RHÔNE-POULENC AG COMPANY RTP, NC

Environmental Chemistry: Method Development

Title:	Bromoxynil: Analytical Method for the Determination of 3,5-Dibromo-4-hydroxybenzoic acid in Cotton Seed and Cotton Gin Trash

Analyte: 3,5-dibromo-4-hydroxybenzoic acid

Substrates: Cotton seed and cotton gin trash, as defined in Pesticide Assessment Guidelines, Subdivision O, September 1995.

Date Issued: REVISED MARCH 26, 1995 (1996?)

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James H. Pittman, Jr. Date

Approval: Russell J. Outcalt 25 March 96
Russell J. Outcalt, Group Leader Date

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Summary Flowchart of Analytical Method

EXTRACTION
20 gram sample.
Heat on high 15 minutes stirring in 200 mL 1 M KOH.
Remove from heat as soon as possible.

↓

CLEAN-UP
Centrifuge and take aliquot.
Reduce volume and acidify.
Place on 100 mL Chem Elut column.
Wait 45 minutes and elute.
Evaporate to dryness.
Add acetonitrile and extract with hexane.

↓

DERIVATIZATION
Add 4 mL solvent and sonicate.
Add 2 mL TMS-diazomethane and sonicate.
Heat to 50 degrees for 1 hour.
Blow sample dry at less than 35 degrees.

↓

COLUMN CHROMATOGRAPHY
Add 0.50 mL methanol and sonicate.
Add 4.5 mL hexane and mix.
Add sample to 5 gram silica column.
Rinse with 10 mL hexane.
Rinse with 15 mL 95% HX/ETOAC.
Elute with 20 mL 90% HX/ETOAC.

↓

GAS CHROMATOGRAPHY
Blow sample dry.
Add 1 mL methanol and sonicate.
Inject on GC-MSD.

Bromoxynil: Analytical Method for the Determination of 3,5-Dibromo-4-hydroxybenzoic Acid in Cotton Seed and Cotton Gin Trash

I. Introduction

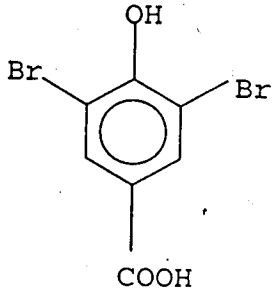
A. Scope

An analytical method is described for the analysis of 3,5-dibromo-4-hydroxybenzoic acid in cotton seed and cotton gin trash, as defined in the Pesticide Assessment Guidelines, Subdivision O.

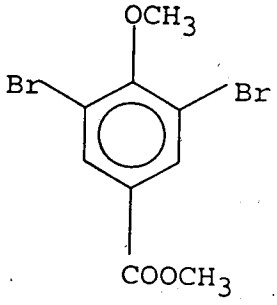
B. Principle

Residues are extracted with 1.0 N KOH that is 50:50(v/v) methanol:water. The extract is made acidic and partitioned with hexane:ethyl acetate(50:50) on a Chem Elut column. Oil is removed with a hexane and acetonitrile partition. The extract is then derivatized with a trimethylsilyl-diazomethane solution. Column chromatography using silica gel is utilized for clean up of all extracts. Quantification of methyl-3,5-dibromo-4-methoxybenzoate is accomplished by gas chromatography using a mass selective detector.

C. Structures



3,5-Dibromo-4-hydroxybenzoic acid



Methyl-3,5-dibromo-methoxybenzoate

II. Materials

Reagents and Solvents were used as received from supplier, unless otherwise noted.
Equivalent reagents and equipment may be substituted where appropriate.

A. Reagents

1. Silica Gel, 40-63 µm particle size, 200-400 mesh, 60 angstrom pore size, Aldrich, Cat. No. 28,859-4. Prepare by drying at ~130 °C for ~24 hours, then deactivate 5% by adding 5 ml water to 95 g of cooled silica and shaking for ~1 hour.

