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VOLUME 110 OF 134 OF SUBMISSION

CGA-293343

TITLE

ANALYTICAL METHOD FOR THE DETERMINATION OF RESIDUES OF CGA-293343 AND THE METABOLITE CGA-322704 IN ANIMAL AND CROP SUBSTRATES BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY WITH DETECTION BY UV AND MASS SPECTROMETRY, INCLUDING VALIDATION DATA

DATA REQUIREMENT

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VOLUME 1 OF 1 OF STUDY

STATEMENT OF NO DATA CONFIDENTIALITY CLAIM

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

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STATEMENT CONCERNING GOOD LABORATORY PRACTICES

The Good Laboratory Practice Compliance Statement regarding the U S Environmental Protection Agency's Good Laboratory Practice Standards (40 CFR Part 160, October 16 1989) provided on page 44, of this submittal volume for AG-675 and signed by the Study Director, is truthful and accurate

Robert K Williams
Manager of Residue Chemistry and
Representative of Submitter/Sponsor

11/6/98
Date

Human Safety Department
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(formerly Ciba Crop Protection)
Greensboro, North Carolina

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I SUMMARY AND INTRODUCTION

A SCOPE

Residues of the insecticide CGA-293343 and its major metabolite, CGA-322704, are analyzed in crop and animal commodities (including processed fractions where applicable) The chemical structures of CGA-293343 and CGA-322704 are shown in Figure 1 Determination of CGA-293343 and CGA-322704 in animal substrates, fruits, vegetables, oils, and grains is possible using a normal phase High Performance Liquid Chromatography/ultraviolet absorption detection (HPLC/UV) method The limit of detection (LOD) for CGA-293343 and CGA-322704 is 1.25 ng, and the limit of quantitation (LOQ) is 0 01 ppm for all substrates except milk and fruit juices The LOQ for milk and juices is reduced to 0.005 ppm

Analysis of cotton substrates, tobacco samples, and forage, fodder and straw of cereal grains and grasses are not possible using the HPLC/UV method at the low quantitation limits These substrates are analyzed using a reverse phase High Performance Liquid Chromatography/Mass Spectrometry (HPLC/MS) method The sample purification procedures for the HPLC/MS method differs slightly from the HPLC/UV method The LOD for the HPLC/MS method 0.25 ng, and the LOQ is 0.01 ppm (LOQ of grass is 0 05 ppm)

Method validation was performed as part of Study 206-97 on representative commodities of animal substrates, vegetables, pome fruits, cereal grains, forage, fodder and straw of cereal grains, grasses, tobacco, and cotton substrates The validation study included analysis of control (untreated) samples, control sample fortified with CGA-293343 and CGA-322704 for determination of method recovery, and analysis of ^{14}C-CGA-293343 treated metabolism samples for determination of extractability and accountability of incurred residues A summary of validation results and representative chromatograms are included in the body of the method Appendix I to Method AG-675 includes all chromatograms from all commodities analyzed, all validation data needed to reproduce calculations, and other information related to the validation study. Appendix II includes validation of Method AG-675 for a potentially significant poultry metabolite Analytical Method AG-675 and the appendices are the final report for Study 206-97

B PRINCIPLE

1 0 Extraction

Ten-gram samples are extracted twice by homogenization in acetonitrile (ACN) water (80.20) Liquid samples such as milk, eggs, and juices are extracted by shaking for 20 minutes in ACN water (80 20) The total extract volume is 200 mL Oil samples are extracted using a liquid-liquid partition

2 0 Analysis Of Animal Substrates, Fruits, Vegetables, Grains, and Oils By HPLC/UV

A 100 mL aliquot is measured (for milk and juices, the entire 200 mL is analyzed) For fruit, vegetable, and grain samples, the aliquot is evaporated until only the aqueous phase remains. For animal substrates, a liquid-liquid partition is performed prior to evaporation. The reduced, aqueous sample is first purified by reverse-phase solid-phase extraction (SPE) by loading onto a phenyl cartridge column After elution from the phenyl SPE column with methanol water (1.1), the sample is evaporated to aqueous and the compounds are partitioned into ethyl acetate The ethyl acetate fraction is evaporated and the sample is further purified by normal phase SPE using both an amino cartridge column and an alumina cartridge column After elution from the alumina column, the samples are evaporated and reconstituted in mobile phase for determination by normal phase HPLC/UV The normal phase column is a Waters Spherisorb S5 NH_2 (250 mm x 4 6 mm I D), with a mobile phase of hexane:ethyl acetate: isopropanol.methanol (11 3 1.1) Confirmation of residues is possible by evaporating the final fraction, reconstituting the sample in ACN·water (10·90) and analyzing using HPLC/MS or HPLC/MS/MS instrumentation.

3 0 Analysis of Cotton, Tobacco, and Forage, Fodder and Straw of Cereal Grains and Grasses By HPLC/MS

A 50 mL aliquot of the extract is measured, and the sample is evaporated to aqueous The aqueous sample is buffered at pH of 7 0 and is purified by passing through a Strong Anion Exchange (SAX) SPE column The eluent from the SAX column is loaded on a phenyl SPE column, the sample is eluted from the phenyl column using water methanol (1 1), and the eluent is evaporated to aqueous The

aqueous sample is partitioned into ethyl acetate, and the sample is further purified by normal phase alumina SPE After elution from the alumina column, the sample is evaporated to dryness and reconstituted in ACN water (10:90) and analyzed using reverse-phase HPLC/MS The reverse phase column is a Zorbax SB-C18 LC-MS column, using a gradient mobile phase of 0.1% acetic acid in acetonitrile and water Should further confirmation of residues be necessary, an HPLC/MS/MS method is provided

II MATERIALS AND METHODS

A APPARATUS/EQUIPMENT

1 0 Bottles, Owens-Brockway square amber, 16-oz with 53 mm wide mouth, or equivalent (Penn Bottle Co)

2 0 Concentration Tubes, conical-bottom 50-mL capacity (Fisher #05-507-5C or equivalent)

3 0 Filter Paper, Whatman Qualitative #5, 7 cm (Whatman #1005070, or equivalent)

4 0 Filter Flask Adapter, neoprene 46 x 29 mm (OD top x bottom) (Fisher #10-18-4, or equivalent)

5 0 Flasks, KIMAX with side-arm, 500-mL (Fisher #10-181E, or equivalent)

6 0 Flasks, Boiling with flat bottom, 125-mL and 500-mL (Fisher #09-552A and 09-552C, or equivalent)

7 0 Flasks, Boiling with round bottom, 50-mL (Fisher #K601000-0124, or equivalent)

8 0 Funnel, Buchner porcelain (Fisher #10-356-C or equivalent)

9 0 Funnel, Separatory, 60-mL and 250-mL (Fisher #10-437-10A and 10-437-10C, or equivalent)

10 0 Graduated cylinder, 10-mL, 50-mL, 100-mL, 250-mL (Fisher #08-551A, 08-551C, 08-551D and 08-551E, or equivalent)

11 0 Homogenizer, Polytron (Brinkman Instruments or equivalent)

12 0 Pasteur pipettes (Kimble 13-678-30B, 13-678-30C, or equivalent)

13 0 Pipettes, volumetric class A, 1-mL, 2-mL and other sizes as needed (Fisher #13-650-2, or equivalent)

14 0 Reservoir, 15-mL for solid phase extraction, (J T Baker #7119-01 or equivalent), with adapter (J T Baker #7122-00, or equivalent)

15 0 Rotary Evaporator, Buchii or equivalent, with a variable temperature water bath (Buchler Instruments, or equivalent), and aspirator pump (Cole-Parmer, or equivalent) The use of a bump trap is suggested (Fisher #K570200-2524, or equivalent), and an adapter for the 50-mL concentration tubes is needed (Fisher # 01-035D, or equivalent)

16 0 Shaker, Orbital, IKA Labortechnik #KS501, or equivalent

17 0 Solid Phase Extraction, Varian MegaBond Elut Phenyl, 6cc/1 0 gram (Varian Part Number 1225-6004)

18 0 Solid Phase Extraction, Varian MegaBond Elut SAX, 6cc/1 0 gram (Varian Part Number 1225-6013)

19 0 Solid Phase Extraction, Varian Bond Elut Amino, 1cc/100 mg (Varian Part Number 1210-2014)

20 0 Solid Phase Extraction, Waters Alumina, 6 cc/1 0 gram (Part Number WAT054580) Note - a 12 cc/2 gram cartridge (Part Number WAT054620) has been used successfully as well

21 0 Ultrasonicator (Heatsystems Inc., or equivalent)

22.0 Vacuum Manifold for solid phase extraction, (Supelco Part Number 5-7250, or equivalent)

23 0 Vials, crimp top borosilicate with Teflon lined caps (Sun Broker, Inc or equivalent)

B REAGENTS

1 0 Acetic Acid, ACS certified (Fisher #A38-500, or equivalent)

2 0 Acetonitrile, HPLC grade (Fisher #A998-4, or equivalent)

3 0 Celite 545 Filter Aid (Fisher #C212-500, or equivalent)

4 0 Ethyl Acetate, HPLC grade (Fisher #E195-4, or equivalent)

5 0 Hexane, HPLC grade (Fisher #H302-4, or equivalent)

6.0 Isopropyl Alcohol (2-propanol), HPLC grade (Fisher #A451-4, or equivalent)

7 0 Methanol, HPLC grade (Fisher # A452-4, or equivalent)

8 0 Sodium Chloride, Certified ACS grade (Fisher #S271-3 or equivalent)

9 0 Sodium Phosphate, Dibasic Anhydrous, ACS Certified (Fisher # S374-500, or equivalent)

10 0 Sodium Phosphate, Monobasid Monohydrate, ACS Certified (Fisher # S369-500, or equivalent)

11 0 Toluene, High Purity Solvent grade (Burdick and Jackson #347-4 or equivalent)

12 0 Water, HPLC grade (picopure, or equivalent) For LC/MS mobile phase, use OPTIMA grade water (Fisher # W7-4, or equivalent)

13 0 Acetonitrile water (v/v) - Prepare 1 liter of (80 20) by adding 800 mL of acetonitrile to 200 mL of water Prepare 1 liter of (10 90) by adding 100 mL of acetonitrile to 900 mL of water

14 0 0 1% Acetic Acid in water (v/v) - Prepare 1 liter by adding 1 mL of acetic acid to 999 mL of OPTIMA grade water 0 1% acetic acid in Acetonitrile - Prepare 1 liter by adding 1 mL of acetic acid to 999 mL of acetonitrile.

15 0 50 mM Sodium Phosphate Buffer, pH approximately 7 0 - Prepare 1 liter by adding 500 mL of 50 mM sodium phosphate monobasic monohydrate (3.45 grams in 500 mL) and 500 mL of 50 mM sodium phosphate dibasic anhydrous (3 55 grams in 500 mL)

16 0 10 mM Sodium Phosphate Buffer, pH approximately 7 0 - Prepare 1 liter by adding 200 ml of 50 mM sodium phosphate buffer (Section II.B 15 0) to 800 mL water

17 0 2% Toluene in Acetonitrile (v/v) - Prepare 1 liter by combining 20 mL of toluene with 980 mL acetonitrile.

18 0 Methanol:water (v/v) - Prepare 1 liter of (50:50) by adding 500 mL methanol to 500 mL water

19 0 Ethyl Acetate Hexane (v/v) - Prepare 1 liter of (20 80) by adding 200 mL of ethyl acetate to 800 mL of hexane Prepare 1 liter of (50 50) by adding 500 mL of ethyl acetate to 500 mL of hexane

20 0 Methanol Ethyl Acetate(v/v) - Prepare 1 liter of (3 97) by adding 30 mL of methanol to 970 mL of ethyl acetate Prepare 1 liter of (10 90) by adding 100 mL of methanol to 900 mL of ethyl acetate

21 0 Hexane Ethyl Acetate Isopropanol Methanol (11:3 1 1) (v/v) - Prepare 2 0 liter by combining 1375 mL hexane, 375 mL ethyl acetate, 125 mL isopropyl alcohol and 125 mL methanol

22 0 Water Saturated with Sodium Chloride - Prepare by adding at least 357 grams sodium chloride per liter of water (at room temperature), and mix well.

23 0 CGA-293343, analytical standard supplied by Chemical Synthesis Group, Novartis Crop Protection, 410 Swing Rd., Greensboro, NC, 27419

24 0 CGA-322704, analytical standard supplied by Chemical Synthesis Group, Novartis Crop Protection, 410 Swing Rd , Greensboro, NC, 27419

C ANALYTICAL PROCEDURE

1 0 Extraction

1 1 Sample Preparation

Animal samples are prepared using the procedures outlined in the current revision of the Novartis Human Safety Standard Operating Procedure (SOP) number 7 27[1] Crop substrates are prepared using the procedures outlined in the current revision of Novartis Biochemistry SOP 7.21[2] These SOP's follow the guidelines of the United States Food and Drug Administration Pesticide Analytical Manual, Volume I, Sections 102 and 203 Solid samples are cut into 1-2 inch pieces and ground in a Hobart food cutter, using dry ice as necessary to keep the sample frozen Liquid samples such as juice, milk, and eggs are fully thawed and well mixed or blended

1 2 Extraction of All Substrates Excluding Eggs, Milk, and Juices and Oils

Weigh a 10-gram sample of homogenized crop samples or animal tissues into a tared extraction bottle Fortify recovery samples as specified in Section II I.2 0 Add 150 mL of ACN:water (80 20) and grind the sample for 1 minute with a Polytron Homogenizer at high speed (~8000-12000 rpm) Next allow the solid material in the extract to settle out (approximately 1-3 minutes) Place a Whatman #5 filter paper in a Buchner funnel on a 500-mL side-arm flask equipped with a filter flask adapter, and attach to a vacuum source Moisten the filter with water, and cover the filter with a bed of Celite Filter Aid (approximately 1-2 cm thick) Decant the solvent and any suspended material onto the Celite and filter, and leave the precipitate in the bottom of the extraction bottle Add 50 mL of ACN water (80:20) to the solid material left in the extraction bottle Homogenize a second time for 1 minute with the Polytron Homogenizer at high speed Filter the second extract through the same filter and into the same flask (pour the solid material into the filter funnel to recover as much solvent as possible)

See section II H 1 0 for possible modifications and potential problems with the extraction procedure

Measure the volume of the extract using a 250-mL stoppered graduated cylinder If necessary, add ACN water (80 20) for a total volume of 200 mL (NOTE - with some substrates, the volume may be greater than 200 mL due to the moisture contained in the sample, and the actual volume should be accounted for in the final calculations) Thoroughly mix the final extract.

1 3 Extraction of Liquid Substrates Such As Eggs, Milk, or Juice

Weigh a 10-gram sample of eggs, milk, or juice into a tared extraction bottle Fortify recovery samples as specified in Section II.I.2 0 Add 150 mL of ACN water (80:20) and shake on an orbital shaker for 20 minutes Place a Whatman #5 filter paper in a Buchner funnel on a 500-mL side-arm flask equipped with a filter adapter (attached to a vacuum system). Moisten the filter with water and cover the filter with an approximately 1 5-cm bed of Celite Filter Aid Decant and filter the entire sample Rinse the extraction bottle with 50 mL of ACN.water (80 20), and filter the rinsate through the same filter and into the same flask

See section II.H 1 0 for modifications to the extraction procedure

Measure the volume of the extract using a 250-mL graduated cylinder If necessary, add ACN water (80.20) for a total volume of 200 mL (NOTE - with some substrates, the volume may be greater than 200 mL due to the moisture contained in the sample, and the actual volume should be accounted for in the final calculations)

1 4 Extraction of Oils

Weigh 5 grams of well mixed oil sample directly into a tared 250-mL separatory funnel that is held upright in a beaker for weighing Fortify as specified in Section II I 2 0, making necessary adjustment for fortification of a 5-gram sample Add 100 mL ACN water (80·20) Proceed to section II C 2 1

2 0 Analysis Of Animal Substrates, Fruits, Vegetables, Grains, and Oils By HPLC/UV

2 1 Liquid-Liquid Partition of Extract for Animal Substrates and Oils

NOTE. THE PROCEDURE IN THIS SECTION IS FOR ANIMAL SUBSTRATES AND OILS WHEN ANALYZING BY HPLC/UV FOR FRUIT, VEGETABLE AND GRAIN SUBSTRATES, SKIP TO SECTION II C.2.2

2 1 1 For all animal substrates except milk, measure a 100-mL aliquot of the extract and pour it into a 250-mL separatory funnel (for oils, the 5-gram sample will have been weighed directly into the separatory funnel, and 100 mL of ACN water (80 20) will have been added as specified in section II C.1 4) Add 5 mL of water saturated with sodium chloride, 2 mL of toluene, and 20 mL of hexane. For milk, pour the entire 200-mL sample extract into a 500-mL separatory funnel, and add 10 mL of water saturated with sodium chloride, 4 mL of toluene, and 40 mL of hexane Partition the samples by shaking for 1 minute Allow the layers to separate after the partition This partition forms three layers, a bottom, aqueous layer (which will contain residual amounts of CGA-293343 and CGA-322704), a middle layer comprised primarily of ACN and toluene (which will contain the majority of CGA-293343 and CGA-322704, as well as some residual water), and a top, hexane layer Drain the lower, aqueous layer and save in a beaker or flask Drain the middle, ACN and toluene layer into a 500-mL flat bottom boiling flask and save Drain the top, hexane layer and discard

2 1 2 Pour the lower aqueous layer (saved previously in a beaker or flask) back into the 250-mL separatory funnel (500-mL separatory funnel for milk) For all substrates (including milk), add 40 mL of 2% toluene in ACN and partition by shaking for 1 minute Allow the layers to separate after the partition This partition forms two layers; a bottom, aqueous layer and a top ACN and toluene layer Drain and discard the bottom,

aqueous layer Save the top layer (ACN and toluene) in the same 500-mL boiling flask used in Section II.C 2 1 1.

2.1 3 Evaporate the sample using a vacuum rotary evaporator (fitted with a bump trap and a water bath of 35-40°C) Evaporate until all of the ACN and toluene has been removed, and only aqueous remains. The ACN and toluene has been removed when the rate of solvent dripping off of the rotary evaporator condensers slows or stops, and when aqueous condensation is observed on the walls of the bump trap (the volume of aqueous remaining is generally about 5 mL) Proceed to section II C 2.3

2 2 Evaporation of Extract of Fruit, Vegetable, and Grain Samples

NOTE THE PROCEDURE IN SECTION II.C 2.2 IS FOR FRUIT, VEGETABLE, AND GRAIN SUBSTRATES ANALYZED BY HPLC/UV ONLY

Pour a 100-mL aliquot of the extract into a 500-mL round bottom flask. For juices, pour the entire 200-mL extract into a 500-mL round bottom boiling flask Evaporate the sample using a vacuum rotary evaporator fitted with a bump trap (water bath of 35-40°C) Evaporate the sample until all of the ACN has been removed and only aqueous remains The ACN has been removed when the rate of solvent dripping off of the rotary evaporator condensers slows or stops, and when aqueous condensation is observed on the walls of the bump trap The final volume of the aqueous sample should be less than 20 mL Proceed to Section II C 2.3

2 3 Phenyl Solid Phase Extraction

Column Conditioning

Attach a 15-mL reservoir to a Varian Bond Elut Phenyl, 6cc/1.0 gram Solid Phase Extraction (SPE) column with an adapter, and place the column on a vacuum manifold system with a stopcock to control flow Condition the column by flushing with 5 mL of methanol followed by 5 mL of water Measure solvents with graduated cylinders (or equivalent

accuracy), and let each solvent used in the following procedure pass through the column until it reaches the top of the phenyl packing Do not let the packing become dry The flow rate through the SPE column should be maintained at about 1 to 2 drops per second with the vacuum manifold system

Sample Addition

Pour the sample from Section II.C 2 1 3 or II C 2.2 into the reservoir above the column Allow the sample to pass through the column without drying the phenyl packing Discard the load eluate.

Column Wash

Add 3 mL of water to the flask that contained the aqueous sample Remove as much material as possible from the side walls of the glassware using sonication (note that for some substrates, material may remain on the walls of the glassware after sonication) Pour the rinse into the column reservoir and allow it to pass through the column without drying the phenyl packing Discard the rinse eluate

Sample Elution

Add 10 mL of methanol:water (50.50) to the boiling flask that contained the sample, swirl and sonicate well to remove all material from the walls of the boiling flask Remove as much material as possible (see section II H.2 0 for possible modification) Place a 50-ml concentration tube under the phenyl column Pour the elution solvent into the column reservoir and allow it to pass through the column Collect the eluate in the 50-mL concentration tube

2.4 Liquid-Liquid Partition of Phenyl Eluate

Evaporate the sample from Section II.C 2 3 using a vacuum rotary evaporator (fitted with an adapter and a water bath at 35-40°C) Evaporate until all the methanol has been removed and only the aqueous layer remains The methanol has been removed when the rate of solvent dripping off of the rotary evaporator condensers slows or stops, and when aqueous

condensation is observed on the walls of the bump trap The volume should be less than or equal to 5 mL after the aqueous portion has been removed.

Add 1 mL of water saturated with sodium chloride to the sample in the concentration tube Add 20 mL of ethyl acetate and stopper the concentration tube with a teflon or plastic stopper. Partition by shaking or vortexing for 1 minute Allow the layers to separate, the top layer is the organic, ethyl acetate layer containing CGA-293343 and CGA-322704 NOTE - as an alternative to performing the partition directly in the concentration tube, the sample may be transferred to a 60-mL separatory funnel for partitioning

Remove the top ethyl acetate layer (e g with a pasteur pipet) and transfer to a 125-mL round bottom flask Remove as much of this layer as possible without transferring any drops of the lower aqueous layer

Add 20 mL of ethyl acetate to the aqueous layer in the concentration tube Partition a second time by shaking or vortexing for one minute Allow the layers to separate, and transfer the top, ethyl acetate layer to the 125-mL round bottom flask Remove as much of this layer as possible without transferring any drops of the lower aqueous layer No water droplets should be transferred into the 125-mL round bottom flask. If any water droplets are transferred, minimize them by removing with a Pasteur pipette Discard the aqueous phase

Evaporate the ethyl acetate sample using a vacuum rotary evaporator (fitted with a bump trap and a water bath at 35-40°C) Evaporate until all of the solvent has been removed If some of the aqueous phase is accidentally carried over from the liquid-liquid partition, it must be evaporated Add 3-5 mL of isopropyl alcohol (or another appropriate solvent) to aid in evaporation of the water, and evaporate to dryness as before. Reconstitute in 5 mL of ethyl acetate.hexane (20 80) Swirl

and sonicate the reconstituted sample The use of sonication is essential to bring CGA-322704 and CGA-293343 into organic solution

2 5 Amino and Alumina Solid Phase Extraction

NOTE The normal phase SPE purification step uses two columns The sample is loaded onto an amino column (= column 1) and the column is washed The load and wash eluates are discarded The compounds are then eluted from column 1 The column 1 eluate is loaded directly onto an alumina column (= column 2), and the load eluate from this column is collected A second, stronger solvent is then used to complete elution from column 2 (column 2 load eluate and final elution solvent are collected and combined) As an option, transfer of the sample from column 1 to column 2 can be accomplished efficiently by stacking column 1 on top of the column 2 prior to elution As the sample elutes from column 1, it will pass directly through column 2 and be collected as it elutes Column 1 is then discarded, and the final elution of the sample from column 2 is performed

Conditioning of Columns

1 Varian Bond Elut Amino SPE column (1 cc/100mg) - Attach a 15-mL reservoir to the top of the alumina column with an adapter Condition the column by flushing with 1 mL of methanol·ethyl acetate (3 97) followed by 1 mL of ethyl acetate.hexane (20 80)

2 Waters Alumina SPE column (6 cc/1g) - Condition column by flushing with 5 of mL methanol ethyl acetate (10.90) followed by 5 of mL methanol ethyl acetate (3 97) Note - If the sample will be transferred from the first column (amino) to the second column (alumina) by stacking the columns, leave at least 2 cm of the last flush solvent above the top of the alumina packing This is needed to ensure that the alumina packing will not go dry during the transfer step

Measure volumes with graduated cylinders (or equivalent accuracy), and let each solvent used in the following procedure pass through the column until it reaches the top of the packing, do not let the packing become dry The flow rate through the SPE columns should be maintained at about 1 to 2 drops per second (a vacuum manifold system may be used, but will not normally be needed)

Sample Addition to the Amino Column

Pour the sample from Section II C 2 4 (in ethyl acetate.hexane 20·80 in a 125-mL round bottom flask) into the reservoir above the amino column (note that material will be left behind on the walls of the round bottom flask for most substrates, which will be removed in subsequent rinse steps below) Allow the sample to pass through the column (stop the flow when the solvent reaches the top of the column frit) Discard the load eluate

Amino Column Wash

Add 1 mL of ethyl acetate:hexane (50 50) to the 125-mL round bottom flask that contained the sample, swirl, and sonicate well. Remove as much material as possible (note that for some substrates, material may remain on the glassware after this wash) Pour into the reservoir above the amino column, and allow the sample to pass through the column (stop the flow when the solvent reaches the top of the column frit) Discard the wash eluate

Sample Elution from the Amino Column and Transfer to the Alumina Column

Add 10 mL of methanol ethyl acetate (3 97) to the 125-mL round bottom flask that contained the sample, swirl, and sonicate to remove material from the walls of the boiling flask NOTE - sonication at this step is essential Pour the elution solvent into the column reservoir and allow it to pass through the amino column Collect the eluate in a beaker or flask Pour the eluate from the amino column (column 1) onto the alumina column (column 2) Collect the load eluate in a 50-mL round bottom flask or a 50-mL concentration tube

Alternatively, the amino column may be stacked on top of the alumina column to transfer the eluate directly If this option is chosen, use the following procedure Ensure that the alumina column (column 2) has at least 2 cm of conditioning solvent (methanol ethyl acetate (3.97)) above the packing Attach the outlet of the amino column (column 1) to the top of the alumina column (column 2) using an adapter (the adapter must create an air-tight fit). Place a 50-mL concentration tube under column 2 to collect all of the load solvent that elutes from column 2. Elute the sample from the amino column as described above Remove and discard the amino column (column 1) after elution onto column 2 is complete

Elution from the Alumina Column

Add 15 mL of methanol.ethyl acetate (10 90) to the 50-mL round bottom flask, swirl, and sonicate well to remove material from the walls of the boiling flask Pour the elution solvent directly into the alumina column (column 2), and collect the eluate in the same 50-mL concentration tube that contains the load eluate

2 6 Preparation of Final Sample

Evaporate the sample from Section II C 2 5 to dryness using a vacuum rotary evaporator (fitted with a bump trap and a water bath of 35-40°C) Use a class A volumetric pipette to reconstitute in the appropriate volume of hexane ethyl acetate isopropanol:methanol (11.3.1.1). A 2-mL final volume is used for samples screened at the method LOQ of 0 01 ppm (0 005 for milk and juices) Sonication and vortex mixing is essential to fully reconstitute the sample in the injection solvent The volume may be increased as needed to bring the residue concentrations within the range of the standards Transfer the sample to an injection vial for automated analysis See Section II D 1 1 for instrumental analysis

3.0 Analysis of Cotton, Tobacco, and Forage, Fodder and Straw of Cereal Grains and Grasses By HPLC/MS

3 1 Evaporation of Extracts

Measure a 50-mL aliquot of the extracts obtained in Section II C 1.2 and pour the aliquot into a 250-mL round bottom boiling flask. Evaporate the sample using a vacuum rotary evaporator (fitted with a bump trap and a water bath of 35-40°C) Evaporate the sample until all of the ACN has been removed and only the aqueous content of the sample remains The ACN has been removed when the rate of solvent dripping off of the rotary evaporator condensers slows or stops, and when aqueous condensation is observed on the walls of the bump trap The final volume of the aqueous sample should be less than 10 mL

3 2 Strong Anion Exchange (SAX) and Phenyl SPE

NOTE This step involves two SPE columns to purify the sample The sample is buffered to pH 7 0 and is passed through an SAX column CGA-293343 and CGA-322704 pass through this column, and the load and flush eluate is collected and loaded directly onto a phenyl SPE column The load eluate of the phenyl column is discarded, and the sample is eluted from the phenyl column with methanol·water (1 1) Transfer of the sample from the SAX column to the phenyl column can be performed by stacking the two columns in series, but stacking of columns is not recommended for substrates that have particulates in the SAX load solvent that may partially clog the first column

Column Conditioning

1 Attach a 15-mL reservoir to a Varian Bond Elut SAX, 6cc/1.0 gram SPE column with an adapter, and condition by flushing the column with 5 mL of 10 millimolar (mM) sodium phosphate buffer

2 Condition a Varian Bond Elut Phenyl, 6cc/1 0 gram SPE column by flushing with 5 mL of methanol followed by 5 mL of 10 mM sodium phosphate buffer If the two

columns are to be stacked in series, add back another 3-5 ml of 10 mM buffer

Measure solvents with graduated cylinders (or equivalent accuracy), and let each solvent used in the following procedure pass through the column until it reaches the top of the SPE packing, and do not let the packing become dry The flow rate through the SPE column should be maintained at about 1 to 2 drops per second with the vacuum manifold system

Pass Sample Through the SAX Column

Add 3 mL of 50-mM sodium phosphate buffer, pH 7 to the aqueous sample in the 250-mL round bottom flask, and mix well Pour the sample into the reservoir above the SAX column and collect the load eluate in a beaker or flask Allow the sample to elute to the top of the SAX packing, and do not let the packing become dry See Section II H 4.0 for note on clogging of the SAX column

Add 5 mL of 10-mM sodium phosphate buffer to the 250-mL round bottom flask, and sonicate well. Pour the 5 mL wash into the reservoir above the SAX and collect the SAX eluate in the same beaker or flask (NOTE - this wash is performed after the load sample has passed through the SAX column) Pour the eluted sample from the SAX column into the reservoir above the phenyl SPE column. Allow the sample to pass through the column and do not let the packing become dry Discard the load eluate.

