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Study Title

**PROPOSED ANALYTICAL ENFORCEMENT METHOD FOR THE
DETERMINATION OF PYRITHIOPAC SODIUM IN COTTON GIN TRASH
USING ASE EXTRACTION AND LC/MS/MS ANALYSIS**

Data Requirement

EEC Directive 91/414/EEC: Annex II 4.2.1

U.S. EPA Pesticide Assessment Guidelines

Subdivision O, 171-4 (c) and (d)

Residue Chemistry 860.1340 Series Final Guidelines

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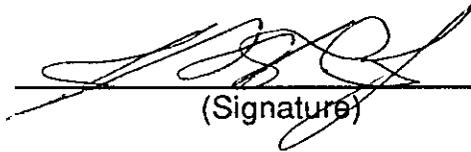
No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA Section 10(d)(1)(A), (B), or (C).

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

The EPA Good Laboratory Practice (GLP) requirements specified in 40 CFR Part 160 and the Council Directive 91/414/EEC of the Council of the European Communities Concerning the Inclusion of Active Substances in Annex I do not require analytical methods to be developed under Good Laboratory Practices (GLP). However, the methods development presented in this report was done under GLP except that no protocol was written, no conduct audit was performed, and no QA audit of the study records was done. Analytical procedures, documentation and archiving of the validation data followed Standard Operating Procedures.

Sponsor

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CERTIFICATION

**PROPOSED ANALYTICAL ENFORCEMENT METHOD FOR THE
DETERMINATION OF PYRITHIOBAC SODIUM IN COTTON GIN TRASH
USING ASE EXTRACTION AND LC/MS/MS ANALYSIS**

We, the undersigned, declare that the work described in this report was performed under our supervision and that this report provides an accurate record of the procedures and results.

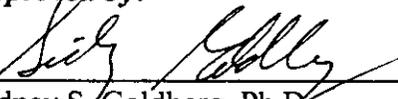
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August 11, 1997

Date Study Completed:

August 28, 1997

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ANALYTICAL METHOD FOR THE DETERMINATION OF PYRITHIOBAC SODIUM IN COTTON GIN TRASH USING ASE EXTRACTION AND LC/MS/MS ANALYSIS

Frederick Q. Bramble, Scott Frizzell, Stephen W. George, Brock A. Peterson, David D. Ranken, James J. Stry, and Sheldon R. Sumpter

1.0 SUMMARY

Pyrithiobac sodium (pyrithiobac, DPX-PE350, KIH-2031, sodium 2-chloro-6-[(4,6-dimethoxypyrimidin-2-yl)thio]benzoate) is extracted from 5 g of cotton gin trash in 35% acetonitrile/65% 0.01M (NH₄)₂CO₃ at 110°C and 1500 psi using a DIONEX ASE™ 200 Extractor. Pyrithiobac is separated from the resulting extract by liquid-liquid partitioning of an aliquot of the sample extract. Pyrithiobac is further separated from coextracts by liquid chromatography (LC) and detected by MS-SIM or MS/MS-SRM detection. For this report, only the MS/MS-SRM data are presented.

The extraction, clean-up, and LC/MS/MS analysis generated acceptable recoveries at all fortification levels. Recoveries for these samples ranged from 70 to 93%. The overall average recovery (± standard deviation) for cotton gin trash fortified at 0.020, 0.040, and 0.20 ppm was 82% (± 10%) with a relative standard deviation of 12% for six samples analyzed. Recovery data from these samples demonstrate that the pyrithiobac sodium residues are stable during the extraction and subsequent purification and analysis steps. The recoveries are acceptable for an analytical method used to support registration. The method limit of detection (LOD) and limit of quantitation (LOQ) are 0.007 and 0.020 µg/g (ppm), respectively which are adequate for enforcement purposes. Extraction efficiency was demonstrated and radiovalidation of the method was performed using standard ¹⁴C methodology.

This method meets U.S. EPA, Subdivision O, 161-4 (c) and (d), Residue Chemistry 860.1340 Series Final Guidelines, and Pesticide Assessment Guideline and EEC Directive 91/414/EEC: Annex II 4.2.1 criteria.

2.0 INTRODUCTION

Pyrithiobac sodium is the active ingredient in Staple® Herbicide which is used to control broad-leaf weeds in cotton. The structure and physicochemical data for pyrithiobac sodium (pyrithiobac, DPX-PE350, KIH-2031, sodium 2-chloro-6-[(4,6-dimethoxypyrimidin-2-yl)thio]benzoate) are found in Appendix 1.

Cotton gin trash is a product of seed cotton processing and an analytical method for the determination of pyrithiobac sodium is required to support registration of Staple® herbicide. Cotton gin trash is made up of seed cotton, cotton seed, twigs and leaves of cotton plants, and even soil from cotton fields; its composition can vary considerably from site to site. Therefore, a vigorous extraction method is required to accommodate this matrix variability and after clean-up of cotton gin trash extracts, a highly selective method of detection is required. Analytical enforcement methods for

the determination of pyriithiobac sodium residues extracted from cotton seed (DuPont Report No. AMR 2816-93, Revision 2, Reference 2) and soil (DuPont Report No. AMR 2745-93, Revision 1, Reference 3) are not applicable to the extraction, clean-up and analysis of cotton gin trash, leading to the development of this method.

As described in this method, pyriithiobac sodium in cotton gin trash is determined using accelerated solvent extraction (ASE), liquid-liquid partition purification, and LC/MS/MS analysis. To test method ruggedness, cotton gin trash from six sites representative of cotton gin trash from where cotton is typically grown, was fortified, extracted, and analyzed. Extraction efficiency was demonstrated and radiovalidation of the method was performed using standard ^{14}C methodology.

3.0 MATERIALS

3.1 *Equipment*

Equivalent equipment may be substituted unless otherwise indicated. Note any specification in the following descriptions before making substitutions. Substitutions should be made only if equivalency/suitability has been verified with acceptable control and fortification recovery data.

ASE™ 200 Extraction Apparatus - extractor and the following parts: 22-mL stainless steel extraction cells, PN 49561; glass fiber filter, PN 47017; 40-mL collection vials, PN 49465, 60-mL collection vials, PN 49466; septa for collection vial lids, PN 49464; O-rings, PN 049457; PEEK seals, PN 049455 DIONEX (Sunnyvale, California)

LC system: HP1100 (Hewlett-Packard, Wilmington, Delaware)

Mass Spectrometer System: Finnigan MAT LCQ™ bench top-ion trap MS with APCI interface and Navigator Version 1.01 data acquisition software (Finnigan Corporation, San Jose, California)

HPLC Column: 4.6 mm i.d. × 15 cm, Zorbax® Rx-C18 analytical column with 5 μm diameter packing, PN 883967-902 (Mac Mod, Chadds Ford, Pennsylvania)

Powder funnel, polypropylene, PN 30255-022 (VWR Scientific Co., Bridgeport, New Jersey)

Centrifuge tubes - 50-mL Pyrex graduated centrifuge tubes PN 21048-050; micro centrifuge tubes, PN 20170-620 (VWR Scientific)

Filter - Gelman Acrodisk 13 CR, PTFE 0.2 μm filter, PN (VWR Scientific)

Balances - Mettler AE240 analytical and PE3600 top-loading balances (Mettler Instrument Corp., Hightstown, New Jersey)

Evaporator - N-Evap® Model 111 laboratory sample evaporator/nitrogen manifold fitted with Teflon®-coated needles (Organomation Associates, South Berlin, Massachusetts). Unit is attached to a dry, clean nitrogen source.

Centrifuge - IEC Clinical Centrifuge (International Equipment Company) with 45° fixed angle rotor PN 20671-213 and six, 50-mL shields PN 20743-008 (VWR Scientific)

Micro Centrifuge - Fisher Model 235B Micro Centrifuge (Fisher Scientific, Pittsburgh, Pennsylvania)

3.2 *Reagents and Standards*

Equivalent reagents may be substituted for those listed below. To determine if substituted reagent impurities interfere with pyriithiobac, appropriate amounts of the solvents should be injected into the HPLC using the chromatographic conditions specified in this report for pyriithiobac.

Water - Deionized water passed through a Milli-Q® water purification system #ZD60 115 UV (Millipore, Bedford, Massachusetts)

Methanol (MeOH)[†] - EM Omni Solv®, HPLC-grade methanol, #MX0488-1 (EM Science, Gibbstown, New Jersey)

Acetonitrile (ACN) - EM Omni Solv®, HPLC-grade acetonitrile, #AX0142-1 (EM Science)

Ethyl Acetate (EA) - EM Omni Solv®, HPLC-grade acetonitrile, #EX0421-1 (EM Science)

Formic acid - EM Omni Solv®, reagent grade, #FX0440-7 (EM Science)

Ammonium Carbonate [(NH₄)₂CO₃] - Baker Analyzed® Reagent, reagent-grade ammonium carbonate #0642-01 (J. T. Baker, Inc., Phillipsburg, New Jersey)

Acetic Acid - Baker Analyzed® glacial acetic acid, #9524-00 (J. T. Baker, Inc.)

