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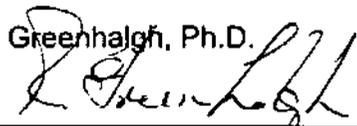
**GAS CHROMATOGRAPHIC DETERMINATION OF AE F039866  
(GLUFOSINATE-AMMONIUM) AND ITS METABOLITES AS RESIDUES IN  
TOLERANT CANOLA, SUGAR BEET TOPS AND ROOTS, CORN AND  
SOYBEAN RAC AND PROCESSED COMMODITIES**

**AVENTIS ANALYTICAL METHOD: BK/01/99**

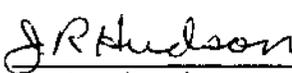
Basis of Method: HRAV-5A (enforcement procedure,  
revised, October 7, 1992)  
AE-24 (November 16, 1994)

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**DATA REQUIREMENT**

*Guideline 171-4(c)*

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Report No. B002939

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## 1.0 INTRODUCTION

The procedure described in this report is designated BK/01/99. It is a further development of the current enforcement method, HRAV-5A for the determination of glufosinate residues in various crops, e.g. apples, grapes, soybeans and tree nuts, which was issued on April 20, 1989 and revised on October 7, 1992.

Historically, the introduction of genetically modified crops necessitated further development of method HRAV-5A to allow determination of the N-acetyl metabolite in field samples of transformed corn and soybean commodities. The genetically modified crops contain an enzyme, which rapidly converts the parent compound to the N-acetyl metabolite. This new procedure called AE-24, allowed determination of the N-acetyl metabolite and the parent compound as separate entities. However, the analysis of numerous genetically modified field samples demonstrated that most of the parent compound was transformed to N-acetyl glufosinate. Thus, the separate determination of residues of the parent and N-acetyl metabolite became less important. The method AE-24 was therefore simplified by removal of the cation exchange separation enabling residues of the parent compound and the N-acetyl metabolite to be expressed as a combined entity.

The procedure BK/01/99 is essentially identical to AE-24 but incorporates several minor modifications which experience has shown to be advantageous in the analysis of various matrices. It describes the standard method that analyses for the combined residues of glufosinate parent and N-acetyl metabolite with the option of the optional of speciated analysis of the two analytes as follows:

- 1: Combined Method (Standard): for the determination of AE F039866 (parent) plus AE F099730 (N-acetyl metabolite) as a combined entity and AE F061517 (MPPA metabolite) separately.
- 2: Speciated Method (Optional): for the determination of AE F039866 (parent), AE F099730 (N-acetyl metabolite) and AE F061517 (MPPA metabolite) as separate entities.

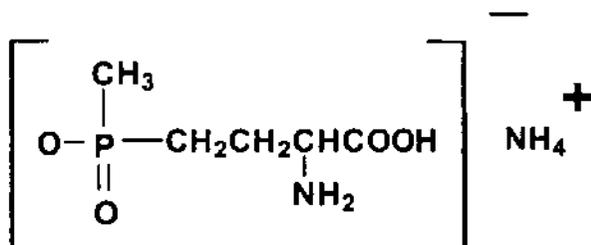
Method BK/01/99 is proposed for the enforcement of glufosinate tolerance levels in corn, soya, canola and sugar beets crops.

## 2.0 CHEMICAL INFORMATION:

### 2.1 AE F039866 (Parent):

Chemical Name: Ammonium-DL-homoalanin-4-yl-(methyl)-phosphate  
(IUPAC, English).

Structure:



#### **AE F039866**

Molecular Formula: C<sub>5</sub>H<sub>15</sub>N<sub>2</sub>O<sub>4</sub>P  
Molecular Weight: 198.2 g/mole  
CAS Number: CA 77182-82-2

### 2.2 Free Acid of AE F039866:

#### 2.2.1 AE F035956

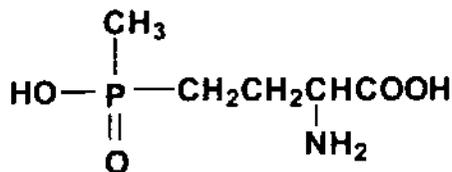
Chemical Name: DL-homoalanin-4-yl-(methyl)phosphinic acid  
(IUPAC, English)

#### 2.2.2 AE F090532 (Purified D-isomer form of AE F035956):

Chemical Name: D-homoalanin-4-yl-(methyl)phosphinic acid  
(IUPAC, English)

Common Name: M-Glufosinate

Structure:



#### **AE F035956**

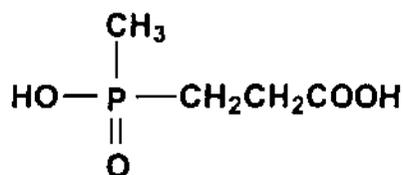
#### **AE F090532**

Molecular Formula: C<sub>5</sub>H<sub>12</sub>NO<sub>4</sub>P  
Molecular Weight: 181.1 g/mole  
CAS Number: CA 51276-47-2 (AE F035956)

2.3 AE F061517 MPPA (Metabolite of AE F039866):

Chemical Name: 3-Methylphosphinico-propionic acid (IUPAC, English)

Structure:



**AE F061517**

Molecular Formula: C<sub>4</sub>H<sub>9</sub>O<sub>4</sub>P  
Molecular Weight: 152.1 g/mole  
CAS Number: CA 15090-23-0

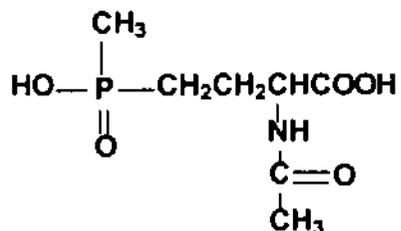
2.4 AE F085355: NAG (Metabolite of AE F039866):

Chemical Name: DL-2-acetamido-4-methylphosphinico-butanoic acid  
(IUPAC, English)

2.4.1 AE F099730: NAG (Purified L-isomer Form of AE F085355)

Chemical Name: Disodium L-2-acetamido-4-methylphosphinico-butyrate  
(IUPAC, English)

Structure.



**AE F085355 (free acid, shown above)**

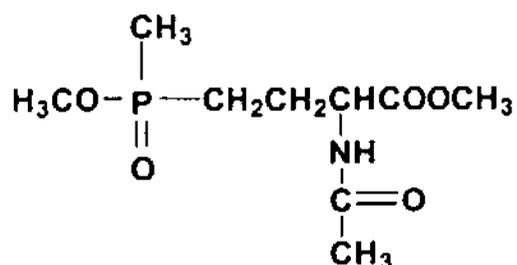
**AE F099730 (disodium salt)**

Molecular Formula: C<sub>7</sub>H<sub>14</sub>NO<sub>5</sub>P (free acid)  
Molecular Weight: 223.2 g/mole (free acid)  
Molecular Weight: 267.1 g/mole (disodium salt)

2.5 AE F064706 (Derivative of AE F039866, AE F035956, AE F085355 and AE F099730):

Chemical Name: Methyl-4-(methoxymethyl)phosphinoyl-2-acetamidobutyrate (IUPAC, English)

Structure:



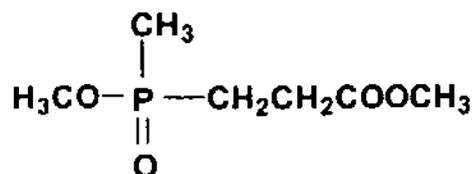
**AE F064706**

Molecular Formula: C<sub>9</sub>H<sub>18</sub>NO<sub>5</sub>P  
Molecular Weight: 251.2 g/mole

2.6 AE F070951 (Derivative of AE F061517):

Chemical Name: Methyl-3-(methoxymethyl)phosphinoyl-propionate (IUPAC, English)

Structure:



**AE F070951**

Molecular Formula: C<sub>6</sub>H<sub>13</sub>O<sub>4</sub>P  
Molecular Weight: 180.1 g/mole

### 3.0 PRINCIPLE OF THE METHOD:

A flow chart of the method is presented in Appendix IV.

With the exception of oil samples, residues of glufosinate (ammonium) and its principal metabolites in glufosinate tolerant canola, sugar beets, field corn and soybeans are extracted from finely ground sample material into distilled water. The aqueous extract is filtered (or centrifuged) to remove non-dissolved solids. Some canola seed samples may require a peptide precipitation step.

An aliquot of the filtrate is passed through an anion exchange resin (hydroxide form) to remove potentially interfering co-extracted matrix components. Such components, which are not bound by the resin, are washed through the column with water. The parent and metabolites are then eluted from the anion exchange resin with a formic acid solution.

The column eluate, containing AE F039866 (or AE F090532), AE F061517 and AE F099730 (or AE F085355) is evaporated to dryness using a rotary evaporator.

#### 3.1 Combined Method (Standard)

##### 3.1.1 All Samples

This version of the method omits the cation resin separation as well as the extraction of the canola, corn or soybean oils. The dry sample residue from the anion resin clean-up or the intact oil sample, is derivatized under reflux for approximately 4.5 hours using trimethylorthoacetate in glacial acetic acid. After a solvent exchange into toluene, the solution is passed through a silica gel SPE cartridge for additional clean up to give only one analytical fraction.

#### 3.2 Speciated Method (Optional)

##### 3.2.1 Oil Samples

In this version of the method, canola, corn or soybean oils are extracted with 5% acetic acid in 1:1 denatured ethanol:water. The extract is defatted with dichloromethane and the aqueous solution is taken to dryness using rotary evaporation.

##### 3.2.2 All Samples

The dry residues from oils (section 3.1.1) as well as the aqueous extract of all other matrices are dissolved in 20 mL of denatured ethanol:water (1:1 v/v). This solution is passed through a cation exchange column (hydrogen form) to separate the metabolites from the parent compound. The column eluate from the loading step is collected and combined with a subsequent (1:1 v/v) denatured ethanol:water wash. This combined eluate is designated "Fraction A" and contains the two metabolites, AE F061517 and AE F099730/ 085355.

### 3.2.2 continued

The cation exchange column is then eluted with an aqueous ammonia solution to remove the parent compound (AE F039866). This eluate is designated "Fraction B".

From this point, Fraction A and Fraction B samples are taken separately through the remaining steps of the method

Each fraction is evaporated to dryness using a rotary evaporator. The sample residue is derivatized under reflux with trimethylorthoacetate in glacial acetic acid for approximately 4.5 hours. After a solvent exchange into toluene, the solution is passed through a silica gel SPE cartridge for additional clean up.

Thus, the analytical solution derived from **Fraction A** contains the derivatives:

- **AE F064706** [methyl-4-(methoxymethyl)phosphinoyl-2-acetamidobutyrate] from the residue of AE F099730 (or AE F085355) and
- **AE F070951** [methyl-3-(methoxymethyl)phosphinoyl-propionate] from the residue of AE F061517.

The analytical solution derived from **Fraction B** contains the derivative:

- **AE F064706** [methyl-4-(methoxymethyl)phosphinoyl-2-acetamidobutyrate] from the residue of AE F039866 (or AE F090532)

### 3.3 Quantitation

Quantitative determination of AE F064706 and/or AE F070951 in the analytical fractions from both the speciated and combined methods, is achieved using GC/FPD, i.e., gas chromatography with flame photometric detection (P-mode). Residues are expressed as AE F035956 (glufosinate free acid) equivalents.

The following analytical standards are used to determine the procedural recoveries: AE F039866, AE F090532, AE F061517 and AE F099730.

**Note:** reference compounds AE F039866 and AE F090532 represent alternatives for use in determining parent compound recovery.

Some soybean seed samples from glufosinate-tolerant plants contain sufficient enzyme activity to convert significant quantities of racemic glufosinate to the N-acetyl metabolite during the extraction. The enzyme does not affect the D-isomer of glufosinate, AE F090532. Similarly, reference compounds AE F085355 and AE F099730 are alternatives for determining recovery of the N-acetyl glufosinate metabolite. The following procedures assume that AE F039866 and AE F099730 reference standards are used as reference compounds. **The exception being soybean seed, when AE F090532 should be used as the fortification standard in the speciated method.**

#### 4.0 EQUIPMENT:

**Note:** Unless otherwise indicated, functionally equivalent equipment may be substituted for the equipment listed below.]

1. Anti-bumping granules: BDH Cat. #B33009. This item can be replaced by glass beads in the derivatization procedure
2. Balances: Mettler Model BB-2440, for sample preparation; Mettler Model AE 240, for standard preparation.
3. Büchner Type Funnel: 7 cm; for vacuum filtration [Fisher Scientific: 10-356B] and glass with coarse frit for resin preparation/regeneration.
4. Centrifuge: Laboratory bench top model, capable of 600-700 x G (2,000 - 3,000 rpm).
5. Centrifuge Bottles/Tubes: Teflon or Glass, 50, 100, 250 mL
6. Collection Tubes, Glass: 16 mm x 100 mm, for collection of silica gel column fractions [Fisher Scientific:14-961-29].
7. Column, Glass: with stopcock and reservoir, 10.5 mm id., 250 mm length; [Lab Glass: LG-4565-T-100], used for anion and/or cation exchange column chromatography.
8. Drying tube, Glass: equipped with a 24/40 ground glass joint, for use with condenser (item 27) [VWR: 26658-025]. To pack, insert glass wool plug then add silicagel dessicant ( ~ 15 g) followed by another glass wool plug. The drying tube is attached to the top of the reflux condenser to prevent moisture entering the reaction flask used for derivatization (see section 7.4).
9. Erlenmeyer Flasks, Glass: 500 mL [VWR: 29140-566].
10. Filter Paper: Whatman No. 934-AH, 1.5  $\mu$ , [e.g., Fisher Scientific: 09-873F (7 cm)], or sized to fit the Büchner funnel (Item 3).
11. Fiat Bottom Flask, Glass. 250 mL capacity [VWR: 29114-045].
12. Food processor: Robot-Coupe.
13. Graduated Cylinders, Glass: various capacities up to 500 mL; for mixing purposes, use a 500 mL vessel or alternatively use a 500 mL volumetric flask.
14. Gas Chromatograph: Varian 3400 GC equipped with a Flame Photometric Detector operating in the Phosphorus mode, or equivalent instrumentation.

4.0 continued

**Note:** According to the application, the GC system is configured for Megabore capillary column operation, with SPI injector. Use of an autosampler device is optional but recommended for optimum reproducibility and efficiency.

15. Gas Chromatography Column: 1.0  $\mu$ m DB-WAX, fused silica, 15 meter x 0.53 mm Megabore column with cross linked (Carbowax 20M) stationary phase, [J & W Scientific: J1257012].

**Note:** Commercially available 30 meter columns may be cut into two shorter columns. Typically, a 10-15 meter column is used for these analyses.

16. Hot Plate: Series 730 PMC Dataplate [Fisher Scientific: 11-495-137].
17. Luer Stopcocks: Varian/Analytichem International; used with SPE silica gel cartridges.
18. Magnetic Stirrer: Variomag Multipoint [Cole-Parmer: G-0465-20].
19. Magnetic Stir Bars: Teflon coated, [VWR: 58948150].
20. Microliter Syringes: 10, 100, 250, 500 microliter ( $\mu$ L), Hamilton.
21. Mill: Retsch ZM-1 or SM 2000 grinder equipped with a 2 mm sieve, depending on the matrix.
22. Millex SR Filters: Millipore No. SLR 025NS; or "Baker" Disposable Filtration Columns, J.T. Baker, Inc., Cat. No. 7121-06.
23. N-Evap Analytical Concentrator: Organomation Associates (see also item 31)
24. Needles: Stainless steel, 100 mm for use with disposable syringes (item 32).
25. Pasteur Pipettes, Glass: 22.9 cm x 0.7 mm O D.
26. Polytron Homogenizer or Ultraturrax: For use with oil extraction procedure (section 7.2.1).
27. Reflux Condensers: High efficiency, 300 mm, equipped with a 24/40 ground glass joint [VWR: 23114-042].
28. Recirculating Chiller: Lauda WK 500, set at 5°C; to circulate cooled antifreeze to the condenser unit of the rotary flash evaporator (item 29).
29. Rotary Flash Evaporator: Buchi, RE-121A [VWR: 27558-354], with a water bath operating at 45-60°C (see also item 31).

4.0 continued

30. Silica Gel SPE Cartridge: 500 mg cartridge with reservoir, [Varian/Analytichem International: No. LR01304].
31. Speed Vac Concentrator: Savant SC110 (or equivalent); for use in place of a rotary evaporator or N-Evaporator, as appropriate, within the method.
32. Syringes: Polypropylene, 10 mL capacity, equipped with a Luer tip [Aldrich Chemical Co.] Unless totally glass, substitution not recommended  
**Note**: avoid the use of rubber-tipped syringe plunger.
33. Ultrasonic Bath: Branson [VWR: 21812-119].
34. Vacuum Box Apparatus: Vac Elut SPS 24 Vacuum Box [Varian/Analytichem International: No. A16500-2]; for use with silica gel SPE cartridges.  
**Note**: Use of a vacuum box is optional. Any equivalent apparatus that provides a uniform and controllable cartridge elution (drip) rate may be used.
35. Vacuum Flask: 500 mL [Fisher 10-180E].
36. Vacuum Pump: Vacuubrand Model MZ2C (or equivalent), for use with the rotary evaporator, Cole Parmer.
37. Volumetric Pipets, Glass: Class A; 0.10, 0.50, 1.0, 2.0 mL capacity.

5.0 **REAGENTS/CHEMICAL SUPPLIES:**

1. Acetic Acid (Glacial): AR.
2. Ammonium Hydroxide: AR [BDH No. ACS 033-037 (28-30% NH<sub>3</sub>), 14.8 M]
3. Anion Exchange Resin: Bio-Rad (140-1431) AG 1X8 ion exchange resin, 8% cross linking, 50-100 dry mesh, in the hydroxide form.

Preparation of Anion Exchange Resin. Mix 100 g of Anion Exchange Resin (item 3) with 1 L of 1M NaOH (item 24) in a 1-2 L beaker and gently stir using a Teflon-coated spatula for approximately 2 minutes. Let stand for 30 minutes or until the resin solution is pH 10-11 as indicated by pH paper. The solution must be stirred slowly to avoid crushing the resin. Filter the resin through a Büchner funnel fitted with a coarse frit disc to remove excess sodium hydroxide. Continue to wash the resin with distilled water until the pH of the filtrate is no longer basic (pH 5.5-7.5). Typically, about 3-5 liters of distilled water are required for 100 g of resin.

4. Cation Exchange Resin: Bio-Rad (142-1431) AG 50W-X8 ion exchange resin, hydrogen form, 8% cross linking, 50-100 dry mesh.

Regeneration of the Cation Exchange Resin: To remove the ammonia elution solvent, wash the used resin with distilled water to neutral pH as indicated by pH paper. Mix 1 volume of resin with 3 volumes of 1 M NaOH (item 24). Stir gently with a Teflon-coated spatula for approximately 2 minutes. Let stand for 30 minutes. Filter the resin through a Büchner funnel fitted with a coarse frit disc to remove excess NaOH. Wash the resin with distilled water to neutral pH. Mix 1 volume of resin with 3 volumes of 1N HCl. Stir gently with a Teflon-coated spatula for approximately 2 minutes. Let stand for 30 minutes. Again filter the resin through the Büchner funnel apparatus to remove excess HCl and continue to wash the resin with distilled water to approximately neutral pH (i.e., pH 5.5-7.5). The resin may be rinsed with acetone to facilitate drying.

5. Dichloromethane: pesticide quality.
6. Ethanol (Denatured): [BDH, Cat. No. B86021], ethanol denatured with wood alcohol (85:15 v/v). Alternatively, an ethanol/methanol (85/15 v/v) mixture of pure reagents can be used.
7. Ethyl Acetate: pesticide quality.
8. Formic Acid: Fisher Scientific (88% w/w), ACS Reagent Grade.
9. Hydrochloric Acid, Concentrated: ACS Reagent Grade (12.4 N), for the regeneration of cation exchange resin.
10. Methyl Acetate: (99%) AR.
11. Methanol: pesticide quality.
12. Silica Gel (Activated): [VWR Scientific 26668-109], grade H 6-16B, blue desiccant, taken from the container to packing the drying tubes (section 4.0, item 8). If pink coloured, re-activate by heating overnight at 110°C.
13. Sodium Hydroxide: AR [VWR 6720-1].
14. Toluene: pesticide quality.
15. Trimethylorthoacetate: Aldrich (99%) [reagent must be dry].

### 5.1 Mixed Solutions

16. 5% Acetic Acid in 1:1 Denatured Ethanol:Water, used for canola, corn or soybean oil extraction).
17. Ammonium Hydroxide (1.5 M): Dilute 100 mL of ammonium hydroxide solution (28-30% NH<sub>3</sub>) to 1000 mL with distilled water, used to elute cation resin).

5.1 continued

18. Ammonium Hydroxide (0.015 M): Dilute 10 mL of 1.5 M ammonium hydroxide solution to 1000 mL with distilled water, used to prepare standard solutions.
19. Denatured Ethanol/Methyl Acetate (1:1 v/v): Mix equal volumes of denatured ethanol and methyl acetate reagents.
20. Denatured Ethanol/Water (1:1 v/v): Mix equal volumes of denatured ethanol and distilled water.
21. Formic Acid (50% w/w): Dilute 570 mL formic acid (88%) to 1 L (final volume) with distilled water. Always add acid to 300-400 mL of water before diluting.
22. HCl (1N): Dilute 81 (±1) mL of concentrated HCl to 1 L with distilled water.
23. Methanol/Methyl Acetate (1:1 v/v): Mix pure reagents in a 1:1 volume ratio of Methanol:Methyl Acetate.
24. Sodium Hydroxide (1M): Dissolve 40 grams of sodium hydroxide [VWR 6720-1] in distilled water to a final volume of 1 liter.
25. Toluene:Methyl Acetate (1:1 v/v): Mix pure reagents in a 1:1 volume ratio of Toluene:Methyl Acetate.

**5.2 Analytical Standards**

25. AE F039866: Ammonium DL-homoalanin-4-yl-(methyl)phosphinate.
26. AE F090532: D-homoalanin-4-yl-(methyl)phosphinic acid.
27. AE F035956: DL-homoalanin-4-yl(methyl)phosphinic acid.
28. AE F061517: 3-Methylphosphinico-propionic acid.
29. AE F085355: DL-2-acetamido-4-methylphosphinico-butanoic acid
30. AE F099730: Disodium L-2-acetamido-4-methylphosphinico-butyrate
31. AE F064706: Methyl-4-(methoxymethyl)phosphinoyl-2-acetamido-butyrate
32. AE F070951: Methyl-3(methoxymethyl)phosphinoyl propionate.

**Note:** Analytical Standards 5.2, item 25; 5.2, item 26; 5.2, item 27; 5.2, item 28; 5.2, item 29; 5.2, item 30; 5.2, item 31 and 5.2, item 32 are available from Aventis CropScience, Aventis Research Center, Pikeville, NC 27863, USA

TABLE I

TYPICAL SERIES OF GC CALIBRATION STANDARDS			
Amount (µL)	Solution ID	Final Dilution Volume (mL)	Final Concentration (ng/µL)
75	B	100.0	0.015
100	B	100.0	0.020
250	B	100.0	0.050
500	B	100.0	0.100
1000	B	100.0	0.200
1500	B	100.0	0.300

**Note:** Final concentrations are expressed as AE F035956 (glufosinate free acid)

### 6.2 Fortification Solutions:

#### AE F039866 (or AE F090532):

Weigh 109 (±1) mg of AE F039866 (or 100 (±1) mg of AE F090532) into a 100 mL volumetric flask. Dissolve and make up to volume with 0.015M ammonium hydroxide. These solutions, Stock Solution C (AE F039866) or Stock Solution C' (AE F090532) contains 1.0 mg of AE F039866 (or AE F090532)/mL, respectively, **expressed as AE F035956 equivalents**. They must be prepared fresh every six months.

#### AE F061517:

Weigh 84 (±1) mg of AE F061517 into a 100 mL volumetric flask. Dissolve and make-up to volume with 0.015 M ammonium hydroxide. This solution (Stock Solution D) contains 1.0 mg of AE F061517/mL, **expressed as AE F035956 equivalents**. It must be prepared fresh every six months.

#### AE F099730:

Weigh 147 (±1) mg of AE F099730 into a 100 mL volumetric flask. Dissolve and make-up to volume with 0.015 M ammonium hydroxide. This solution (Stock Solution E) contains 1.0 mg of AE F099730/mL, **expressed as AE F035956 equivalents**. It must be prepared fresh every six months.

#### Combined Standards:

Transfer 1.0-mL aliquots of Stock Solutions C and D to the same 100 mL volumetric flask. Dilute to volume with 0.015 M ammonium hydroxide (or with denatured ethanol/methyl acetate (1:1) for the fortification of oil samples). This solution (Stock Solution F) contains 10.0 µg of AE F039866 and AE F061517/mL, **expressed as AE F035956 equivalents**. It must be prepared fresh every six months.

## 6.0 PREPARATION OF STANDARD SOLUTIONS:

**Note:** The calibration and fortification standard amounts in the examples below are given for pure (100%) materials. The actual amounts used should be adjusted for certified percent purity. Store all solutions in a refrigerator.]

### 6.1 Calibration Solutions:

#### AE F064706 and AE F070951:

Weigh 139 ( $\pm 1$ ) mg of AE F064706 and 100 ( $\pm 1$ ) mg of AE F070951 into separate 100 mL volumetric flasks. Dissolve in pure methanol and make each up to volume. These Stock Solutions A1 and A2, contain 1.0 mg of AE F064706/mL and 1.0 mg of AE F070951/mL, respectively, **expressed as AE F035956 equivalents**. They must be prepared fresh every six months.

Transfer a 2.0-mL aliquot from both Stock Solution A1 and Stock Solution A2 to the same 100 mL volumetric flask. Dilute to volume with pure methyl acetate. This solution (Solution B) contains 20.0  $\mu\text{g}$  of AE F064706 and AE F070951/mL, **expressed as AE F035956 equivalents**. It must be prepared fresh every six months.

Make dilutions of Solution B every month (or as needed) for calibration of the gas chromatograph. A typical set of dilutions for GC calibration is shown in Table I.

