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Study Title

Split Analytical Method for the Quantitation
of DPX-66037 in Sugar Beets

Data Requirements

U. S. EPA Pesticide Assessment Guidelines
Subdivision O, 171-4

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GOOD LABORATORY PRACTICE STATEMENT

The EPA Good Laboratory Practice (GLP) requirements specified in 40 CFR Part 160 are not applicable to analytical methods development. However, the methods validation work, contained in this report, was conducted in the spirit of compliance with the GLP regulations.

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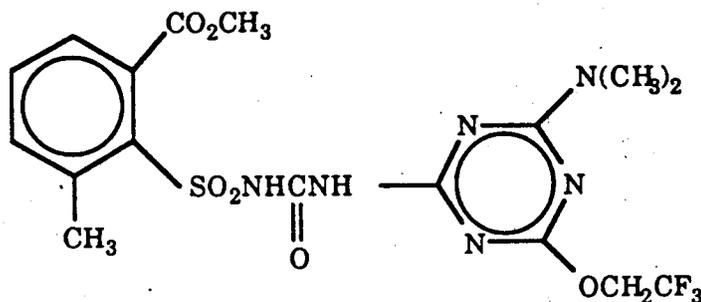
SUMMARY/INTRODUCTIONA. Scope

This method uses two high performance liquid chromatographic separations (HPLC) with UV detection at 232 nm for the determination of DPX-66037 residues in sugar beet foliage and roots. Sample clean-up is achieved through reversed phase chromatography and analysis is achieved through a second chromatographic separation. This approach has been successfully applied to several sulfonylurea herbicides in many matrices (References 1, 2, and 3). The method provides a means to quantitate DPX-66037 in sugar beet foliage and roots at levels as low as 0.05 ppm based on a 10-gram sample. DPX-66037 may be detected at levels as low as 0.01 ppm.

Sugar beet foliage and root samples from 4 U.S. locations were fortified with concentrations of DPX-66037 ranging from 0.05 ppm to 0.500 ppm and analyzed using this split analytical method. Recoveries ranged from 83% to 108% for foliage and 88% to 112% for roots with a standard deviation of 7% in each case.

The method has been designed for increased simplicity and productivity by eliminating manually intensive operations such as liquid/liquid extraction and evaporations. Concentration of analyte and clean-up of sample matrix are performed by chromatography. Only slight modification to an existing HPLC is required to perform this method.

The Chemical Abstracts name for DPX-66037 is Methyl 2-[[[4-(dimethyl amino)-6-(2,2,2-trifluoroethoxy)-1,3,5-triazin-2-yl]amino]carbonyl]amino]sulfonyl]-3-methylbenzoate. Its structure is shown below.



B. Introduction

This method describes the use of two HPLC separations for the quantitation of DPX-66037 in sugar beet foliage and roots. The first separation is identified as the "Clean-up Chromatograph" and removes most sample coextractives. Sample clean-up and analyte concentration are achieved by reversed phase chromatography on a Zorbax® Phenyl analytical column. The second separation, the "Analytical Chromatograph", separates the remaining sample components allowing quantitation of DPX-66037 on a Zorbax® R_x-C18™ column. Alternatively, a single instrument may be used for both functions by cleaning all the samples and then changing the column and eluent to allow for analysis. This two system approach is referred to as the Split Method of analysis. The Split Method is suggested for enforcement purposes.

DPX-66037 is extracted from chopped foliage or roots into a solution of 20% acetonitrile, 80% pH 8 ammonium carbonate buffer. Centrifugations and a drop in pH to below 3 are followed by solid phase extraction on a phenyl Bond-Elut® cartridge. Extracts are then ready for chromatography.

Two mL of Sample Extract is injected onto the phenyl column and chromatographed in an eluent of 60% methanol buffered at pH 3.5. DPX-66037, which is predominantly uncharged in the pH 3 sample solution (26% methanol), concentrates at the head of the column during injection. As chromatography continues, DPX-66037 is separated from a large number of polar compounds which could interfere in the subsequent analytical step. Beginning at a fixed time before the analyte would elute, the effluent is collected in a 10-mL volumetric flask for a period of four minutes. After collection from the "Clean-up Chromatograph", the fraction is further acidified with concentrated phosphoric acid and diluted to a 10 mL with deionized water (to cause the protonated DPX-66037 to again concentrate at the head of the R_x-C18 column).

Two mL of this solution is injected onto the Analytical Chromatograph. The analyte is chromatographed with an eluent at higher pH (7.00) and lower methanol concentration (42%) on a Zorbax R_x-C18 column. Since the DPX-66037 is ionized at this pH, it has reduced affinity for the column, and as a result is separated from the sample components collected from the Clean-up HPLC, which are now

comparatively less polar. DPX-66037 is detected in both systems with a UV detector at 232 nm. This allows for quantitation at 0.05 ppm on the Analytical HPLC and detection at 0.01 ppm.

MATERIALS/METHODS

A. Equipment (Equivalent apparatus may be substituted)

Coarse Food Processor - Hobart model 84145 or 84186 food processor (Hobart Corp., Troy, Ohio).

Balance - Mettler model PM4600 (Mettler Instrument Corporation, Hightstown, New Jersey).

Homogenizer - Tekmar SDT Tissumizer® model SDT-1810 with model SDT-182EN shaft and generator (Tekmar Co., Cincinnati, Ohio).

Centrifuge - Du Pont Sorvall® model RC-5C refrigerated centrifuge (Du Pont Instruments, Wilmington, Delaware).

Centrifuge Rotors - Du Pont models HS4 and SS34 (Du Pont Instruments) or equivalent compatible with centrifuge and required force.

Centrifuge Bottles - 250-mL polypropylene, IEC Maxiforce® #2050, or 250-mL polypropylene, Nalge™ #21020-028 (VWR Scientific, Bridgeport, New Jersey) or equivalent compatible with centrifuge rotor and force.

Centrifuge Tubes - Sepcor® 29mm x 103mm, 40-mL, polypropylene (VWR Scientific) or equivalent compatible with centrifuge rotor and force.

Narrow-Range pH Paper - EM® ColorpHast® Indicator Strips, Narrow range, pH 2.5-4.5 (VWR Scientific).

pH Meter - Beckman model ϕ -11™ (Beckman Instruments, Inc., Fullerton, California).

Solid Phase Extraction (SPE) Cartridges - Bond Elut® 3 cc Phenyl SPE cartridges, package of 50, #1210-2032 (Analytichem International, Harbor City, California).

SPE Reservoirs - Bond Elut® 25-mL reservoirs, package of 50, #602500 (Analytichem International).

SPE Adaptors - Bond Elut® adaptors for connection of reservoirs with cartridges, package of 15, #636001 (Analytichem International).

SPE Vacuum Manifold - Visiprep™ SPE vacuum manifold #5-7030H (Supelco, Inc., Bellefonte, Pennsylvania).

Filters - 0.22 µm, 47 mm cellulose ester filter, Millipore Type GS, #GSWP 047 00 (Millipore, Inc, Bedford, Massachusetts).

Syringes - 30-mL or 50-mL glass syringes with Luer connection.

Liquid Chromatographs (Equivalent Chromatographic apparatus may be substituted)

Clean-up Chromatograph:

Pump:	Waters Model 590 Programmable Solvent Delivery Module (Millipore, Inc.)
Detector:	Kratos Spectroflow model 783 programmable absorbance detector (ABI Analytical)
Recorder:	Linseis model L4000 strip chart recorder (Linseis Company)
HPLC Column:	Du Pont Zorbax Phenyl 4.6mm x 150mm, 5µm analytical column, #883952-712 (MAC-MOD)
Column Oven:	Waters model WAT038039 and Temperature control module (Millipore, Inc.)
Eluent Selection Valve:	Rheodyne model 5302 three-way slider valve with model 5300 pneumatic actuator (Rheodyne, Inc.)
Injection Valve:	Rheodyne model 7000 high pressure six-port, two-position valve (Rheodyne Inc.)
Solenoid Air Valves:	Rheodyne model 7163 set for four-way operation (Rheodyne, Inc.)
Stream Switching Valve:	Rainin three-way valve #38-082 (Rainin Instrument Co.)

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Analytical Chromatograph:

Pump: Waters model 600E solvent delivery system
(Millipore, Inc.)

Detector: Waters model 481 UV absorbance
spectrophotometer (Millipore, Inc.)

Recorder: Waters model 745B data module (Millipore, Inc.)

HPLC Column: \downarrow Du Pont Zorbax R_x-C18, 4.6mm x 250mm, 5 μ m
analytical column, #880967-902 (MAC-MOD)

Column Oven: Waters model WAT038039 and Temperature
control module (Millipore, Inc.)

Injector: Waters model 712 refrigerated Wisp automated
injector (Millipore, Inc.)

B. Reagents and Standards (Equivalent reagents may be substituted, but they must meet the criteria of the chemicals listed)

Water - Deionized water passed through a Milli-Q[®] Water Purification System (Millipore, Corp.).

Methanol - EM[®] Omnisolv[®] #MX0488-1 (EM Science).

Acetonitrile - Fisher, HPLC-grade #NA1648 (Fisher Scientific, Fair Lawn, New Jersey).

K₂HPO₄ - "Baker Analyzed"[®] Reagent #3252-01 (J.T. Baker Chemical Co., Phillipsburg, New Jersey).

KH₂PO₄ - "EM[®] low absorbance grade #PX 1566-2 (EM Science).

85% H₃PO₄ - Fisher o-phosphoric acid 85%, HPLC grade #A260-500 or equivalent. H₃PO₄ - "Baker Analyzed"[®] Reagent #0260-02 (J.T. Baker Chemical Co.) may be substituted with appropriate adjustments for concentration.

