

US EPA ARCHIVE DOCUMENT

**TRADE SECRET**

*Study Title*

**IMPROVED ANALYTICAL ENFORCEMENT METHOD FOR THE  
DETERMINATION OF KIH-2031 (DPX-PE350) RESIDUES  
IN COTTONSEED USING COLUMN-SWITCHING LIQUID  
CHROMATOGRAPHY**

*Data Requirement*

US EPA Pesticide Assessment Guidelines  
Subdivision O, 171-4

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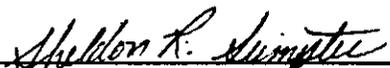
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**IMPROVED ANALYTICAL ENFORCEMENT METHOD FOR THE DETERMINATION OF KIH-2031 (DPX-PE350) RESIDUES IN COTTONSEED COLUMN-SWITCHING LIQUID CHROMATOGRAPHY**

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**PURPOSE FOR REVISION**

The purpose for this revision is to correct the part number for the guard column on page 10 of the original report. In addition, the method, AMR 2816-93, calls for potassium citrate in the *Preparation of Solutions and Extractions Procedure for Cottonseed* sections of the report. However, the source of potassium citrate was not mentioned in the *Reagents and Standards* section of the report. Therefore, the potassium citrate and its source were added to the report.

**ABSTRACT**

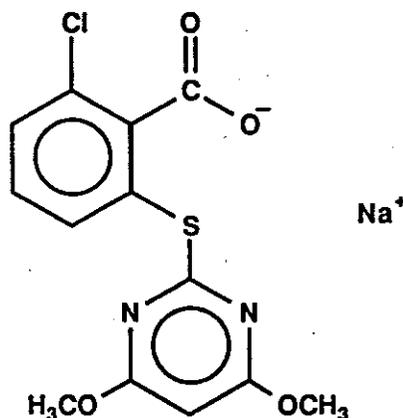
An improved analytical enforcement method was developed and is described for the determination of KIH-2031 (sodium 2-chloro-6-[(4,6-dimethoxypyrimidin-2-yl)thio]benzoate, DPX-PE350) in cottonseed. After extracting DPX-PE350 from whole cottonseed, the extract is cleaned-up using liquid-liquid extraction and solid-phase extraction techniques. The instrumental method is based on the use of column-switching HPLC with UV absorbance detection at 254 nm. The method quantitation limit for DPX-PE350 in cottonseed is 0.010 ppm.

The chromatographic separation requires one switching valve, and two analytical columns in series. The first column is used as a "clean-up" column from which DPX-PE350 is transferred to the second column. The transfer occurred at  $\pm 0.5$  minutes related to the average retention time for DPX-PE350 standards. After transfer to the second column, the analytical separation of DPX-PE350 is performed.

The average recovery ( $\pm$  standard deviation), as determined using HPLC/UV, for 11 cottonseed samples was  $84 \pm 14\%$  giving a relative standard deviation of 17%. The validity of this method was confirmed by standard  $^{14}\text{C}$  methodology; extraction efficiency recoveries ranged from 78 to 87% and the average recovery ( $\pm$  standard deviation) for 6 samples was  $83 \pm 3\%$  giving a relative standard deviation of 4%. This method meets U.S. EPA Subdivisión O, 171-4 Pesticide Assessment Guideline criteria.

## INTRODUCTION

This analytical method was developed to satisfy U.S. EPA registration requirements (including tolerance enforcement) for Staple® Herbicide. This method determines residues of DPX-PE350 in whole cottonseed. Staple® Herbicide is a post-emergent broadleaf herbicide for cotton that contains a single, active ingredient: KIH-2031 (sodium 2-chloro-6-[(4,6-dimethoxypyrimidin-2-yl)thio]benzoate, DPX-PE350). KIH-2031 is referred to as DPX-PE350 in this report. The chemical structure for DPX-PE350 is shown below:



DPX-PE350

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/mL
Methanol	270 g/mL
Acetone	813 mg/mL
Acetonitrile:	347 mg/mL
Partition Coefficient, n-octanol/pH 7 water:	0.14
Dissociation Constant, pK <sub>a</sub>	2.34

Fortified cottonseed matrix was homogenized and extracted in 2:1 acetonitrile:0.01 M (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub> and then cleaned up prior to HPLC analysis by the use of liquid-liquid, and solid-phase extraction. For solid-phase extraction, two Bond Eluts® were

used. The first Bond Elut® was a 20-cc SAX column from which DPX-PE350 was transferred to a 12-cc C<sub>18</sub> Bond Elut® column. The solution was then evaporated to dryness and reconstituted in 0.5 mL of 28% ACN:72% pH 3, 30 mM phosphate buffer.

For HPLC analysis, the following procedure was used. A 0.1-mL sample was injected on a Zorbax® SB-CN column. At ± 0.5 minutes relative to the average retention time of DPX-PE350 on the SB-CN column, a switching valve was positioned to allow DPX-PE350 to be loaded and concentrated onto the second column, a Zorbax® SB-C<sub>18</sub> analytical column. After the transfer of DPX-PE350 to the second column, the SB-CN column was cleaned out with 75% acetonitrile/25% Milli-Q® water and equilibrated to the mobile phase composition of the second column. Further separation was accomplished with both columns in series.

The following table demonstrates the important differences that exist between AMR 2689-93 (see Reference 2) and AMR 2816-93 (this improved, more rugged method).

Comparison of Cottonseed Extraction and Clean-up Methods

AMR 2689-93	AMR 2816-93
<u>Clean-up</u>	
1. Use 20% MeOH in 1 M Na <sub>2</sub> SO <sub>4</sub> at pH 3 to elute DPX-PE350 off of SAX column.	1. Use 25% MeOH in 1 M potassium citrate to elute DPX-PE350 off of SAX column
2. Final volume = 5.0 mL	2. Final volume = 0.5 mL
<u>HPLC</u>	
1. Injection volume = 1.0 mL	1. Injection volume = 0.10 mL
2. Column II = R <sub>X</sub> -C <sub>18</sub>	2. Column II = SB-C <sub>18</sub>
3. Cut window = 0.4 min which takes a fraction of the DPX-PE350 peak	3. Cut window = 1.0 min. The entire DPX-PE350 peak is transferred from Column I to Column II.
4. Aqueous mobile phase is 0.1 M HOAc (pH 2.8)	4. Aqueous mobile phase is 30 mM phosphate buffer at pH 3
<u>Results</u>	
1. Several peaks from coextracts elute near the retention time of DPX-PE350 using a 0.4 min cut window.	1. Chromatograms have fewer peaks eluting at the retention time of DPX-PE350 using a 1.0 min cut window.
2. Average recovery = 86% Relative Standard Deviation = 14%	2. Average recovery = 84% Relative Standard Deviation = 17%

The method, AMR 2689-93, works well and generates similar results to the improved method, AMR 2816-93. Therefore, the average recoveries and relative standard deviations for the two methods are similar. However, the improved method is more robust due to three important changes.

First, the improved method uses a potassium citrate solution instead of a sodium sulfate solution to remove DPX-PE350 from the SAX Bond Elut<sup>®</sup>. Sodium sulfate precipitates out of solution at temperatures less than about 30°C. Therefore, solutions have to be kept warm. If precipitation occurs while the sodium sulfate solution passes through the Bond Elut<sup>®</sup>, the flow through the Bond Elut<sup>®</sup> stops. The potassium citrate solution used in the improved method does not have the solubility problems that the sodium sulfate solution has.

Second, the improved method uses an injection volume of 0.1 mL instead of a 1.0 mL. In the improved method, the final volume is decreased from 5 to 0.5 mL to compensate for the 0.10 mL injection volume that is used. A 0.10 mL injection volume has the advantage of being compatible with more autosamplers than a 1.0 mL injection.

Finally, a combination of the Zorbax SB-C18 column and the phosphate buffer provide a better selectivity for DPX-PE350 and the cottonseed coextracts. The added selectivity of this mobile phase and column allows up to a 1.0 min cut window to be used. Compared to the analytical method described in AMR 2689-93 that uses a 0.4 min cut window, less coextract elutes near the retention time of DPX-PE350 using the method described in AMR 2816-93. A benefit of the longer cut window is that the retention time of DPX-PE350 does not have to be as precise from run to run.

## **EXPERIMENTAL**

### ***Equipment***

Equivalent equipment may be substituted unless otherwise indicated.

### ***Liquid Chromatograph***

HPLC System, Waters (Millipore, Inc., Milford, Mass.):

- Pump control module, Waters;
- Three pumps, Waters, Model 510;

- Photo diode array detector, Waters, Model 996;
- Millennium 2010 v1.10 software run on a NEC 486/33 computer, Waters;
- Auto injector, Waters, Model 717 equipped with a 2.5-mL syringe;
- Temperature control module, Waters;
- Column heater module, Waters; and
- Six-port switching valve, (Valco Inst., Houston, Tex., Model E60, #EC6W)

HPLC Columns

- Pre-Column 1 DuPont Zorbax® SB-CN 4.0 x 12.5 mm, 5- $\mu$  Reliance Cartridge Guard Column, #820674-916 and column end-fittings, #820529-901 (MAC-MOD Analytical Inc., Chadds Ford, Pa.), do not substitute.
- Column 1 DuPont Zorbax® SB-CN 4.6 x 150 mm, 5- $\mu$  analytical column, #883975-905 (MAC-MOD Analytical Inc., Chadds Ford, Pa.), do not substitute.
- Column 2 DuPont Zorbax® SB-C<sub>18</sub> 4.6 x 250 mm, 5- $\mu$  analytical column, #880975-902 (MAC-MOD Analytical Inc., Chadds Ford, Pa.), do not substitute.

Separatory Funnels

250-mL separatory funnel, #Z14,306-5 (Aldrich Chemical Co., Milwaukee, Wis.).

Solid-Phase Extraction Apparatus

Solid-phase extraction manifold, #5-7044M, with disposable Teflon® solvent guides, #5-7059 (Supelco, Bellefonte, Pa.).

Solid-Phase Extraction Cartridges and Adapters

20-cc SAX Mega Bond Elut® #1225-6029, 12-cc C<sub>18</sub> Mega Bond Elut® #1225-6015, and 12-20-mL Adapter #1213-1003 (Varian Sample Preparation Products, San Fernando, Calif.), do not substitute. These Bond Eluts® may be used without calibration.

Evaporator

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

Mobile Phase Filters

For pH 3, 30 mM phosphate buffer, use 0.45- $\mu$ m pore, Cat. No. HATF 047 00, Type HA filters (Millipore, Inc., Milford, Mass.). For acetonitrile, use 0.5- $\mu$ m pore, Cat. No. FHUP 047 00, Type FH filters (Millipore, Inc., Milford, Mass.). For premixed mobile phases: 28% acetonitrile/72% pH 3, 30 mM phosphate buffer and 43% acetonitrile/57% pH 3, 30 mM phosphate buffer, use 0.45- $\mu$ m pore, Cat. No. HVHP 047 00, Type HV filters (Millipore, Inc., Milford, Mass.).

Centrifuge

Sorvall® Superspeed RC2-B centrifuge equipped with a GSA 6-place, 250-mL rotor (Ivan Sorvall, Inc., Newtown, Conn.).

Centrifuge Bottles

250-mL, polypropylene, "IEC Maxiforce" VWR #21018-037;  
15-mL, Pyrex, graduated conical centrifuge tube with #13 stopper, VWR #21048-027 (VWR Scientific, Bridgeport, N.J.).

Scintillation Counter

Tracor® Analytic Mark III, Model 16881 (Liquid-Scintillation System, Elk Grove Village, Ill.).

Scintillation Fluid

DuPont Atomlight® Solution #NEF968 (NEN Research Product, Boston, Mass.).

Homogenizer

Tekmar® SKT Tissumizer® Model SDT-1810 with a Model SDT-182EN shaft and generator (Tekmar Co., Cincinnati, Ohio).

Syringes

5.0-mL disposable plastic syringe "Fortuna", Part No. Z11685-8 (Aldrich Chemical Co., Milwaukee, Wis.); Hamilton 100- and 500- $\mu$ L syringes, #80600 and #80800, respectively (Hamilton, Reno, Nev.).

pH Meter

Beckman model PHI 11 (Beckman Instruments, Inc., Fullerton, Calif.).