**Note:** To ensure accuracy and verify reference solution stability, freshly prepared calibration standard solutions are to be cross-checked with the corresponding, previously prepared calibration solutions by side-by-side GC/FPD analysis (e.g., just prior to the expiration date of the solution). The new and old standards should agree, i.e., GC/FPD analyte response to within  $\pm 10\%$  (rel.) Otherwise, standard preparation accuracy must be re-verified and/or appropriate problem solving measures implemented. In addition, ongoing monitoring of GC/FPD response of calibration factors is also recommended to provide an indication of analytical system control, including adequate calibration standard stability.]

GC Calibration: [See also sections 8.2 and 8.3.]

The calibration solutions shown below (Table I) have been found to provide an adequate linear working range for GC/FPD analysis. Other concentration of standards may be used according to specific analysis requirements. alternate dilution schemes may also be used. However, the working range of the calibration plot from the lowest to highest standard concentration utilized, should not exceed an approximate factor of 10 to 20.

## 6.2 continued

Transfer 1.0-mL aliquots of Stock Solutions D and E to the same 100 mL volumetric flask. Dilute to volume with 0.015 M ammonium hydroxide (or with denatured ethanol/methyl acetate (1:1) for fortification of oil samples). This solution (Stock Solution G) contains 10.0 µg of AE F061517 and AE F099730/mL, **expressed as AE F035956 equivalents**. It must be prepared fresh every six months.

Transfer 1.0-mL aliquots of Stock Solutions C (or C'), D and E to the same 100 mL volumetric flask. Dilute to volume with 0.015 M ammonium hydroxide (or with denatured ethanol/methyl acetate (1:1) for fortification of oil samples). This solution, Stock Solution H (with AE F039866) or Stock Solution H' (with AE F090532) contains 10.0 µg of AE F039866 (or AE F090532), AE F061517/mL and AE F099730/mL, **expressed as AE F035956 equivalents**. It must be prepared fresh every six months.

The 10.0 µg/mL stock solutions H or H', containing AE F039866 (or AE F090532), AE F061517 and AE F099730 will be used to fortify a control sample for recovery purposes when the speciated method is used.

The 10.0 µg/mL stock solutions (F and G) containing AE F039866 plus AE F061517 or AE F061517 plus AE F099730 will be used to separately fortify two control samples for recovery purposes when using the non-speciated/combined method.

**Note:** For the determination of procedural recoveries, fortify glufosinate tolerant (transgenic) soybean seed, hulls, and meal samples with solutions containing AE F090532. All other sample matrices may be fortified with solutions containing AE F039866.

Additional fortification solutions may be prepared as required to cover the range of residues found in actual field samples. Stock solutions and/or fortification solutions may have standard concentration levels which differ from those specified above (see also section 11.0).

## 7.0 **ANALYTICAL PROCEDURE: COMBINED METHOD**

### 7.1 **Sample Preparation:**

Analytical samples (solids) are to be finely ground using a Retsch grinder (or equivalent) or homogenized in a food processor (or equivalent) with dry ice (as necessary). It is important that the laboratory sample be finely ground (e.g., to a particle size  $\leq 2$  mm diameter) and homogeneous before subjecting it to the analytical procedures described below. Liquid analytical samples (e.g., oils), which are homogeneous, require no additional preparation.

## 7.2 Extraction:

### 7.2.1 All Matrices Except Oils (See section 7.2.2 for extraction of oils)

Into a 500-mL Erlenmeyer flask, accurately weigh 12.5 ( $\pm 0.1$ ) g of the analytical sample (Ws) prepared as described in section 7.1. Add 200 ( $\pm 1$ ) mL of distilled water and a magnetic stirring bar.

**Note:** See also section 13.2 (Extraction).

Place the flask containing the sample and distilled water onto a magnetic stirring plate. Stir vigorously at room temperature for 0.5 hour. Transfer the entire contents of the extraction flask to a clean 500 mL graduated mixing cylinder. Add sufficient distilled water to achieve a 500 mL final extraction volume (V1) and mix well. The diluted extracts derived from all solid matrices are filtered (see section 7.2.1.1) except that from canola seed, which is centrifuged to facilitate the separation of any oil (see section 7.2.1.2).

#### 7.2.1.1 Filtration (All matrices except canola seeds)

Vacuum filter the mixture through a Whatman 934-AH filter paper contained in a Buchner funnel, collecting the aqueous phase in a clean 1 L flask.

Except for grain dust, transfer a 100.0 mL aliquot (V2) to a 250 mL flat bottom flask. Proceed to section 7.3.

For grain dust samples, transfer a 50.0 mL aliquot (V2) to a 250 mL flat bottom flask. Adjust the volume to 100.0 mL by adding 50.0 mL of distilled water. Proceed to section 7.3.

**Note:** For all matrices, especially soybean seed, hull and meal, if fine particulate matter present clogs the filter paper or passes through the filter paper, the sample may be centrifuged prior to filtration. Centrifuge a 150 to 200 mL portion of the extract (e.g., 600-700 x G, 2,000 - 3,000 rpm) for 10-15 min in a 250 mL Teflon centrifuge bottle.

#### 7.2.1.2 Centrifugation and Peptide Precipitation (Canola seed)

In the case of canola seed, centrifuge 150 to 200 mL portion of the extract (e.g., 600-700 x G, 2,000 - 3,000 rpm) for 10 to 15 minutes in a 250 mL Teflon centrifuge bottle. Remove the top fatty layer using a Pasteur pipet.

Transfer a 100 mL aliquot of aqueous extract to a 250-mL Teflon centrifuge bottle. Add 100-mL of acetone to the extract aliquot, mix by swirling. Let stand for approximately 5 minutes, preferably in a refrigerator. Centrifuge the mixture for 5-10 minutes (600 x G, at 2000 - 3000 rpm). Remove a 96 mL aliquot (50%), (equivalent to 50 mL of the aqueous extract, considering the volume change on mixing the water and acetone) of the supernatant solution and transfer to a 250-mL flat bottom flask. Using a rotary evaporator (50°C),

### 7.2.1.2 continued

evaporate this solution to remove the acetone solvent. The volume of the aqueous extract is 50.0 mL (V2) at this point ] Add 50 mL of distilled water to the flask. Proceed to section 7.3.

### 7.2.2 Canola, Corn and Soybean Oil

In the combined method, the oil matrices are not extracted but are derivatized directly

Into a 250-mL flat bottom flask, weigh 2.5 ( $\pm 0.1$ ) g (Ws) of oil. To determine the procedural recovery, a control oil sample is fortified at this point, using a standard solution prepared in 1:1/ethanol:methyl acetate [see section 6.2 above]. Proceed to section 7.4.1 (derivatization step).

**Note:** It is recommended to fortify oil samples with  $\leq 0.2$  mL of standard solution. If a volume  $\geq 0.2$  mL is used, evaporate the fortification solution solvent using a rotary evaporator or stream of nitrogen gas, before carrying out the derivatization step.

### 7.3 Extract Clean Up Using Anion Exchange: (see section 13.3)

Use anion exchange resin that has been conditioned as described in section 5.0. Slurry pack a calibrated glass chromatography column (~10.5 mm id) with resin, using distilled water to assist in the transfer and packing the resin into the column. The following volumes of resin ( $\pm 0.5$  mL) are used according to sample type:

- Canola, Sugar Beet and Corn Samples: 12 mL
- Soybean Samples: 18 mL

**Note:** For convenience, the glass chromatography column is calibrated prior to use. Add a measured volume of water to an empty column containing a glass wool plug and mark the volume level on the column exterior accordingly

Load the column with the 100.0 mL (total) aqueous extract from section 7.2.

**Note:** V2 = 100.0 mL for all matrices except for canola seed and grain dust where V2 = 50.0 mL. Pass this solution through the resin under gravity flow and wash the resin with 50 ( $\pm 5$ ) mL distilled water. Regulate the effluent flow with the column stopcock valve to give a flow rate of about one to two drops per second. Discard the column eluate that contains residual sample matrix. Elute both the parent compound (AE F039866 or AE F090532) and metabolites (AE F061517 and AE F099730) with 100 ( $\pm 10$ ) mL of 50% formic acid, collecting the eluate directly into a clean 250 mL flat bottom flask. Use a drip rate of about one to two drops per second.

### 7.3 continued

Evaporate the formic acid eluate to dryness using a rotary evaporator (50-55°C). It is recommended that rotary evaporators be operated with a diaphragm pump and chilled antifreeze (-5°C) in the condensers. Add 10 (±1) mL of water to the dried sample extract and again evaporate to dryness as above to remove all residual formic acid. Add 10 (±1) mL of methanol to the dried sample extract and again evaporate to dryness.

**Note:** Operate the rotary evaporator at a moderately slow speed, to avoid excessive dispersion of the sample inside of the flat bottom flask.

### 7.4 Derivatization:

Add 3 (±0.5) mL of glacial acetic acid to the flat bottom flask containing the sample residue (section 7.3 or section 8.4 for the speciated method). Sonicate at room temperature for approximately one minute or until all visible sample residue is dissolved and/or dislodged from the wall of the flask. Add 12 (±1) mL of trimethylorthoacetate and a few glass beads or anti-bumping granules. Mix by swirling with sonication. When mixing is complete, reflux the reaction mixture for 4.5 hours, using a condenser fitted with a drying tube (see also section 13.4). The drying tube has been found to be necessary during periods of high humidity. It prevents atmospheric moisture getting into the reaction vessel and inhibiting the derivatization reaction. Proceed to section 7.5

#### 7.4.1 Derivatization of Oil Samples

Add 6.0 (±0.5) mL of glacial acetic acid to the flat bottom flask containing the oil sample (from section 7.2 2). Sonicate at room temperature for about one minute. Add 24 (±1) mL of trimethylorthoacetate and some glass beads or anti-bumping granules. Mix by swirling with sonication. When mixed, reflux the reaction mixture for 4.5 hours as above. Proceed to section 7.5.

### 7.5 Reconstitution

After the reflux period, allow the sample to cool to room temperature. Disconnect the flask from the reflux condenser and add 15 (±1) mL of toluene.

**Note:** The procedure may be stopped when the sample has cooled or after addition of toluene. The flask is capped for storage at room temperature.

The cooled derivatized sample is reduced in volume by evaporating the contents of the flat bottom flask to a final volume of approximately 2 mL. This can be achieved using a rotary evaporator operating with a water bath temperature of 45°C or an equivalent technique, e.g., Speed Vac apparatus.

## 7.5 continued

Add successive 15 ( $\pm 1$ ) mL portions of toluene and repeat the evaporation procedure. At least three 15 mL portions of toluene are added and evaporated to an approximate final volume of 0.5 - 1 mL, to remove all traces of the derivatization solution.

**Note:** It is important that the solution is not evaporated to dryness during the reconstitution procedure. The derivatized parent and/or metabolite compounds may be lost should this occur.

The final volume of the extract is adjusted to no more than 4 mL of toluene.

## 7.6 Post Derivatization Clean Up:

The derivatized sample extract undergoes additional clean up prior to GC/FPD analysis. This final clean-up step is carried out using commercially available silica gel SPE cartridges.

The SPE clean-up technique may require verification of the retention and elution characteristics of the silica gel material utilized. The characterization procedure is described in section 7.6.2.

### 7.6.1 Silica Gel SPE Cartridge Clean Up:

Silica Gel SPE cartridges are normally used with a vacuum box assembly as described in the Equipment section 4.0. The manufacturer's operating instructions for the particular device used should be noted (see section 7.6.2.)

Immediately before use, the SPE cartridges are conditioned by passing ~10 mL of degassed (sonicated) methyl acetate:toluene (1:1 v/v) through each cartridge. Do not allow the cartridges to become dry at this point. The flow rate through the SPE cartridge is adjusted to one to two drops per second for all conditioning, washing and eluting operations.

Add 4.0 ( $\pm 0.1$ ) mL methyl acetate to the toluene extract from section 7.5. The solution is sonicated to dislodge any residual sample adhering to the flask.

To load the SPE cartridge, attach a 100 mm syringe needle to a 10 mL disposable (polypropylene) syringe [section 4.0, item 32] and draw the sample into the syringe. Adjust the final volume in the syringe to 8.0 ( $\pm 0.2$ ) mL with toluene, achieving a 1:1 methyl acetate:toluene solution. **Save the empty flask at this point.**

Invert the syringe i.e. plunger end down, remove and save the needle. Attach a 0.45  $\mu\text{m}$  disposable filter to the tip of the syringe. Load the contents of the syringe onto the top of the SPE cartridge using the syringe/disposable filter apparatus. Elute to waste but do not allow the cartridge to become dry. Remove and save the filter from the disposable syringe, re-attached the 100 mm. syringe needle. Wash saved empty flask with 10 mL methyl acetate.

## 7.6.1 continued

Draw this final wash solution into the disposable syringe, remove the needle and reconnect the filter assembly and apply this wash solution to the SPE cartridge. Elute to waste; the cartridge is dried using vacuum suction at this point.

**Note:** alternate procedure (see section 13.5)

Elute the AE F064706 and AE F070951 residues with 5.5 ( $\pm 0.1$ ) mL of methanol:methyl acetate (1:1 v/v) solvent. Collect approximately 5 mL of eluate in a clean collection tube (section 4.0, Item 6) which is typically contained in the vacuum box assembly. Approximately 5 mL of eluate should be collected for each sample analyzed. Transfer the eluate into a 50 mL round bottom flask (see Note below). Wash the tube with approximately 10 mL of methyl acetate. Add the wash to the round bottom flask. Go to section 7.6.3.

**Note:** If solvent exchange (7.6.3) is to be carried out by an alternate technique, e.g. using an N-Evap concentrator, a more appropriate collection vessel may be used in place of the round bottom flask.

### 7.6.2 Silica Gel SPE Cartridge Characterization:

Typically, the commercially available SPE cartridges specified in section 4.0, item 30, will perform according to the procedure described above. However, if low analyte recovery occurs, additional characterization of the SPE cartridges should be considered as part of a problem-solving process. Such characterization may be necessary to account for possible variability in silica gel raw materials as well as to verify the conditioning and elution procedures described in section 7.6.1.

To characterize column performance, add AE F070951 and AE F064706 standard solutions to a control sample not previously fortified, which has undergone procedural work-up through the derivatization step (section 7.4). The fortification level of AE F070951 and AE F064706 should provide a representative amount of test compound, i.e., 200-250 ng in a 5.0 mL final GC/FPD test solution volume. Continue the analytical procedure (section 7.6) and observe the "recovery" of the test compounds after GC/FPD analysis (see below). The methanol:methyl acetate solvent strength and/or elution volume may need to be optimized to maximize recovery (90-100%) for both test substances. As needed, adjust the final eluate volume accordingly (section 7.6.3) to obtain a 5.0 mL (typical) volume in methyl acetate solvent.

**Note:** the analytical standards are prepared in methyl acetate.

### 7.6.3 Solvent Exchange:

Using a rotary evaporator with a water bath set at approximately 35°C or an N-Evap Analytical Concentrator operating at approximately 50°C, evaporate the silica gel column eluate to approximately 0.5 - 1.0 mL. **Caution:** Care must be

### 7.6.3 continued

taken to avoid excessive evaporation, do not evaporate to a volume < 0.5 mL at any point.

Using methyl acetate, quantitatively transfer this solution to an appropriate (e.g., 5.0 mL) volumetric flask or graduated tube and adjust to final volume (V3) with methyl acetate. Typically, a final volume of 5.0 ( $\pm 0.1$ ) mL (2.5 mL for canola seed and grain dust) provides adequate sensitivity for GC/FPD analysis.

**Note:** The volume of the solution for GC analysis may be reduced to compensate for particular GC/FPD sensitivity limitations. However, avoid excessive evaporation, i.e. to volumes < 0.5 mL, to minimize evaporative losses of derivatized compounds.

## 8.0 ANALYTICAL PROCEDURE: SPECIATED METHOD

### 8.1 Sample Preparation

As described in section 7.1.

### 8.2 Extraction:

#### 8.2.1 All Matrices Except Oils

As described in section 7.2.1

#### 8.2.2 Extraction of Canola, Corn and Soybean Oils

Into a 250-mL Teflon centrifuge bottle, weigh 10.0 ( $\pm 0.1$ ) g (Ws) of the oil sample. For procedural recovery, a control oil sample is fortified at this point, using a standard solution prepared in 1/1 ethanol/methyl acetate (see section 6.2). Add 200 ( $\pm 1$ ) mL (V1) of 5% acetic acid in 1:1 denatured ethanol:water. Homogenize for approximately 5 minutes using a Polytron operating at ~4,000 rpm or use an equivalent device. Let the solution stand in the centrifuge bottle at room temperature for at least one hour. Using 50 - 100 mL glass or Teflon centrifuge tubes, centrifuge for 5-10 minutes at medium speed, e.g., achieving 600 - 700 x G at 2,000 - 3,000 rpm.

Remove the oil layer from the top of the liquid, using a Pasteur pipet. Transfer a 50.0 mL aliquot (V2) of the extract to a 250 mL separatory funnel. Add 20 mL of dichloromethane and gently shake for 30 seconds. After separation of the layers, drain the organic (bottom) layer into a clean 50 mL beaker and save this solution. Transfer the aqueous phase to a clean 250 mL flat bottom flask. Return the contents of the 50-mL beaker (organic phase) to the separatory funnel and add 10 mL of water. Shake for a few seconds and allow the layers

## 8.2.2 continued

to separate. Remove and discard the bottom (organic) layer. Add remaining aqueous layer to the flat bottom flask containing the initial aqueous extract. Evaporate to dryness using a rotary evaporator (50-55°C) [or equivalent]. Add 10 mL of water and again evaporate to dryness. Proceed to section 8.4

### 8.3 Extract Clean Up Using Anion Exchange:

As described in section 7.3.

### 8.4 Cation Exchange: Clean Up and Separation of Analytes (see section 13.3)

The cation exchange separation is only used with the speciated method.

Slurry pack a calibrated glass chromatography column (~10.5 mm id) with 12 mL of cation exchange resin (see section 5.0). Use denatured ethanol water (1:1 v/v) to assist in transferring and packing the resin into the column.

Dissolve the dry residue from 8.2.1 and 8.3 in 20.0 mL of denatured ethanol:water (1:1 v/v) and sonicate. Load onto the cation column. Allow this solution to pass through the resin under gravity flow, collecting the eluate, designated **Fraction A**, in a clean 250 mL flat bottom flask. **Fraction A** contains the metabolites, AE F061517 and AE F099730

At this point, wash the resin with 30 mL of denatured ethanol:water (1:1 v/v), collecting this wash eluate in the same flat bottom flask. The flow of column effluent is regulated with the column stopcock valve such that a flow rate of approximately one to two drops per second is attained.

Into a separate clean 250 mL flat bottom flask, elute the parent compound, AE F039866 (or AF E090532), with 100 mL of 1.5M aqueous ammonium hydroxide, **Fraction B**. Use a drip rate of one to two drops per second.

In separate procedures, both Fraction A and Fraction B are taken to dryness using a rotary evaporator set at 50-55°C. Add 5 to 10 mL of ethyl acetate or methanol to the dried extract and again evaporate to dryness as above.

**Note: Fractions A and B are taken through the remaining steps of the method separately, in parallel fashion. It is important that laboratory equipment, e.g., glassware, be appropriately identified as corresponding to either Fraction A or Fraction B of the sample(s). This precaution is required to ensure accurate identification of the sample residue component.]**

Continue the speciated procedure as described for the combined method in sections 7.4 (derivatization), 7.5 (reconstitution) and 7.6 (post derivatization clean up).

## 9.0 DETERMINATION BY GAS CHROMATOGRAPHY:

The derivatives of the parent (AE F039866 or AE F090532) → AE F064706) and the metabolites (AE F099730 → AE F064706 and AE F061517 → AE F070951) are analyzed by gas chromatography with flame photometric detection (P-mode). A fused silica megabore column (0.53 mm id) is used. It is important that the exact final volume (V3) of the GC test solution is known, typically 5.0 (±0.1) mL in methyl acetate. The speciated method gives two fractions (A and B) that are injected separately. With the combined method, only one fraction is obtained for analysis. The instrumentation and conditions reported here have given satisfactory results for a wide variety of matrices. However, they should be regarded only as a guide and the analyst should adjust the conditions to give the best results for the samples being analyzed and the equipment used.

**Note: The alternate conditions, with 3 microliter or less injection volume are preferred.**

### 9.1 Gas Chromatography Instrumentation.

A Varian Model 3400 Gas Chromatograph equipped with a Flame Photometric Detector operating in the phosphorus selective mode (P-mode) is adequate for the determination of 0.05 ppm levels of the test compounds. Other GC/FPD systems which are found to have equivalent or superior performance, e.g., signal-to-noise (S/N), repeatability, and sensitivity, may be used. Data acquisition is carried out using the Varian Star Workstation Software.

#### 9.1.1 Chart Recorder/Electrometer:

Operate in the linear mode measuring either peak area or height. For manual peak height measurement, attenuate as required to obtain chart recorder peak heights  $\geq 10$  mm (S/N  $\geq 3$ ) for a level corresponding to 0.05 ppm of each test compound, determined as AE F035956 equivalents. When using an electronic integrator, the S/N ratio must also be  $\geq 3$  for the peak(s) of interest, with a minimum peak height of 5 mm displayed on the chromatogram.

#### 9.1.2 Quantitation Limit (Minimum).

The validated LOQ of this method is **0.05 ppm of each test compound**, quantified as AE F035956 equivalents. At this level: i) fortification recoveries should be within a range of 70% to 120%, ii) the analyte GC/FPD signal should be  $\geq 3$  times the background GC/FPD signal, and iii) the intra-laboratory reproducibility as indicated by the relative standard deviation ( $n \geq 3$ ) obtained from replicated analyses should fall within 20% (rel.) of the averaged result.

## 9.2 DB-Wax Megabore Capillary Column Chromatography:

Column: 15 meter x 0.53 mm id fused silica Megabore column with 1.0 micron DB-Wax stationary phase (J&W Scientific)

Temperatures: Inlet: 225°C  
Detector: 230°C

### Column Temperature Program:

	<b>General</b>	<b>Alternate</b>
Initial Temp:	145°C	70°C
Initial Time:	1.5 min.	0.5 min
Rate1:	2.5°C/min.	7.0°C/min
Final Temp1:	150°C	100°C
Rate2:	5°C/min.	8.0°C/min
Final Temp2:	240°C	240°C
Final Time:	5 min.	8 min.

### Gas Flows:

Carrier: 20 mL/min. (Helium)  
Make-up: 10 mL/min. (Nitrogen)  
Detector: 85-100 mL/min. (Hydrogen)  
70-120 mL/min. (Air)

### Injection Parameters:

Injector Type: Varian SPI, equipped with a buffered liner (Restek #20850) hand-packed with silanized glass wool  
Volume: 5 µL (typical) – Vinj. Use the lowest injection volume that yields acceptable performance  
Rate: 5.0 µL/second

### Approximate Retention Time:

	<b>General</b>	<b>Alternate</b>
(AE F070951)	6 - 7 min.	13 min.
(AE F064706)	21 - 22 min.	24 min.

**Note:** The above retention times are estimated for a 15-meter DB-Wax GC column operated as above. Other operating conditions may be used according to the specifications of alternative instrumentation and/or laboratory variables including GC column length, supplier, etc. Although permissible within the scope of this methodology, alternative GC/FPD operating conditions must produce adequate performance, i.e., reproducible retention times, sensitivity, S/N, and component resolution. Note the use of fused silica Megabore GC column lengths > 15 meters is not recommended.

Representative chromatograms are included in Appendix I.

### 9.3 GC/FPD Response Calibration:

The GC/FPD response is calibrated as follows using the representative GC/FPD operating conditions outlined in section 9.2 (above)

Determine the GC/FPD response in peak area/height units for AE F064706 or AE F070951 for a series of analytical standards as prepared in section 6.1.

The lowest level analytical standard must correspond to a sample residue level approximately 50% to 70% of the 0.05 ppm quantitation limit.

For example, assuming a 12.5 g sample (V1 = 500 mL), a sample aliquot of 100 mL (V2), a final volume (V3) of 5 mL, and a 5 µL injection volume (Vinj), successful GC/FPD analysis of a 0.025 ng/µL standard is required to approximate 0.05 ppm of test compound. All concentration levels are expressed as AE F035956 equivalents.

During each analytical sequence, a standard calibration curve is drawn and a linear least square regression calculation is carried out:

$$Y = mX + b$$

$$X = \frac{Y - b}{m}$$

where: Y = analyte (GC/FPD) peak area (or height) from the injected sample  
m = slope of the regression line (X coefficient)  
X = amount of analyte found in the sample (ng)  
b = constant (Y-intercept)

For verification of stable GC/FPD response, construct a standard curve which includes the levels of interest (see Note below). Typical calibration curves are provided for AE F064706 and AE F070951 in Appendix II. Every 2 to 3 sample injections within an analytical sequence are to be followed by an analytical standard. This practice serves as an ongoing quality control check of detector sensitivity/drift and component retention time (column) stability. Always analyze a calibration standard before the first analytical sample and after the last analytical sample within a sequence.

Residue results must not be determined by extrapolation of calibration data outside of the concentration range of the analyzed calibration standards (using ±10% tolerance, typically).

**Note:** Before standards or samples are analyzed within a sequence, two to three sample injections are recommended. These pre-sequence analyses serve to condition the GC/FPD system, e.g., to mask active sites.<sup>2,3</sup> Instrument response should be stabilized such that the GC/FPD peak areas/heights from replicate injections (n ≥ 3) agree to within ±10% relative.]

9.4 Sample Analysis:

Inject an aliquot, typically 5 µL, of the prepared sample from section 7.6.3, noting the injection volume (V<sub>inj</sub>). For practical purposes, the use of equivalent injection volumes for standards and samples is highly recommended.

Compute the integrated or accurately measured peak area/height of AE F064706 and/or AE F070951 in the chromatogram. Compare the component peak area/height values with the analytical standard amount found on the corresponding calibration curve (section 9.3). Samples and standards must be analyzed under stabilized GC conditions and within the same analytical sequence.