Concentrated HCl (36.5-38%) - "Baker Analyzed"[®] Reagent #9535-01.

(NH₄)₂CO₃ - "Baker Analyzed"[®] Reagent #0642-01.

DPX-66037 - DPX-66037 Reference Standard (Du Pont Agricultural Products Department, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19880-0402).

C. Preparation of Solutions

✓ 1 M KH_2PO_4 : Dissolve 136 g of KH_2PO_4 in 800 mL of water and dilute to 1 L. Filter through a 0.22 μm filter.

✓ 1 M K_2HPO_4 : Dissolve 174 g of K_2HPO_4 in 800 mL of water and dilute to 1 L. Filter as above.

0.1 M KH_2PO_4 : Mix 100 mL of 1 M KH_2PO_4 with 900 mL of water.

0.1 M K_2HPO_4 : Mix 100 mL of 1 M K_2HPO_4 with 900 mL of water.

17 mM H_3PO_4 : Mix 1.153 mL of 85% H_3PO_4 with 1 L of water.

1 M $(\text{NH}_4)_2\text{CO}_3$: Dissolve 96.09 g $(\text{NH}_4)_2\text{CO}_3$ in ~800 mL of water and dilute to 1 L. Filter through a 0.22 μm filter.

0.1 M $(\text{NH}_4)_2\text{CO}_3$: Mix 100 mL of 1 M $(\text{NH}_4)_2\text{CO}_3$ with 900 mL of water. Adjust the pH of this solution to 8.0 (calibrated pH meter) by addition of 85% H_3PO_4 .

✓ Sample Extraction Solution: 20% acetonitrile/ 80% 0.1 M $(\text{NH}_4)_2\text{CO}_3$ buffer

Mix 200 mL of acetonitrile with 800 mL of 0.1 M $(\text{NH}_4)_2\text{CO}_3$ (pH 8). Final pH measures 8.5-8.6.

Solid Phase Extraction (SPE) Solutions:

SPE Wash Solution: 50% methanol, 50% ~ 0.05 M Potassium Phosphate, pH 2.75

Combine 500 \pm 10 mL of 0.017 M H_3PO_4 and 500 \pm 10 mL 0.1 M KH_2PO_4 solutions. While monitoring pH of the mixture, add additional H_3PO_4 or KH_2PO_4 solution as needed to reach a pH of 2.75, as measured by a pH meter. Mix 500 mL of this ~ 0.05 M solution with 500 mL of methanol. Adjust the pH of the final solution to 3.2 \pm 0.2 by addition of concentrated HCl.

✓ SPE Elution Solution: 65% methanol, 35% 0.1 M Potassium Phosphate, pH 6.8
Combine 500 ± 5 mL of 0.1 M KH_2PO_4 and 500 ± 5 mL of 0.1 M K_2HPO_4 solutions. While monitoring pH of the mixture, add additional K_2HPO_4 or KH_2PO_4 until a pH of 6.8 is reached, as measured by a pH meter. Mix 700 mL of this 0.1 M solution with 1300 mL of methanol. No further pH adjustment is required; the pH of the final solution measures about 8.5 (± 0.2).

✓ Standard Dilution Solution: 40% SPE Elution Solution, 60% water. Mix 800 mL of SPE Elution Solution with 1200 mL of water.

Chromatographic Eluents:

Eluent 1: 0.01 M Potassium Phosphate in 60% methanol, pH 3.5
Add 20 mL of 1 M KH_2PO_4 to 800 mL water. Add 1200 mL of methanol and mix. Adjust the pH of this solution to 3.5 (as measured by a calibrated pH meter) by addition of 85% H_3PO_4 . Sparge briefly (about 5 minutes) with helium to degas; further sparging may change the methanol concentration due to evaporation.

✓ Eluent 2: 0.01 M Potassium Phosphate in 42% methanol, pH 7.0
Add 20 mL of 1 M K_2HPO_4 to 1160 mL water. Add 840 mL methanol and mix. Adjust the pH of this solution to 7.0 (as measured by a calibrated pH meter) by addition of 85% H_3PO_4 . Sparge briefly as above.

✓ Eluent 3: 90% methanol
Mix 1800 mL methanol with 200 mL water. Sparge briefly as above.

Standards:

Stock Standard Solution

✓ Accurately weigh about 50 mg of DPX-66037 Reference Standard and bring to 100-mL volume with acetonitrile to make stock standard solution at about 500 $\mu\text{g}/\text{mL}$. Make an intermediate dilution from the stock standard to 10.0 $\mu\text{g}/\text{mL}$ in acetonitrile; this will be used for fortification of samples and preparation of chromatographic standards. Keep the stock standard solution in the freezer for storage; the 10 $\mu\text{g}/\text{mL}$ standard may be kept in the refrigerator for short-term storage.

Chromatographic Standard Solutions

Prepare chromatographic standards ranging from 0.003 $\mu\text{g}/\text{mL}$ to 0.05 $\mu\text{g}/\text{mL}$ in Standard Dilution Solution from the 10 $\mu\text{g}/\text{mL}$ standard solution above. The concentration of acetonitrile in these final dilutions is kept at or below 2% (acetonitrile may be evaporated if required to reduce its volume). Keep all chromatographic standards at or below 4°C following preparation. These standards should be stable for at least

2-3 days. Before chromatography, acidify aliquots of these standards to a pH of 2.5 to 3. Once acidified, the stability of the standards is greatly reduced; the acidified solutions should be kept on ice at all times and chromatographed within the next 18 hours.

D. Analytical Procedure

1. Preparation of Sample

Frozen beet foliage or beet root samples are chopped in a Hobart chopper with dry ice. The dry ice is allowed to sublime, and samples are stored at -20°C until sampled for analysis.

2. Fortification

Place $10 (\pm 0.1)$ grams of untreated chopped sample into a 250-mL centrifuge bottle. Keep samples frozen at all times. For a 0.100 ppm fortification add 0.100 mL of the $10 \mu\text{g}/\text{mL}$ DPX-66037 standard solution in acetonitrile to the 10-g sample. Fortify at levels covering the range of expected levels in field samples and at the quantitation limit (0.05 ppm) of this analytical method, generally 0.05 to 0.5 ppm. Allow the acetonitrile to evaporate (under a stream of nitrogen). Keep samples frozen until ready to go on to the next step.

3. Extraction

Accurately weigh $10 (\pm 0.1)$ grams of frozen chopped sugar beet roots or foliage into a 250-mL centrifuge bottle. Fortify as in step 2 if required. Keep samples frozen if extraction is to be delayed. Add $100 (\pm 2)$ mL of Sample Extraction Solution; cap bottle and shake briefly to mix. Let soak for about 10 minutes. Homogenize with a Tissumizer[®] for 1 minute at a speed low enough to prevent excessive foaming. Wait 5 minutes and repeat the Tissumizing-rest procedure two more times.

Centrifuge the sample-buffer mixture in the Sorvall[®] RC-5C centrifuge (HS- rotor) for 10 minutes at 7000 rpm, equivalent to a relative centrifugal force (RCF) of 9,500 g (brake on, refrigeration optional). If using an alternate speed,

centrifuge until most particulates have separated from the Sample Extraction Solution.

Remove about 20 mL of supernatant with a glass syringe and large bore needle and transfer into a 40-mL centrifuge tube. The extraction may be interrupted at this point if required. Samples should be stable enough at this stage for overnight, refrigerated storage.

Acidify contents of centrifuge tube by dropwise addition of 85% H₃PO₄ until a pH between 2 and 3 is reached (as determined by narrow-range pH paper). Once a sample has been acidified, proceed directly through the clean-up procedure which follows, as DPX-66037 is not stable at this pH at room temperature.

Centrifuge in the Sorvall® RC-5C centrifuge (SS-34 rotor) for 10 minutes at 20,000 rpm, equivalent to a RCF of 47,800 g (brake on; 2-10°C).

4. Clean-Up

Solid phase extraction (SPE) of the Sample Extracts is carried out on 3 cc phenyl Bond-Elut® SPE cartridges. Solutions are held in 25-mL reservoirs above the SPE cartridges and are drawn through the cartridges by use of a vacuum manifold. Flow should be dropwise at a rate of about 1 drop per second. The column bed is not allowed to dry out between application of solutions.

While samples are undergoing the last centrifugation, precondition SPE cartridges by drawing about 5 mL of methanol followed by about 5 mL of water through each cartridge. Ten mL each supernatant from the last centrifugation is removed with a 10-mL serological pipet, transferred to a SPE reservoir, and drawn through a cartridge. Each cartridge is washed by drawing 10 mL of SPE Wash Solution through it. Do not let the column bed dry out, but make sure that there is minimal liquid standing above the packing material at this point.

Place 10-mL volumetric flasks inside the vacuum manifold under each cartridge for collection of the analyte. Four mL of SPE Elution Solution is passed through each cartridge, allowing the column bed to dry.

Each volumetric flask is brought to volume with HPLC-quality water, capped, and stored refrigerated if analysis is to be delayed. These samples may be stored overnight if necessary.

5. Chromatography

When ready to chromatograph samples add three drops of 85% H_3PO_4 to the contents of each 10-mL volumetric flask and mix. Check that the pH of the extract is less than 3 with narrow-range pH paper. Acidify and chill chromatographic standards in the same manner (three drops of H_3PO_4 per 10-mL of standard solution). All acidified samples and standards are kept on ice while awaiting and during chromatography as DPX-66037 is not stable at this pH at room temperature.

Chromatographic conditions and instrumentation are described in the following section.

