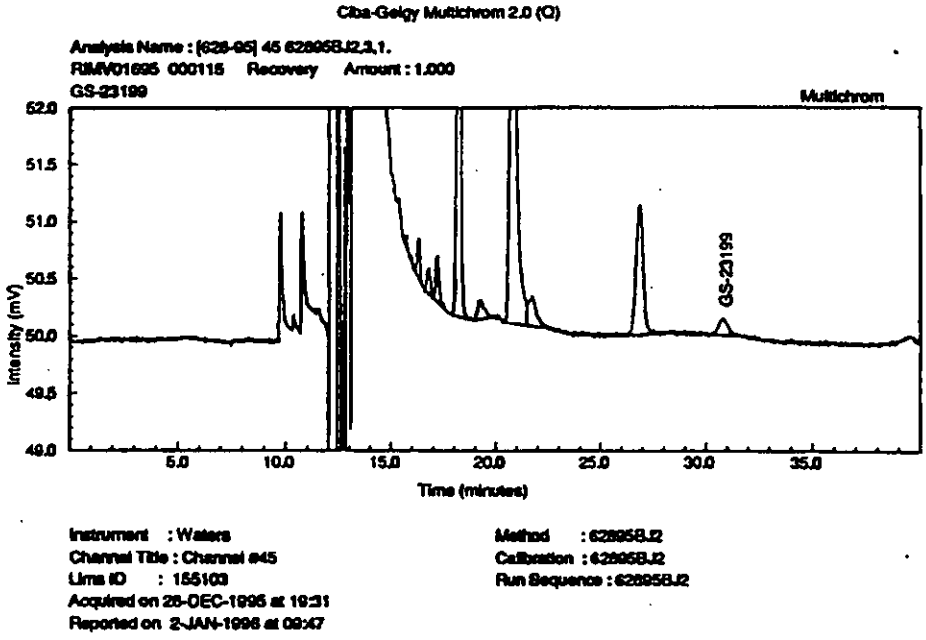


7. TW.20A, 0.20 ppm fortification, 50 mg injected, 6.81 ng GS-23199 found, 0.14 ppm GS-23199 found, 68% recovery

FIGURE 9. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATO DRY POMACE FOR GS-23199

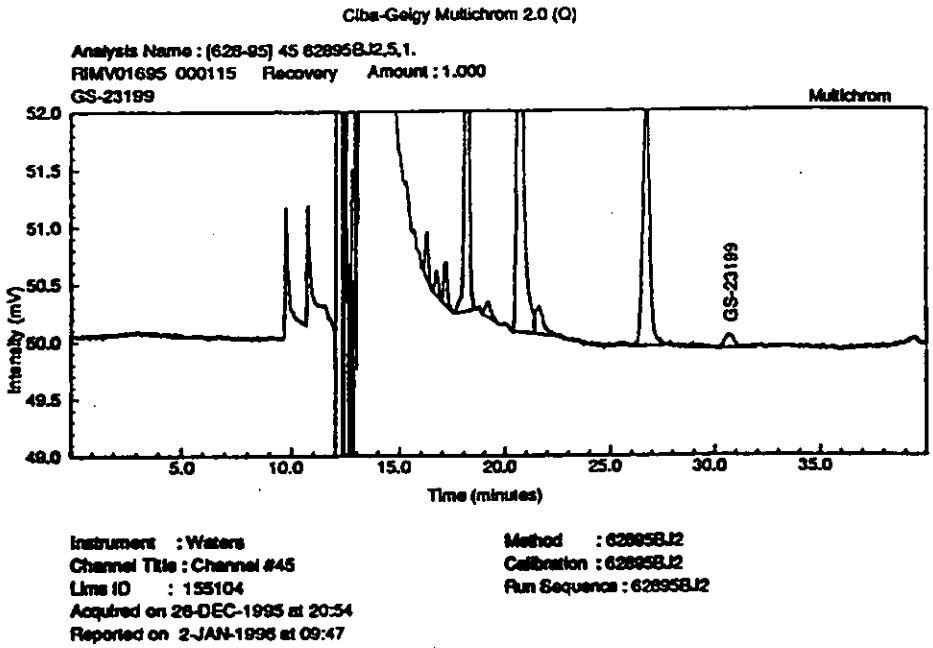


1. TD.CA, control, 50 mg injected, <1.0 ng found, <0.02 ppm GS-23199 found

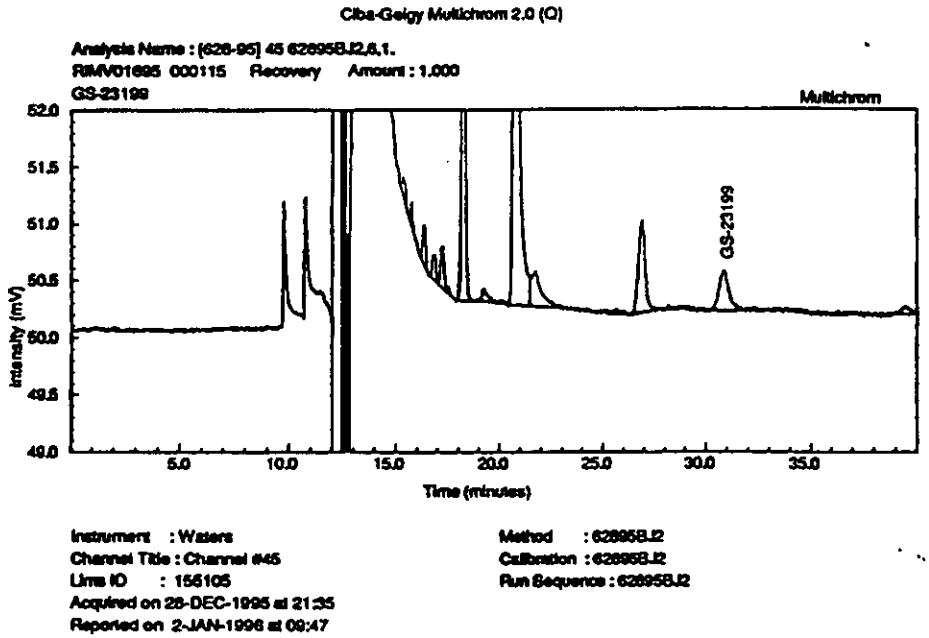


2. TD.02A, 0.02 ppm fortification, 50 mg injected, 0.94 ng GS-23199 found, 0.019 ppm GS-23199 found, 93% recovery

FIGURE 9. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATO DRY POMACE FOR GS-23199 (Continued)

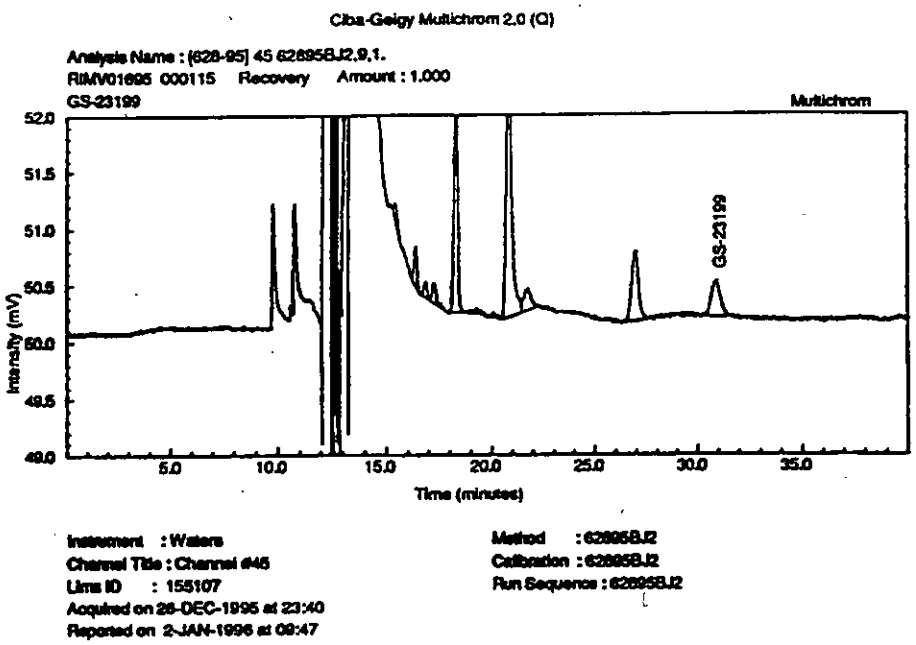


3. TD.02B, 0.02 ppm fortification, 51 mg injected, 0.81 ng GS-23199 found, 0.016 ppm GS-23199 found, 80% recovery

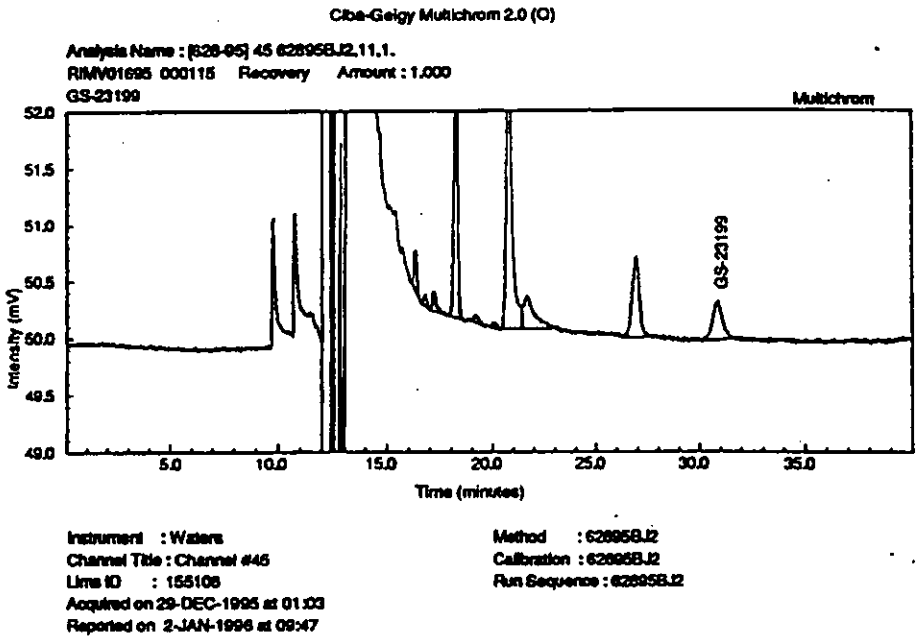


4. TD.05A, 0.05 ppm fortification, 50 mg injected, 2.10 ng GS-23199 found, 0.042 ppm GS-23199 found, 85% recovery

FIGURE 9. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATO DRY POMACE FOR GS-23199 (Continued)