10.0 CALCULATION OF THE RESIDUE:

10.1 Percent Recovery:

Following the GC/FPD analysis described in section 8.4, determine the residue of test compound in the sample as follows:

Method	Fraction	Analyte Measured (GC/FPD)	Residue Compound Determined
Speciated	A	AE F070951	AE F061517 – Metabolite
		AE F064706	AE F099730 – Metabolite
	B	AE F064706	AE F039866 – Parent
Combined	One	AE F070951	AE F061517 – Metabolite
		AE F064706	AE F039866 (or AE F090532) plus AE F099730 – Parent plus N-acetyl metabolite

Parts per million (ppm) of AE F035956 (free acid) equivalents = **Amt ÷ B**

where:

**Amt** = ng (X) of AE F064706 or AE F070951 found from the standard curve, expressed as AE F035956 equivalents (See 7.3)

**B** = mg of sample injected

$$= \frac{W_s \text{ (g)} \cdot V_2 \text{ (mL)} \cdot V_{inj} \text{ (}\mu\text{L)}}{V_1 \text{ (mL)} \cdot V_3 \text{ (mL)}}$$

Percent recovery is calculated as follows:

$$\% \text{ Recovery} = \frac{\text{ppm found as AE F035956 equivalents} \cdot 100}{\text{ppm added as AE F035956 equivalents}}$$

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## 10.2 Sample Residue (see example residue calculation sheet in Appendix III)

Using corn grain (lab sample ID: GR01-F1-B) in this example (**Fraction B**):

AE F064706 Found:

Parts per million (ppm) of AE F035956 (free acid) equivalents = **Amt ÷ B**

Amt = ng found from curve = 0.117 ng (using the example in Appendix III)

$$B = \frac{25.0 \text{ g} \cdot 25 \text{ mL}}{250 \text{ mL}} + \frac{5 \mu\text{L}}{5 \text{ mL}} = 2.50 \text{ mg}$$

AE F035956 (free acid) equivalents =  $0.117 \text{ ng} \div 2.50 \text{ mg} = 0.047 \text{ ppm}$

Since the AE F064706 analyte was found in **Fraction B**, the residue corresponds to the parent compound, ammonium-DL-homoalanin-4-yl-(methyl)-phosphinate (reference compound: AE F039866).

**Note:** In the above example, a 25.0 g sample ( $W_s$ ) was extracted into a 250 mL final volume ( $V_1$ ), from which a 25 mL aliquot ( $V_2$ ) was taken. For corn grain, this is equivalent to the procedure specified in section 9.3. See also section 13.2.

## 11.0 QUALITY CONTROL PROCEDURES:

### 11.1 Laboratory Fortifications:

To assure the quality of the data, laboratory fortifications should be run with each set of residue samples. These fortified samples must cover the range of expected residues in the set and at least 10% of the set must be laboratory fortifications. Spiking of quality control samples should be made directly onto the analytical sample prior to extraction. Preparation of fortification standards is described in section 6.2. Spiking volumes may range from 100  $\mu\text{L}$  to 1 mL using the 10  $\mu\text{g}/\text{mL}$  spiking solutions of AE F039866 (or AE F090532) + AE F061517 + AE F099730 (speciated method) or AE F039866 (or AE F090532) + AE F061517 or AE F061517 + AE F099730 (combined method).

For example, 0.50 mL of Stock Solution F [10.0  $\mu\text{g}/\text{mL}$  (each) of AE F039866 and AE F061517, expressed as AE F035956 equivalents] added to 12.5 g of control sample corresponds to 0.40 ppm of AE F035956 (free acid) equivalents of each compound, in the form of AE F039866 (parent) and AE F061517 (metabolite).

### 11.2 Sample Storage:

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## 11.2 Sample Storage:

All residue samples should be stored frozen until analysed. After sampling for analysis, the remaining sample should be promptly re-frozen and stored until authorized for disposal.

## 12.0 CONFIRMATORY TECHNIQUES:

At least two sources of data provide confidence in the identification of the GC/FPD peaks assigned to the derivatized parent and metabolite test compounds. First, analysis cross-checks carried out using four different GC columns have provided results consistent with the assigned GC peaks.(1)

Secondly, GC/MS analysis yields characteristic EI mass spectra for both the derivatized parent and metabolite compounds. The GC/MS retention times of the verified test compounds were the same as those for GC/FPD chromatography

In addition, GC/MS operation in the Selected Ion Monitoring mode can be used to verify characteristic relative mass abundance ratios using the following ions: m/z 149 and 165 (for AE F070951) and m/z 150 and 192 (for AE F064706). A Hewlett Packard 5890A GC interfaced to a Model 5970 Mass Selective Detector has been utilized successfully for such confirmatory analysis.(4)

## 13.0 MODIFICATIONS AND/OR POTENTIAL PROBLEM AREAS:

### 13.1 Fortification:

**Speciated method:** fortification solutions containing a combination of AE F039866 (or AE F090532), AE F099730 and AE F061517 may be utilized to determine recovery efficiency.

**Combined method** samples should be fortified with reference standards containing either the parent [e.g., AE F039866 (or AE F090532) or the N-acetyl metabolite (AE F099730). These should not be added as a mixture because these substances are derivatized to a common moiety (AE F064706). As a result, combined residue of parent and N-acetyl metabolite is determined from one chromatography peak. Fortification solutions containing either the parent compound or its N-acetyl glufosinate metabolite (prepared as described in section 6.2) are therefore recommended.

For troubleshooting purposes, e.g. verification of the cation exchange separation step, samples should be fortified with reference standards containing either the parent, e.g., AE F039866 (or AE F090532) or the N-acetyl metabolite (AE F099730). These substances must be fortified separately on individual quality control (QC) samples for method troubleshooting purposes.

### 13.2 Extraction:

When considering the potential variability among the sample matrices covered by this procedure, **sample moisture content is generally accounted for** by adjusting the final sample extraction volume (V1) to 500 ( $\pm 1$ ) mL. This step, combined with the use of a 12.5 g sample of homogeneous material, also minimizes bulk volume contribution from the sample itself, which may be significant for samples of hulls. Equivalent procedures that adequately account for sample moisture content as well as bulk sample volume may be utilized on a case-by-case basis. For example, a 25.0 g corn grain sample can be successfully analyzed, using a 25 mL aliquot (V2) taken from a 250 mL (V1) total extract volume. However, it is essential that the sample extract aliquot (V2) which is carried through the procedure accurately reflects the analyzed sample mass. Indications of the moisture content of representative samples from the EPA Livestock Feeds Table <sup>5</sup> are shown below:

COMMODITY	PERCENT DRY MATTER
<b>Field Corn</b>	
Grain	88
Forage	40
Fodder (stover)	83
Milled Products	85
<b>Soybeans</b>	
Seed	89
Meal	92
Hulls	90
Forage	35
Hay	85

### 13.3 Anion and Cation Exchange Columns

The elution profiles of the anion or cation exchange columns may be experimentally verified or calibrated using the appropriate reference substances, including C-14 materials. Column profiles should be established with fortified matrix extracts rather than solvent blanks. This will account for extracted sample matrix components that may affect column characteristics.

On occasion, some samples may require anion exchange clean up of a 50 mL extract aliquot (V2), i.e., instead of a 100 mL aliquot. In this case, the procedure used for the grain dust samples should be followed. This troubleshooting option is recommended, as may be required to optimize procedural recovery of parent and metabolite compounds

#### 13.4 Derivatization:

Variable and/or low derivatization yields may be the result of spurious water contamination of the derivatization mixture. All reagents and glassware used to carry out the derivatization reaction must be dry. Typically, contamination by water in this step more affects the recovery of AE F061517 as compared to that of parent compound)

The risk of contamination by water can be reduced by the use of a silica gel (section 5.0, item 12) drying tube (section 4.0, item 8) on the reflux condenser

Certain sample matrices, i.e., corn grain, soybean seed and meal, may require additional time for sonication prior to derivatization (section 7.4). Sonication should continue until all sample residue dissolves, including any material that may adhere to the flask wall. In some cases, a five-minute sonication with acetic acid followed by a 5-10 minute sonication after addition of trimethylorthoacetate. Sample should be capped during this procedure.

#### 13.5 Silica Gel Clean-up Procedure

An alternate way of loading the silica gel SPE cartridge (section 7.6.1) follows:

Transfer the toluene extract (section 7.5) to a 10 mL graduated cylinder, adjust the volume to 4.0 mL with toluene, and return this solution to the original flask. Add 4.0 mL of methyl acetate. Filter this solution through a disposable filtration column (section 4.0, item 22), which is "piggy-backed" onto the silica gel clean-up column. Rinse the flask with 10 mL of methyl acetate and elute this solution through the filtration column and SPE cartridge. Both the loading and rinse solutions are eluted to waste. Remove the filtration column and dry the SPE cartridge using vacuum suction as described in the method. Continue with elution of the SPE cartridge (section 7.6.1).

#### 13.6 Chromatography:

Normally, there are some residual matrix components in samples analyzed that may lead to a gradual deterioration of GC column performance. For fused silica Megabore GC columns, performance may often be restored by cutting and discarding the first six to twelve inches of GC column (injector end). The use of a retention gap of pure fused silica may also be advantageous.

It is also important to utilize a gas chromatography system (i.e., including the injector, column, and detector) which has been adequately conditioned just prior to initiating a sample analysis sequence. This essentially involves masking any active sites in the system (see Note, section 9.3). Further, GC instrument problems may individually or collectively contribute to poor quantitative data.

### 13.6 continued

Factors to consider in this regard include: i) contaminated carrier gas, e.g., with oxygen, ii) excessive GC column stationary phase bleed, iii) non-optimized column connections (e.g., fractured column ends, split ferrules, exposed external column coating material, etc., and iv) minor gas leaks.

### 14.0 SAFETY:

Derivatization reactions (section 7.4), evaporation steps, etc. should be carried out in a fume hood or high efficiency condensers must be used to prevent escape of reagent fumes into the laboratory.

Rotary evaporators should be set up in a fume hood or exhaust fumes should be vented adequately. Alternatively, a cold trap may be used, especially when evaporating formic acid.

### 15.0 REFERENCES

- 1 HRAV-5A Analytical Enforcement Method, accepted by EPA
- 2 Froberg, J. E. and Doose, G. M. (1986) In "Analytical Methods for Pesticides and Plant Growth Regulators, Volume XIV", (G. Zweig and J. Sherma, eds.), pp55-56, Academic Press, Inc., New York.
- 3 Cretney, W. J., McLaughlin, F. A., and Fowler, B. R., Journal of High Resolution Chromatography and Chromatography Communications, 10, (1987) 428.
- 4 August 5<sup>th</sup> 1999 Reply to EPA RAB1/HED Review (9 July 1999) of Glufosinate-Ammonium Tolerance Petition, EPA Tolerance Petition 7F910
- 5 Livestock Feeds Table, Subdivision O (Residue Chemistry) of the Pesticide Assessment Guidelines; Proposed changes issued June 2, 1994, US EPA,

## APPENDIX I

### Representative Chromatograms

Chroma- togram #	Description	Page
1	0.015 µg/mL AE F070951 + AE F064706	37
2	0.020 µg/mL AE F070951 + AE F064706	37
3	0.050 µg/mL AE F070951 + AE F064706	38
4	0.100 µg/mL AE F070951 + AE F064706	38
5	0.200 µg/mL AE F070951 + AE F064706	39
6	0.300 µg/mL AE F070951 + AE F064706	39
<b>EXAMPLE CHROMATOGRAMS USING SPECIATED METHOD<sup>1</sup></b>		
7	Control corn grain, not fortified; Fraction A: all residues <0.05 ppm	40
8	Control corn grain, not fortified; Fraction B: all residues <0.05 ppm	40
9	Control corn grain, fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F085355, Fraction A Recoveries: AE F061517 = 80%, AE F085355 = 102%	41
10	Control corn grain sample, fortified at 0.25 ppm with AE F061517 and at 0.25 ppm with AE F039866, Fraction B Recovery AE F039866 = 87%	41
11	Control corn silage sample, not fortified; Fraction A: all residues <0.05 ppm	42
12	Control corn silage sample, not fortified, Fraction B: all residues <0.05 ppm	42
13	Control corn silage sample, fortified at 1.0 ppm with AE F061517 and at 1.0 ppm with AE F085355, Fraction A Recovery: AE F061517 = 90%, AE F085355 = 117%	43
14	Control corn silage sample, fortified at 1.0 ppm with AE F061517 and at 1.0 ppm with AE F039866, Fraction B. Recovery AE F039866 = 100%	43
15	Control corn oil sample, not fortified; Fraction A: all residues <0.05 ppm	44
16	Control corn oil sample, not fortified, Fraction B: all residues <0.05 ppm	44
17	Control corn oil sample, fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F085355, Fraction A. Recoveries: AE F061517 = 95%, AE F085355 = 107%	45
18	Control corn oil sample, fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F039866, Fraction B. Recovery AE F039866 = 90%	45
19	Control soybean seed sample, not fortified; Fraction A: all residues <0.05 ppm	46
20	Control soybean seed sample, not fortified; Fraction B: all residues <0.05 ppm	46
21	Control soybean seed sample, fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F085355, Fraction A. Recoveries: AE F061517 = 112%, AE F085355 = 107%	47
22	Control soybean seed sample, fortified at 0.25 ppm with AE F061517 and at 0.25 ppm with, AE F090532, Fraction B. Recovery AE F090532 = 91%	47

<sup>1</sup> Corn and soybean validation data taken from Xenos project XEN3-19A (HRAV Study 93-0027)

## APPENDIX I (continued)

Chroma- togram #	Description	Page
<b>EXAMPLE CHROMATOGRAMS USING COMBINED METHOD<sup>1,2</sup></b>		
41	Control canola seed sample, not fortified; all residues <0.05 ppm	57
42	Control canola seed sample, fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F099730 Recoveries. AE F061517 = 102%, AE F099730 = 111%	57
43	Control canola seed sample, fortified at 0.50 ppm with AE F061517 and at 0.50 ppm with AE F039866 Recoveries. AE F061517 = 111%, AE F039866 = 97%	58
44	Control canola straw sample, not fortified; all residues <0.05 ppm	58
45	Control canola straw sample, fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F039866 Recoveries AE F061517 = 72, AE F039866 = 100%	59
46	Control canola straw sample, fortified at 0.50 ppm with AE F061517 and at 0.50 ppm with AE F099730. Recoveries: AE F061517 = 89%, AE F099730 = 104%	59
47	Control sugar beet root sample, not fortified; all residues <0.05 ppm	60
48	Control sugar beet root sample, fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F099730. Recoveries: AE F061517 = 92%, AE F099730 = 90%	60
49	Control sugar beet root sample, fortified at 0.50 ppm with AE F061517 and at 0.50 ppm with AE F039866 Recoveries AE F061517 = 90%, AE F039866 = 83%	61
50	Control sugar beet top sample, not fortified, all residues <0.05 ppm	61
51	Control sugar beet top sample, fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F039866 Recoveries AE F061517 = 90, AE F039866 = 70%	62
52	Control sugar beet top sample, fortified at 0.50 ppm with AE F061517 and at 0.50 ppm with AE F099730. Recoveries: AE F061517 = 104%, AE F099730 = 93%	62

<sup>1</sup> Canola validation data taken from Xenos project XEN95-07 (AgrEvo German Study ER95ECN552).

<sup>2</sup> Sugar beet roots and tops data taken from Xenos project XEN97-05 (AgrEvo Study BK-97R-04)

## APPENDIX I (continued)

Chroma- togram #	Description	Page
<b>EXAMPLE CHROMATOGRAMS USING SPECIATED METHOD<sup>1,2</sup></b>		
23	Control soybean hay sample, not fortified, Fraction A: all residues <0.05 ppm	48
24	Control soybean hay sample, not fortified; Fraction B: all residues <0.05 ppm	48
25	Control soybean hay sample, fortified at 1.0 ppm with AE F061517 and at 1.0 ppm with AE F085355, Fraction A. Recoveries: AE F061517 = 101%, AE F085355 = 100%	49
26	Control soybean hay sample, fortified at 1.0 ppm with AE F061517 and at 1.0 ppm with AE F039866, Fraction B. Recovery: AE F039866 = 82%	49
27	Control canola seed, not fortified; Fraction A: all residues <0.05 ppm	50
28	Control canola seed, not fortified; Fraction B: all residues <0.05 ppm	50
29	Control canola seed, fortified at 0.05 ppm with AE F061517, at 0.05 ppm with AE F099730 and at 0.05 ppm with AE F039866, Fraction A. Recoveries: AE F061517 = 114%, AE F099730 = 120%	51
30	Control canola seed, fortified at 0.50 ppm with AE F061517, at 0.50 ppm with AE F099730 and at 0.50 ppm with AE F039866, Fraction B. Recovery: AE F039866 = 93%	51
<b>EXAMPLE CHROMATOGRAMS USING COMBINED METHOD<sup>3,4</sup></b>		
31	Control corn grain, not fortified, all residues <0.05 ppm	52
32	Control corn grain, fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F099730. Recoveries: AE F061517 = 91%, AE F099730 = 100%	52
33	Control corn grain, fortified at 0.10 ppm with AE F061517 and at 0.10 ppm with AE F039866. Recoveries: AE F061517 = 90%, AE F039866 = 83%	53
34	Control corn fodder, not fortified; all residues <0.05 ppm	53
35	Control corn fodder, fortified at 0.60 ppm with AE F061517 and at 6.0 ppm with AE F099730. Recoveries: AE F061517 = 98%, AE F099730 = 92%	54
36	Control soybean seed, not fortified; all residues <0.05 ppm	54
37	Control soybean seed, fortified at 2.0 ppm with AE F061517 and at 2.0 ppm with AE F039866. Recoveries: AE F061517 = 72%, AE F039866 = 87%	55
38	Control soybean hay, not fortified; all residues <0.05 ppm	55
39	Control soybean hay, fortified at 0.20 ppm with AE F061517 and at 0.20 ppm with AE F099730. Recoveries: AE F061517 = 105%, AE F099730 = 100%	56
40	Control soybean hay, fortified at 0.20 ppm with AE F061517 and at 0.20 ppm with AE F039866. Recoveries: AE F061517 = 103%, AE F039866 = 93%	56

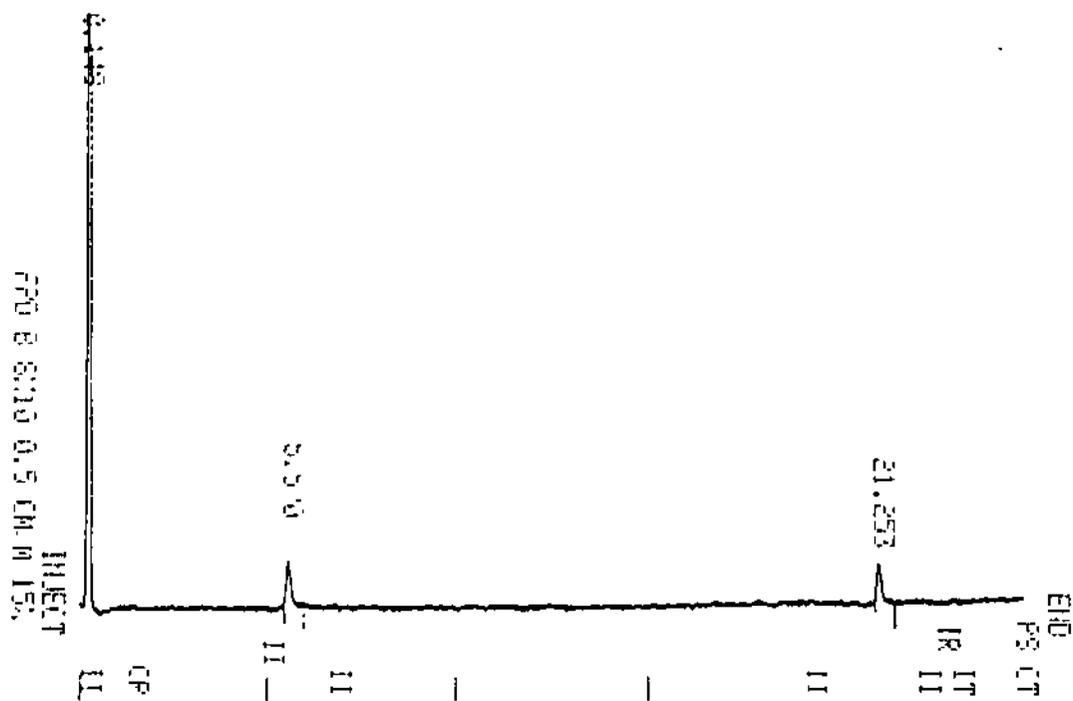
<sup>1</sup> Soybean validation data taken from Xenos project XEN3-19A (HRAV Study 93-0027)

<sup>2</sup> Canola data taken from Xenos project XEN97-49 (AgrEvo Canada Study 97AC14)

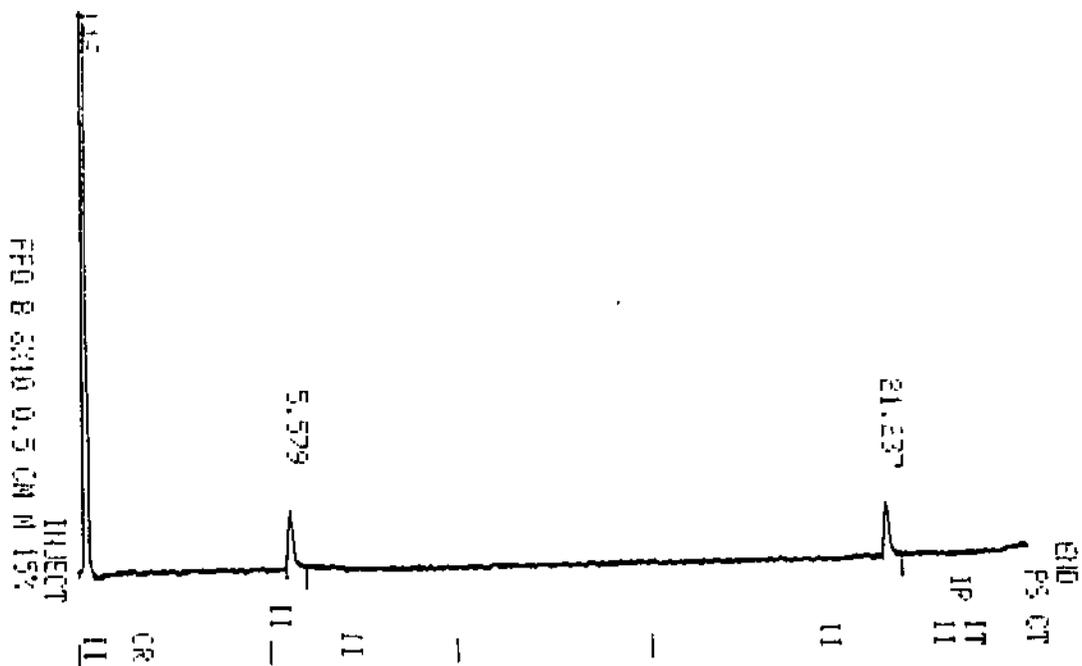
<sup>3</sup> Corn validation data taken from Pan-Ag study 94354.