E. Instrumentation

1. Description

The Clean-up chromatograph is a modular, high pressure, reverse-phase chromatograph equipped with a high pressure six-port, two-position valve which allows the injection of 2-mL samples. A three-way slider valve with pneumatic actuator allows selection of two different eluents, Eluent 1, the chromatographic eluent, and Eluent 3, the cleaning eluent. The system is also equipped with a three-way stream switching valve which enables collection of the DPX-66037 peak as it elutes off the Clean-up Chromatograph. A schematic of the HPLC system is shown in Figure 2. The chromatographic conditions are summarized in Table III.

The valves may be controlled through the programming capability available in many HPLC components. We use the programming capability of the Kratos detector to perform these operations. The schematic for these valves are shown in Figure 3. See Key to Detector Programming section (page 33) and Table IV for programming details.

Sample Extract clean-up is carried out on a Zorbax Phenyl analytical column with an eluent of 60% methanol buffered at pH 3.5. A DPX-66037 standard solution is first chromatographed to determine the analyte's retention time, a stream-switching valve following the detector is actuated so effluent may be collected. Collection continues for 4 minutes, resulting in about 6.0 mL eluent in a 10-mL volumetric flask. (The timing of collection is not critical and may be done manually; an error of ± 10 seconds will not significantly affect the results of the analysis). The volumetric flask for eluent collection is kept on ice at all times to reduce DPX-66037 decomposition in the acidic chromatographic eluent. After collection, three drops of concentrated phosphoric acid (100 μ L) is added to further reduce the pH to between 2 and 3, and the contents of the flask brought to volume (10 mL) with deionized water to ensure that DPX-66037 will concentrate at the head of the R_x-C18 analytical column during subsequent injection. This results in a five-fold dilution of DPX-66037 in the original sample since only 2-mL of sample extract was injected onto the Clean-Up Chromatograph.

After effluent collection, the Zorbax Phenyl column is flushed with 90% methanol (Eluent 3) for about 10 minutes by switching the Eluent Selection Valve. After 10 minutes, the system is returned to its original state and the column allowed to reequilibrate with Eluent 1 (60% methanol, pH 3.5) for 10 minutes.

The Phenyl column on the Clean-Up HPLC must be evaluated daily by chromatography of a standard solution to ensure adequate peak shape and retention time for complete analyte collection.

The peak width at baseline for this standard injection should be no greater than 2 - 3 minutes to ensure quantitative collection of the analyte. Peak tailing was not seen to be a major problem since the DPX-66037 peak is completely collected and then diluted, acidified and reinjected into the analytical chromatograph. The dilution and acidification ensure that DPX-66037 concentrates at the head of the second chromatograph ensuring adequate peak shape. However, the Phenyl column can be replaced following 40 to 50 chromatographic runs (excluding standards). When a new phenyl column is installed, the retention time of the DPX-66037 peak must be determined and Detector program #7 modified to reflect the change in retention time.

The Analytical Chromatograph consists of a pump, a WISP refrigerated autosampler, column oven and UV detector. It is equipped with a 2-mL sample loop and the ability to select between two eluents. Chromatographic conditions are listed in Table III. The eluent obtained from the Clean-Up Chromatograph is injected on a Zorbax R_x-C18 column with Eluent 2, one of lower methanol concentration (42%) and higher pH (7.0) than in the Clean-Up Chromatograph. After DPX-66037 has eluted, the column is flushed with 90% methanol (Eluent 3) for 5 minutes following each sample run to remove any highly retained sample components. This flush is not necessary after chromatography of a standard solution.

2. Operating Conditions

See Table III for a list of chromatographic operating conditions for both the Clean-Up and Analytical Chromatographs. Note that a minimum of 5 mL of sample or standard solution is required to adequately flush and fill a 2-mL sample loop.

Clean-Up Chromatograph:

- a. Load Detector Program #7 into Kratos detector. This program will chromatograph a sample on the Phenyl column with Eluent 1.
- b. Prepare a DPX-66037 standard solution at or above 0.01 µg/mL concentration. Acidify, vortex and sparge the standard solution.
- c. Place acidified standard solution in an ice bath for at least 10 minutes to drop the temperature.
- d. Inject 2 mL of the standard solution. Determine the retention time of the DPX-66037 peak in Eluent 1.
- e. Determine the effluent collection time: Two minutes prior to the analyte retention time, a stream-switching valve following the detector is actuated so effluent may be collected. Collect the analyte and eluent in a volumetric flask placed in an ice bath. Collection continues for 4 minutes, resulting in about 6 mL of effluent in a 10-mL volumetric flask. Effluent collection should begin 2 minutes prior to DPX-66037 retention time and should end 2 minutes after.

- f. After collection, the pH is reduced by addition of three drops of concentrated phosphoric acid (100 μ L) and the contents of the flask brought to a volume of 10 mL with water to ensure that the DPX-66037 will concentrate at the head of the analytical column during subsequent injection. This results in a five-fold dilution of the DPX-66037 in the original sample since only 2 mL was injected.
- g. After effluent collection, the Clean-up Column is washed with 90% methanol for 10 minutes. After 10 minutes, the system is returned to its original state and the column allowed to reequilibrate with Eluent 1 (60% methanol, pH 3.5).

Analytical Chromatograph:

- a. Inject 2 mL of the acidified sample from the Clean-up Chromatograph onto the Zorbax R_X-C18 column. Chromatograph with Eluent 2 (42% Methanol, pH 7.0).
- b. After the DPX-66037 peak has eluted, the column is flushed with Eluent 3 (90% Methanol) for 5 minutes following each sample run to remove any highly retained sample components.
- c. The system is allowed to reequilibrate with Eluent 2 (42% Methanol, pH 7.0) for about 10 minutes.

Chromatography of Samples and Standards:

Edit Detector Program #7 to contain the t-2 minute and t+2 minute times determined above for the Clean-up Chromatograph. Load Detector Program #7.

Chromatograph prepared standards and samples through the Clean-up and Analytical Chromatographs. All samples and standards should be at 0°C in an ice bath. At least 5 mL of sample or standard solution is required for each run.

3. Calibration Procedure:

Prepare DPX-66037 chromatographic standard solutions by diluting the 10.0 μ g/mL stock standard with Standard Dilution Solution as specified in the Reagents and Standards section. Make 4 or 5 standards covering the range of levels expected in analyses of samples. Typically 0.001 μ g/mL, 0.005 μ g/mL, 0.010 μ g/mL, 0.025 μ g/mL, and 0.050 μ g/mL standards are chromatographed.

Chromatographic standards must be acidified and chilled before chromatography, as detailed in steps above. Chromatograph each standard solution in the Clean-up Chromatograph followed by the Analytical Chromatograph. This is to ensure that the standards are run in the same fashion as the samples. DPX-66037 has a retention time of 22 - 26 minutes in the Clean-up and Analytical Chromatographs.

Measure the DPX-66037 peak height from each chromatogram, recording appropriate attenuations. A plot of peak height versus concentration should be linear and pass through the origin (see Figure 1).

Calculate a calibration factor (CF) for each standard solution chromatographed.

4. Determination of DPX-66037 in Sample:

Chromatograph each acidified, chilled sample extract. Identify the presence or absence of DPX-66037 based on its retention time determined in the calibration runs. Measure the DPX-66037 peak height, if present, as done for calibration, recording appropriate attenuations.

Calculate the concentration of DPX-66037 in each sample using the equation described under Methods of Calculations.

F. Interferences

Several sulfonylurea herbicide residue methods (Reference 2,3) have been developed using the same technique, and are selective and relatively free of significant interference. A number of pesticides which might be encountered were tested using this analytical method. Pesticide test solutions corresponding to 0.20 ppm active ingredient of 12 different compounds were processed from the solid phase extraction step through both chromatographic steps. Chromatograms from the Analytical HPLC are shown in Figures 8 and 9. A chromatogram of DPX-66037 is shown for comparison. None of the chromatograms showed any interference in the region of DPX-66037 elution. Table V lists the pesticides tested.

If a significant interference is apparent, the pH of eluent 2 may be adjusted slightly to selectively move the DPX-66037 relative to the interference. An adjustment of 0.2 pH units or less in either direction is suggested.

G. Confirmatory Techniques

The presence of DPX-66037 may be confirmed by substituting a Zorbax R_x-C8 4.6 mm x 250 mm column or Zorbax Phenyl 4.6 mm x 250 mm column for the Zorbax R_x-C18 column and slightly increasing the methanol concentration in Eluent 2 to maintain the retention time of DPX-66037 on the Analytical Chromatograph.

The acid-base character of DPX-66037 may also be used to allow confirmation. Changing the pH of eluent 2 by 0.2 to 0.5 pH units in either direction should selectively change the retention time for the DPX-66037 peak. (Do not exceed a pH of 7.5 to avoid column degradation.) If the pH is raised, the methanol concentration may need to be decreased to obtain the desired retention. Similarly, a decrease in eluent pH may require an increase in methanol concentration to ensure elution before Eluent 3 is selected for column cleaning.

H. Time Required for Analysis

Sample processing is relatively rapid; typically 4 to 6 sample extracts can be prepared in less than two hours. When the split approach is used, sample clean-up requires about 45 minutes per sample and analysis requires about 55 minutes (25 minutes of which is required for syringe loading and injection of the sample by the WISP). If a manual injector was substituted in the Analytical Chromatograph, analysis time could be reduced by 25 minutes. However, with our system sample throughput can be expected to be from 8 to 10 total samples (excluding standards).

If only one chromatograph is used for both tasks, the same number of samples can be processed but clean-up and analysis will require an extra day. It is suggested a batch of samples be processed through the Clean-Up HPLC on one day, stored overnight at 4°C and chromatographed on the reconfigured Analytical HPLC the following day.