2. Potassium Hydroxide pellets, Reagent grade, ACS, VWR Scientific, Cat. No. VW5040-5.

3. Hydrochloric Acid, Reagent grade, ACS, VWR Scientific, Cat. No. VW3110-3.

4. Sodium Sulfate, anhydrous, granular, J.T. Baker, Cat. No. 3375-05

5. N-Ethyldiisopropylamine, 97%, Fluka, Cat. No. 03440

6. (Trimethylsilyl)diazomethane, 2.0M solution in hexanes, Aldrich, Cat. No. 36,283-2

B. Solvents

1. Acetonitrile, EM OMNISOLV, VWR Scientific Cat. No EM-AX0155-6

2. Methanol, EM OMNISOLV, VWR Scientific Cat. No EM-MX0484-1

3. Water, Purified by Millipore Milli-Q water purification system.

4. Hexane, EM HPLC, VWR Scientific Cat. No EM-HX0290-1

5. Ethyl Acetate, EM Anhydrous, VWR Scientific Cat. No EM-EX0240-5

C. Solutions

1. 1.0 N Potassium Hydroxide Solution, mix 66.01 g of potassium hydroxide (85% assay) in 500 ml of purified water in a 1 liter volumetric flask. Mix and fill to line with methanol

2. 3.0 M Hydrochloric Acid Solution, mix 500 ml of concentrated hydrochloric acid with 1500 ml of purified water

3. 50:50 Hexane:Ethyl Acetate Solution, mix 1000 ml of hexane with 1000 ml of ethyl acetate

4. 9:1 Acetonitrile:Methanol Solution that is 5% in N-Ethyldiisopropylamine, mix 90 ml of acetonitrile with 10 ml of methanol. Mix 95 ml of this solution with 5 ml of N-ethyldiisopropylamine

5. 95:5 Hexane:Ethyl Acetate Solution, mix 950 ml of hexane with 50 ml of ethyl acetate

6. 90:10 Hexane:Ethyl Acetate Solution, mix 900 ml of hexane with 100 ml of ethyl acetate

D. Equipment

1. Aluminum Crimp-Top Seal, 11 mm TFE/RUB Septum, Sun Brokers, Inc., Cat. No. 200 100

2. Analytical Balance

3. Autosampler Vials, 1 ml, clear, Wheaton, Cat. No. 223682

4. Chromatography Columns, 15 mm i.d., 250 ml Reservoir, Kontes

5. Disposable Pasteur Pipettes

6. Polypropylene Copolymer Centrifuge Bottle, 250 ml, Nalgene, Cat. No. 3141-0250

7. Glasswool

8. Graduated Cylinders, calibrated to contain, appropriate sizes

9. Boiling Flasks, 500 ml with 24/40 joint

10. Centrifuge, capable of holding 250 ml bottles and spinning at 4000 RPM

11. Erlenmeyer Flasks, 125 and 250 ml

12. Condensers, Allihn with 24/40 joint

13. Separatory Funnels, 250 ml

14. Chem Elut columns, 100 ml, Varian, Cat. No. 1219-8010

15. Sonicator, Model 5200, Branson, Cat. No. B5210DTH

16. Volumetric Flasks, 100 ml, class A

17. Volumetric Pipettes, appropriate sizes, class A

18. Hewlett-Packard 5890 Series II GC equipped with Mass Selective Detector (refer to Section V of this document for details)

19. Capillary Column, DB-XLB, 30 m X 0.25 mm i.d., 0.25 µm film thickness, J & W Scientific, Cat. No. 122-1232 (a DB-5ms same diminsions may be used if DB-XLB is not available)

20. Heating Stir Plates, Corning Low Profile, Cat No. 33920-219

21. Magnetic Stir Bars, 2 X 3/8 inches Teflon coated

22. Optional: Repeat-a-pet, appropriate sizes, Markson

E. Analytical Standards

Analytical Standards available from Rhône-Poulenc Ag Company

1. 3,5-Dibromo-4-Hydroxybenzoic Acid

2. MB 14057 Methyl-3,5-Dibromo-4-Methoxybenzoate

III. Standard Solution Preparation

A. General

1. The concentrations of standard solutions should be adjusted to account for the purity of the neat solid standards.

2. After preparation, standards should be transferred from the volumetric flasks into screw-capped amber bottles to prevent possible photodegradation.

3. Store standard solutions in the refrigerator at or below 10 °C when not in use.

B. Fortification and Calibration Standard Solutions

The following is provided as an example of how standard solutions may be prepared.
Other concentrations may be used as appropriate.