Balances

Allied Fisher Scientific Model 2200 analytical balance (Denver Instrument Co., Denver, Colo.).

Ultrasonic Bath

Branson Model 2200 ultrasonic bath (VWR Scientific Co., Bridgeport, N.J.).

Mixer

Vortex Genie 2 (VWR Scientific Co., Bridgeport, N.J.).

Pipettes

Pipetman #P-1000 adjustable pipette and EDP-Plus pipette #EP-10ML (Rainin, Emeryville, Calif.).

Autosampler Vials

Waters 4-mL vials #72710 with low volume glass inserts and springs #72704 (Millipore, Milford, Mass.).

**Reagents and Standards**

Equivalent reagents may be substituted for those listed below. To determine if substituted reagent impurities interfere with DPX-PE350, appropriate amounts of the solvents should be injected into the HPLC using the chromatographic conditions specified in this report for DPX-PE350.

Water

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

Potassium Phosphate, Monobasic, Crystal (KH<sub>2</sub>PO<sub>4</sub>)

Baker analyzed reagent #3246-05 (J. T. Baker, Phillipsburg, N.J.).

Dichloromethane (DCM)

EM Omni Solv®, residue grade, #DX0831-1 (EM Science, Gibbstown, N.J.).

Methanol (MeOH)

EM Omni Solv®, HPLC grade, #MX0488-1 (EM Science, Gibbstown, N.J.).

Acetonitrile (ACN)

EM Omni Solv®, HPLC grade, #AX0142-1 (EM Science, Gibbstown, N.J.).

Ammonium Carbonate ((NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>)

Baker Analyzed Reagent, reagent grade, Ammonium Carbonate #0642-01 (J. T. Baker, Inc., Phillipsburg, N.J.).

Phosphoric Acid (H<sub>3</sub>PO<sub>4</sub>), concentrated

Baker Analyzed Reagent, reagent-grade concentrated phosphoric acid, #0260-01 (J. T. Baker, Inc., Phillipsburg, N.J.).

Sodium Chloride (NaCl)

Reagent grade sodium chloride crystals, #5X04201-1 (EM Science, Gibbstown, N.J.).

50% (w/w) Sodium Hydroxide (NaOH) Solution

Baker Analyzed Reagent, reagent grade, #3727-01 (J. T. Baker, Inc., Phillipsburg, N.J.).

Sodium Azide, Practical

Baker #3V015-05 (J. T. Baker, Inc., Phillipsburg, N.J.). See warning in Special Precautions section of this report.

Potassium Citrate, Monohydrate

Baker Analyzed Reagent, potassium citrate, monohydrate 99%, #JT3066-1 (VWR Scientific, Bridgeport, N.J.).

DPX-PE350

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 DPX-PE350, NEN #2764-067, HOTC #370, 99.0% pure. Specific Activity: 70.210  $\mu\text{Ci}/\text{mg}$ . Radiolabel location: pyrimidine-2- $^{14}\text{C}$ .

Cottonseed Samples

This method was developed and validated using control cottonseed samples from Prattville, Ala. (DuPont sample number S00043136-B) obtained from DuPont Agricultural Products.

Preparation of Solutions

pH 3, 30 mM Phosphate Buffer

Add 7.21 g  $\text{KH}_2\text{PO}_4$ , 0.2 g sodium azide, and 0.40 mL of 85%  $\text{H}_3\text{PO}_4$  to 2.0 L of fresh HPLC-grade water in a beaker and dissolve the salt using a magnetic stirrer. *Note: see Special Precautions section of this report for sodium azide warning.* Adjust the pH to 3.00 with 85%  $\text{H}_3\text{PO}_4$  if the pH is higher than pH 3.00 or with 50% NaOH if the pH is lower than pH 3.00. This solution should be prepared fresh weekly and should be filtered daily before use with a 0.45- $\mu\text{m}$  pore filter.

0.01 M Ammonium Carbonate

Dissolve 0.78 g of  $(\text{NH}_4)_2\text{CO}_3$  in about 800-mL distilled water and dilute to 1.00 L in a volumetric flask. This solution should be made weekly.

1.0 M Potassium Citrate

Weigh 162.2-g potassium citrate and dissolve in 500-mL distilled water. This solution should be made weekly.

25% MeOH/75% 1.0 M Potassium Citrate

Add 125-mL MeOH to 375-mL 1.0 M potassium citrate solution. This solution should be made weekly.

pH 2.40 Water

Add concentrated  $\text{H}_3\text{PO}_4$  to 1 L of distilled water and adjust to pH 2.40 ( $\pm 0.05$ ) using a pH meter. This solution should be made weekly.

30% MeOH, pH 2.4 Water

Add 30 mL of MeOH to 70 mL of pH 2.40 ( $\pm 0.05$ ) water. A new solution should be prepared weekly.

28% ACN/72% pH 3, 30 mM Phosphate Buffer

Add 140-mL acetonitrile to 360-mL pH 3, 30 mM phosphate buffer. A new solution should be prepared weekly.

**HPLC Eluents**

Eluent A: 100% acetonitrile;

Eluent B: 100% pH 3, 30 mM phosphate buffer;

Eluent C: 100% Milli-Q<sup>®</sup> water.

**Standards**

Use class A volumetric flasks when preparing standard solutions.

Stock Standard Solution

Prepare a standard stock solution by accurately weighing 10 mg of DPX-PE350 into a 100-mL volumetric flask on an analytical balance. Record the weight 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 2 months stored at room temperature when covered with aluminum foil.

Intermediate Standard Solution

Prepare an intermediate standard solution by pipetting 1.00 mL of the 100- $\mu\text{g}/\text{mL}$  DPX-PE350 stock standard into a 100-mL volumetric flask. Bring to volume using HPLC-grade methanol. The concentration of this solution is 1- $\mu\text{g}/\text{mL}$  DPX-PE350 in methanol. This standard solution is stable for 2 months stored at room temperature when covered with aluminum foil.

Chromatographic Standard Solutions

The 1- $\mu\text{g}/\text{mL}$  DPX-PE350 in methanol fortification solution is used to make the following standards.

Prepare an 800-ng/mL DPX-PE350 standard by pipetting 8.00 mL of the fortification standard (1  $\mu\text{g}/\text{mL}$ ) into a 10-mL volumetric flask. Evaporate the methanol (to dryness) using an N-Evap. Add 28% acetonitrile/72% pH 3, 30 mM phosphate buffer to the volumetric flask and dilute to 10.00 mL.

Prepare a 400-ng/mL DPX-PE350 standard by pipetting 4.00 mL of the fortification standard (1  $\mu\text{g}/\text{mL}$ ) into a 10-mL volumetric flask. Evaporate the methanol (to dryness) using an N-Evap. Add 28% acetonitrile/72% pH 3, 30 mM phosphate buffer to the volumetric flask and dilute to 10.00 mL.

Prepare a 200-ng/mL DPX-PE350 standard by pipetting 2.00 mL of the fortification standard (1  $\mu\text{g}/\text{mL}$ ) into a 10-mL volumetric flask. Evaporate the methanol (to dryness) using an N-Evap. Add 28% acetonitrile/72% pH 3, 30 mM phosphate buffer to the volumetric flask and dilute to 10.00 mL.

Prepare a 100-ng/mL DPX-PE350 standard by pipetting 1.00 mL of the fortification standard (1  $\mu\text{g}/\text{mL}$ ) into a 10-mL volumetric flask. Evaporate the methanol (to dryness) using an N-Evap. Add 28% acetonitrile/72% pH 3, 30 mM phosphate buffer to the volumetric flask and dilute to 10.00 mL.

Prepare a 50-ng/mL DPX-PE350 standard by pipetting 0.50 mL of the fortification standard (1  $\mu\text{g}/\text{mL}$ ) into a 10-mL volumetric flask. Evaporate the methanol (to dryness) using an N-Evap. Add 28% acetonitrile/72% pH 3, 30 mM phosphate buffer to the volumetric flask and dilute to 10.00 mL.

Prepare a 30-ng/mL DPX-PE350 standard by pipetting 0.30 mL of the fortification standard (1  $\mu\text{g}/\text{mL}$ ) into a 10-mL volumetric flask. Evaporate the methanol (to dryness) using an N-Evap. Add 28% acetonitrile/72% pH 3, 30 mM phosphate buffer to the volumetric flask and dilute to 10.00 mL.

Fortification Standard Solutions

The 1- $\mu\text{g}/\text{mL}$  intermediate standard solution is used for all fortifications of samples analyzed by HPLC.

Analytical Procedure

Storage and Preparation of Samples

Control cottonseed samples are stored at 4°C or frozen until analysis. Frozen samples should be thawed and thoroughly mixed before use.

Cottonseed Fortification Procedure

Fortify selected 5-g whole cottonseed samples with an appropriate amount of the fortification standard solution. For HPLC method validation, samples were fortified at a level shown in the following table:

<u>Volume of Standard</u>	<u>Standard Conc.</u>	<u>Sample Weight</u>	<u>Fortification Level</u>
<u>(<math>\mu\text{L}</math>)</u>	<u>(<math>\mu\text{g}/\text{mL}</math>)</u>	<u>(g)</u>	<u>(ppm)</u>
50	1.0	5.0	0.010
150	1.0	5.0	0.030
500	1.0	5.0	0.100

Extraction Procedure for Cottonseed

Figure 1 shows a flow diagram of the extraction procedure for cottonseed.

1. Weigh 5 g ( $\pm 0.1$  g) of sample into a 250-mL polypropylene centrifuge bottle and fortify. *Record weights for each sample.*
2. Fortify (if applicable) by adding "X"  $\mu\text{L}$  of the 1- $\mu\text{g}/\text{mL}$  intermediate stock solution (see the table on the previous page for the amount added to the cottonseed). *Record the volume of the fortification standard used.*
3. After fortification, using a graduated cylinder add 100.0 mL ( $\pm 0.6$  mL) of 2:1 acetonitrile (ACN):0.01 M  $(\text{NH}_4)_2\text{CO}_3$ . *Record the volume of 2:1 extraction solvent used.*
4. Homogenize each sample for 3 min with the Tissumizer®.

5. After homogenizing the cottonseed, rinse the Tissumizer® probe with 10.0 mL ( $\pm 0.6$  mL) of the 2:1 ACN:0.01 M  $(\text{NH}_4)_2\text{CO}_3$  solution. *Record the volume of 2:1 solution used to rinse probe.* Use a clean probe for each sample that is homogenized (see *Cleaning Procedures* section of this report).
6. Centrifuge the sample for 15 min at room temperature at 3,000 RPM (1525-g relative centrifugal force).
7. Pour 60.0 mL ( $\pm 0.6$  mL) of sample extract from the centrifuge bottle into a graduated cylinder. Use care when pouring so that only the liquid is transferred to the graduated cylinder. *Record the volume of extract poured into the graduated cylinder.*
8. Add the 60 mL of extract to a 250-mL separatory funnel.
9. Add 50-mL dichloromethane (DCM) to the separatory funnel and shake for 1 minute.
10. Discard the bottom layer (the DCM layer). Keep the aqueous phase in the separatory funnel. If an emulsion persists after waiting 15 min, retain it in the separatory funnel with the aqueous phase.
11. Add 0.05-g NaCl to each separatory funnel.
12. Add 50-mL DCM to each separatory funnel and shake for 1 minute.
13. Discard the lower phase and keep the upper phase in the separatory funnel.
14. Condition each SAX Bond Elut® column to be used by passing 20-mL MeOH followed by 20 mL of distilled water and then 20-mL 0.01 M  $(\text{NH}_4)_2\text{CO}_3$  through the columns. *Do not allow the SAX columns to dry after conditioning them.*
15. Add approximately 10 mL of the extracted samples to the SAX columns by draining the separatory funnels into the Bond Elut® cartridges.
16. Allow the extract to pass through the SAX column at a rate of about 2 mL/min and add the rest of the extract to the SAX column.
17. Rinse the separatory funnels with 5 mL of 0.01 M  $(\text{NH}_4)_2\text{CO}_3$ .
18. Add the rinsate to the respective SAX column.
19. Condition each C<sub>18</sub> Bond Elut® column to be used by passing 5 mL of MeOH, followed by 10 mL of distilled water and then 20 mL of 25% MeOH in 1 M potassium citrate

through the columns. Leave about 10 mL of the 25% MeOH in 1 M potassium citrate solution above the top of the C<sub>18</sub> packing and stop the flow through the C<sub>18</sub> columns. This prevents the C<sub>18</sub> Bond Elut<sup>®</sup> from drying before eluent from the SAX column begins to drip into the C<sub>18</sub> column in the following step.