Pyriithiobac Sodium (DPX-PE350, KIH-2031) - Reference substance used for HPLC analysis: analytical standard grade DPX-PE350, Lot #4, 98.7% pure (prepared by Kumiai/Ihara Chemical Co. for DuPont Agricultural Products, Global Technology Division, E. I. du Pont de Nemours and Company)

Radioactive pyriithiobac sodium (DPX-PE350), NEN #2764-099, HOTC #370, 99.0% pure. Specific Activity: 70.210 μCi/mg. Radiolabel location: [pyrimidine-2-¹⁴C] (DuPont Agricultural Products, Global Technology Division, E. I. du Pont de Nemours and Company)

3.3 *Safety and Health*

No unusually hazardous materials are used in this method. All appropriate material safety data sheets should be read and followed, and proper personal protective equipment should be used.

Caution: Extraction cells used for this method are extremely hot (110°C) after the extraction. Allow the cells to cool for at least 15 minutes before handling to avoid burns.

4.0 METHODS

4.1 *Principles of the Analytical Method*

To accommodate analysis of the wide variety of cotton gin trash samples that are generated from seed cotton processing, both a vigorous extraction method and a highly selective means of detection are required. Analytical enforcement methods for the determination of pyriithiobac sodium residues extracted from cotton seed (DuPont Report No. AMR 2816-93, Revision 2, Reference 2) and soil (DuPont Report No. AMR 2745-93, Revision 1, Reference 3) are not applicable to the extraction, clean-up and analysis of cotton gin trash, leading to new method development.

The Dionex ASE™ 200 efficiently extracts aged pyriithiobac residues from cotton gin trash with the use of 35% acetonitrile/65% 0.01M (NH₄)₂CO₃ at 1500 psi, 110°C. However, this rigorous extraction generates samples that must be extensively cleaned-up before analysis. Extracts were purified using liquid-liquid partition, liquid-solid chromatography and sorption techniques. After cleaning up samples, single-column and column-switching, reversed-phase LC/UV were used to analyze extracts. The LC/UV methods were not adequate, as coextracts interfered with the quantitation of pyriithiobac at the 0.020 ppm level.

An elegant solution to overcome the interference problems that LC/UV had is to analyze pyriithiobac in cotton gin trash by LC/MS or LC/MS/MS. After ASE, an aliquot of the sample extract is purified using liquid-liquid partition. Processed extracts are separated by liquid chromatography (LC) and pyriithiobac is selectively detected by MS-SIM or MS/MS-SRM. For this report, only the MS/MS-SRM data are presented.

A flow diagram of the analytical method from extraction to analysis is shown in Figure 1.

4.2 *Analytical Procedure*

4.2.1 *Glassware and Equipment Cleaning*

Glassware and extraction cells should be scrubbed by brush with a soap solution, rinsed two to five times with water, and rinsed with acetone or other suitable solvents. Distilled or deionized water may be added to the rinse sequence. The glassware and extraction cells are air-dried.

4.2.2 *Preparation of Solutions*

The following solutions should be prepared weekly and stored at room temperature unless stated otherwise:

0.01 M ammonium carbonate - Dissolve 0.96 g of (NH₄)₂CO₃ in about 800-mL distilled water and dilute to 1.00 L in a volumetric flask.

0.10 M acetic acid - Add 5.75 mL of glacial acetic acid to 500 mL of Milli-Q® water; dilute to 1 L.

35% acetonitrile/65% 0.01 M ammonium carbonate - add 350 mL of acetonitrile to 650 mL of 0.01 M ammonium carbonate.

20% methanol/80% 0.1 M acetic acid - add 200 mL methanol to 800 mL of 0.1 M acetic acid.

4.2.3 Preparation and Stability of Stock Standard

Use Class A volumetric flasks when preparing standard solutions.

Prepare a standard stock solution by accurately weighing 10 mg of pyriithiobac into a 100-mL volumetric flask on an analytical balance. Record the weight (to three significant figures) of the standard used to make the stock solution. Dissolve the standard in approximately 75 mL of HPLC-grade methanol. After dissolving, bring the solution to 100.00-mL volume using HPLC-grade methanol. This standard solution is stable for approximately 8 months when stored at approximately 4°C. The concentration of this solution is 100-µg/mL pyriithiobac sodium in methanol.

4.2.4 Preparation and Stability of Fortification Standards

Use Class A volumetric flasks when preparing standard solutions.

Prepare two fortification standard solutions: in 1 µg/mL and 10 µg/mL. Prepare the 10-µg/mL pyriithiobac sodium in methanol standard by pipetting 10.00 mL of the 100-µg/mL pyriithiobac stock standard into a 100-mL volumetric flask. Bring to volume using 100% HPLC-grade methanol. Prepare the 1-µg/mL pyriithiobac sodium in methanol standard by pipetting 1.00 mL of the 100-µg/mL pyriithiobac stock standard into a 100-mL volumetric flask. Bring to volume using 100% HPLC-grade methanol. These standard solutions are stable for approximately 8 months stored at approximately 4°C.

4.2.5 Preparation and Stability of Chromatographic Standards

Use Class A volumetric flasks when preparing standard solutions.

The 1-µg/mL pyriithiobac sodium in 100% methanol fortification standard is used to prepare the chromatographic standards. Prepare the standards by pipetting volumes of the 1-µg/mL fortification standard solution of pyriithiobac into a 25-mL volumetric flask, as shown in the following table:

Desired Standard Concentration ($\mu\text{g/mL}$)	Volume of 1 $\mu\text{g/mL}$ Standard Required (mL)
0.100	2.50
0.0500	1.25
0.0250	0.625
0.0100	0.250
0.00500	0.125
0.00200	0.050

Evaporate the methanol (to dryness) in each of the 25-mL volumetric flasks using an N-Evap[®]. Dilute to volume with 20% MeOH/80% 0.1M acetic acid. These standard solutions are stable for approximately 6 months stored at 4°C.

4.2.6 Source of Samples

Cotton gin trash used for method validation was generated in DuPont Study No. AMR 3295-95. Seed cotton was harvested from five states known for cotton production and processed to cotton gin trash at Texas A&M University. Seed cotton used for the method validation was from control plots from the locations listed in the following table.

Test Site No./ Sample I.D.	Test Site Location	
	City	State
1/S00048256	Seven Springs	North Carolina
2/S00100612	Greenville	Mississippi
3/S00088395	Olton	Texas
4/S00088398	Edmondson	Texas
5/S00085203	Dill City	Oklahoma
6/S00052438	Madera	California

4.2.7 Storage and Preparation of Samples

Cotton gin trash samples should be received frozen and should be stored in a freezer until they are ready to be prepared for analysis. Prepare cotton gin trash samples by chopping in a Hobart[®] Mixer.

4.2.8 Sample Fortification Procedure

Generally, fortified samples are prepared using a 1.0- $\mu\text{g/mL}$ or 10.0- $\mu\text{g/mL}$ pyriithiobac sodium in methanol fortification standard solution. Fortify samples by adding 0.10 to 0.50 mL of the fortification standard to the cotton gin trash sample. Let sample air dry for 15 minutes.

4.2.9 Analyte Extraction Procedure

1. Secure cap with frit on one end of 22-mL ASE extraction cell, seat a cellulose filter into the extraction cell against the cap frit - this is the bottom cap (see Section 5.3, Special Precautions). Weigh 5-g sample of Cotton Gin Trash in 22-mL ASE extraction cell using a polypropylene funnel.
2. Fortify with DPX-PE350 standard at appropriate level if the sample is to be a Fortified Control sample. Let stand for 15-30 minutes for solvent to evaporate.
3. Place top cap with frit onto extraction cell and mark as the top.
4. Place extraction cell along with a collection vial on ASE and extract with 35% ACN/ 65% 0.01M ammonium carbonate at the following conditions:

110 degC

1500 psi

60% flush

90 second purge

2 cycles

5 min static time (heat time of 6 min is set by firmware)

(Extraction time/sample = 20 minutes)

Caution: Extraction cells are extremely hot (110°C) after the extraction. Allow the cells to cool for at least 15 minutes before handling.

4.2.10 Analyte Purification Procedure

Each extract was cleaned-up using the following procedure before LC/MS/MS analysis.