Alternative to collecting in a beaker or flask, the sample may be loaded directly onto the phenyl column by stacking the two columns is series If this option is chosen, perform the following procedure Prior to loading of the sample onto the SAX column, ensure that the phenyl column has at least 3-5 mL of conditioning solvent (10-mM sodium phosphate buffer) above the packing Attach the outlet of the SAX column to the top of the phenyl column using an adapter (the adapter must create an air-tight fit) Pass the sample through the SAX column as described above, and allow the sample

eluting from the SAX to pass through the phenyl column Perform the 5 mL column rinse with 10 mM sodium phosphate buffer (as described above)

When this is complete, CGA-293343 and CGA-322704 will have passed through the SAX column and will be trapped on the phenyl column. Remove and discard the SAX column, and discard the aqueous solvent that has eluted from the phenyl column.

Elution From the Phenyl SPE

Add 10 mL of methanol:water (1 1) to the boiling flask that contained the aqueous sample. Remove as much material as possible using sonication. Place a 50-mL concentration tube under the phenyl column Pour the elution solvent into the column reservoir and allow it to pass through the column. Collect the eluate in a 50-mL concentration tube

3 3 Liquid-Liquid Partition of Phenyl Eluate

Evaporate the sample from Section II C 3.2 using a vacuum rotary evaporator (fitted with an adapter and a water bath at 35-40°C). Evaporate until all the methanol has been removed and only the aqueous layer remains The methanol has been removed when the rate of solvent dripping off of the rotary evaporator condensers slows or stops, and when aqueous condensation is observed on the walls of the glassware. The volume should be ≤5 mL after the methanol has been removed

Add 1 mL of water saturated with sodium chloride to the sample in the concentration tube Add 20 mL of ethyl acetate (measured with a graduated cylinder) and stopper the concentration tube with a Teflon or plastic stopper Partition by shaking or vortexing for 1 minute Allow the layers to separate, the top layer is the organic ethyl acetate layer containing CGA-293343 and CGA-322704 NOTE - as an alternative to performing the partition directly in the concentration tube, the sample may be transferred to a 60-mL separatory funnel for partitioning

Remove the top ethyl acetate layer (e g. with a Pasteur pipet) and transfer to a 125-mL round bottom flask Remove as much of this layer as possible without transferring any drops of the lower aqueous layer

Add 20 mL of ethyl acetate to the aqueous layer in the concentration tube Partition a second time by shaking or vortexing for one minute. Allow the layers to separate, and remove the top ethyl acetate layer and transfer to the same 125-mL round bottom flask Remove as much of this layer as possible without transferring any drops of the lower aqueous layer No water droplets should be transferred into the 125-mL round bottom flask If any water droplets are transferred, minimize them by removing with a Pasteur pipette Discard the aqueous phase

Evaporate the ethyl acetate sample using a vacuum rotary evaporator (fitted with a bump trap and a water bath at 35-40°C) Evaporate until all of the solvent has been removed If some of the aqueous phase is accidentally carried over from the liquid-liquid partition, it must be removed Add 3-5 mL of acetonitrile to aid in evaporation of the water, and evaporate to dryness as before. Reconstitute in 5 mL of ethyl acetate hexane (20:80). Swirl and sonicate the reconstituted sample The use of sonication is essential to bring CGA-322704 and CGA-293343 into organic solutions

3.4 Alumina Solid Phase Extraction

Column Conditioning

Condition a 6 cc/1g Waters alumina SPE column (with a stopcock) by flushing with 5 mL methanol ethyl acetate (10 90) followed by 5 mL ethyl acetate hexane (20 80)

Measure each volume with graduated cylinders (or equivalent accuracy), and let each solvent used in the following procedure pass through the column until it reaches the top of the packing, and do not let the packing become dry The use of a vacuum manifold will not normally be needed

Sample Addition to the Alumina SPE Column

Pour the sample from Section II C 3 3 into the alumina SPE and allow it to pass through the column Discard the load eluate

Column Wash

Add 5 mL of ethyl acetate hexane (20 80) to the 125-mL round bottom flask and sonicate well NOTE· sonication is essential at this step to prevent losses. Pour the rinse eluate into the alumina column and allow it to pass through the column. Discard the wash eluate

Sample Elution

Add 10 mL of methanol ethyl acetate (10.90) to the 125-mL round bottom flask and sonicate and mix well Place a 50-mL concentration tube (or a 50-mL round bottom boiling flask) under the alumina column Pour the solvent into the alumina SPE, and allow it to pass the column Collect the eluate in a 50-mL concentration tube

3 5 Preparation of Final Sample

Evaporate the sample from Section II C 3 4 to dryness using a vacuum rotary evaporator fitted with an adapter (water bath of 35-40°C) Use a class A volumetric pipette to reconstitute in the appropriate volume of acetonitrile water (10 90). Sonication and vortex mixing is essential to fully reconstitute the sample in the injection solvent. A final volume of 1 mL is used for samples screened at the method LOQ of 0 01 ppm. The volume may be increased as needed to bring the residue concentrations within the range of the standards Transfer the sample to an injection vial for automated analysis See Section II D 1 2 for instrumental analysis

D INSTRUMENTATION

1 0 Description and Operating Conditions

1 1 Normal Phase HPLC/UV Analysis

Residues of CGA-293343 and CGA-322704 are determined by normal-phase HPLC/UV using a Waters Spherisorb NH_2 analytical column (4.6 x 250-mm). The mobile phase is Hexane:Ethyl Acetate: Isopropyl Alcohol Methanol (11 3 1·1), using a flow rate of 1 5 mL/minute Detection is by UV at 255 nm, and a 100-uL injection volume is used The run time for this system is about 30 minutes, but should be increased if late eluting peaks are observed The instrumental conditions for this system are detailed in Table I

1 2 Reverse Phase HPLC/MS Conditions

Residues of CGA-293343 and CGA-322704 are determined by reverse-phase HPLC/MS using a Zorbax LC-MS SB-C18 analytical column (2.1 x 50-mm) The mobile phase is a gradient using 0 1% acetic acid in water versus 0 1% acetic acid acetonitrile, which is detailed in Table II A flow rate of 0 2 mL/minute is used, and the flow is split post-column at a 3 1 ratio (waste·mass spectrometer) A 20-uL injection volume is used Detection of CGA-293343 is accomplished by scanning for the $M+H^+$ ion of mass:charge ratio of 292 Detection of CGA-322704 is accomplished by scanning for the $M+H^+$ ion of 250 (alternatively, the $M-H^-$ ion of mass·charge of 248 can be used for CGA-322704) Validation of this method was performed using a Micromass Platform LC (for HPLC/MS analyses) and a PE-Sciex API III (for HPLC/MS/MS analyses) See Table II for the instrumental conditions using the Micromass Platform LC Table 3 shows the instrumental conditions when using the PE-Sciex API III for analysis by LC/MS/MS

2 0 Standardization

2.1 Standardization of the HPLC/UV System

Standardize the HPLC system by injecting 100-µL aliquots of standard solutions containing CGA-293343 and CGA-322704 in a range of 1 25 to 100.0 ng (injected on the column) Generate a linear regression from the data by comparing detector response and ng injected See Section II.I 1 1 for preparation of analytical standards It is necessary to run a set of standards with each analytical set to achieve accurate quantitation of residues

2 2 Standardization of the HPLC/MS Systems

Standardize the HPLC/MS system by injecting 20-µL aliquots of standard solutions containing CGA-293343 and CGA-322704 in a range of 0 25 to 10 0 ng (injected on the column) Generate a linear regression from the data by comparing detector response and ng injected See Section II.I.1 2 for preparation of analytical standards It is necessary to run a set of standards with each analytical set to achieve accurate quantitation of residues The same procedure is used when analyzing by HPLC/MS/MS

E INTERFERENCES

Highly specific confirmatory methods are described in Section F of this method to distinguish CGA-322704 and CGA-293343 from any interfering peaks, should they be observed No major interferences were noted that would affect analysis of samples at the method LOQ during the validation study

Over the course of the usage of this method on numerous crops as part of magnitude of the residue studies, minor co-eluting interferences were observed in a few of the field trials near the method LOQ of 0 01 ppm when analyzing by HPLC/UV At the time of issue of this method, such minor interferences were observed only in broccoli and cabbage field trials (no interferences were observed in any fruits, vegetables or animal substrates) Re-analysis of these samples by HPLC/MS (using procedures specified in section II F 1 0) led to a more specific analysis with no co-eluting interferences in any case

F CONFIRMATORY TECHNIQUES

1 0 Confirmation of Samples After Primary Analysis By HPLC/UV

Residues found in substrates analyzed by normal-phase HPLC/UV can be confirmed by re-injecting the final sample on the reverse phase HPLC/MS system described in Table II. The solvent from 1 mL of the final fraction of these samples must first be evaporated (using nitrogen evaporation or vacuum rotary evaporation in a 50-mL concentration tube), and the sample reconstituted in 1 mL of acetonitrile.water (10·90)

2 0 Confirmation of Samples After Primary Analysis By HPLC/MS

The HPLC/MS system used for primary analysis is a very highly specific method, since detection is performed by scanning for the actual molecular ion of each analyte The analysis by HPLC/MS is considered to be a self-confirming technique If further confirmation is desired, the samples can be analyzed using HPLC/MS/MS. In this technique, the molecular ion is further fragmented by collision-induced dissociation, and a resulting fragment ion is analyzed on a second quadrupole mass analyzer The fragment ions used for CGA-293343 and CGA-322704 are 211 and 169, respectively See Table III for the instrumental conditions for analysis by HPLC/MS/MS Example chromatograms and validation data are provided in Figure 13 and Table VII, respectively

G TIME REQUIRED

The extraction and cleanup of a set of 6 samples may be completed within a time period of approximately 8-12 hours HPLC analyses should take 3 - 6 hours using automatic injection

H MODIFICATIONS AND POTENTIAL PROBLEMS

1 0 Extraction Procedure

Some of the crop substrates are very light, such as undelinted cotton seed and straw, and a 10 gram sample leaves a large filter cake in the Buchner funnel in Section II.C 1 2 The large filter cake holds up a

significant amount of extraction solvent. To prevent losses at this step, rinse the extraction bottle and filter cake with 15-20 mL ACN·water (80 20)

The use of Celite Filter Aid (section II C 1.2 and II.C.1 3) ensures fast filtration of all samples, and the use of Celite is essential with some samples to prevent clogging The use of Celite may be omitted with certain substrates if it is not needed to achieve efficient filtration.

Some substrates will have a lot of material that floats on top of the extraction solvent (e.g. cotton gin trash, straw) after the first extraction in Section II C 1 2 This material should be left in the extraction bottle as best as possible so that it experiences the second extraction. Pour off the extraction solvent slowly, and use a spatula to hold back the floating materials

Some substrates have solid materials that do not settle out easily after the first extraction (e.g undelinted cotton seed, grass) in Section II.C 1 2. Pour off as much solvent as possible from these samples, and leave behind the solid material so that it experiences the second extraction This will leave behind a larger than normal volume of the first extract

2.0 Phenyl SPE Column

The phenyl purification step relies on the use of the elution solvent (methanol water, 1 1) to rinse the round bottom flask of Sections II C 2 3 and II.C.3 2 in order to achieve a complete transfer of the sample No losses were observed if this rinse is performed as specified, even if some of the sample matrix is still visibly left behind on walls of the glassware An option that will prevent any residual matrix from adhering to the walls of the round bottom flask is to first add 5 mL of methanol, sonicate and swirl, followed by 5 mL of water (instead of a premixed elution solvent of 10 mL of methanol water 1:1) The transfer of CGA-293343 and CGA-322704 was found to occur efficiently using either option, but the use of premixed methanol water (1 1) as a rinse may leave behind some of the sample matrix
Certain grains were found to overload the phenyl SPE columns, resulting in low recoveries This was remedied by adding an 0 5 gram phenyl column (Varian Bond Elut #1210-2032) stacked below the 1 gram phenyl This is suggested when analyzing grain samples such as barley

3 0 Amino and Alumina SPE Columns

In the purification step of Sections II.C.2 5 and II.C.3 4, the two SPE columns may be stacked in series to directly elute the sample from the amino to the alumina column. It is valid to collect the eluent from the amino column in a separate flask, and load it onto the alumina column without stacking the two columns

It is important to rinse the round bottom flask that contained the sample with the rinse and elution solvents prior to loading them on the columns This ensures that the entire sample is transferred to the columns As specified in Section II C.2 5 and II.C.3.4, sonication is essential to achieve efficient transfer of the sample

4 0 Clogging of SAX Columns

Certain substrates will have particles come out of solution as the acetonitrile is evaporated off of the extract aliquot in preparation for the SAX purification step in Section II C.3.2. This may result in clogging of the SAX column in some cases This can be avoided by placing a small glass wool plug (<1/2 inch thick) in the bottom of the reservoir that is attached to the top of the SAX column. The glass wool will retain the particles before they reach the SAX column

I PREPARATION OF STANDARD SOLUTIONS AND SAMPLE FORTIFICATION PROCEDURES

1 0 Preparation of Fortification and Analytical Standards

1 1 Standards For Analysis by HPLC/UV

Weigh 10 mg of CGA-293343 analytical standard into a 100-mL volumetric flask and dilute to the mark with hexane ethyl acetate isopropyl alcohol methanol (11 3 1 1) Repeat for CGA-322704 to obtain two separate stock solutions of 100 ng/µL Add 10 mL of each to a 100-mL volumetric flask and dilute to the mark with hexane ethyl acetate isopropyl alcohol methanol (11 3 1 1) to give a stock solution containing 10 ng/µL of both analytes. Alternatively, weigh the CGA-293343 and CGA-322704 into the same 100-mL volumetric flask and dilute to the line with hexane ethyl acetate isopropyl alcohol methanol (11.3 1.1) to obtain a stock solution containing 100 ng/uL of both analytes Make serial

dilutions of the stock solutions with hexane.ethyl acetate isopropyl alcohol·methanol (11:3:1:1) to give a series of fortification/analytical standards in a range of 0 025 to 1.0 ng/µL. Store the standards in a refrigerator in capped amber bottles when not in use.

NOTE - sonication is required to dissolve CGA-293343 and CGA-322704 in hexane ethyl acetate isopropyl alcohol.methanol (11 3·1 1) It was found that in some instances, complete dissolution was difficult to achieve As an alternative, make the 100 ng/µL stock solution in ethyl acetate, and make the serial dilutions using hexane:ethyl acetate isopropyl alcohol methanol (11 3 1 1)

1 2 Standards For Analysis by HPLC/MS

Weigh 10 mg of CGA-293343 analytical standard into a 100-mL volumetric flask and dilute to the mark with acetonitrile water (10 90) Repeat for CGA-322704 to obtain two separate stock solutions of 100 ng/µL Add 10 mL of each to a 100-mL volumetric flask and dilute to the mark with acetonitrile.water (10:90) to give a stock solution containing 10 ng/µL of both analytes. Alternatively, weigh the CGA-293343 and CGA-322704 into the same 100-mL volumetric flask and dilute to the line with acetonitrile water (10 90) to obtain a stock solution containing 100 ng/µL of both analytes Make serial dilutions of the stock solutions with acetonitrile water (10.90) to give a series of fortification/ analytical standards in a range of 0 0125 to 0 5 ng/µL Store the standards in a refrigerator in capped amber bottles when not in use

2 0 Sample Fortification Procedures

Add 1 mL of 0 1 ng/µL to 10 grams of substrate to obtain a sample fortified at 0.01 ppm. Other volumes and standard concentrations may be used for fortification, though the total volume used for fortification should not exceed 2 mL See Section II J 2.0 for recovery calculation procedures

J METHODS OF CALCULATION

1 0 Determination of Sample Residues

Inject aliquots of the final fractions into the HPLC system under the same conditions as for the standards Compare the peak heights of the unknown samples to the standard curve or enter the peak heights into a least squares program to determine the nanograms of CGA-293343 and CGA-322704 in the injected aliquot

To calculate the residue results, the mg injected must first be calculated as follows:

$$(1)\ \text{mg inj} = \frac{(G)\,(V_a)\,(V_i)}{(V_e + W)\,(Vf)}$$

G = milligrams sample extracted
V_a = aliquot volume
V_e = extraction volume
V_i = injection volume
Vf = total volume of final injection solution (mL)
W = weight of sample in grams

To determine ppm of each analyte found in samples, use equation 2

$$(2)\ \text{ppm} = \frac{(\text{ng analyte found})\,(100)}{(\text{mg sample injected})\,(R\%)}$$

R% = recovery ratio given by equation 5 below

The R% value is used when generating residue data and validation data Residues are corrected only when R% is less than 100% The use of controls and fortified controls to determine R% are not required for tolerance enforcement purposes

To convert the ppm found for CGA-322704 to parent CGA-293343 equivalents, multiply the results from equation 2 by the ratio of the molecular weights, defined as F

$$(3)\ F = \frac{\text{MW parent}}{\text{MW metabolite}} = \frac{292}{250}$$

2 0 Determination of Procedural Recoveries

2 1 Determine the ng of CGA-322704 and CGA-293343 injected in the sample, and calculate the mg injected using the procedures described in Section II J 1 0. Calculate the final ppm values of the control and fortified samples according to the following equation·

$$(4)\ \text{ppm} = \frac{\text{ng analyte found}}{\text{mg sample injected}}$$

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 Calculate the recovery factor as a percentage (R) by the equation

$$(5)\ R\% = \frac{(\text{ppm analyte found} - \text{ppm control})}{\text{ppm analyte added}} \times 100$$

Note Analysis of controls, correction of recovery results for small control values, and use of the recovery factor is not required for tolerance enforcement purposes. They are used for method validation and generation of residue data where applicable

III METHOD VALIDATION RESULTS AND DISCUSSION

The objective of Study Protocol 206-97 was to validate Analytical Method AG-675 for the determination of residues of the insecticide CGA-293343 and the metabolite CGA-322704 in crop and animal substrates The precision and accuracy of the method was verified by analyzing control (untreated) samples fortified with CGA-293343 and CGA-322704 in the following representative substrates

Dairy (Cow)	Fat, Kidney, Liver
Dairy (Goat)	Leg Muscle, Milk
Poultry	Fat, Eggs
Pome Fruit	Fruit (Pears), Wet Pomace (Apples), Juice (Apples)
Tuberous Vegetables	Tubers, Wet Peels
Cucurbits	Cucumbers
Fruiting Vegetables	Fruit (Tomato), Tomato paste, Green Peppers
Leafy Vegetables	Spinach
Brassica Vegetables	Broccoli

Cotton	Undelinted Seed, Cotton Seed Oil
Cereal Grains	Corn Grain, Wheat Grain
Forage, Fodder, Straw of Cereal Grains	Corn Fodder, Sorghum Forage
Turf.	Grasses (Bermuda Grass from Georgia, Kentucky Blue Grass from New Jersey)

Method precision, the extractability of incurred residues from weathered samples, and the accountability expected for this method was studied by the analysis of the following ^{14}C-CGA-293343 treated metabolism samples

Pears	Fruit from Metabolism Study 198-96[3]
Corn	Grain and Fodder from Metabolism Study 95PSA41PR2[4]
Cucumbers,	Fruit (soil treatment sample, and soil + foliar treated sample) from Metabolism Study 282-95[5]
Goat	Leg Muscle and Milk from Metabolism Study 027AM03[6]

The Limit of Quantitation (LOQ), as determined by the smallest acceptable fortification level, is 0 01 ppm for CGA-293343 and 0 01 ppm for CGA-322704 for all substrates except milk, juices, and grasses The LOQ for milk and juices is 0.005 ppm for each analyte, and for grasses is 0 05 ppm The Limit of Detection (LOD), as determined by the smallest acceptable standard injected, is 1 25 ng for samples analyzed by HPLC/UV, and 0.25 ng for samples analyzed by HPLC/MS Typical standard chromatograms analyzed by HPLC/UV are shown in Figure 5, standards analyzed by HPLC/MS are shown in Figure 6, and standards analyzed by HPLC/MS/MS are shown in Figure 7 Calibration plots from these analyses are shown in Table VIII. The results from these standard injections are typical of the results observed during the validation of method AG-675. Chromatograms from the analysis of reagent blanks analyzed by method AG-675 are shown in Figure 8 Representative chromatograms from the analysis of vegetable samples are shown in Figures 9 Representative chromatograms from the analysis of fruit samples are shown in Figures 10 Representative chromatograms from the analysis of animal samples are shown in Figures 11 Representative chromatograms from the analysis of cotton, tobacco, and forage, fodder and straw of cereal grains and grasses are shown in Figures 12

Additional analyses to confirm the identity and quantity of residues of CGA-293343 and CGA-322704 using HPLC/MS and HPLC/MS/MS instrumentation were successfully validated on representative samples Representative chromatograms are from the confirmatory analyses are shown in Figure 13

Results of the validation study (Protocol 206-97) are summarized in the following sections Appendix I to method AG-675 contains all chromatograms from the validation study and all the data necessary to reproduce calculations of results (as directed by Residue Chemistry Test Guidelines OPPTS860 1340 Residue Analytical Method), as well as the study elements required under the general GLP guidelines

A ACCURACY AND PRECISION

Table IV shows the individual results obtained from the analysis of controls and control samples fortified with CGA-293343 and CGA-322704 over a range of 0 005 ppm to 2 0 ppm The overall mean for all recoveries for CGA-293343 was 86% (n = 119, standard deviation = 9.8, coefficient of variation (%CV) = 11%). The overall mean for all recoveries for CGA-322704 was 87% (n=118, standard deviation = 9 6, %CV = 11%)

Below is a statistical summary of the results obtained by fortification level Note that the 0.005 ppm level is the LOQ for milk and juice only The accuracy expected at each recovery level, indicated by the average recovery values, ranged from 78% to 98% for CGA-293343, and from 77% to 89% for CGA-322704 The precision expected for each recovery level, indicated by the %CV values, ranged from 7 2% to 14% for CGA-293343 and from 4 6% to 15% for CGA-322704.

Recovery	# Of	CGA-293343			CGA-322704		
Level	Analyses	Average	St Dev	%CV	Average	St Dev	%CV
0 005	4	98	12	12	86	11	13
0 01	45	89	11	13	89	13	15
0 05	5	80	11	14	77	11	14
0 10	12	86	6 3	7 2	87	5 5	6 3
0 20	6	84	9 4	11	87	6 6	7 6
0 50	22	85	6 6	7 7	87	5 8	6 7
1 0	16	84	6 9	8 3	87	4 0	4 6
2 0	9	78	8 4	11	83	6 7	8 0

Provided below is a statistical summary of results obtained for each technique (HPLC/UV, HPLC/MS, or HPLC/MS/MS). The analyses of samples by HPLC/MS, performed on the Micromass Platform LC instrument, included primary analysis of some substrates, as well as confirmation analyses on representative samples that were analyzed by HPLC/UV The analysis of samples by HPLC/MS/MS, performed on the Sciex API III instrument, also included both primary analysis of samples (grasses) and confirmation analysis on representative substrates that had been analyzed by HPLC/MS

Analytical Technique	# of Analyses	CGA-293343 Average	CGA-293343 St Dev	CGA-293343 %CV	CGA-322704 Average	CGA-322704 St Dev	CGA-322704 %CV
HPLC/UV	91	87	9 6	11	(n=90) 88	9 7	11
HPLC/MS	18	81	11	13	80	7 5	9 3
HPLC/MS/MS	10	87	8 0	9 1	86	6 6	7 7

Table V shows the statistical calculations (mean, range, standard deviation, %CV) from the triplicate analysis of samples that were treated with ^{14}C-CGA-293343 as part of metabolism studies Below is a summary of the precision expected (in terms of %CV) from triplicate analysis of the same sample containing incurred residues Included is the %CV determined for CGA-293343, CGA-322704, and the resulting precision when results are presented in terms of total CGA-293343 equivalents (the adjustment of the ppm determined for the metabolite CGA-322704 based on molecular weight, added to the ppm determined for CGA-293343) The %CV for all triplicate analyses were less than 20% except for the determination of CGA-293343 in the soil treated + foliar spray treated cucumbers A coefficient of variation of 27% for this analysis occurred when one of the triplicate samples had 0 022 ppm CGA-293343, compared to 0 036 and 0 038 ppm for the other two replicates.

Substrate	%CV for CGA-293343	%CV for CGA-322704	% CV for CGA-293343 Parent Equivalents
Pears	4 0	3 1	3 7
Corn Grain	Residues <0 01 ppm	Residues <0 01 ppm	not applicable
Corn Fodder	6 5	15	11
Cucumbers[1]	9 5	Residues <0 01 ppm	9 5
Cucumbers[2]	27	15	11
Goat Meat	18	13	18
Goat Milk	16	16	16

[1] Soil-treated only cucumber sample
[2] Soil-treated plus foliar spray cucumber sample

B EXTRACTABILITY AND ACCOUNTABILITY

Samples from metabolism studies that were treated with ^{14}C-CGA-293343 were analyzed by Analytical Method AG-675 to determine the extractability and accountability from incurred, weathered samples Table VI shows the extractability and accountability results from triplicate analysis of these samples, and compares the results from AG-675 with those obtained from the metabolism studies The extractability for all the samples was within 18% of the extractable residues (as determined in the metabolism study) with the exception of soil - only treated cucumbers The accountability observed when the samples are analyzed for CGA-293343 and CGA-322704 by Analytical Method AG-675 are within 11% of the expected accountability (as determined in the metabolism studies), with the exception of the same cucumber samples The low extractability and accountability observed in the soil only treated cucumbers is

likely a result of some anomaly in the subsample obtained for the validation study, and is not caused by a problem with the method itself. The soil treatment + foliar spray cucumber samples showed good extractability and accountability

C CONFIRMATORY METHOD

Method AG-675 is a highly specific method for the extraction, purification, chromatographic separation and detection of CGA-293343 and CGA-322704 This method additionally provides for the qualitative and quantitative confirmation of residues by re-analysis of the samples by HPLC/MS or HPLC/MS/MS The majority of substrates of this method are analyzed by normal phase HPLC/UV as the primary analysis, and re-analysis by reverse phase HPLC/MS gives confirmation by obtaining data from a second separation mechanism and a detection system that is specifically tuned to detect and quantify the $M+H^+$ ions of CGA-293343 (m/z = 292) and CGA-322704 (m/z = 250) Interferences will be extremely rare in such a system, and would not be expected to occur in both the normal phase HPLC/UV and the reverse phase HPLC/MS system Also included in Method AG-675 is an HPLC/MS/MS method, where the $M+H^+$ ions of CGA-293343 and CGA-322704 undergo collision induced disassociation, and the instrument is tuned to detect and quantify one specific resulting fragment ion from each compound (m/z = 211 for CGA-293343 and 169 for CGA-322704) This specific method can be used as definitive confirmation for sample residues detected by the HPLC/UV and the HPLC/MS methods

Table VII shows a comparison of the results obtained from the primary analysis of samples to the mass spectroscopy confirmatory analysis from representative sample sets Results were in excellent agreement in all cases

IV CONCLUSION

Method AG-675 is a valid and accurate method for the determination of residues of CGA-293343 and CGA-322704 in crops and animal substrates This conclusion is based upon the accuracy, precision, extractability and accountability obtained during the validation study

V CERTIFICATION

The reports and experimental results included in this study, Laboratory Project ID AG-675, are certified to be authentic accounts of the experiments

Daniel D. Campbell

Daniel D Campbell
Scientist I
Residue Chemistry
Human Safety Department

9-18-98
Date

QUALITY ASSURANCE STATEMENT

Report Title Analytical Method for the Determination of Residues of CGA-293343 and the Metabolite CGA-322704 in Animal and Crop Substrates by High Performance Liquid Chromatography with Detection by UV and Mass Spectrometry, Including Validation Data

Study Director D Campbell

Report Number: Analytical Method No AG-675

Study Number 206-97 and amendments

Pursuant to Good Laboratory Practice Regulations, this statement verifies that the aforementioned study was inspected and/or audited and the findings reported to Management and to the Study Director by the Quality Assurance Unit on the dates listed below

INSPECTION/AUDIT TYPE	INSPECTION/AUDIT DATE(S)	REPORTING DATE
Protocol Audit	5/30/97	5/30/97
In-Progress Inspection	4/6/98	4/6/98
Final Report, Analytical Method No AG-675	9/9-11/98	9/11/98

Prepared by Karen S. Price Date 9/11/98

Karen S Price
Senior Quality Assurance Auditor
Quality Assurance Unit
Novartis Crop Protection

VII TABLES AND FIGURES

TABLE I HPLC OPERATING PARAMETERS FOR THE DETERMINATION OF CGA-293343 AND CGA-322704 RESIDUES BY NORMAL PHASE HPLC/UV

Instrument	Perkin-Elmer LC-250 Isocratic Pump, with a Perkin-Elmer ISS-200 Autosampler and Kratos 783 UV Detector (or equivalent instrumentation)
Column	Spherisorb 5 NH_2, 250 x 4 6 mm (Metachem Part #0190-250x46, or equivalent)
Mobile phase	Hexane Ethyl Acetate Isopropanol Methanol (11.3 1 1)
Flow Rate	1 5 mL/minute
Injection	100 µL injection volume
Detection	UV at 255 nm
Run Time	30 minutes (the run time will need to be increased if late eluting peaks are observed)
Retention Time	CGA-322704 = ~12 minutes CGA-293343 = ~20 minutes (these times will vary with variation in column batches and mobile phase composition)
Data Acquisition	Microvax II (Q) Operating System, VMS Version 5 3-1 Application Software VG Multichrom Version 2 0 Worksheet Version Ws pas 1 3 1 (or equivalent)

TABLE II HPLC OPERATING PARAMETERS FOR THE DETERMINATION OF CGA-293343 AND CGA-322704 RESIDUES BY REVERSE PHASE HPLC/MS

Instrument ESI/APCI quadrupole LC/MS, Micromass Platform LC, with a Hewlett-Packard HP-1100 HPLC inlet system (autosampler, solvent degasser, quaternary pump, column oven, and in-line UV detector)

Column Zorbax LC-MS SB-C18 (50 mm x 2 1 mm I D , 5 0 um, Part No 86095 902)
Column oven temperature = 25°C

Mobile phase Solvent A = 0 1% acetic acid in water
Solvent B = 0 1% acetic acid in acetonitrile
Mobile Phase Gradient Program (total run time 28 minutes)

Time	A %	B %
0 0	95	5
4 0	90	10
13	80	20
16	40	60
19	40	60
23	95	5
28	95	5

Flow Rate 0 2 mL/minute, flow split post-column by a ratio of 3 1 (waste MS)