20. Connect the SAX columns to the top of the C<sub>18</sub> columns using the #1213-1003 adapter.
  21. Elute DPX-PE350 from the SAX column to the C<sub>18</sub> column by passing 20 mL of 25% MeOH in 1 M potassium citrate through the stacked Bond Eluts<sup>®</sup>.
  22. Allow the Bond Eluts<sup>®</sup> to dry by passing air through them for at least 10 min to dry off adsorbed water.
  23. Dispose of the SAX Bond Eluts<sup>®</sup> and take the connector off of the top of the C<sub>18</sub> Bond Eluts<sup>®</sup>.
  24. Rinse the C<sub>18</sub> Bond Eluts<sup>®</sup> with 10-mL distilled water, 10 mL of pH 2.4 water and then 10-mL 30% MeOH in pH 2.4 water.
  25. Dry the C<sub>18</sub> columns by allowing air to pass through them for 15 minutes.
  26. Elute DPX-PE350 from the C<sub>18</sub> Bond Elut<sup>®</sup> by eluting with 10-mL MeOH into 15-mL Pyrex centrifuge tubes.
  27. Using an N-Evap<sup>®</sup> at 50°C, evaporate samples to dryness.
- The procedure may be stopped at this point and continued later. Samples extracted in this fashion and cleaned up through the Bond Eluts<sup>®</sup> should be stored in a refrigerator at approximately 4°C until analyzed. Samples stored as described are stable for at least 2 weeks.
28. Add 0.5 mL of 28% ACN/72% pH 3, 30 mM phosphate buffer to the containers holding the DPX-PE350 samples.  
*Record final volumes.*
  29. Mix the samples with a Vortex<sup>®</sup> mixer for approximately 5 seconds and sonicate for 15 minutes.

When filling the auto sampler tray, every 2-3 samples are bracketed by a standard. Although sample carry-over from one sample to another was not observed, samples analyzed for this report were run from lowest to highest concentration.

For samples having unknown concentrations, sample vials should be loaded on the auto sampler in a nonsystematic fashion. Samples should be intermixed with standards (bracket every 2-3 samples with a standard). The levels of standards should be

selected to bracket the expected DPX-PE350 levels (if known) in the samples analyzed.

Liquid Chromatography

The HPLC system components have already been described in the *Equipment* section of this report. Common conditions for the HPLC method are shown in the following table:

Wavelength	254 nm
Column Temperature	40.0°C
Injection Volume	0.10 mL
Mobile Phase A	100% ACN
Mobile Phase B	100% pH 3, 30 mM phosphate buffer
Mobile Phase C	100% Milli-Q® water

Isocratic multi-dimensional HPLC was used with the columns listed in the *Equipment* section of this report. (For a review of multi-dimensional, column-switching HPLC, see References 3 and 4). A diagram of the column-switching valve arrangement is shown in Figure 2, where Column I and Column II are Zorbax® SB-CN and Zorbax® SB-C18 analytical columns, respectively. See the *Special Precautions* section of this report for a discussion of steps required prior to initial use of these HPLC columns.

With the valve in Position 1, the effluent from Column I leaves the column through the valve, enters a bypass loop, flows back through the valve, and then flows to the detector. With the valve in Position 2, the effluent from Column I goes (via the valve) to Column II, back to the valve, and then to the detector. *All tubing connecting the switching valve to the analytical columns and detector should be 0.005-inch internal diameter tubing to minimize dead volume.*

Before injection, the valve is put in Position 1, so that the HPLC flow bypasses Column II. Pump 28% ACN/72% pH 3, 30 mM phosphate buffer at 1.0 mL/min through Column I only. When DPX-PE350 starts to elute from Column I, the valve is switched to Position 2 in order to trap the peak on Column II. After the peak is collected at the head of Column II, the valve is switched back to Position 1.

Since cottonseed matrix affects the pre-column (guard column), a new pre-column should be conditioned by injecting 0.10 mL of extracted, cleaned-up cottonseed extract as described in the *Special Precautions* section of this report.

Valve-switching times (the "time window" or "cut window") are set at  $\pm 0.50$  min around the average retention time for three DPX-PE350 standards on Column I only. The retention time (through Column I) relative standard deviation ( $RSD = 100 * \text{Std. Dev.}/\text{Avg.}$ ) for the standards injected should be no greater than 3%.

A shorter time window (<1 min and >0.4 min) may be required for some cottonseed samples. If separations of extracted and cleaned-up unfortified cottonseed samples (cottonseed with no DPX-PE350 added) have interfering peaks at the retention time of DPX-PE350, then the time window should be made shorter. The required time window is determined immediately before the analysis of a sample set is started. A sample set may consist of unfortified and fortified samples, as well as treated samples.

To assure the time window is adequate for an entire sample set, no more than 12-15 runs (including standards) should be made before reevaluating the average retention time of DPX-PE350. When the average retention time is being determined, the peak shape of DPX-PE350 should be observed. If the peak tails badly or recoveries are poor, the pre-column and/or analytical column may need to be replaced before further analysis (see *Special Precautions* section of this report). For this work, the pre-columns needed to be replaced after approximately twelve injections of cottonseed extract samples (not including standards).

The mobile phase used to determine the average retention time of the standards is 28% ACN/72% pH 3, 30 mM phosphate buffer at a flow rate of 1 mL/min. A column temperature of 40°C is used. Using this mobile phase composition, typical DPX-PE350 peak widths for standards injected ranged from 0.5 to 0.7 min, depending on the SB-CN column used and the DPX-PE350 retention time on the SB-CN column. The DPX-PE350 standards had a capacity factor of about 4 ( $k' \approx 4$ ) using the above stated conditions.

The following column-switching routine is used to separate DPX-PE350 from coextracted compounds (see Figure 2 and Tables I.A and I.B). A 0.10-mL sample is injected into Column I. The initial mobile-phase concentration is 28% ACN/72% pH 3, 30 mM phosphate buffer at a flow rate of 1 mL/min. At the beginning of the determined time window (the time window is typically about 8 to 10 min from the point of injection), the valve is switched from Position 1 to Position 2 and DPX-PE350 is transferred to Column II. At the end of the time window, the valve is switched from Position 2 to Position 1. The solvent delay time is about 1.8 min on Column I.

After DPX-PE350 is trapped on Column II and the valve is switched back to Position 1, the mobile phase is changed from 28% ACN/72% pH 3, 30 mM phosphate buffer to 75% ACN/25% Milli-Q® water, and the flow rate is increased from 1 to 2.0 mL/min, to quickly clean off Column I (a 5-min wash). After cleaning Column I, the column is conditioned 10 min with 43% ACN/57% pH 3, 30 mM phosphate buffer at 2.0 mL/min (through Column I only). Column I is then reequilibrated 1 min at 1.0 mL/min for 1 min using this mobile-phase composition. Following these steps, Column I is in the correct mobile phase to complete the analytical separation on Column II.

After setting Column I at Column II conditions, the valve is switched to Position 2, to elute DPX-PE350 from Column II using the 43% ACN/57% pH 3, 30 mM phosphate buffer mobile phase. DPX-PE350 elutes from Column II at a retention time of about 37 min from the start of the run. After DPX-PE350 elutes from Column II, 75% ACN/25% Milli-Q® water is passed through both columns at 1 mL/min for 15 min to clean off Column II. Then, 43% ACN/57% pH 3, 30 mM phosphate buffer is passed through both columns at 1 mL/min to set Column II at the conditions required for the next separation. The valve is switched to Position 1 and 28% ACN/72% pH 3, 30 mM phosphate buffer is passed through Column I only at 2 mL/min for 5 min. The flow rate is reduced to 1 mL/min and the system is allowed to run for another one minute. At this time, Column I and Column II are both ready for the next injection.

#### <sup>14</sup>C Method Validation

Six, 5-g cottonseed samples were fortified with [<sup>14</sup>C]DPX-PE350 standard according to the following table:

<u>Volume of Standard</u> <u>(<math>\mu</math>L)</u>	<u>Standard Conc.</u> <u>(<math>\mu</math>g/mL)</u>	<u>Sample Weight</u> <u>(g)</u>	<u>Fortification Level</u> <u>(ppm)</u>
50	0.99	5.0	0.010
150	0.99	5.0	0.030
500	0.99	5.0	0.099

After allowing the fortified samples to stand for 72 hours at room temperature, they were extracted using Steps 3 through 29 of the *Extraction Procedure* of this report with the following exceptions.

In Steps 19 and 21, a 20% MeOH in 1 M Na<sub>2</sub>SO<sub>4</sub> at pH 3 solution was used to elute the DPX-PE350 off of the SAX column, and in Step 28, a 5-mL final volume was used. A 0.5-mL aliquot of each sample was taken from the 5.0-mL final extract (from Step 30 of the *Extraction Procedure* section of this report) solution and added to a separate labeled scintillation vial. Ten milliliters of scintillation counting fluid were added to the 0.5-mL extract aliquot.

A blank sample consisting of 10 mL of scintillation fluid was analyzed for each sample set. The counts from the blank were subtracted from the fortified samples and the percent extraction efficiency was calculated. Two standards for the extraction efficiency study were prepared by taking 0.050 and 0.500 mL of 0.99 µg/mL [<sup>14</sup>C]DPX-PE350 and adding these amounts to 10 mL of scintillation fluid.

### **Calculations**

For this report, chromatographic peak heights were measured and the amounts of DPX-PE350 found were determined with a Millennium 2010 v1.10 automated integration and external standard quantitation method. The process used is briefly discussed below and examples are shown.

Known concentrations (ng/mL) for the external standards used were entered into the Millennium database by the HPLC operator. A Millennium automated method was used to determine peak heights for DPX-PE350 peaks. Millennium software was used to plot the response (peak heights) vs. the amount (concentration in ng/mL) of the standards.

The amount of DPX-PE350 in a sample was found using a calibration curve. To accomplish this, Millennium software generated a multi-level, linear least squares fit calibration curve for the data. The equation for the line is  $y = mx + b$ , where  $y$  is the peak height,  $x$  is the amount (ng/mL) of DPX-PE350,  $m$  is the slope of the line, and  $b$  is the  $y$ , (ordinate) intercept. The solution to the equation for this line gives the amount of DPX-PE350 found (ng/mL) as a function of the peak height.

Millennium software gives a correlation coefficient and values for the slope and intercept in the resulting printouts. It uses a different notation for slope and intercept: the slope = A1, and the  $y$  intercept = A0.

Concentration of the Analyte in the Sample (ppm found)

The parts per million (ppm) DPX-PE350 found in cottonseed is given by:

$$\text{ppm found} = \frac{(A)(FV)}{(W)(AF)(1000 \text{ ng} / \mu\text{g})}$$

where  $A$  is the amount, ng/mL, of DPX-PE350 found,  $FV$  is the sample final volume, mL,  $W$  is the weight, g, of the cottonseed extracted, and  $AF$  is the aliquot factor (60 mL/110 mL in all cases). The aliquot factor takes into account the fact that only part of the extract (see Steps 3-7 in the *Extraction Procedure* of this report) was cleaned-up before HPLC analysis.

Fortification Level (ppm)

The fortification level (ppm) is the amount of DPX-PE350 that is applied to cottonseed. This is given by:

$$\text{ppm fortification level} = \frac{(VS)(CS)}{W}$$

where  $VS$  is the volume of the fortification standard that was pipetted onto the cottonseed,  $CS$  is the concentration of the fortification standard, and  $W$  is the sample weight that was fortified.