1. Decant ASE cotton gin trash extracts into 50- or 100-mL graduated cylinder, rinse ASE collection vial two times with 3-5 mL of the extraction solvent each time, adding rinsate to graduated cylinder.
2. Bring the extract to 50 mL with the extraction solvent. Mix well.
3. Measure 10-mL aliquot of the extract with graduated cylinder and decant into a fresh 40-mL ASE collection vial. Pipet 0.5 mL of formic acid into the 10-mL aliquot of the extract.
4. Add 10 mL of ethyl acetate to 40-mL ASE collection vial, cap and shake vigorously (or vortex mix) for 10-20 seconds. Centrifuge the sample at 4000 rpm for 5 min. If an emulsion persists, centrifuge the sample again at 4000 rpm for 5 min. Pipet the top (ethyl acetate) layer into a 50-mL glass centrifuge tube.
5. Repeat Step 4.
6. Evaporate the ethyl acetate extract to dryness under a stream of nitrogen in a water bath at 50°C.

7. Add 1-mL methanol to the 50-mL centrifuge tube containing the extract and vortex mix. Transfer the solution to a 5-mL volumetric flask. Add 1-1.5 mL of 0.1M acetic acid to 50-mL centrifuge tube and vortex mix. Transfer the solution to the 5-mL volumetric flask. Add another 1-1.5 mL of 0.1 M acetic acid to the 50-mL centrifuge tube and vortex mix, transferring the solution to the 5-mL volumetric flask. Bring the sample to volume with 0.1M acetic acid.
8. Add 1.25 mL (use tube gradations) of the sample to a micro centrifuge tube and centrifuge the sample at $\geq 10,000$ rpm for 10 min.
9. Filter 0.5 to 1 mL of the centrifuged sample into an LC autosampler vial using a 0.2 μ m filter. Analyze by LC/MS as described in the next section.

Note: Steps 8 and 9 are required to minimize particulates which if not removed will plug the LC system.

4.3 Chromatography .

Minimum requirements of the HPLC system are described in the Equipment section above. This is an gradient-elution, reversed phase analysis on a C18 column designed for use with low-pH mobile phases. Conditions used for analysis are summarized below.

HPLC Conditions:

System	Hewlett-Packard HP1100 HPLC		
Column:	4.6 mm i.d. \times 15 cm, Zorbax [®] Rx-C18 analytical column with 5 μ m diameter packing		
Column Temperature	30 °C		
Injection Volume:	0.100 mL		
Flow Rate:	1.0 mL/min		
Conditions:	Time	%A	%B
(see Section 5.3, Special Precautions)	0	85	15
	1	85	15
	17	40	60
	18	10	90
	25	10	90
	35	End Run	
	A. 1% aqueous acetic acid B: acetonitrile		
	Post-Column time was set at 10 min to allow reequilibration of column at 85% A and 15% B.		
PE350 Retention Time:	~ 15 min		
Total Run Time.	~ 35 min		

4.4 LC/MS/MS Analysis

4.4.1 Description

LC/MS or LC/MS/MS employing atmospheric pressure chemical ionization (APCI) on a bench top-ion trap instrument is described for the quantitative analysis of pyriithiobac sodium residues in cotton gin trash at levels down to 0.02 ppm (LOQ).

During the method development process, a tune file was created for pyriithiobac sodium by infusing the analyte into the mobile phase stream (flow and composition were adjusted to approximate conditions expected at the on-column elution time for pyriithiobac sodium). During the tuning process, the protonated chlorine-isotope cluster $(MH)^+$ of the free acid form of pyriithiobac sodium (m/z 327+329) was detected in the acidic aqueous mobile phase and fragmented in MS/MS-SRM to produce $(M-OH)^+$; retaining the chlorine-isotope cluster (m/z 309+311). Analysis spectra acquired in MS/full-scan and MS/MS-full scan modes are presented in Figure 2 to illustrate the ion response detected, including the characteristic ion abundance ratio ($\sim 3/1$) in the isotope cluster for a monochlorinated molecule.

The instrument was operated in MS-SIM and MS/MS-SRM positive ion modes for quantitative analysis. The peak response for pyriithiobac sodium in the base peak (m/z 327 for MS-SIM and m/z 309 for MS/MS-SRM) ion chromatogram was integrated and the peak area used for quantitation. The selective and specific MS/MS fragmentation of the protonated molecular ion of pyriithiobac sodium coupled with the characteristic chlorine-isotopic cluster present in the product ions can be used to confirm the identity of a peak eluting at the pyriithiobac retention time.

4.4.2 Operating Conditions

The conditions outlined below are representative of those used for the particular instrument upon which this method was developed and evaluated.

APCI-LC/MS/MS Mass Spectrometer Conditions:

Source Type.	APCI	
Polarity:	positive	
Data Type.	centroid	
Vaporizer Temperature	550°C	
Sheath Gas Flow:	47 (in arbitrary units)	
Aux Gas Flow:	0.25 (in arbitrary units)	
Heated Capillary Temperature:	150°C (see Section 5.3, Special Precautions)	
Discharge Current:	8.75 μ A	
Discharge Voltage:	4.9 kV	
Capillary Voltage.	5.00 V	
Tube Lens Offset:	30.00 V	
Octapole RF Amplifier	400 Vp-p	
Octapole 1 Offset:	-1.75 V	
Octapole 2 Offset.	-6.50 V	
InterOctapole Lens Voltage:	-21.80 V	
Trap DC Offset Voltage:	-9.75 V	
Multiplier Voltage.	-781 V	
Run Time:	18.00 min	
Divert Flow until	13.00 min (note: valve connections to interface and waste are reversed - divert until 0.00, resume at 13.00)	
Resume Divert at:	18.00 min	
Segments:	1	
Scan Events:	1	
Segment 1 Scan Events:	m/z 328 \pm 3 amu \rightarrow m/z 308.5-311.5 (MS/MS-SRM) m/z 328 \pm 3 amu (MS-SIM)	
Source CID:	off	
Relative CID (Ion Trap).	18 %	(MS/MS-SRM only)

4.4.3 Sample Analysis

The calibration solutions and sample extract preparation for LC/MS/MS analysis are described in sections 4.2.5 and 4.2.10, respectively. At least one analysis (standard or analyte-containing sample) should be run prior to beginning the analysis set. This is to insure the source is equilibrated to mobile phase addition, and to evaluate analyte/system response.

The run order for calibration standards and sample extracts should be standard-sample extract-standard so that standards bracket sample extracts. The range of calibration

standards should be from 0.002 µg/mL to 0.1 µg/mL, and include a minimum of 4 concentrations.

4.4.4 Calibration Procedures

For the data in this report, the external standard calibration technique was used to quantitate the amount of pyriithiobac sodium in cotton gin trash samples. The average and standard deviation of the response factors (analyte peak area/analyte concentration) for the calibration standard runs immediately before and after a sample extract analysis are determined. If the relative standard deviation (RSD, standard deviation/average) for the two calibration standards is <20%, then the analyte response is determined as ppm found is equal to the analyte peak area response (ion counts) for the sample divided by the average response factor (ion counts/µg/mL) multiplied by the final extract volume dilution volume (25 mL) divided by sample weight (5 g).

Samples having detector responses for pyriithiobac sodium greater than the highest accompanying standard should be diluted to fall within the range of standards and reanalyzed.

Duplicate analyses is recommended, but optional.

4.4.5 Sample Analysis

Sample analysis should be conducted as outlined below.

Each set of samples analyzed for investigation purposes should include at least one unfortified sample (a sample which matches the investigation samples as closely as possible, preferably from an untreated plot). Cotton gin trash, preferably from an untreated plot, should be fortified with the pyriithiobac at a known level, and carried through the procedure to verify recovery.

For the analysis, a standard should be injected before and after each sample. If analysis is delayed, samples should be stored refrigerated or frozen until analysis. Extracted and cleaned-up samples should be stable for at least two weeks if kept refrigerated, and for at least five weeks if kept frozen.

The face of the heated capillary becomes blackened during the analysis of samples, so this should be inspected frequently and cleaned as needed to maintain instrument performance.