<sup>4</sup> Soybean validation data taken from Xenos project XEN94-29V (AgrEvo Study BK-94R-02)

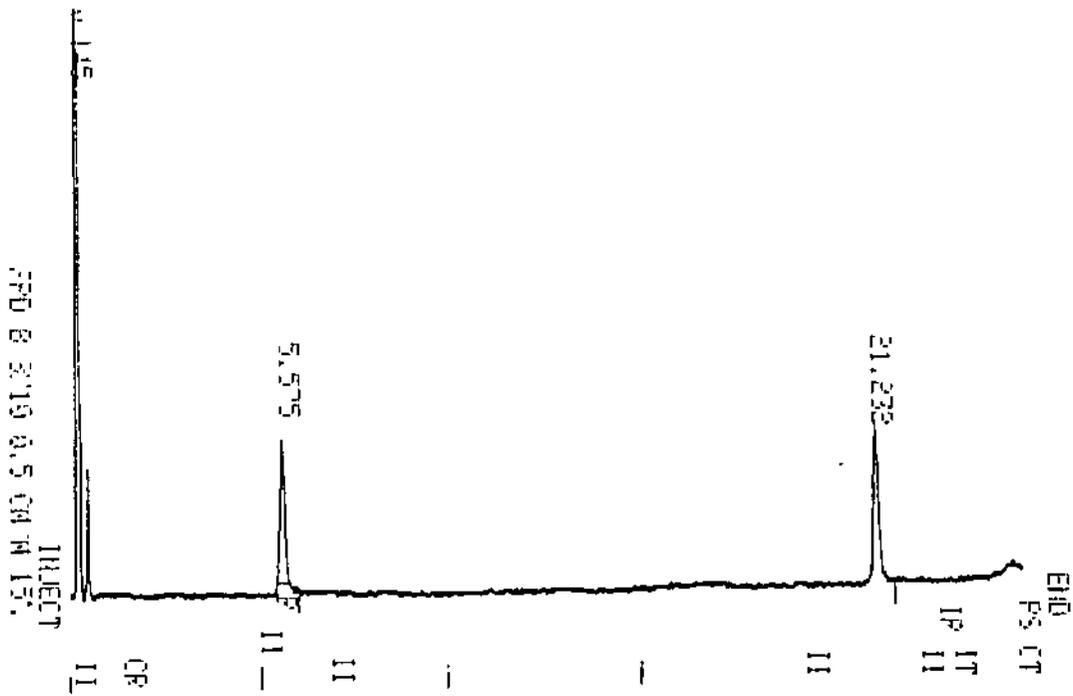
Chromatogram 1: 0.015 ng/ $\mu$ L AE F070951 + AE F064706



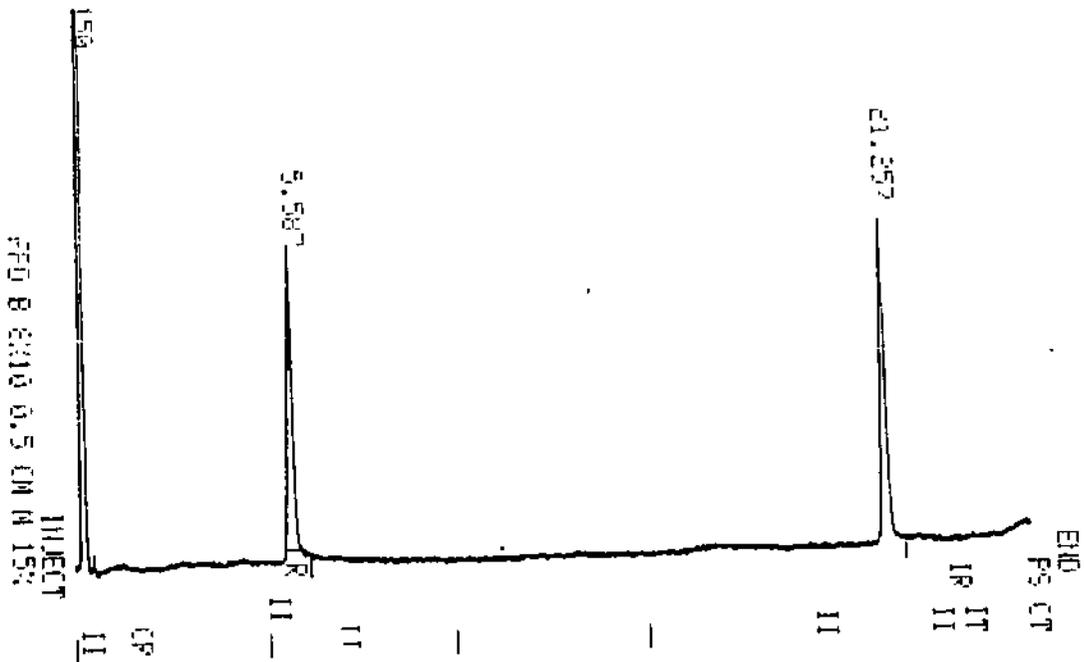
Chromatogram 2: 0.020 ng/ $\mu$ L AE F070951 + AE F064706



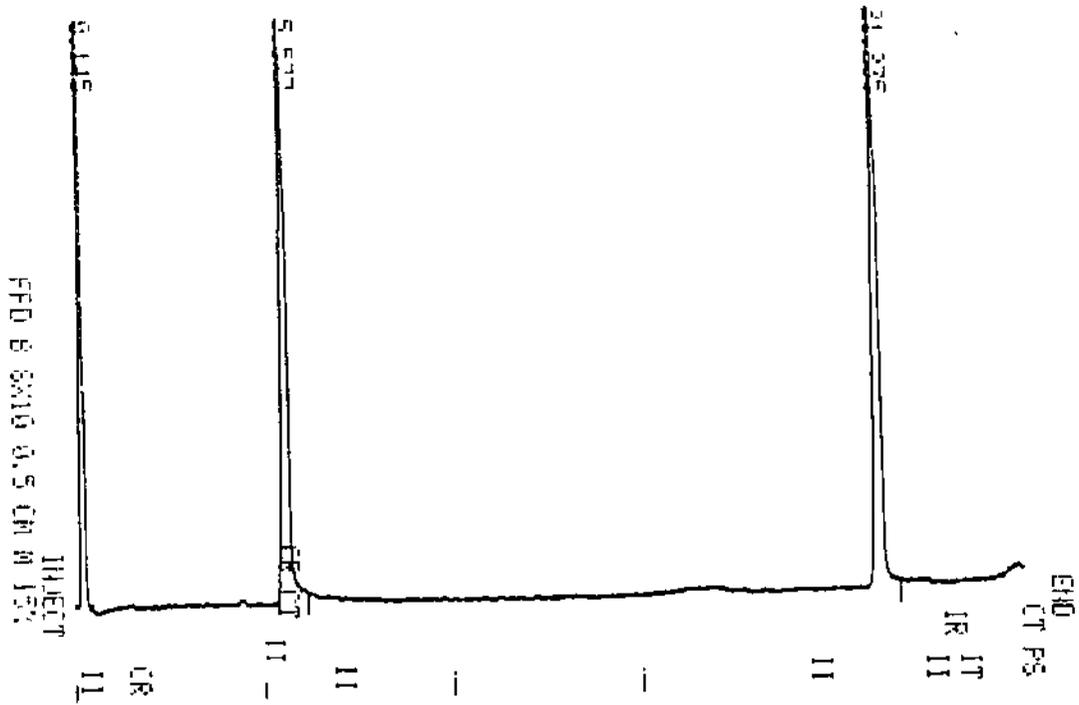
Chromatogram 3: 0.050 ng/ $\mu$ L AE F070951 + AE F064706



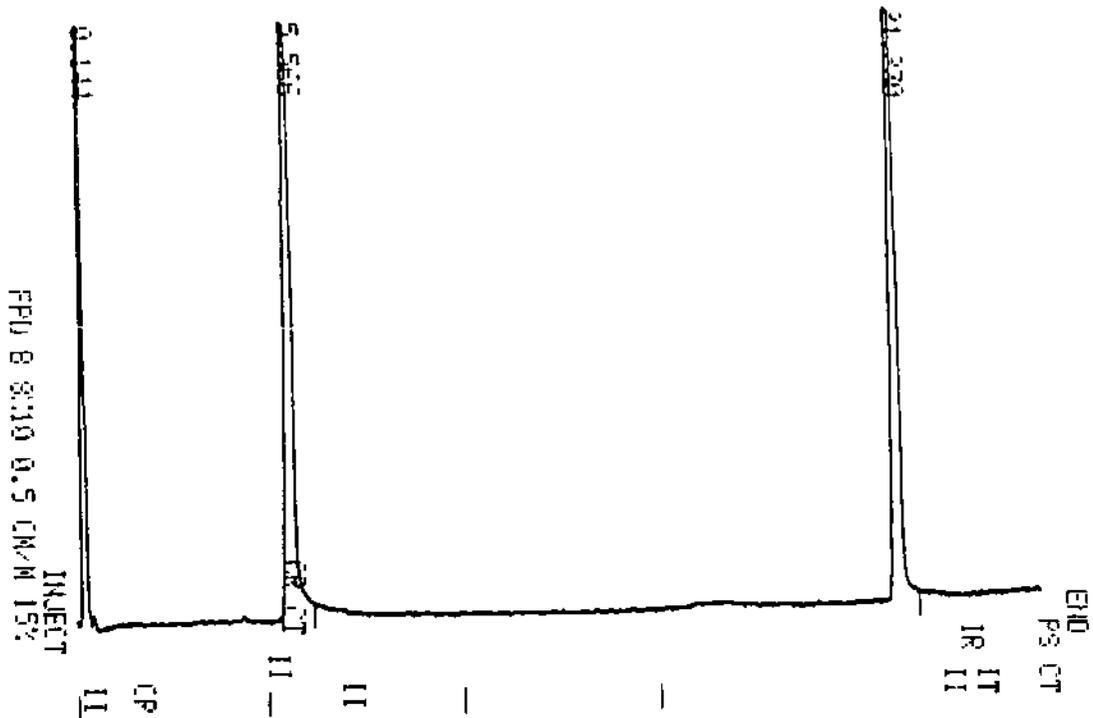
Chromatogram 4: 0.100 ng/ $\mu$ L AE F070951 + AE F064706



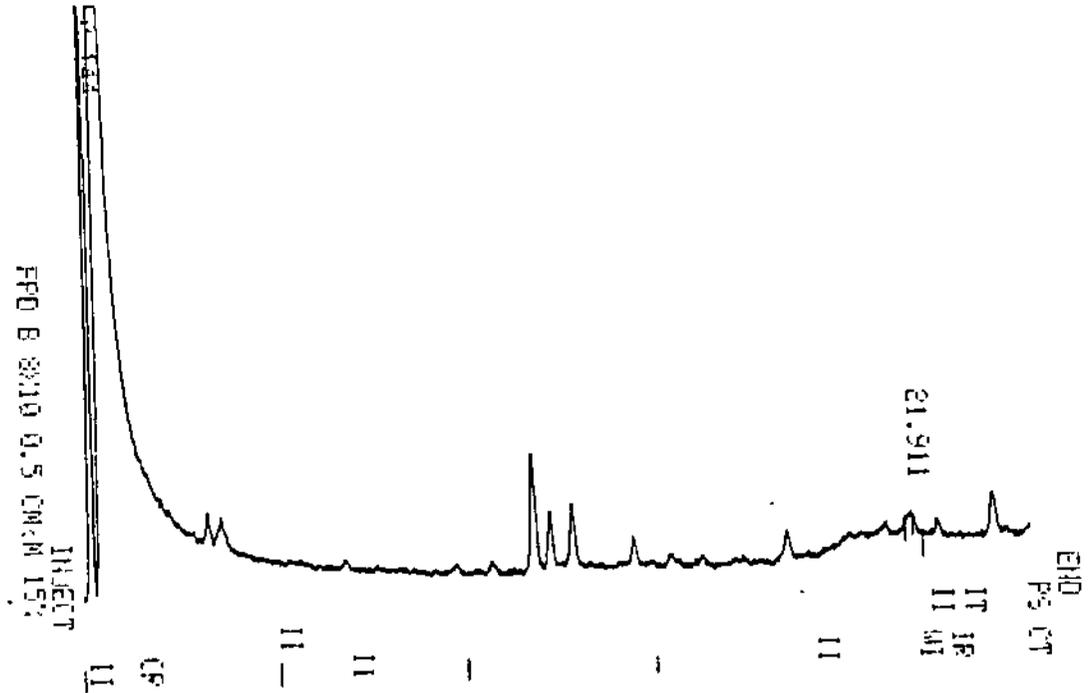
Chromatogram 5: 0.200 ng/ $\mu$ L AE F070951 + AE F064706



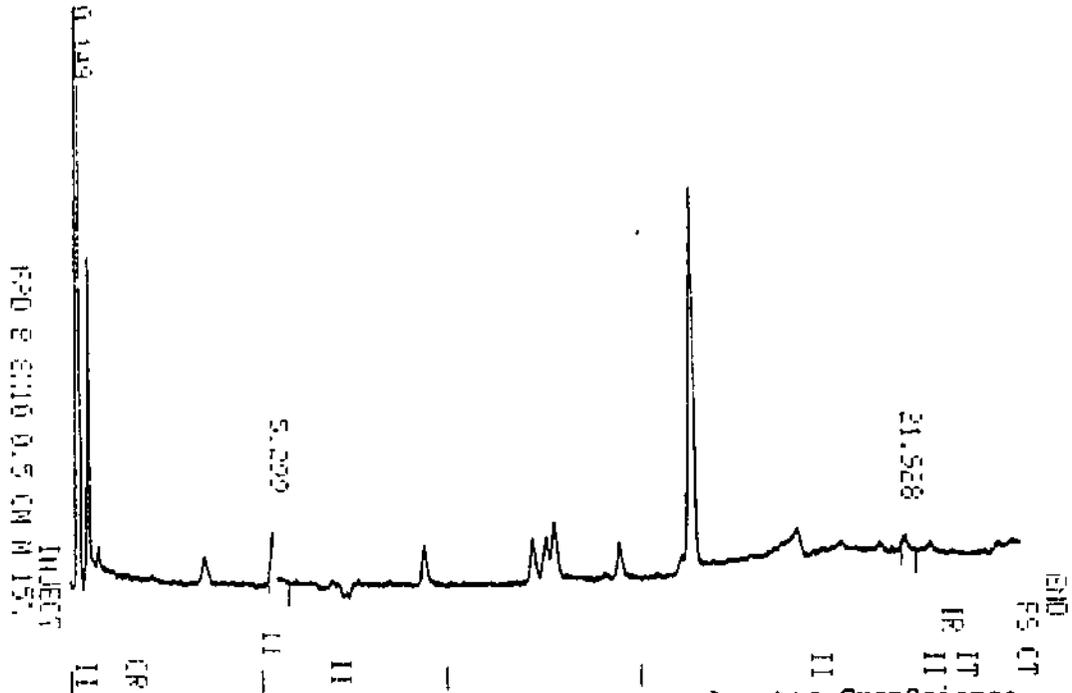
Chromatogram 6: 0.300 ng/ $\mu$ L AE F070951 + AE F064706



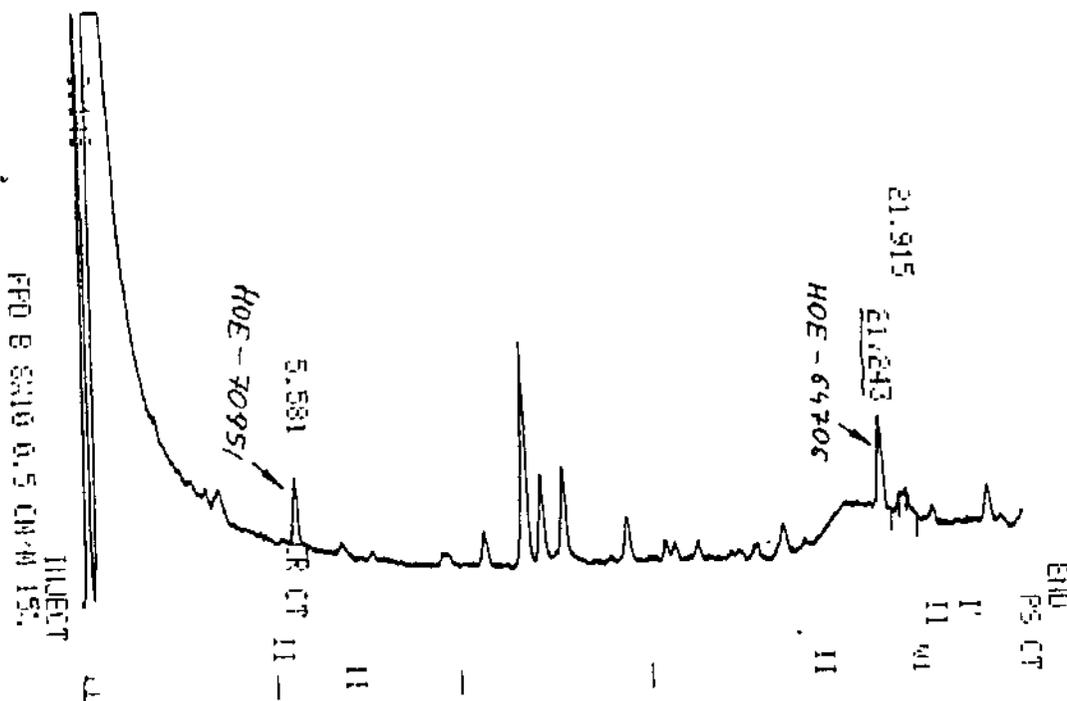
Chromatogram 7: Control Corn Grain; Fraction A; All Residues <0.05 ppm  
GR02C-A



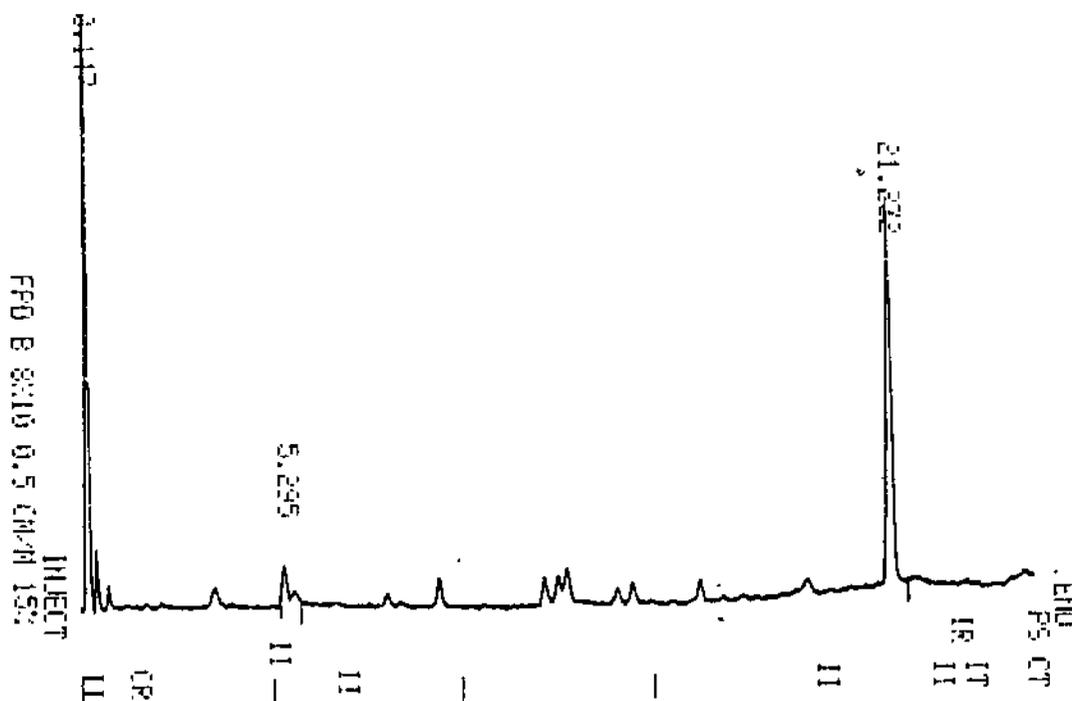
Chromatogram 8: Control Corn Grain; Fraction B; All Residues <0.05 ppm  
GR02C-B



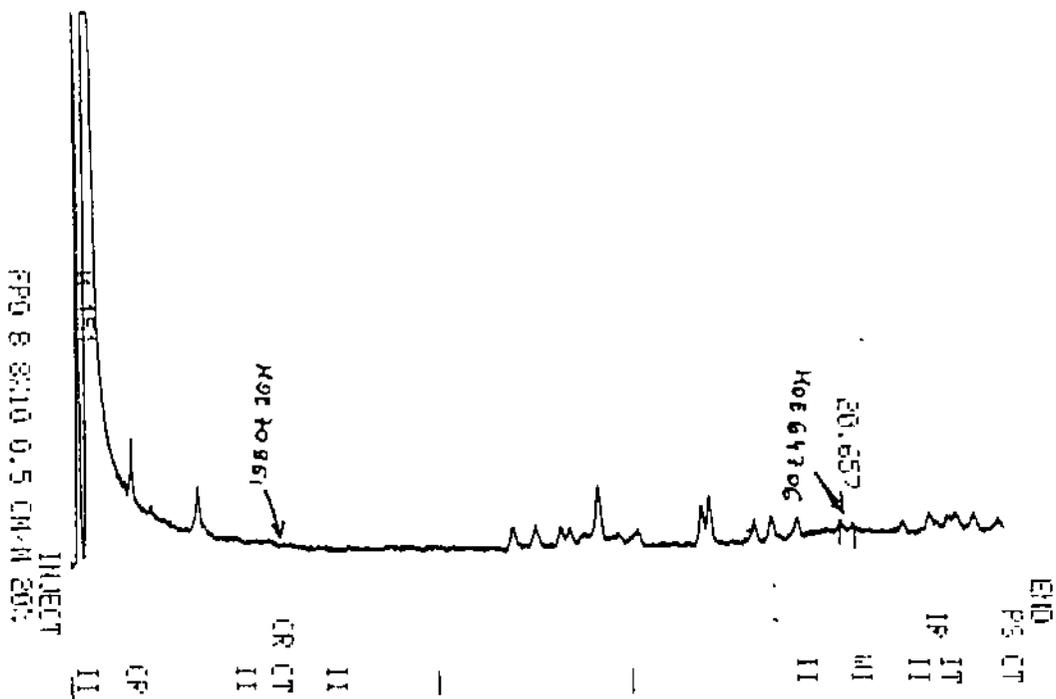
**Chromatogram 9:** Control Corn Grain Fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F085355; Fraction A; Recoveries: AE F061517 = 79.6%, AE F085355 = 102%  
**GR01F2-A**



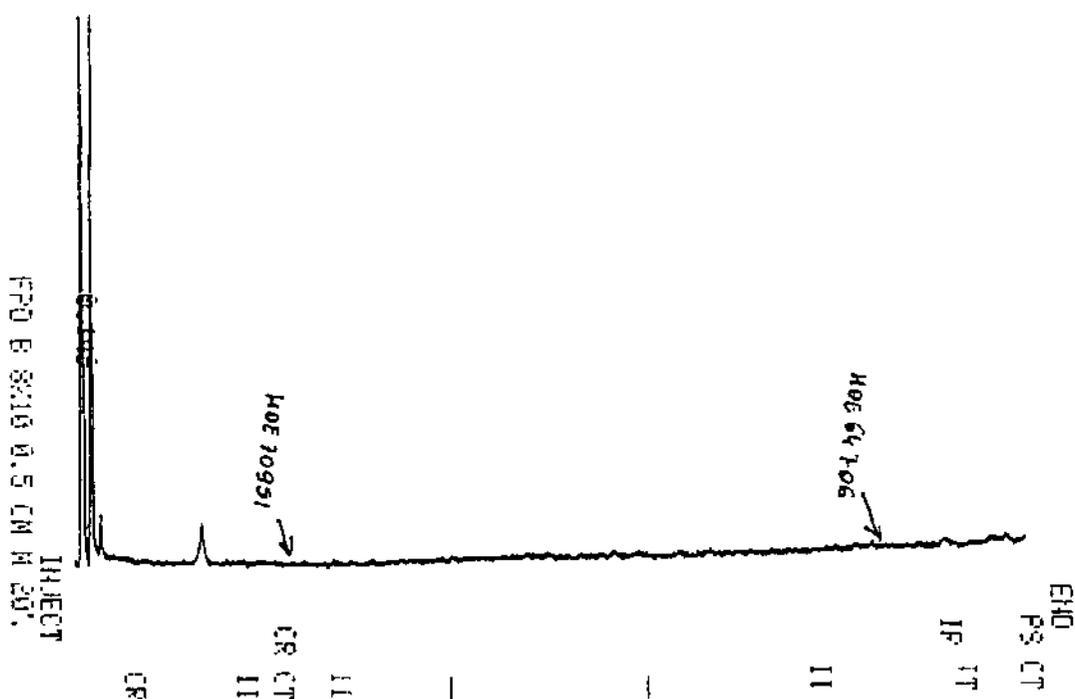
**Chromatogram 10:** Control Corn Grain Fortified at 0.25 ppm with AE F061517 and at 0.25 ppm with AE F039866; Fraction B; Recovery AE F039866 = 87.4%  
**GR01F5-B**



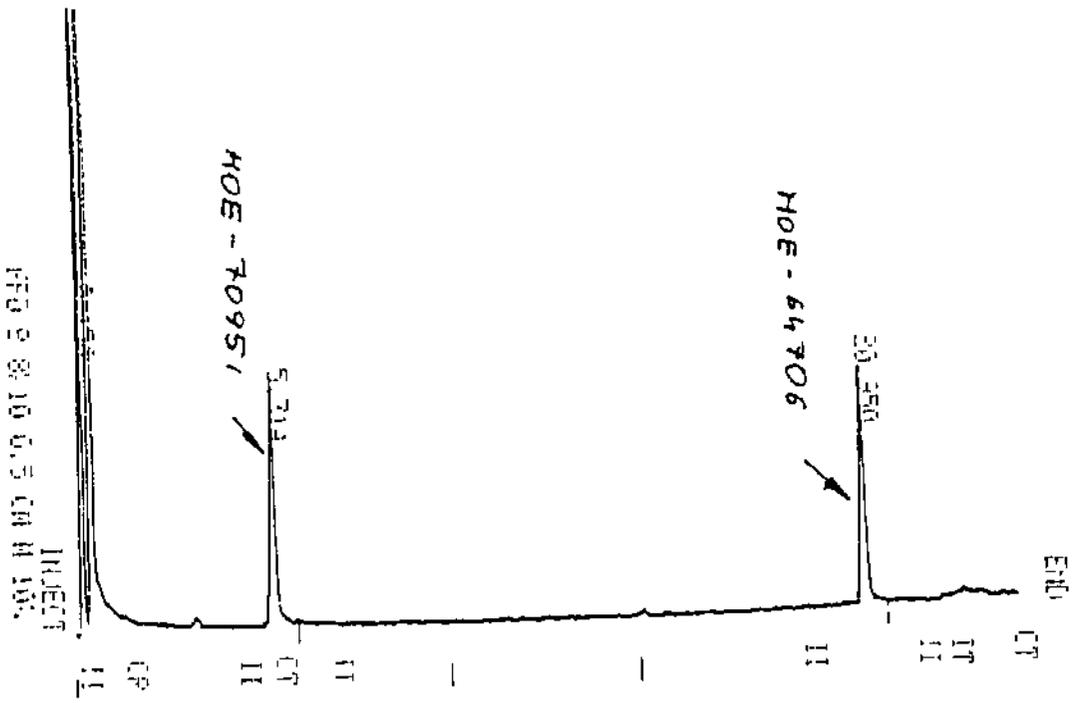
Chromatogram 11: Control Corn Silage; Fraction A; All Residues <0.05 ppm  
SI01C-A



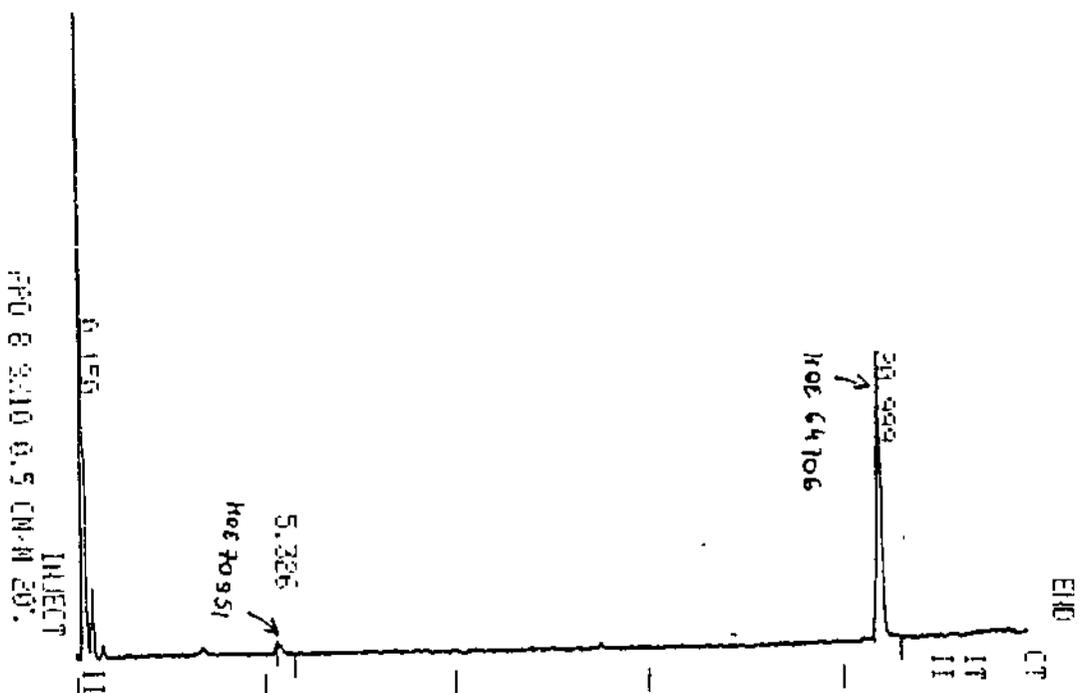
Chromatogram 12: Control Corn Silage; Fraction B; All Residues <0.05 ppm  
SI01C-B