Retention Time CGA-293343 ~ 11 minutes
CGA-322704 = ~ 13 minutes
(these times will vary with variation in column batches and mobile phase composition)

Injection 20 µL injection volume

TABLE II HPLC OPERATING PARAMETERS FOR THE DETERMINATION OF CGA-293343 AND CGA-322704 RESIDUES BY REVERSE PHASE HPLC/MS (Continued)

MS Detection

Set Points - The capillary and cone voltages may need to be optimized to maximum sensitivity of a particular instrument by infusion of a CGA-293343 and CGA-322704 standard (1 ng/uL in mobile phase)

Typical Set Points (used for method validation)

Ionization Mode	Electrospray
Probe Capillary	3.5 kV
Sample Cone	22 V
Ion energy	1 3 V
Source Heater	110°C

Monitoring ions -

Compound	Ion Polarity	Mass/Charge (m/z)
CGA-293343	Positive	292 [m+H$^+$]
CGA-322704	Positive	250 [m+H$^+$]

Data Acquisition

Acquisition, system control, peak integration, and quantitation was performed using Micromass Masslynx v2 3 Recovery and residue calculations performed using VG Multichrome Version 2 0 Worksheet Version Ws pas 1 3 1 (or equivalent)

TABLE III OPERATING PARAMETERS FOR THE DETERMINATION OF CGA-293343 AND CGA-322704 RESIDUES BY REVERSE PHASE HPLC/MS/MS

Instrument PE Sciex API-III+ Triple Quadrupole Mass Spectrometer IonSpray Liquid Introduction Interface, with an HPLC inlet consisting of a Perkin-Elmer Series 4 Gradient Pump, Perkin-Elmer Series 200 Autosampler, and a Eppendorf Model CH-30 Column Heater

Column Aquasil C-18, 5µm 150x4 6 mm (cat# 155-775, Keystone Scientific, Inc)

Mobile Phase A = 0 1% acetic acid in water
B = 0 1% acetic acid in acetonitrile

Mobile Phase Gradient Program (setting for the PE Series 4 pump)

Time (min)	% A	% B	Curve
0	90	10	
4	90	10	
14	30	70	1
17	30	10	
17 1	90	10	0
25 1	90	10	

Flow Rate· 1 5 mL/minute, flow split post-column by a ratio of 3 1 (waste MS)

Retention Time CGA-293343 ~ 9 5 minutes
CGA-322704 = ~ 10.5 minutes
(these times will vary with variation in column batches and mobile phase composition)

Injection 50 µL injection volume

TABLE III OPERATING PARAMETERS FOR THE DETERMINATION OF CGA-293343 AND CGA-322704 RESIDUES BY REVERSE PHASE HPLC/MS/MS (Continued)

MS Detection Set Points -

Typical State File Values

	Q1 293343 positive ion	Monitoring State file
ISV	4500 00	4500 00
IN	650 00	650 00
OR	50 00	60 00
R0	30 00	30 00
M1	1000 00	150 00
RE1	118 00	122 50
DM1	0 20	0 15
R1	27 50	26 00
L7	-35 00	22 00
R2	-20 00	19 00
M3	1000 00	150 00
RE3	124 60	120 00
DM3	0 13	0 15
RX	-10 00	0 00
R3	-70 0	13 00
L9	-250 00	-250 00
FP	-250 00	-250 00
MU	-4600 00	-4600 00
CC	10	1
CGT	off	ca. 225-235

State file values will vary slightly from instrument to instrument These values may need slight adjustment during regular instrument optimization procedures

TABLE III OPERATING PARAMETERS FOR THE DETERMINATION OF CGA-293343 AND CGA-322704 RESIDUES BY REVERSE PHASE HPLC/MS/MS (Continued)

Monitoring ions -

Compound	Ion Polarity	Molecular Ion (m/z)	Product Ion Monitored (m/z)
CGA-293343	Positive	292 [m+H$^+$]	211
CGA-322704	Positive	250 [m+H$^+$]	169

Data Acquisition Acquisition performed using Apple System 7 5, program RAD v2 6, quantitation by Macquan v1 3, and calibration and mass tuning by Tune v2 5 Recovery and residue calculations performed using VG Multichrome Version 2 0 Worksheet Version Ws pas 1 3 1 (or equivalent)

TABLE IV RECOVERY RESULTS FOR CONTROL AND CGA-322704 AND CGA-293343 FORTIFIED CONTROL SUBSTRATES

SUBSTRATE	SAMPLE NUMBER	ANALYSIS METHOOD	SPIKE LEVEL	CGA-293343 RECOVERY	CGA-293343 STATISTICS AVG	STDEV	%CV	CGA-322704 RECOVERY	CGA-322704 STATISTICS AVG	STDEV	%CV
Apple Wet Pomace	1	UV	0 00	(control)							
Apple Wet Pomace	2	UV	0 01	69 3				80 7			
Apple Wet Pomace	3	UV	0 01	76 9				73 4			
Apple Wet Pomace	4	UV	0 20	73 7				79 2			
Apple Wet Pomace	5	UV	1 00	72 2				78 2			
Apple Wet Pomace	6	UV	1 00	lost	73 0	2 7	3 8	lost	77 9	2 7	3 5
Apple Juice	7	UV	0 00	(reagent blank)							
Apple Juice	8	UV	0 00	(control)							
Apple Juice	9	UV	0 005	89 1				75 8			
Apple Juice	10	UV	0 005	87 9				77 4			
Apple Juice	11	UV	0 1	82 4				83 9			
Apple Juice	12	UV	0 5	85 1				85 8			
Apple Juice	13	UV	0 5	83 5	85 6	2 6	3 0	87 7	82 1	4 7	5 7
Pears	14	UV	0	(control)							
Pears	15	UV	0 01	103 6				108 3			
Pears	16	UV	0 01	102 7				101 7			
Pears	17	UV	0 50	89 1				91 2			
Pears	18	UV	0 50	85 3	95 2	8 1	8 5	88 2	97 4	8 1	8 3
Potato Tubers	22	UV	0 00	(reagent blank)							
Potato Tubers	23	UV	0 00	(control)							
Potato Tubers	24	UV	0 01	96 1				99 1			
Potato Tubers	25	UV	0 01	94 3				99 7			
Potato Tubers	26	UV	0 10	85 1				87 8			
Potato Tubers	27	UV	0 50	84 7				89 8			
Potato Tubers	28	UV	0 50	83 4	88 7	5 3	6 0	88 8	93 0	5 2	5 6
Potato Wet Peel	29	UV	0 00	(control)							
Potato Wet Peel	30	UV	0 01	78 7				78 0			
Potato Wet Peel	31	UV	0 01	82 9				79 4			
Potato Wet Peel	32	UV	0 20	85 6				89 6			
Potato Wet Peel	33	UV	1 00	88 2				90 3			
Potato Wet Peel	34	UV	1 00	85 2	84 1	3 2	3 8	88 8	85 2	5 3	6 3

TABLE IV RECOVERY RESULTS FOR CONTROL AND CGA-322704 AND CGA-293343 FORTIFIED CONTROL SUBSTRATES (Continued)

SUBSTRATE	SAMPLE NUMBER	ANALYSIS METHOOD	SPIKE LEVEL	CGA-293343 RECOVERY	CGA-293343 STATISTICS AVG	STDEV	%CV	CGA-322704 RECOVERY	CGA-322704 STATISTICS AVG	STDEV	%CV
Cucumber	35	UV	0 00	(control)							
Cucumber	36	UV	0 01	87 0				92 3			
Cucumber	37	UV	0 01	91 3				(lost)			
Cucumber	38	UV	0 50	83 4	87 2	3 2	3 7	89 9	91 1	1 2	1 3
Tomato (Fruit)	45	UV	0 00	(control)							
Tomato (Fruit)	46	UV	0 01	89 2				97 6			
Tomato (Fruit)	47	UV	0 01	110 3				104 7			
Tomato (Fruit)	48	UV	0 50	85 2				86 8			
Tomato (Fruit)	49	UV	1 00	68 2				92 8			
Tomato (Fruit)	50	UV	1 00	74 8	85 5	14 5	16 9	90 4	94 5	6 2	6 6
Tomato Paste	51	UV	0 00	(control)							
Tomato Paste	52	UV	0 01	76 6				77 0			
Tomato Paste	53	UV	0 01	78 6				83 6			
Tomato Paste	54	UV	0 20	77 8				81 4			
Tomato Paste	55	UV	2 00	76 4				80 5			
Tomato Paste	56	UV	2 00	79 1	77 7	1 1	1 4	82 8	81 0	2 3	2 9
Green Pepper	57	UV	0 00	(control)							
Green Pepper	58	UV	0 01	93 6				92 4			
Green Pepper	59	UV	0 01	94 7				90 9			
Green Pepper	60	UV	0 10	88 2				91 3			
Green Pepper	61	UV	1 00	84 5				88 6			
Green Pepper	62	UV	1 00	85 1	89 2	4 2	4 8	86 4	89 9	2 1	2 4
Spinach	63	UV	0 00	(control)							
Spinach	64	UV	0 01	117 2				77 4			
Spinach	65	UV	0 01	109 0				42 2			
Spinach	66	UV	0 10	94 6				93 2			
Spinach	67	UV	0 50	89 6				90 0			
Spinach	68	UV	0 50	79 3	97 9	13 6	13 9	83 0	77 1	18 3	23 7

TABLE IV RECOVERY RESULTS FOR CONTROL AND CGA-322704 AND CGA-293343 FORTIFIED CONTROL SUBSTRATES (Continued)

SUBSTRATE	SAMPLE NUMBER	ANALYSIS METHOOD	SPIKE LEVEL	CGA-293343 RECOVERY	CGA-293343 STATISTICS AVG	STDEV	%CV	CGA-322704 RECOVERY	CGA-322704 STATISTICS AVG	STDEV	%CV
Broccoli	69	UV	0 00	(control)							
Broccoli	70	UV	0 01	101 2				94 9			
Broccoli	71	UV	0 01	89 8				92 8			
Broccoli	72	UV	0 10	76 3				81 6			
Broccoli	73	UV	0 20	lost				lost			
Broccoli	74	UV	0 20	83 8	87 8	9 1	10 4	90 1	89 8	5 0	5 6
Fat (Cow, Omental)	75	UV	0 00	(control)							
Fat (Cow, Omental)	76	UV	0 01	80 1				85 2			
Fat (Cow, Omental)	77	UV	0 01	85 8				86 9			
Fat (Cow, Omental)	78	UV	0 20	82 6				86 5			
Fat (Cow, Omental)	79	UV	2 00	86 3				90 0			
Fat (Cow, Omental)	80	UV	2 00	79 1	82 8	2 9	3 5	85 4	86 8	1 8	2 0
Kidney (Cow)	81	UV	0 00								
Kidney (Cow)	82	UV	0 01	87 8				89 9			
Kidney (Cow)	83	UV	0 01	91 4				94 4			
Kidney (Cow)	84	UV	0 10	82 8				87 2			
Kidney (Cow)	85	UV	1 00	82 9				87 0			
Kidney (Cow)	86	UV	1 00	lost	86 2	3 6	4 2	lost	89 6	3 0	3 3
Liver (Cow)	87	UV	0 00	(control)							
Liver (Cow)	88	UV	0 01	85 2				91 6			
Liver (Cow)	89	UV	0 01	84 3				91 0			
Liver (Cow)	90	UV	0 10	85 8				88 0			
Liver (Cow)	91	UV	0 50	90 1				90 1			
Liver (Cow)	92	UV	0 50	84 6	86 0	2 1	2 5	86 0	89 3	2 1	2 3
Meat (goat muscle)	93	UV	0 00	(control)							
Meat (goat muscle)	94	UV	0 01	86 1				88 1			
Meat (goat muscle)	95	UV	0 01	86 0				88 3			
Meat (goat muscle)	96	UV	1 00	88 1	86 7	1 0	1 2	89 1	88 5	0 4	0 5

TABLE IV RECOVERY RESULTS FOR CONTROL AND CGA-322704 AND CGA-293343 FORTIFIED CONTROL SUBSTRATES (Continued)

SUBSTRATE	SAMPLE NUMBER	ANALYSIS METHOD	SPIKE LEVEL	CGA-293343 RECOVERY	CGA-293343 STATISTICS AVG	STDEV	%CV	CGA-322704 RECOVERY	CGA-322704 STATISTICS AVG	STDEV	%CV
Milk (goat)	100	UV	0 00	(control)							
Milk (goat)	101	UV	0 005	112 6				95 9			
Milk (goat)	102	UV	0 005	104 1				95 8			
Milk (goat)	103	UV	0 50	87 8	101 5	10 3	10 1	89 7	93 8	2 9	3 1
Eggs	107	UV	0 00	(control)							
Eggs	108	UV	0 01	lost				lost			
Eggs	109	UV	0 01	91 9				94 8			
Eggs	110	UV	0 20	81 2				85 4			
Eggs	111	UV	2 00	82 8				88 1			
Eggs	112	UV	2 00	84 2	85 0	4 1	4 8	88 9	89 3	3 4	3 8
Fat (poultry)	113	UV	0 00	(control)							
Fat (poultry)	114	UV	0 01	97 9				93 0			
Fat (poultry)	115	UV	0 01	85 1				93 7			
Fat (poultry)	116	UV	0 10	90 1				94 0			
Fat (poultry)	117	UV	1 00	83 3				89 2			
Fat (poultry)	118	UV	1 00	86 3	88 5	5 2	5 8	93 3	92 6	1 7	1 9
Cotton Seed	119	LC/MS	0 00	(reagent blank)							
Cotton Seed	120	LC/MS	0 00	(control)							
Cotton Seed	121	LC/MS	0 01	70 0				89 0			
Cotton Seed	122	LC/MS	0 01	72 0				76 0			
Cotton Seed	123	LC/MS	0 50	74 0				75 3			
Cotton Seed	124	LC/MS	2 00	57 8				69 6			
Cotton Seed Oil	125	LC/MS	2 00	75 4	69 8	6 3	9 0	76 2	77 2	6 4	8 3
Cotton Seed Oil	126	UV	0 00	(reagent blank)							
Cotton Seed Oil	127	UV	0 00	(control)							
Cotton Seed Oil	128	UV	0 01	89 6				96 0			
Cotton Seed Oil	129	UV	0 01	100 1				107 6			
Cotton Seed Oil	130	UV	0 05	61 8				60 2			
Cotton Seed Oil	131	UV	0 50	66 4				68 5			

TABLE IV RECOVERY RESULTS FOR CONTROL AND CGA-322704 AND CGA-293343 FORTIFIED CONTROL SUBSTRATES (Continued)

SUBSTRATE	SAMPLE NUMBER	ANALYSIS METHOD	SPIKE LEVEL	CGA-293343 RECOVERY	CGA-293343 STATISTICS AVG	STDEV	%CV	CGA-322704 RECOVERY	CGA-322704 STATISTICS AVG	STDEV	%CV
Grain (Corn)	132	UV	0 50	93 7	82 3	15 3	18 6	95 8	85 6	18 1	21 1
Grain (Corn)	133	UV	0 00	(control)							
Grain (Corn)	134	UV	0 01	86 6				107 6			
Grain (Corn)	135	UV	0 01	93 3				117 4			
Fodder (Corn)	136	UV	0 10	lost	90 0	3 3	3 7	lost	112 5	4 9	4 4
Fodder (Corn)	140	LC/MS	0 00	(control)							
Fodder (Corn)	141	LC/MS	0 01	72 0				64 0			
Fodder (Corn)	142	LC/MS	0 01	74 9				73 8			
	143	LC/MS	1 00	85 3	77 4	5 7	7 4	80 9	72 9	6 9	9 5
Grain (wheat)											
Grain (wheat)	147+148	UV	0 00	(control + reagent blank, accidentally combined during workup)							
Grain (wheat)	149	UV	0 01	102 2				93 3			
Grain (wheat)	150	UV	0 01	89 3				87 4			
Grain (wheat)	151	UV	0 10	78 3				77 9			
Grain (wheat)	152	UV	0 50	89 0				88 7			
	153	UV	0 50	94 5	90 6	7 8	8 6	94 3	88 3	5 8	6 6
Forage (Sorghum)	154	LC/MS	0 00	(reagent blank)							
Forage (Sorghum)	155	LC/MS	0 00	(control)							
Forage (Sorghum)	156	LC/MS	0 01	68 0				78 0			
Forage (Sorghum)	157	LC/MS	0 01	82 0				96 0			
Forage (Sorghum)	158	LC/MS	0 10	83 6				85 4			
Forage (Sorghum)	159	LC/MS	1 00	86 0				86 4			
Tobacco (green)	160	LC/MS	1 00	83 6	80 6	6 4	8 0	84 0	86 0	5 8	6 8
Tobacco (green)	161	LC/MS	0 00	(control)							
Tobacco (green)	162	LC/MS	0 01	96 1				80 7			
Tobacco (green)	163	LC/MS	0 01	94 0				81 9			
Tobacco (green)	164	LC/MS	0 10	91 6				77 5			
Tobacco (green)	165	LC/MS	1 00	92 2				85 2			

TABLE IV RECOVERY RESULTS FOR CONTROL AND CGA-322704 AND CGA-293343 FORTIFIED CONTROL SUBSTRATES (Continued)

SUBSTRATE	SAMPLE NUMBER	ANALYSIS METHOOD	SPIKE LEVEL	CGA-293343 RECOVERY	CGA-293343 STATISTICS AVG	STDEV	%CV	CGA-322704 RECOVERY	CGA-322704 STATISTICS AVG	STDEV	%CV
Grass (Georgia)	166	LC/MS	1 00	95 3	93 8	1 7	1 9	85 0	82 1	2 9	3 5
Grass (Georgia)	167	LC/MS/MS	0 00	(control)							
Grass (Georgia)	168	LC/MS/MS	0 05	80 6				77 6			
Grass (Georgia)	169	LC/MS/MS	0 05	80 6				75 2			
Grass (Georgia)	170	LC/MS/MS	0 10	97 1				90 4			
Grass (Georgia)	171	LC/MS/MS	0 50	92 4				84 8			
Grass (New Jersey)	172	LC/MS/MS	0 50	89 3	88 0	6 5	7 4	88 3	83 3	5 9	7 1
Grass (New Jersey)	173	LC/MS/MS	0 00	(control)							
Grass (New Jersey)	174	LC/MS/MS	0 05	83 6				81 5			
Grass (New Jersey)	175	LC/MS/MS	0 05	91 6				89 6			
Grass (New Jersey)	176	LC/MS/MS	0 20	101 0				97 6			
Grass (New Jersey)	177	LC/MS/MS	0 50	80 1				90 2			
	178	LC/MS/MS	0 50	78 0	86 8	8 5	9 7	86 9	89 1	5 2	5 8

overall statistics		CGA-293343	CGA-322704
	average	86 0	87 0
standard	deviation	9 8	9 6
	%cv	11 4	11 0
	n	119 0	118 0

TABLE V PRECISION FROM THE TRIPLICATE ANALYSIS OF ^{14}C-CGA-293343 TREATED METABOLISM SAMPLES BY METHOD AG-675

Substrate and Sample Numeric ID	Metabolism Reference (Study # / Numeric ID)	206-97 Sample Number	HPLC Results CGA-293343	CGA-322704	Total CGA-293343 Equivalents (ppm)
Pears (fruit) 205659	US study 198-96 sample #168412	19	0 15	0 081	0 24
		20	0 18	0 092	0 29
		21	0 17	0 088	0.27
		Mean	0 18	0 090	0.28
		Range	0 15 0 18	0 081 0 092	0.24 - 0.29
		Standard Deviation	0 007	0 003	0 010
		CV (%)	4 04	3 14	3 70
Corn Grain 203234	Basle study 95PSA41 2	137	<0 01	<0 01	not applicable
		138	<0 01	<0 01	not applicable
		139	<0 01	<0 01	not applicable
		Mean	not applicable	not applicable	not applicable
		Range	- -	- -	- -
		Standard Deviation	- -	- -	- -
		CV (%)	- -	- -	- -
Corn Fodder 203233	Basle study 95PSA41.2	144	0 022	0 017	0 042
		145	0 024	0 021	0 049
		146	0 025	0 023	0 052
		Mean	0 024	0 020	0 047
		Range	0 022 0 025	0 017 0 023	0 042 - 0 052
		Standard Deviation	0 002	0 003	0 005
		CV (%)	6 454	15 025	10 747
Cucumbers	U S study 282-95 159393 (pre-foliar spray	39	0 043	<0 01	0 043
		40	0 051	<0 01	0 051
		41	0 044	<0 01	0 044
		Mean	0 046	not applicable	0 046
		Range	0 043 - 0 051	- -	0 043 0 051
		Standard Deviation	0 004	- -	0 004
		CV (%)	9 48	- -	9 48
Cucumbers	U S study 282-95 159394 (post-foliar spra	42	0 022	0 018	0 043
		43	0 036	0 015	0 054
		44	0 038	<0 01*	0 050
		Mean	0 032	0 014	0 05
		Range	0 022 - 0 038	<0 01 - 0 018	0 043 - 0 054
		Standard Deviation	0 009	0 002	0 005
		CV (%)	27.24	14 80	10.90
			*value taken as 0 01 ppm for calculations		
Goat Meat	Basle Study 027AM03	97	0 77	0 057	0 841
		98	0 56	0 044	0 612
		99	0 79	0 054	0 848
		Mean	0 707	0 052	0 77
		Range	0 56 0 79	0 044 - 0 057	
		Standard Deviation	0 126	0 007	0 134
		CV (%)	17 87	13 17	17 46
Goat Milk	Basle Study 027AM03	104	0 087	0 17	0 282
		105	0 064	0 12	0 206
		106	0 085	0 16	0 270
		Mean	0 079	0 149	0.25
		Range	0 064 - 0 087	0 12 - 0 17	
		Standard Deviation	0 013	0 024	0 040
		CV (%)	16.20	15 98	16 03

TABLE VI RESULTS FROM THE ANALYSIS OF ^{14}C-CGA-293343 TREATED SAMPLES BY METHOD AG-675 AND COMPARISON TO RESULTS OBTAINED FROM THE METABOLISM STUDIES

	Preliminary Results from Metabolism Studies					Results from Analysis by AG-675 (triplicate analysis)				
Substrate	Reference	% Extracted	CGA-293343 (ppm)	CGA-322704 (ppm)	% of TRR	Reference	%Extracted	CGA-293343 (ppm)	CGA-322704 (ppm)	% of TRR
Pears (fruit) - 2 foliar sprays @ 150 gal/Ha (300 gal/Ha total)	Study 198-96 #168412	83 2	0 20	0 14	48 2	Sample # 205659	82 4	0 15	0 081	34 8
								0 18	0 092	41 0
								0 17	0 088	38 9
									Average	38 2
Corn Grain -seed treated @500 gal/Ha	Basle Seed Treatment 95PSA41 2	38 5	0 006	0 007	17 1	Sample # 203234	33 9	<0 01	<0 01	na
								<0 01	<0 01	na
								<0 01	<0 01	na
Corn Fodder -seed treated @500 gal/Ha	Basle Seed Treatment 95PSA41 2	57 8	0 03	0 05	9 2	Sample # 203233	40 06	0 022	0 017	4 7
								0 024	0 021	5 5
								0 03	0 025	5 9
									Average	5 4
Cucumbers - soil sprayed @1500 gal/Ha	Study 282-95 #159393	99 5	0 14	0 01	42 58	Sample # 205657	57 02	0 043	<0 01	13 1
								0 051	<0 01	15 5
								0 047	<0 01	13 4
									Average	14 0
Cucumbers - soil sprayed @1500 gal/Ha + 500 gal/Ha foliar spray	Study 282-95 #159394	86 9	0 02	0 003	14 9	Sample # 205658	73 48	0 022	0 018	13 3
								0 023	0 021	16 6
								0 038	0 01	15 7
									Average	15 2
Goat Meat 4 doses of 100 ppm (feed)	Basle study 95PSA41 2 (TRR = 2 1 ppm)	94 7	1 0	0 12	54 1	Sample # 210166 (TRR = 1 7 ppm)	87 9	0 77	0 057	49 3
								0 56	0 047	35 9
								0 79	0 057	49 8
									Average	45 0
Goat Milk 4 doses of 100 ppm (feed)	Basle study 95PSA41 2 (TRR = 1 2 ppm)	91 2	0 37	0 44	69	Sample # 210165 (TRR = 0 44 ppm)	86 4	0 087	0 17	64 7
								0 061	0 12	47 4
								0 085	0 16	61 8
									Average	58 0

TABLE VII RESULTS FROM THE CONFIRMATORY LC/MS ANALYSIS OF REPRESENTATIVE SUBSTRATES AND COMPARISON TO THE RESULTS FROM THE PRIMARY ANALYSIS

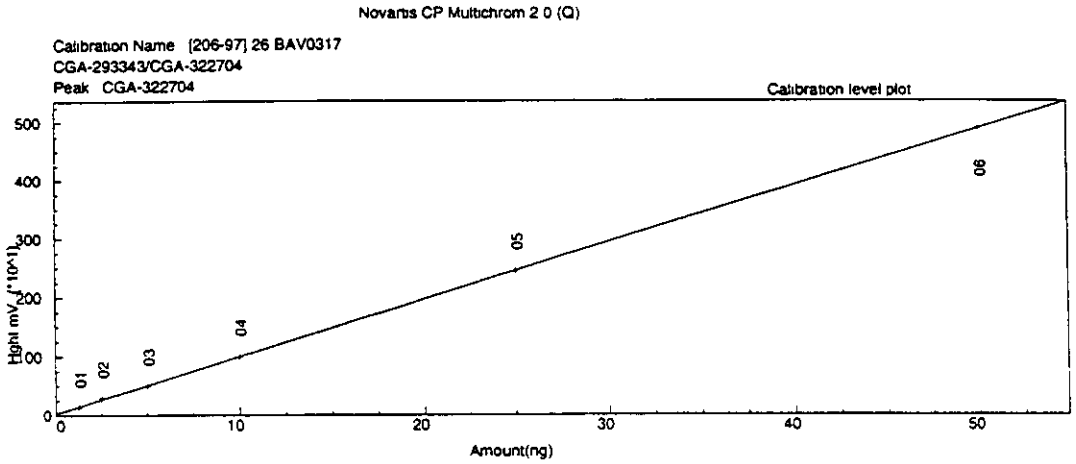
			CGA-293343 Results				CGA-322704 Results			
	Protocol	Recovery	Primary Analysis		Confirmatory Analysis		Primary Analysis		Confirmatory Analysis	
Substrate*	Sample #	Level	ppm	% recovery	ppm	% recovery	ppm	% recovery	ppm	% recovery
Cucumbers	35	Control	<0 01		<0 01		<0 01		<0 01	
	36	0 01	0 009	87	0 01	116	0 009	92	0 009	94
	37	0 01	0 009	91	0 01	104	**	**	0 009	90
	38	0 5	0 42	83	0 42	84	0 449	90	0 44	87
	39	^{14}C metabolism sample	0 043		0 044		<0 01		<0 01	
	40	^{14}C metabolism sample	0 051		0 047		<0 01		<0 01	
	41	^{14}C metabolism sample	0 044		0 044		<0 01		<0 01	
	42	^{14}C metabolism sample	0 022		0 025		0 018		<0 01	
	43	^{14}C metabolism sample	0 036		0 037		0 015		<0 01	
	44	^{14}C metabolism sample	0 038		0 033		<0 01		<0 01	
Goat Milk	100	Control	<0 01		<0 01		<0 01		<0 01	
	101	0 005	0 006	113	0 006	122	0 005	96	0 006	120
	102	0 005	0 005	104	0 005	102	0 005	96	0 006	106
	103	0 50	0 44	88	0 46	92	0 45	90	0 45	89
	104	^{14}C metabolism sample	0 087		0 098		0 17		0 16	
	105	^{14}C metabolism sample	0 064		0 077		0 12		0 13	
	106	^{14}C metabolism sample	0 085		0 095		0 16		0 15	
Corn Fodder	140	Control	<0 01		<0 01		<0 01		<0 01	
	141	0 01	0 007	72	0 008	78	0 009	64	0 006	56
	142	0 01	0 007	75	0 008	83	0 01	74	0 006	64
	143	1 0	0 85	85	0 76	75	0 81	81	0 80	80
	144	^{14}C metabolism sample	0 022		0 022		0 017		0 012	
	145	^{14}C metabolism sample	0 024		0 024		0 021		0 014	
	146	^{14}C metabolism sample	0 025		0 025		0 023		0 014	
Sorghum forage	154	Reagent Blank	<0 01		<0 01		<0 01		<0 01	
	155	Control	<0 01		<0 01		<0 01		<0 01	
	156	0 01	0 007	68	0 01	96	0 012	78	0 009	93
	157	0 01	0 008	82	0 009	85	0 014	96	0 009	89
	158	0 1	0 084	84	0 092	92	0 09	85	0 081	81
	158	1 0	0 86	86	0 86	86	0 87	86	0 88	88
	160	1 0	0 84	84	0 90	90	0 84	84	0 86	86
Tobacco (Green Leaves)	161	Control	<0 01		<0 01		<0 01		<0 01	
	162	0 01	0 01	96	0 008	73	0 009	81	0 007	75
	163	0 01	0 009	94	0 009	76	0 009	82	0 008	78
	164	0 1	0 092	92	0 081	80	0 078	77	0 079	79
	165	1 0	0 92	92	0 85	85	0 85	85	0 87	87
	166	1 0	0 95	95	0 88	88	0 85	85	0 90	90

* Cucumbers and goat milk were analyzed by HPLC/UV and confirmed by HPLC/MS The rest of the substrates listed were analyzed by HPLC/MS and confirmed by HPLC/MS/MS