1. Weigh 0.1000 g (±0.1 mg) of each analytical standard individually into 100 ml volumetric flasks. Dissolve in methanol and mix well. Dilute to final volume with methanol. Concentration of each standard is 1000 µg / ml.

2. Withdraw a 10.0 ml aliquot from each of the 1000 µg / ml individual standards and add to a 100 ml volumetric flask. Dilute to volume with methanol. The concentration of this standard is 100 µg / ml for each analyte in the methanol solution.

3. Withdraw a 10.0 ml aliquot from the 100 µg / ml mixed standard and add to a 100 ml volumetric flask. Dilute to volume with methanol. The concentration of this standard is 10 µg / ml for each analyte in methanol.

4. By further dilution of the 10 µg / ml mixed standard with methanol, prepare a series of standards to serve as fortification standards or calibration standards.

IV. Methods of Analysis

Stopping points in the method are left to the discretion of the analyst performing the method.
The author has placed the diamond symbol (♦) throughout the method to indicate places that are convenient to stop. Overnight storage in a refrigerator is suitable, however, a freezer is recommended for longer time periods. Samples should be allowed to warm / thaw prior to use.

The tilde symbol (~) indicates 'approximately'.

A. Sample Preparation

Use samples as received from processor.

B. Extraction

1. Weigh ~20 g of cotton seed or cotton gin trash into a 500 ml boiling flask.

2. Fortify as necessary and then let stand at least 10 minutes.

3. Add 200 ml of 1.0 N potassium hydroxide solution in 50:50 water:methanol and heat on setting 10, stirring for 15 minutes. Remove from heat and as soon as possible transfer an aliquot of about 100 ml to a Polypropylene Copolymer Centrifuge Nalgene® bottle.

C. Centrifuge

1. Centrifuge at ~4000 RPM for 15 minutes.

2. Decant 75 ml (7.5 g equivalent) into a 100 ml graduated cylinder, then pour into a 250 ml erlenmeyer flask.

3. Rinse graduated cylinder with 10 ml methanol twice.

D. Chem Elut

1. Reduce volume of extract to less than 50 ml by heating to 50 degrees in a constant temperature bath and blowing a strong stream of nitrogen into the flask.

2. Add 30 ml of 3 M hydrochloric acid and mix.

3. Put solution on 100 ml Chem Elut column.

4. Rinse flask with ~10 ml water twice and put on Chem Elut column.

5. After 45 minutes, elute extract with 250 ml of hexane:ethyl acetate (50:50) into a 500 ml boiling flask.♦

E. Derivatization

1. Evaporate sample to dryness. (Some oil will remain but all solvent must be removed.)

2. Add 50 ml acetonitrile to sample and sonicate to dissolve.

3. Transfer sample to a 250 ml separatory funnel and extract three times with 75 ml hexane. (Rinse the 500 ml boiling flask with the hexane. Discard the hexane after each extraction. The acetonitrile is the bottom layer. Let out the acetonitrile into the original 500 ml boiling flask each time.)♦

4. Evaporate the sample to dryness at less than 35 degrees C. This can be done with a rotovap or a stream of nitrogen.

5. Add 4.0 ml 9:1 acetonitrile:methanol that is 5% in N-ethyldiisopropylamine. Sonicate to dissolve residues.

6. Add 2.0 ml 2.0 M trimethylsilyl-diazomethane in hexane. Sonicate about 10 seconds.

7. Heat to 50 degrees C for 1 hour.

8. With a stream of nitrogen, blow sample dry at less than 35 degrees C.

F. Column Chromatography

1. Plug a 15 mm i.d. glass chromatography column with glass wool. Add approximately 5.0 g of 40-63 µm particle size silica gel (previously dried at ~130 °C for ~24 h, then deactivated 5% by adding 5ml water to 95 g of cooled silica and shaking for ~1 h.) followed by ~3/4 inch of anhydrous sodium sulfate. Tap the column after the addition of each compound to settle the packing. Wash the column with approximately 25 ml of hexane, eluting until the level of the solvent just reaches the top of the sodium sulfate. Discard the solvent wash.