4.5 Calculations

4.5.1 Methods

Quantitation of the amount of pyriithiobac sodium found in extracted cotton gin trash was accomplished using external standards. Known pyriithiobac concentrations (µg/mL) and responses (peak area) from these standards were used to calculate a response factor (peak area / analyte concentration). The analysis data was entered into an Excel® spreadsheet designed to use functions: STDEV (standard deviation),

AVERAGE (average), and IF (if, then logic statement) to calculate ppm found and percent recovery. If the percent relative standard deviation (%RSD) was less than 20% for response the factors of the 2 standards bracketing the sample analysis, then the ppm found is determined in cotton gin trash samples by the following calculation.

$$\text{ppm found} = \left(\frac{\text{Analyte Peak Area, ion counts}}{\text{Average Response Factor, ion counts} / \mu\text{g} / \text{mL}} \right) \times \left(\frac{\text{Dilution Volume, mL}}{\text{Sample Weight, g}} \right)$$

The dilution factor accounts for the 10 mL aliquot of 50 mL of the sample extract taken and a final volume of 5 mL. The dilution volume is 5 mL (50 mL/10 mL) = 25 mL. If the percent relative standard deviation (%RSD) was greater than or equal to 20% for response the factors of the 2 standards bracketing the sample analysis, then the samples were reanalyzed. The percent recovery for fortified samples is determined by the following calculation.

$$\text{Percent Recovery} = \frac{\text{ppm found} \times 100}{\text{ppm fortified}}$$

4.5.2 Example

0.02-ppm fortified cotton gin trash sample (Sample 81197-4P, LC/MS/MS analysis PE350_11Aug203, Table 1):

$$\text{Average Response Factor} = \frac{71763338 + 72365130 \text{ ion counts} / \mu\text{g} / \text{mL}}{2} = 72064234$$

$$\text{ppm found} = \left(\frac{266702 \text{ ion counts}}{72064234 \text{ ion counts} / \mu\text{g} / \text{mL}} \right) \times \left(\frac{25 \text{ mL}}{5 \text{ g}} \right) = 0.0185 \mu\text{g} / \text{g}$$

(ppm values are rounded to two significant figures using a Microsoft Excel rounding method in Tables 1 and 2 of this report)

For this sample, the percent recovery found was calculated as follows:

$$\% \text{ Recovery} = 100(0.0185 \mu\text{g}/\text{g} / 0.020 \mu\text{g}/\text{g}) = 93\%$$

(percent recoveries are rounded to the nearest whole number in Tables 1 and 2 of this report, without rounding the concentration or ppb found)

5.0 RESULTS AND DISCUSSION

5.1 Method Validation Results

5.1.1 Detector Response

Pyriithiobac sodium standard solutions used to generate calibration curves ranged from 0.002 to 0.1- $\mu\text{g}/\text{mL}$ in concentration. The response of the standards detected was linear over this concentration range (see Figure 3). Cotton gin trash fortified

from 0.02 ppb to 0.2 ppb were successfully extracted, cleaned-up and analyzed by the LC/MS/MS method.

Representative chromatograms of pyriithiobac sodium standards and of cotton gin trash samples fortified with pyriithiobac are shown in Appendix 2.

5.1.2 Unfortified Samples

Interference peaks in unfortified sample chromatograms were less than the LOD at the retention time for pyriithiobac sodium.

5.1.3 Recoveries

Cotton gin trash samples fortified with pyriithiobac sodium were analyzed by APCI LC/MS/MS following extraction and processing. The data are found in Table 1.

The method generated acceptable recoveries at levels which might be expected in cotton gin trash. Cotton gin trash samples were fortified at several levels: 0.02, 0.04, and 0.2 ppm. Recoveries for these samples ranged from 70 to 93%. The overall average recovery (\pm standard deviation) for cotton gin was 82% (\pm 10%) with a relative standard deviation (RSD) of 12% for 6 samples analyzed. Recovery data from these samples demonstrate that the pyriithiobac sodium residues are stable during the extraction and subsequent clean-up and analysis steps and that the recoveries are acceptable for this analytical method to be used to support registration.

5.1.4 Extraction Efficiency/Radiovalidation

The extraction efficiency of this method was confirmed by standard ^{14}C methodology. Radiovalidation was performed by analyzing fortified samples that had been extracted and cleaned-up using the method by APCI LC/MS/MS. Six samples were fortified and aged 11 days at room temperature in the laboratory before extraction. Liquid scintillation counting (LSC) results of the raw extract indicated that the extraction efficiency ranged from 80 to 90% with an average of 86% (\pm 4% standard deviation) and an RSD of 5%. After extraction and clean-up the samples were analyzed again just before LC/MS/MS analysis. The average of 83% (\pm 1% standard deviation) and an RSD of 1%. The results from the analysis of these samples are shown in Table 3. The average recovery of the six samples extracted, purified, and analyzed by LC/MS/MS was 83%, in agreement with the LSC results. This indicates that LC/MS/MS provides accurate quantitative data.

The fact that the method extracted 80-90% of the radioactivity from the 11-day laboratory aged cotton gin trash indicates that this method has acceptable extraction efficiency.

The data above along with the data from the cotton gin trash samples fortified with the nonradiolabeled pyriithiobac sodium demonstrate that pyriithiobac sodium residues are successfully extracted and stable throughout the extraction and purification steps and detectable by nonradiochemical means: by a mass spectrometer detector.

5.1.5 *Method Detection Limit and Limit of Quantitation*

The method limit of detection (LOD) and limit of quantitation (LOQ) for the LC/MS/MS method are 0.007 and 0.020 $\mu\text{g/g}$ (ppm), respectively.

The limit of quantitation (LOQ) by LC/MS/MS analysis for pyriithiobac sodium extracted from cotton gin trash was determined to be 0.02 ppm. This quantitation limit is defined as the lowest fortification level evaluated at which acceptable average recoveries (70-120%, RSD < 20%) were achieved. This quantitation limit also reflects the fortification level at which an analyte peak was consistently generated at a level approximately 10 times the signal at pyriithiobac's retention time in the chromatograms of unfortified control samples.

The method limit of detection (LOD) was estimated to be 0.007 ppm. An LOD value should be estimated by each lab using this method. The estimate of the method detection limit is defined as the concentration of pyriithiobac sodium determined by extrapolation of the calibration curve for an unfortified soil sample at three times the worst-case chromatographic baseline noise that was analyzed to validate this method (Reference 4). The chromatographic noise was measured near the pyriithiobac sodium retention time in unfortified samples. The complete residue method was used to generate the samples that were analyzed for the LOD determination.

5.2 *Time Required for Analysis*

Typically six to eight samples can be prepared during the course of a normal eight-hour day. With the equipment used in this study, LC/MS/MS required 35 minutes per sample or standard (including reequilibration time). These analyses were run unattended overnight.

5.3 *Modifications or Special Precautions*

Before an extraction, check the white O-rings installed in the exterior end of each extractor cell cap and in the ends of the rinse tubes. These O-rings should be pressed into place or replaced as needed. Before loading an extraction cell, the PEEK seals for the cell should be checked to avoid leaks during an extraction. Worn PEEK seals are discolored and often have deep grooving on the surface. Replace worn PEEK seals before extraction.

Low pressure mixing LC instruments that use a proportioning valve to mix solvents may not be adequate for this method. Therefore, the mobile phases may need to be premixed. The need for premixing is determined by unstable retention times, or baseline fluctuations during the gradient. Mobile phases should be degassed, particularly when low pressure mixing systems are used.

The face of the heated capillary on the LC/MS/MS instrument becomes blackened during the analysis of samples, so this should be inspected frequently and cleaned as needed to maintain instrument performance.

5.4 *Method Ruggedness*

5.4.1 Stability and Ruggedness Testing

The stability of pyriithiobac sodium in standards and extracts has been stated in the respective sections of this report. The stability of reagents used in this method have also been stated.

Several variables were explored to establish the ruggedness of this method from sample extraction through LC/MS/MS analysis. Samples of cotton gin trash from six different sites typical of where cotton is grown were used to validate this method. Two different analysts extracted and cleaned up the validation samples. This method was validated in the LC/MS or the LC/MS/MS mode on the Finnigan MAT LCQ™ bench top-mass spectrometer. This method was also validated in a separate laboratory by different analysts using LC/MS and LC/MS/MS on a system made by different manufacturer.

5.4.2 Specificity/Potential Interference

Due to the mass selective nature of the detection of this method, interference is less than the LOD at the retention time of pyriithiobac.

6.0 CONCLUSIONS

This method for the determination of pyriithiobac sodium residues extracted from cotton gin trash meets U.S. EPA Pesticide Assessment Guidelines Subdivision O, 171-4 (c) and (d), Residue Chemistry 860.1340 Series Final Guidelines and EEC Directive 91/414/EEC: Annex II 4.2.1.

Pyriithiobac sodium (pyriithiobac, DPX-PE350, KIH-2031, sodium 2-chloro-6-[(4,6-dimethoxypyrimidin-2-yl)thio]benzoate) is efficiently extracted from 5 g of cotton gin trash by 35% acetonitrile/ 65% 0.01M (NH₄)₂CO₃ at 110°C and 1500 psi using a DIONEX ASE™ 200 Extractor. This method was radiovalidated demonstrating that aged pyriithiobac sodium residues are extracted, cleaned-up and detected using non radiochemical means.

The method limit of detection (LOD) and limit of quantitation (LOQ) for the LC/MS and LC/MS/MS method are 0.007 and 0.02 µg/g (ppm), respectively, and are sufficiently justified.

At the retention time of pyriithiobac, the LC/MS/MS method is free of interference at the LOD in unfortified cotton gin trash samples that were extracted and analyzed using the method.

The method generated acceptable recoveries at levels expected in cotton gin trash extracted and cleaned-up by two different analysts and two different LC/MS and LC/MS/MS systems.