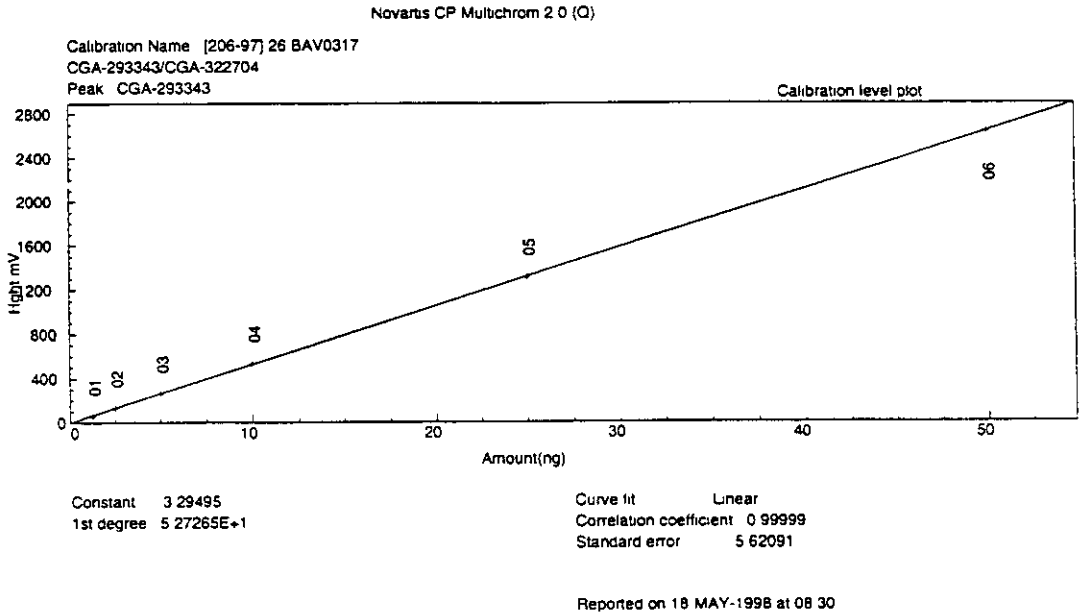
** Recovery of 173% occurred for CGA-322704 analysis of this sample by HPLC/UV Reason for large peak for this analyte on this chromatogram is unknown (see this chromatogram in Figure ### of Appendix I) Confirmation by HPLC/MS gave result of 90%

TABLE VIII REPRESENTATIVE STANDARD CALIBRATION CURVES

ANALYSIS BY HPLC/UV (see Figure 5 for chromatograms of these standards)



1 CGA-322704



2 CGA-293343

TABLE VIII REPRESENTATIVE STANDARD CALIBRATION CURVES (Continued)

ANALYSIS BY HPLC/MS (see Figure 6 for chromatograms of these standards)

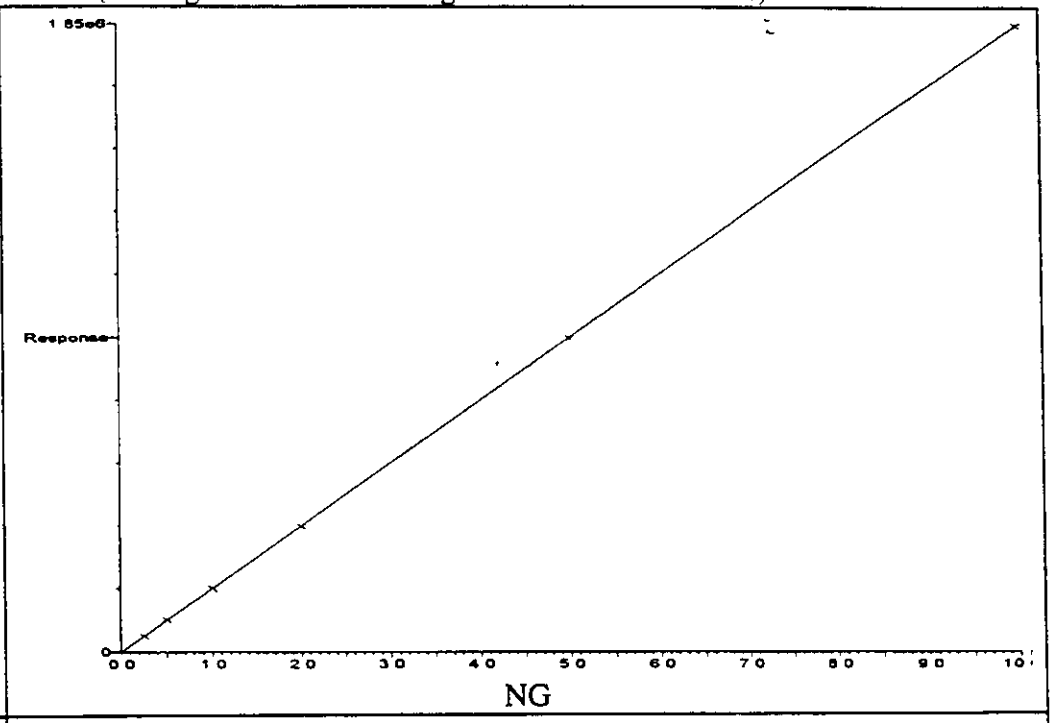
CGA-322704

Slope = 184871 9

Y-intercept = 313 9

Correlation Coefficient = 0 999997

Curve Fit = Linear



CGA-293343

Slope = 129042 9

Y-intercept = 7203 5

Correlation Coefficient = 0 999718

Curve Fit = Linear

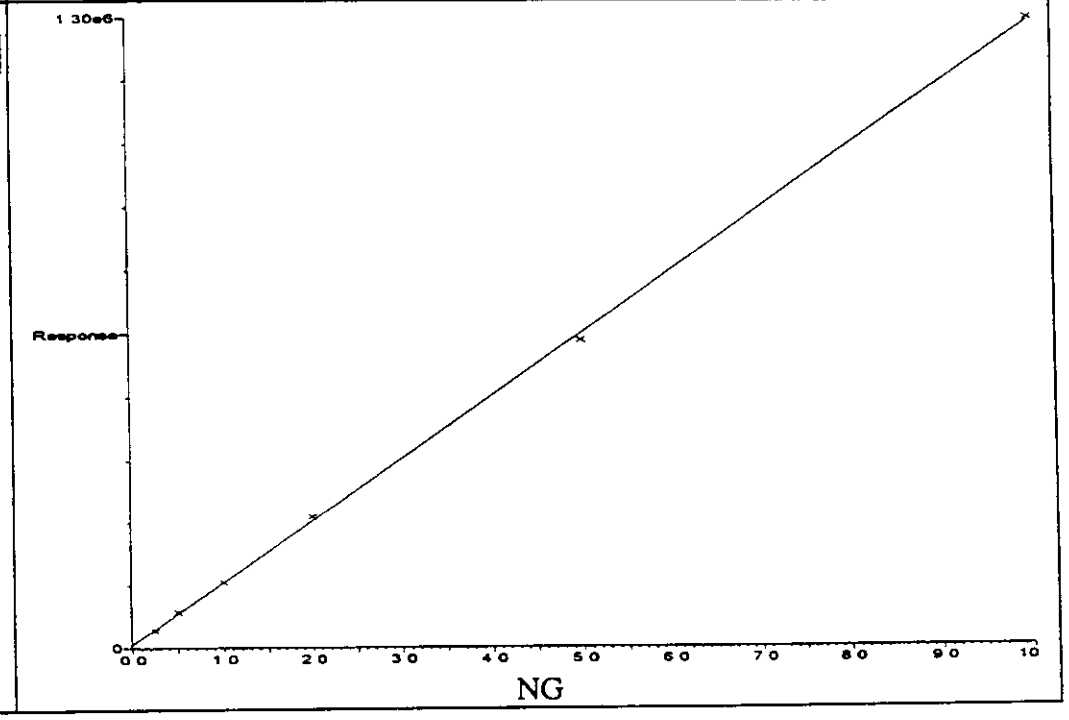


TABLE VIII REPRESENTATIVE STANDARD CALIBRATION CURVES (Continued)

ANALYSIS BY HPLC/MS/MS (see Figure 7 for chromatograms of these standards)

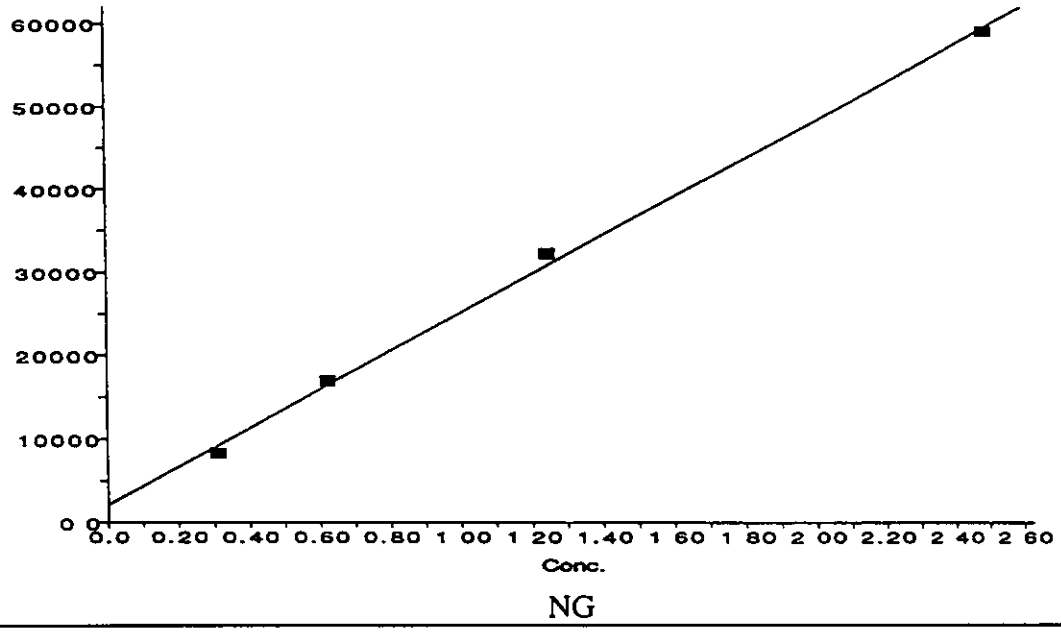
CGA-322704

Slope = 23089 5

Y-intercept = 2066 8

Correlation Coefficient = 0 99895

Curve Fit = Linear



CGA-293343

Slope = 23024 6

Y-intercept = 2382 0

Correlation Coefficient = 0 99869

Curve Fit = Linear

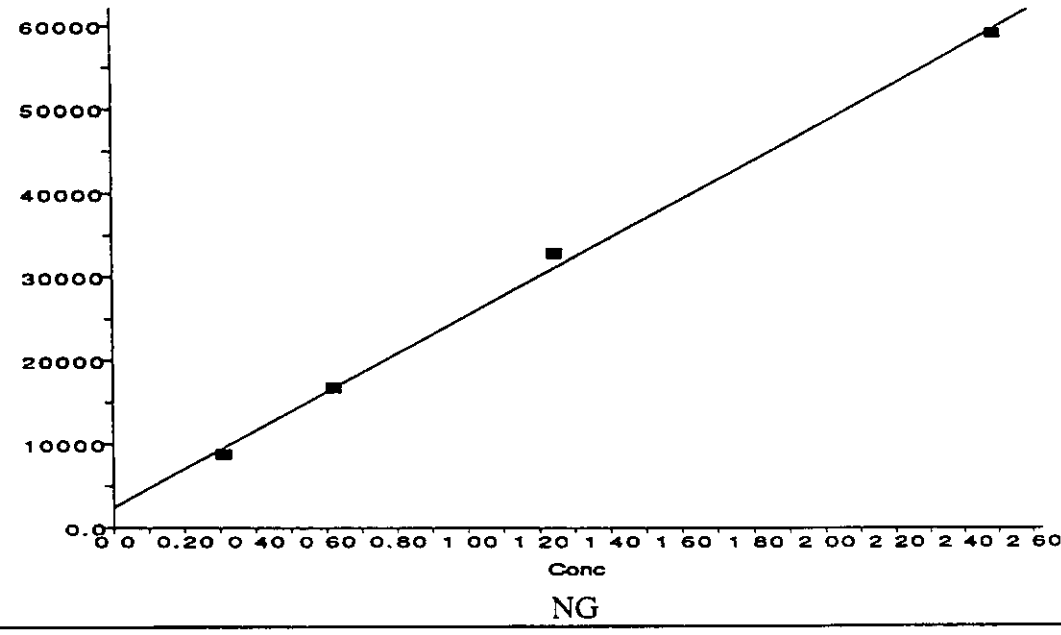
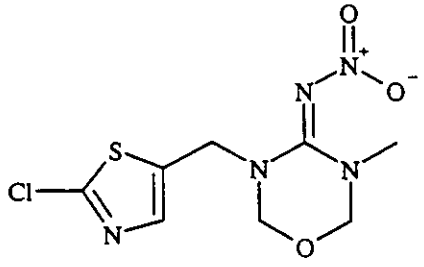


FIGURE 1 STRUCTURES AND CHEMICALS NAMES

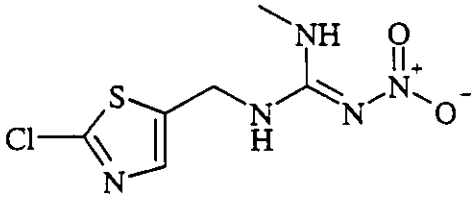


CGA-2933434

CAS Name *4H*-1,3,5-Oxadiazin-4-imine, 3-[(2-chloro-5-thiazolyl)methyl] tetrahydro-5-methyl-N-nitro-

CAS Number. 153719-23-4

Molecular Weight = 291.02



CGA-322704

CAS Name Guanidine, N-[(2-chloro-5-thiazolyl) methyl]-N'-methyl-N''-nitro-

CAS Number 131748-59-9

Molecular Weight = 249 8

FIGURE 2 ANALYTICAL PROCEDURE FLOW CHART FOR THE ANALYSIS OF CROP SAMPLES BY HPLC/UV

EXTRACTION -

- Add 150-mL ACN H_2O (80 20) to 10 gram sample - Polytron 1 minute
- Allow solid material to settle Decant and Filter solvent through Whatman 5 filter with Celite (1-2 cm thickness)
- Add 50-mL ACN H_2O (80 20) to remaining solid material - Polytron 1 minute and filter Combine the two extracts
- If necessary, rinse the extraction bottle and filter cake with 10-mL ACN H_2O (80 20)
- Pour combined extract into 250-ml graduated Cylinder; bring to 200-mL with ACN H_2O (80 20)
- Transfer 100-mL aliquot into a 500-mL round bottom boiling flask (RB), and evaporate sample to aqueous (less than or equal to 20-mL)

PHENYL SOLID-PHASE EXTRACTION (under vacuum with reservoir and stopcock)

- CONDITION COLUMN (BondElut 1g/6cc, Varian Part#1225-6004) 5-mL Methanol → 5-mL H_2O
- LOAD SAMPLE Discard eluate
- WASH Add 3-mL H_2O to RB, swirl and SONICATE WELL Apply to column, and discard eluate
- ELUTE SAMPLE Rinse RB with 10-mL of methanol H_2O (1 1), and add to column Collect eluate in 50-mL concentration tube
- Evaporate sample to aqueous (less than or equal to 5 mL)

PARTITION -

- Add 1-mL H_2O saturated with sodium chloride and 20-mL ethyl acetate (EtoAc) to the concentration tube
- Partition for 1 minute (shake or vortex) Allow 2 layers to form
- Remove top layer with a pipet, and transfer to a 125-mL RB
- Repeat for second 20-mL EtoAc partition (combine with first EtoAc layer)
- Evaporate sample to dry and reconstitute in 5-mL EtoAc Hexane(20 80) SONICATE WELL

NORMAL PHASE SOLID-PHASE EXTRACTION (Gravity only, with reservoir and stopcock)

- CONDITION COLUMNS
  AMINO COLUMN Varian Bondelut 100mg/1cc(Varian Part#1210-2014) with reservoir and stopcock 1) 1-mL 3 97 MeOH EtoAc → 2) 1-mL 20 80 EtoAc Hexane
  ALUMINA COLUMN Waters 1g/6 cc (Waters Part #WAT054620) 1) 5-mL 10 90 MeOH EtoAc → 2) 5-mL 3 97 MeOH EtoAc If stacking columns, leave ~ 2cm of solution on top of packing
- LOAD SAMPLE ON AMINO COLUMN
  Load the sample (in EtoAc Hexane(20 80) and discard eluate
- WASH COLUMN Wash RB (SONICATE WELL) and column with 1-mL EtoAc Hexane(50 50) Discard eluate
- STACK COLUMNS Attach the AMINO cartridge to the ALUMINA cartridge (with adapter)
- ELUTE FROM AMINO INTO ALUMINA (COLLECT ALUMINA ELUATE)
  Add 10-mL 3 97 MeOH EtoAc to RB, SONICATE WELL, and add to column Allow to drip through both the amino and the alumina columns, and collect in a 50-mL concentration tube (Option - collect alumina eluate separately without stacking columns, and load it onto the alumina Collect alumina eluate)
- ELUTE REMAINING SAMPLE FROM ALUMINA
  Disconnect the two columns, and discard the amino column Add 10-mL 10 90 MeOH EtoAc to the RB as a rinse(SONICATE WELL), add to alumina column to elute remainder compounds Collect in same 50-mL conc tube

FINAL SAMPLE

Evaporate sample to dry and reconstitute in Hexane EtoAc isopropyl alcohol methanol (11 3 1 1)
Analyze by HPLC/UV

FIGURE 3 ANALYTICAL PROCEDURE FLOW CHART FOR THE ANALYSIS OF ANIMAL SAMPLES BY HPLC/UV

EXTRACTION -

- Add 150-mL ACN H_2O (80 20) to 10 gram sample - Polytron 1 minute
- Allow solid material to settle Decant and Filter solvent through Whatman 5 filter with Celite (1-2 cm thickness)
- Add 50-mL ACN H_2O (80 20) to remaining solid material - Polytron 1 minute and filter Combine the two extracts
- Pour combined extract into 250-ml graduated Cylinder; bring to 200-mL with ACN H_2O (80 20)
- Transfer 100-mL aliquot into a 250-mL separatory funnel (SF) (note - for oil samples, weigh 5 gram sample directly into this funnel, and add 100-mL of ACN H_2O (80 20)

PARTITION OF EXTRACT

- Add following to sample 25-mL hexane, 5-mL H_2O saturated with sodium chloride, 2-mL toluene
- Shake 1 minute Allow 3 layers to separate Drain and save bottom aqueous layer Save middle ACN/Toluene layer in a 500-mL round bottom boiling flask (RB) Discard top hexane layer Return the bottom aqueous layer to the SF
- Add 40-mL Toluene ACN (2 98), shake 1 minute Drain and discard bottom aqueous layer Combine the ACN/Toluene layers in the 500-mL RB, and evaporate Sample to aqueous

PHENYL SOLID-PHASE EXTRACTION (under vacuum with reservoir and stopcock)

- CONDITION COLUMN (BondElut 1g/6cc, Varian Part#1225-6004) 5-mL Methanol → 5-mL H_2O
- LOAD SAMPLE Discard eluate
- WASH Add 3-mL H_2O to RB, swirl and SONICATE WELL Apply to column, and discard eluate
- ELUTE SAMPLE Rinse RB with 10-mL of methanol H_2O (1 1), and add to column Collect eluate in 50-mL concentration tube
- Evaporate sample to aqueous (less than or equal to 5 mL)

PARTITION -

- Add 1-mL H_2O saturated with sodium chloride and 20-mL ethyl acetate (EtoAc) to the concentration tube
- Partition for 1 minute (shake or vortex) Allow 2 layers to form
- Remove top layer with a pipet, and transfer to a 125-mL RB
- Repeat for second 20-mL EtoAc partition (combine with first EtoAc layer)
- Evaporate sample to dry and reconstitute in 5-mL EtoAc Hexane(20 80) SONICATE WELL

NORMAL PHASE SOLID-PHASE EXTRACTION (Gravity only, with reservoir and stopcock)

- CONDITION COLUMNS
  AMINO COLUMN Varian Bondelut 100mg/1cc(Varian Part#1210-2014) with reservoir and stopcock
  1) 1-mL 3 97 MeOH EtoAc → 2) 1-mL 20 80 EtoAc Hexane
  ALUMINA COLUMN Waters 1g/6 cc (Waters Part #WAT054620) 1) 5-mL 10 90 MeOH EtoAc → 2) 5-mL 3 97 MeOH EtoAc If stacking columns, leave ~ 2cm of solution on top of packing
- LOAD SAMPLE ON AMINO COLUMN
  Load the sample (in EtoAc Hexane(20 80) and discard eluate
- WASH COLUMN Wash RB (SONICATE WELL) and column with 1-mL EtoAc Hexane(50 50) Discard eluate
- STACK COLUMNS Attach the AMINO cartridge to the ALUMINA cartridge (with adapter)
- ELUTE FROM AMINO INTO ALUMINA (COLLECT ALUMINA ELUATE)
  Add 10-mL 3 97 MeOH EtoAc to RB, SONICATE WELL, and add to column Allow to drip through both the amino and the alumina columns, and collect in a 50-mL concentration tube (Option - collect alumina eluate separately without stacking columns, and load it onto the alumina Collect alumina eluate)
- ELUTE REMAINING SAMPLE FROM ALUMINA
  Disconnect the two columns, and discard the amino column Add 10-mL 10 90 MeOH EtoAc to the RB as a rinse(SONICATE WELL), add to alumina column to elute remainder compounds Collect in same 50-mL conc tube

FINAL SAMPLE. Evaporate sample to dry and reconstitute in Hexane EtoAc isopropyl alcohol methanol (11 3 1 1) Analyze by HPLC/UV

FIGURE 4 ANALYTICAL PROCEDURE FLOW CHART FOR THE ANALYSIS OF SAMPLES BY HPLC/MS

EXTRACTION -

- Add 150-mL ACN H_2O (80 20) to 10 gram sample - Polytron 1 minute
- Allow solid material to settle Decant and Filter solvent through Whatman 5 filter with Celite (1-2 cm thickness)
- Add 50-mL ACN H_2O (80 20) to remaining solid material - Polytron 1 minute and filter Combine the two extracts
- If necessary, rinse the extraction bottle and filter cake with 10-mL ACN H_2O (80 20)
- Pour combined extract into 250-ml graduated Cylinder; bring to 200-mL with ACN H_2O (80 20)
- Transfer 50-mL aliquot into a 250-mL round bottom boiling flask (RB), and evaporate sample to aqueous (less than or equal to 20-mL)

SAX / PHENYL SOLID-PHASE EXTRACTION (under vacuum with reservoir and stopcock)

- CONDITION COLUMNS
  SAX COLUMN 6 cc/ 1 gram (Varian part #1225-6013) 10-mL of 10mM potassium phosphate buffer
  PHENYL COLUMN 6 cc/ 1 gram (Varian part#1225-6004) 5-mL methanol → 10-ml 10mM buffer If stacking columns, leave ~ 5-mL of buffer above phenyl packing
- Stack SAX on top of phenyl with adapter
- LOAD SAMPLE Add 3-mL 50mM Buffer to sample, mix well and apply to column Allow to drip through both the SAX and the phenyl Discard eluate
- Wash 250-mL RB flask with 5-mL 10mM buffer and apply to column Allow to drip through both the SAX and the phenyl Discard eluate Discard SAX column
- ELUTE SAMPLE FROM PHENYL Rinse RB with 10-mL of methanol H_2O (1 1), and add to column Collect eluate in 50-mL concentration tube
- Evaporate sample to aqueous (less than or equal to 5 mL)

PARTITION -

- Add 1-mL H_2O saturated with sodium chloride and 20-mL ethyl acetate (EtoAc) to the concentration tube
- Partition for 1 minute (shake or vortex) Allow 2 layers to form
- Remove top layer with a pipet, and transfer to a 125-mL RB
- Repeat for second 20-mL EtoAc partition (combine with first EtoAc layer)
- Evaporate sample to dry and reconstitute in 5-mL EtoAc Hexane(20 80) SONICATE WELL

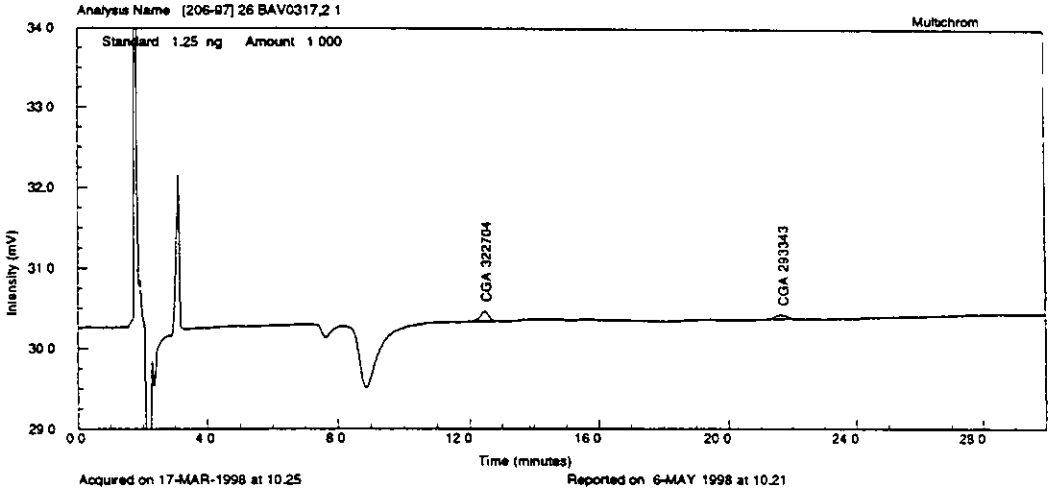
ALUMINA NORMAL PHASE SOLID-PHASE EXTRACTION

- CONDITION COLUMN (Waters Part #WAT054620) 1) 5-mL 10 90 MeOH EtoAc → 2) 5-mL EtoAc Hexane(20 80)
- LOAD SAMPLE Discard load eluate
- WASH Add RB 5-mL EtoAc Hexane(20 80) to RB (SONICATE WELL) and apply to column Discard wash eluate
- ELUTE SAMPLE Add 10-mL mL MeOH EtoAc (10 90) to RB, SONICATE, and add to the alumina column to elute sample Collect in 50-mL concentration tube

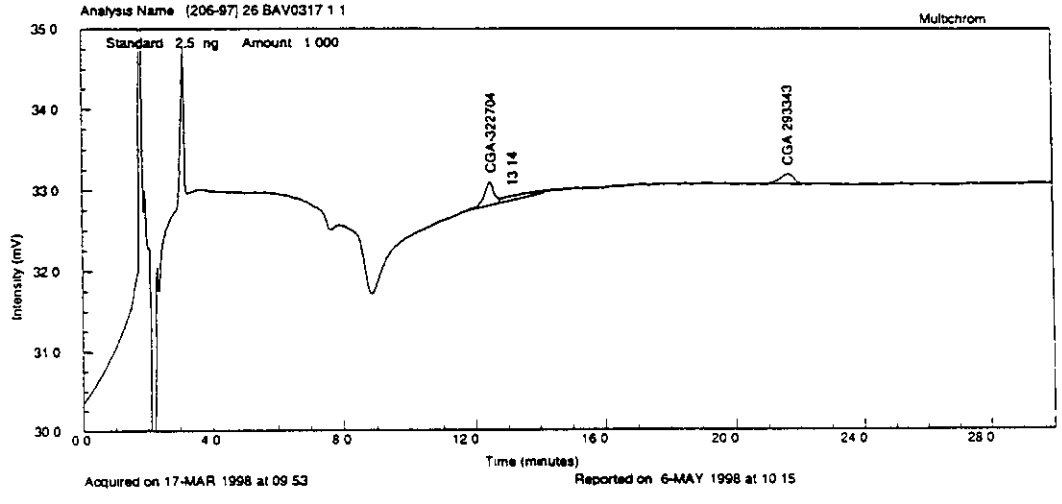
FINAL SAMPLE: Evaporate sample to dry and reconstitute in ACN H2O (10 90) Analyze by HPLC/MS

FIGURE 5 REPRESENTATIVE STANDARD CHROMATOGRAMS ANALYSIS BY HPLC/UV

Standards from the analysis of wheat grain (samples 148-153)



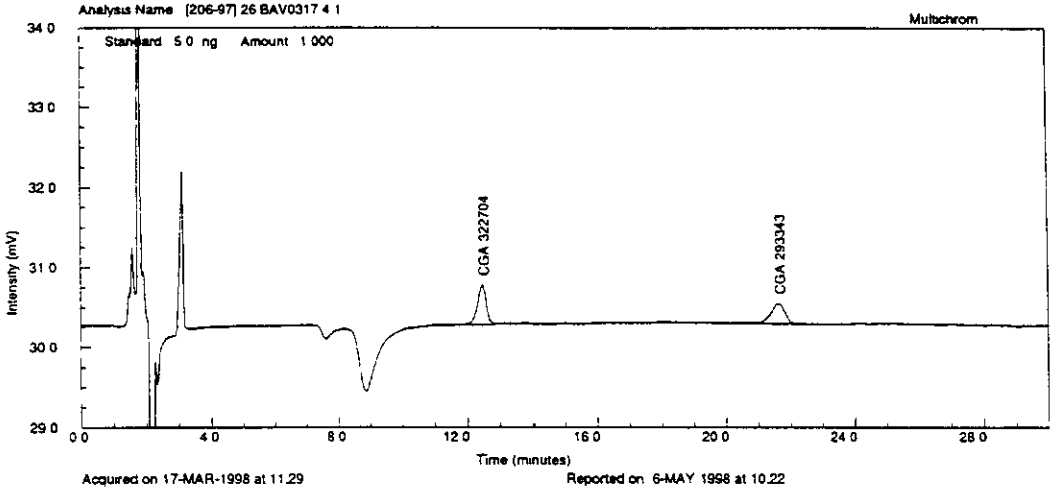
1 0 0125 ng/µL standard, 1 25 ng injected