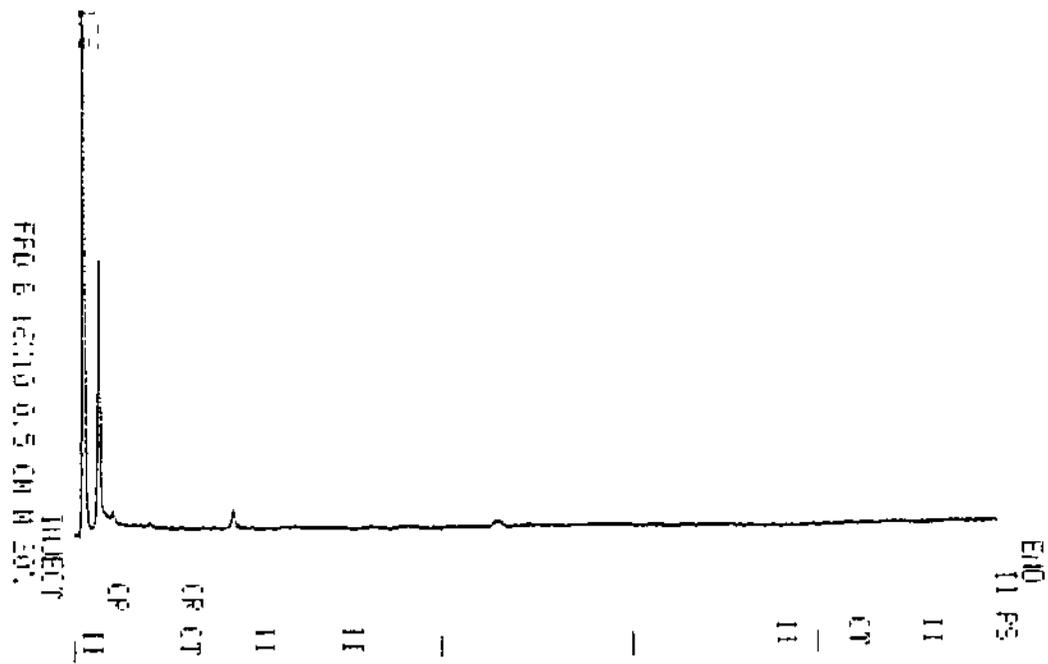
**Chromatogram 13: Control Corn Silage Fortified at 1.00 ppm with AE F061517 and at 1.00 ppm with AE F085355; Fraction A; Recoveries: AE F061517 = 90.2%, AE F085355 = 117%**  
**SI01F5-A**



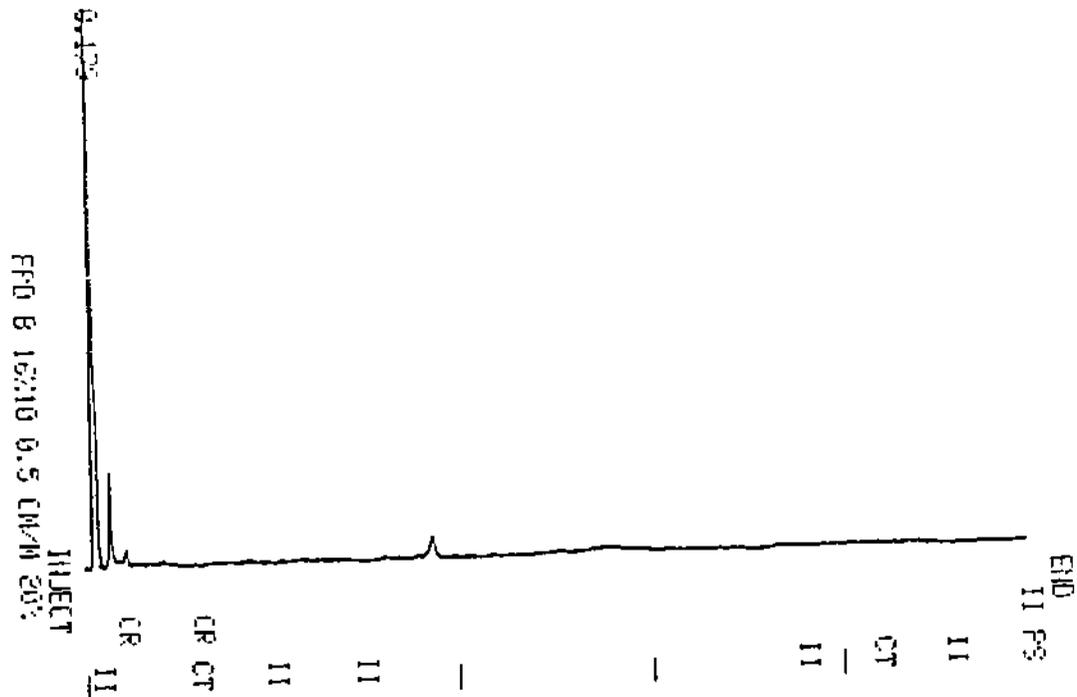
**Chromatogram 14: Control Corn Silage Fortified at 1.00 ppm with AE F061517 and at 1.00 ppm with AE F039866; Fraction B; Recovery AE F039866 = 100%**  
**SI01F4-B**



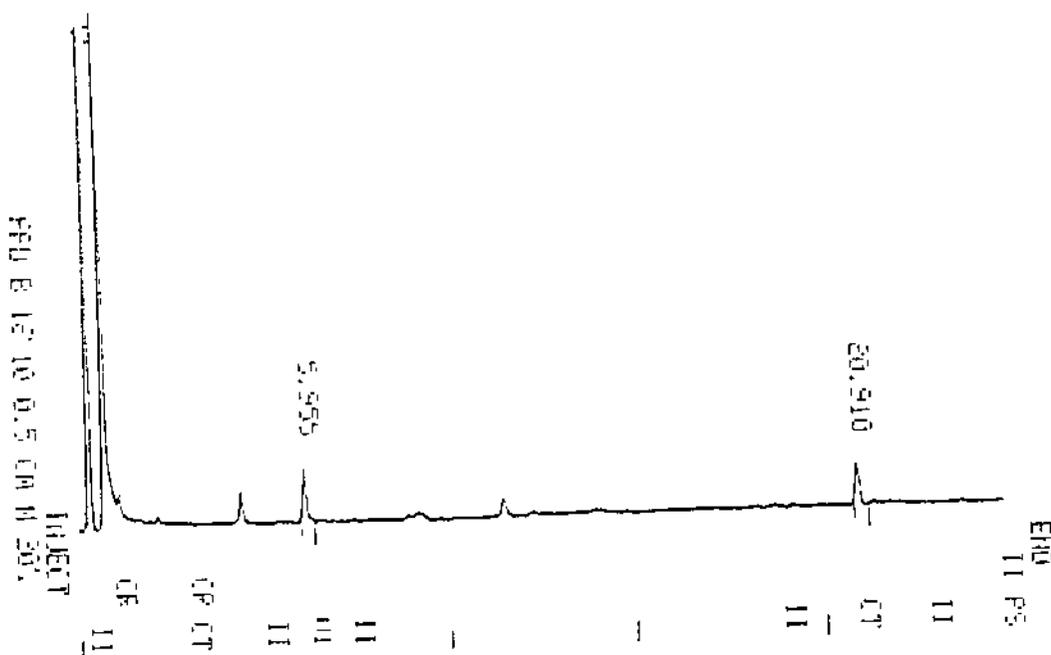
Chromatogram 15: Control Corn Oil; Fraction A; All Residues <0.05 ppm  
PO-01-C-A



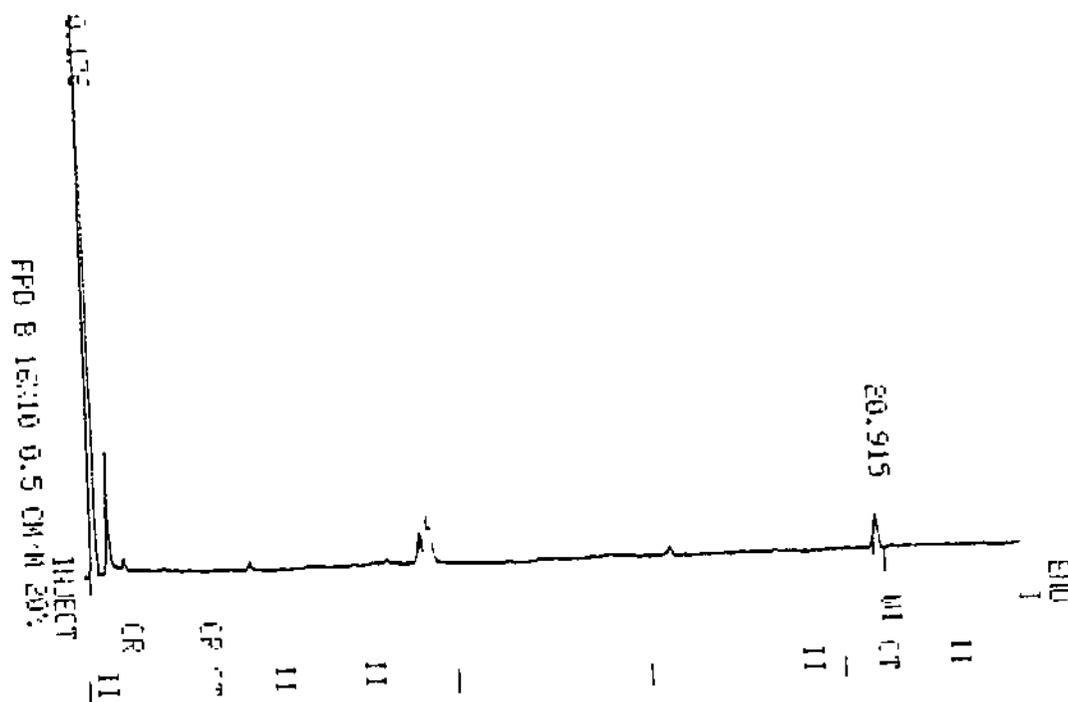
Chromatogram 16: Control Corn Oil; Fraction B; All Residues <0.05 ppm  
PO-01-C-B



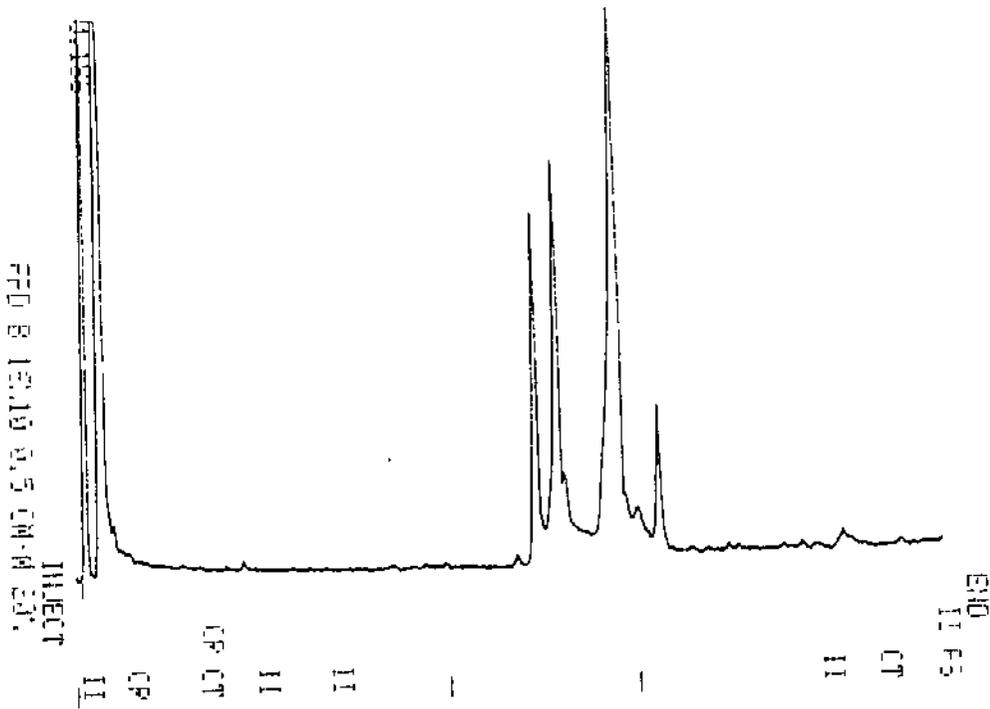
**Chromatogram 17:** Control Corn Oil Fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F085355; Fraction A; Recoveries: AE F061517 = 94.6%, AE F085355 = 107%  
**PO-01-F2-A**



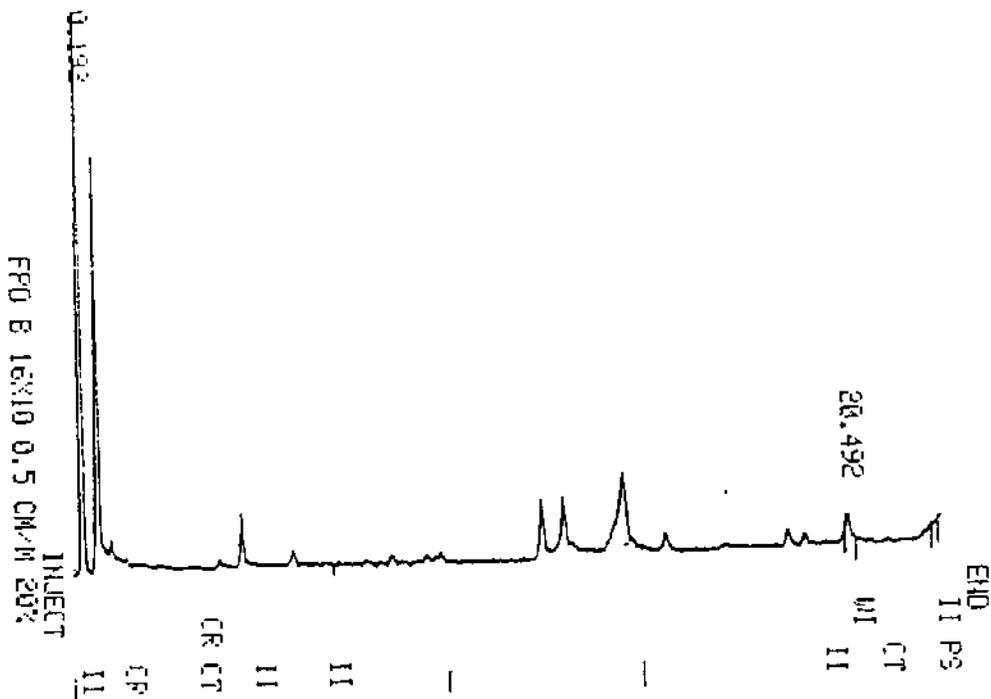
**Chromatogram 18:** Control Corn Oil Fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F039866; Fraction B; Recovery AE F039866 = 90.4%  
**PO-01-F1-B**



Chromatogram 19: Control Soybean Seed; Fraction A; All Residues <0.05 ppm  
SS-02-C-A

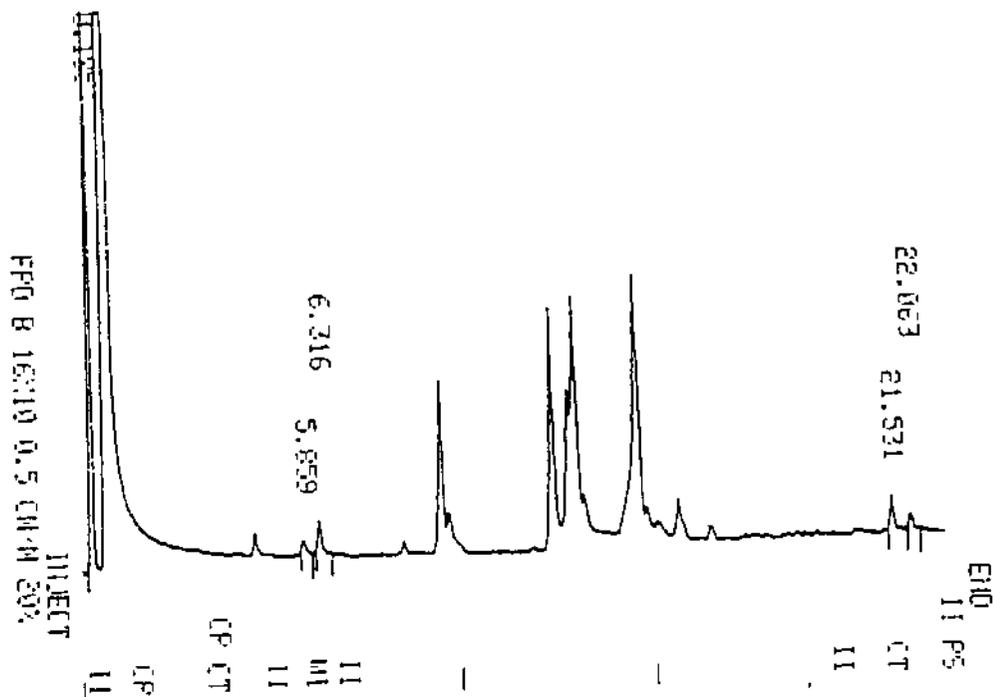


Chromatogram 20: Control Soybean Seed; Fraction B; All Residues <0.05 ppm  
SS-01-C-B



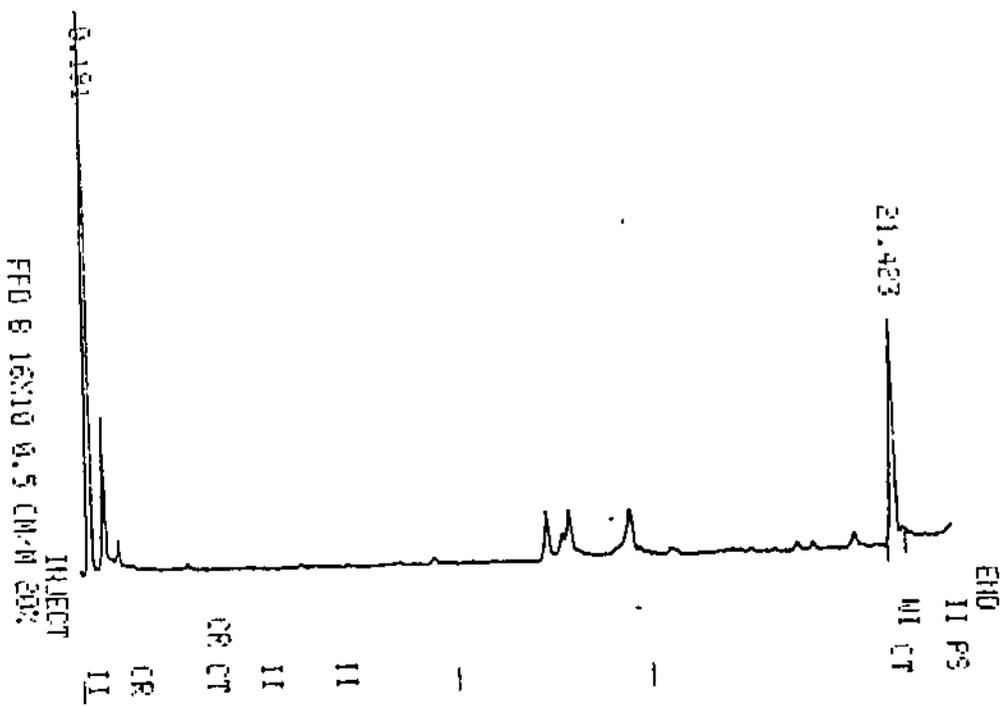
**Chromatogram 21:** Control Soybean Seed Fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F085355; Fraction A; Recoveries: AE F061517 = 112%, AE F085355 = 107%

**SS-01-F2-A**

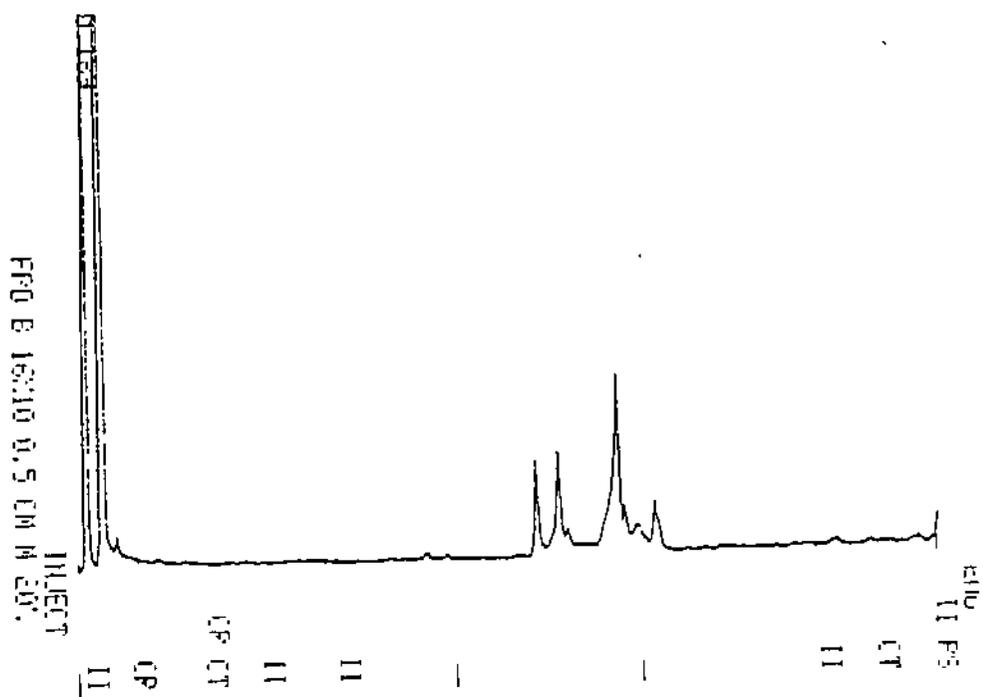


**Chromatogram 22:** Control Soybean Seed Fortified at 0.25 ppm with AE F061517 and at 0.25 ppm with AE F090532; Fraction B; Recovery AE F090532 = 91.0%

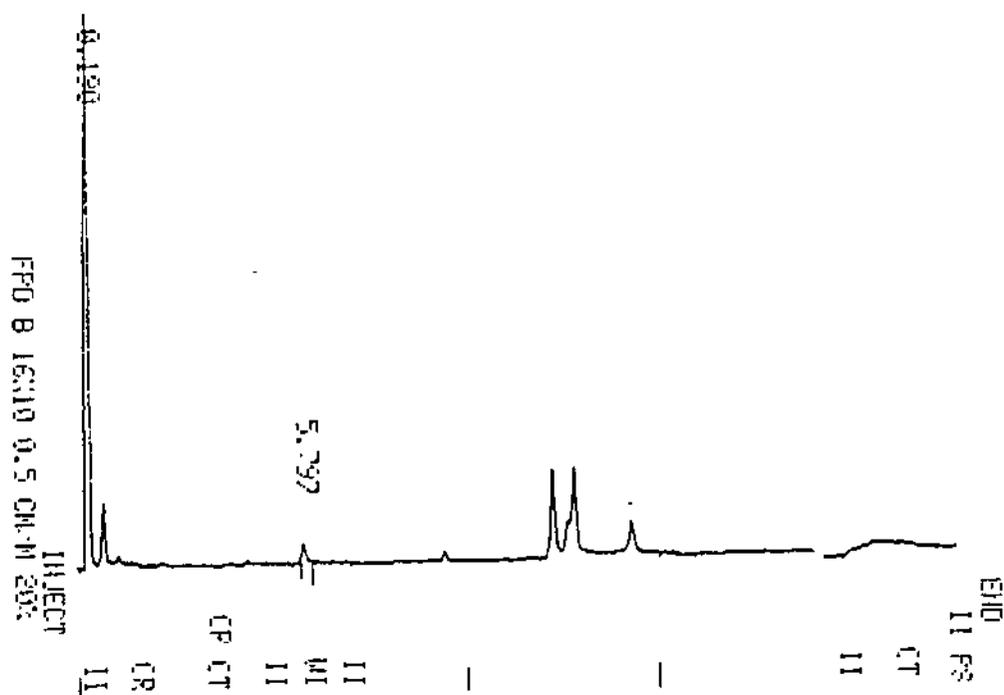
**SS-01-F5-B**



Chromatogram 23: Control Soybean Hay; Fraction A; All Residues <0.05 ppm  
SH-02-C-A

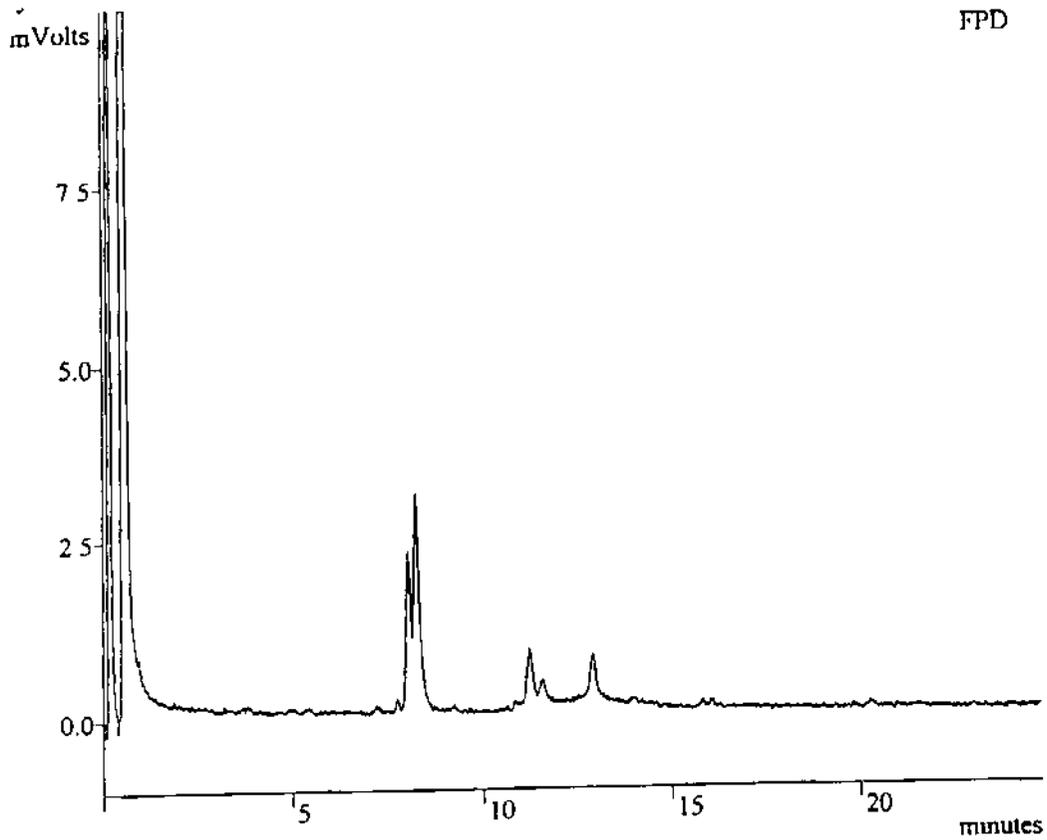


Chromatogram 24: Control Soybean Hay; Fraction B; All Residues <0.05 ppm  
SH-02-C-B