7.0 RETENTION OF RECORDS

Originals or exact copies of all raw data and pertinent information, including the original protocol, any amendments, and the final report will be retained at:

E. I. du Pont de Nemours and Company
DuPont Agricultural Products
Global Technology Division
Experimental Station
Wilmington, DE 19880-0402

8.0 REFERENCES

1. Bates, M., "Determination of the Physico-Chemical Properties of KIH-2031 (DPX-PE350) According to EPA Requirements", DuPont Report No. AMR 2506-92, DuPont Agricultural Products, E. I. du Pont de Nemours and Company, Wilmington, DE.
2. Sumpter, S.R., Peterson, B.A., Wadsley, M.P., "Improved Analytical Enforcement Method for the Determination of KIH-2031 (DPX-PE350) Residues in Cottonseed Using Column-Switching Liquid Chromatography", DuPont Report No. AMR 2816-93, Revision 2, DuPont Agricultural Products, E. I. du Pont de Nemours and Company, Wilmington, DE.
3. Sumpter, S.R., Peterson, B.A., Mulderig, L.J., and Ledeker, K.W., "Analytical Method for the Determination of Pyriithiobac Sodium in Soil Using Subcritical Water Extraction, Graphitized Carbon Clean-Up, and Column-Switching LC/UV Analysis with Confirmation by LC/MS", DuPont Report No. AMR 2745-93, Revision 1, DuPont Agricultural Products, E. I. du Pont de Nemours and Company, Wilmington, DE.
4. Cairns, T., and Rogers, W. M., "Acceptable Analytical Data for Trace Analysis," *Anal. Chem.*, **55**, 54A (1983).

TABLE 1
METHOD VALIDATION RECOVERIES FOR PYRITHIOBAC SODIUM EXTRACTED
FROM COTTON GIN TRASH

Run #	Sample ID	Pk Area PE350	Resp. Factor %RSD	ppm Found	%Recovery
PE350_11Aug201	0.002 µg/mL Std	105933	52966505		
PE350_11Aug202	control 81197-1P		21%	< LOD	na
PE350_11Aug203	0.004 µg/mL Std	287053	71763338		
PE350_11Aug204	0.02 ppm fort81197-4P	266702	1%	0.019	93%
PE350_11Aug205	0.01 µg/mL Std	723651	72365130		
PE350_11Aug206	control 81197-2P		1%	< LOD	na
PE350_11Aug207	0.05 µg/mL Std	3591909	71838187		
PE350_11Aug208	0.04 ppm fort81197-5P	560931	11%	0.036	90%
PE350_11Aug209	0.1 µg/mL Std	8429625	84296246		
PE350_11Aug210	control 81197-3P		3%	< LOD	na
PE350_11Aug211	0.002 µg/mL Std	161389	80694465		
PE350_11Aug212	0.2 ppm fort81197-6P	2491366	11%	0.17	83%
PE350_11Aug213	0.004 µg/mL Std	275811	68952690		
PE350_11Aug214	control 81197-1		3%	< LOD	na
PE350_11Aug215	0.01 µg/mL Std	720421	72042074		
PE350_11Aug216	0.02 ppm fort81197-2	242188	2%	0.017	85%
PE350_11Aug217	0.05 µg/mL Std	3521254	70425077		
PE350_11Aug218	control 81197-3		5%	< LOD	na
PE350_11Aug219	0.1 µg/mL Std	7601207	76012070		
PE350_11Aug220	0.04 ppm fort81197-4	417322	4%	0.028	71%
PE350_11Aug221	0.002 µg/mL Std	142889	71444605		
PE350_11Aug222	control 81197-5		7%	< LOD	na
PE350_11Aug223	0 004 µg/mL Std	257770	64442423		
PE350_11Aug224	0.2 ppm fort81197-6	1994261	14%	0.14	70%
PE350_11Aug225	0.01 µg/mL Std	782943	78294344		

Calculations:

Response Factor = Peak Area / Standard Concentration,
 ppm Found = If %RSD > 20% then "NT-%RSD" is displayed

If %RSD 20% or less, then:

Peak Area × Average (Resp. Factor above, Resp. factor below) × 25 / 5
 % Recovery = ppm Found × 100 / ppm fortified

TABLE 2
METHOD VALIDATION RECOVERY SUMMARY FOR PYRITHIOPAC SODIUM
EXTRACTED FROM COTTON GIN TRASH

	Sample ID	Fortification Level (ppm)	Amount Found (ppm)	%Recovery
control	81197-1P	0.0	< LOD	Not Applicable
control	81197-2P	0.0	< LOD	Not Applicable
control	81197-3P	0.0	< LOD	Not Applicable
control	81197-1	0.0	< LOD	Not Applicable
control	81197-3	0.0	< LOD	Not Applicable
control	81197-5	0.0	< LOD	Not Applicable
ppm fort	81197-2	0.02	0.017	85%
ppm fort	81197-4P	0.02	0.019	93%
				Average
				Standard Dev.
				RSD
				89%
				6%
				6%
ppm fort	81197-4	0.04	0.028	71%
ppm fort	81197-5P	0.04	0.036	90%
				Average
				Standard Dev.
				RSD
				81%
				13%
				17%
ppm fort	81197-6	0.2	0.14	70%
ppm fort	81197-6P	0.2	0.17	83%
				Average
				Standard Dev.
				RSD
				77%
				9%
				12%
				Overall Average
				Standard Dev.
				RSD
				n = 6
				82%
				10%
				12%

TABLE 3
METHOD RADIOVALIDATION AND EXTRACTION EFFICIENCY SUMMARY FOR
PYRITHIOBAC SODIUM EXTRACTED FROM COTTON GIN TRASH

Run #	Sample ID	Pk Area PE350	Resp. Factor %RSD	ppm Found	%Recovery
PE35014AugRadVal01	0 01 µg/mL Std	373317	37331700		
PE35014AugRadVal02	control 81297-1P		13%	< LOD	na
PE35014AugRadVal03	0 05 µg/mL Std	2254820	45096400		
PE35014AugRadVal04	control 81297-2P		1%	< LOD	na
PE35014AugRadVal05	0 1 µg/mL Std	4578308	45783080		
PE35014AugRadVal06	0.18 ppm fort 81297-3P	1478073	9%	0.15	84%
PE35014AugRadVal07	0.2 µg/mL Std	10432480	52162400		
PE35014AugRadVal08	0 18 ppm fort 81297-4P	1450408	14%	0 15	85%
PE35014AugRadVal09	0 01 µg/mL Std	428786	42878600		
PE35014AugRadVal10	0.18 ppm fort 81297-5P	1332215	1%	0 15	86%
PE35014AugRadVal11	0.05 µg/mL Std	2164859	43297180		
PE35014AugRadVal12	0 18 ppm fort 81297-6P	1367402	3%	0 15	86%
PE35014AugRadVal13	0 1 µg/mL Std	4507516	45075160		
PE35014AugRadVal14	0 18 ppm fort 81297-7P	1403607	10%	0.15	81%
PE35014AugRadVal15	0.2 µg/mL Std	10323090	51615450		
PE35014AugRadVal16	0.18 ppm fort 81297-8P	1420702	4%	0 13	74%
PE35014AugRadVal17	0.2 µg/mL Std	10902177	54510885		

Average = 83%
Standard Dev. = 5%
RSD = 6%

Calculations:

Response Factor = Peak Area / Standard Concentration,

ppm Found = If %RSD > 20% then "NT-%RSD" is displayed

If %RSD 20% or less then Peak Area / Average (Resp Factor above, Resp factor below) × 25 / 5

% Recovery = ppm Found × 100 / ppm fortified

LOD = 0 007 ppm, LOQ = 0 02 ppm

FIGURE 1
FLOW DIAGRAM OF THE ANALYTICAL METHOD FOR THE DETERMINATION OF
PYRITHIOBAC SODIUM EXTRACTED FROM COTTON GIN TRASH