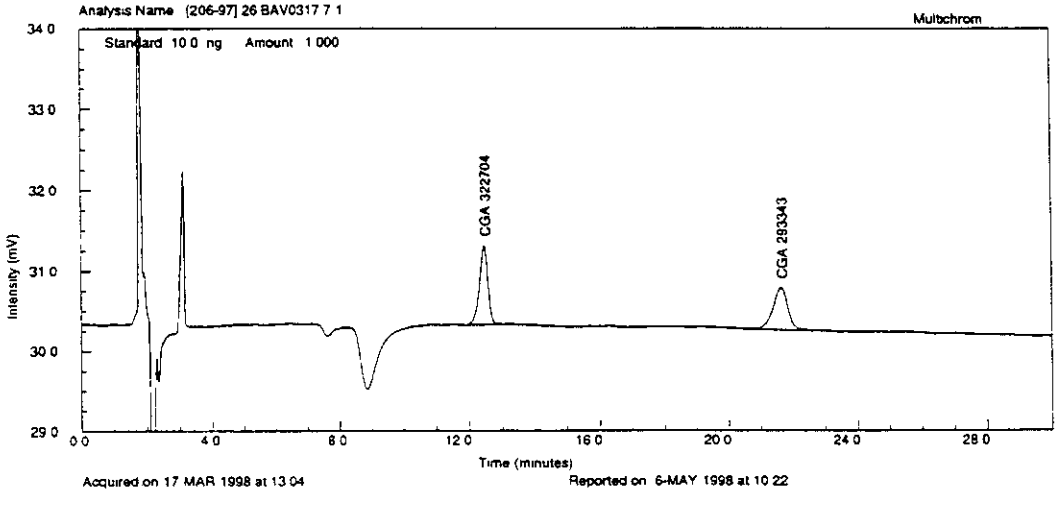
2 0 025 ng/µL standard, 2 5 ng injected

FIGURE 5 REPRESENTATIVE STANDARD CHROMATOGRAMS ANALYSIS BY HPLC/UV (Continued)

Standards from the analysis of wheat grain (samples 148-153)



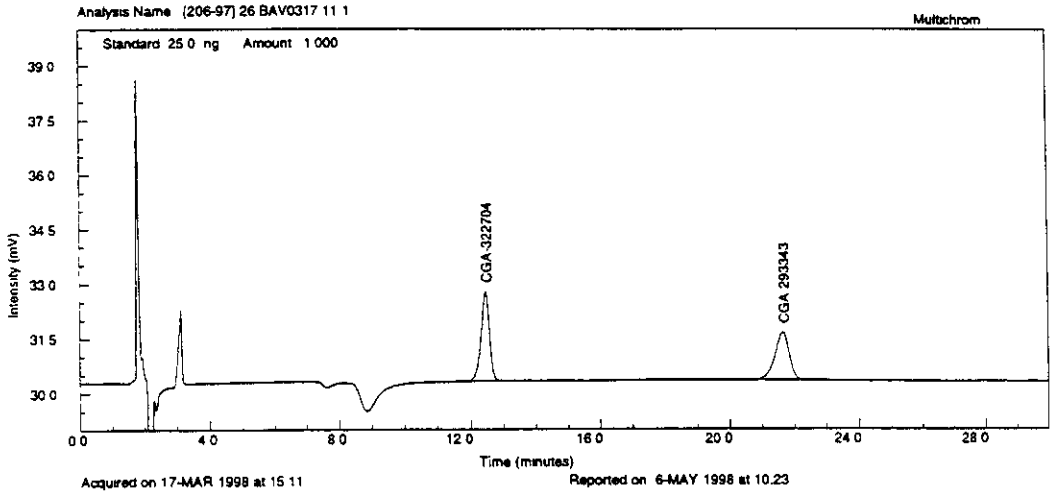
3 0 05 ng/µL standard, 5 0 ng injected



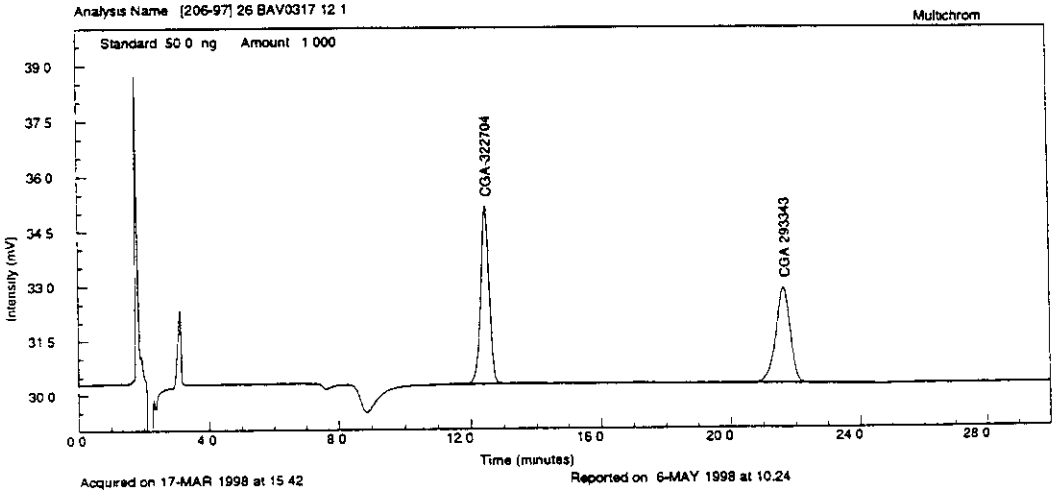
4 0 1 ng/µL standard 10 ng injected

FIGURE 5 REPRESENTATIVE STANDARD CHROMATOGRAMS. ANALYSIS BY HPLC/UV (Continued)

Standards from the analysis of wheat grain (samples 148-153)



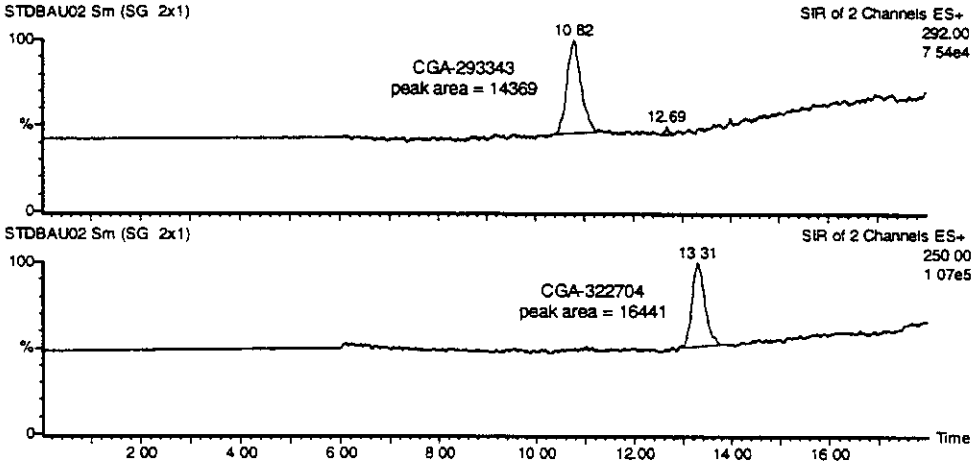
5 0 25 ng/µL standard, 25 ng injected



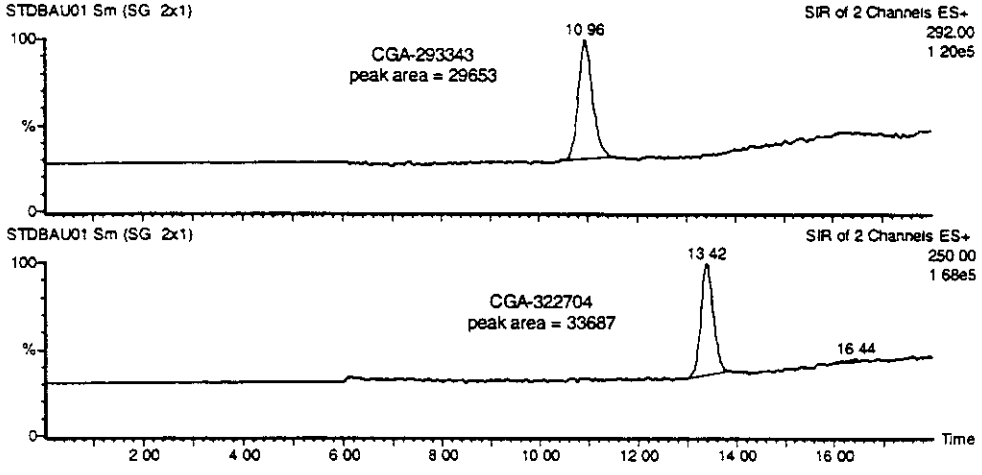
6 0 5 ng/µL standard, 50 ng injected

FIGURE 6 REPRESENTATIVE STANDARD CHROMATOGRAMS ANALYSIS BY HPLC/MS

Standards from the analysis of corn fodder (samples 140-146)

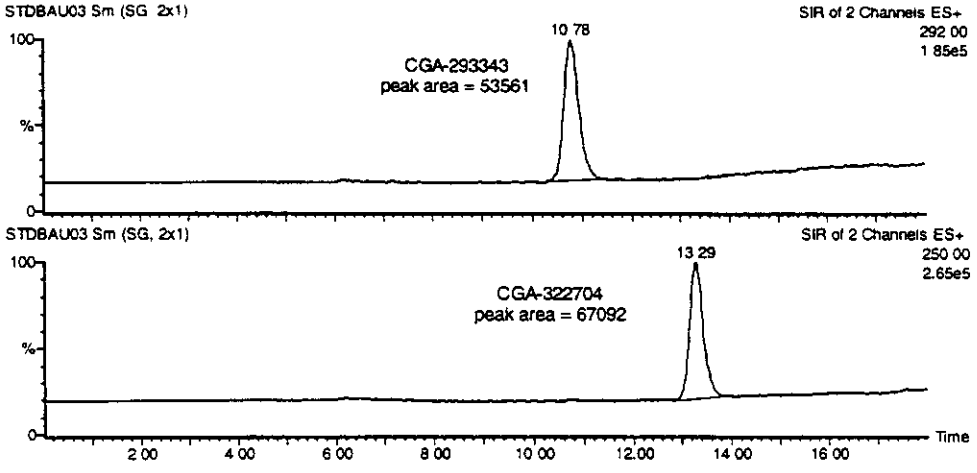


1 0 0125 ng/µL standard, 0 25 ng injected 0 23 ng determined for CGA-293343, 0 25 ng determined for CGA-322704

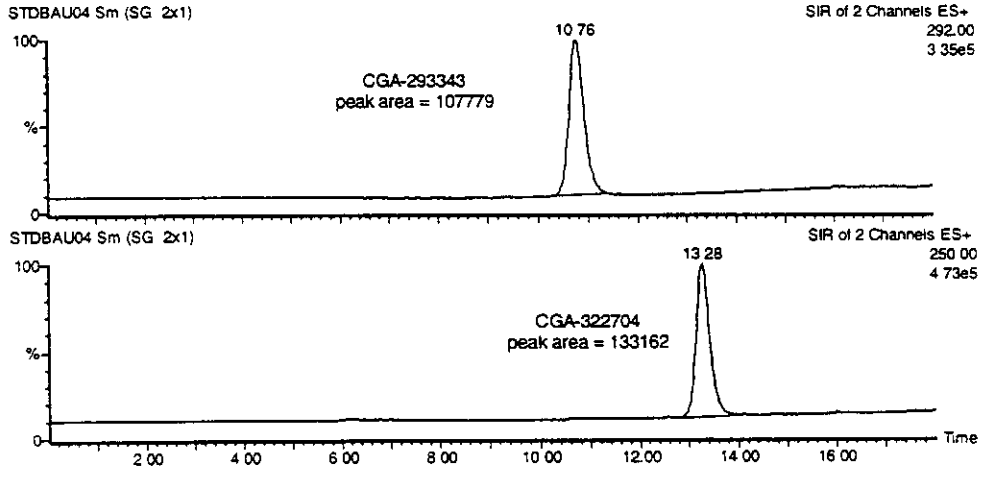


2 0 025 ng/µL standard, 0 50 ng injected 0 52 ng determined for CGA-293343, 0 51 ng determined fo r CGA-322704

FIGURE 6 REPRESENTATIVE STANDARD CHROMATOGRAMS ANALYSIS BY HPLC/MS (Continued)

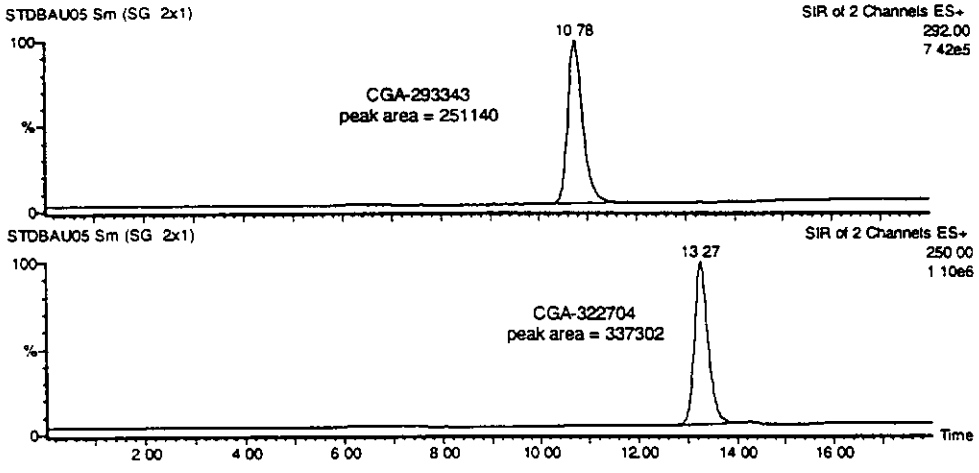


3 0 05 ng/µL standard, 1 0 ng injected 1 0 ng determined for CGA-293343, 1 0 ng determined for CGA-322704

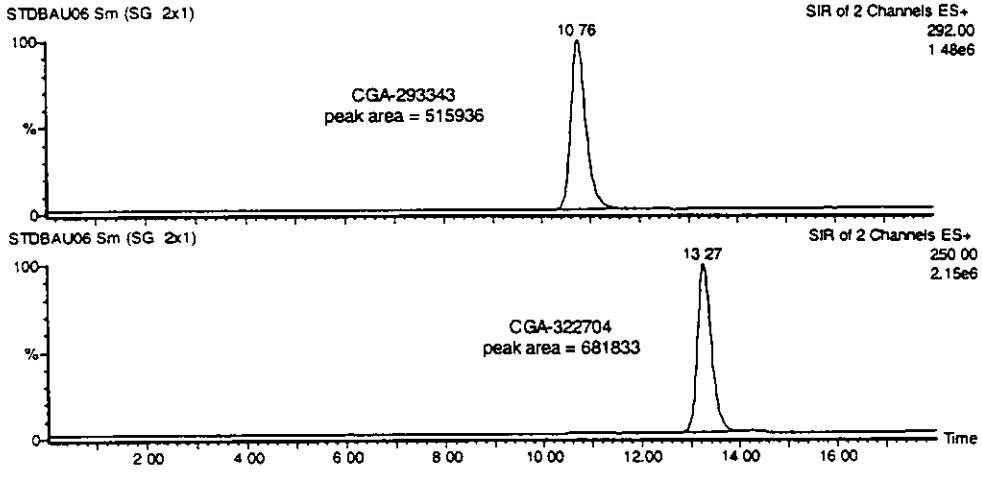


4 0 10 ng/µL standard, 2 0 ng injected 2 07 ng determined for CGA-293343, 1 99 ng determined for CGA-322704

FIGURE 6 REPRESENTATIVE STANDARD CHROMATOGRAMS ANALYSIS BY HPLC/MS (Continued)



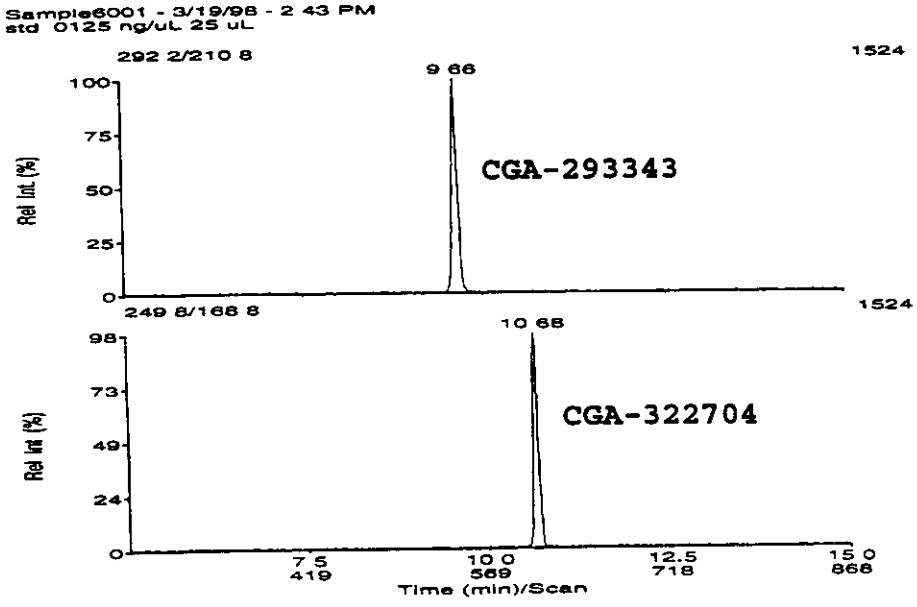
5 0 25 ng/µL standard, 5 0 ng injected 4 89 ng determined for CGA-293343, 4 99 ng determine d for CGA-322704



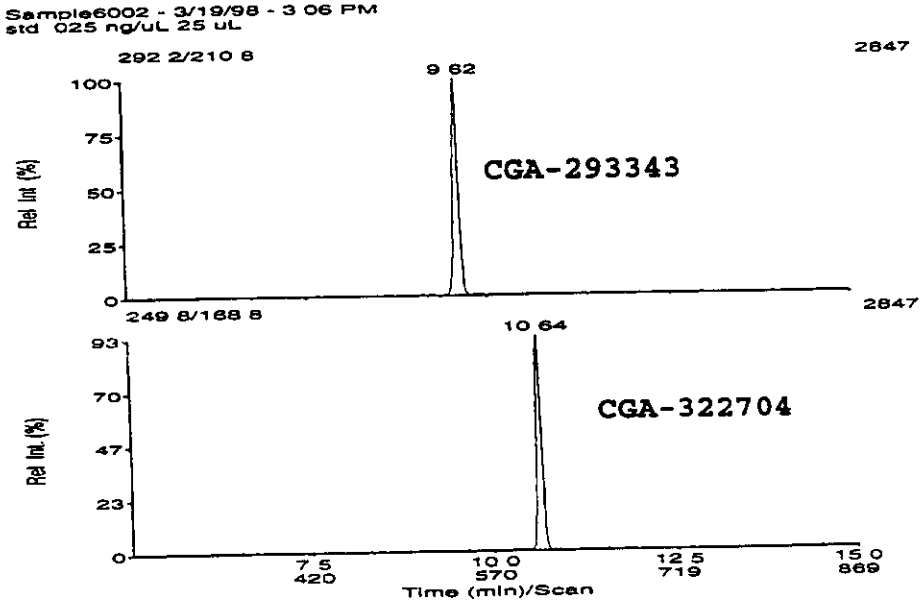
6 0 50 ng/µL standard, 10 0 ng injected 10 04 ng determined for CGA-293343, 10 01 ng determined for CGA-322704

FIGURE 7 REPRESENTATIVE STANDARD CHROMATOGRAMS ANALYSIS BY HPLC/MS/MS

Standards from the analysis of Georgia grass (samples 167 - 172).

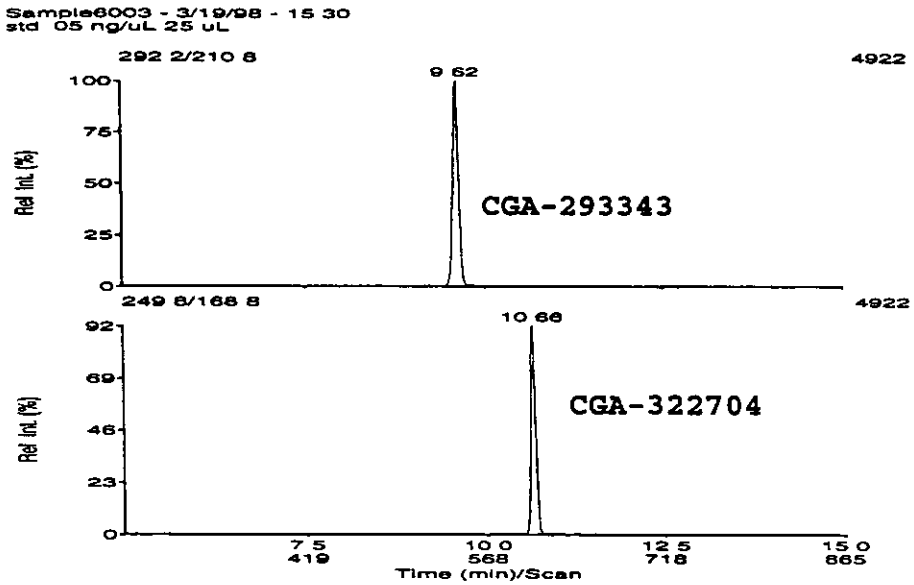


1 0 0125 ng/µL standard, 0 3125 ng injected 0 274 ng determined for CGA-293343 (top chromatogram, peak area = 8694), 0 268 ng determined for CGA-322704 (bottom chromatogram, peak area = 8253)

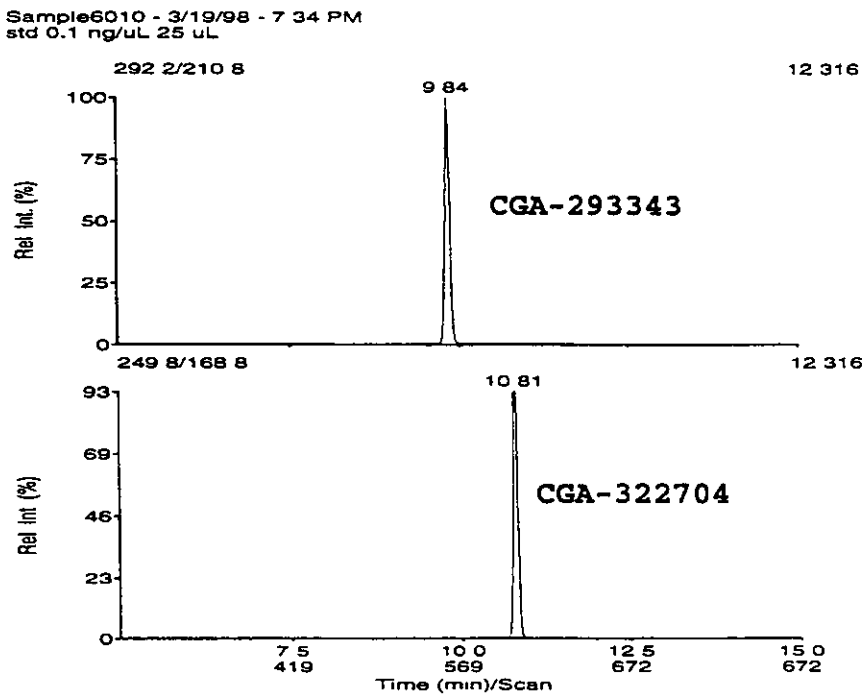


2 0 025 ng/µL standard, 0 625 ng injected 0 623 ng determined for CGA-293343 (top chromatogram, peak area = 16719), 0 640 ng determined for CGA-322704 (bottom chromatogram, peak area = 16854)

FIGURE 7 REPRESENTATIVE STANDARD CHROMATOGRAMS ANALYSIS BY HPLC/MS/MS (Continued)

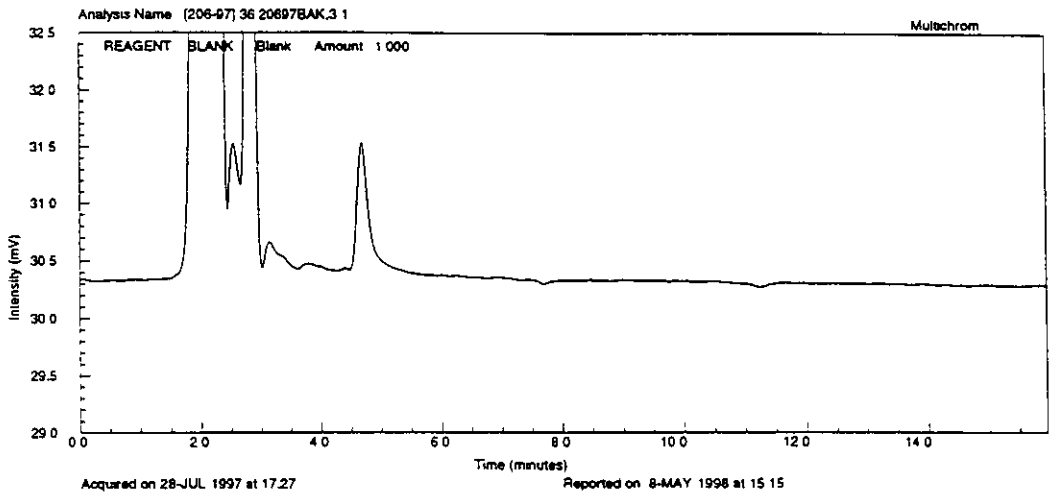


3 0 05 ng/µL standard, 0 125 ng injected 1 321 ng determined for CGA-293343 (top chromatogram, peak area = 32788), 1 305 ng determined for CGA-322704 (bottom chromatogram, peak area = 32196)

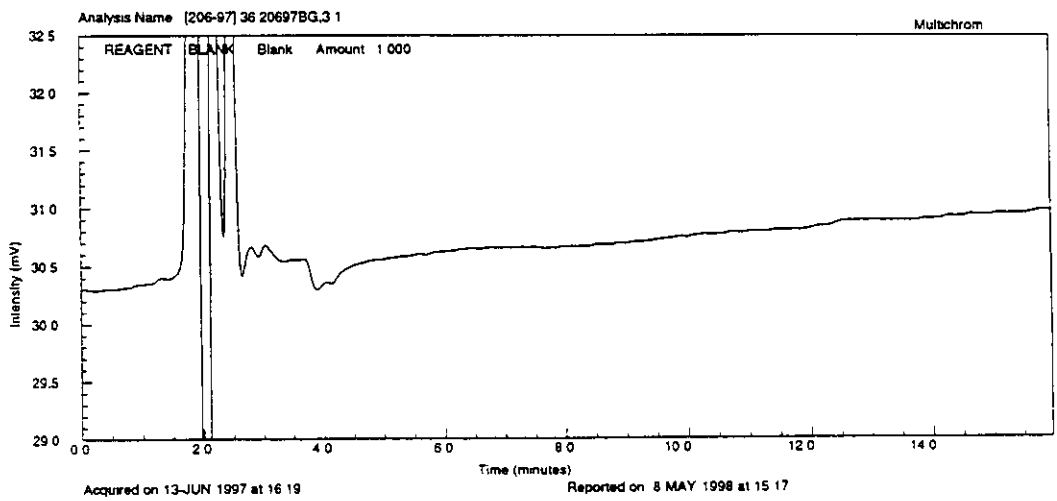


4 0 25 ng/µL standard, 2 5 ng injected 2 470 ng determined for CGA-293343 (top chromatogram, peak area = 59255), 2 474 ng determined for CGA-322704 (bottom chromatogram, peak area = 59197)

FIGURE 8 CHROMATOGRAMS FROM THE ANALYSIS OF REAGENT BLANKS

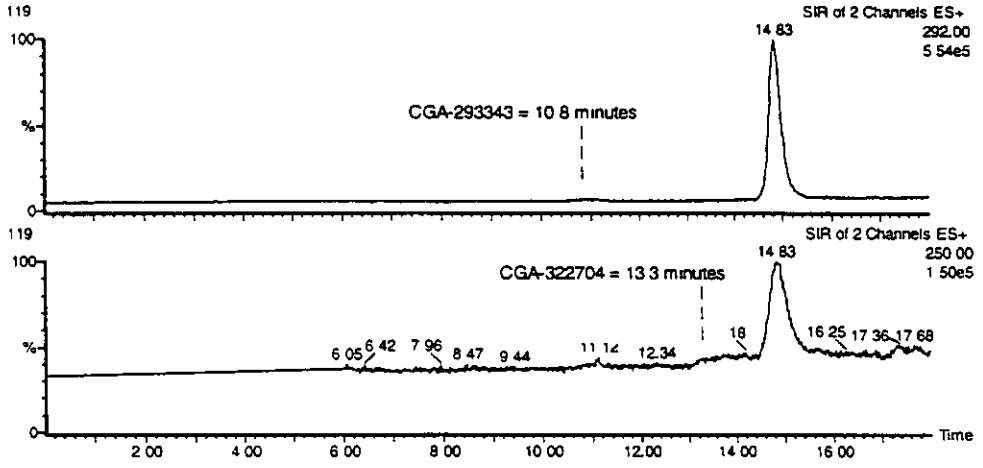


1 Reagent Blank Sample # 7- Analyzed with apple juice analytical set (shaker extraction, LOQ = 0 005 ppm, HPLC/UV analysis) Equivalent of 500 mg injected <0 005 ppm CGA-293343 and <0 005 ppm CGA-322704 detected

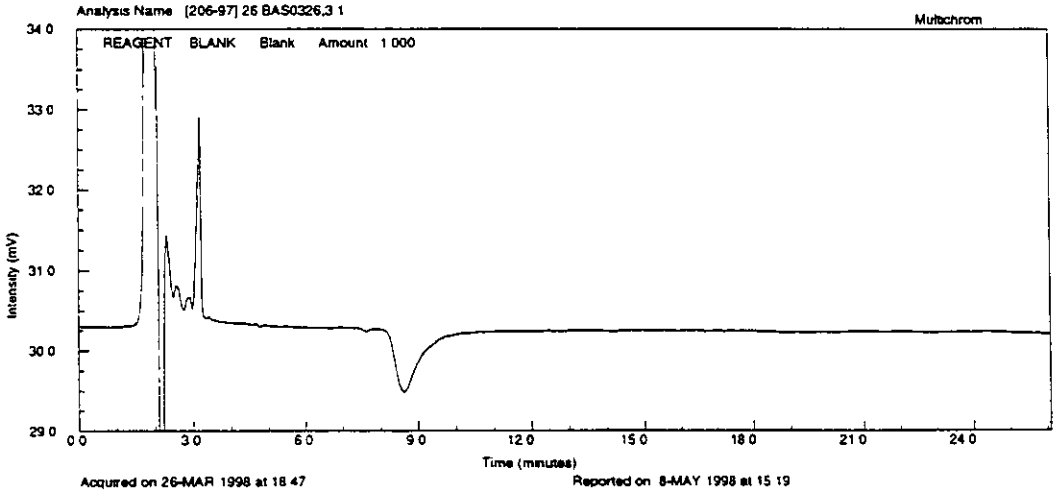


2 Reagent Blank Sample # 22 - Analyzed with potato tuber analytical set (polytron extraction, LOQ = 0 01 ppm, HPLC/UV analysis) Equivalent of 250 mg injected <0 01 ppm CGA-293343 and <0 01 ppm CGA-322704 detected