I. Modifications or Potential Problems

Greater ease of operation can be obtained by automating the valve sequences with the programming devices available on some HPLC components. We used the programming capability of the Kratos detector (see Detector Programming Section). Alternatively, valve actuation may be done manually with acceptable precision.

If equipment is limited, it is possible to reconfigure the Clean-Up HPLC for operation as the Analytical HPLC. Following clean-up, the samples may be stored at -4°C overnight to allow time for conversion. The column and eluent must be changed for the chromatograph to become the Analytical HPLC. The minimum changes required would be replacement of Eluent 1 with Eluent 2 and a change in columns from Zorbax Phenyl to an analytical Zorbax Rx-C18. Cleaned samples may be analyzed the following day.

A 5-mL sample loop for the Clean-Up Chromatograph can be substituted for the 2-mL sample loop used in this method. This would ensure a higher signal-to-noise level for DPX-66037 if a 2-mL sample loop gave unsatisfactory signal-to-noise ratio.

Slight variation in the retention time of DPX-66037 may occur each time new eluents are prepared. Care must be taken during measurement of solutions and pH adjustment.

If interferences from the sample matrix are present and resolution is insufficient on the Zorbax Rx-C18 column, Eluent 2 may be adjusted by changing its pH and/or methanol concentration. The change in pH should selectively move the DPX-66037 peak relative to an interference; the methanol concentration may then require adjustment to provide suitable retention.

I. Method of Calculation

1. Calibration Factor (CF)

The CF is the ratio of detector response (peak height or area) to the DPX-66037 concentration. It is calculated for each chromatographic standard solution

chromatographed. A standard is typically run after every 3 to 4 samples. The average of CFs from the standard runs preceding and following a group of samples is used for calculation of DPX-66037 concentrations in those samples.

$$CF = \frac{\text{DPX-66037 peak height or area}}{\text{DPX-66037 concentration } (\mu\text{g/mL}) \text{ of the standard}}$$

2. Concentration of Analyte in the Sample

$$\text{ppm DPX-66037 in sample} = [M \times (V + B)] + [SW \times \text{Avg. CF}]$$

M = Height or area of the DPX-66037 peak measured in the same units as those used for the CF calculation above.

V = Volume of Sample Extraction Solution (100 mL)

B = Volume of water contributed by the sample

B = % water in sample \times SW \times 1 mL/g water
% water in Sugar Beet = 80%

SW = Sample weight in grams (10 g)

AVG. CF = Calibration Factor described above

RESULTS/DISCUSSION

A. Accuracy

Untreated sugar beet foliage and root samples from four sources were fortified at levels ranging from 0.050 to 0.50 ppm and analyzed to assess the accuracy of this analytical method. Samples were processed and analyzed over a period of two weeks. DPX-66037 recoveries averaged 95% for foliage and roots (see Tables I and II, Reference 4). Extraction efficiency of DPX-66037 was estimated by the analysis of sugar beets treated in the field with ^{14}C -DPX-66037 at 100 oz.ai/acre seven days before sampling. The extraction efficiency was estimated to be between 93 and 101% (Reference 5).

A potential source of error in this method is the decomposition of DPX-66037 in the sample or standard under acidic conditions. Decomposition is minimized by keeping acidified samples and standards on ice while awaiting chromatography. If an autosampler is used, it must be capable of refrigeration. Less

than 4% degradation over 20 hours is seen when DPX-66037 in acidified solution is kept at 0°C (Reference 6). Lowered recoveries may result from the solid phase extraction step and also if the effluent collection window in the Clean-up Chromatograph is too narrow relative to the baseline width of the DPX-66037 peak. The collection time cannot be lengthened significantly because of the need to allow for dilution of the collected fraction before injection into the Analytical Chromatograph. The Phenyl column should be evaluated daily to ensure retention time, peak shape, and size for analyte collection.

Allowance for the contribution by water in the plant tissues to the total extraction volume has been included in the calculations. Inaccurate estimation of water content may produce an error of a few percent.

B. Precision

Recoveries ranged from 83% to 108% for foliage and 88% to 112% for roots, with standard deviation of 7% each for DPX-66037 fortifications ranging from 0.05 to 0.50 ppm (see Tables I and II, Reference 4). Decomposition of the analyte and variability in sample water content are slight contributors to the variability.

C. Limits of Detection and Quantitation

The limit of quantitation is determined by the signal-to-noise ratio of the detector and the size of any peaks not fully resolved. The limit of quantitation is at least 0.05 ppm (approximately 12 times the baseline noise) for our particular instrument. The detection limit, defined here as three times the baseline noise, is at least 0.01 ppm (Reference 4). The detection and quantitation limits are those for the HPLC system we used, which employs a very sensitive detector. We expect similar levels to be realized using comparable equipment. Chromatograms of 0.050 ppm DPX-66037 fortified samples can be seen in Figures 4 and 7.

D. Ruggedness

This method uses reversed phase liquid chromatography with UV detection; both are well understood and known to be stable and reliable. The effluent collection window is adequate (4 minutes) relative to the base line width of

the DPX-66037 peak (typically 2-3 minutes). This allows for variability in DPX-66037 retention time and non-ideal peak shape on the phenyl column (band broadening or peak tailing). Solid phase extraction conditions are conservative, and this processing step should be sufficiently rugged to permit use of different lots without validation of each. The minimal number of processing steps also contributes to the ruggedness of this method.

We easily achieved the quantitation limit stated using our instrumentation; a system with greater baseline noise should still provide adequate quantitation at 0.05 ppm.

CONCLUSION

This analytical method is suitable for the quantitation of DPX-66037 in sugar beet foliage and roots at levels down to 0.05 ppm. It adequately extracts and allows quantitation of DPX-66037 at that level and detection down to 0.01 ppm.

This method is suitable as an enforcement method whereby only a few number of samples require analysis. It requires operator assistance during the injection of extracted samples in the Clean-Up Chromatograph but operation of the Analytical Chromatograph can be fully automated.

CERTIFICATION

ANALYTICAL ENFORCEMENT METHOD FOR THE
QUANTITATION OF DPX-66037 IN SUGAR BEETS

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

Report by: Jennifer S. Amoo October 11, 1991
Jennifer S. Amoo Date
Study Director

Approved by: Richard F. Sauers October 11, 1991
Richard F. Sauers Date
Research Supervisor

Date Study Initiated: May 20, 1991

Date Study Completed: October 11, 1991

Notebook References: E70858 and E70677

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Wilmington, Delaware 19880-0402
and/or the
Du Pont Records Management Center
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Sponsor: E. I. du Pont de Nemours and Company
Agricultural Products Department
Research and Development Division
Experimental Station
Wilmington, Delaware 19880-0402

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TABLE I

RECOVERY DATA FOR METHOD VALIDATION

Sample Matrix	Test Site	DPX-66037 Fortification (ppm)	DPX-66037 Recovery (ppm)	% Recovery
Sugar Beet Roots	Grandin, ND	0.500	0.458	92
		0.500	0.454	91
		0.250	0.234	94
		0.250	0.221	88
		0.100	0.096	96
		0.100	0.091	91
		0.050	0.049	98
		0.050	0.049	98
		0.500	0.443	89
		0.500	0.440	88
Roots	Billings, MT	0.250	0.223	89
		0.250	0.219	88
		0.100	0.090	90
		0.100	0.094	94
		0.050	0.048	96
		0.050	0.048	96
		0.500	0.461	92
		0.500	0.461	92
		0.250	0.227	91
		0.250	0.224	89
Roots	Filer, ID	0.100	0.094	94
		0.100	0.094	94
		0.050	0.056	112
		0.050	0.056	112
		0.500	0.530	106
		0.500	0.521	104
		0.250	0.257	103
		0.250	0.249	99
		0.100	0.092	92
		0.100	0.092	92
Roots	Madera, CA	0.050	0.048	95
		0.050	0.048	95

Average Recovery = 95.0%
 Standard Deviation = 7%

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TABLE II
RECOVERY DATA FOR METHOD VALIDATION

Sample Matrix	Test Site	DPX-66037 Fortification (ppm)	DPX-66037 Recovery (ppm)	% Recovery		
Sugar Beet Tops	Grandin, ND	0.500	0.495	99		
		0.500	0.448	90		
		0.250	0.247	99		
		0.250	0.242	97		
		0.100	0.085	85		
		0.100	0.090	90		
		0.050	0.054	108		
		0.050	0.049	98		
		Tops	Billings, MT	0.500	0.463	93
				0.500	0.458	92
0.250	0.234			94		
0.250	0.229			92		
0.100	0.090			90		
0.100	0.108			108		
0.050	0.050			100		
0.050	0.050			100		
Tops	Filer, ID			0.500	0.445	89
				0.500	0.464	93
		0.250	0.232	93		
		0.250	0.227	91		
		0.100	0.093	93		
		0.100	0.093	93		
		0.050	0.054	108		
		0.050	0.049	98		
		Tops	Madera, CA	0.500	0.469	94
				0.500	0.469	94
0.250	0.234			94		
0.250	0.234			94		
0.100	0.083			83		
0.100	0.083			83		
0.050	0.047			94		
0.050	0.047			94		

Average Recovery = 95%
 Standard Deviation = 7%

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TABLE IIISUMMARY OF CHROMATOGRAPHIC CONDITIONSCLEAN-UP CHROMATOGRAPH

HPLC Column: Du Pont Zorbax Phenyl 4.6 mm x 150 mm, 5 μ m analytical column (MAC-MOD)

Column Temperature: 40°C

Eluents: 1 60% Methanol/40% 10 mM KH₂PO₄, pH 3.5,
3 90% Methanol

Eluent Flow-rate: 1.5 mL/min

Sample Volume: 2.0 mL

Detector Settings: Wavelength - 232 nm
Sensitivity - 0.010 AUFS

Recorder Settings: Chart Speed - 10 cm/hr
Attenuation - 10 mV

ANALYTICAL CHROMATOGRAPH

HPLC Column: Du Pont Zorbax Rx-C18, 4.6mm x 250mm, 5 μ m analytical column (MAC-MOD)

Column Temperature: 40°C

Eluents: 2 42% Methanol/60% 10mM K₂HPO₄, pH 7.0
3 90% Methanol

Eluent Flow-rate: 1.5 mL/min

Sample Volume: 2.0 mL

Detector Settings: Wavelength - 232 nm
Sensitivity - 0.005 AUFS

Integrator Settings: Chart Speed - 0.25 cm/min
Attenuation - 4

KEY TO DETECTOR PROGRAMMING

The events which occur on the Split LC during sample clean-up are controlled through the programming of the Kratos Spectroflow detector (see Figure 2). The detector program #7 is listed below. The program controls 4 internal relays which switch 12-volt power supplies to activate solenoid air valves which, in turn, activate chromatographic valves 1,2 and 4. Relay 1 controls the sample injection valve; Relay 2 controls the eluent selection valve ; Relay 4 controls the stream-switching valve (not pneumatically actuated); Relay 3 is not in use.