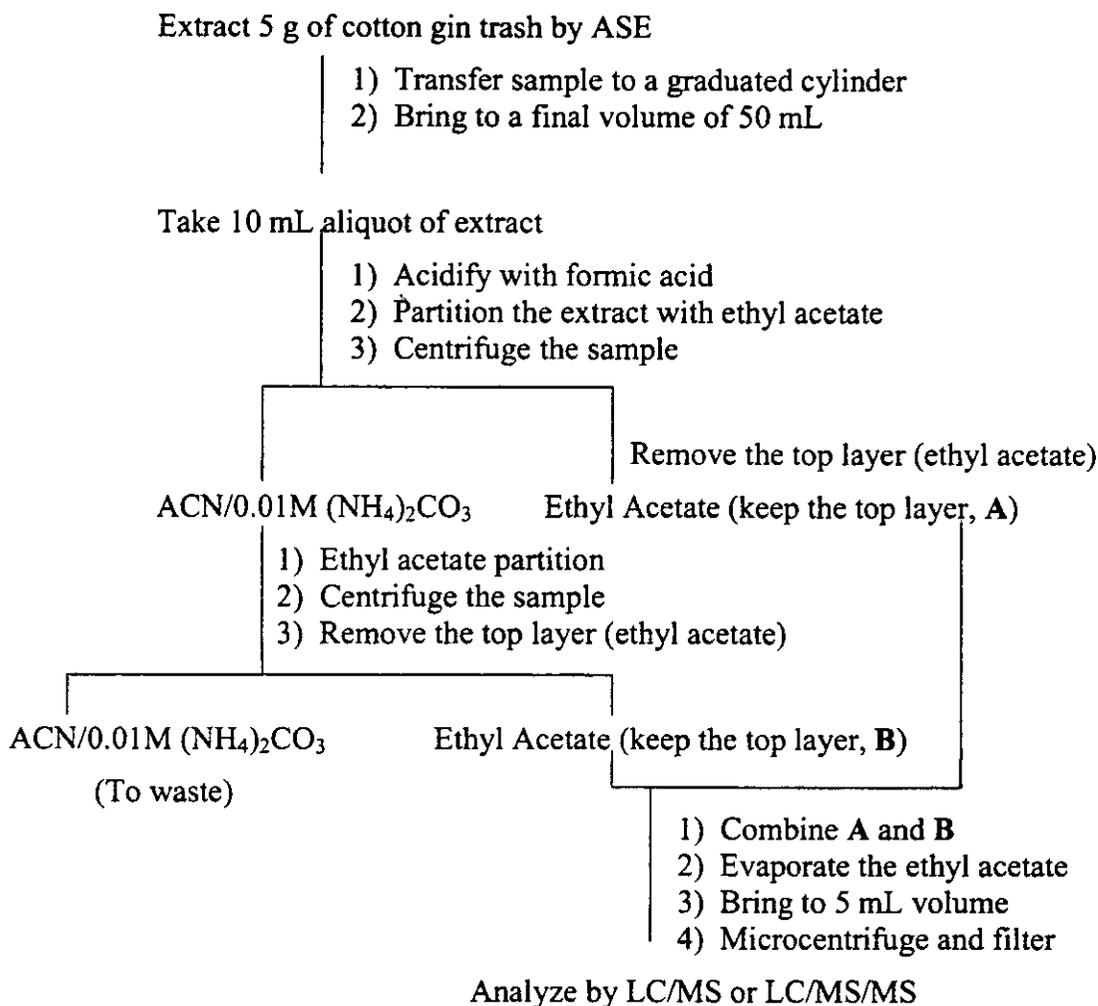
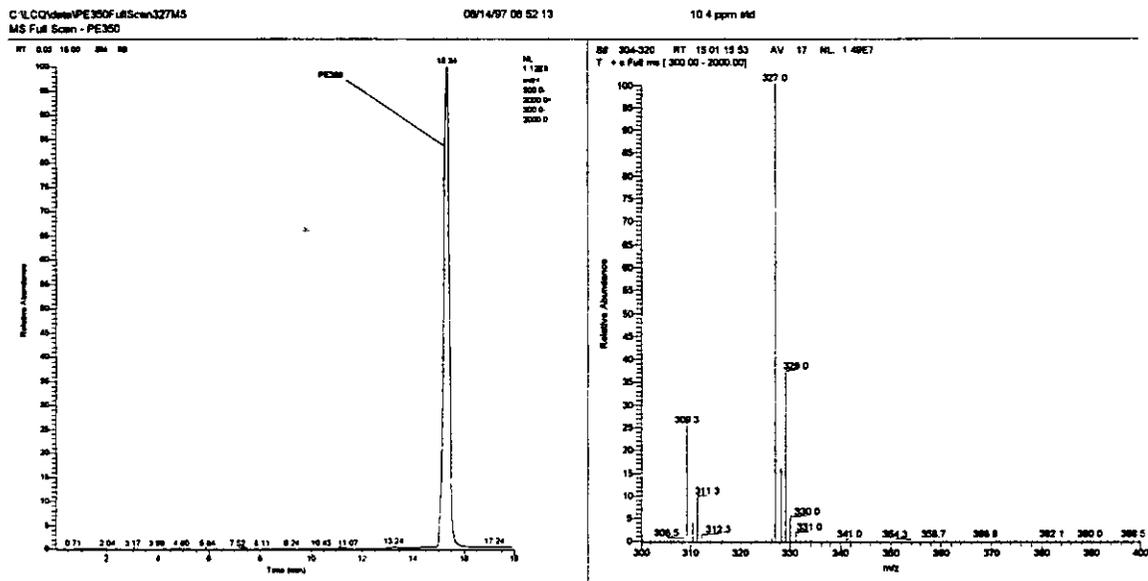


FIGURE 2
CHROMATOGRAMS AND MASS SPECTRA FOR PYRITHIOPAC SODIUM IN
MS-FULL SCAN AND MS/MS-FULL SCAN MODES

MS-Full Scan Chromatogram and Spectrum



MS/MS-Full Scan Chromatogram and Spectrum

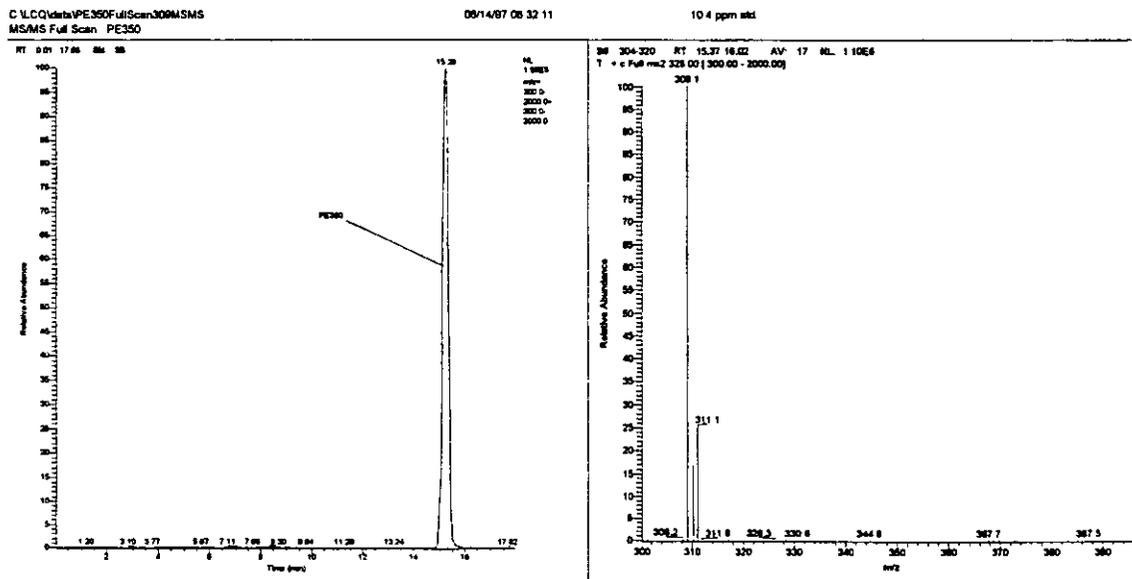
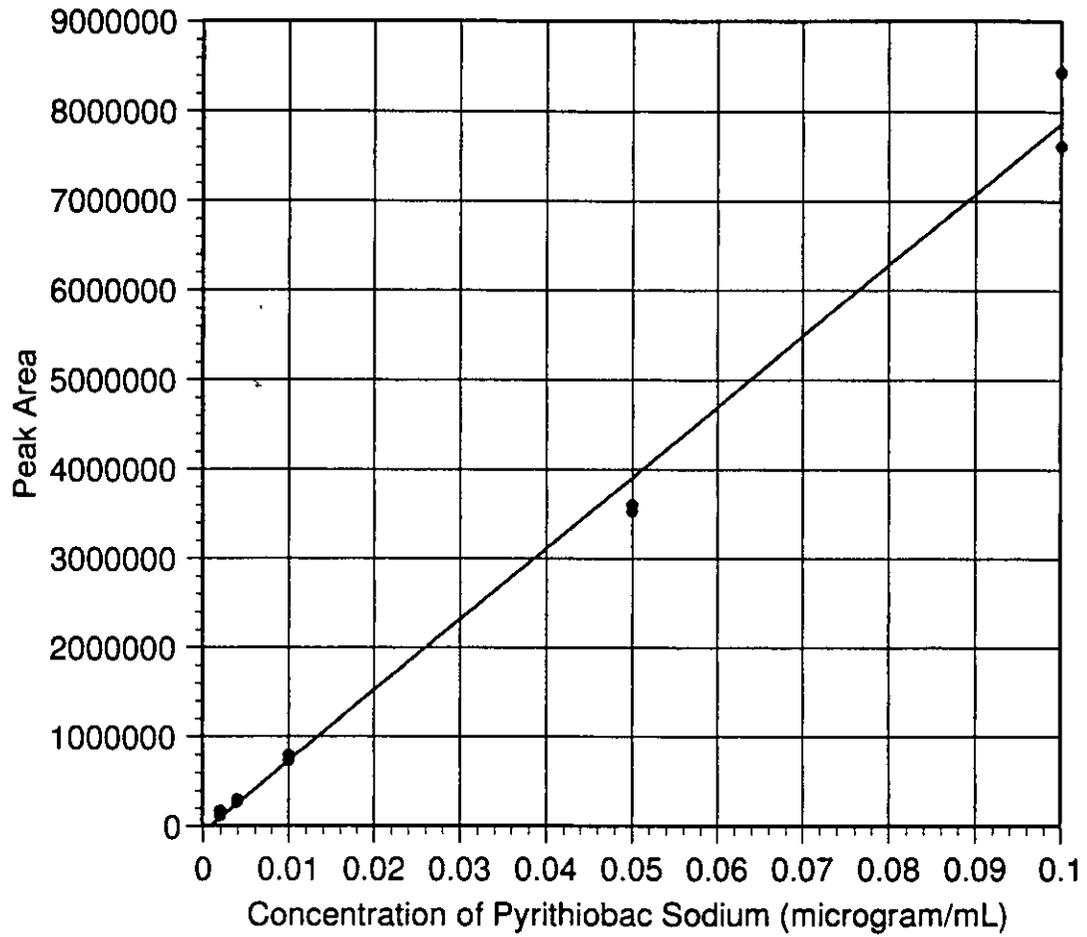
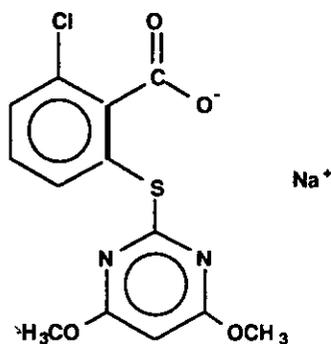