2. Add 0.5 ml methanol to sample and sonicate to dissolve residues.

3. Add 4.5 ml hexane to sample and sonicate.

4. Add sample to chromatography column and allow level to drain to the top of the sodium sulfate.

5. Rinse derivatization flask with 10 ml hexane, sonicate, add to chromatography column, and allow level to drain to the top of the sodium sulfate.

6. Rinse derivatization flask with 15 ml 95:5 hexane:ethyl acetate, sonicate, add to chromatography column, and allow level to drain to the top of the sodium sulfate.

7. Elute sample into a 125 ml erlenmeyer flask with 20 ml 90:10 hexane:ethyl acetate.♦

8. With a stream of nitrogen, blow sample dry at less than 35 degrees C. Dilute the residue to desired volume with methanol and sonicate about ten seconds. Load samples into vials for analysis and seal. ♦ Analyze by gas chromatography.

G. General Method Notes / Potential Problems

1. After the reflux step, transfer only enough sample to take the 75 ml aliquot after the centrifuge step.

2. The longer the extract remains in the reflux solution (1.0 KOH) , the more compound will begin to decompose. The author puts a tygon tube with a fast stream of nitrogen into the 250 ml flask to blow down to less than 50 ml.

3. If sample aliquot volume is not reduced to less than 50 ml the Chem Elut columns can be overloaded. If Chem Elut columns are overloaded, water will seep through. This will cause evaporation to be difficult but it can be accomplished if methanol is added to azeotrope.

4. If the Chem Elut columns are very slow to elute try loosening the tip with pliers.

5. Before addition to chromatography columns some separation may occur. Try to minimize the time between sonication and addition to the column.

6. If chromatography columns stop flowing due to air pockets in silica or sodium sulfate, tap on sides until air bubbles are released.

V. Gas Chromatography

A. Instrumentation

1. Gas Chromatograph:	Hewlett-Packard 5890 Series II GC, 7673 Autosampler, 18594B Sampler Controller, Split/Splitless Injector, or an equivalent system
2. Detector:	Mass Selective Detector, Hewlett-Packard Model 5972 or equivalent
3. Data Acquisition:	Hewlett-Packard ChemStation
4. Column:	J & W Scientific DB-XLB 30 m X 0.25 mm i.d., 0.25 µm film thickness

B. Gas Chromatograph Conditions

1. Carrier Gas:	Helium, Head Pressure set at 15 PSI
2. Inlet Liner:	4-mm i.d. nominal volume 900 µl, borosilicate glass with single taper on GC end (HP part #5181-3316)
3. Injector Temperature:	250 °C
4. Detector Temperature:	300 °C
5. Oven Temperatures:	Initial: 100 °C, hold 2 minute Ramp 30 °C / min to 180 °C, hold 1 minute Ramp 5 °C / min to 230 °C, hold 0 minutes Ramp 40 °C / min to 300 °C, hold 5 minutes
6. Injection Volume:	1.0 µL

7. Splitless injection with split vent off for 60 seconds.

C. Mass Selective Detector Parameters

1. Tune File	Maximum Sensitivity Autotune

2. Solvent Delay	8.0 minutes	
3. EM voltage	600 over tune	
4. Acquisition Mode	SIM	
5. SIM Parameters	GROUP 1	Methyl-3,5-dibromo-4-methoxybenzoate
	Low Resolution -	Yes
	Dwell time per ion	100 msec
	Starting time	10.0 min
	Quantitation ion	292.85
	Qualifier ions	290.85
		323.85

D. General Chromatography Notes / Potential Problems

1. Several standards should be injected prior to actual analysis using a new column or after the GC has set idle for any considerable length of time to condition and/or to remove any contaminants.

2. A gold plated seal is used at the interface of the glass liner and column. Hewlett-Packard Part No. 18740-20885.

3. The GC parameters are guidelines and can be optimized for the instrument and column actually used. Record the actual GC conditions used for data acquisition and include in report.

4. The detector ion amu values are nominal. Exact values should be determined from a scan run after each tune.

VI. Quantification of Residues

A. Calibration Curves

1. Linear regression should be used to generate a calibration curve for the analyte. At least five different standard concentrations

should be run with each set of samples. Standards should be interspersed with samples to compensate for any minor change in instrument response. Extracts should be diluted such that the peak areas obtained are within the area range between the lowest and highest standards injected.