Time: The time (in minutes) after the run begins at which each event occurs.

Relay (1-4): Each relay may be in one of two positions, 1 = on and 0 = off. Only relays 1,2, and 4 are operational and they actuate the following:

Relay 1 - Valve 1, Sample Injection Valve; 1 = valve counterclockwise for sample injection onto phenyl column.

Relay 2 - Valve 2, Eluent Selection valve; 1 = Eluent 1 selected, 0 = Eluent 3 selected for forward-flush.

Relay 4 - Valve 4, Stream-Switching valve; 1 = Eluent stream switched to allow for collection into flask.

TABLE IV

DETECTOR PROGRAM #7 FOR CLEAN-UP CHROMATOGRAPH

LC Conditions: 1.5 mL/min., 40°C
 Detector: 0.010 AUFS, 232 nm

STEP	TIME (min)	RELAY				STATUS	EVENT
		1	2	3	4		
0	0.00	0	0	0	0	L 1 . .	Sample loop in LOAD position
1	1.33	1	0	0	0	I 1 . .	Sample loop in INJECT position
2	5.33	0	0	0	0	L 1 . .	Reset to LOAD
3	*	0	0	0	1	L 1 . C	Begin Eluent collection
4	*	0	0	0	0	L 1 . E	End Eluent collection
5	26.00	0	1	0	0	L 0 . .	Start forward- flush Eluent 3
6	35.00	0	0	0	0	L 1 . .	End forward- flush

* Step 3: DPX-66037 retention time minus 2 minutes
 Step 4: DPX-66037 retention time plus 2 minutes

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TABLE V

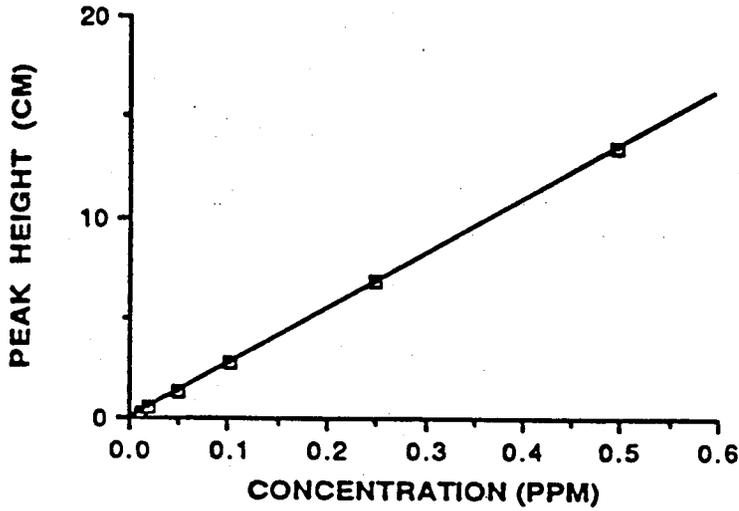
PESTICIDES TESTED

<u>Batch No.</u>	<u>Pesticide</u>
1	Nortron Poast Betanal
2	Diazinon Malathion Lontrel
3	Desmedipham Diethatly Ethyl Chlorpyrifos
4	Methomyl Quizalop-Ethyl Triadimefon

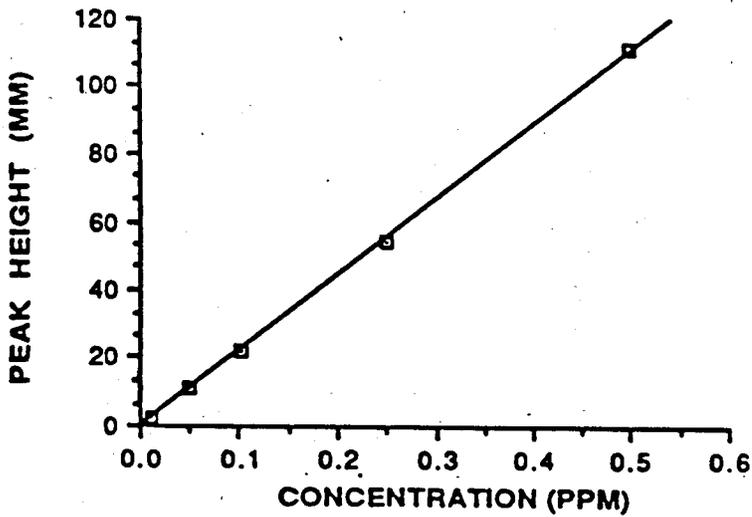
FIGURE 1

CALIBRATION CURVE FROM METHOD VALIDATION

DPX-66037 STANDARD CURVE (5/20/91)



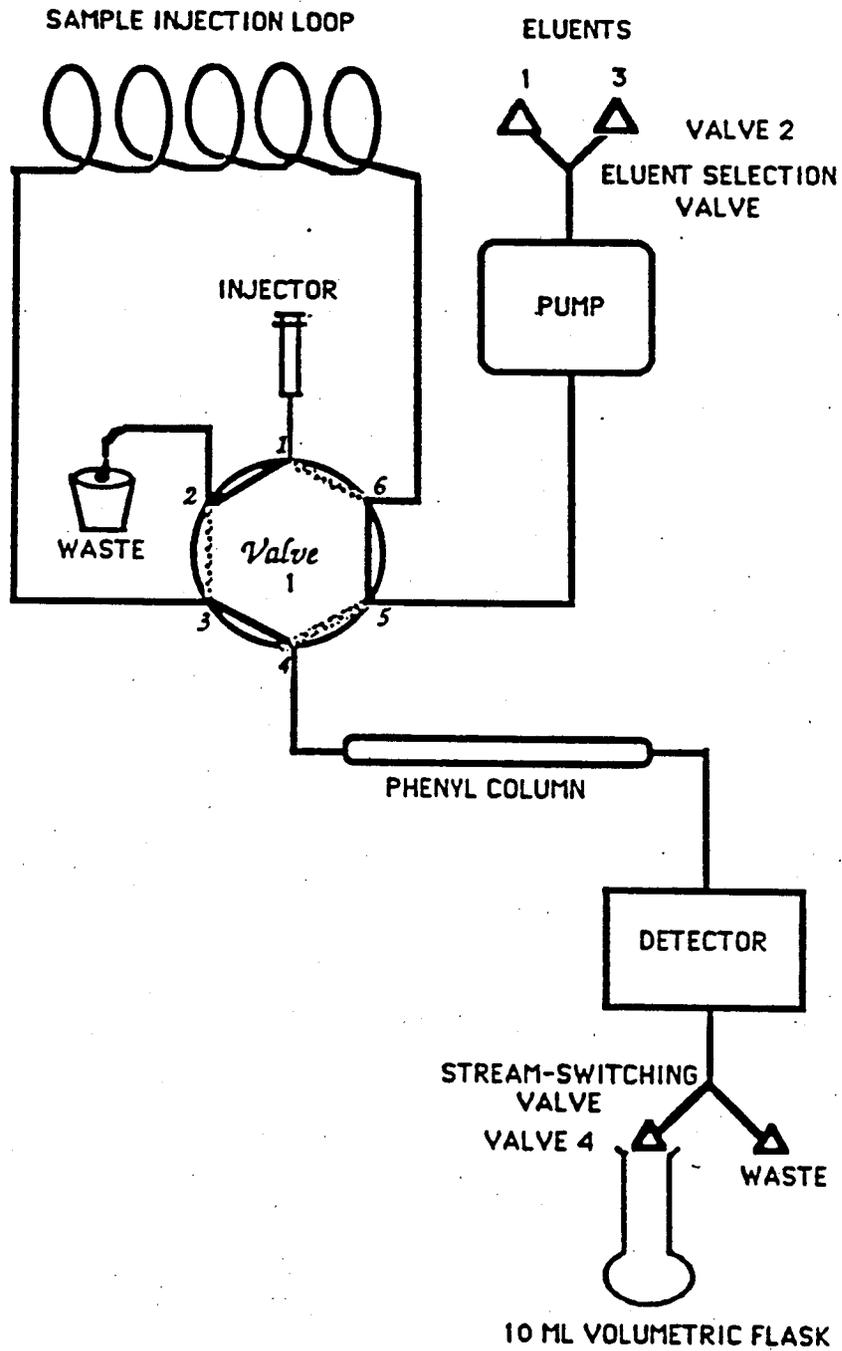
DPX-66037 STANDARD CURVE (6/18/91)