FIGURE 8 CHROMATOGRAMS FROM THE ANALYSIS OF REAGENT BLANKS (Continued)

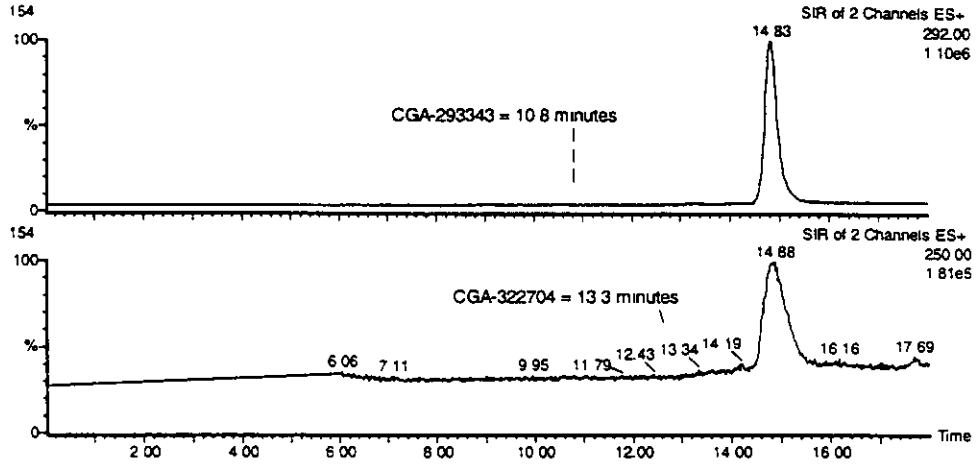


3 Reagent Blank Sample # 119 - Analyzed with undelinted cotton seed analytical set (polytron extraction, LOQ = 0.01 ppm, HPLC/MS procedures) Equivalent of 50 mg injected <0.01 ppm CGA-293343 and <0.01 ppm CGA-322704 detected



4 Reagent Blank Sample # 126 - Analyzed with cotton seed oil analytical set (partition extraction, LOQ = 0.01 ppm, HPLC/UV analysis) Equivalent of 250 mg injected <0.01 ppm CGA-293343 and <0.01 ppm CGA-322704 detected

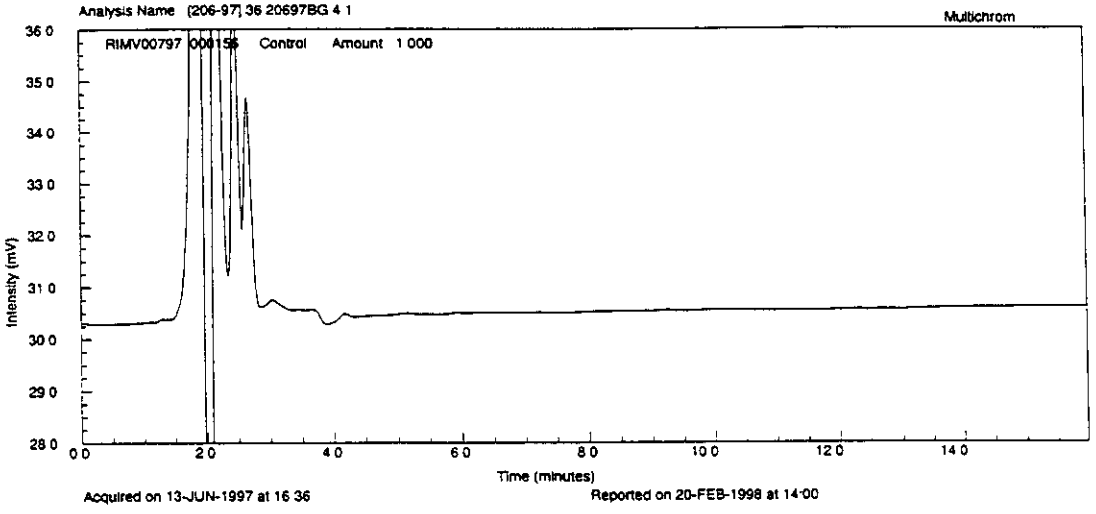
FIGURE 8 CHROMATOGRAMS FROM THE ANALYSIS OF REAGENT BLANKS (Continued)



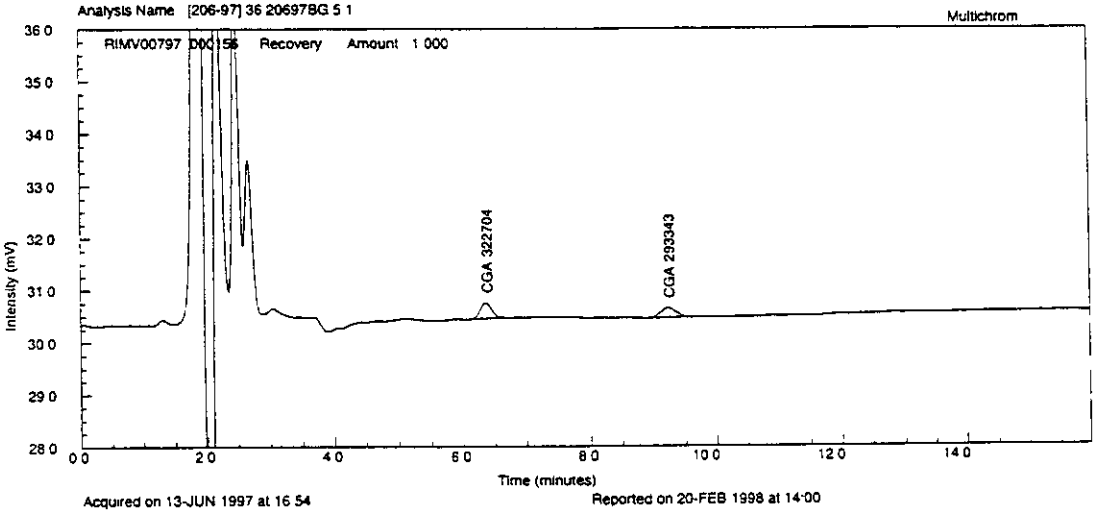
5 Reagent Blank Sample # 119 - Analyzed with sorghum forage analytical set (polytron extraction, LOQ = 0 01 ppm, HPLC/MS procedures) Equivalent of 50 mg injected <0 01 ppm CGA-293343 and <0 01 ppm CGA-322704 detected

FIGURE 9 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF VEGETABLES

POTATOES



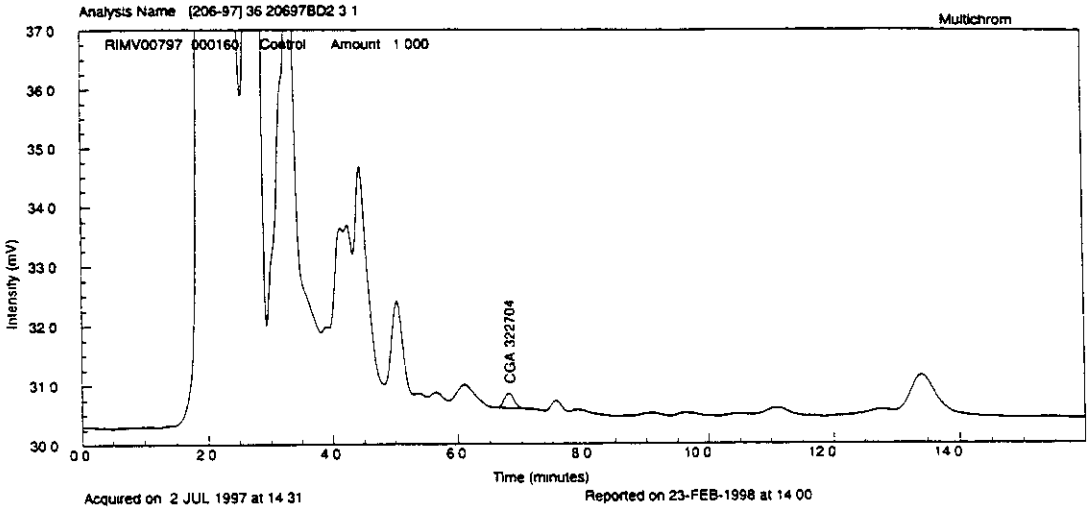
1 Potato Tubers - Sample #23, Control 250 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined (<0 01 ppm)



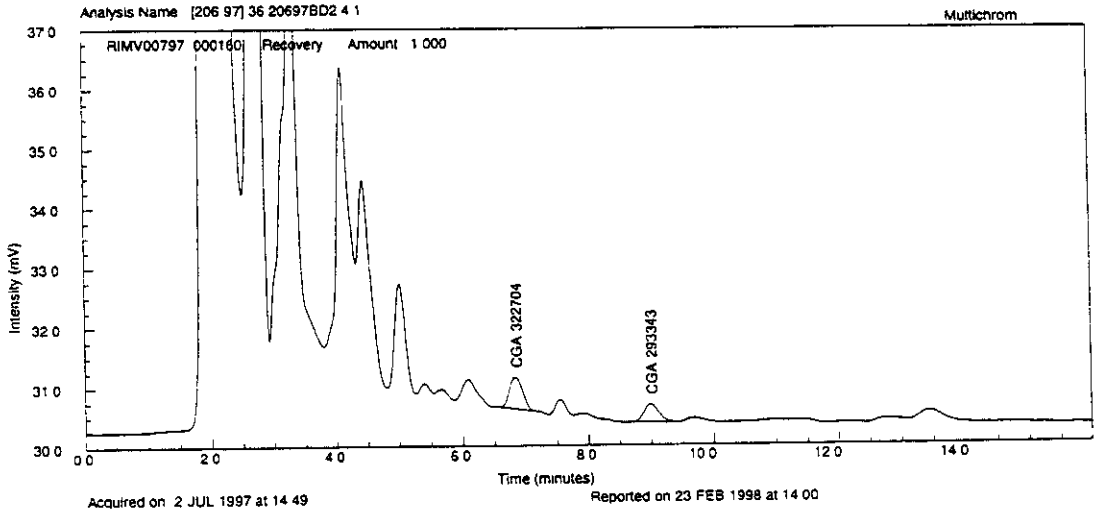
2 Potato Tubers - Sample #24, Control + 0 01 ppm CGA-293343 and 0 01 ppm CGA-322704 250 mg injected 2 4 ng CGA-293343 determined, 0 01 ppm, 96 % recovery 2 5 ng CGA-322704 determined, 0 01 ppm, 99% recovery

FIGURE 9 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF VEGETABLES (Continued)

SPINACH.



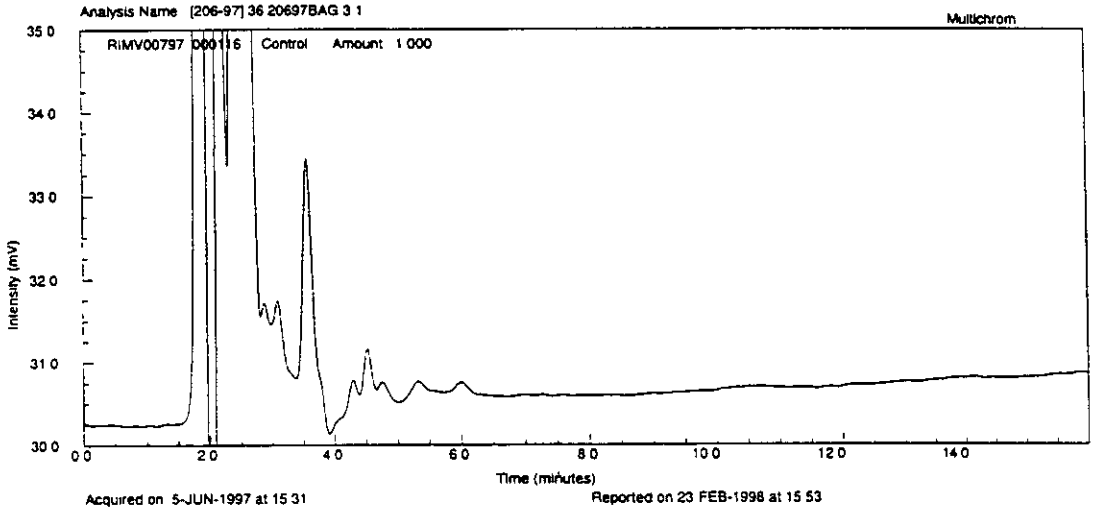
3 Spinach Leaves - Sample #63, Control 250 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) 1 7 ng CGA-322704 determined, 0 007 ppm



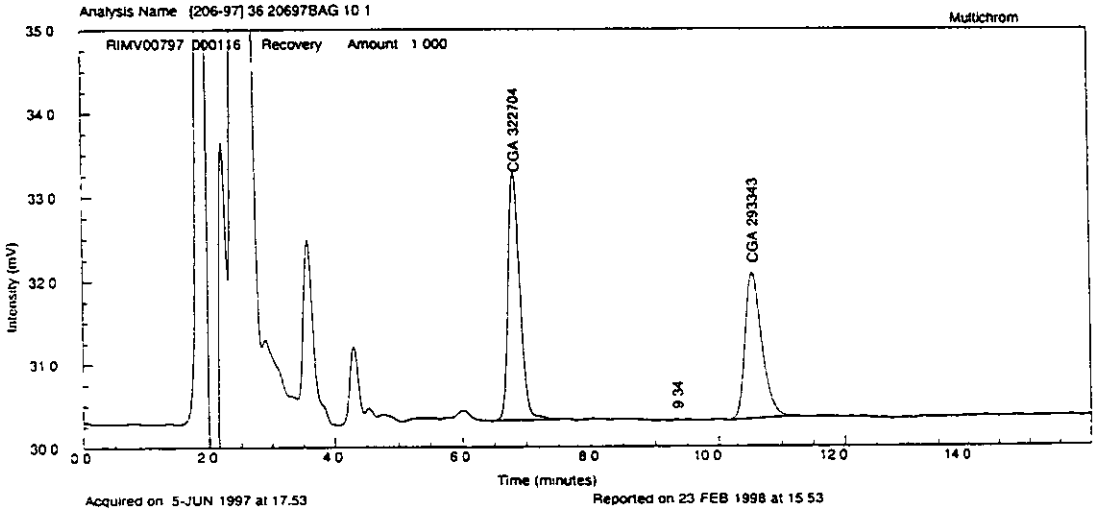
4 Spinach Leaves - Sample #64, Control + 0 01 ppm CGA-293343 and 0 01 ppm CGA-322704 250 mg injected 2 9 ng CGA-293343 determined, 0 01 ppm, 117% recovery 3 6 ng CGA-322704 determined, 0 015 ppm, 0 008 ppm after subtraction of control peak observed in Figure 9, chromatogram 3 above, 77% recovery

FIGURE 9 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF VEGETABLES (Continued)

BROCCOLI



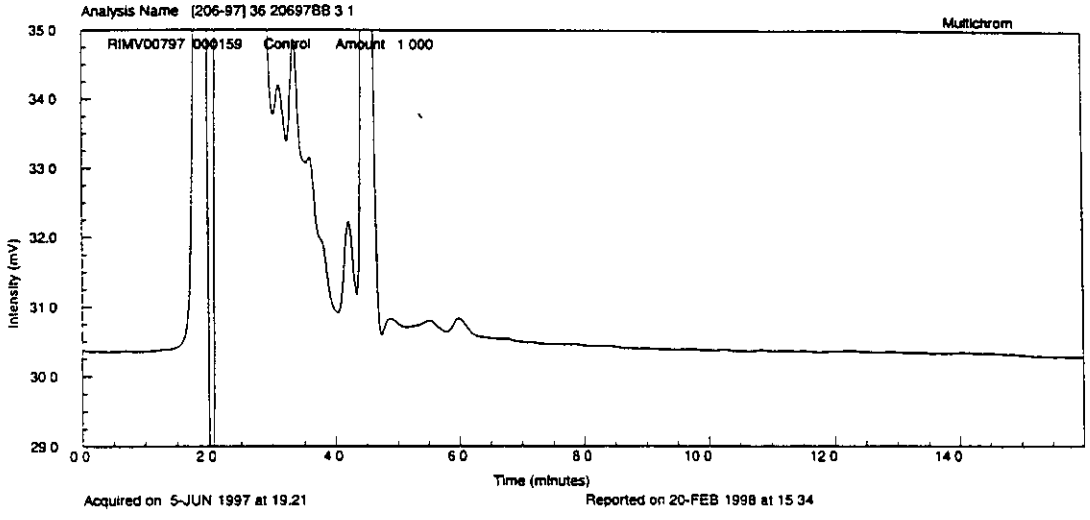
5 Broccoli - Sample #69, Control 250 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined, (<0 01 ppm)



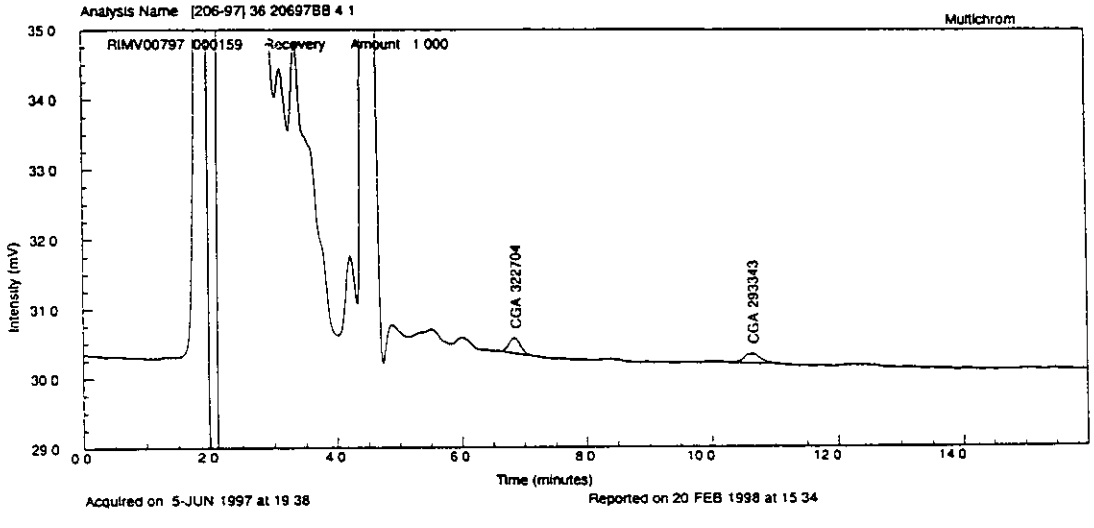
6 Broccoli - Sample #74, Control + 0 20 ppm CGA-293343 and 0 20 ppm CGA-322704 125 mg injected 21 ng CGA-293343 determined, 0 17 ppm, 84% recovery 23 ng CGA-322704 determined, 0 18ppm, 90% recovery

FIGURE 9 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF VEGETABLES (Continued)

TOMATO PASTE.

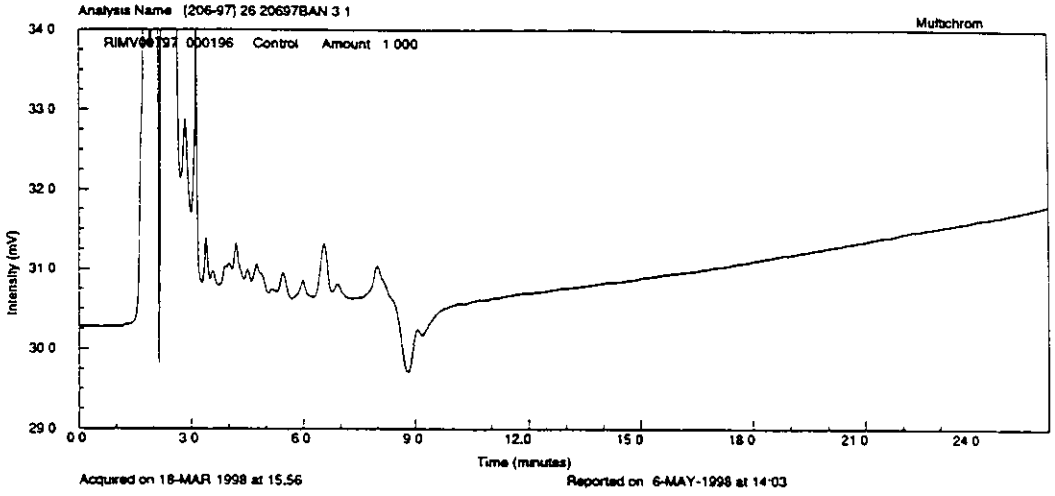


7 Tomato Paste - Sample #51, Control 250 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm)

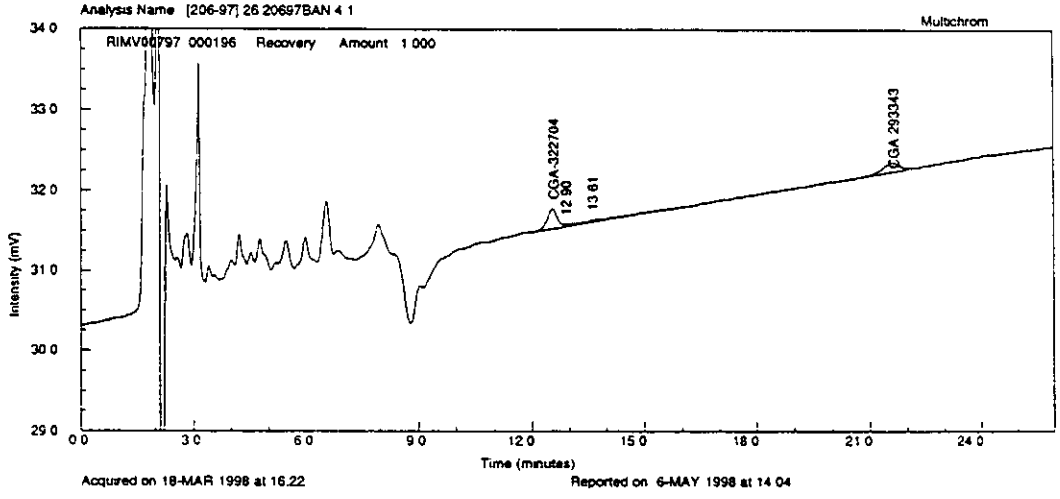


8 Tomato Paste - Sample #52, Control + 0 01 ppm CGA-293343 and 0 01 ppm CGA-322704 250 mg injected 1 9 ng CGA-293343 determined, 0 008ppm, 77% recovery 1 9 ng CGA-322704 determined, 0 008ppm, 77% recovery

FIGURE 10 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF FRUIT SAMPLES

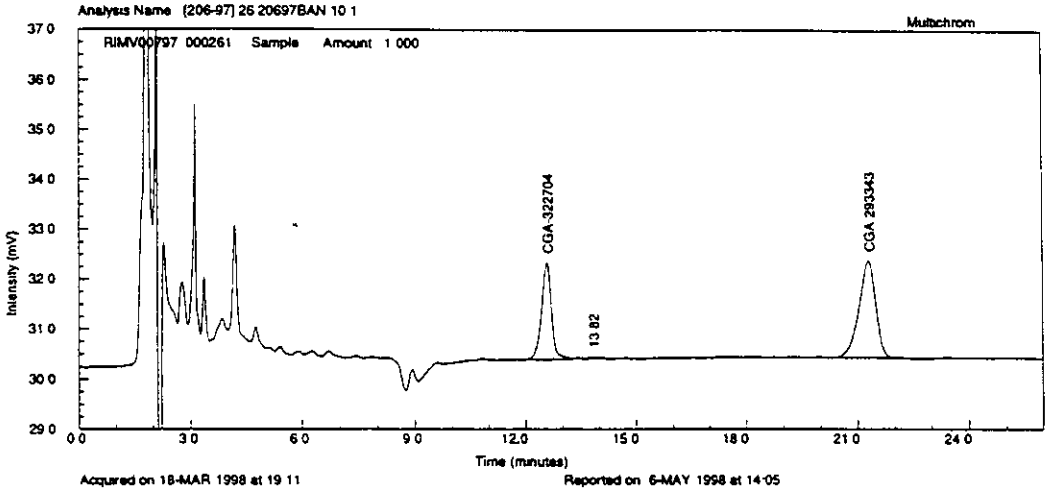


1 Pear Fruit - Sample #14, Control 250 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined (<0 01 ppm)

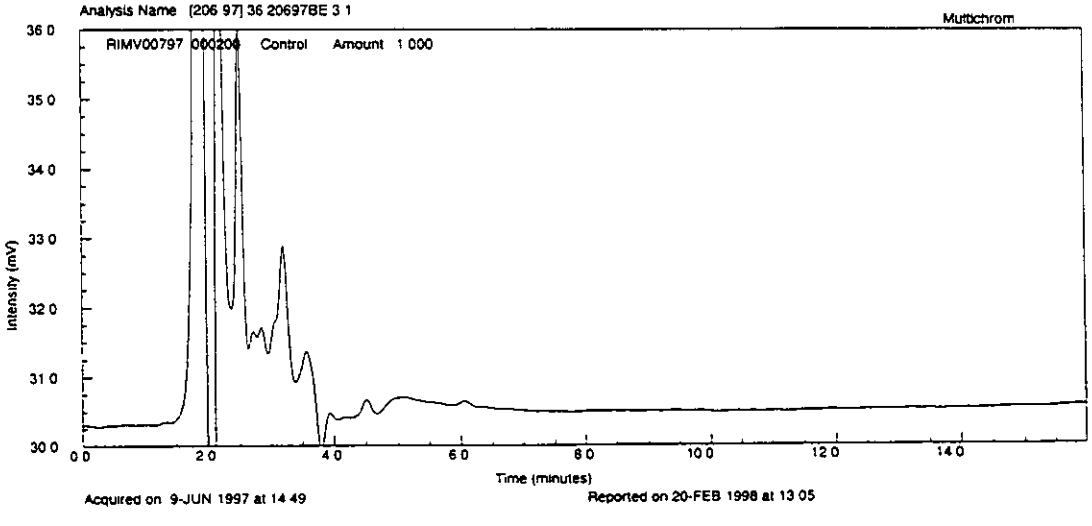


2 Pear Fruit - sample #15, Control + 0 01 ppm CGA-293343 and 0 01 ppm CGA-322704 250 mg injected 2 59 ng CGA-293343 determined 0 01 ppm, 104 % recovery 2 71 ng CGA-322704 determined 0 01 ppm, 108% recovery

FIGURE 10 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF FRUIT SAMPLES (Continued)

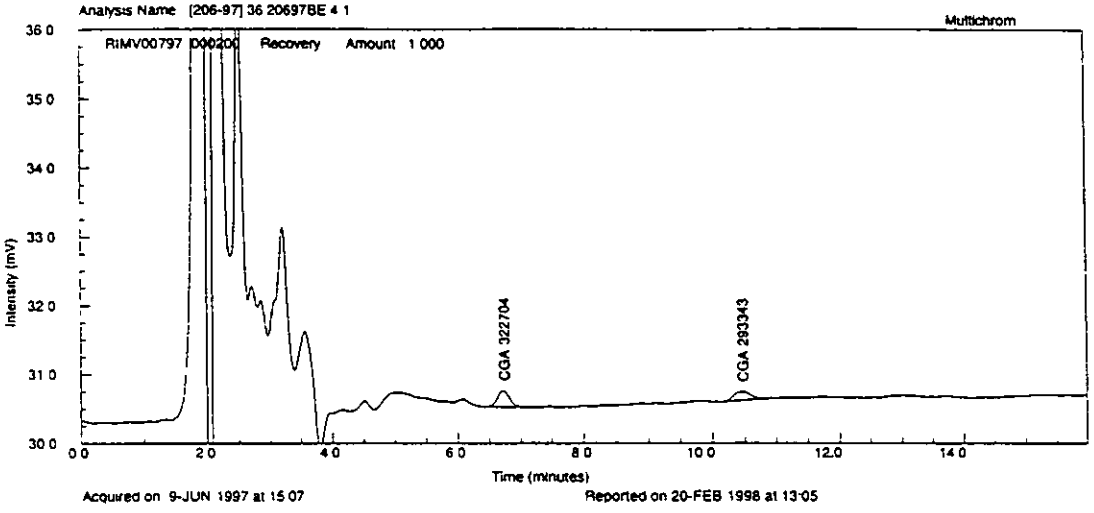


3 Pear Fruit -Sample #19, ^{14}C-CGA-293343 treated sample 250 mg injected 35 5 ng CGA-293343 determined 0 14 ppm, 0 15 ppm corrected for set recovery 19 7 ng CGA-322704 determined 0 079 ppm 0 081 ppm corrected for set recovery

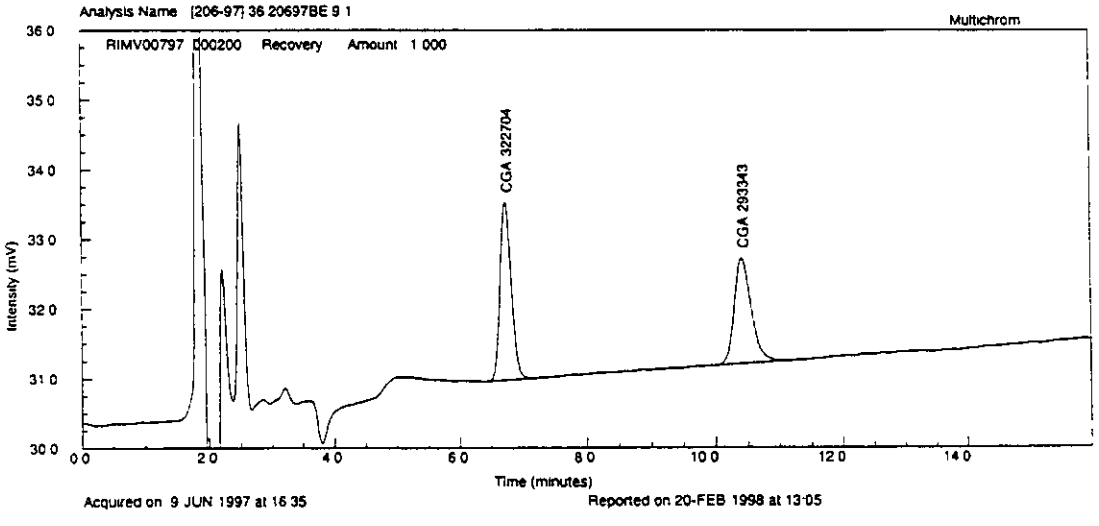


4 Apple Wet Pomace - Sample #1, Control 250 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined (<0 01 ppm)

FIGURE 10 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF FRUIT SAMPLES (Continued)