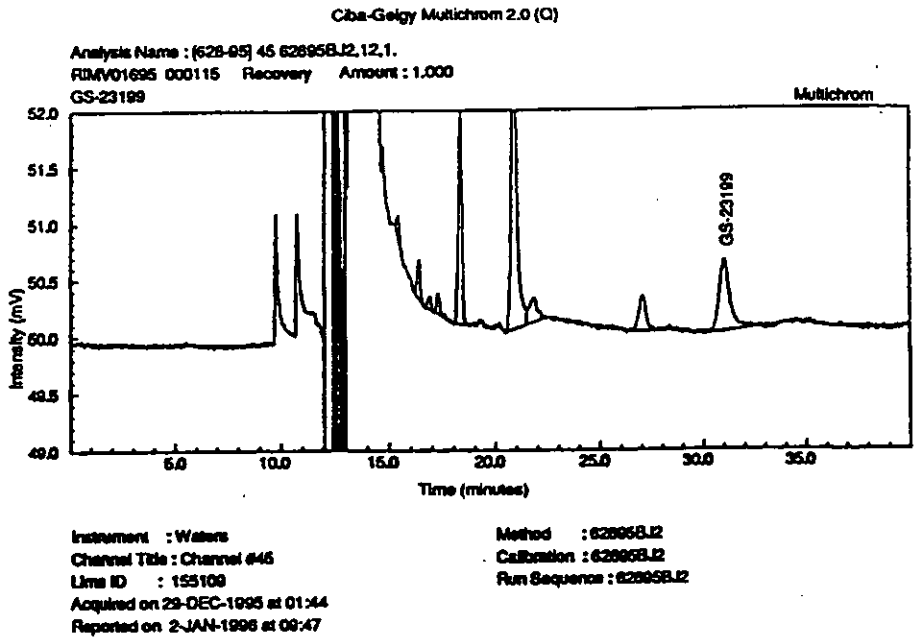


5. TD.10A, 0.10 ppm fortification, 25 mg injected, 1.90 ng GS-23199 found, 0.077 ppm GS-23199 found, 77% recovery



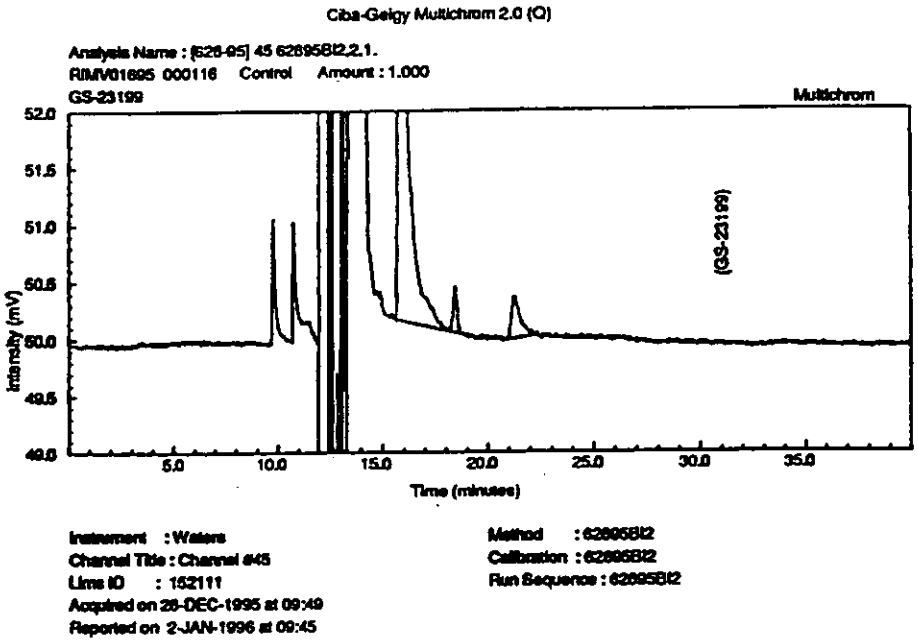
6. TD.10B, 0.10 ppm fortification, 25 mg injected, 2.04 ng GS-23199 found, 0.081 ppm GS-23199 found, 81% recovery

FIGURE 9. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF TOMATO DRY POMACE FOR GS-23199 (Continued)

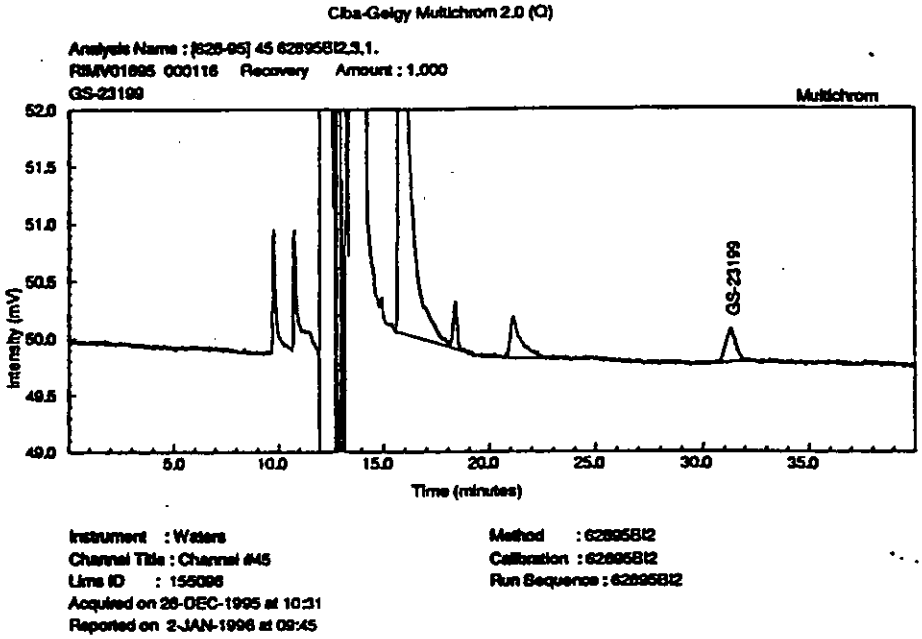


7. TD.20A, 0.20 ppm fortification, 25 mg injected, 3.95 ng GS-23199 found, 0.16 ppm GS-23199 found, 79% recovery

FIGURE 10. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF PEPPERS FOR GS-23199