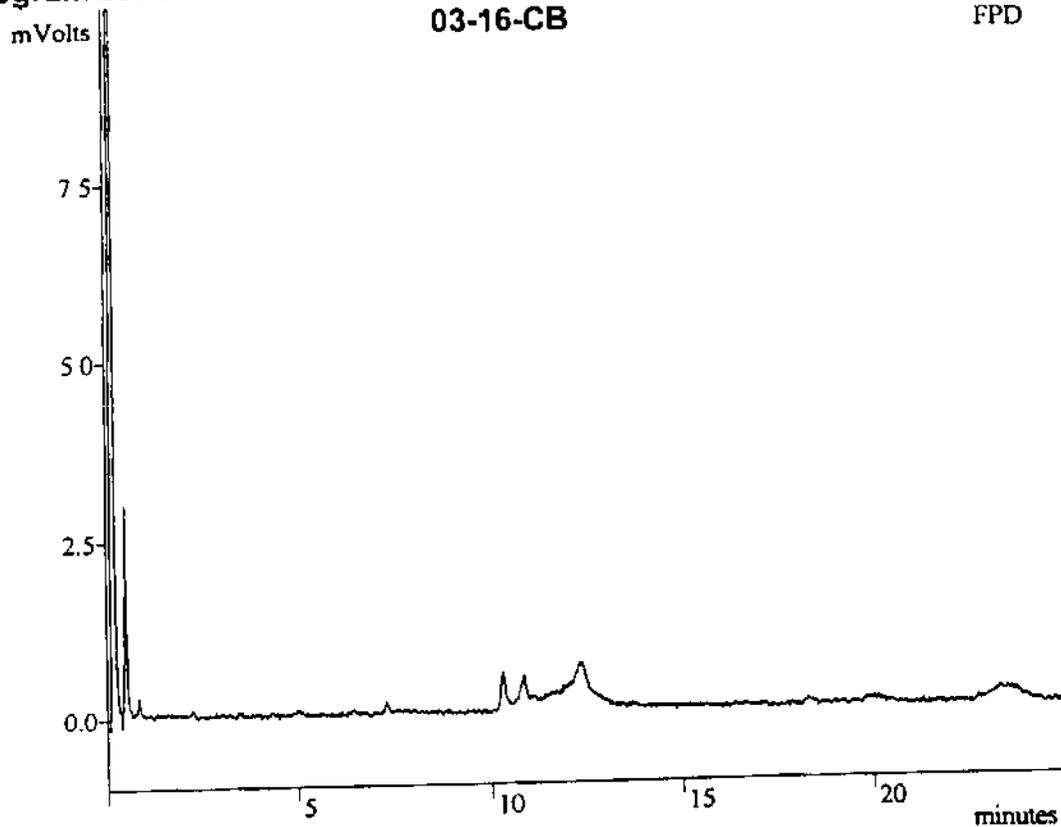




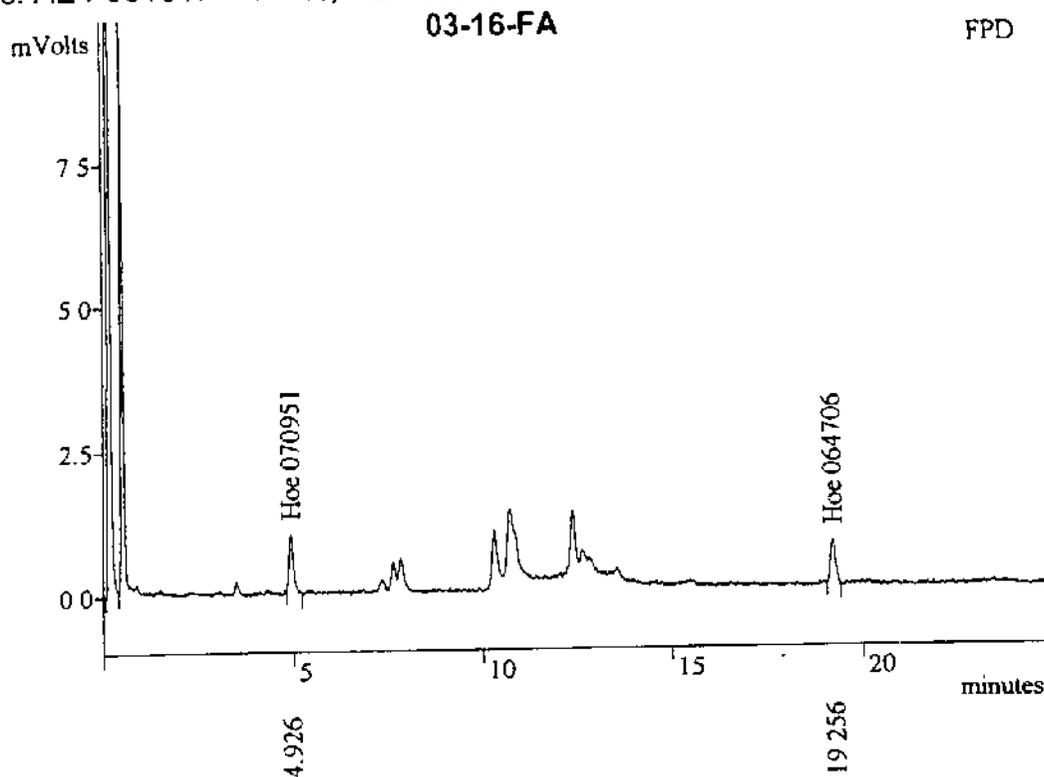
Chromatogram 27: Control Canola Seed; Fraction A; All Residues <0.05 ppm  
02-01C-A



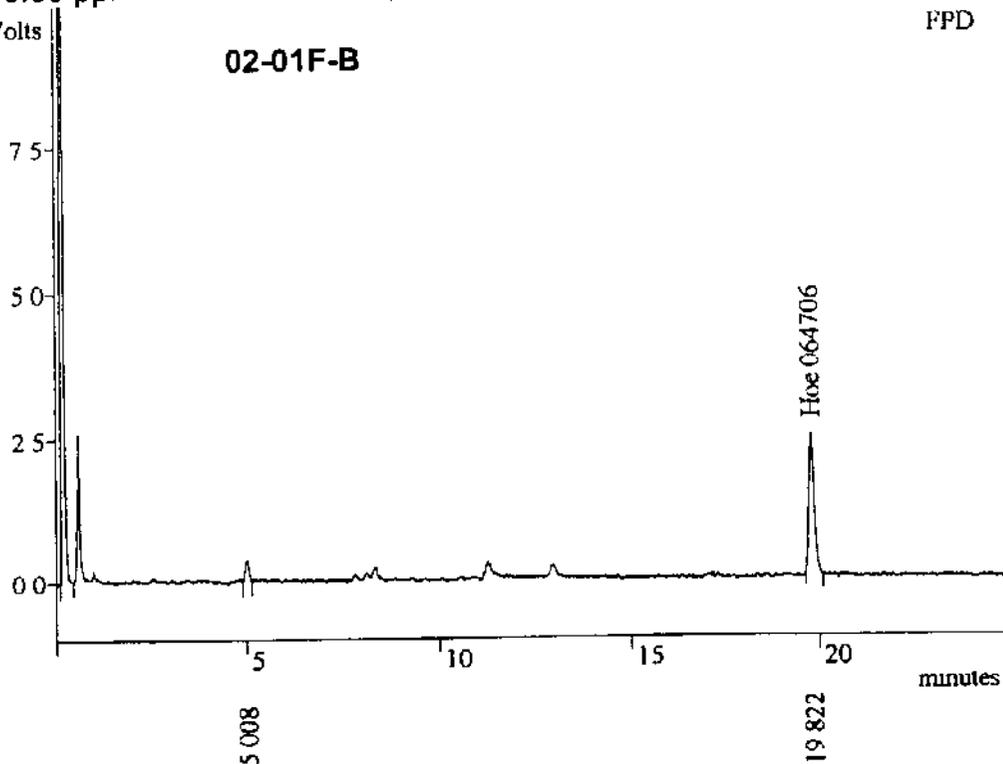
Chromatogram 28: Control Canola Seed; Fraction B; All Residues <0.05 ppm  
03-16-CB



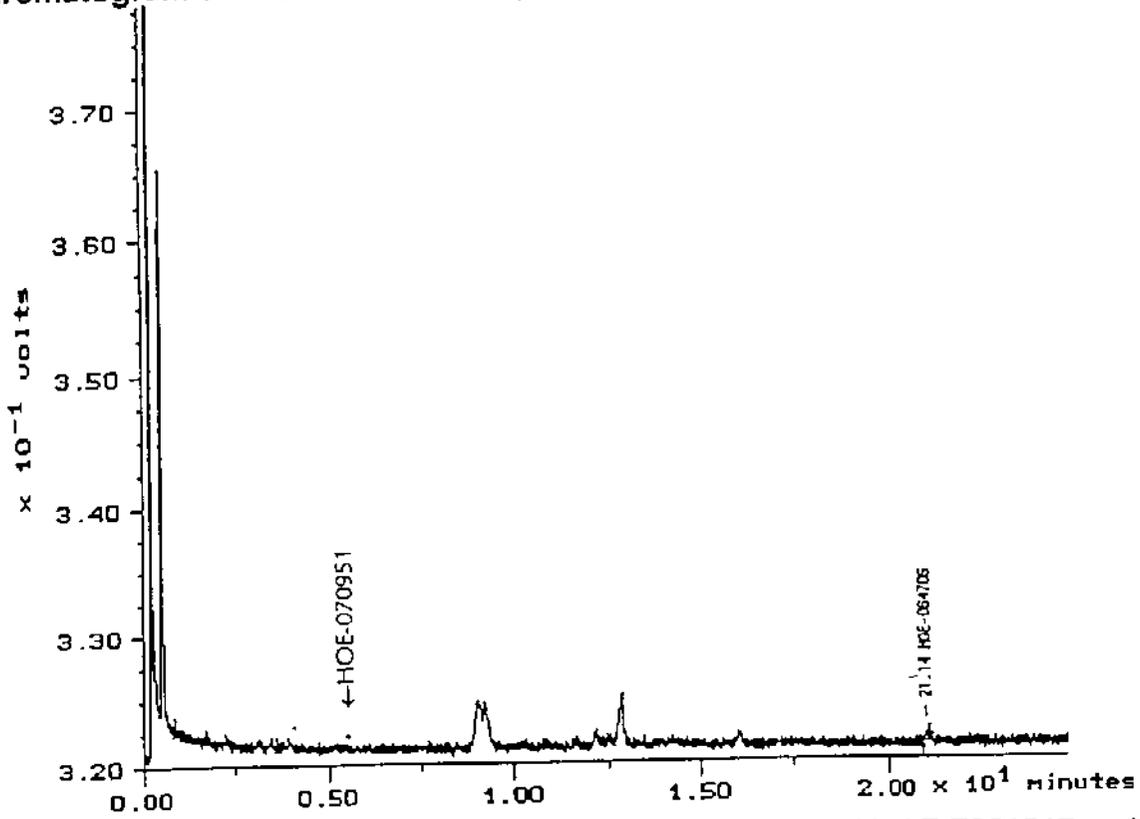
**Chromatogram 29:** Control Canola Seed Fortified at 0.05 ppm with AE F061517, 0.05 ppm with AE F099730 and at 0.05 ppm with AE F039866, Fraction A; Recoveries: AE F061517 = 114%, AE F099730 = 120%



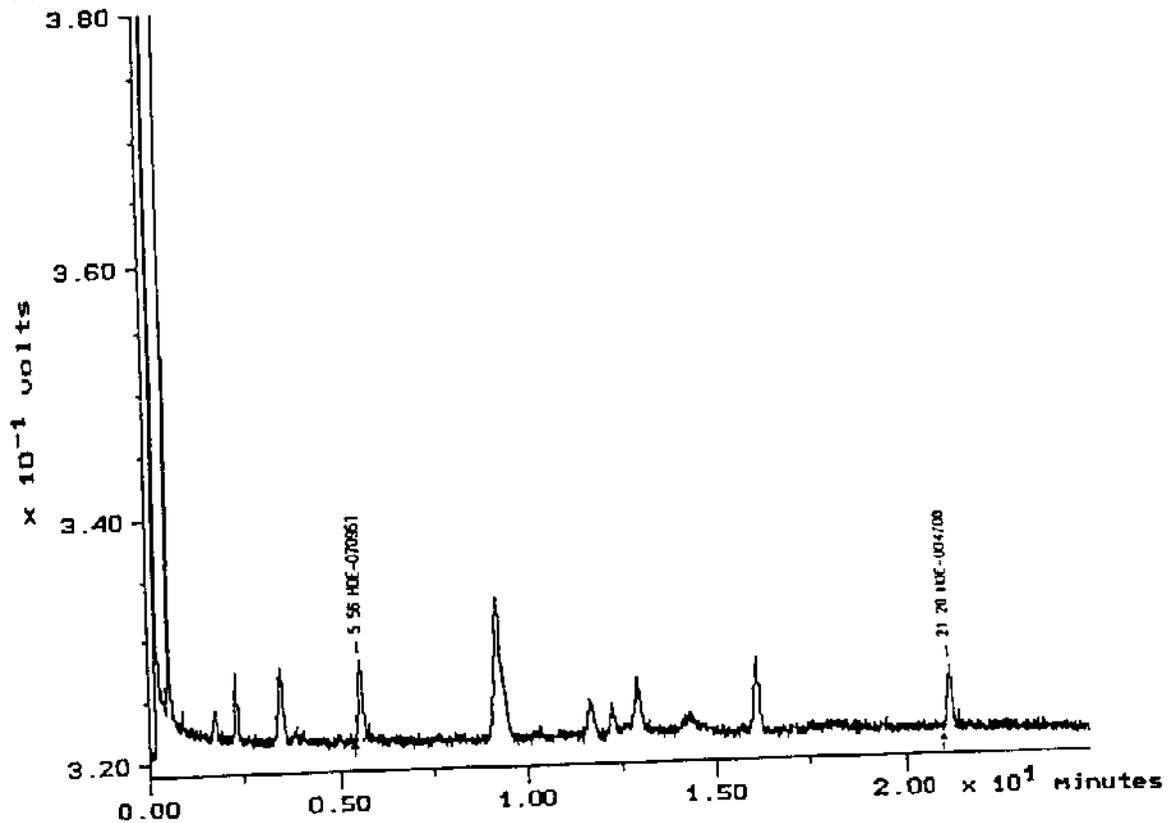
**Chromatogram 30:** Control Canola Seed Fortified at 0.50 ppm with AE F061517, 0.50 ppm with AE F099730 and at 0.50 ppm with AE F039866; Fraction B; Recovery AE F039866 = 92.6%



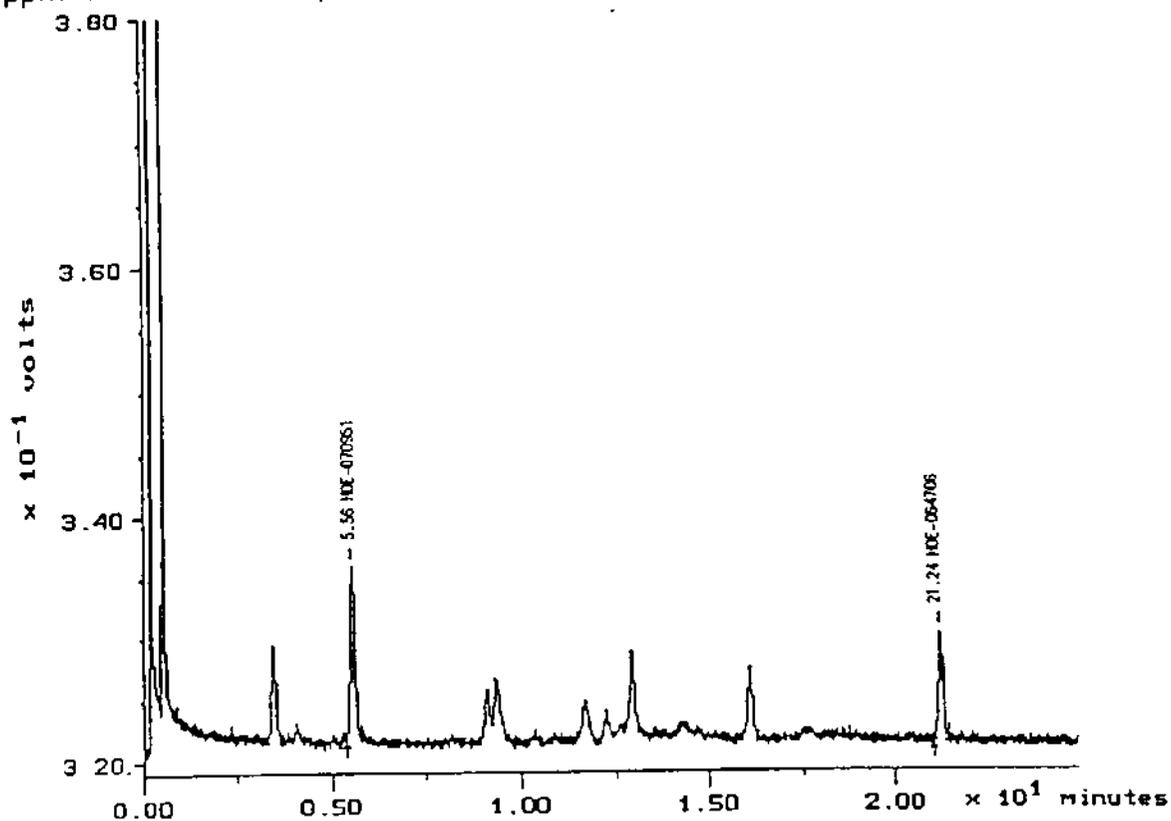
Chromatogram 31: Control Corn Grain; All Residues <0.05 ppm



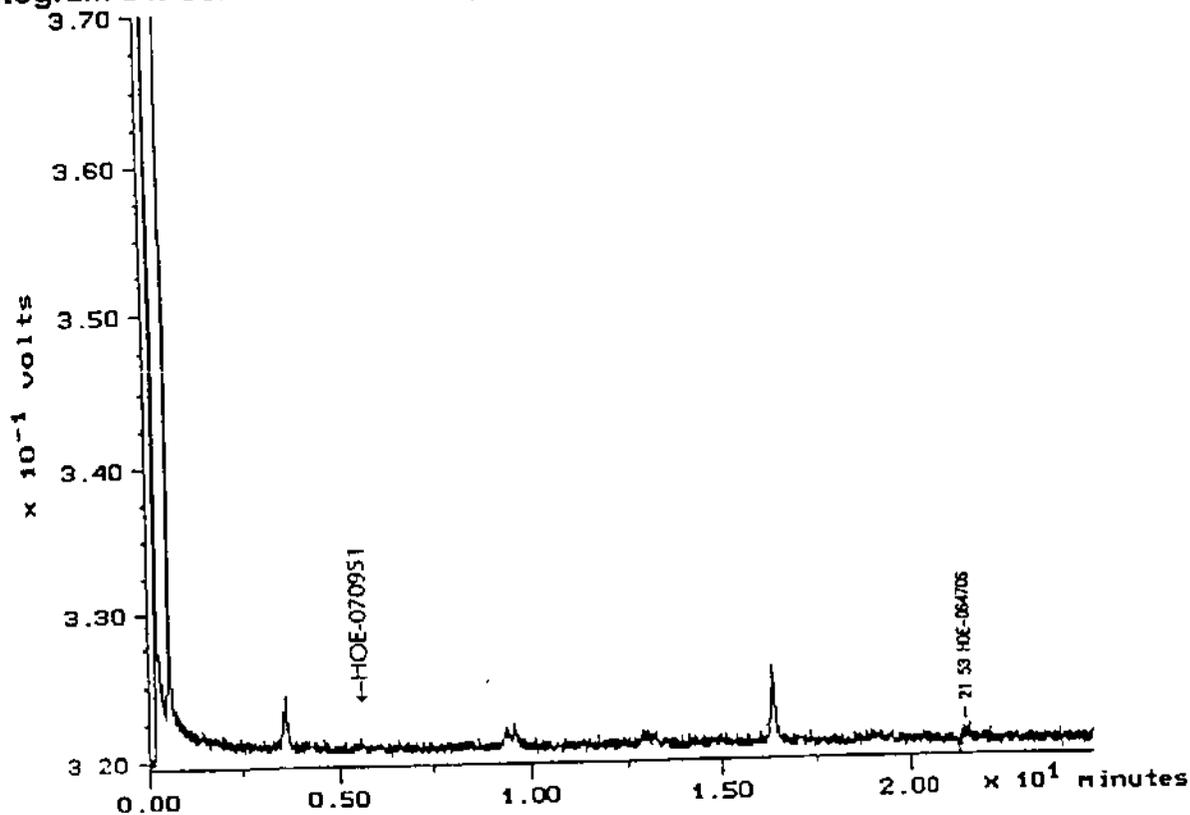
Chromatogram 32: Control Corn Grain Fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F099730; Recoveries: AE F061517 = 91%, AE F099730 = 100%



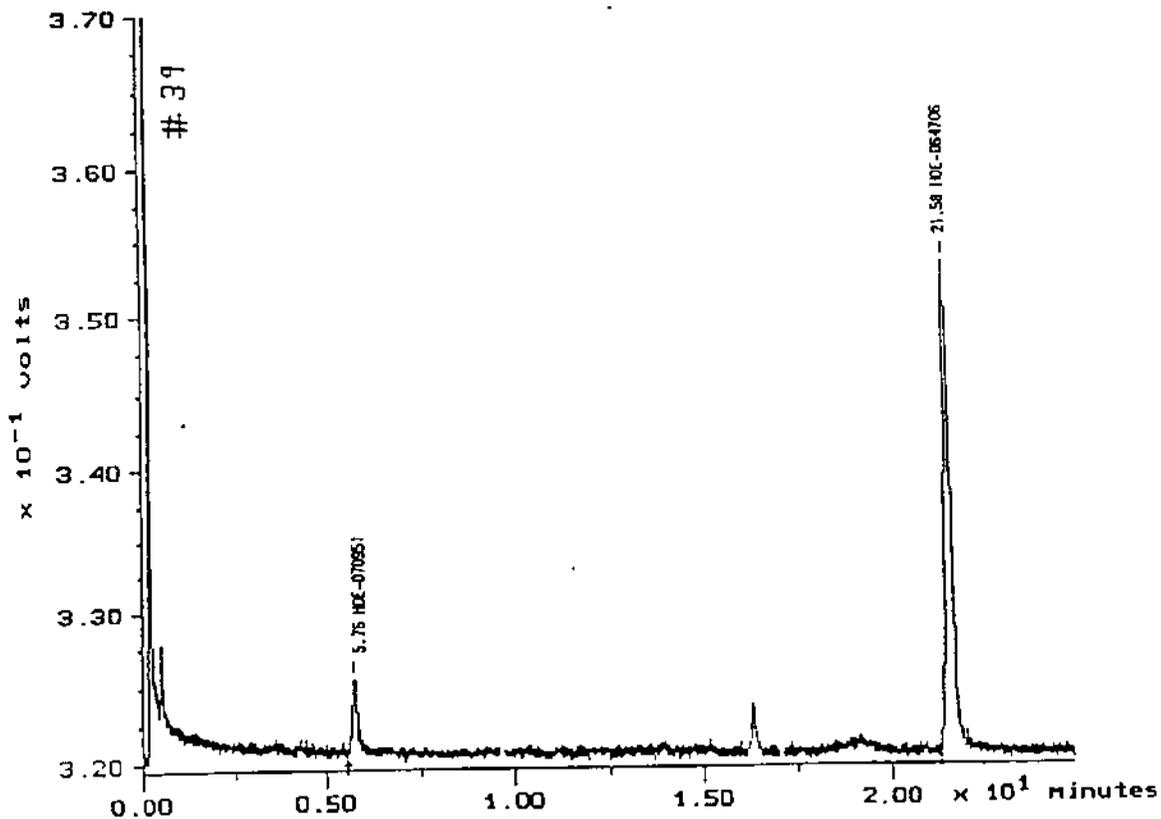
Chromatogram 33: Control Corn Grain Fortified at 0.10 ppm with AE F061517 and at 0.10 ppm with AE F039866; Recoveries: AE F061517 = 90%, AE F039866 = 83%