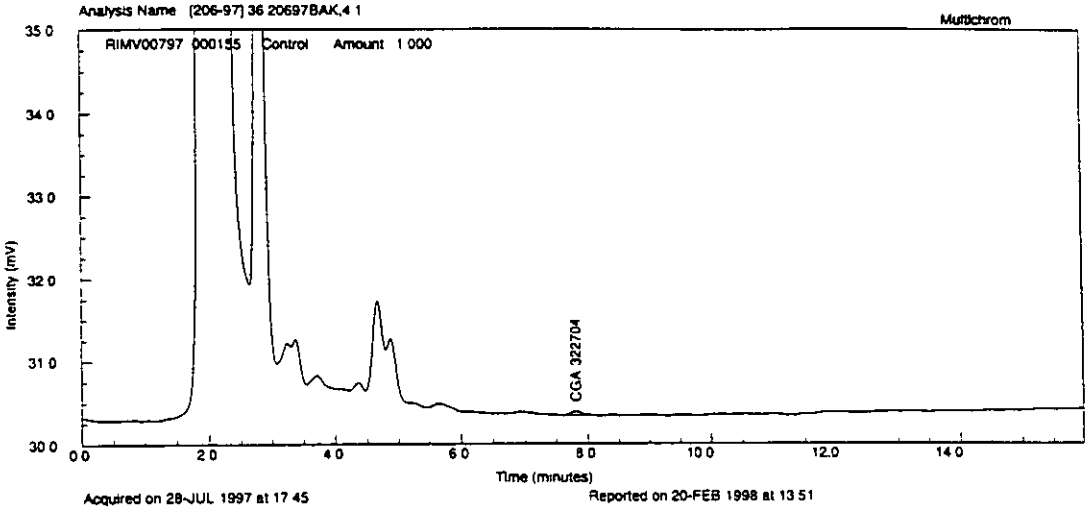


5 Apple Wet Pomace - Sample # 2, Control + 0 01 ppm CGA-293343 and 0 01 ppm CGA-322704 250 mg injected 1 7 ng CGA-293343 determined, 0 007 ppm, 69% recovery 2 0 ng CGA-322704 determined, 0 008 ppm, 81% recovery

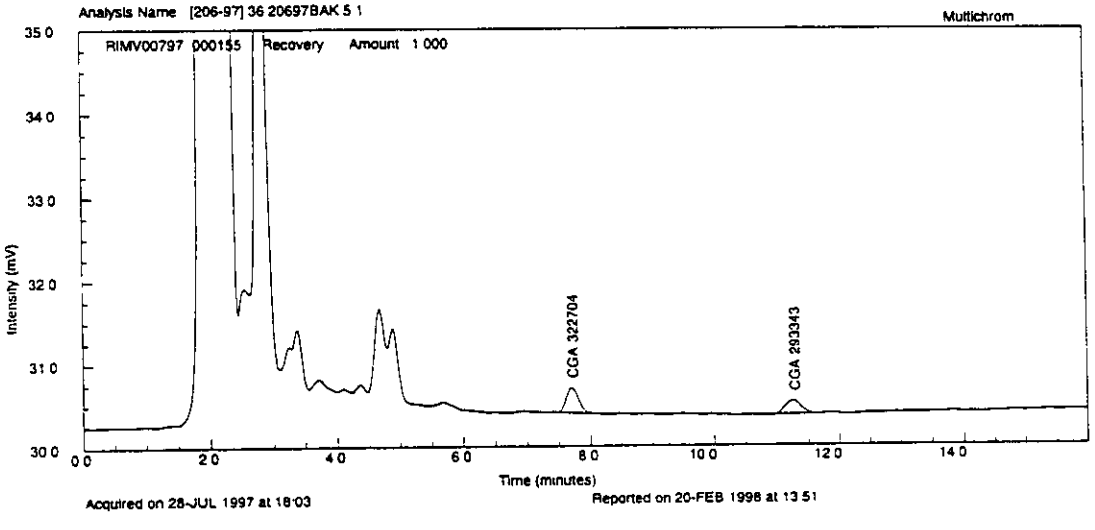


6 Apple Wet Pomace - Sample # 5, Control + 1 0 ppm CGA-293343 and 1 0 ppm CGA-322704 25 mg injected 18 ng CGA-293343 determined, 0 72 ppm, 72% recovery 20 ng CGA-322704 determined 0 78ppm, 78% recovery

FIGURE 10 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF FRUIT SAMPLES (Continued)

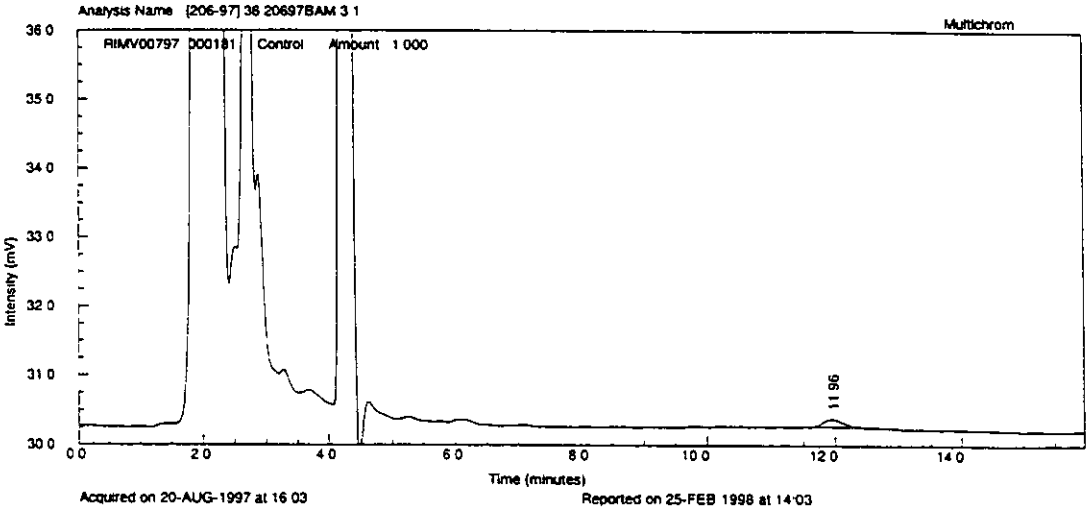


7 Apple Juice - Sample #8, Control 500 mg injected <1 25 ng CGA-293343 determined (<0 005 ppm) <1 25 ng CGA-322704 determined (0 6 ng observed), <0 01 ppm

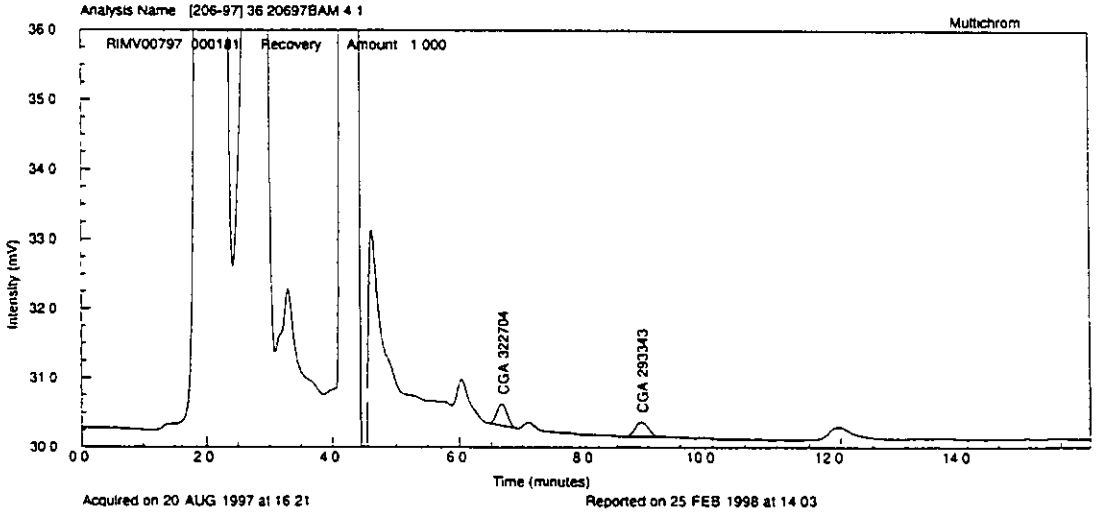


8 Apple Juice - Sample #9, Control + 0 005 ppm CGA-293343 and Cga-322704 500 mg injected 2 2 ng CGA-293343 determined, 0 004 ppm, 89% recovery 2 5 ng CGA-322704 determined, 0 005 ppm, 76% recovery after subtraction of control peak observed in Figure 10, chromatogram 7 above

FIGURE 11 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF DAIRY AND POULTRY SAMPLES

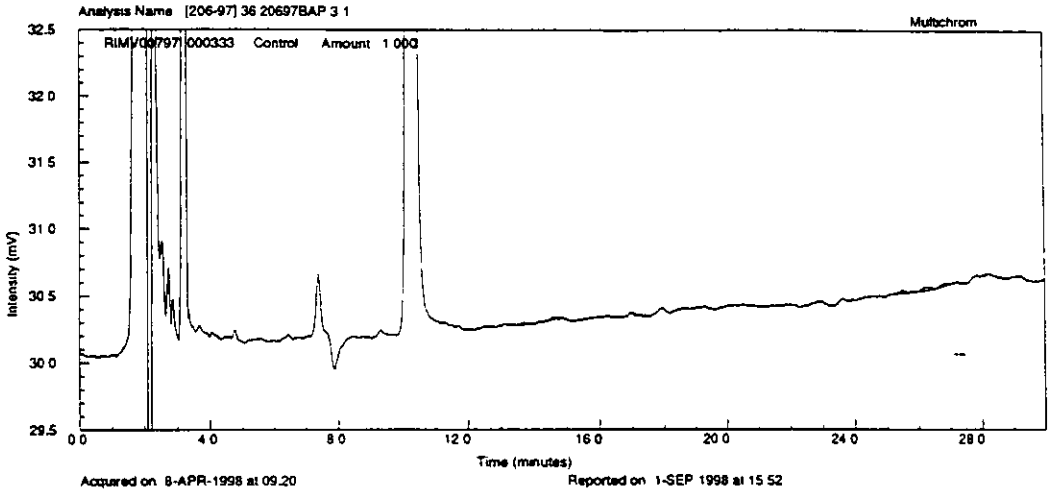


1 Cow Liver - Sample # 87, Control 250 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined (<0 01 ppm)

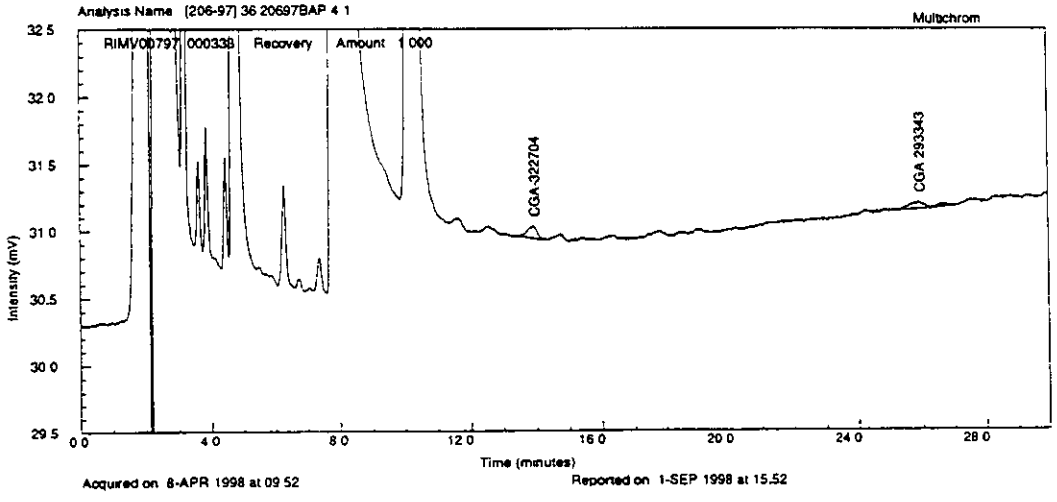


2 Cow Liver - Sample # 88, Control + 0 01 ppm 250 mg injected 2 1 ng CGA-293343 determined, 0 009 ppm 85% recovery 2 3 ng CGA-322704 determined 0 01 ppm, 92% recovery

FIGURE 11 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF DAIRY AND POULTRY SAMPLES (Continued)

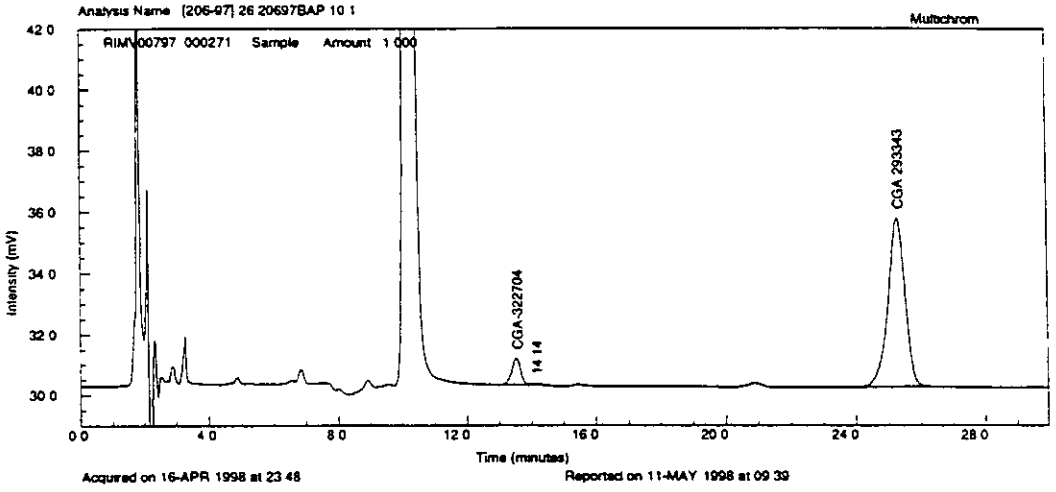


3 Goat Leg Muscle - Sample # 93, Control 250 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined (<0 01 ppm)

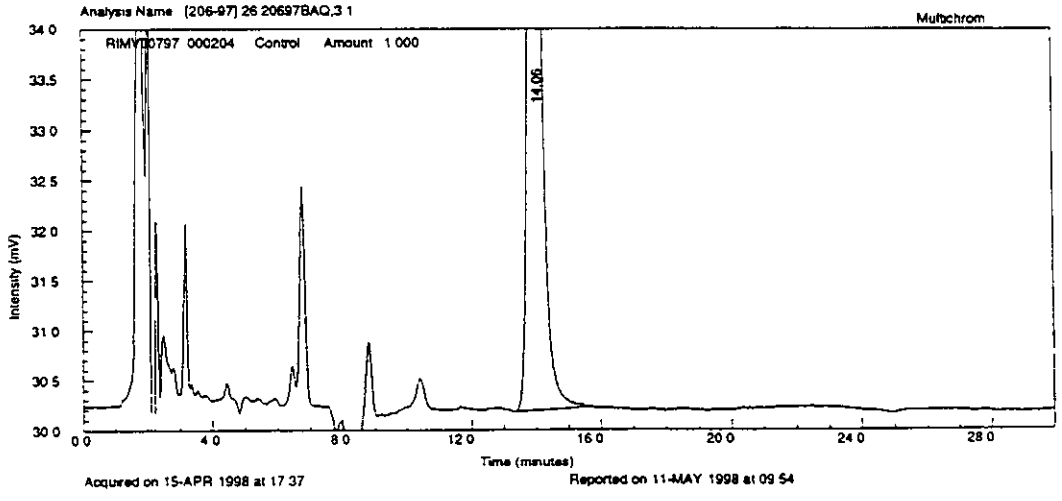


4 Goat Leg Muscle - Sample # 94, Control + 0 01 ppm CGA-293343 and CGA-322704 250 mg injected 2 15 ng CGA-293343 determined, peak height = 71, 0 009 ppm, 86% recovered 2 20 ng CGA-322704 determined, peak height = 183, 0 009 ppm, 88% recovered

FIGURE 11 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF DAIRY AND POULTRY SAMPLES (Continued)

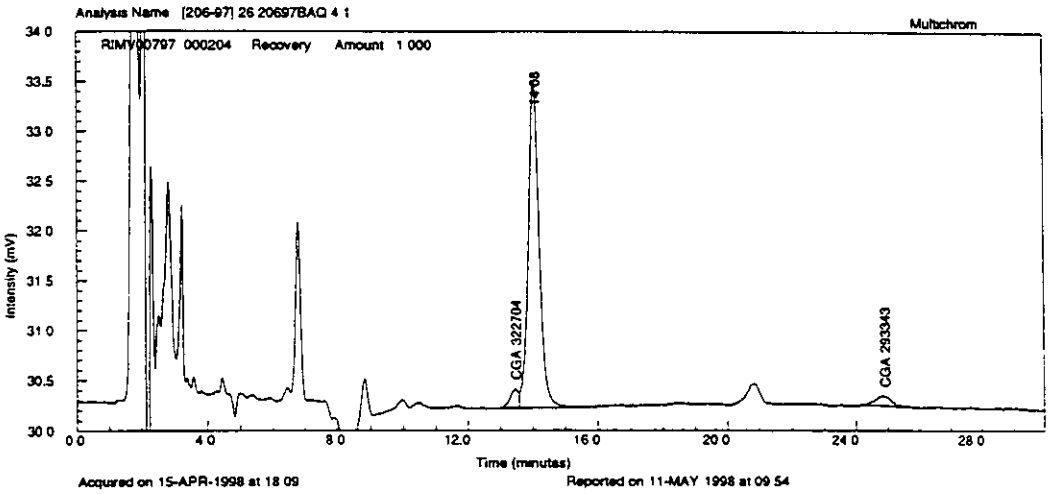


5 Goat Leg Muscle - Sample #97, ^{14}C-CGA-293343 treated goat 250 mg injected 168 ng CGA-293343 determined, 0 67 ppm, 0 77 ppm corrected for set recovery 12 6 ng CGA-322704 determined 0 05 ppm, 0 06 ppm corrected for set recovery

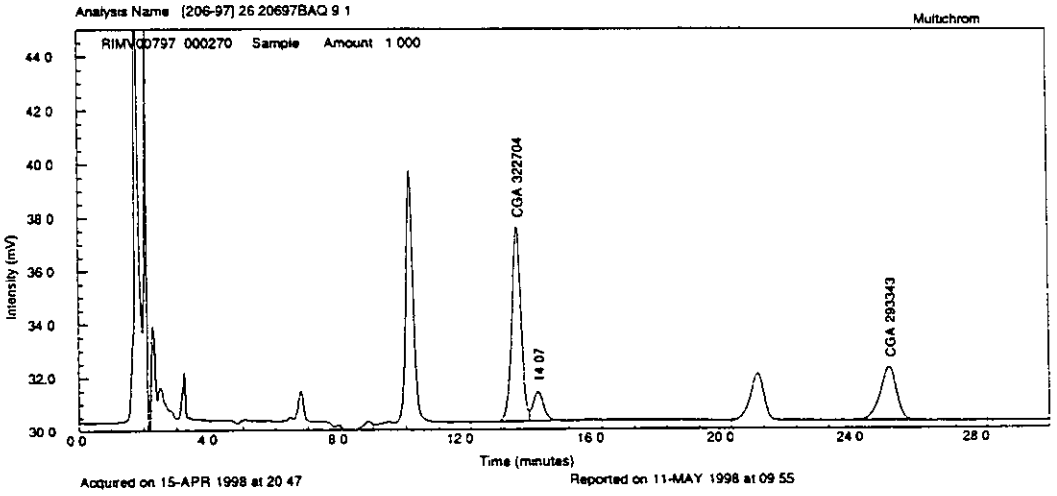


6 Goat Milk - Sample #100, Control 501 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined (<0 01 ppm)

FIGURE 11 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF DAIRY AND POULTRY SAMPLES (Continued)

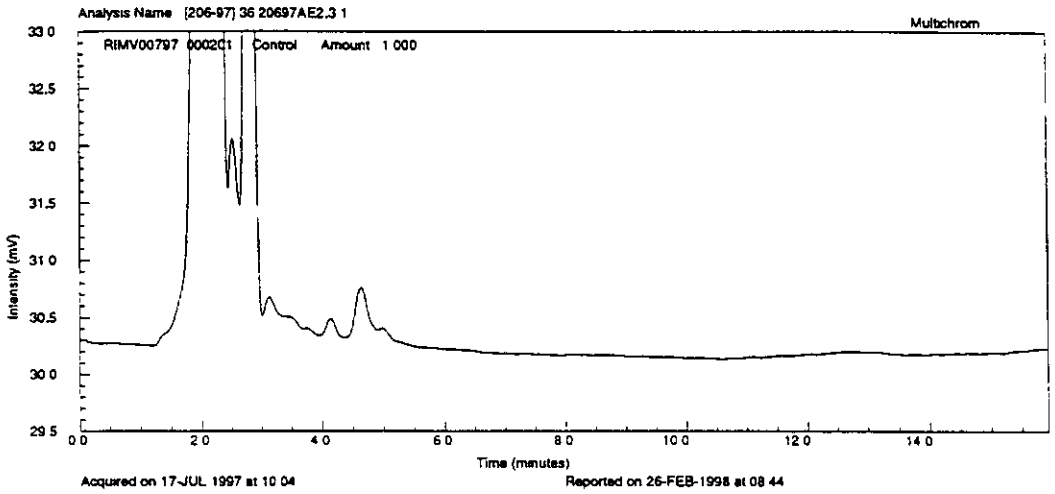


7 Goat Milk - Sample #101, Control + 0 005 ppm 500 mg injected 2 8 ng CGA-293343 determined, 0 006 ppm, 113 % recovery 2 4 ng CGA-322704 determined 0 005 ppm, 96 % recovery

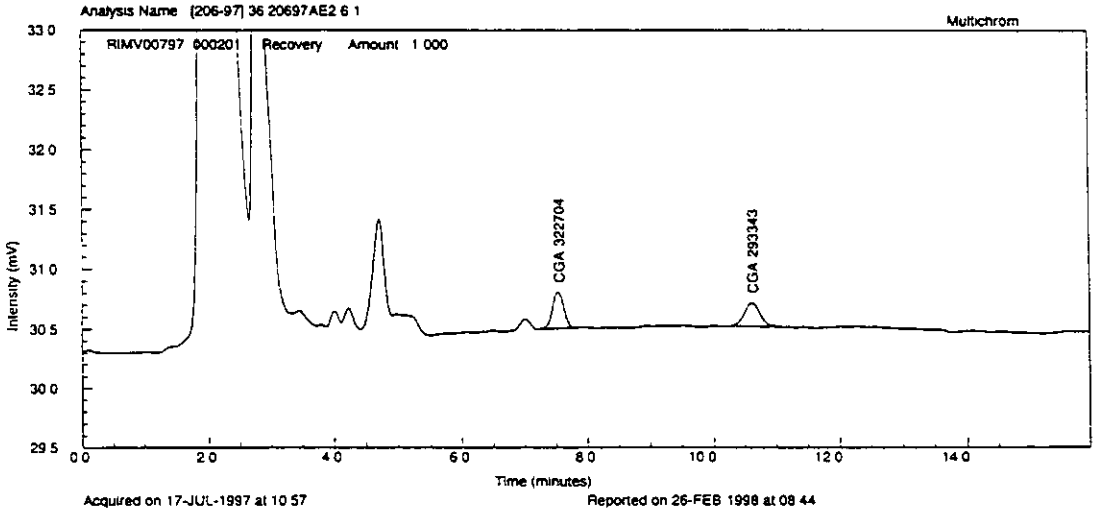


8 Goat Milk - Sample #104, ^{14}C-CGA-293343 treated goat 501 mg injected 43 7 ng CGA-293343 determined, 0 087 ppm, 0 087 ppm corrected for set recovery 78 6 ng CGA-322704 determined 0 16 ppm, 0 17 ppm corrected for set recovery

FIGURE 11 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF DAIRY AND POULTRY SAMPLES (Continued)

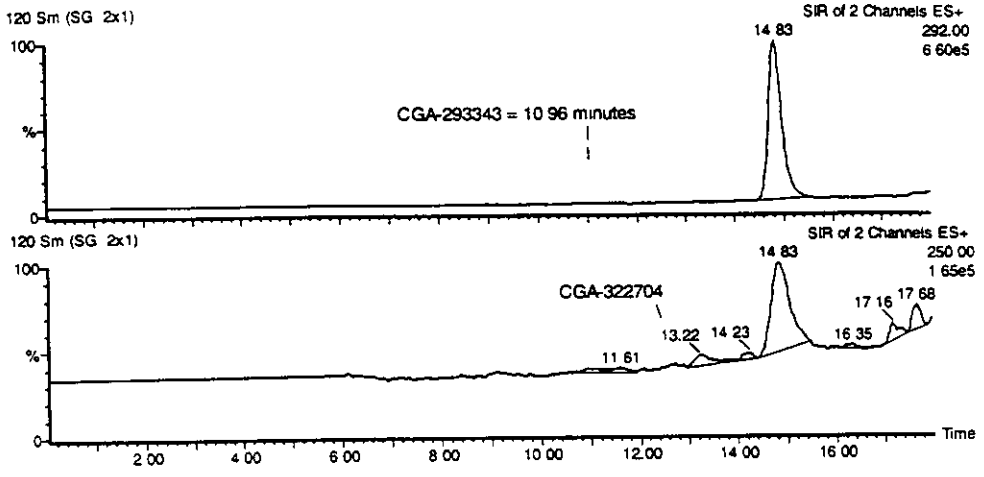


9 Poultry Eggs - Sample #107, Control 250 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined (<0 01 ppm)

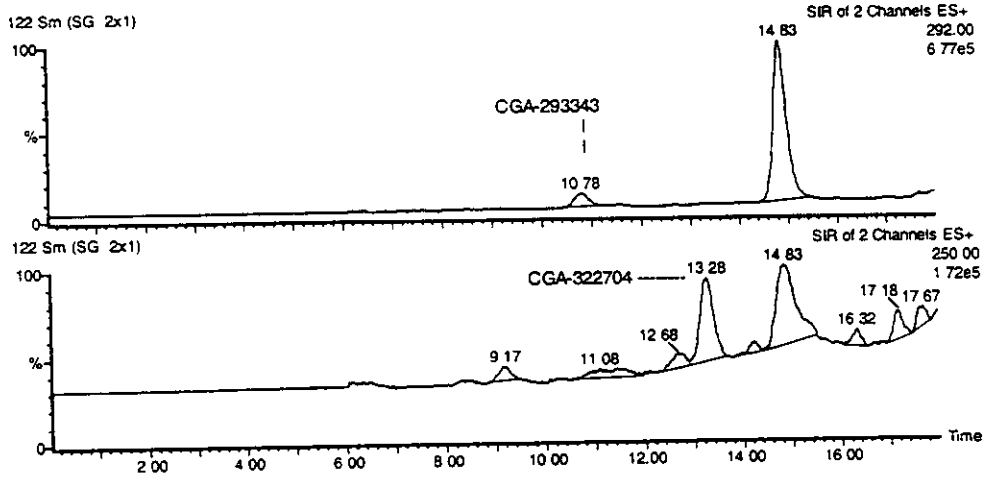


10 Poultry Eggs - Sample # 109, Control + 0 01 ppm CGA-293343 and 0 01 ppm CGA-322704 250 mg injected 2 3 ng CGA-293343 determined, 0 01 ppm, 92% recovery 2 4 ng CGA-322704 determined, 0 01 ppm, 95% recovery

FIGURE 12 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF COTTON, TOBACCO, GRASSES, AND CEREAL GRAIN SUBSTRATES

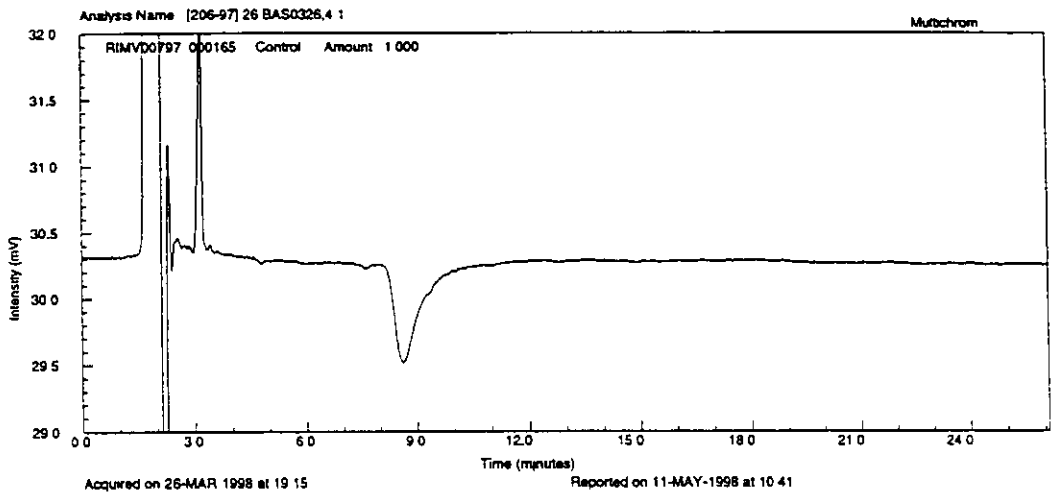


1 Cotton Undelinted Seed - Sample # 120, Control 50 mg injected <0 25 ng CGA-293343 determined <0 25 ng CGA-322704 determined (0 03 ng, 0 001 ppm)