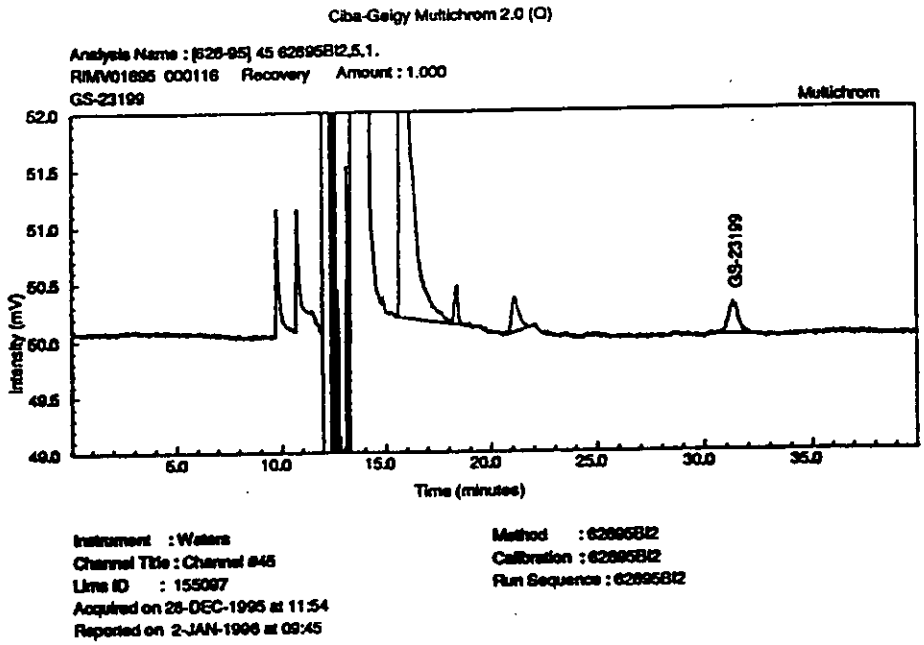


1. PP.CA, control, 100 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

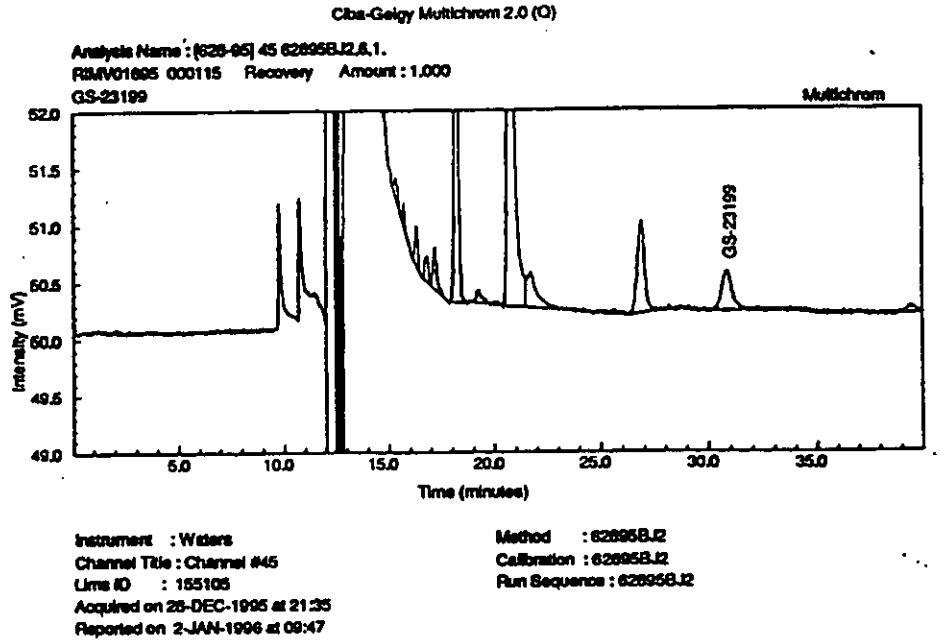


2. PP.02A, 0.02 ppm fortification, 99 mg injected, 1.81 ng GS-23199 found, 0.018 ppm GS-23199 found, 92% recovery

FIGURE 10. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF PEPPERS FOR GS-23199 (Continued)

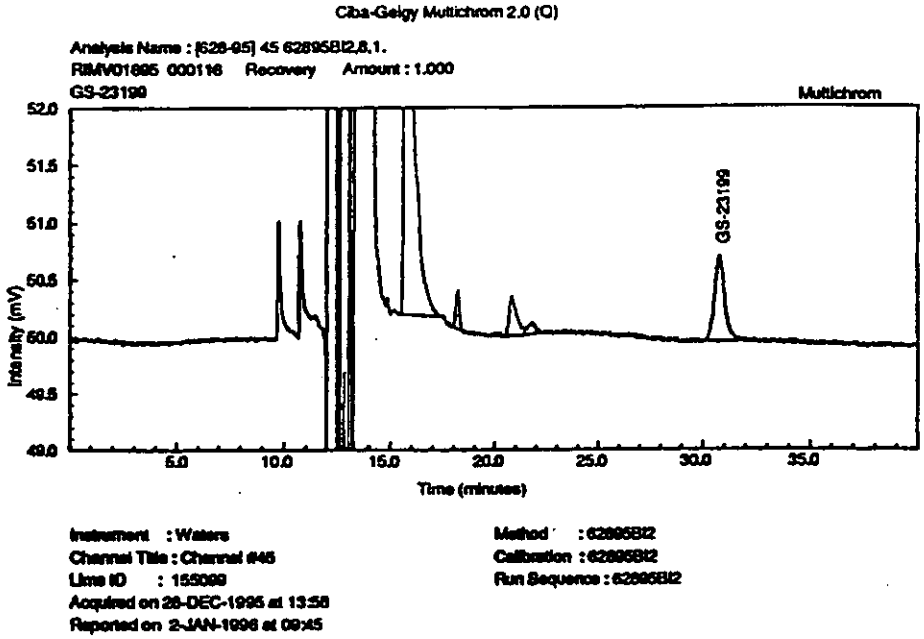


3. PP.02B, 0.02 ppm fortification, 99 mg injected, 1.78 ng GS-23199 found, 0.018 ppm GS-23199 found, 89% recovery

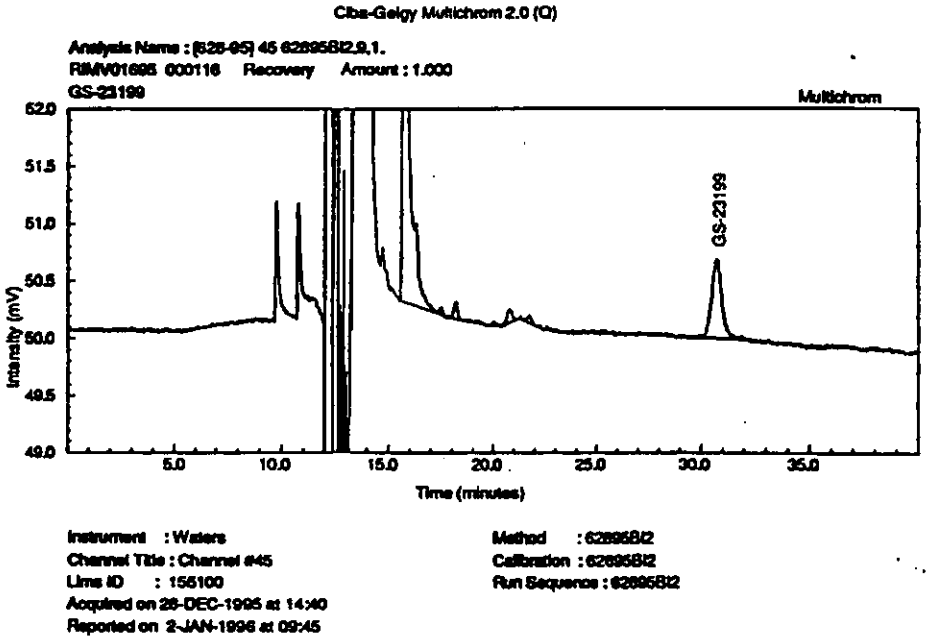


4. PP.05A, 0.05 ppm fortification, 99 mg injected, 4.17 ng GS-23199 found, 0.042 ppm GS-23199 found, 84% recovery

FIGURE 10. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF PEPPERS FOR GS-23199 (Continued)

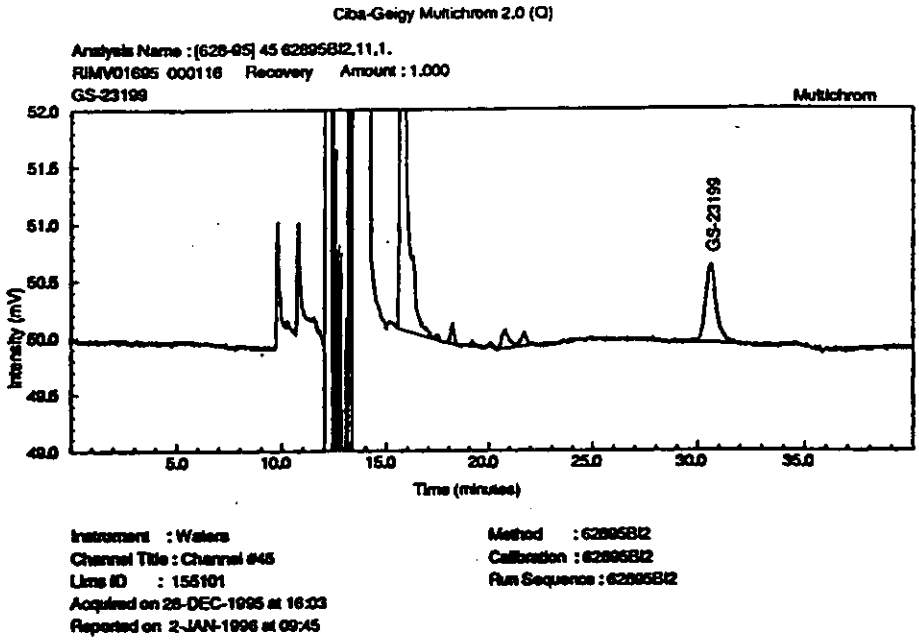


5. PP.05B, 0.05 ppm fortification, 100 mg injected, 4.37 ng GS-23199 found, 0.044 ppm GS-23199 found, 88% recovery

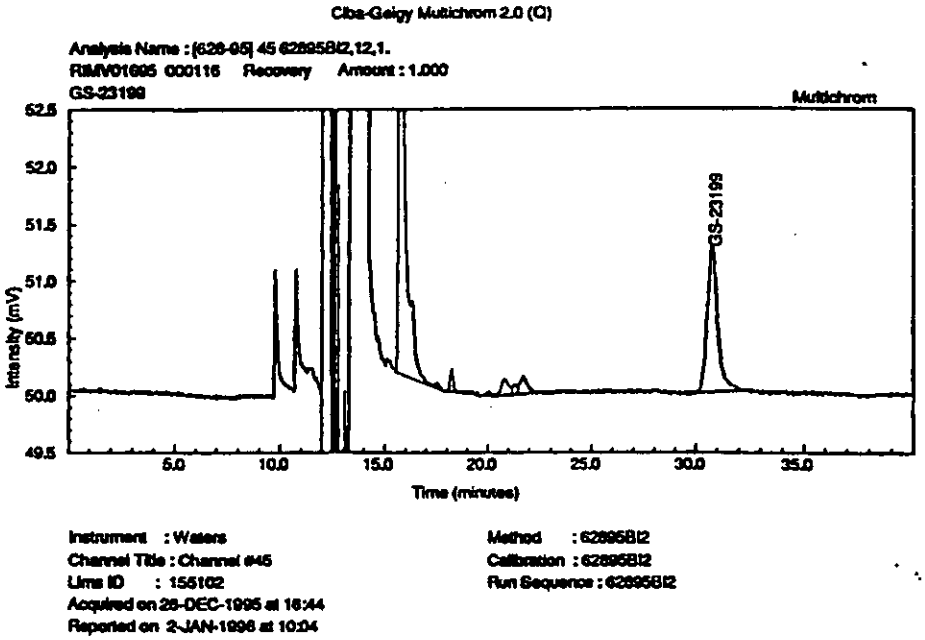


6. PP.10A, 0.10 ppm fortification, 50 mg injected, 4.04 ng GS-23199 found, 0.081 ppm GS-23199 found, 81% recovery

FIGURE 10. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF PEPPERS FOR GS-23199 (Continued)