2. Linear regression coefficients should be calculated from 'peak area' (or 'peak height') versus 'nanogram / ml injected'. Data from the analytical standards should be fit to the linear equation, $y = a + bx$.

where: y = peak area or height
a = calibration line intercept
b = calibration line slope
x = conc of analyte in inj soln

B. Quantification of Residues

1. Methyl-3,5-dibromo-4-methoxybenzoate should be quantified by comparison to the standard curves obtained from a linear regression analysis of the data.

2. Equations

2.1 Concentration of analyte in sample in ppb (parts per billion).

$$z = (y - a) / b \times c / d$$

where: y = peak area (or height), response of analyte of interest
a = intercept of calibration line from linear regression (area or height)
b = slope of calibration curve from linear regression (response per ng/ml)
c = final volume of sample (ml)
d = sample weight (g)
z = conc of analyte in sample (ppb)

2.2 Corrected concentration of analyte in sample in ppb.

$$Z' = z / C$$

where: Z' = corrected concentration
z = concentration found from curve
C = conversion factor

for conversion of

3,5-dibromo-4-hydroxybenzoic acid to
methyl-3,5-dibromo-4-methoxybenzoate C = 1.095

2.3 Percent recovery

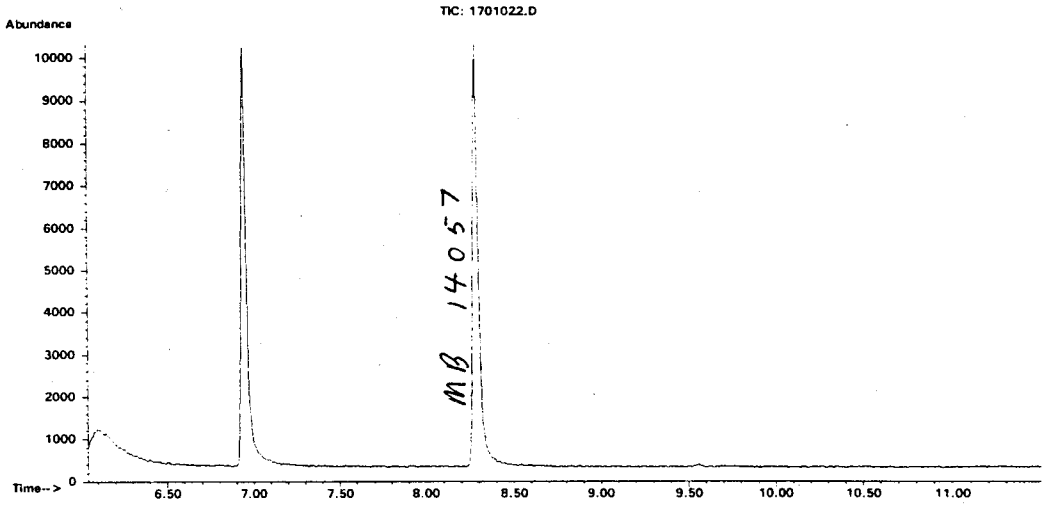
$$\% \text{ recovery} = \frac{(\text{ppb found in fort sample} - \text{ppb found in UTC})}{\text{actual fortification level in ppb}} \times 100\%$$