COPY

FIGURE 2

SCHEMATIC OF THE CLEAN-UP CHROMATOGRAPH

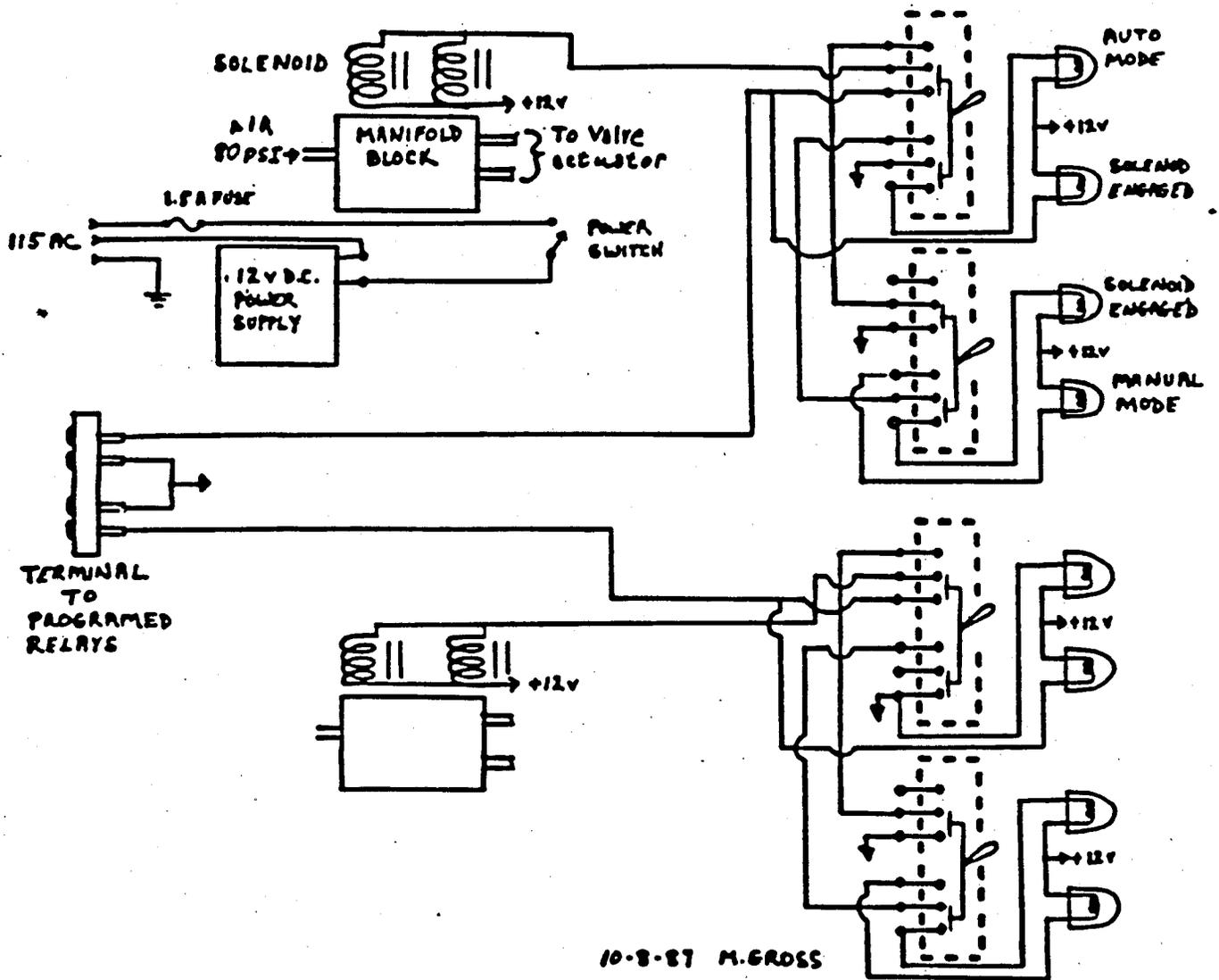


————— Valve Off (Counterclockwise)
- - - - - Valve On (Clockwise)