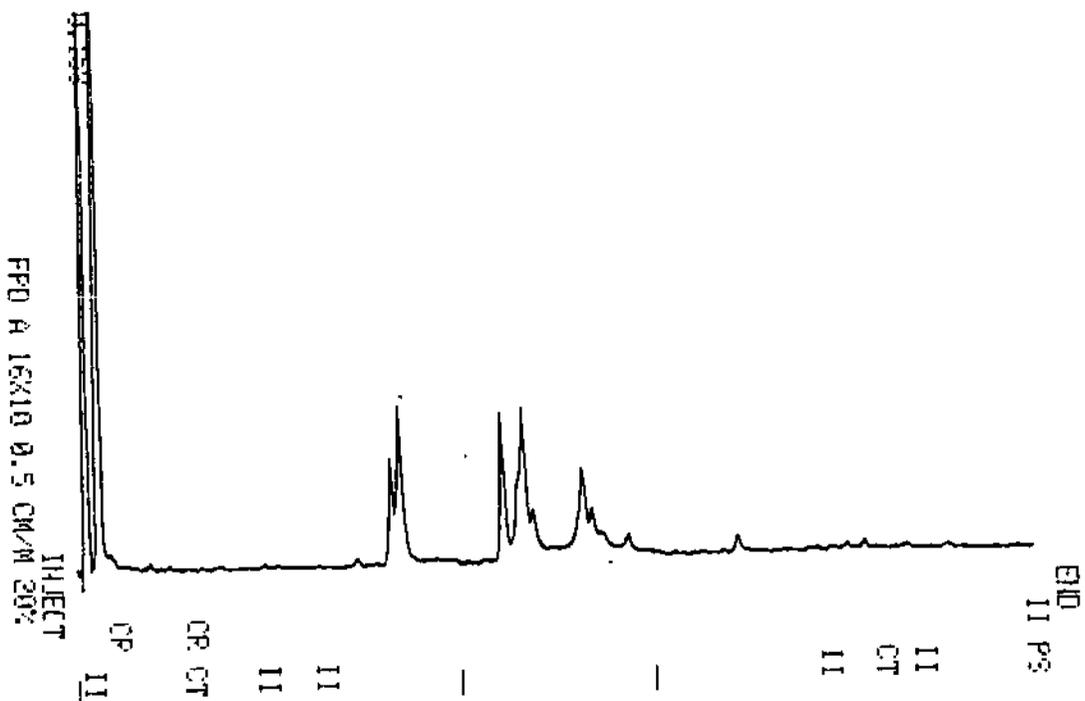
Chromatogram 34: Control Corn Fodder; All Residues <0.05 ppm



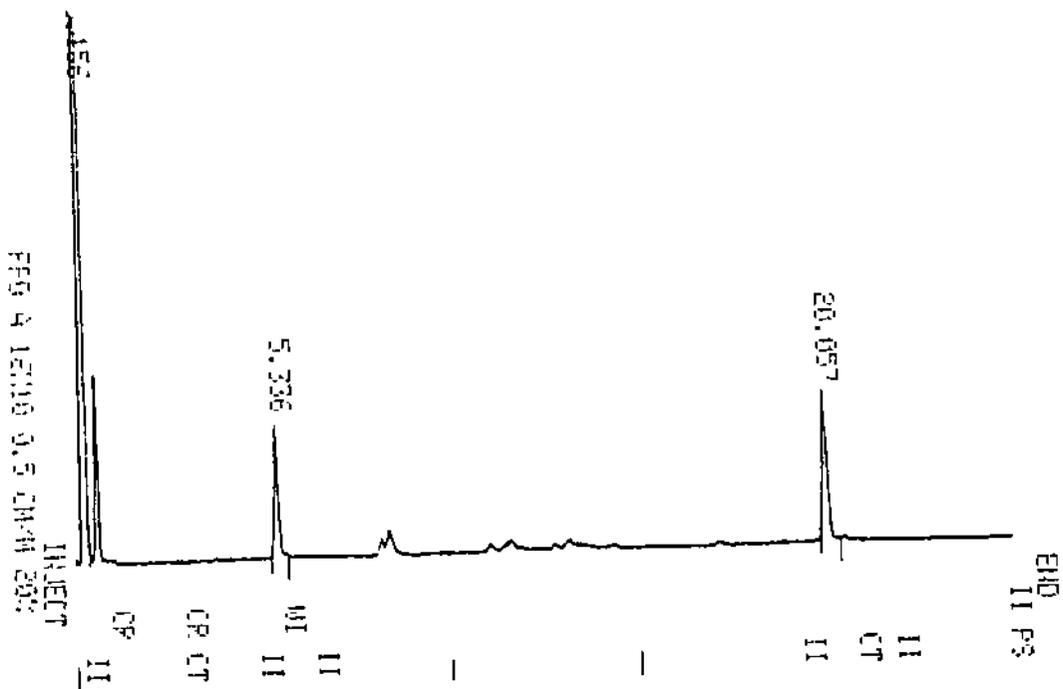
**Chromatogram 35:** Control Corn Fodder Fortified at 0.60 ppm with AE F061517 and at 6.02 ppm with AE F099730; Recoveries: AE F061517 = 98%, AE F099730 = 92%



**Chromatogram 36:** Control Soybean Seed; All Residues <0.05 ppm  
SS-CONTROL



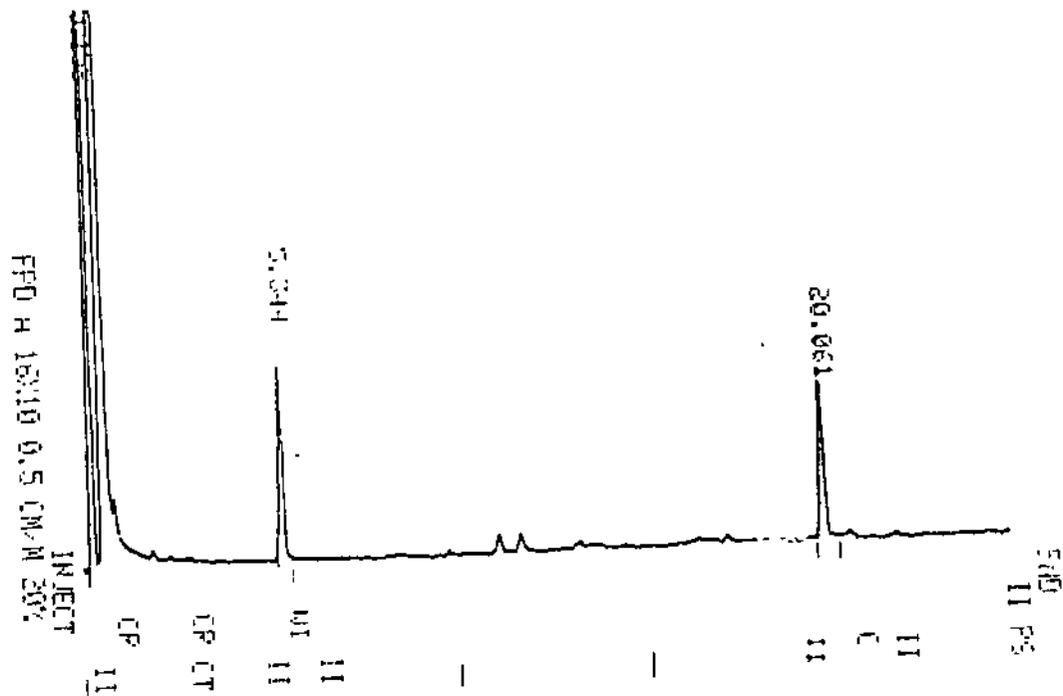
**Chromatogram 37: Control Soybean Seed Fortified at 2.00 ppm with AE F061517 and at 2.00 ppm with AE F039866; Recoveries: AE F061517 = 72.3%, AE F039866 = 87.3%**  
**SS-F5**



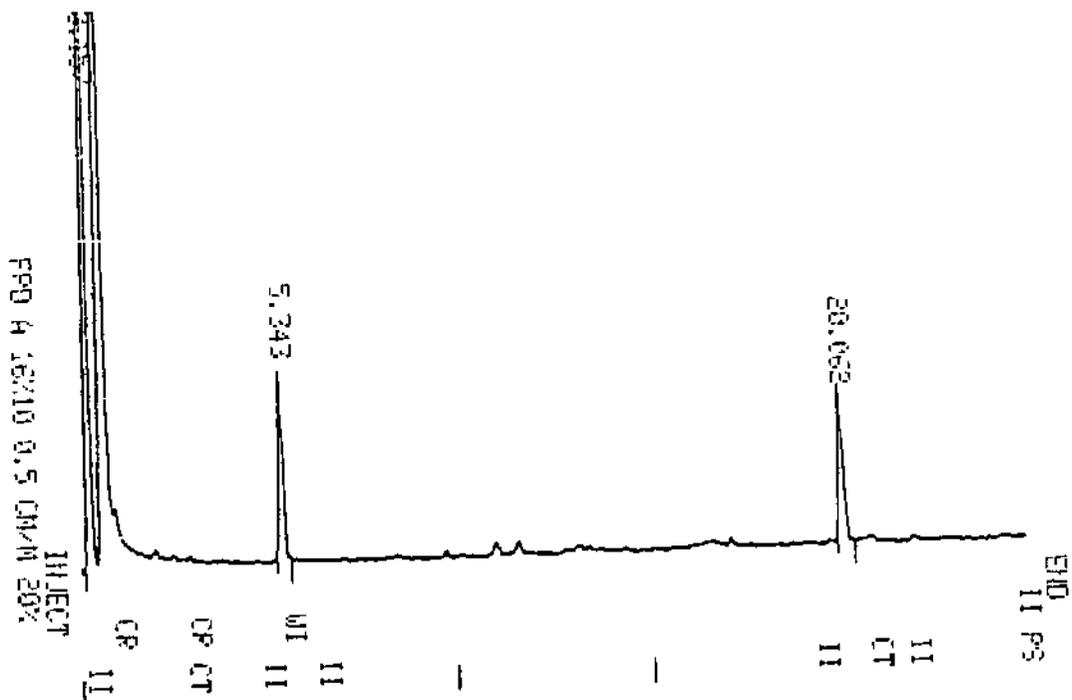
**Chromatogram 38: Control Soybean Hay; All Residues <0.05 ppm**  
**SH-C**



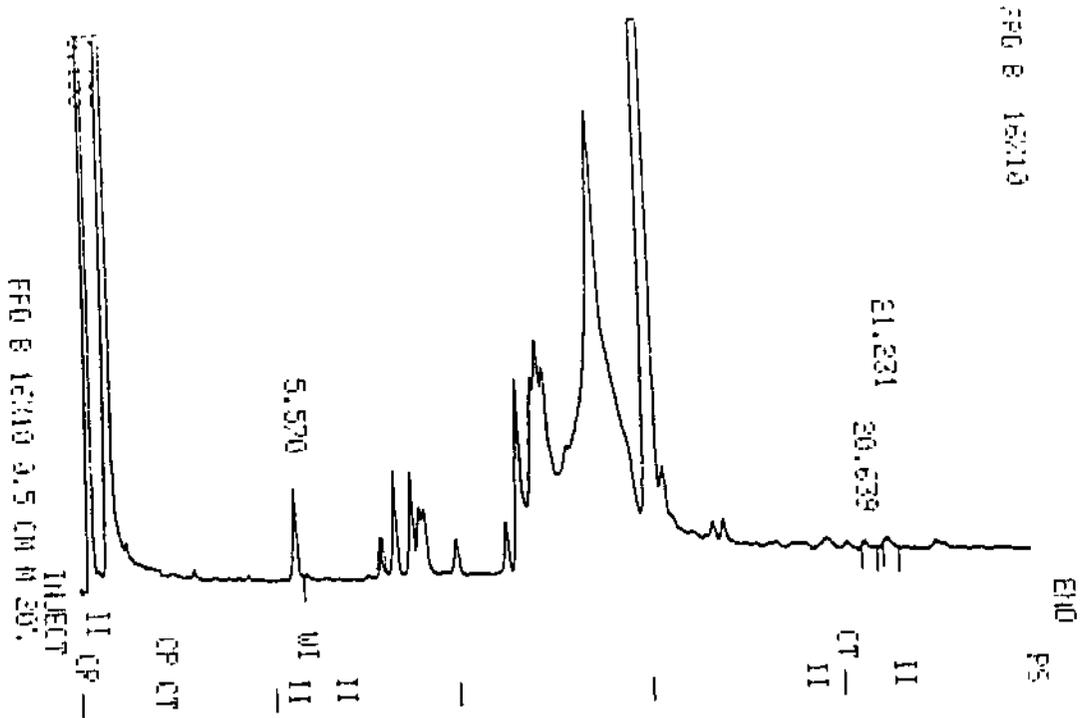
**Chromatogram 39:** Control Soybean Hay Fortified at 0.20 ppm with AE F061517 and at 0.20 ppm with AE F099730; Recoveries: AE F061517 = 105%, AE F099730 = 100%  
**SH-F2**



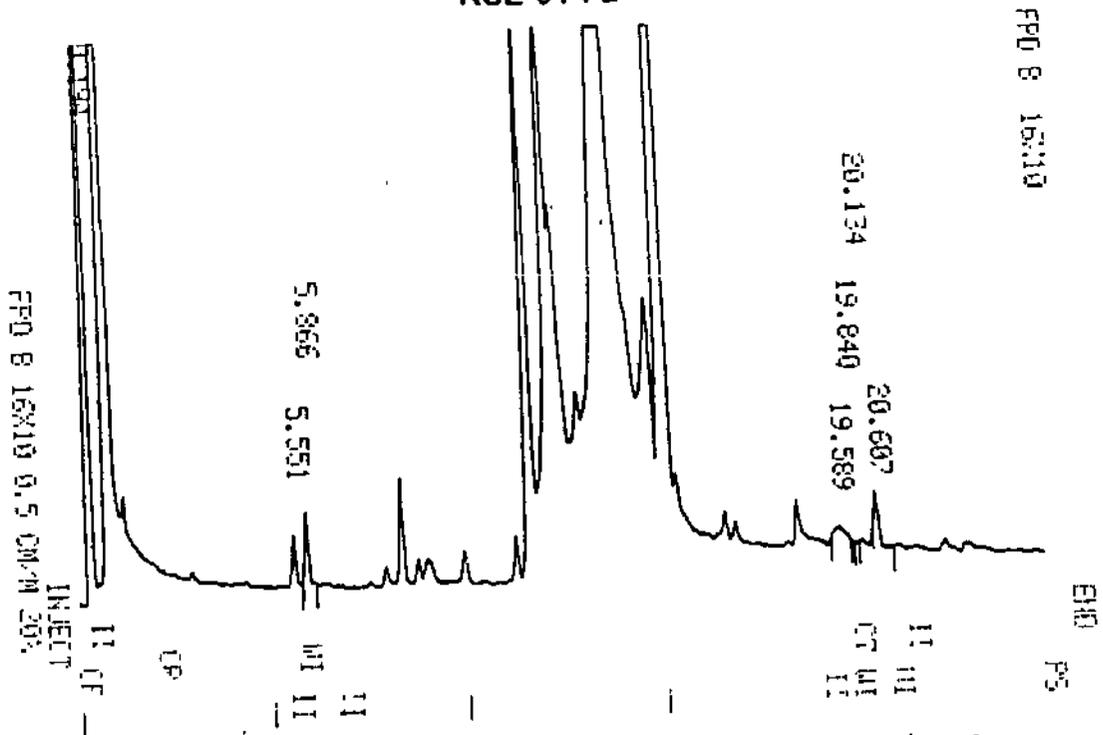
**Chromatogram 40:** Control Soybean Hay Fortified at 0.20 ppm with AE F061517 and at 0.20 ppm with AE F039866; Recoveries: AE F061517 = 103%, AE F039866 = 92.7%  
**SH-F1**



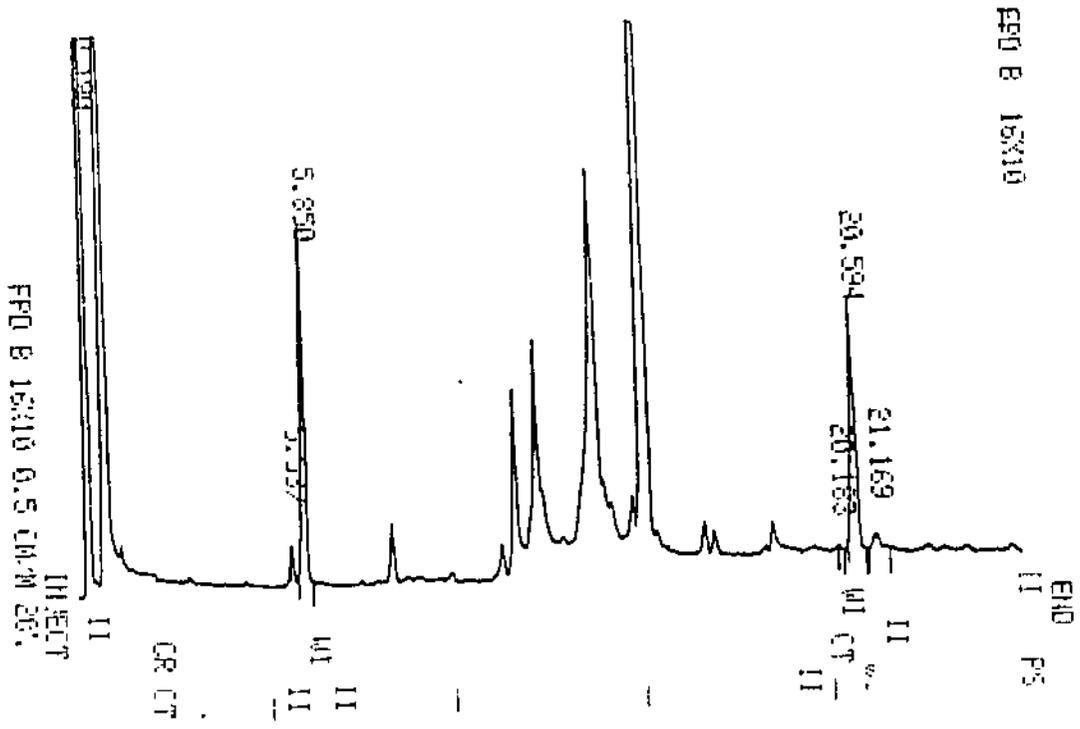
**Chromatogram 41: Control Canola Seed; All Residues <0.05 ppm**  
**RSE-01-C**



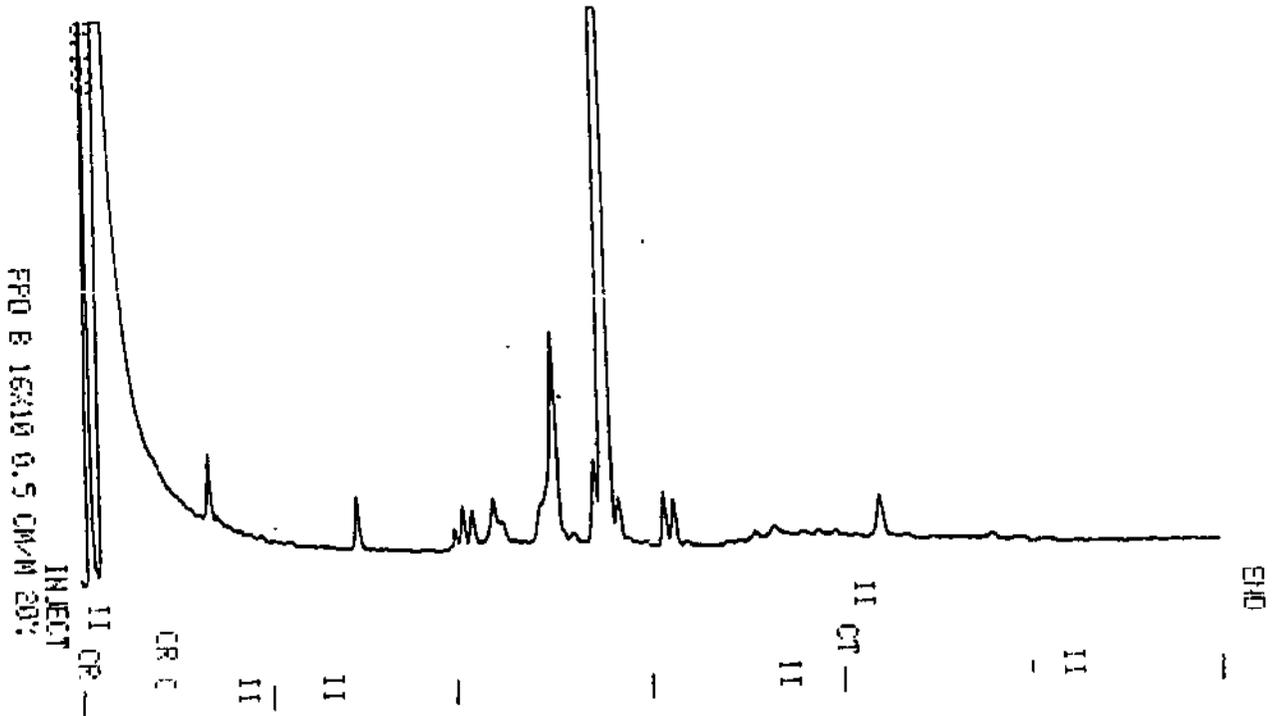
**Chromatogram 42: Control Canola Seed Fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F099730; Recoveries: AE F061517 = 102%, AE F099730 = 111%**  
**RSE-01-F2**



**Chromatogram 43: Control Canola Seed Fortified at 0.50 ppm with AE F061517 and at 0.50 ppm with AE F039866; Recoveries: AE F061517 = 111%, AE F039866 = 96.7%**  
**RSE01F3**

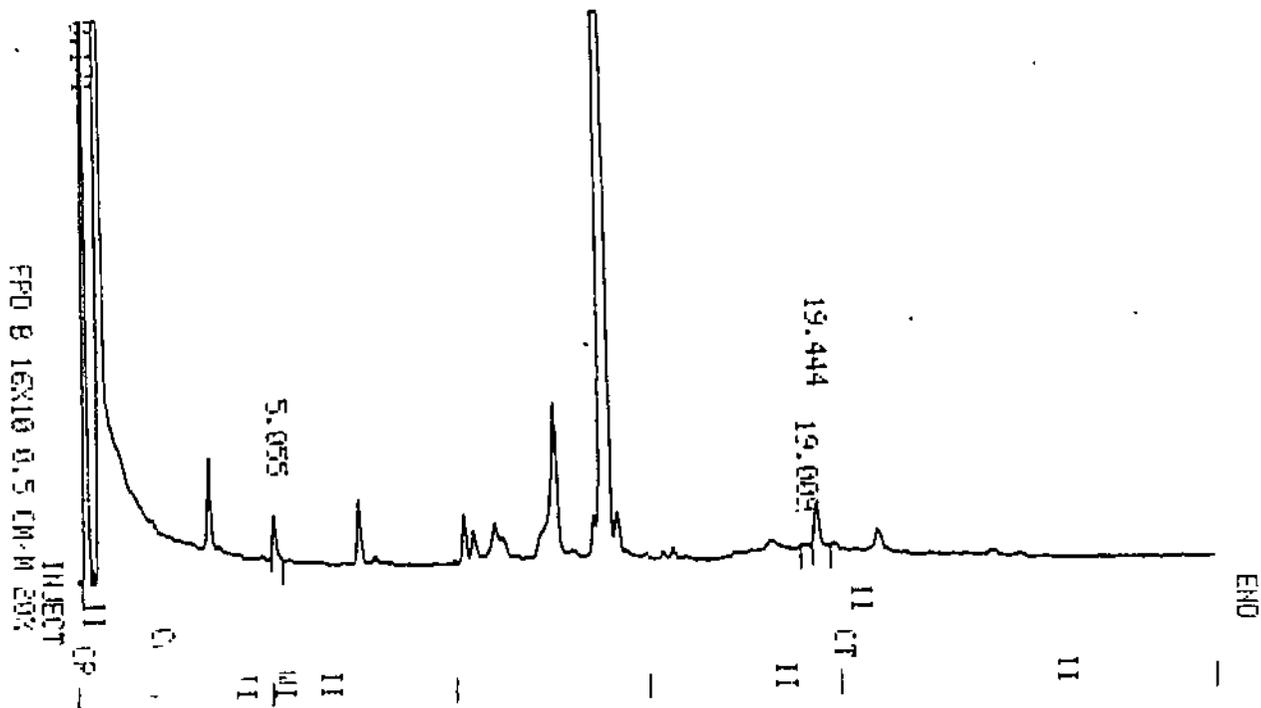


**Chromatogram 44: Control Canola Straw; All Residues <0.05 ppm**  
**RHA-01-C**



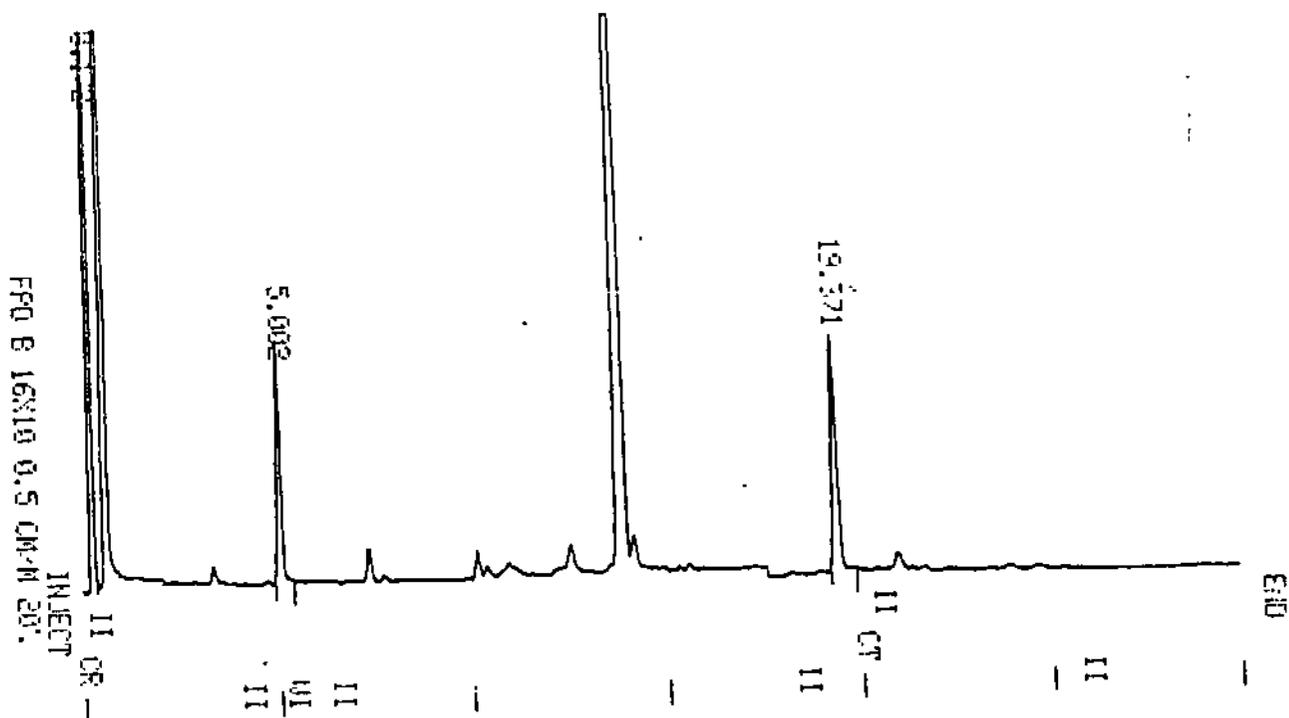
**Chromatogram 45:** Control Canola Straw Fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F039866; Recoveries: AE F061517 = 71.9%, AE F039866 = 99.9%