3. Residues shall be reported as 3,5-dibromo-4-hydroxybenzoic acid equivalents.

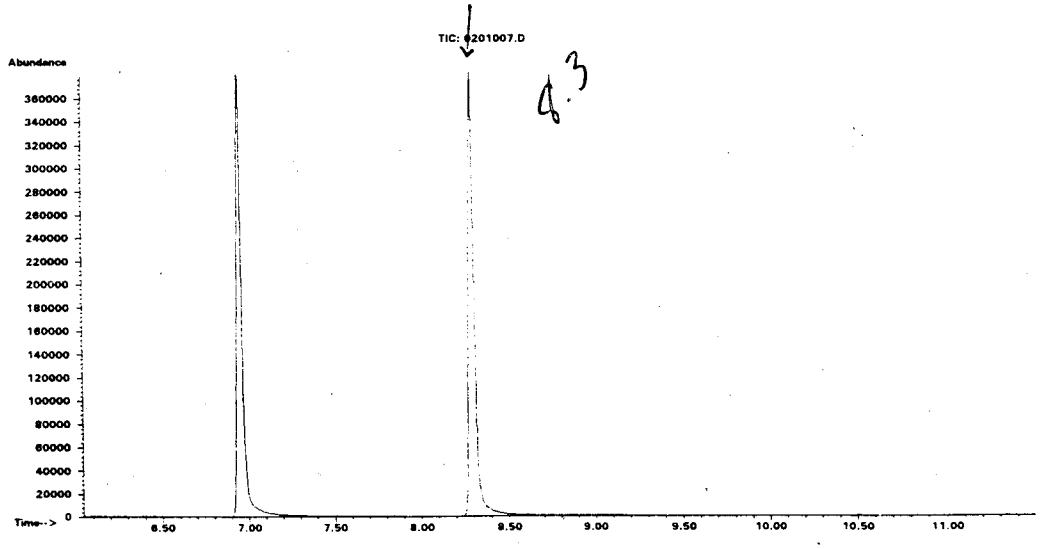
VII. Example Chromatograms

A. Standards

1. Standard, MB 14057, 0.025 ug/ml in methanol

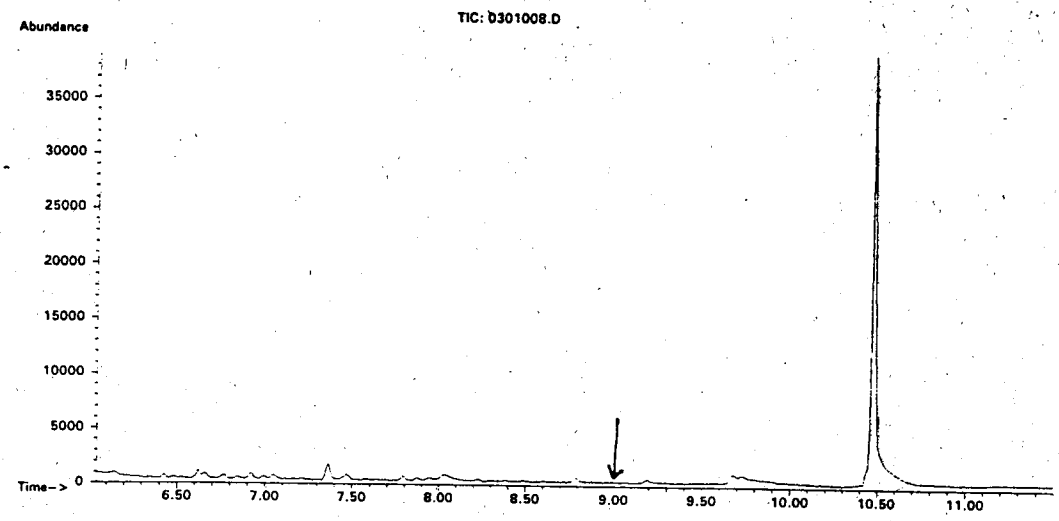