Recovery

The recovery is given by

$$\% \text{ Recovery} = 100 \left( \frac{\text{ppm found}}{\text{ppm fortification level}} \right)$$

Sample Calculations

Using the data for the Spike 1 cottonseed sample that was extracted August 10 (See Data Sheet 1), the following sample calculations were prepared:

$$\begin{aligned} \text{ppm found} &= \frac{(36.4 \text{ ng} / \text{mL})(0.50 \text{ mL})}{(5.07 \text{ g})(60.0 \text{ mL} / 110 \text{ mL})(1000 \text{ ng} / \mu\text{g})} \\ &= 0.0066 \text{ ppm} \end{aligned}$$

$$\begin{aligned}\text{ppm fortification level} &= \frac{(0.0500 \text{ mL})(1.05 \mu\text{g} / \text{mL})}{5.07 \text{ g}} \\ &= 0.0104 \text{ ppm}\end{aligned}$$

$$\% \text{ Recovery} = 100 \frac{(0.0066 \text{ ppm})}{(0.0104 \text{ ppm})} = 63\%$$

#### Extraction Efficiency

$$\% \text{ Extraction Efficiency} = 100 \frac{(\text{Raw - Blank Counts, dpm})(\text{Final Volume, mL})(\text{Aliquot Factor})}{(\text{Standard counts, dpm})(\text{Volume Sampled, mL})}$$

where the aliquot factor is the initial sample volume divided by the aliquot volume of the sample (110 mL/60 mL in all cases).

*Example - (Spike 5 in Data Sheet 4 of Appendix)*

$$\% \text{ Extraction Efficiency} = 100 \left[ \frac{(3503 \text{ dpm})(5.0 \text{ mL})(110 \text{ mL} / 60 \text{ mL})}{(0.500 \text{ mL})(158534 \text{ dpm} / \text{mL})(0.50 \text{ mL})} \right] = 81\%$$

#### Method Quantitation Limit (MQL) Determination

The method quantitation limit (MQL) of DPX-PE350 in cottonseed is 0.010 ppm which is 3 to 5 times the average chromatographic noise. The chromatographic noise is the combination of instrumental noise from the LC pump and detector, and the background signal from the cottonseed coextracts eluting at the retention time of DPX-PE350. Cottonseed samples were fortified at the 0.010 ppm level, and then extracted and analyzed.

#### Special Precautions

- A. **Warning-** Sodium azide is, combustible, shock sensitive and can explode. Sodium azide is an extreme health hazard and should not be swallowed, inhaled, or absorbed through the skin and should be disposed of properly. Read the material safety data sheet for this compound before use.

Sodium azide is used in this method as a growth inhibitor for bacteria. It does not need to be used if the phosphate buffer is filtered daily, prepared fresh weekly and stored in the dark. If sodium azide is not used, the solvent lines from the reservoir to the pump should be purged with acetonitrile weekly to control bacterial growth.

- B. Mobile phases should be thoroughly degassed daily. Solvents are degassed by filtering them through a Millipore® vacuum filtering apparatus while sonicating the apparatus. If a low pressure mixing HPLC is used, mobile phases should be sparged at 30 mL/min.
- C. If new analytical columns are used or if columns have not been used for a day or more, they are conditioned in the following way. First, 100% ACN is passed through both columns at 1 mL/min. The baseline is monitored during this process. After achieving a stable baseline, further conditioning is performed to set the columns in the mobile phases for the analysis.

Second, the columns are conditioned in the mobile phases that are used in the analysis. Column II (the SB-C18 column) is conditioned with 43% acetonitrile/57% pH 3, 30 mM phosphate buffer for 30 min at 1 mL/min by passing the mobile phase through both columns. Then, the valve is switched to Position 1 and Column I is conditioned with 28% acetonitrile/72% pH 3, 30 mM phosphate buffer for 5 min at 2 mL/min.

After conditioning the columns, the auto sampler is purged with 28% acetonitrile/72% pH 3, 30 mM phosphate buffer for 3 min at 1 mL/min.

- D. Injection of cottonseed extract into the guard and SB-CN analytical column affects the peak shape and may affect the retention time of DPX-PE350. When a new guard is installed, condition it by injecting an extracted and cleaned-up cottonseed sample.

To condition the guard column, assure that the guard column and Column I are connected in series. Inject 0.1 mL of the control cottonseed extract sample that has been cleaned up using Steps 1-30 in the extraction procedure and allow it to pass through the guard column and Column I using a 28% ACN/72% pH 3, 30 mM phosphate buffer mobile phase. Allow the mobile phase to run for 10 min at 1 mL/min. The guard column and Column I are then cleaned by passing 75% ACN/25% Milli-Q® water through them for 5 min at 2 mL/min. *If the guard column is not conditioned with cottonseed, the cut window established by injecting standards may miss the front of the DPX-PE350 peak in samples injected after the first cottonseed extract is injected.*

If peak tailing is observed when standards elute from the SB-CN column, the SB-CN guard column is replaced. This

usually occurs after about 12 injections of cottonseed extract (not including standards).

The following procedure is followed when a pre-column is replaced. After the pre-column is replaced, it is purged with 100% ACN for 5 min at 2 mL/min before connecting the pre-column to the analytical column. After conditioning the pre-column, the pre-column and SB-CN analytical column are purged with 100% ACN for 5 min at 2 mL/min and the column oven chamber is reequilibrated to 40.0°C. Then, the pre-column and analytical column are conditioned with 28% ACN/72% pH 3, 30 mM phosphate buffer for 5 min at 2 mL/min. Finally, the pre-column is conditioned with cottonseed extract as already explained.

- E. In some circumstances, depending on the HPLC instrumentation, premixed solvents may be required to generate reproducible retention times. Relative standard deviations for DPX-PE350 standards injected and detected after eluting from Column I to establish the cut window should not be greater than 3%.
- F. Procedural standards (extraction solvent fortified with DPX-PE350 without the sample matrix, but containing all reagents and using all analysis procedures) will probably fail to show adequate recoveries. Sample matrix is important for a successful liquid-liquid extraction in Steps 9 and 12 of the *Extraction Procedure for Cottonseed*.

#### ***Cleaning Procedures***

All glassware can be cleaned by means of any approach that is consistent with trace organic analysis.

Generally, the following is done. Glass items are initially rinsed with acetone (technical grade), followed by a thorough scrubbing with an aqueous soap solution (prepared in tap water). Then, glassware is rinsed with tap water, followed by another acetone rinse. Tekmar® Tissumizer® probes were disassembled according to the manufacturer's directions and cleaned by the above procedure.

## RESULTS AND DISCUSSION

### *Detector Response*

DPX-PE350 has an absorbance maximum at 254 nm and this wavelength is used in this method. Table II shows the results for a series of standards that were injected. The UV detector response at 254 nm was linear over the range of standards analyzed, 30-800 ng/mL, (see Figure 3).

### *Sample Chromatograms*

Figure 4 shows a chromatogram from a cottonseed sample using the column-switching method described in the *Experimental* section of this report that was fortified at the 0.020-ppm level. This figure has the cut time (i.e., time window) and column clean-up and analysis times labeled. Figures 5-9 show typical chromatograms resulting from 0.10-mL injections of standards, and cleaned-up control and fortified cottonseed samples.

### *Recoveries of Cottonseed by HPLC/UV Analysis*

After extraction, cottonseed extracts fortified with DPX-PE350 were analyzed by HPLC using UV detection. The data, from which recoveries were calculated, are found in the Appendix (Data Sheets 1-3).

Tables III and IV summarize recovery data obtained for cottonseed fortified with DPX-PE350. Recoveries were acceptable over the fortification range employed (0.01-0.1 ppm).

An error that could have caused a 45% recovery for a cottonseed sample fortified at 0.0210 ppm could not be determined. The result is an outlier for the 0.0210 ppm fortification level. The 45% recovery can be rejected from the 0.0210 ppm fortification level data with a 5% risk of a false rejection (see the Dixon Test in Reference 5). Including the outlier, the average recovery for the 0.0210 ppm fortified samples is 66% (Std. Dev. = 14%) giving an RSD of 22%. Not including the outlier, the average recovery for the 0.0210 ppm fortified samples is 73% (Std. Dev. = 3%) giving an RSD of 4%.

Not including the outlier, the average recovery ( $\pm$  Std. Dev.) for all of the fortified samples at all levels was  $84 \pm 14\%$ ; the relative standard deviation was 17%. This result is in good agreement with the  $86 \pm 12\%$  ( $\pm$  Std. Dev.) recoveries and an RSD of 14% reported in AMR 2689-93.

The method quantitation limit (MQL) was found to be 0.010 ppm. The MQL is based on a signal 3 to 5 times the chromatographic noise of cottonseed fortified at 0.010 ppm and then extracted using the *Extraction Procedure for Cottonseed* and then analyzed by HPLC. Recoveries were adequate for the three samples that were fortified, extracted, and analyzed at the 0.010-ppm level.

A plot of measured *vs.* known ppm for all of the samples analyzed by HPLC/UV is shown in Figure 10. This plot demonstrates the variability of the recoveries at the various fortification levels.

#### ***<sup>14</sup>C Method Validation***

In order to demonstrate the validity of this analytical method, control cottonseed was fortified with [<sup>14</sup>C]DPX-PE350, stored 72 hours at room temperature, and then extracted and analyzed using the procedures described in the *Experimental* section of this report. Standards for the fortification levels were prepared by spiking scintillation cocktail with an appropriate amount of [<sup>14</sup>C]DPX-PE350 (see Data Sheet 4 in Appendix). As can be seen in Table V, acceptable extraction efficiency for the 6 samples was demonstrated (mean extraction efficiency was  $83 \pm 3$  % with % RSD = 4%). Data for this study are found in the Appendix (see Data Sheet 4).

#### ***Timing***

The time required for sample preparation and analysis was dependent on the number of samples being analyzed at one time. Normally, six samples were run, taking 6-8 hours for sample clean-up. HPLC automated runs required 75 min per sample to complete; thus, six samples required 7.5 hours.

#### ***Method Ruggedness***

The method uses reversed-phase liquid chromatography with UV detection, both of which are well understood and known to be stable and reliable. A variety of different lots of columns (pre-column, Column I, and Column II) were used and very little change in the HPLC method was needed. Also, different batches of Bond Eluts® were used successfully during the development of this method.

Optimizing the clean-up and the equilibration times for the SB-CN column is very important. If the equilibration times for Columns I and II are too short, the baseline drifts and retention

times shift. Zorbax® SB-CN and SB-C<sub>18</sub> columns should be used to assure that this method works as described and so that column stability is satisfactory at the mobile-phase pH used.

### **CONCLUSIONS**

This analytical method is suitable for the measurement of DPX-PE350 extracted from cottonseed at an MQL of 0.010 ppm and it meets the criteria put forth in EPA Subdivision O, 171-4 Pesticide Assessment Guidelines. In addition, this method has been designed to be run on relatively simple, conventional, and commercially available HPLC equipment (auto sampler, UV detector; and switching valve). Automation allows unattended analysis.

**ACKNOWLEDGMENTS**

Helpful discussions during the development of this method with Robert Dietrich are greatly appreciated. The method is more rugged thanks to his encouragement and suggestions.

**CERTIFICATION**

**IMPROVED ANALYTICAL ENFORCEMENT METHOD FOR THE DETERMINATION OF KIH-2031 (DPX-PE350) RESIDUES IN COTTONSEED USING COLUMN-SWITCHING LIQUID CHROMATOGRAPHY**

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.

**Report by:**

Sheldon R. Sumpter  
Sheldon R. Sumpter  
Study Director

28 Jan 1994  
Date

**Approved by:**

Sidney S. Goldberg  
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28-Jan-94  
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**TABLES I.A AND I.B  
TYPICAL HPLC PUMP AND COLUMN-SWITCHING TIMING SEQUENCE**

Table I.A. Times and Values for Column Switching

#	Time (min)	Event	Function	Explanation
1	0.00	Event 3	On	Start run through Column I only
2	0.00	Event 4	Off	
3	7.89	Event 4	On	Start column switch; DPX-PE350 is transferred
4	7.89	Event 3	Off	
5	8.89	Event 3	On	End-column switch; Clean Column I
6	8.89	Event 4	Off	
7	28.00	Event 4	On	Start analytical separation on Column II
8	28.00	Event 3	Off	
9	58.90	Event 3	On	Set Column I to initial conditions
10	58.90	Event 4	Off	

The Waters pump control module has four external contact closure (TTL to GND) events that are activated using the Millennium 2010 software. The values of Event 3 and Event 4 (on and off times) control the Valco column switching valve: Event 3 off, Event 4 on = valve in Position 1; Event 3 on, Event 4 off = valve in Position 2. The Valco valve wiring is hooked up in the following way to the pump control module: red coated wire to Event 3, black coated wire to Event 4, and green coated wire to a Waters 12-V power supply negative position. If both events are turned on at the same time, the valve continues to rotate; therefore, flow through the system stops.