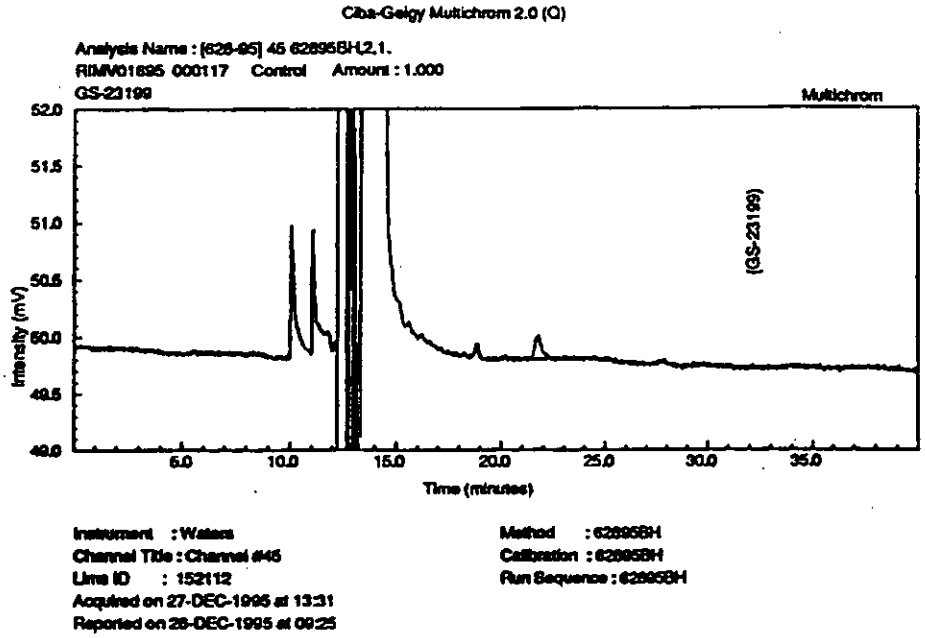


7. PP.10B, 0.10 ppm fortification, 50 mg injected, 4.20 ng GS-23199 found, 0.084 ppm GS-23199 found, 84% recovery

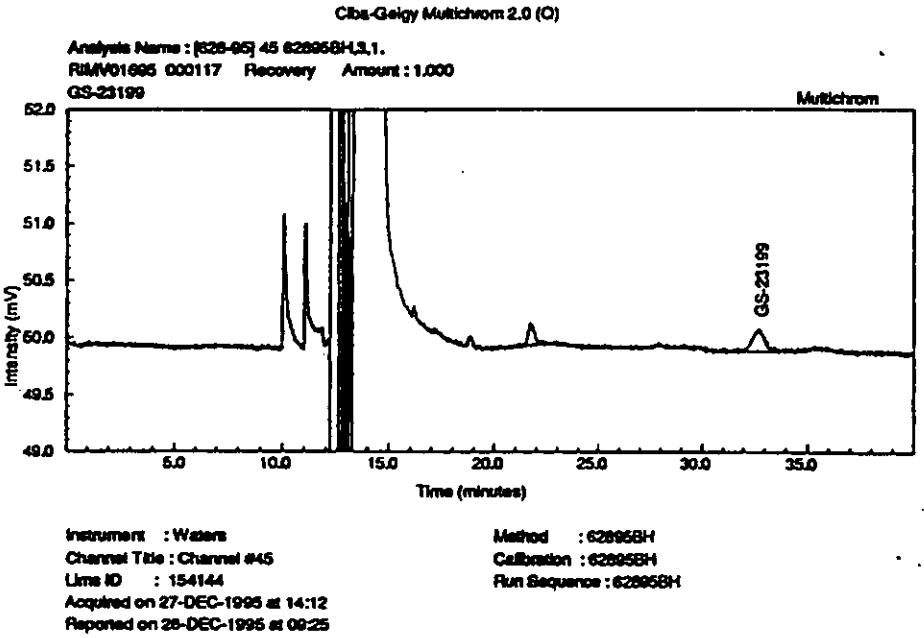


8. PP.20A, 0.20 ppm fortification, 50 mg injected, 7.36 ng GS-23199 found, 0.15 ppm GS-23199 found, 74% recovery

FIGURE 11. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF GREEN TOBACCO FOR GS-23199

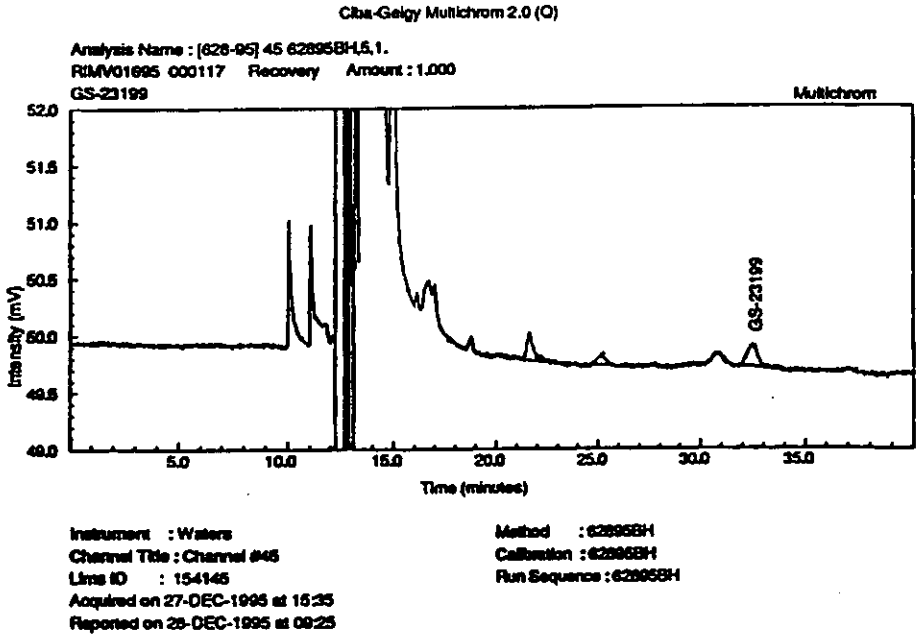


1. TG.CA, control, 100 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

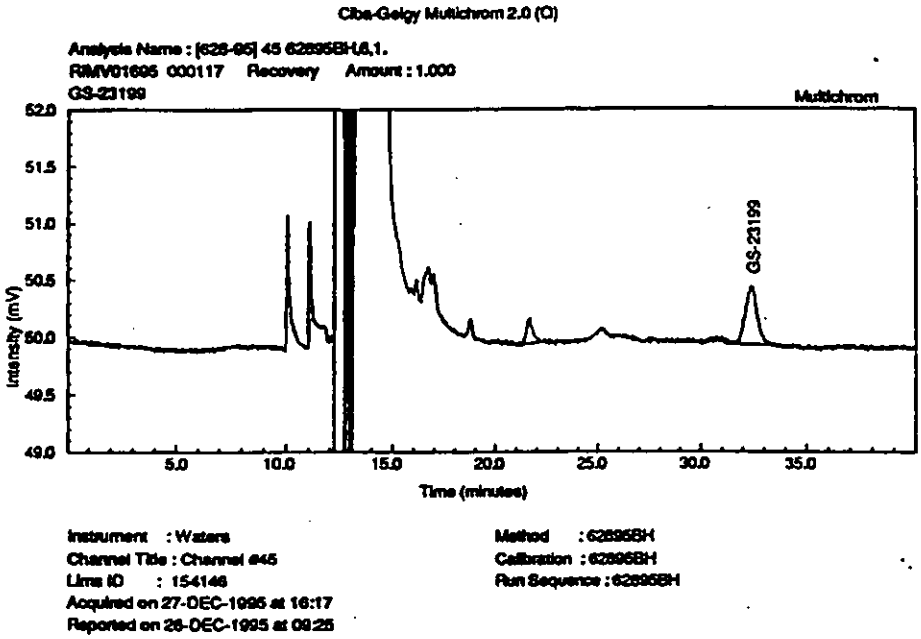


2. TG.02A, 0.02 ppm fortification, 100 mg injected, 1.44 ng GS-23199 found, 0.014 ppm GS-23199 found, 72% recovery

FIGURE 11. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF GREEN TOBACCO FOR GS-23199 (Continued)