FIGURE 3
TYPICAL APCI LC/MS/MS CALIBRATION CURVE



$$f(x) = 7.934298E+7 \cdot x + -7.765413E+4$$
$$R^2 = 9.937320E-1$$

APPENDIX 1
STRUCTURE AND PHYSICO-CHEMICAL PROPERTIES OF PYRITHIOPAC SODIUM

Pyriproxyfen Sodium

Bates (see Reference 1) has determined the following physico-chemical properties for DPX-PE350:

Melting Point: 233.8-234.2°C

Solubility:

Water	728 g/L
Methanol	270 g/L
Acetone	812 mg/L
Acetonitrile:	347 mg/L

Partition Coefficient,
n-octanol/pH 7 water: 0.14

Dissociation constant, pKa 2.34

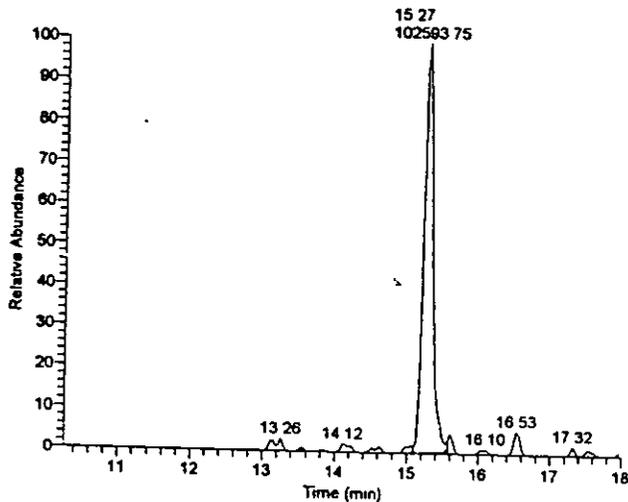
APPENDIX 2
REPRESENTATIVE CHROMATOGRAMS

LC/MS/MS Chromatograms Shown at Original Size

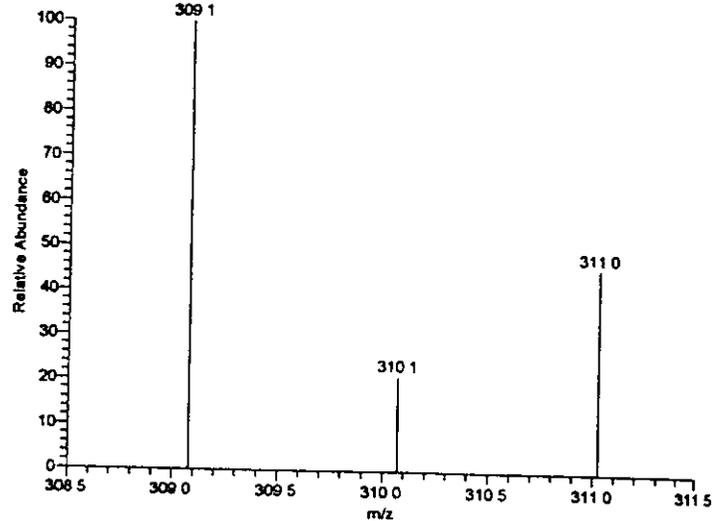
LC/MS/MS CHROMATOGRAMS OF PYRITHIOPAC SODIUM STANDARDS ANALYZED DURING METHOD VALIDATION