Table I.B. Times and Values of Mobile-Phase Mixing and Flow Rate Using the Waters Pump Control Module (PCM)

#	Time (min)	Flow (mL/min)	%A	%B	%C	Curve Type	Explanation
1	0.00	1.00	28.0	72.0	0.0	0	Start analysis on Column I only
2	12.00	2.00	75.0	0.0	25.0	11	Clean off Column I
3	17.00	2.00	43.0	57.0	0.0	11	Set Column I to Column II cond.
4	27.00	1.00	43.0	57.0	0.0	11	Set proper flow rate
5	39.00	1.00	75.0	0.0	25.0	11	Clean off Column II
6	54.00	1.00	43.0	57.0	0.0	11	Set Column II at initial cond.
7	69.00	2.00	28.0	72.0	0.0	11	Set Column I at initial cond.
8	74.00	1.00	28.0	72.0	0.0	11	Set at initial flow rate

Curve Type 0 on the Waters HPLC system is the starting condition for the analysis. Curve Type 11 on the Waters HPLC system is a step gradient that begins at the specified time. Mobile phases A, B, and C are 100% ACN and 100% pH 3, 30 mM phosphate buffer, and Milli-Q® water, respectively.

**TABLE II**  
**DATA FOR SERIES OF DPX-PE350 STANDARDS**

Peak Heights and Retention Times for DPX-PE350 Standards  
from Data Sheet Number 3 in the Appendix

<u>Conc.</u> <u>(ng/mL)</u>	<u>Peak Height</u> <u>(<math>\mu</math>V)</u>	<u>Retention Time</u> <u>(min)</u>
30.0	785	36.915
50.0	757	36.923
100.0	1518	36.918
200.0	3097	36.933
400.0	6175	36.922
800.0	12213	36.908

**TABLES III.A, III.B, AND III.C  
RECOVERIES AT THREE FORTIFICATION LEVELS USING HPLC/UV ANALYSIS**

Table III.A. Recoveries for cottonseed spiked at 0.010 ppm level with DPX-PE350

Name of Sample	Data Sheet	Fortification Level (ppm)	Peak Height ( $\mu$ V)	ppm Found	Recovery (%)
Spike 1, 9 Aug	1	0.0104	630	0.0066	63
Spike 2, 9 Aug	1	0.0104	801	0.0084	81
Spike 3, 9 Aug	1	0.0105	963	0.0102	97

Average Recovery = 80% (Std. Dev. = 17%) RSD = 21%.

Table III.B. Recoveries for cottonseed spiked at 0.021 ppm with DPX-PE350

Name of Sample	Data Sheet	Fortification Level (ppm)	Peak Height ( $\mu$ V)	ppm Found	Recovery (%)
Spike 1, 10 Aug	2	0.0210	1593	0.0158	75
Spike 2, 10 Aug	2	0.0210	1047	0.0094	45*
Spike 1, 16 Aug	3	0.0210	1409	0.0157	75
Spike 2, 16 Aug	3	0.0210	1322	0.0147	70

\* The 45% recovery is an outlier in this group. Including the outlier, the average recovery is 66% (Std. Dev. = 14%) giving an RSD of 22%. Not including the outlier, the average recovery is 73% (Std. Dev. = 3%) giving an RSD of 4%. The 45% recovery can be rejected from the 0.0210 ppm fortification level data with a 5% risk of a false rejection (see the Dixon Test in Reference 5).

Table III.C. Recoveries for cottonseed spiked at 0.10 ppm with DPX-PE350

Name of Sample	Data Sheet	Fortification Level (ppm)	Peak Height ( $\mu$ V)	ppm Found	Recovery (%)
Spike 3, 10 Aug	2	0.104	9137	0.103	99
Spike 4, 10 Aug	2	0.104	9557	0.108	104
Spike 3, 16 Aug	3	0.104	7463	0.0888	85
Spike 4, 16 Aug	3	0.100	8280	0.0989	94

Average Recovery = 96% (Std. Dev. = 8%) RSD = 8%.

**TABLES IV.A AND IV.B  
RECOVERIES USING HPLC/UV ANALYSIS**

Table IV.A. Recoveries for cottonseed spiked with DPX-PE350

<u>Name of Sample</u>	<u>Data Sheet</u>	<u>Fortification Level (ppm)</u>	<u>Peak Height (<math>\mu</math>V)</u>	<u>ppm Found</u>	<u>Recovery (%)</u>
Spike 1, 9 Aug	1	0.0104	630	0.0066	63
Spike 2, 9 Aug	1	0.0104	801	0.0084	81
Spike 3, 9 Aug	1	0.0105	963	0.0102	97
Spike 1, 10 Aug	2	0.0210	1593	0.0158	75
Spike 2, 10 Aug	2	0.0210	1047	0.0094	45*
Spike 1, 16 Aug	3	0.0210	1409	0.0157	75
Spike 2, 16 Aug	3	0.0210	1322	0.0147	70
Spike 3, 10 Aug	2	0.104	9137	0.103	99
Spike 4, 10 Aug	2	0.104	9557	0.108	104
Spike 3, 16 Aug	3	0.104	7463	0.0888	85
Spike 4, 16 Aug	3	0.100	8280	0.0989	94

\* As shown in Table III.B, the poor recovery for this sample indicates that it is an outlier compared to the recoveries for the other samples. The average recovery for all of these samples, not including the outlier, is 84% (Std. Dev. = 14%) and the relative standard deviation is 17%. Including this sample, the average recovery for all of the samples is 81% (Std. Dev. = 18%) and the relative standard deviation is 22%.

Table IV.B. Peak height at retention time of DPX-PE350 for cottonseed control samples

<u>Name of Sample</u>	<u>Data Sheet</u>	<u>Peak Height (<math>\mu</math>V)</u>
Control 1, 9 Aug	1	LBN
Control 2, 9 Aug	1	917*
Control 3, 9 Aug	1	LBN
Control 1, 10 Aug	2	LBN
Control 2, 10 Aug	2	LBN
Control 1, 16 Aug	3	LBN
Control 2, 16 Aug	3	LBN

LBN = Level of Baseline Noise.

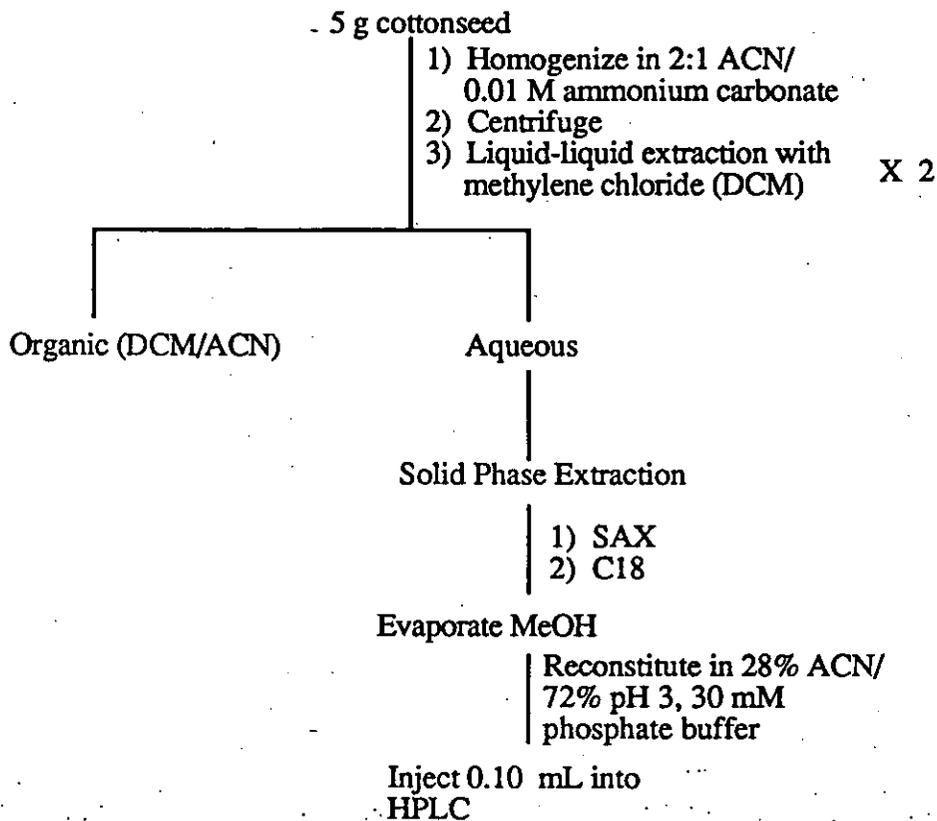
\*This control sample was the only one that had an interfering peak near the retention time of DPX-PE350.

**TABLE V**  
**EXTRACTION EFFICIENCY OF [<sup>14</sup>C]DPX-PE350**

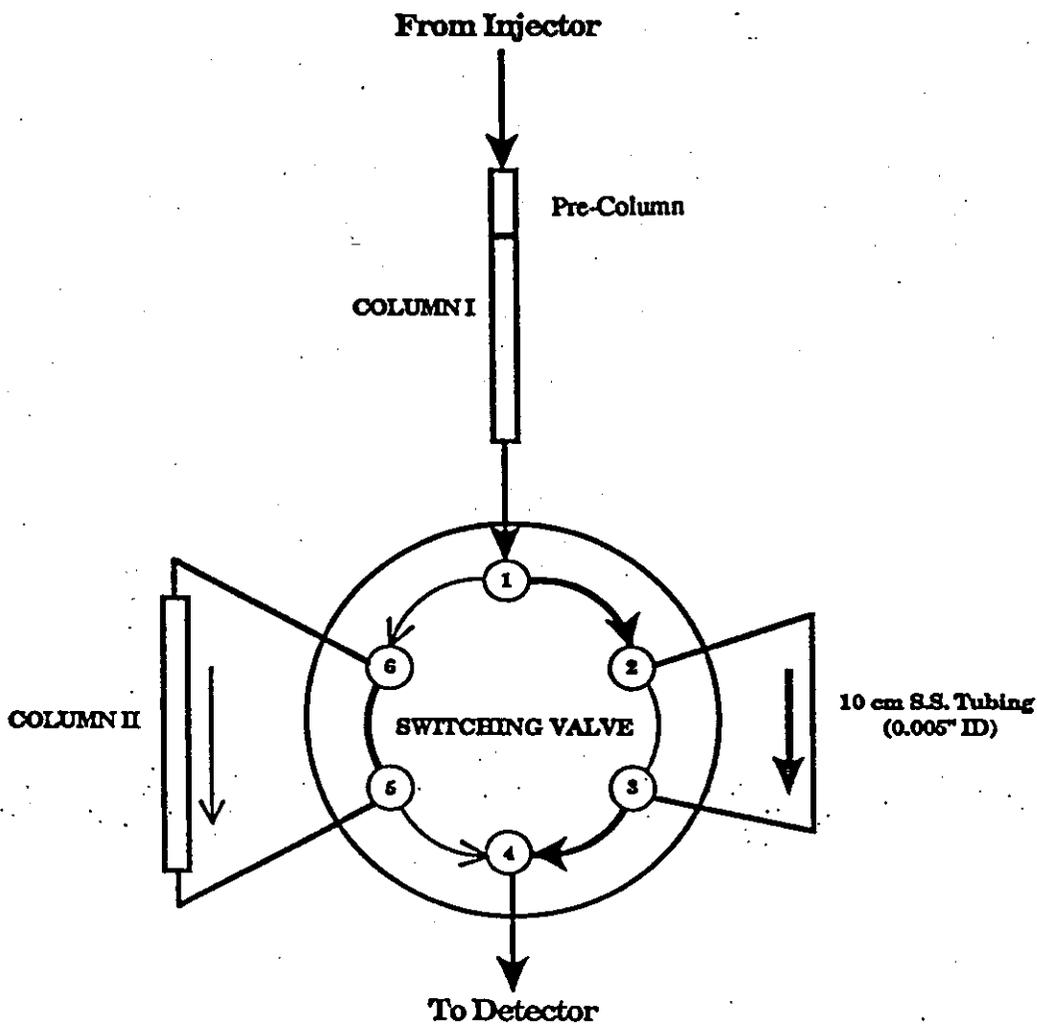
<u>Name of Sample</u>	<u>Data Sheet</u>	<u>Fort. Level (ppm)</u>	<u>% Extract. Effic.</u>
Spike 1 3/30	4	0.010	86
Spike 2 3/29	4	0.010	87
Spike 3 3/29	4	0.030	85
Spike 4 3/29	4	0.030	82
Spike 5 3/29	4	0.099	81
Spike 6 3/29	4	0.099	78