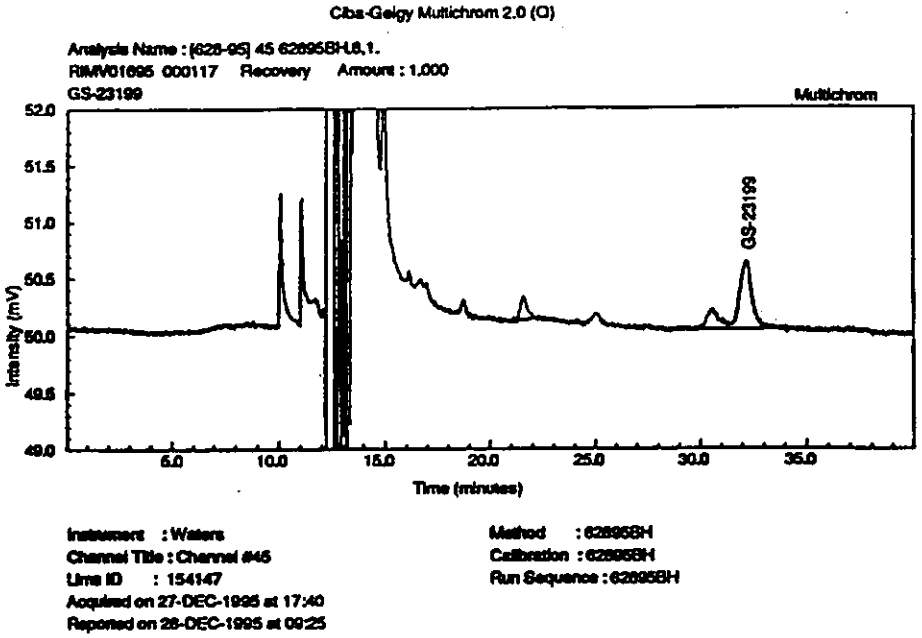


3. TG.02B, 0.02 ppm fortification, 100 mg injected, 1.25 ng GS-23199 found, 0.013 ppm GS-23199 found, 63% recovery

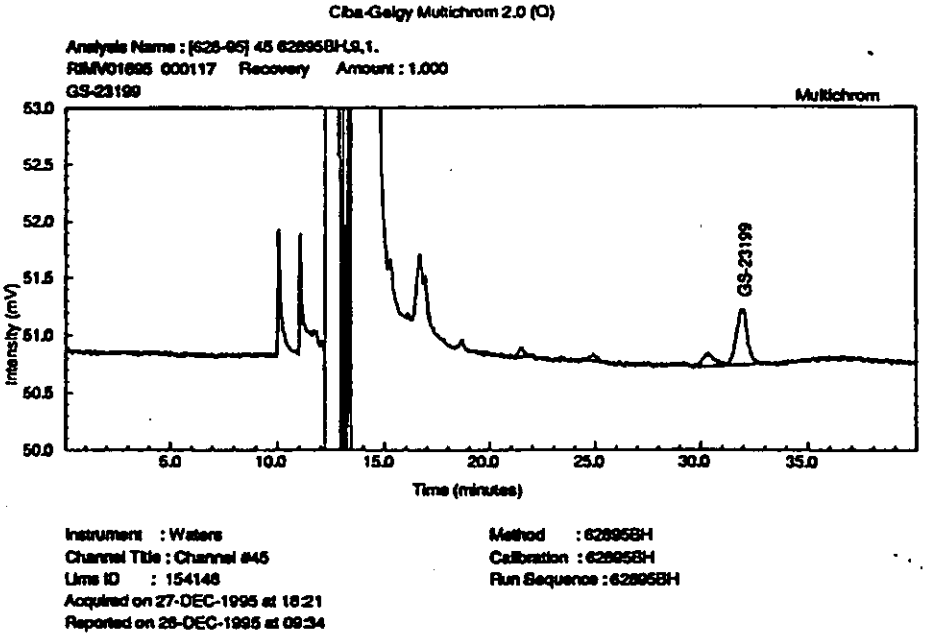


4. TG.05A, 0.05 ppm fortification, 100 mg injected, 3.57 ng GS-23199 found, 0.036 ppm GS-23199 found, 71% recovery

FIGURE 11. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF GREEN TOBACCO FOR GS-23199 (Continued)

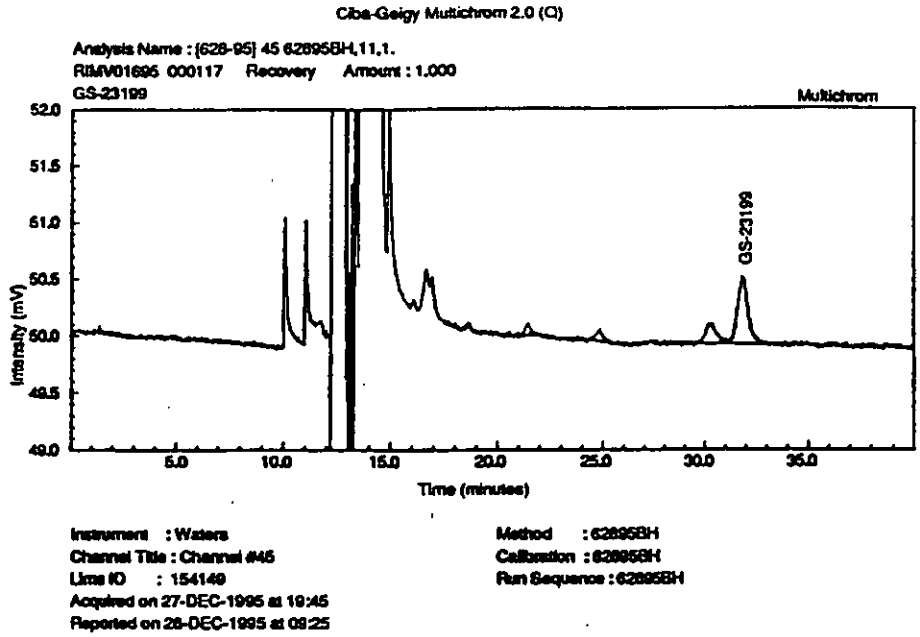


5. TG.05B, 0.05 ppm fortification, 100 mg injected, 4.21 ng GS-23199 found, 0.042 ppm GS-23199 found, 84% recovery

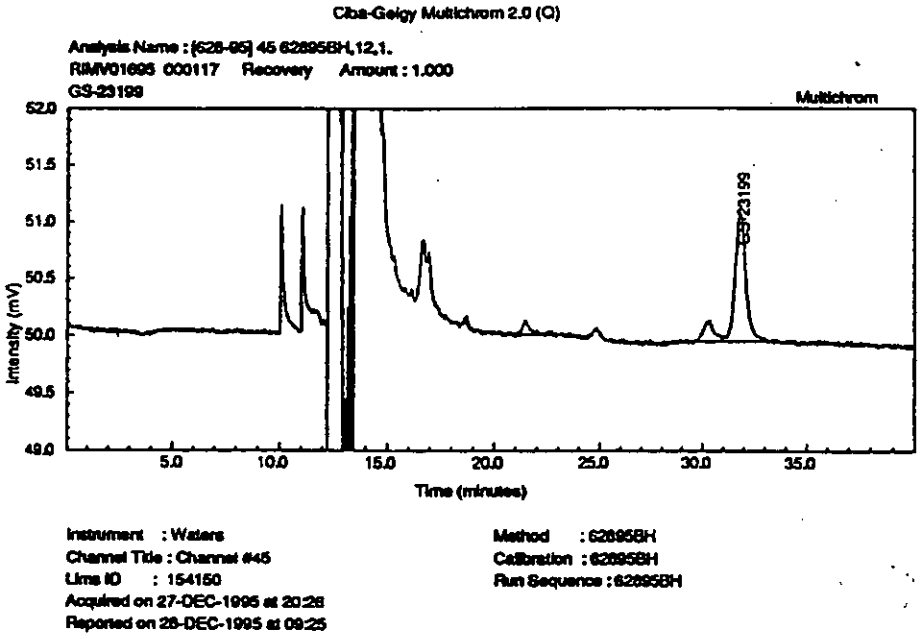


6. TG.10A, 0.10 ppm fortification, 50 mg injected, 3.09 ng GS-23199 found, 0.062 ppm GS-23199 found, 62% recovery

FIGURE 11. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF GREEN TOBACCO FOR GS-23199 (Continued)