2. Standard, MB 14057, 1.0 ug/ml in methanol

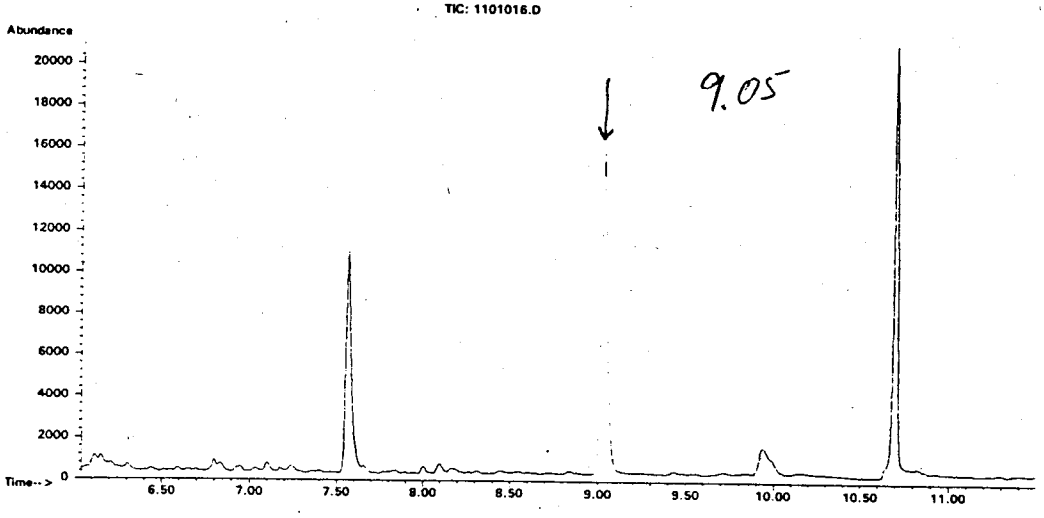


B. Cotton Samples

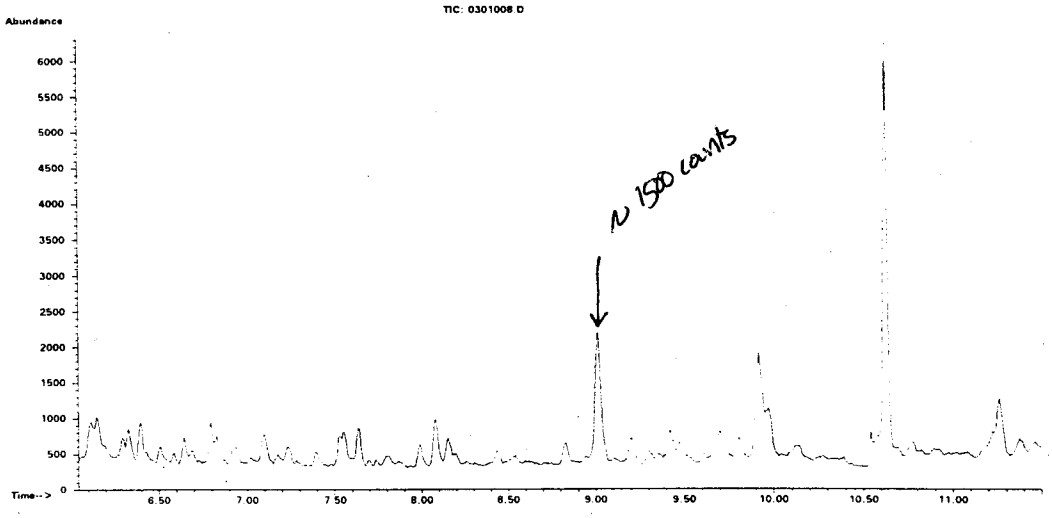
1. Cotton Seed, Untreated Control



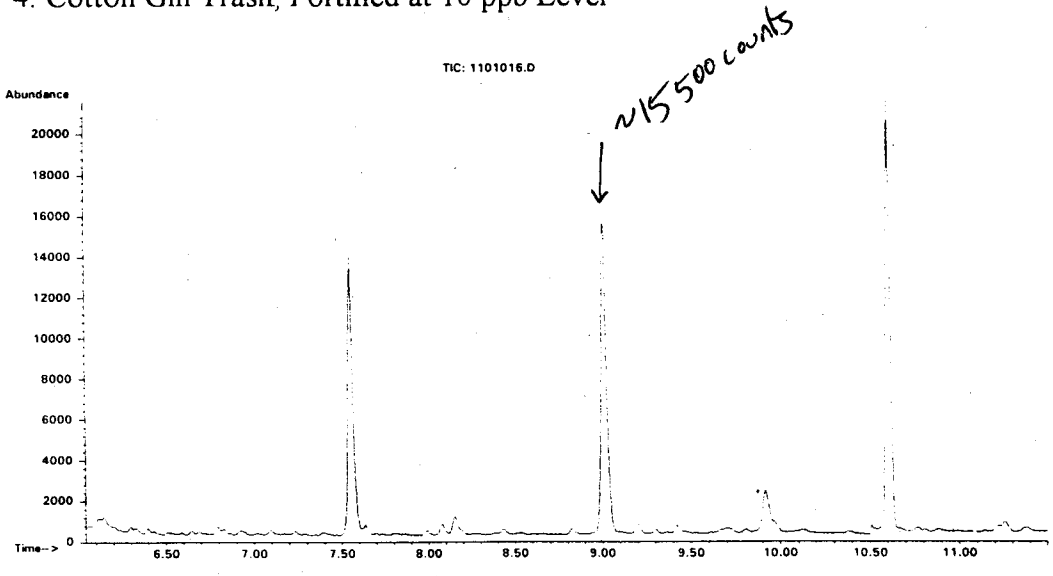
2. Cotton Seed, Fortified at 10 ppb Level