Mean Recovery = 83% (Std. Dev. = 3%)  
% RSD = 4%

**FIGURE 1**  
**DPX-PE350 EXTRACTION FROM COTTONSEED FLOW DIAGRAM**



**FIGURE 2**  
**PLUMBING DIAGRAM FOR COLUMN SWITCHING**

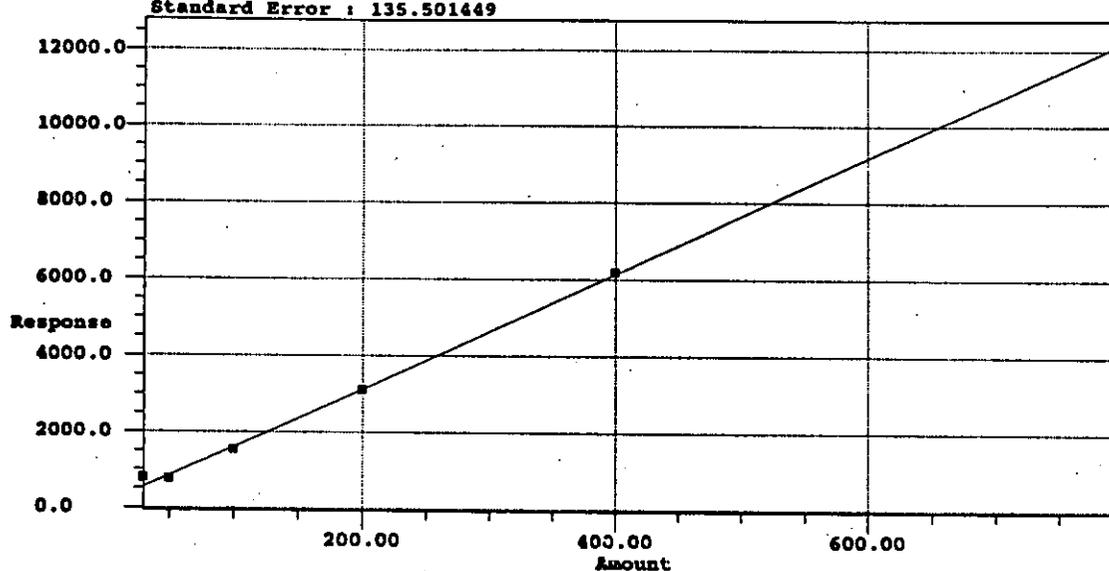


Flow path - valve Position 1:  
(Event 3 = on, Event 4 = off) →

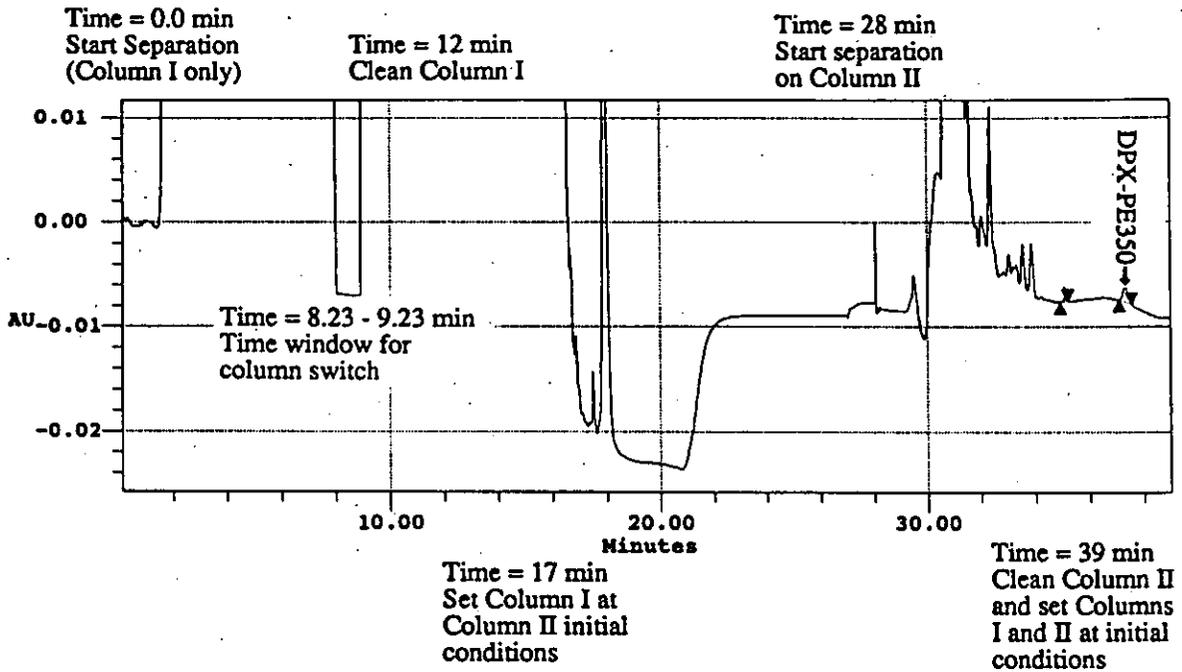
Flow path - valve Position 2:  
(Event 3 = off, Event 4 = on) →

**FIGURE 3**  
**STANDARD CURVE FOR DPX-PE350 STANDARDS USING HPLC/UV**

Processing Method : NewProcMeth1  
System : System3 RAD\_PDA Channel : 254nm  
Date : 02-SEP-93 Type : LC Name : DPX-PE350  
Retention Time : 37.000 Order : 1 AO : 110.819206  
A1 : 15.113849 A2 : 0.000000  
A3 : 0.000000 A4 : 0.000000  
A5 : 0.000000 Correlation Coeff : 0.999633  
Standard Error : 135.501449

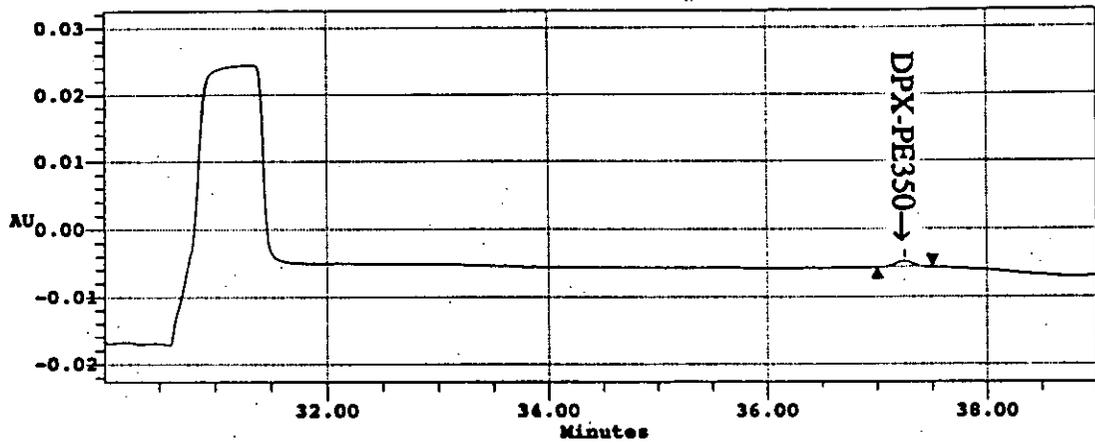


**FIGURE 4**  
**CHROMATOGRAM OF A COTTONSEED SAMPLE FORTIFIED AT THE 0.020-PPM**  
**LEVEL WITH DPX-PE350**

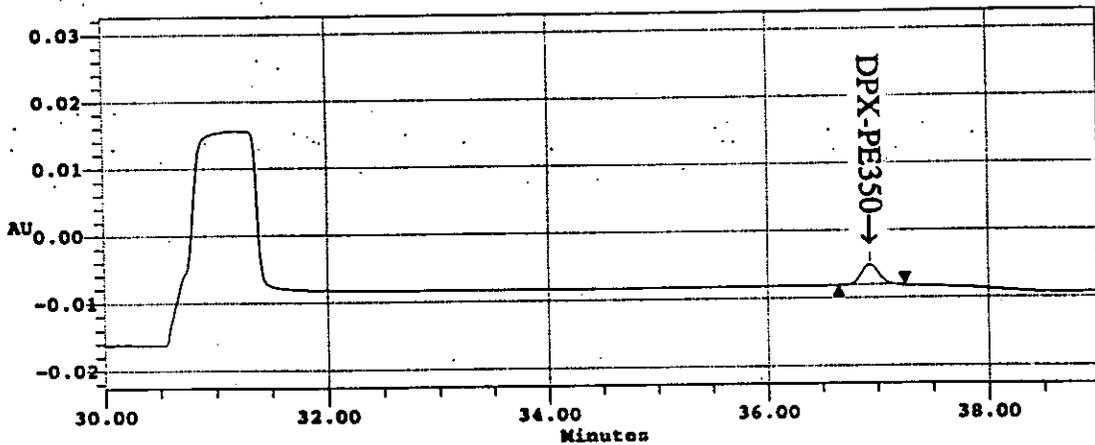


**FIGURE 5**  
**CHROMATOGRAMS OF DPX-PE350 STANDARDS**

- A. A 0.10-mL injection of a 30-ng/mL DPX-PE350 Standard.  
Chromatographic conditions are described in the text.

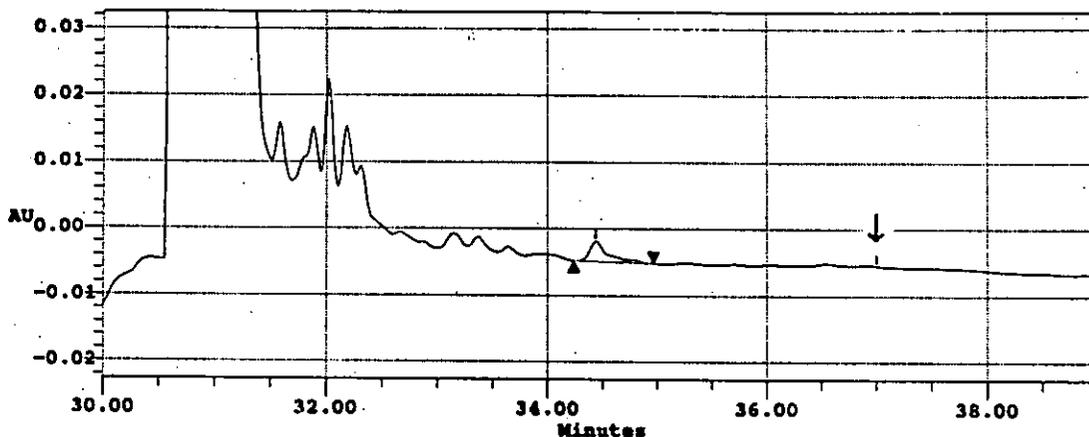


- B. A 0.10-mL injection of a 200-ng/mL DPX-PE350 Standard.  
Chromatographic conditions are described in the text.