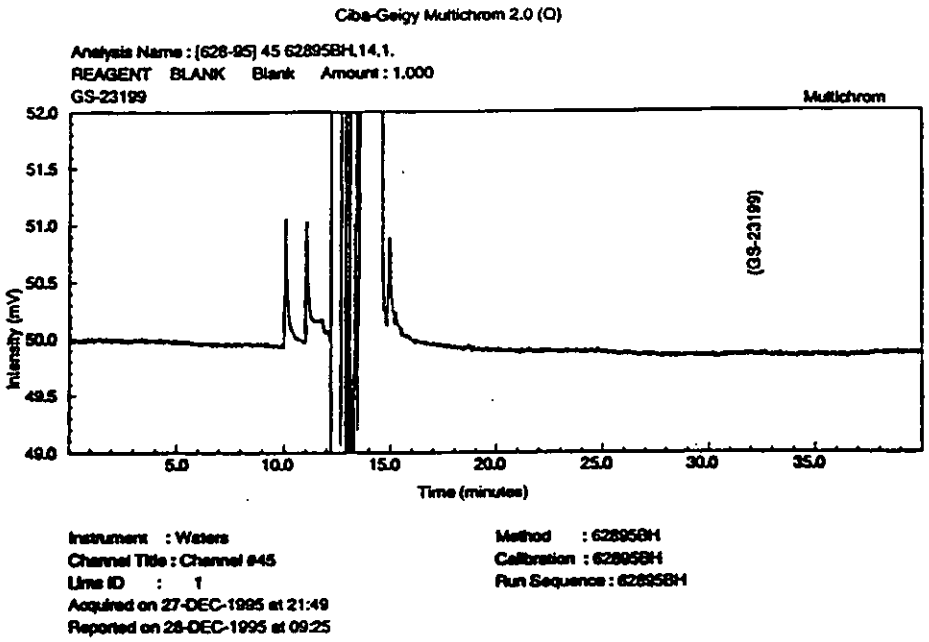


7. TG.10B, 0.10 ppm fortification, 50 mg injected, 3.72 ng GS-23199 found, 0.074 ppm GS-23199 found, 74% recovery



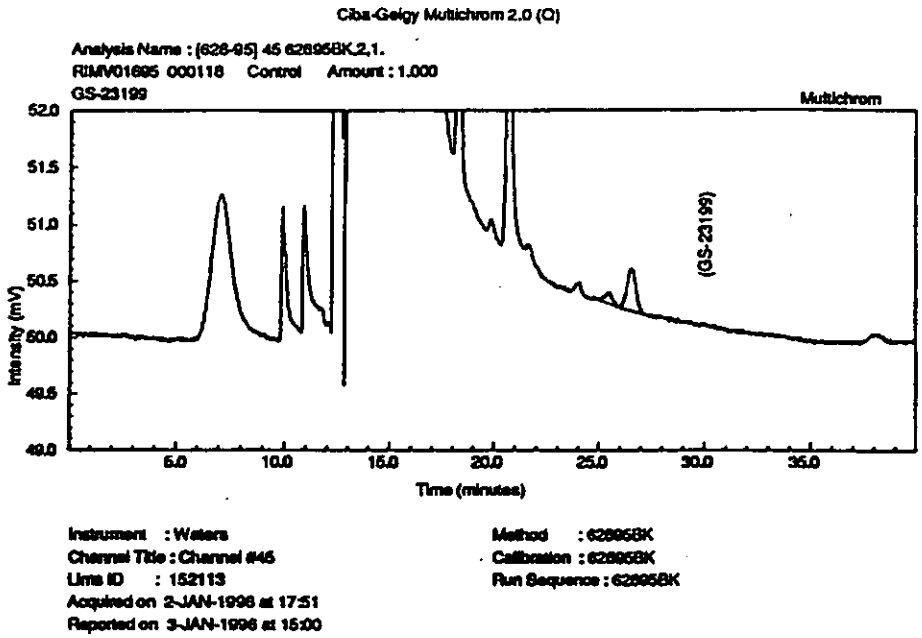
8. TG.20A, 0.20 ppm fortification, 50 mg injected, 7.24 ng GS-23199 found, 0.14 ppm GS-23199 found, 72% recovery

FIGURE 11. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF GREEN TOBACCO FOR GS-23199 (Continued)

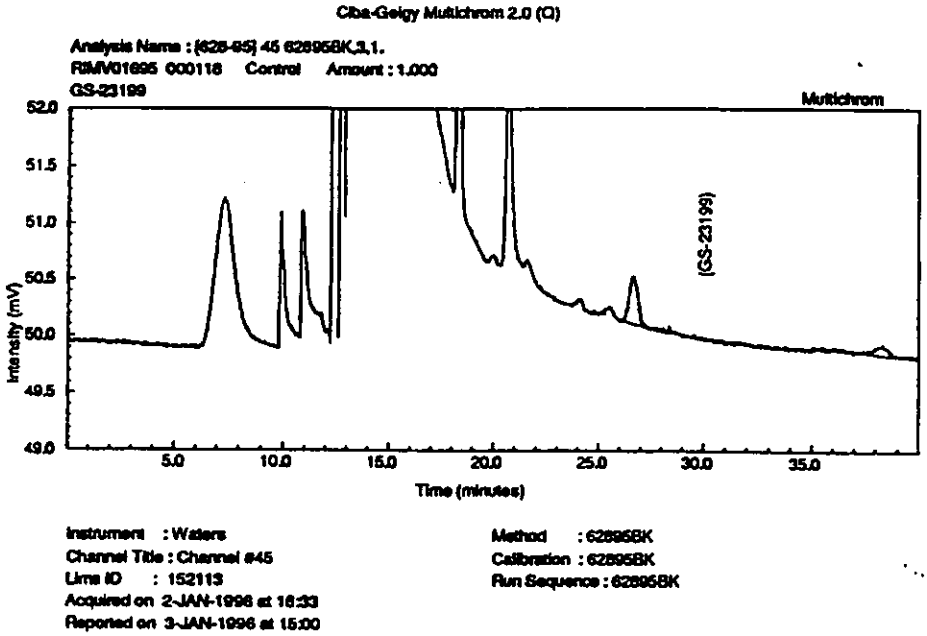


9. TG.RB, 50 mg injected, <2.0 ng found, <0.02 ppm GS-23199 found

FIGURE 12. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CURED TOBACCO FOR GS-23199

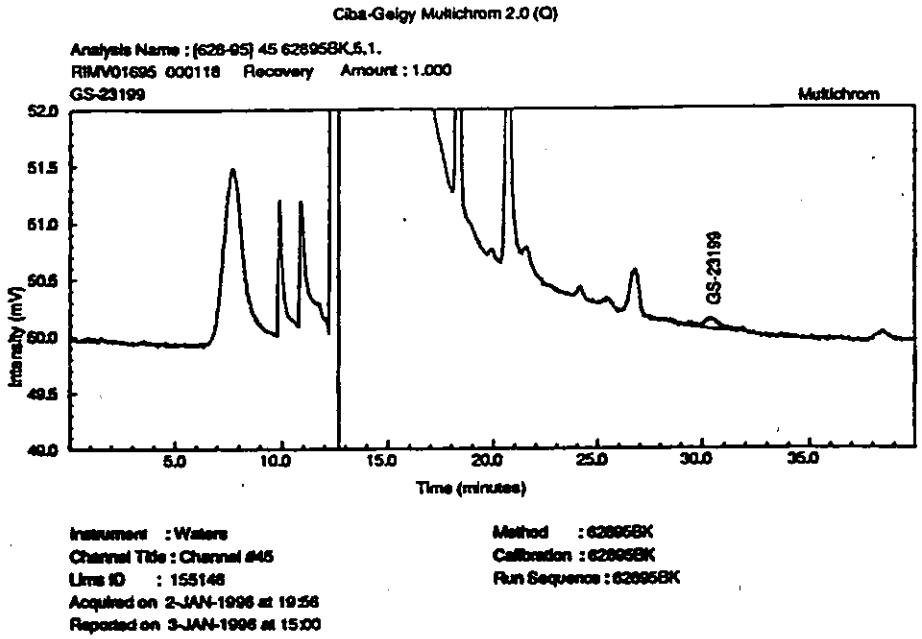


1. TC.CA, control, 51 mg injected, <1.0 ng found, <0.02 ppm GS-23199 found

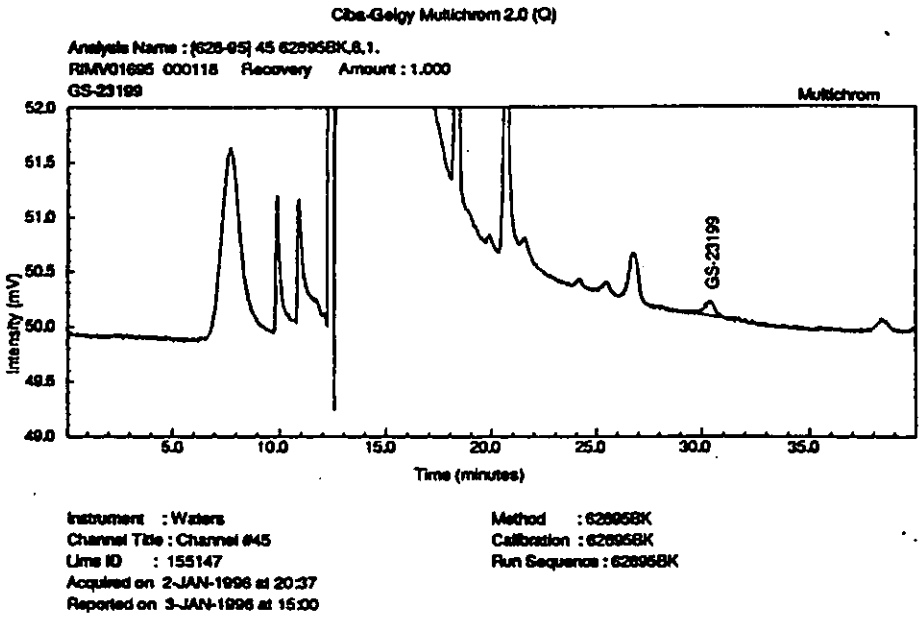


2. TC.CB, control, 50 mg injected, <1.0 ng found, <0.02 ppm GS-23199 found

FIGURE 12. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CURED TOBACCO FOR GS-23199