**RHA-01-F1**

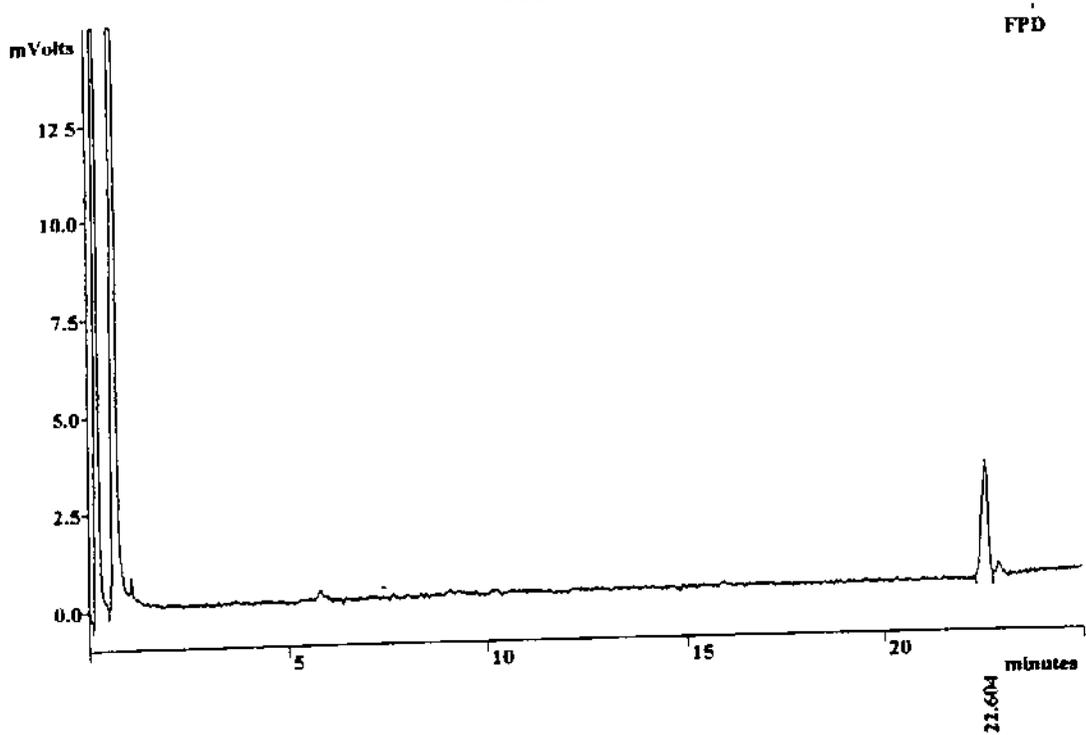


**Chromatogram 46:** Control Canola Straw Fortified at 0.50 ppm with AE F061517 and at 0.50 ppm with AE F099730; Recoveries: AE F061517 = 89.3%, AE F099730 = 104%

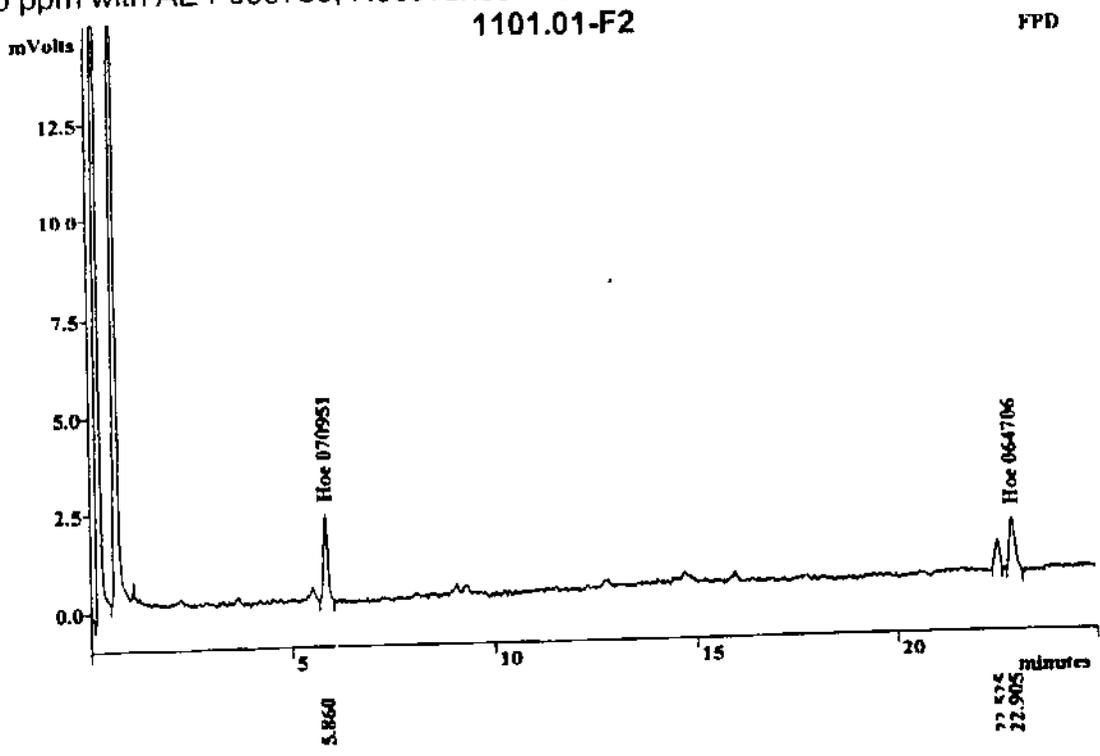
**RHA01F4**



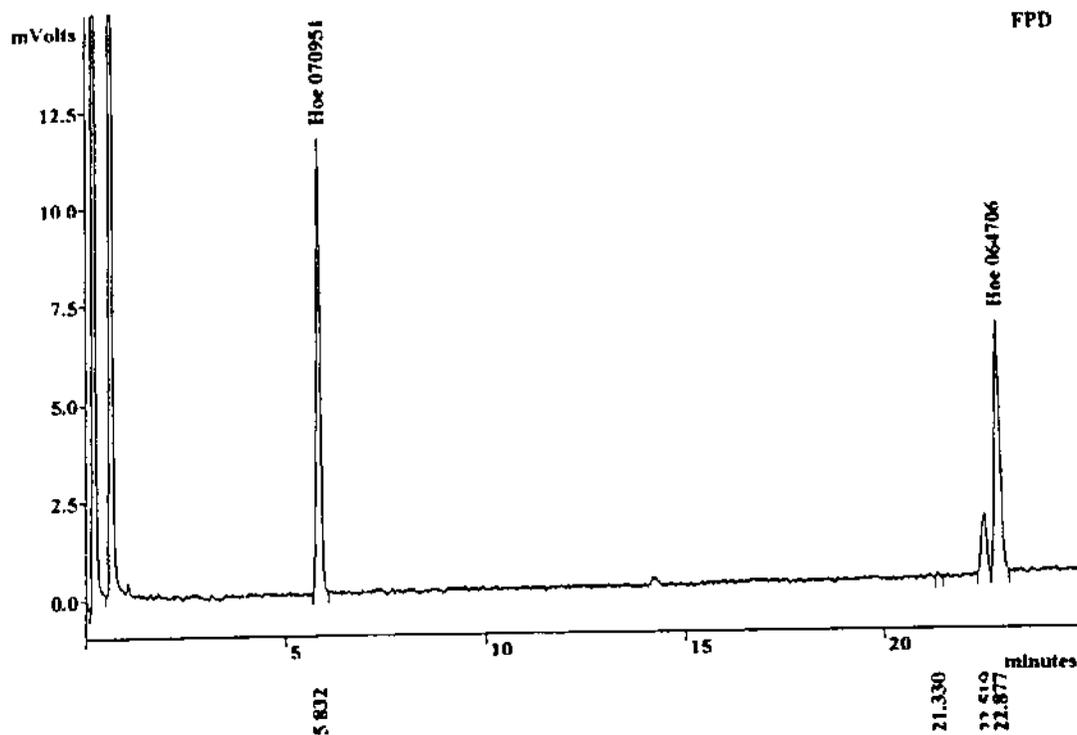
Chromatogram 47: Control Sugar Beet Root; All Residues <0.05 ppm  
0501.01C



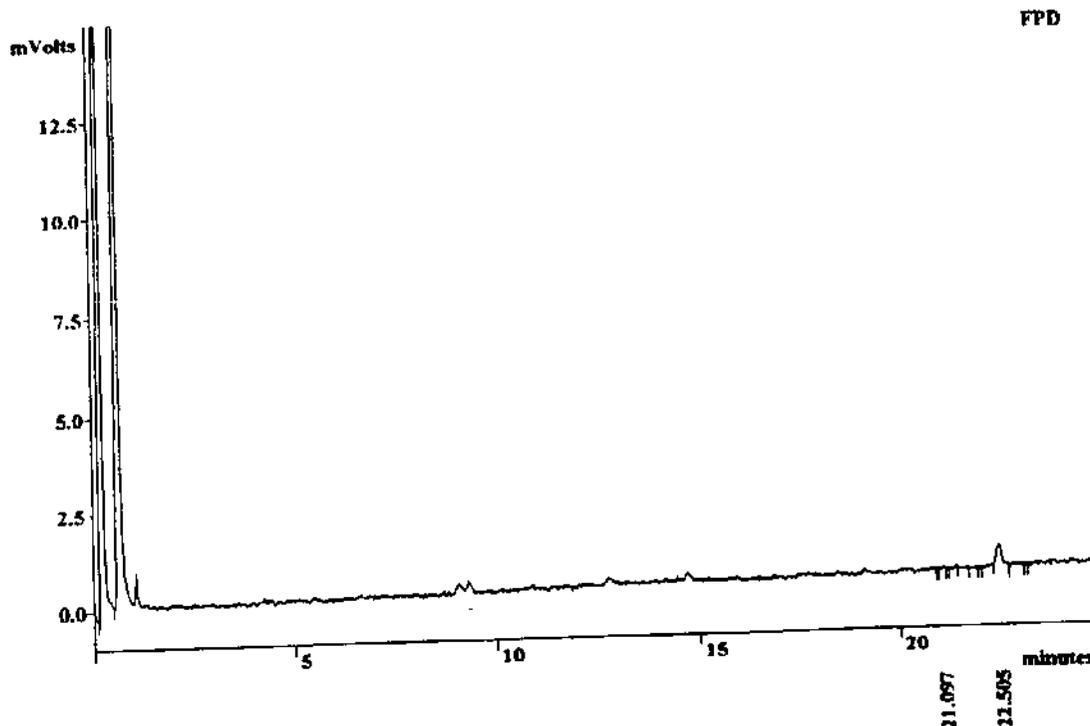
Chromatogram 48: Control Sugar Beet Root Fortified at 0.05 ppm with AE F061517 and  
at 0.05 ppm with AE F099730; Recoveries: AE F061517 = 91.8%, AE F099730 = 89.8%



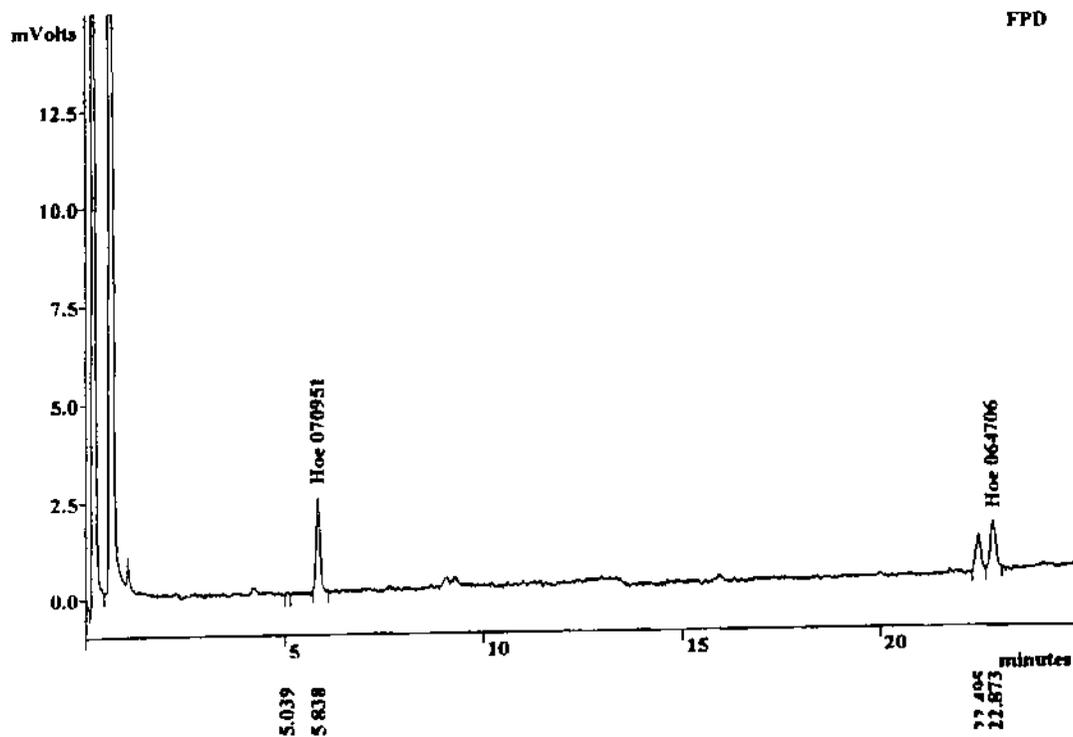
**Chromatogram 49: Control Sugar Beet Root Fortified at 0.50 ppm with AE F061517 and at 0.50 ppm with AE F039866; Recoveries: AE F061517 = 90.0%, AE F039866 = 83.2% 0501.01F3**



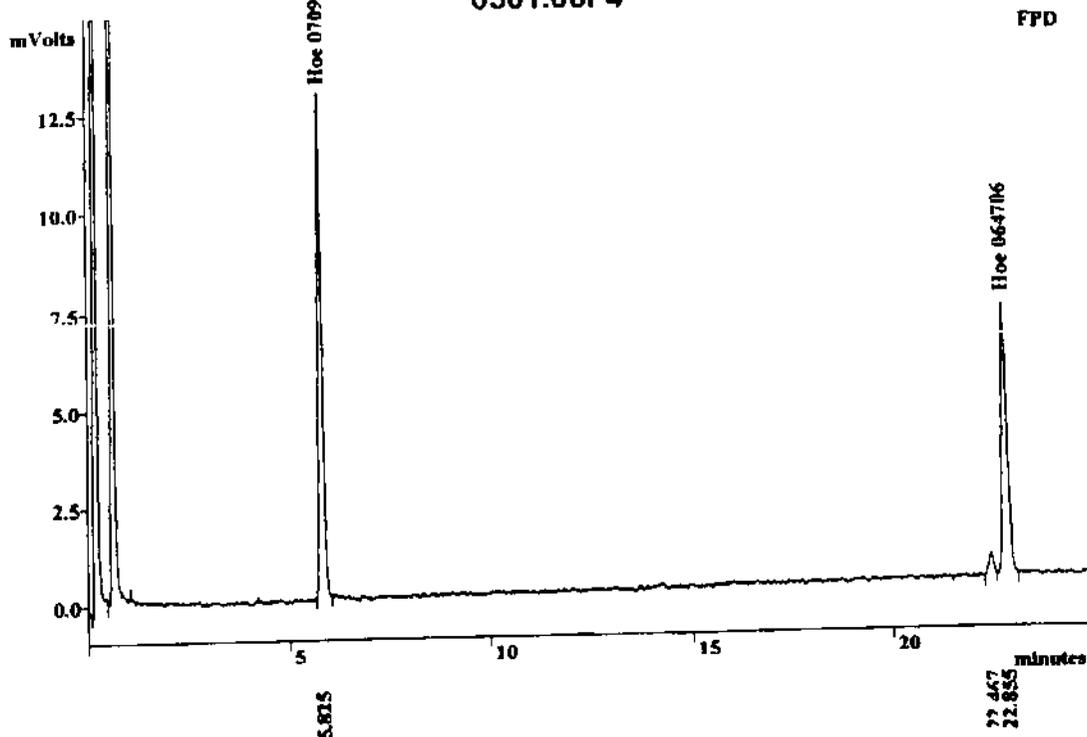
**Chromatogram 50: Control Sugar Beet Top; All Residues <0.05 ppm 1101.06-C**



**Chromatogram 51:** Control Sugar Beet Top Fortified at 0.05 ppm with AE F061517 and at 0.05 ppm with AE F039866; Recoveries: AE F061517 = 90.4%, AE F039866 = 69.9%  
**0501.06-F1**



**Chromatogram 52:** Control Sugar Beet Top Fortified at 0.50 ppm with AE F061517 and at 0.50 ppm with AE F099730; Recoveries: AE F061517 = 104%, AE F099730 = 92.6%  
**0501.06F4**

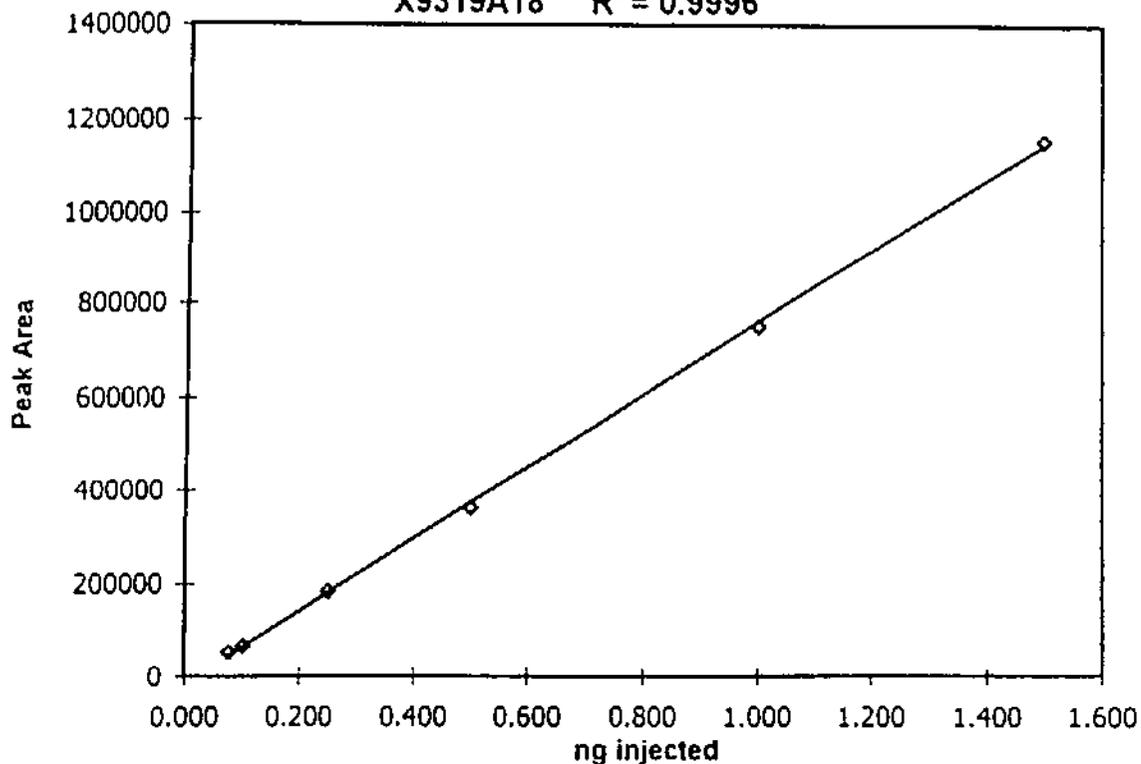


## APPENDIX II

### Typical Calibration Curves

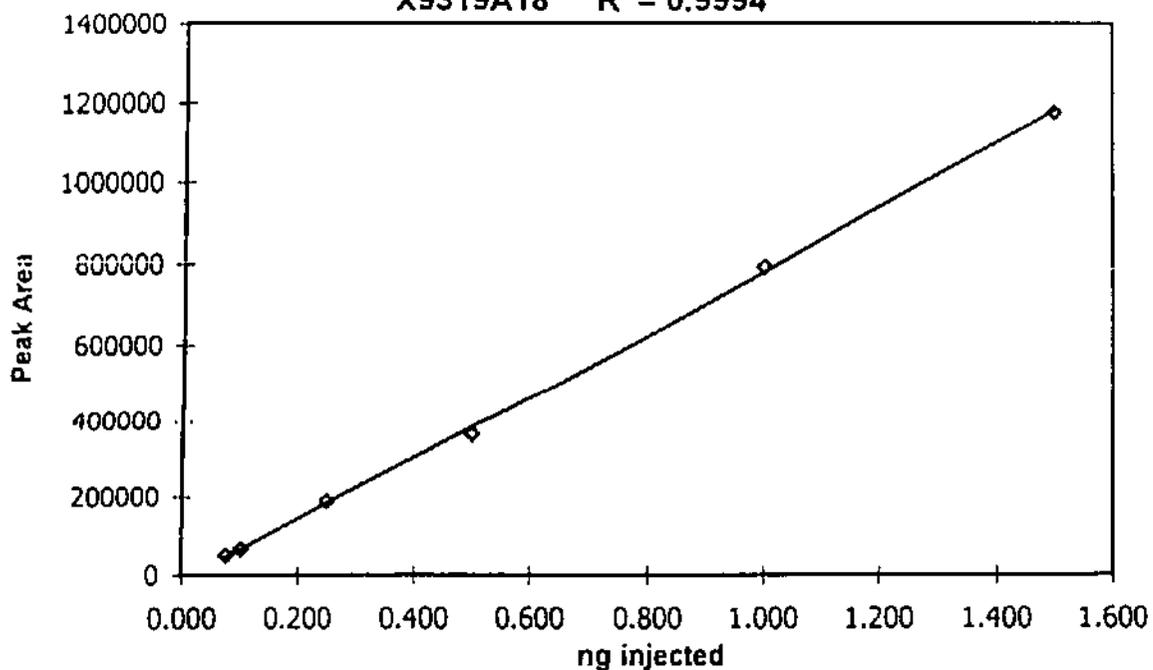
Calibration Curve for AE F070951

X9319A18  $R^2 = 0.9996$



Calibration Curve for AE F064706

X9319A18  $R^2 = 0.9994$



## APPENDIX III

### Example Calculation Sheet

# GAS CHROMATOGRAPHIC DATA SHEET

Compound: Hoe 039866, Hoe 085355 and Hoe 061517

Xenos Project No.: XEN93-19A  
Sponsor Study No.: 93-0027

Extracted: December 03, 1993  
Injected: December 06, 1993  
Analyst: Nicole Bertrand

Matrix: Corn Grain

Data Sheet No.: X9319A18

Verified:

GC Run #	Sample	Extract		Aliquot		Injected		Peak Area		Injected/Found (ng)		Found (ppm)		Spike added (ppm)		Recovery (%)	
		g	mL	g	mL	uL	mg	070951	064706	070951	064706	039866	085355	061517	039866	085355	061517
232	Methyl Acetate					5		ND	ND	ND	ND	ND	ND				
233	0.015 ng/ $\mu$ L			5		5	52388	48326	0.075	0.075	0.075	0.075	0.075				
234	0.020 ng/ $\mu$ L			5		5	66441	63915	0.100	0.100	0.100	0.100	0.100				
235	GR01C-A	25.0	200	2.50	5	5	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	85.1
236	GR01F1-A	25.0	200	2.50	5	5	69247	ND	0.106	ND	ND	ND	0.043	0.050	0.050	0.050	86.9
237	GR02F1-A	25.0	200	2.50	5	5	70960	ND	0.109	ND	ND	ND	0.043	0.050	0.050	0.050	
238	0.050 ng/ $\mu$ L			5		5	183003	187484	0.250	0.250	0.250	0.250	0.250				
239	GR01F3-A	25.0	200	2.50	5	5	141924	ND	0.201	ND	ND	ND	0.080	0.100	0.100	0.100	80.3
240	GR01F5-A	25.0	200	2.50	5	5	381618	ND	0.512	ND	ND	ND	0.205	0.250	0.250	0.250	81.9
241	GR01C-B	25.0	200	2.50	5	5	ND	ND	ND	ND	ND	ND	ND				
242	0.100 ng/ $\mu$ L			5		5	360336	362841	0.500	0.500	0.500	0.500	0.500				
243	GR01F1-B	25.0	200	2.50	5	5	ND	77267	ND	0.117	0.117	0.117	0.117	0.050	0.050	0.050	93.7
244	GR02F1-B	25.0	200	2.50	5	5	ND	86740	ND	0.129	0.129	0.129	0.129	0.050	0.050	0.050	103
245	GR01F3-B	25.0	200	2.50	5	5	ND	156677	ND	0.217	0.217	0.217	0.217	0.100	0.100	0.100	86.8
246	0.200 ng/ $\mu$ L			5		5	748171	792230	1.000	1.000	1.000	1.000	1.000				
247	GR01F5-B	25.0	200	2.50	5	5	ND	418484	ND	0.546	0.546	0.546	0.546	0.250	0.250	0.250	87.4
248	0.300 ng/ $\mu$ L			5		5	1153488	1174093	1.500	1.500	1.500	1.500	1.500				

### GC Instrument

Model: Varian 3400, S.N. 10821  
Detector: FPD  
Column: DB-WAX, 15 m x 0.53 mm, 1  $\mu$ m film  
Column No: 3791911

### Temperatures

Column: initial 130°C  
hold 1.5 min  
Program: 130-140°C @ 2.5°C/min  
140-240°C @ 5°C/min  
Inlet: hold 0 min  
Program: 225°C, hold 23 min  
225-245°C @ 50°C/min  
Detector: hold 2 min  
230°C

### Regression Output

Hoe 070951    Hoe 064706  
R Square    0.9996    0.9994  
Intercept    -12818    -15917  
X Variable    771017    795442  
Observations    6    6

### Data System

Model: IBDH Integrator  
Chart Speed: 0.5 cm/min  
Attenuation: 8  
Range: 10

### Note

ND = no peak detected  
O/R = peak area outside of calibration range.

# APPENDIX IV

## Method Flow Chart