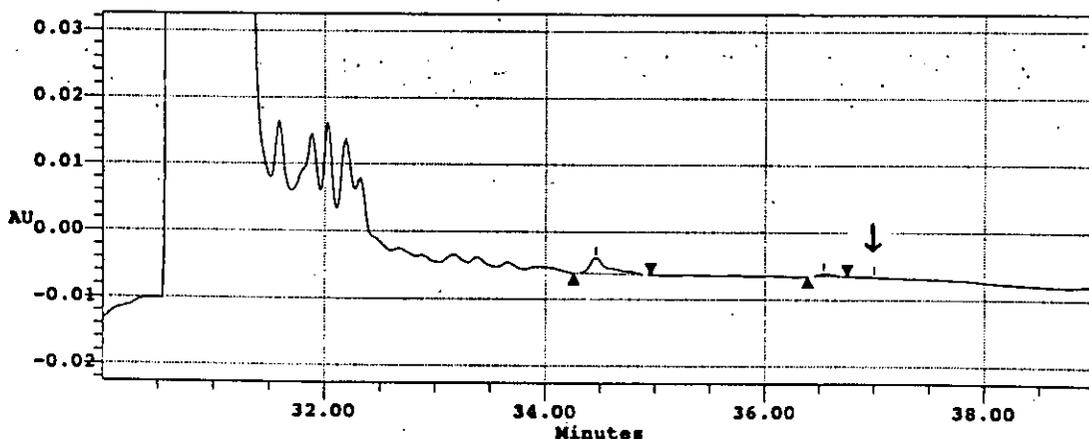


**FIGURE 6**  
**CHROMATOGRAMS OF CONTROL COTTONSEED SAMPLES**

- A. Cottonseed control sample, Control 1 8/16, see Data Sheet 3. The arrow indicates the retention time of DPX-PE350. Chromatographic conditions are described in the text.

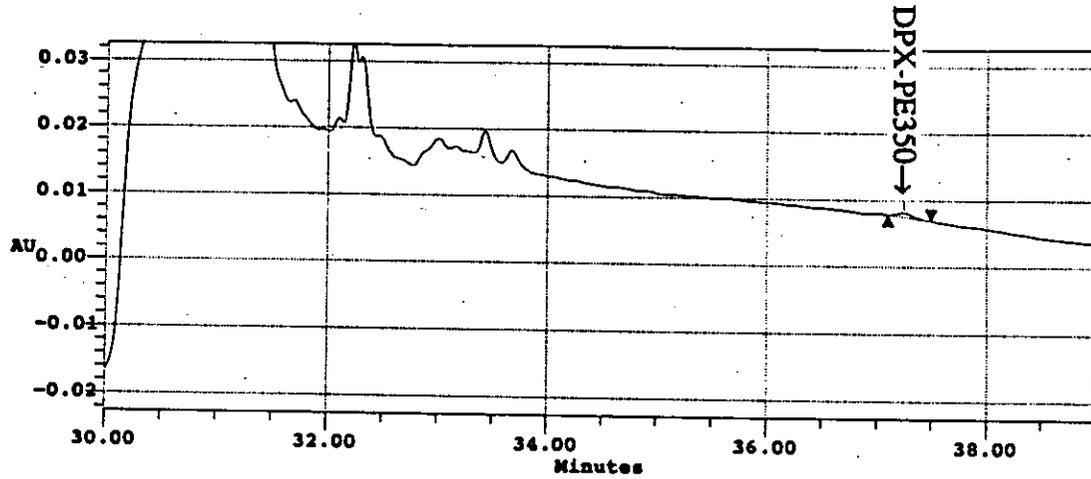


- B. Cottonseed control sample, Control 2 8/16, see Data Sheet 3. The arrow indicates the retention time of DPX-PE350. Chromatographic conditions are described in the text.

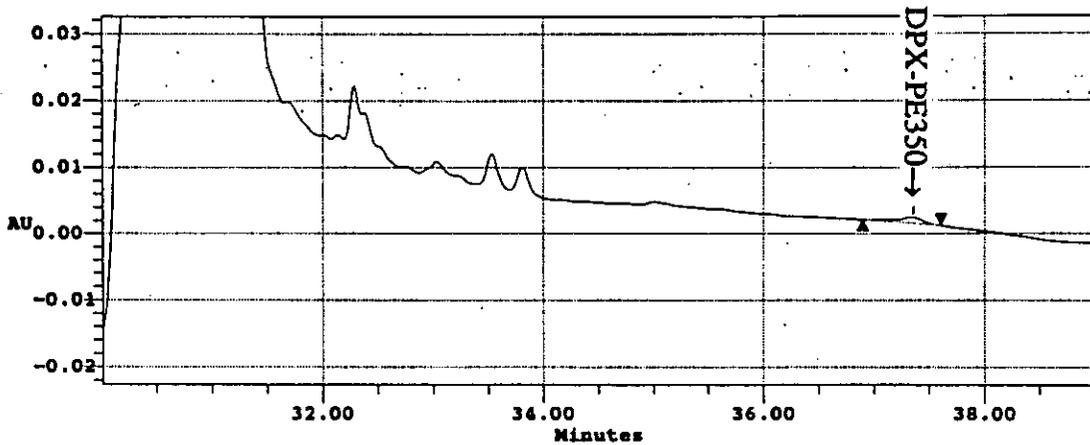


**FIGURE 7**  
**CHROMATOGRAMS OF COTTONSEED SAMPLES FORTIFIED AT 0.010 PPM WITH**  
**DPX-PE350**

- A. Cottonseed sample fortified at 0.010 ppm, Spike 1 8/9, Data Sheet 1.  
Chromatographic conditions are described in the text.

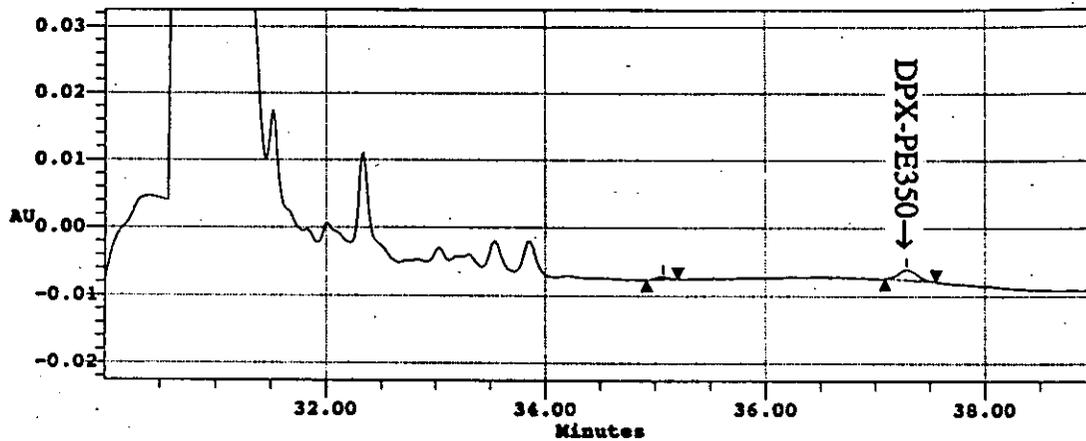


- B. Cottonseed sample fortified at 0.010 ppm, Spike 3 8/9, Data Sheet 1.  
Chromatographic conditions are described in the text.

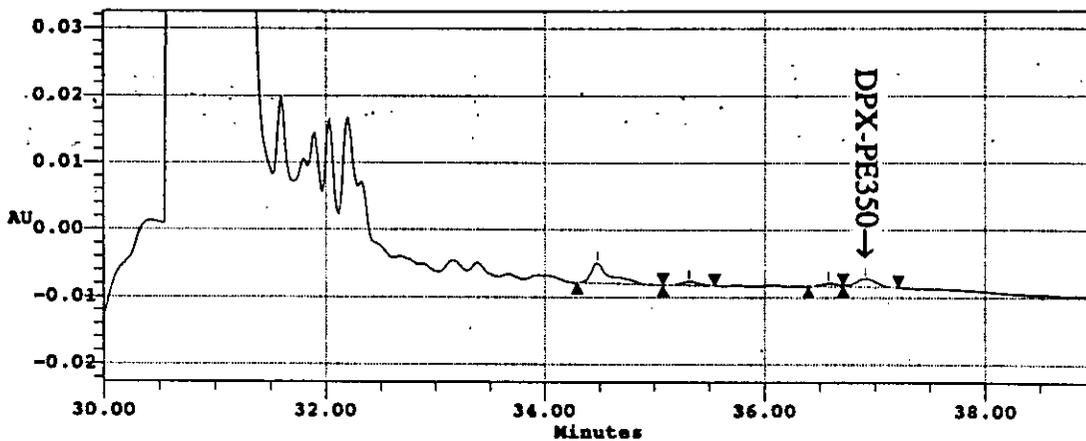


**FIGURE 8**  
**CHROMATOGRAMS OF COTTONSEED SAMPLE FORTIFIED AT 0.021 PPM WITH**  
**DPX-PE350**

- A. Cottonseed sample fortified at 0.021 ppm, Spike 1 8/10, Data Sheet 2.  
Chromatographic conditions are described in the text.

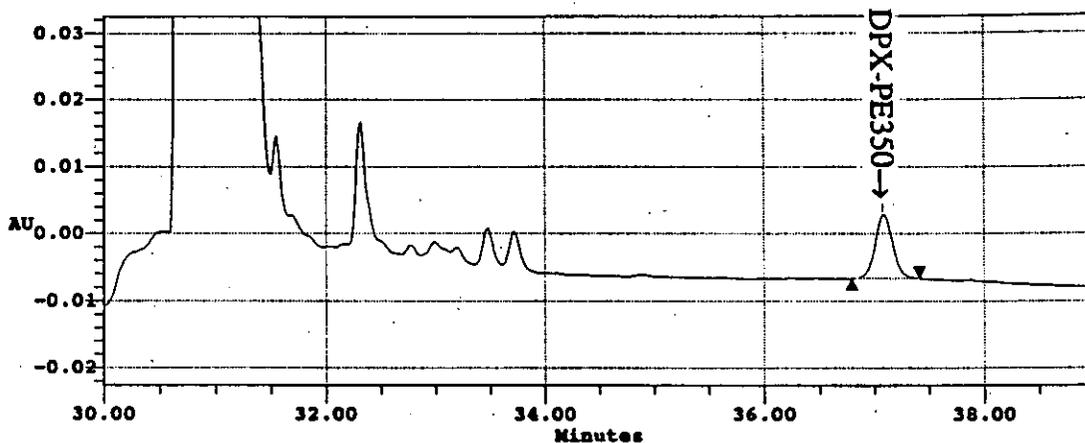


- B. Cottonseed sample fortified at 0.021 ppm, Spike 2 8/16, Data Sheet 3.  
Chromatographic conditions are described in the text.