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FIGURE 3

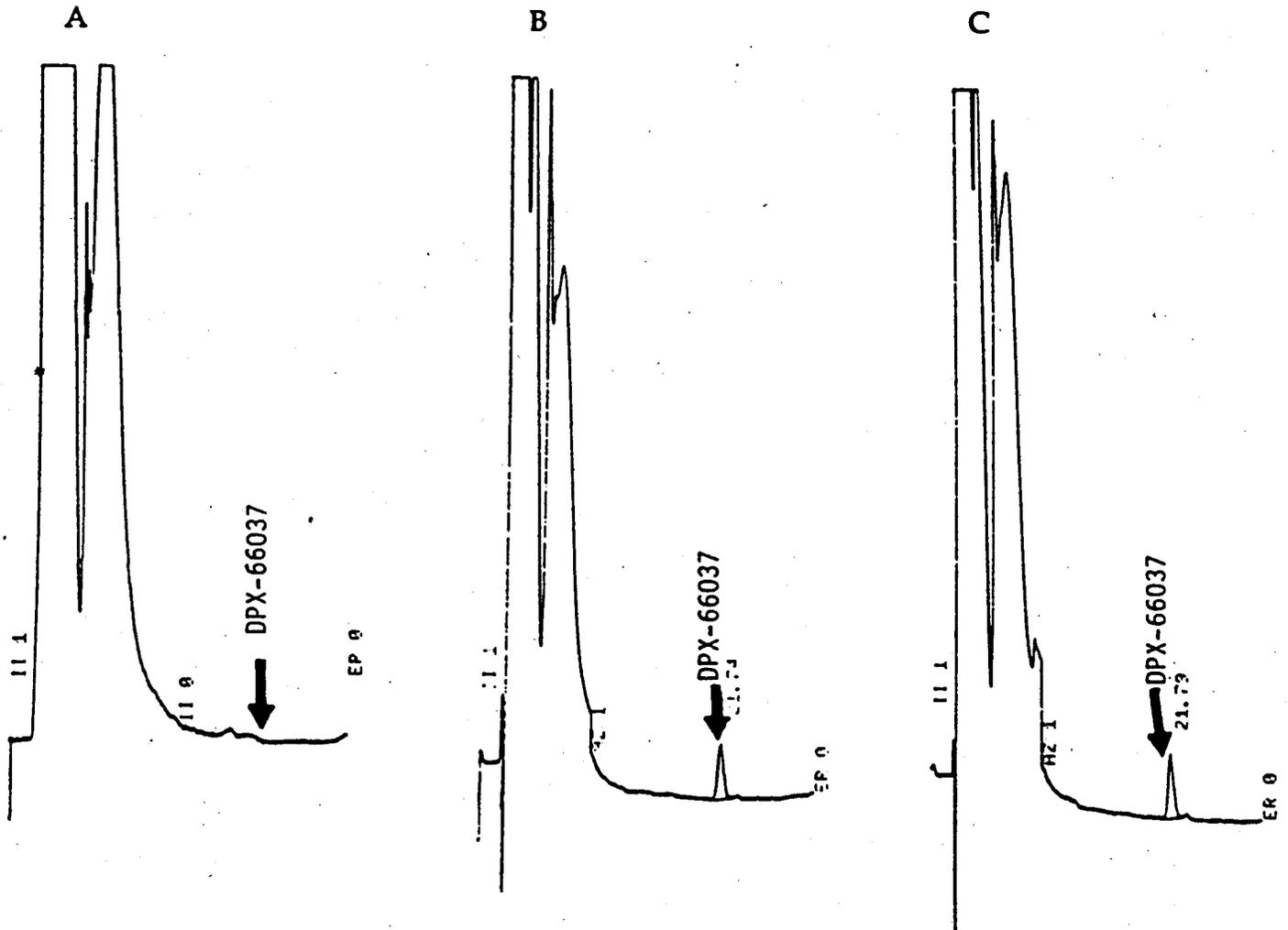
SCHEMATIC OF PNEUMATIC VALVE-SWITCHING STATIONS



COPY

FIGURE 4

CHROMATOGRAMS OF SUGAR BEET ROOTS FROM MADERA, CALIFORNIA

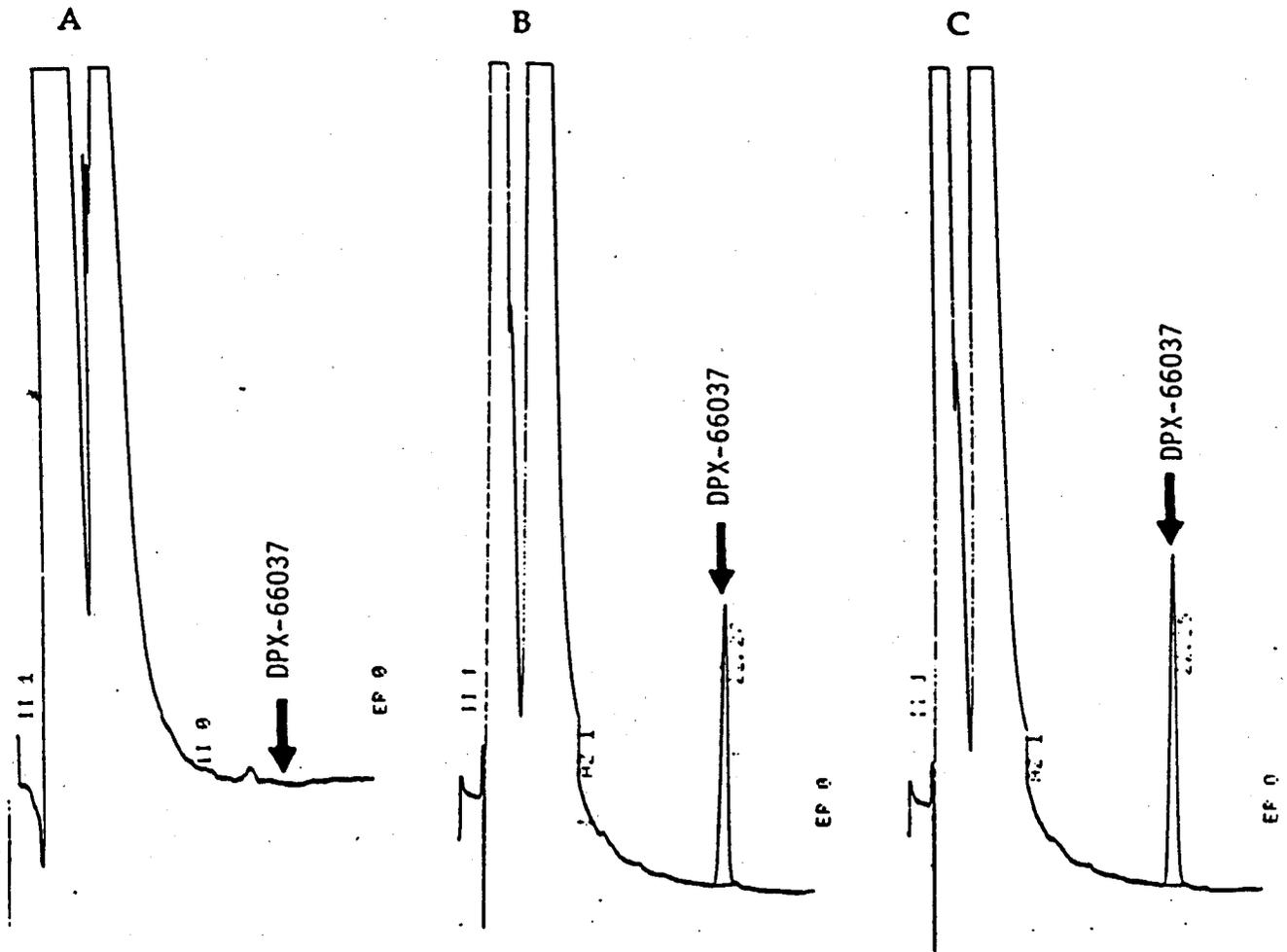


- A. Control sugar beet root
- B. Control sugar beet root spiked with 0.05 ppm DPX-66037
- C. 0.005 $\mu\text{g}/\text{mL}$ Standard Solution.

COPY

FIGURE 5

CHROMATOGRAMS OF SUGAR BEET ROOTS FROM FILER, IDAHO

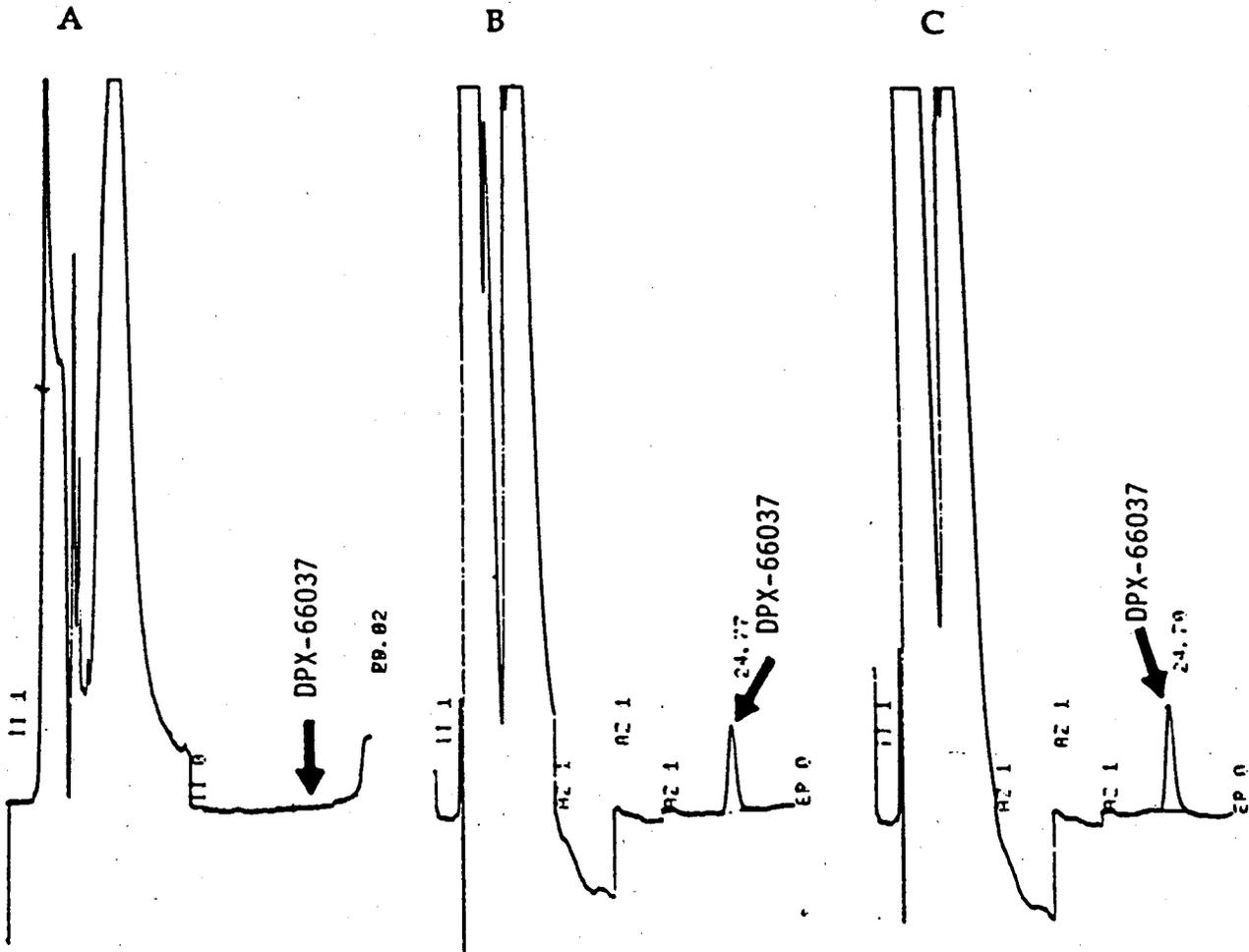


- A. Control sugar beet root
- B. Control sugar beet root spiked with 0.250 ppm DPX-66037
- C. 0.025 $\mu\text{g}/\text{mL}$ Standard Solution.

COPY

FIGURE 6

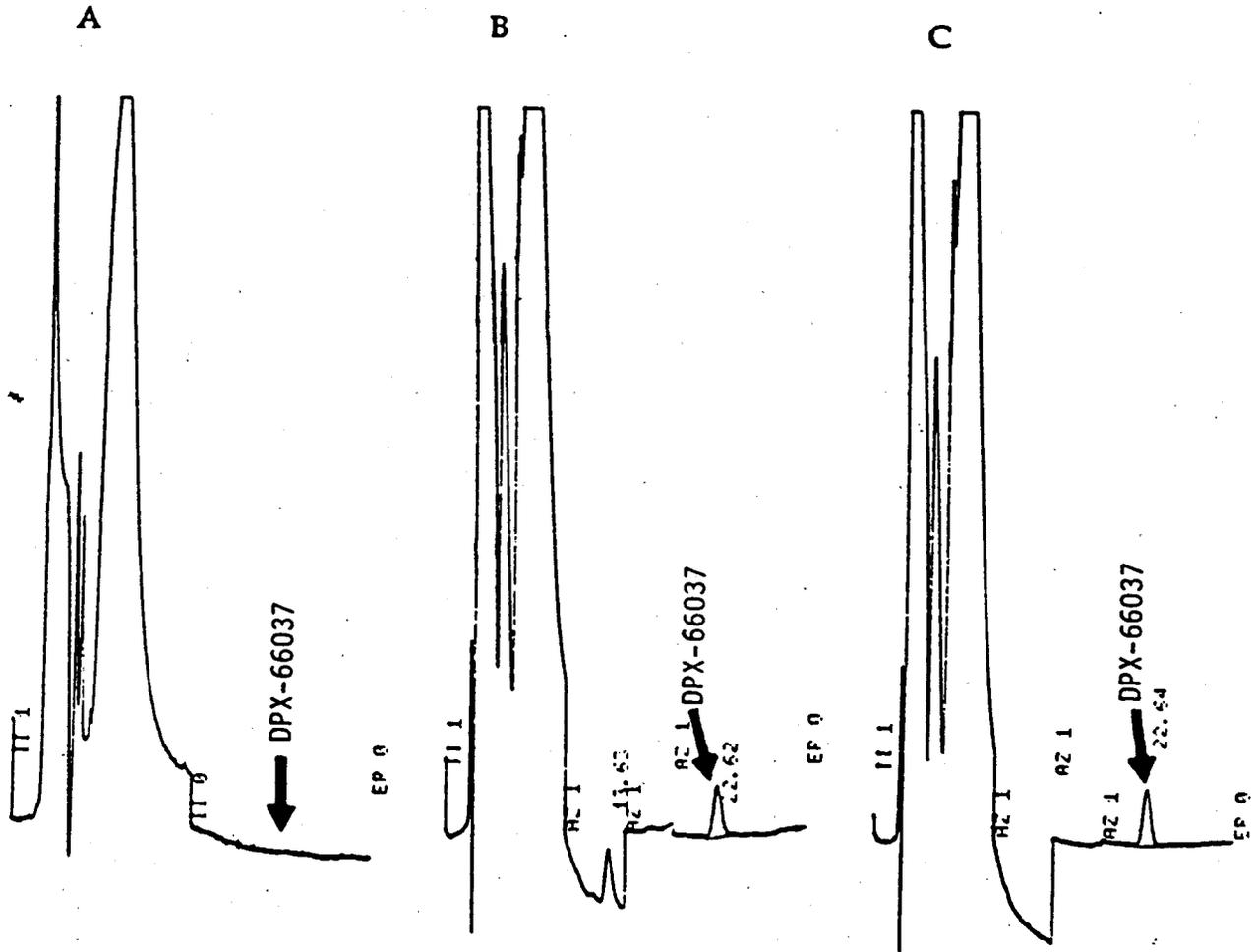
**CHROMATOGRAMS OF SUGAR BEET FOLIAGE FROM
GRANDIN, NORTH DAKOTA**



- A. Control sugar beet foliage
- B. Control sugar beet foliage spiked with 0.10 ppm DPX-66037
- C. 0.0100 $\mu\text{g}/\text{mL}$ Standard Solution.