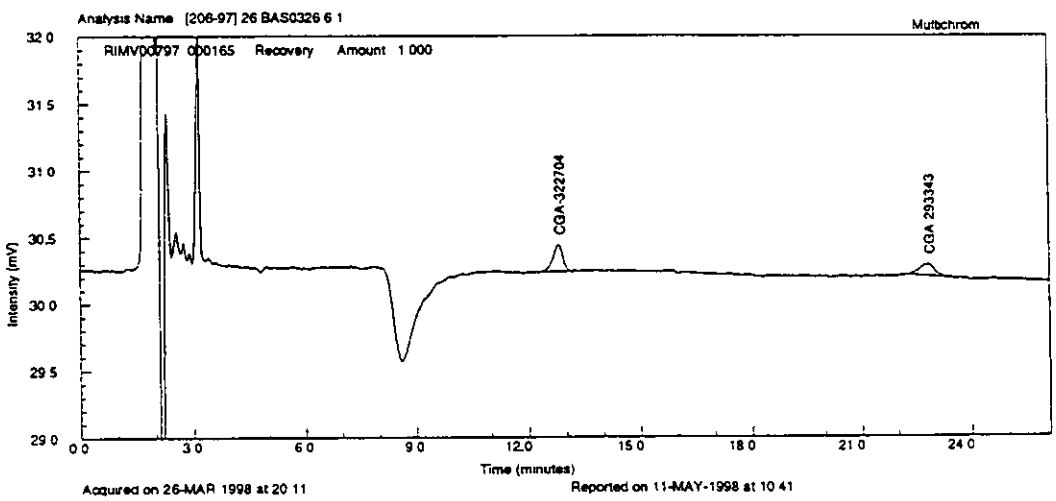


2 Cotton Undelinted Seed - Sample # 122, Control + 0 01 ppm CGA-293343 and 0 01 ppm CGA-322704 50 mg injected 0 36 ng CGA-293343 determined, 0 007 ppm, 72% recovery 0 41 ng CGA-322704 determined, 0 008 ppm, 76% recovery

FIGURE 12 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF COTTON, TOBACCO, GRASSES, AND CEREAL GRAIN SUBSTRATES (Continued)

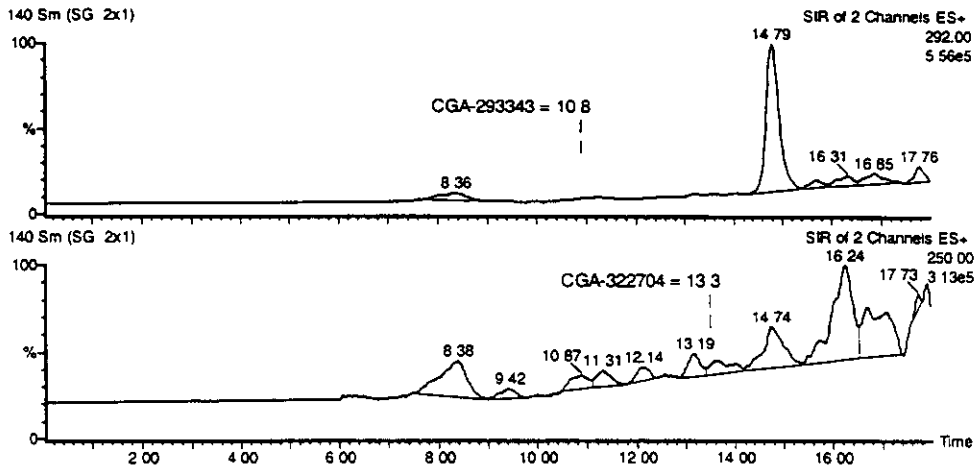


3 Cotton Seed Oil - Sample #127, Control 251 5 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined (<0 01 ppm)

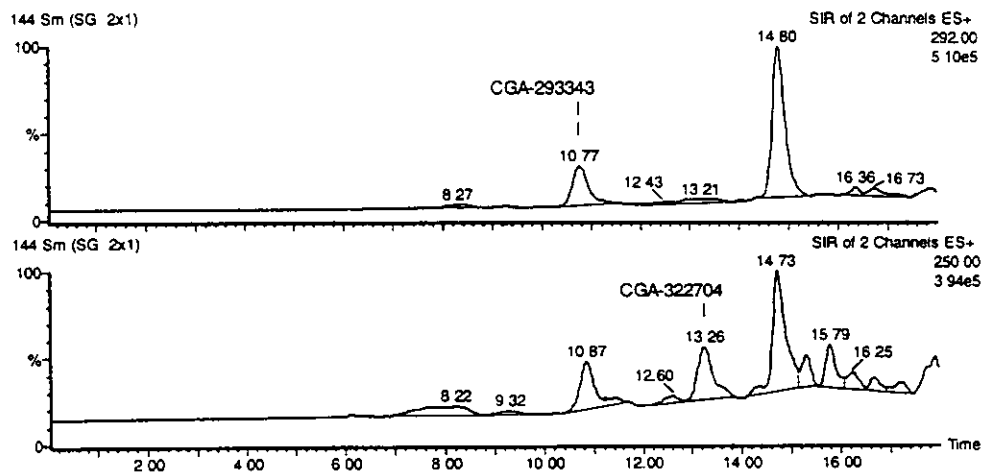


4 Cotton Seed Oil - Sample #128, Control + 0 01 ppm CGA-293343 and CGA-322704 251 mg injected 2 2 ng CGA-293343 determined, 0 009 ppm, 90 % recovery 2 4 ng CGA-322704 determined, 0 01 ppm, 96% recovery

FIGURE 12. REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF COTTON, TOBACCO, GRASSES, AND CEREAL GRAIN SUBSTRATES (Continued)

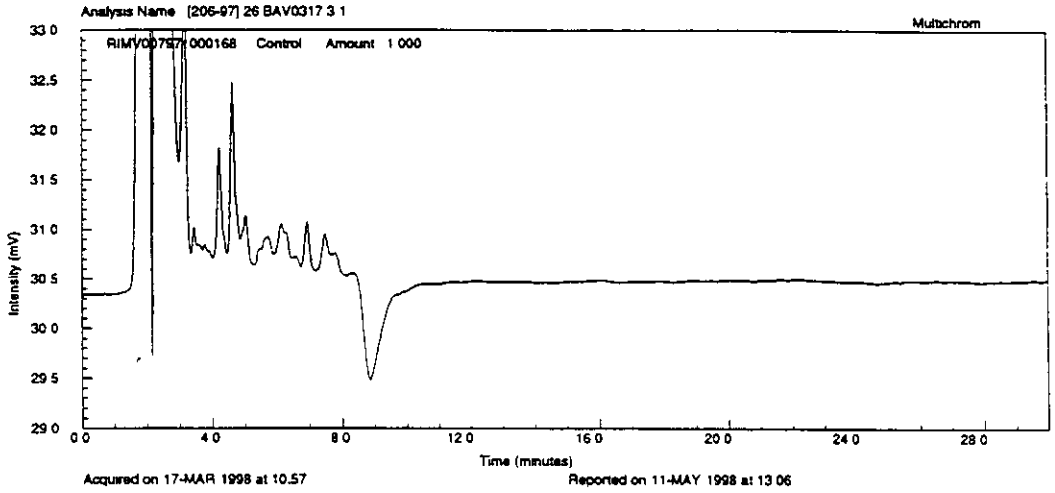


5 Corn Fodder - Sample #140, Control 50 mg injected <0 25 ng CGA-293343 determined, <0 01 ppm <0 25 ng CGA-322704 determined, <0 01ppm (0 13 ng, 0 003 ppm)

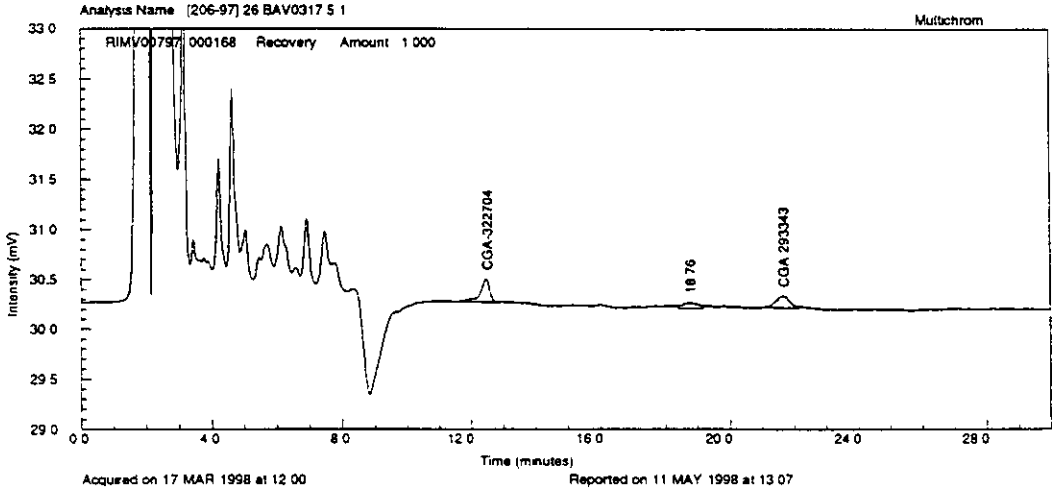


6 Corn Fodder - Sample #144 , ^{14}C-CGA-293343 treated corn fodder sample 45 mg injected 0 78 ng CGA-293343 determined, 0 017 ppm, 0 022 ppm corrected for set recovery 0 57 ng CGA-322704 determined, 0 013 ppm, 0 017 ppm corrected for set recovery

FIGURE 12 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF COTTON, TOBACCO, GRASSES, AND CEREAL GRAIN SUBSTRATES (Continued)

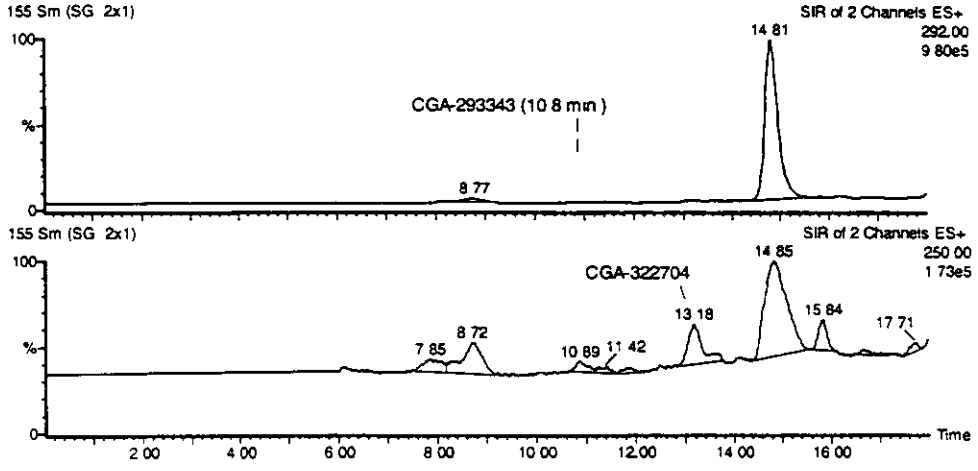


7 Wheat Grain - Sample #148 Control 247 5 mg injected <1 25 ng CGA-293343 determined (<0 01 ppm) <1 25 ng CGA-322704 determined (<0 01 ppm)

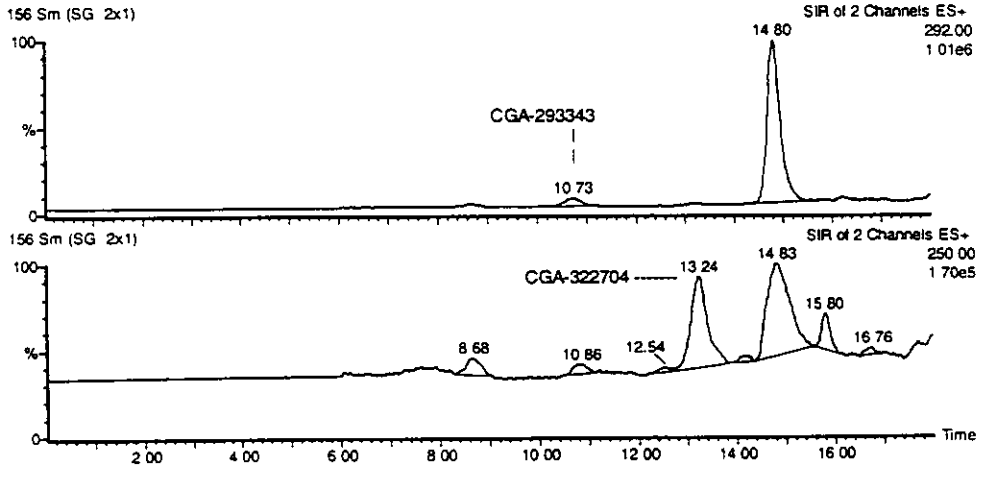


8 Wheat Grain - Sample #149, Control + 0 01 ppm CGA-293343 and 0 01 ppm CGA-322704 233 2 mg injected 2 4 ng CGA-293343 determined, 0 01 ppm, 102 % recovery 2 2 ng CGA-322704 determined, 0 009 ppm, 93 % recovery

FIGURE 12 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF COTTON, TOBACCO, GRASSES, AND CEREAL GRAIN SUBSTRATES (Continued)

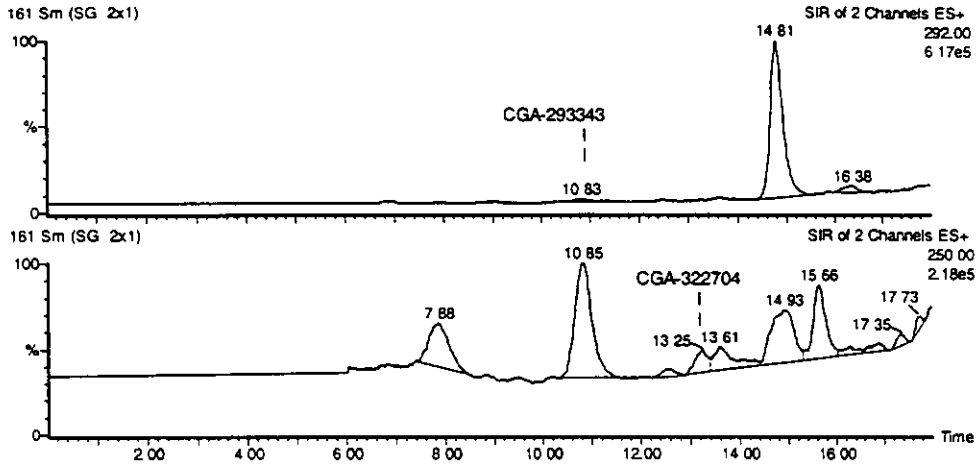


9 Sorghum forage - Sample #155, Control 50 mg injected <0 25 ng CGA-293343 determined, <0 01 ppm <0 25 ng CGA-322704 determined, <0 01 ppm (0 21 ng observed, 0 004 ppm)

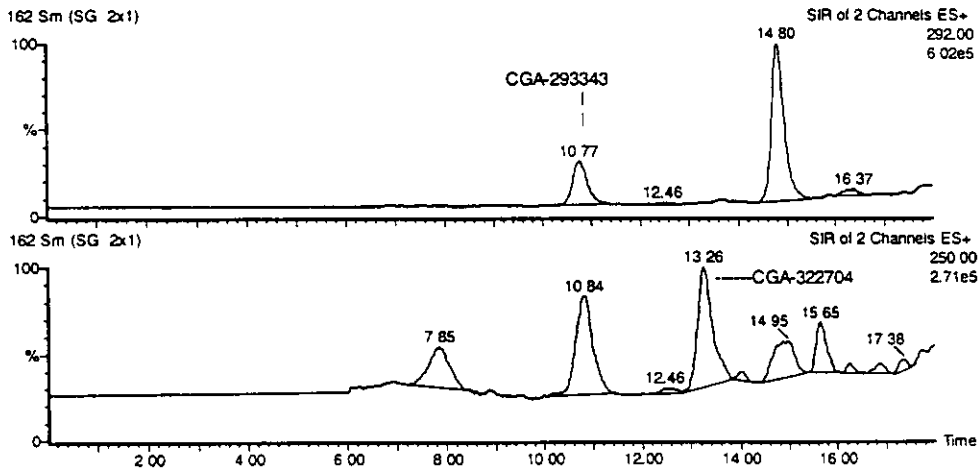


10 Sorghum forage - Sample #156, Control 50 mg injected 0 34 ng CGA-293343 determined, 0 007 ppm, 68 % recovery 0 60 ng CGA-322704 determined, 0 012 ppm, 78% recovery (after subtraction of control peak shown in chromatogram 9, above)

FIGURE 12 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF COTTON, TOBACCO, GRASSES, AND CEREAL GRAIN SUBSTRATES (Continued)

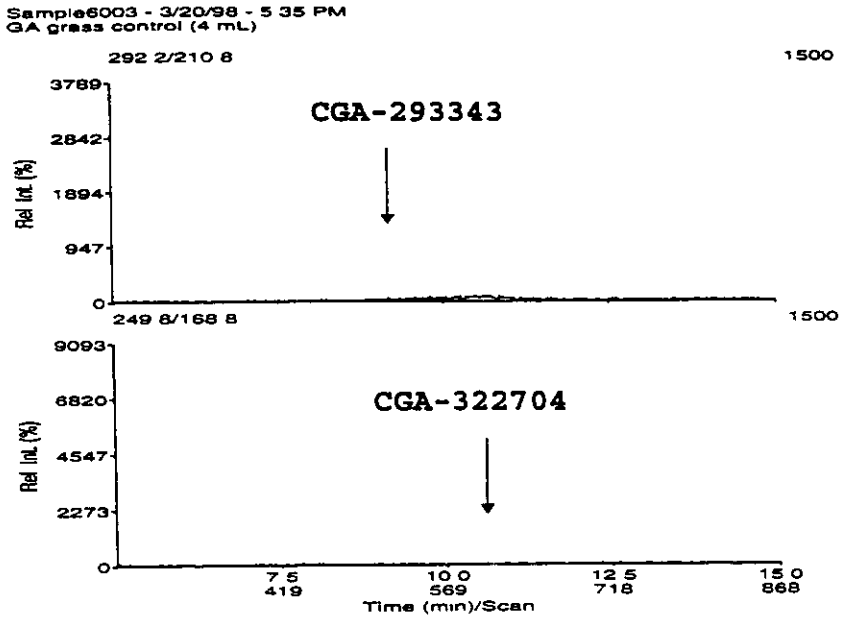


11 Tobacco (green leaves) - Sample #161, Control 99 mg injected <0 25 ng CGA-293343 determined, <0 01 ppm <0 25 ng CGA-322704 determined, <0 01 ppm (0 07 ng observed, 0 001 ppm)

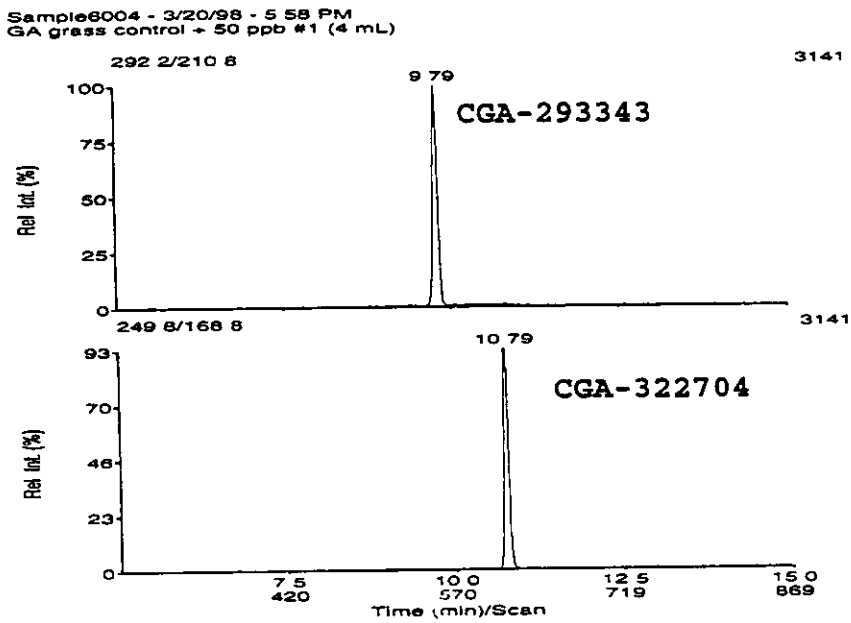


12 Tobacco (green leaves) - Sample #162, Control 95 7 mg injected 0 92 ng CGA-293343 determined, 0 01 ppm, 96% recovery 0 84 ng CGA-322704 determined, 0 009 ppm, 81% recovery after subtraction of control peak observed in #11 above

FIGURE 12 REPRESENTATIVE CHROMATOGRAMS FROM THE ANALYSIS OF COTTON, TOBACCO, GRASSES, AND CEREAL GRAIN SUBSTRATES (Continued)

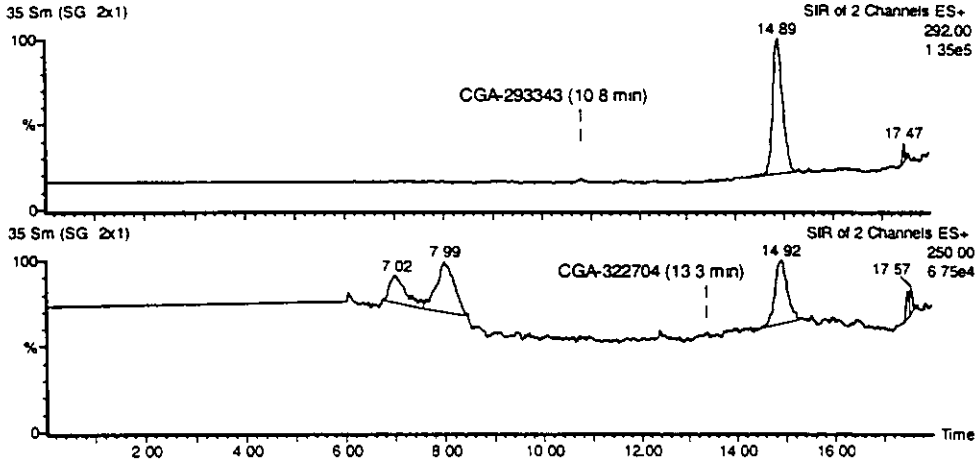


13 Grass (Georgia) - Sample #167, Control 16 mg injected <0 25 ng CGA-293343 determined (top chromatogram), <0 05 ppm <0 25 ng CGA-322704 determined (bottom chromatogram), <0 05 ppm

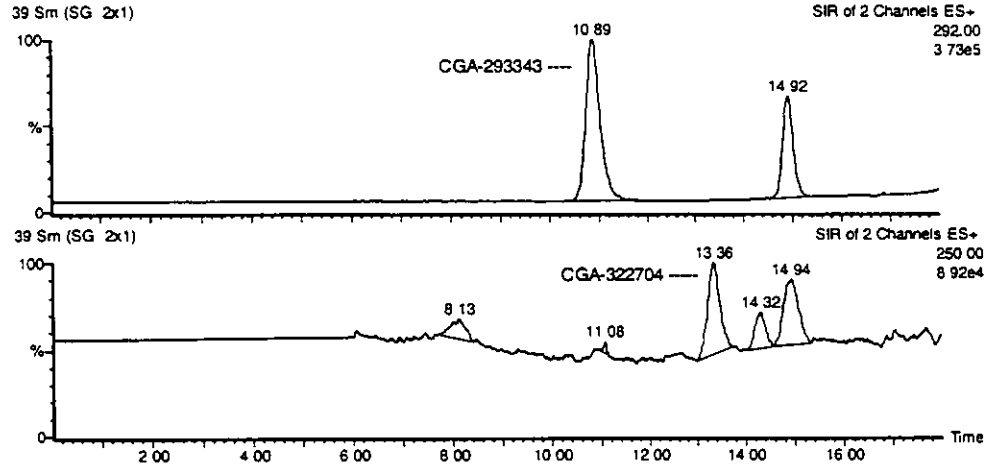


14 Grass (Georgia) - Sample #168, Control + 0 05 ppm CGA-293343 and 0 05 ppm CGA-322704 15 mg injected 0 61 ng CGA-293343 determined (top chromatogram), 0 04 ppm, 81% recovery 0 59 ng CGA-322704 determined (bottom chromatogram), 0 04 ppm, 78% recovery

FIGURE 13 REPRESENTATIVE CHROMATOGRAMS FROM CONFIRMATORY ANALYSES

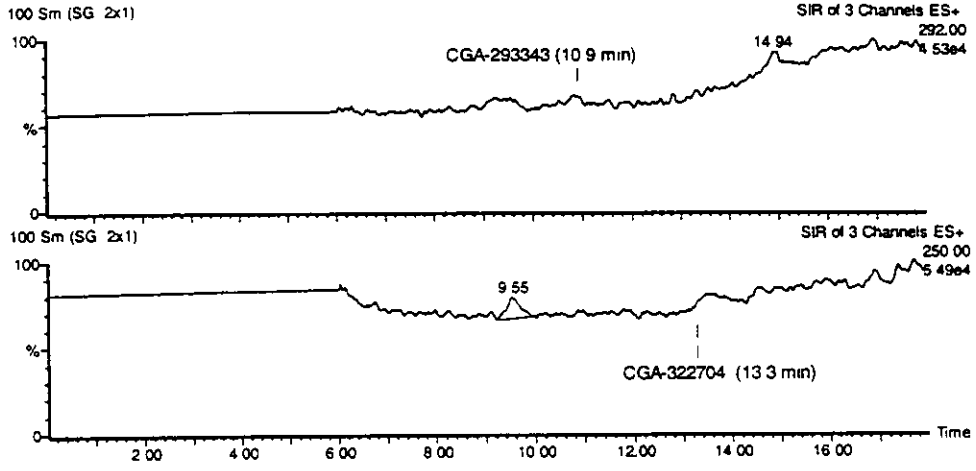


1 Confirmation of Cucumber Analysis - Sample #35, Control 50 2 mg injected <0 25 ng CGA-293343 determined (<0 01 ppm) <0 25 ng CGA-322704 determined (<0 01 ppm)

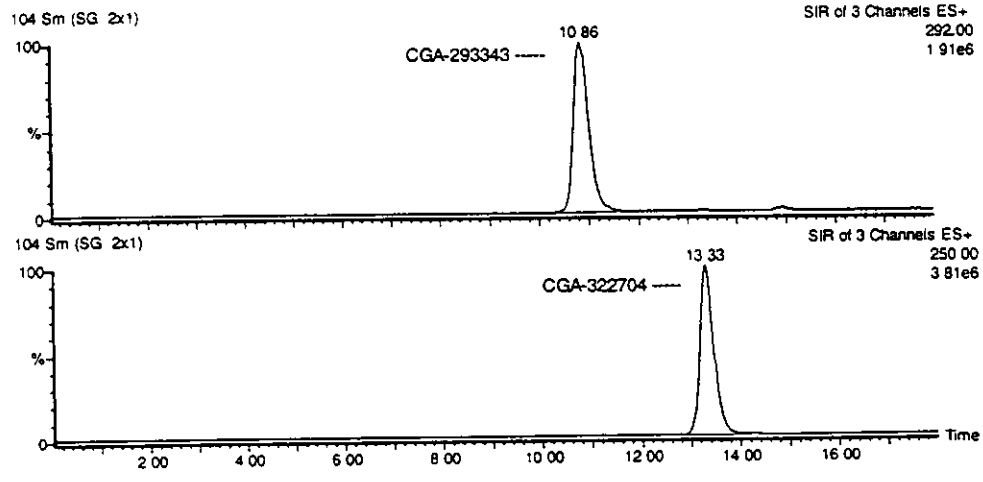


2 Confirmation of Cucumber Analysis - Sample #39, ^{14}C-CGA-293343 treated field sample 50 mg injected 2 2 ng CGA-293343 determined 0 044 ppm <0 25 ng CGA-322704 determined (0 21 ng), <0 01 ppm (0 004 ppm)

FIGURE 13 REPRESENTATIVE CHROMATOGRAMS FROM CONFIRMATORY ANALYSES (Continued)



3 Confirmation of Goat Milk Analysis - Sample #100, Control 100 1 mg injected < 25 ng CGA-293343 determined (<0 005 ppm) <0 25 ng CGA-322704 determined (<0 005 ppm)



4 Confirmation of Goat Milk Analysis - Sample #104, ^{14}C-CGA-293343 treated field sample 100 2 mg injected 9 8 ng CGA-293343 determined, 0 098 ppm 16 1 ng CGA-322704 determine, 0 16 ppm

VIII REFERENCES

1 Alemanni, A , "Preparation of Animal Tissues/ Products for Residue Analysis," Novartis Biochemistry SOP Number 7 27, Revision 2, November 11, 1994, MRID# 42928403.

2 Moore, M E , "Preparation of Crop Samples For Residue Analysis," Novartis Biochemistry SOP Number 7 21, Revision 2, May 5, 1997

3 Capps, T , ^{14}C-CGA-293343· Nature of the Residue in Pears, Novartis Crop Protection, Study Number 198-96, Report Number ABR-98041, July 7, 1998

4 Sandmeier, P , Metabolism of [Thiasol-2-^{14}C] CGA-293343 in Corn, Novartis Crop Protection, Study Number 95PSA41, Project Report 95PSA41PR2, October 17, 1997

5 Capps, T , ^{14}C-CGA-293343 Nature of the Residue in Field Grown Cucurbits, Novartis Crop Protection, Study Number 282-95, Report Number ABR-98048, In Progress

6 R Rumbeli, The Metabolism of [Thiazol-2-^{14}C] CGA-293343 After Multiple Oral Administration to Lactating Goats, Novartis Crop Protection, Study Number 027AM03, In Progress

EPA ADDENDUM for the Residue Analytical Method

1) Subtraction of control values are not recommended by the residue chemistry guidelines for tolerance enforcement methods and should not be used in gathering magnitude of the residue data. Analysts should disregard these instructions in their calculations. (See calculations in method on page 39, index 2.0; 2.1)

2) A moisture percentage of 0% was used for correction of the gram aliquot size for all samples since the extract volume is adjusted to a known volume before aliquotting.