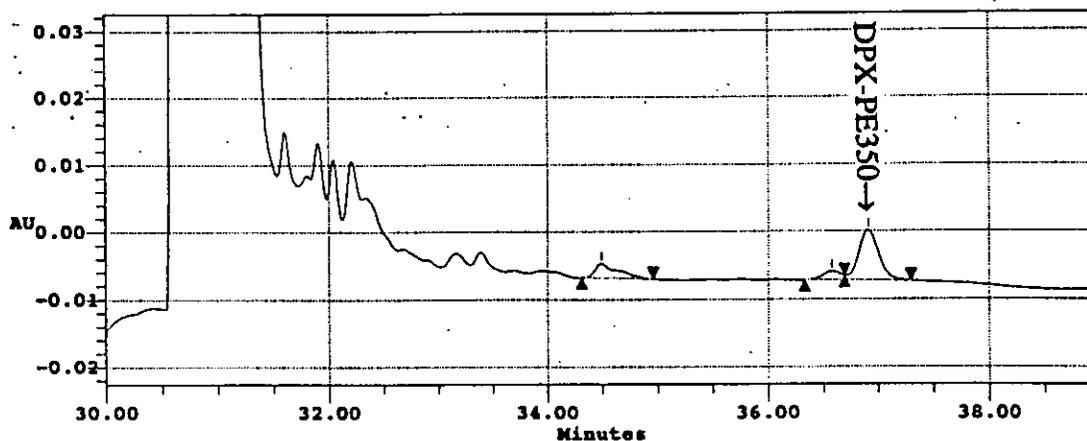


**FIGURE 9**  
**CHROMATOGRAMS OF COTTONSEED SAMPLE FORTIFIED AT 0.10 PPM WITH**  
**DPX-PE350**

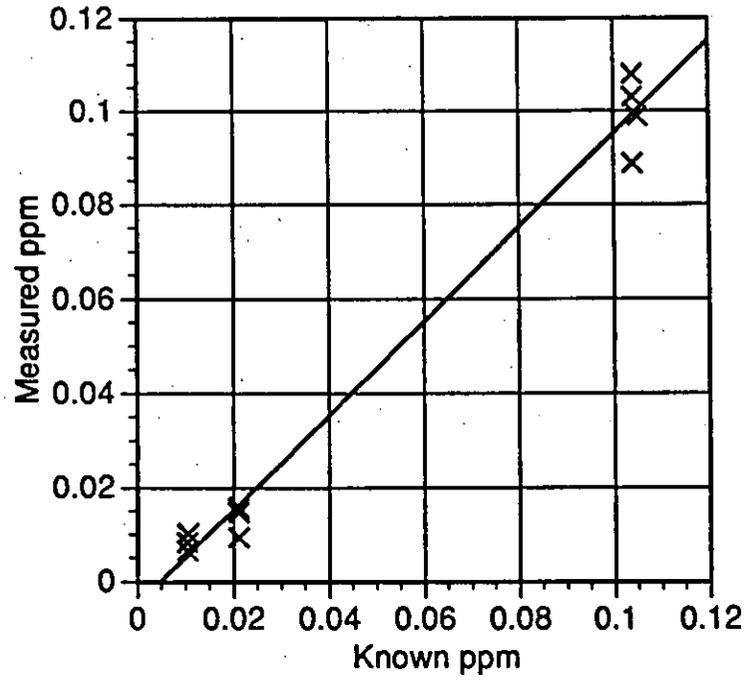
- A. Cottonseed sample fortified at 0.10 ppm, Spike 4 8/10, Data Sheet 2.  
Chromatographic conditions are described in the text.



- B. Cottonseed sample fortified at 0.10 ppm, Spike 3 8/16, Data Sheet 3.  
Chromatographic conditions are described in the text.



**FIGURE 10**  
**PLOT OF MEASURED VS. KNOWN PPM DPX-PE350**



$$f(x) = 9.992865E-1 \cdot x + -4.765475E-3$$
$$R^2 = 9.860539E-1$$

### 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, Del.
2. Sumpter, S. R.; Peterson, B. A.; Wadsley, M. P., "Analytical Enforcement Method for the Determination of KIH-2031 (DPX-PE350) Residues in Cottonseed Using Column-Switching Liquid Chromatography," DuPont Report No. AMR 2689-93.
3. Ramsteiner, K. A., *J. Chromatogr.* 1988, 456, 3-20.
4. Snyder, L. R.; Kirkland, J. J., "Introduction to Modern Liquid Chromatography", 2nd ed.: John Wiley & Sons, Inc.: New York, 1979: Chapter 16.
5. Taylor, J. K., "Quality Assurance of Chemical Measurements", Lewis Publishers, Inc.: Chelsea, Mich., 1987, p. 35.

**APPENDIX I**  
**DATA SHEETS NUMBERS 1-4**

**APPENDIX I  
DATA SHEET NUMBER 1**

Study No. AMR 2816-93

Matrix/Crop: Cottonseed, Prattville, AL,  
S00043136-B

Data Sheet Number 1

Standard 8/23/93

Extracted by: Brock Peterson Date: 9 Aug 1993

Analyzed by: Brock Peterson Date: 24, 25 Aug 1993

**STANDARDS**

Standard	C	PH	RT
1	30.0	954	37.257
2	50.0	914	37.208
3	100.0	1347	37.507
4	200.0	3336	37.242
5	400.0	6940	37.425
6	800.0	14017	37.192

**NOTATION:**

C = Standard concentration, ng/mL  
PH = Peak height,  $\mu$ V  
RT = Retention time, min  
VS = Volume, mL, of fortification standard  
CS = Fortification standard concentration,  $\mu$ g/mL  
W = Weight of cottonseed, g  
A = Amount found, ng/mL  
AF = Aliquot factor = 60.0 mL/110 mL = 0.5454  
FV = Final volume  
IV = Injection volume

**FORTIFIED SAMPLES**

Sample	VS	CS	W	RT	PH	A	ppm Found	ppm Fortification	% Recovery
Spike 1	0.0500	1.05	5.07	37.250	630	36.4	0.0066	0.0104	63
Spike 2	0.0500	1.05	5.02	37.357	801	46.3	0.0084	0.0104	81
Spike 3	0.0500	1.05	5.00	37.358	963	55.5	0.0102	0.0105	97

ppm found = (A)(FV) / (W)(AF)(1000)

ppm fortification level = (VS)(CS) / W

% Recovery = 100 ppm found / ppm fortification level

**CONTROL SAMPLES**

Sample	W	PH
Control 1	5.01	LBN
Control 2	5.05	917*
Control 3	5.02	LBN

LBN = Level of Baseline Noise

\*RT = 37.092 min

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Du Pont Agricultural Products  
Experimental Station  
Wilmington, DE 19880-0402

Data calculated and entered by: Mullen R. Sumpter

Date: 14 Oct 1993

**APPENDIX I (CONTINUED)  
DATA SHEET NUMBER 2**

Study No. AMR 2816-93 Matrix/Crop: Cottonseed, Prattville, AL, Data Sheet Number 2  
S00043136-B  
 Standard 8/23/93 Extracted by: Brock Peterson Date: 10 Aug 1993  
 Analyzed by: Brock Peterson Date: 26, 27 Aug 1993

**STANDARDS**

Standard	C	FH	RT
1	30.0	865	36.807
2	50.0	985	36.632
3	100.0	1690	37.025
4	200.0	2833	37.057
5	400.0	7375	37.018
6	800.0	12647	36.947

**NOTATION:**

C = Standard concentration, ng/mL  
 PH = Peak height,  $\mu$ V  
 RT = Retention time, min  
 VS = Volume, mL, of fortification standard  
 CS = Fortification standard concentration,  $\mu$ g/mL  
 W = Weight of cottonseed, g  
 A = Amount found, ng/mL  
 AF = Aliquot factor = 60.0 mL/110 mL = 0.5454  
 FV = Final volume  
 IV = Injection volume

**FORTIFIED SAMPLES**

Sample	VS	CS	W	RT	FH	A	ppm Found	ppm Fortification	% Recovery
Spike 1	0.1000	1.05	5.01	37.288	1593	86.2	0.0158	0.0210	75
Spike 2	0.1000	1.05	5.04	37.143	1047	51.7	0.0094	0.0210	45
Spike 3	0.5000	1.05	5.02	37.042	9137	562.39	0.103	0.104	99
Spike 4	0.5000	1.05	5.02	37.080	9557	588.9	0.108	0.104	104

ppm found = (A)(FV) / (W)(AF)(1000)  
 ppm fortification level = (VS)(CS) / W  
 % Recovery = 100 ppm found / ppm fortification level

**CONTROL SAMPLES**

Sample	W	FH
Control 1	5.07	LBN
Control 2	5.07	LBN

LBN = Level of Baseline Noise

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 Du Pont Agricultural Products  
 Experimental Station  
 Wilmington, DE 19880-0402

Data calculated and entered by: Melvin R. Sanyal Date: 14 Oct 1993

**APPENDIX I (CONTINUED)  
DATA SHEET NUMBER 3**

Study No. AMR 2816-93

Matrix/Crop: Cottonseed, Prattville, AL,  
S00043136-B

Data Sheet Number 3

Standard 8/23/93

Extracted by: Brock Peterson Date: 16 Aug 1993

Analyzed by: Brock Peterson Date: 1,2 Sept 1993

**STANDARDS**

Standard	C	PH	RT
1	30.0	785	36.915
2	50.0	757	36.923
3	100.0	1518	36.918
4	200.0	3097	36.933
5	400.0	6175	36.922
6	800.0	12213	36.908

**NOTATION:**

C = Standard concentration, ng/mL  
PH = Peak height,  $\mu$ V  
RT = Retention time, min  
VS = Volume, mL, of fortification standard  
CS = Fortification standard concentration,  $\mu$ g/mL  
W = Weight of cottonseed, g  
A = Amount found, ng/mL  
AF = Aliquot factor = 60.0 mL/110 mL = 0.5454  
FV = Final volume  
IV = Injection volume

**FORTIFIED SAMPLES**

Sample	VS	CS	W	RT	PH	A	ppm Found	ppm Fortification	% Recovery
Spike 1	0.1000	1.05	5.01	36.893	1409	85.9	0.0157	0.0210	75
Spike 2	0.1000	1.05	5.00	36.912	1322	80.1	0.0147	0.0210	70
Spike 3	0.5000	1.05	5.02	36.910	7463	486.45	0.0888	0.104	85
Spike 4	0.5000	1.05	5.01	36.947	8280	540.5	0.0989	0.105	94

ppm found = (A)(FV) / (W)(AF)(1000)  
ppm fortification level = (VS)(CS) / W  
% Recovery = 100 ppm found / ppm fortification level

**CONTROL SAMPLES**

Sample	W	PH
Control 1	5.03	LBN
Control 2	5.04	LBN

LBN = Level of Baseline Noise

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Du Pont Agricultural Products  
Experimental Station  
Wilmington, DE 19880-0402

Data calculated and entered by: Sheldon R. Sumpter Date: 14 Oct 1993

**APPENDIX I (CONTINUED)  
DATA SHEET NUMBER 4**

Study Number AMR 2689-93  
Standard NB E74794, p. 53  
Standard Lot Number 2764-067

Matrix/Crop: Cottonseed, Milan, TN, S00049961-B Date Sheet Number 4  
Extracted by: Brock Peterson Date: 29 March 1993  
Analyzed by: Brock Peterson Date: 30 March 1993  
Date Spiked: 26 March 1993 - let stand 72 hr at room temp.

Standard	Vol. of Std. (mL)	Std. Conc. (µg/mL)	Std. Counts (dpm)	Correct Counts (dpm)	Std. Counts (dpm)/Vol. of Std. (mL)
1	0.050	0.99	8250	8237	164740
2	0.500	0.99	76177	76164	152328

Avg. Std. Counts/mL: 158534 dpm/mL

Initial Volume, mL 110.0  
Aliquot Volume, mL 60.0  
Final Volume, mL 5.0  
Volume Sampled, mL 0.50

**FORTIFIED SAMPLE**

Sample	Vol. of Std. (mL)	Std. Conc. (µg/mL)	Sample Weight (g)	Fortified Level (ppm)	Raw Counts (dpm)	Correct Counts (dpm)	% EE
Spike 1	0.050	0.99	5.0	0.010	386	373	86
Spike 2	0.050	0.99	5.0	0.010	389	376	87
Spike 3	0.150	0.99	5.0	0.030	1112	1099	85
Spike 4	0.150	0.99	5.0	0.030	1083	1070	82
Spike 5	0.500	0.99	5.0	0.099	3516	3503	81
Spike 6	0.500	0.99	5.0	0.099	3390	3377	78

% EE = % Extraction Efficiency =  $100 \times (\text{Correct Counts}) / (\text{Std. Counts}) \times (\text{Vol. Sampled}) / (\text{Final Vol.}) \times (\text{Aliquot Factor})$   
 Fortification Level =  $(\text{Volume of standard}) \times (\text{Standard Concentration}) / \text{Sample weight}$   
 \*Std. Counts =  $(\text{vol. of std. for fort., mL}) \times (\text{Avg. std. counts, dpm/mL})$   
 Corrected Counts = Raw sample counts - Blank cocktail counts  
 Aliquot Factor = Initial volume / Aliquot volume  
 Blank cocktail counts: 13 dpm

Data calculated and entered by: Walker R. Hunter Date 21 May 1993

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Du Pont Agricultural Products  
Experimental Station  
Wilmington, DE 19880-0402