0.002- $\mu\text{g/mL}$ pyriithiobac sodium standard

RT 10 27 - 18 01 SM 5G

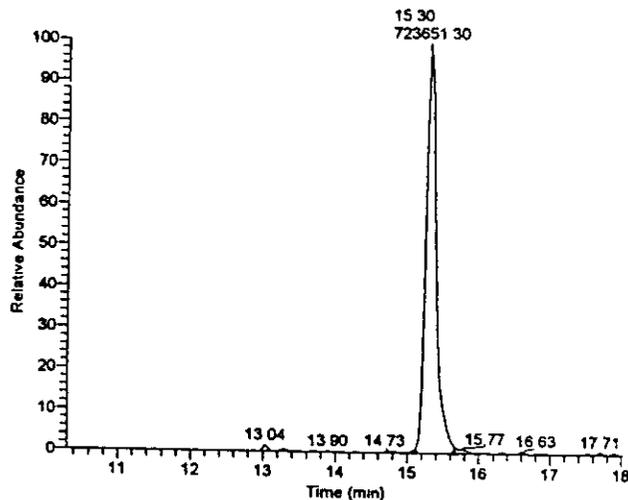


NL
1 15E4
m/z=
308 5-
309 5

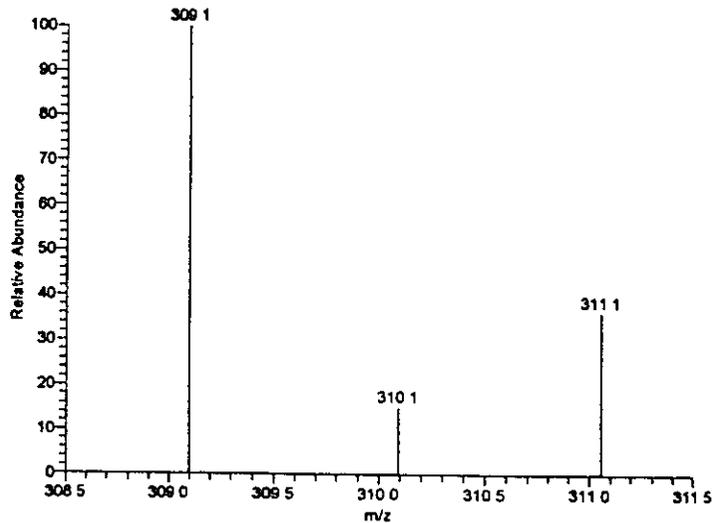


0.01- $\mu\text{g/mL}$ pyriithiobac sodium standard

RT 10 30 - 18 03 SM 5G

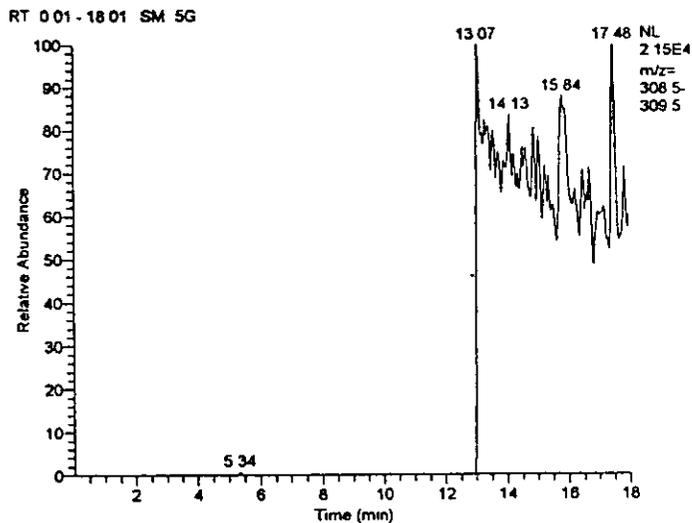


NL
7 22E4
m/z=
308 5-
309 5

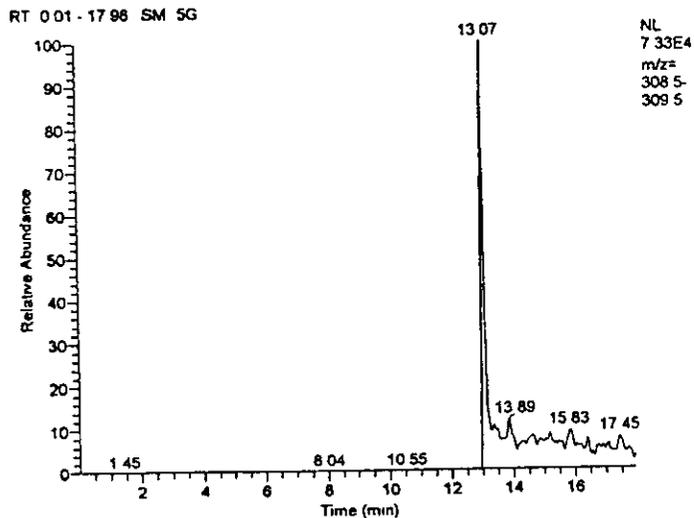


LC/MS/MS CHROMATOGRAMS OF UNFORTIFIED COTTON GIN TRASH SAMPLES ANALYZED DURING METHOD VALIDATION

Sample: 81197-1P, unfortified cotton gin trash from Site 1, ppm pyriithiobac sodium found < LOD.



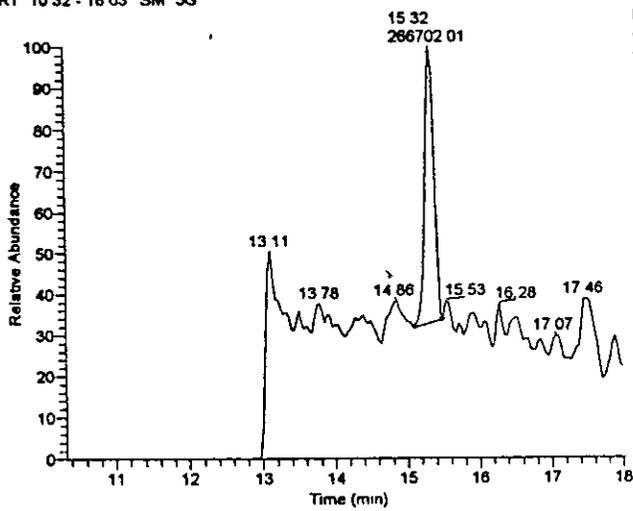
Sample: 81197-3P, unfortified cotton gin trash from Site 3, ppm pyriithiobac sodium found < LOD.



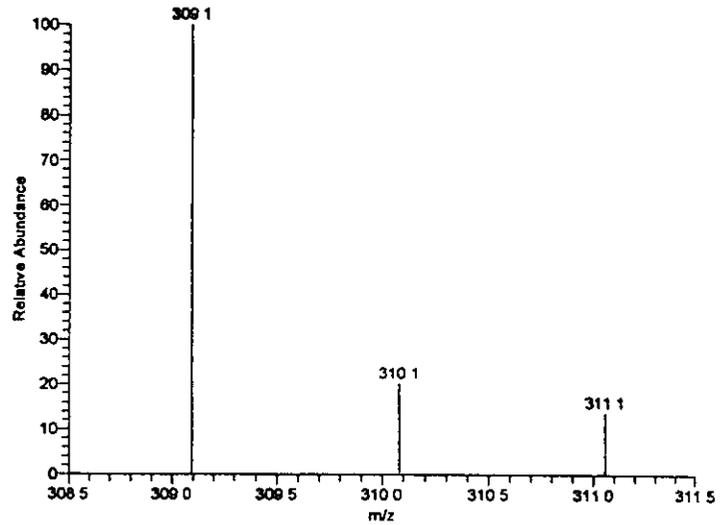
LC/MS/MS CHROMATOGRAMS OF FORTIFIED COTTON GIN TRASH SAMPLES ANALYZED DURING METHOD VALIDATION

Sample: 81197-4P fortified cotton gin trash, Site 1, 0.0185-ppm pyriithiobac sodium found, 93% recovery for 0.02-ppm fortification.

RT 10.32 - 18.03 SM 5G

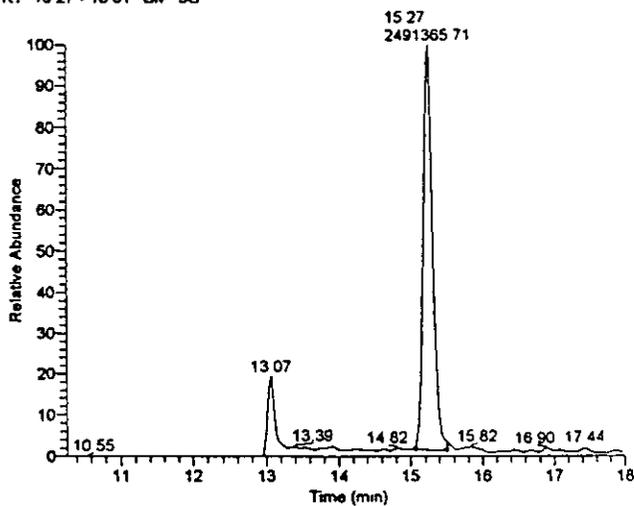


NL
4.55E4
m/z=
308.5
309.5

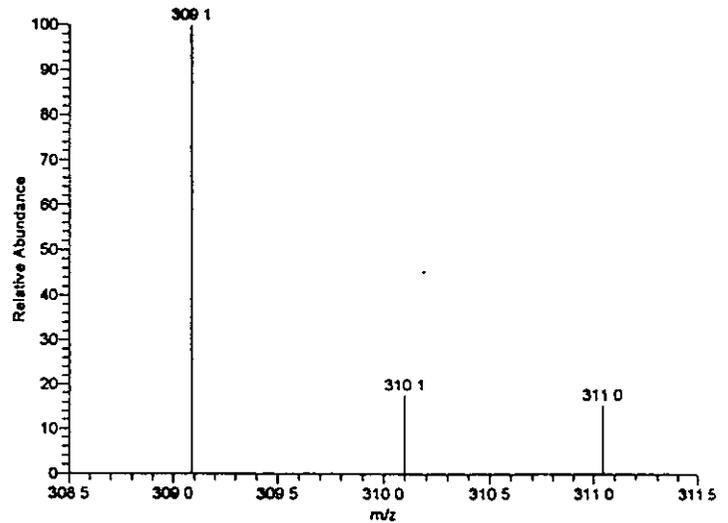


Sample: 81197-6P, fortified cotton gin trash, Site 3, 0.17 ppm pyriithiobac sodium found, 83% recovery for 0.2-ppm fortification.

RT 10.27 - 18.01 SM 5G



NL
2.84E5
m/z=
308.5
309.5



**EPA ADDENDUM For Residue Analytical Method
Pyrethiobac Sodium on Cotton Commodities
PP# 0F04391**

1) The method called for determining pyrethiobac sodium by LC/MS/MS using an ion trap mass spectrometer. However, since ACB does not have an ion trap, ACB validated the method by LC/MS/MS using a triple stage quadrupole instrument.

2) ACB made slight modifications to the LC/MS/MS instrument parameters as follows:

LC/MS/MS Instrumentation: Waters 2695 HPLC and Micromass Quattro Micro Triple Stage Quadrupole Mass Spectrometer,
HPLC Column. Zorbax Eclipse XDB-C8,
Gradient: Exactly as written in method,
Source APCI Positive Mode,
Corona: 8 μ A,
Cone Voltage: 25 V,
Source Temp.: 120°C,
Desolvation Temp.: 600°C,
Cone Gas Flow: 100 L/Hr,
Desolvation Gas Flow. 500 L/Hr,
Collision Energy: 17,

The following LC/MS/MS ion transitions pairs are monitored for pyrethiobac sodium: m/z 327>309, m/z 328>310, and m/z 329>311. The m/z 327>309 transition is used for quantitation.

3) The method as written suggested that the final extract be diluted to a volume of 5 mL, for the lowest fortification level. With a 5 mL final volume, the ACB noticed that signal enhancement occurred (i.e. apparent recoveries of 200%). The ACB found that the problem with signal enhancement was alleviated when diluting the sample to a final volume of at least 20 mL. This phenomenon is often observed with APCI, when injecting dirty extracts