3. Cotton Gin Trash, Untreated Control



4. Cotton Gin Trash, Fortified at 10 ppb Level



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3. Withdraw a 10.0 ml aliquot from the 100 µg / ml mixed standard and add to a 100 ml volumetric flask. Dilute to volume with methanol. The concentration of this standard is 10 µg / ml for each analyte in methanol.

4. By further dilution of the 10 µg / ml mixed standard with methanol, prepare a series of standards to serve as fortification standards or calibration standards.

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1. Weigh ~20 g of cotton seed or cotton gin trash into a 500 ml boiling flask.

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3. Add 200 ml of 1.0 N potassium hydroxide solution in 50:50 water:methanol and heat on setting 10, stirring for 15 minutes. Remove from heat and as soon as possible transfer an aliquot of about 100 ml to a Polypropylene Copolymer Centrifuge Nalgene® bottle.

C. Centrifuge

1. Centrifuge at ~4000 RPM for 15 minutes.

2. Decant 75 ml (7.5 g equivalent) into a 100 ml graduated cylinder, then pour into a 250 ml erlenmeyer flask.

3. Rinse graduated cylinder with 10 ml methanol twice.

D. Chem Elut

1. Reduce volume of extract to less than 50 ml by heating to 50 degrees in a constant temperature bath and blowing a strong stream of nitrogen into the flask.

2. Add 30 ml of 3 M hydrochloric acid and mix.

3. Put solution on 100 ml Chem Elut column.

4. Rinse flask with ~10 ml water twice and put on Chem Elut column.

5. After 45 minutes, elute extract with 250 ml of hexane:ethyl acetate (50:50) into a 500 ml boiling flask.♦

E. Derivatization

1. Evaporate sample to dryness. (Some oil will remain but all solvent must be removed.)

2. Add 50 ml acetonitrile to sample and sonicate to dissolve.

3. Transfer sample to a 250 ml separatory funnel and extract three times with 75 ml hexane. (Rinse the 500 ml boiling flask with the hexane. Discard the hexane after each extraction. The acetonitrile is the bottom layer. Let out the acetonitrile into the original 500 ml boiling flask each time.)♦

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F. Column Chromatography

1. Plug a 15 mm i.d. glass chromatography column with glass wool. Add approximately 5.0 g of 40-63 µm particle size silica gel (previously dried at ~130 °C for ~24 h, then deactivated 5% by adding 5ml water to 95 g of cooled silica and shaking for ~1 h.) followed by ~3/4 inch of anhydrous sodium sulfate. Tap the column after the addition of each compound to settle the packing. Wash the column with approximately 25 ml of hexane, eluting until the level of the solvent just reaches the top of the sodium sulfate. Discard the solvent wash.

2. Add 0.5 ml methanol to sample and sonicate to dissolve residues.

3. Add 4.5 ml hexane to sample and sonicate.

4. Add sample to chromatography column and allow level to drain to the top of the sodium sulfate.

5. Rinse derivatization flask with 10 ml hexane, sonicate, add to chromatography column, and allow level to drain to the top of the sodium sulfate.

6. Rinse derivatization flask with 15 ml 95:5 hexane:ethyl acetate, sonicate, add to chromatography column, and allow level to drain to the top of the sodium sulfate.

7. Elute sample into a 125 ml erlenmeyer flask with 20 ml 90:10 hexane:ethyl acetate.♦

8. With a stream of nitrogen, blow sample dry at less than 35 degrees C. Dilute the residue to desired volume with methanol and sonicate about ten seconds. Load samples into vials for analysis and seal. ♦ Analyze by gas chromatography.

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6. If chromatography columns stop flowing due to air pockets in silica or sodium sulfate, tap on sides until air bubbles are released.

V. Gas Chromatography

A. Instrumentation