FIGURE 7

CHROMATOGRAMS OF SUGAR BEET FOLIAGE FROM BILLINGS, MONTANA

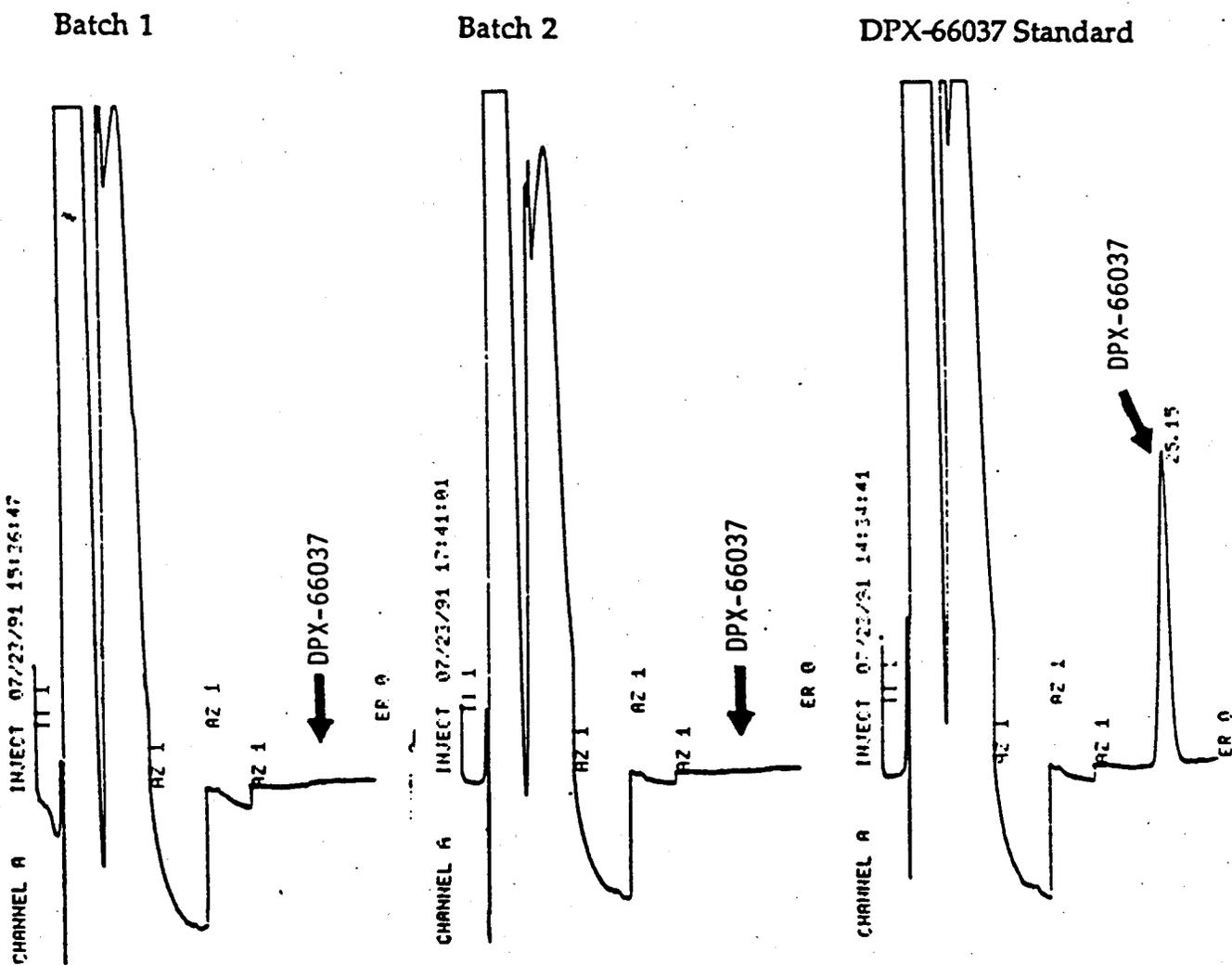


- A. Control sugar beet foliage
- B. Control sugar beet foliage spiked with 0.05 ppm DPX-66037
- C. 0.005 $\mu\text{g}/\text{mL}$ Standard Solution

COPY

FIGURE 8

CHROMATOGRAMS OF PESTICIDES IN BATCH 1 AND 2
CHROMATOGRAM OF A 0.020 µg/mL (0.200 ppm) DPX-66037
STANDARD SOLUTION

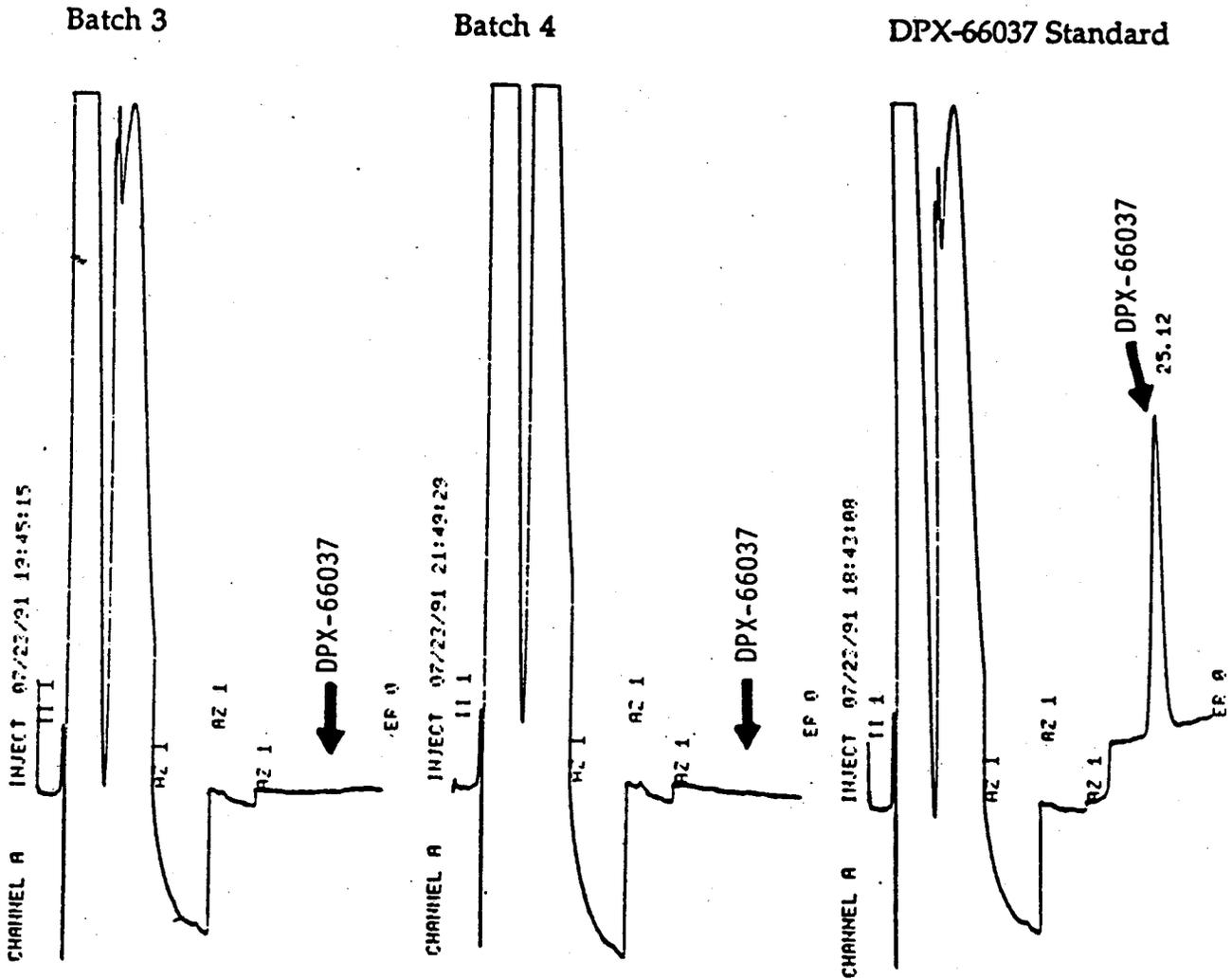


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FIGURE 9

CHROMATOGRAMS OF PESTICIDES IN BATCH 3 AND 4

CHROMATOGRAM OF A 0.020 µg/mL (0.200 ppm) DPX-66037
STANDARD SOLUTION



COPY

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5. L. J. Major, P. G. Rossi, "Extraction Efficiency of DPX-66037 from Sugar Beet Foliage and Roots," contained in the Study Records of AMR-2021-91.
6. L. J. Majors, "Stability Study: DPX-66037 in Acidified Standard Dilution Solution", contained in the Study Records for AMR-2021-91.