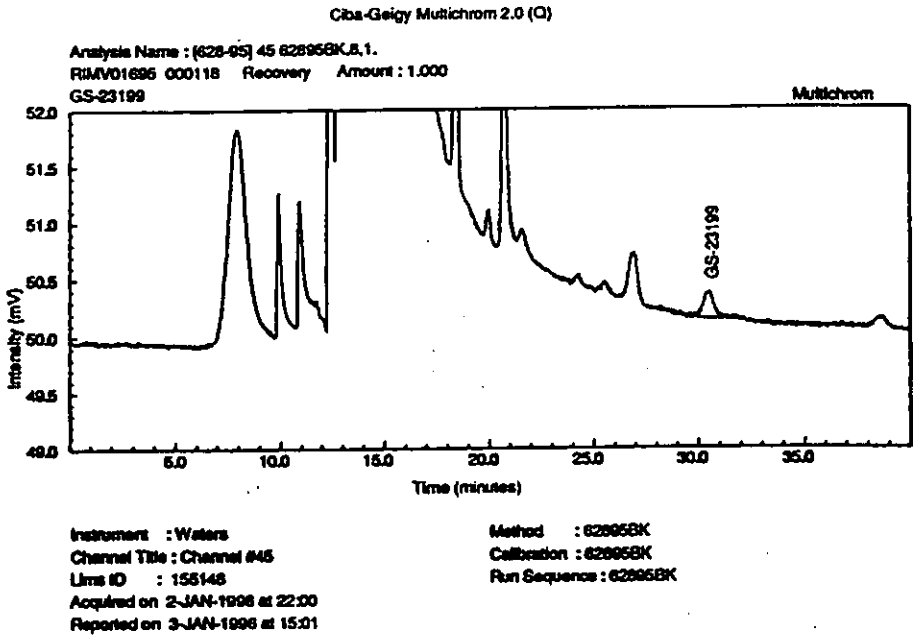


3. TC.02A, 0.02 ppm fortification, 50 mg injected, 0.73 ng GS-23199 found, 0.014 ppm GS-23199 found, 72% recovery

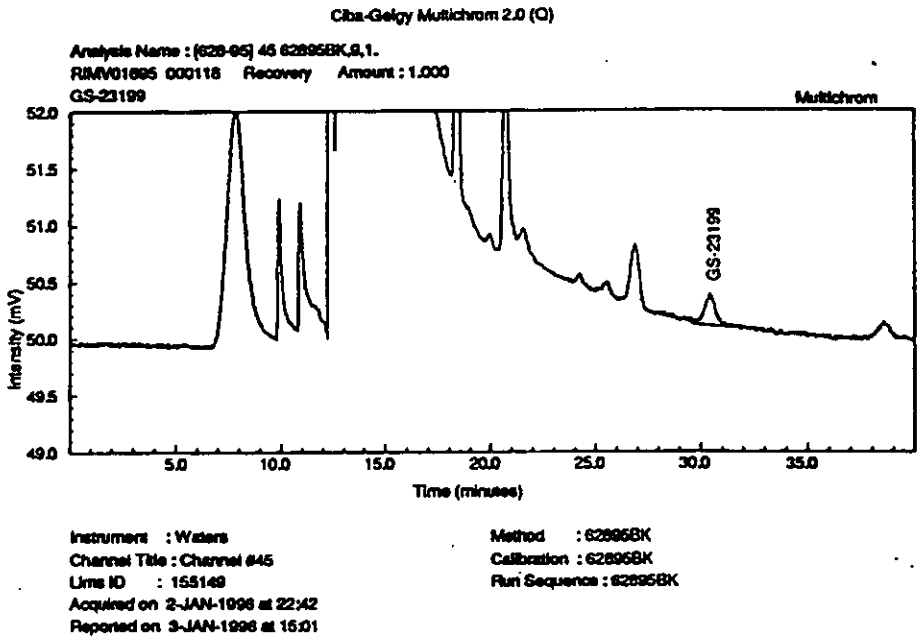


4. TC.02B, 0.02 ppm fortification, 50 mg injected, 0.61 ng GS-23199 found, 0.012 ppm GS-23199 found, 61% recovery

FIGURE 12. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CURED TOBACCO FOR GS-23199 (Continued)

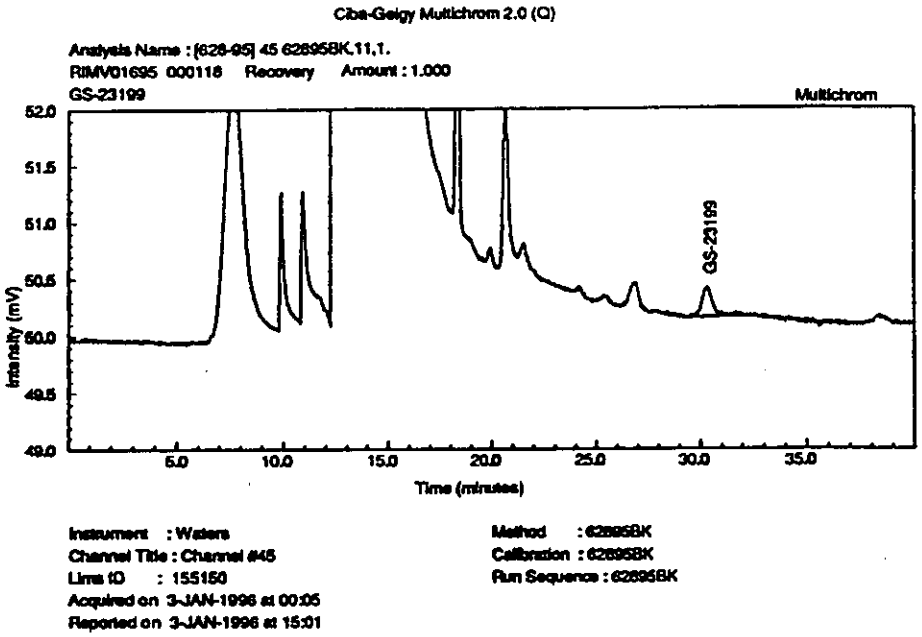


5. TC.05A, 0.05 ppm fortification, 50 mg injected, 1.51 ng GS-23199 found, 0.030 ppm GS-23199 found, 60% recovery

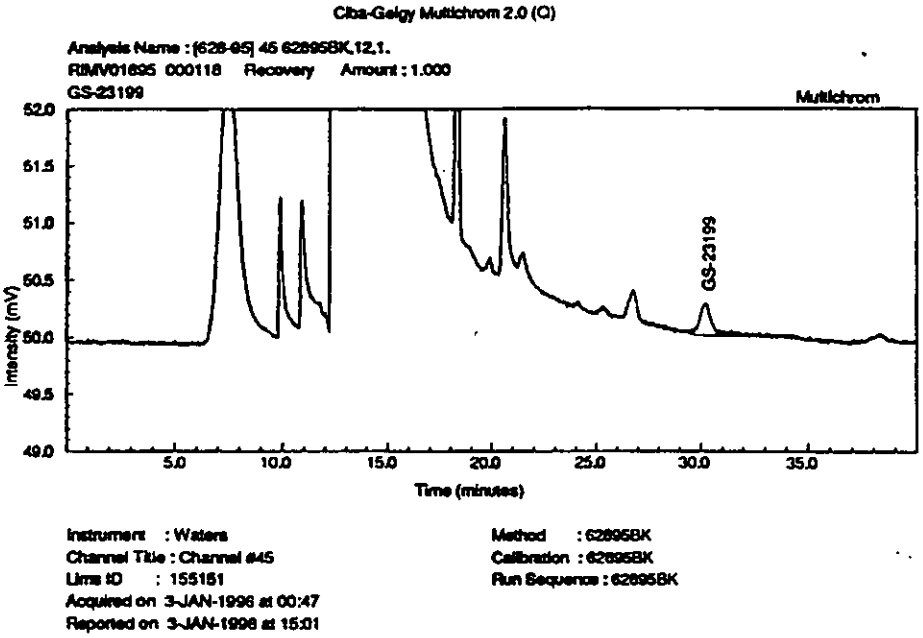


6. TC.05B, 0.05 ppm fortification, 50 mg injected, 1.63 ng GS-23199 found, 0.033 ppm GS-23199 found, 65% recovery

FIGURE 12. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CURED TOBACCO FOR GS-23199 (Continued)

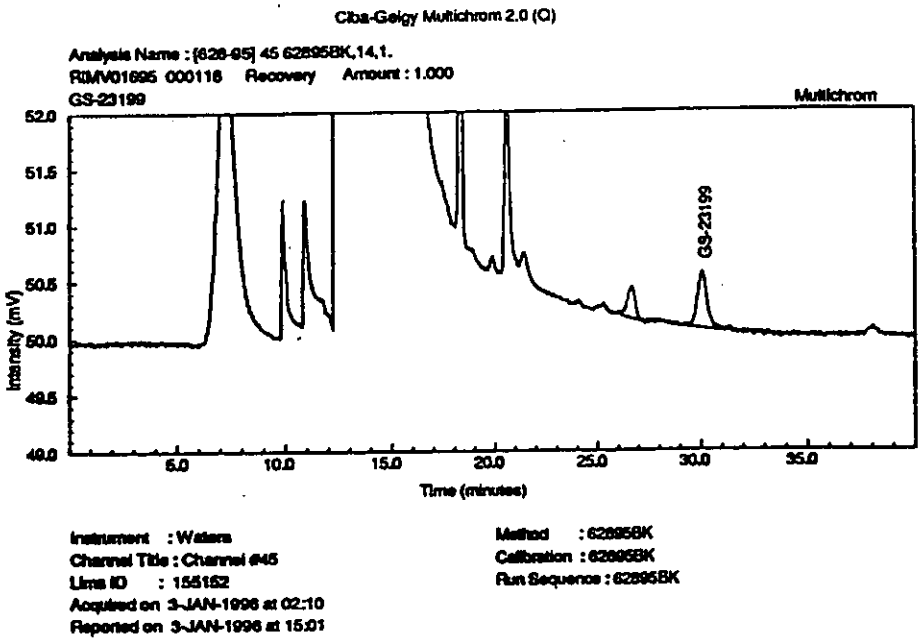


7. TC.10A, 0.10 ppm fortification, 25 mg injected, 1.48 ng GS-23199 found, 0.059 ppm GS-23199 found, 59% recovery

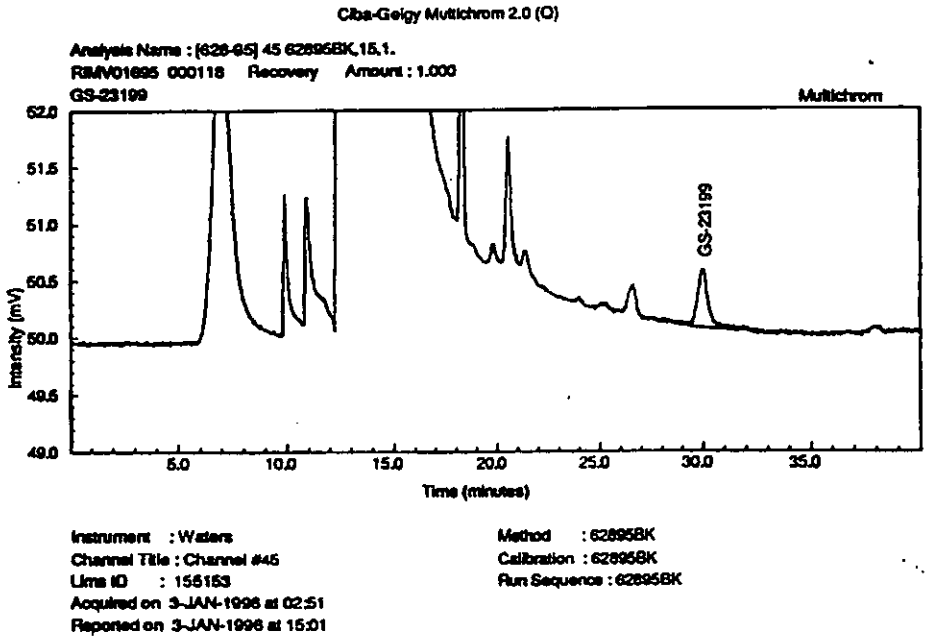


8. TC.10B, 0.10 ppm fortification, 25 mg injected, 1.80 ng GS-23199 found, 0.072 ppm GS-23199 found, 72% recovery

FIGURE 12. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CURED TOBACCO FOR GS-23199 (Continued)

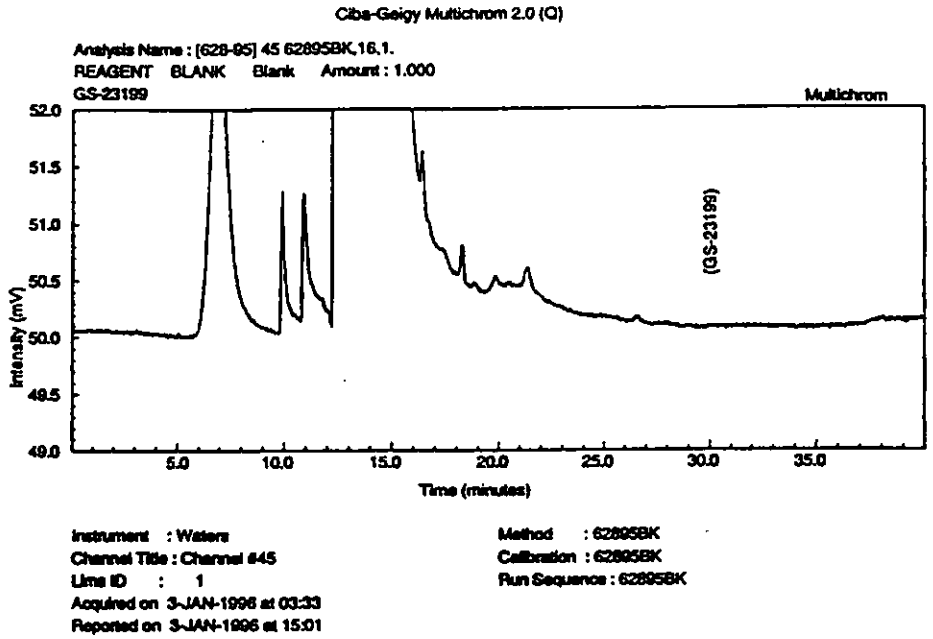


9. TC.20A, 0.20 ppm fortification, 25 mg injected, 2.62 ng GS-23199 found, 0.10 ppm GS-23199 found, 52% recovery



10. TC.20B, 0.20 ppm fortification, 25 mg injected, 3.12 ng GS-23199 found, 0.13 ppm GS-23199 found, 62% recovery

FIGURE 12. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF CURED TOBACCO FOR GS-23199 (Continued)



11. TC.RB, reagent blank, 50 mg injected, <1.0 ng found, <0.02 ppm GS-23199 found

VIII. REFERENCES

1. Tribolet, R., REM 154.05, "Determination of Metabolite GS-23199 by Single Column High Performance Liquid Chromatography"

2. Joseph, T. A., Thorn, T., Analytical Method AG-643, "Analytical Method for the Determination of CGA-215944 in Crops by HPLC."

3. Joseph, T. A., Thorn, T., Ciba Protocol 628-95, "Analytical Method for the Determination of GS-23199 in Crops by HPLC."

4. Joseph, T. A., Thorn, T., Residue Test Report RI-MV-016-95.

EPA ADDENDUM

1) ACB substituted equivalent equipment and materials.

A. The HPLC columns used were Zorbax 7u CN, 250 x 4.6 mm, 7um as in the method and Sphereclone 5u NH2, 250 mm x 4.6mm, 5um replacing Spherisorb 5 NH2, 250 x 4.6 mm, 5um described in the method as per communication with the petitioner .

B. To improve separation of GS-23199 on CN column, ACB increased the percentage of methanol in the mobile phase from 5 to 10 parts (90:10:5:1, hexane:methanol:isopropanol:acetonitrile) and increasing the retention time from ~ 6 min. to ~9 min.

2) If the petitioner wishes, the following can be added to the method as alternate supplies. ACB used a Hewlett Packard (HP) 1100 LC with diode array detector and a HP 1050 pump module for the second column. The sample was introduced on the first column, at approximately 8.5 min. a valve was opened to allow flow from first column directly onto second column, 1.5 min. later the valve was closed. The eluant from first column goes to waste (except when valve is in open position), the eluant from second column goes to the detector,

Column 1: Zorbax 7u CN, 250 x 4.6 mm, 7um
Mobile Phase 1: 90:10:5:1, hexane:methanol:isopropanol:acetonitrile
Column 2: Sphereclone 5u NH2, 250 mm x 4.6mm, 5um
Mobile Phase 2: 70:20:10:1, methanol:acetonitrile:isopropanol:hexane
Flow Rate: 1 ml/ min
Injection volume: 100 ul
Detector parameters
- Sample signal: 260 nm
- Sample bandwidth: 20 nm
- Slit: 8 nm
- Reference signal: 380 nm
- Reference bandwidth: 80 nm

3) Sample calculation should be corrected to read more clearly as:

$$\text{mg sample injected} = \frac{(\text{sample weight, g})(\text{aliquot taken, ml})(\text{volume injected, ml})(1000\text{mg/g})}{(\text{total extract volume, ml})(\text{final volume, ml})(\text{dilution factor})}$$

4) A correction needs to be made in section II.I.1.0, the concentration of the stock solution is 200 ug/ml not "mg/ml".

5) A correction in Table I needs to be made. The detection wavelength is 260 nm. There is an odd spacing in the written method.

Hops Method

Rec'd directly from Petitioner

GS-23199 Crop Method

EXTRACTION

Weigh 5 g semi-frozen sample into 8 oz. wide mouth jar and fortify.
Add 8-mL 0.05M sodium borate and 80-mL MeOH. Cap and shake at approx. 250 osc/hr for 1 hr.
Vacuum filter through Whatman 5 into 250-mL side arm flask. Rinse filter cake with 10-mL MeOH.
Transfer to 100-mL volumetric, adjust to volume with MeOH, and shake.

C18 SOLID PHASE (Use full vacuum for all SPE)

First C18: Precondition a 6-mL, 1000-mg BakerBond SPE C18 cartridge with 6-mL of methanol followed by 6-mL of water. Add 3 -mL of water to a 10-mL aliquot of the sample extract and pass through the cartridge under vacuum, collecting the eluate in a 50-ml concentration tube. Wash the cartridge with 5-mL of 70/30 methanol/water, collecting the eluate in the same tube as initial load. Allow the cartridge to dry under full vacuum for at least 30 seconds. Quantitatively transfer eluate to a 50ml RBF with MeOH. Rotovap to aqueous (sample should become cloudy when it reaches aqueous).

Second C18: Add 3.5-ml of MeOH to eluate (sample should become clear). Precondition a 6-mL, 1000-mg BakerBond SPE C18 cartridge with 6-mL of methanol followed by 6-mL of water. Pass extract through the cartridge under vacuum, collecting the eluate. Wash the cartridge with 5-mL of 39/61 methanol/water, collecting the eluate in the same tube as initial load. Allow the cartridge to dry under full vacuum for at least 30 seconds. Quantitatively transfer eluate to a 100ml RBF with MeOH. Rotovap to aqueous.

PARTITION

Transfer the solution to a 60-mL sep funnel. Rinse round bottom with 1.5-ml H_2O and add to sep funnel.
Adjust pH to approx. 4.5 with 0.5 M H_3PO_4. Partition for 30 secs. with 20-mL EtOAc (if an emulsion forms, apply heat using warm air such as a hair dryer). Drain lower, aqueous fraction back into rb and drain EtOAc through approx. 10 g of anhydrous Na_2SO_4 in a fritted glass funnel into a 100-mL rb.
Repeat partition 2 X 15 mL EtOAc and combine the organic phases. Rinse the Na_2SO_4 with 5-mL EtOAc and collect.
Add two drops 95/5 (v/v) decane/ diethylene glycol diethyl ether Evap. to dryness at <30°.

Reconstitute in 60/20/20 (v/v/v) HX/MeOH/IPA. Sonicate, mix well before vialing.

Sample ID	Final Volume		Sample ID	Final Volume

Ch:

WS: