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Attachment #2

42

TRADE SECRET

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Study Title

ANALYTICAL METHOD FOR THE QUANTITATION OF DPX-E9636 IN CORN (FORAGE AND GRAIN)

Data Requirement

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

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Study Completed On

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Registration Specialist Leon W Hershberger
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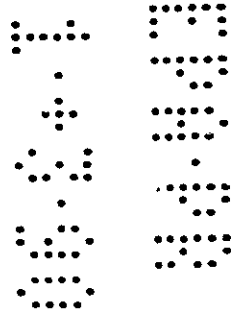

GOOD LABORATORY PRACTICE STATEMENT

The GLP requirements specified in 40 CFR Part 160 are not applicable to residue data chemistry requirements at the time of submission.

This study was conducted in the spirit of good laboratory practices.

Sponsor: E. I. du Pont de Nemours and Company, Inc.

Submitter: E. I. du Pont de Nemours and Company, Inc.

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I. SUMMARY/INTRODUCTION

A. Summary

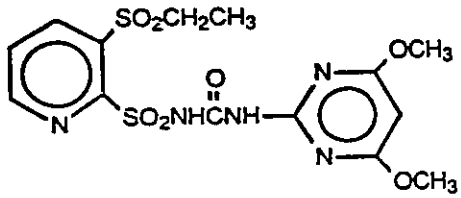
An HPLC method for the quantitation of DPX-E9636 in corn forage and grain has been developed which uses two HPLCs with UV detection at 254 nm. One chromatograph performs sample clean-up and the second allows sample analysis. Sample clean-up is achieved through reverse-phase chromatography enlisting eluent-switching between the two HPLCs. Column-switching, achieved by transfer of effluent from one HPLC to the next, provides the resolution required for quantitation of DPC-E9636. Alternately, these steps can be carried out by more elaborate instrumentation allowing fully automated control. Both approaches have been successfully applied to several sulfonylurea herbicides in many matrices Both are described herein for quantitation of DPX-E9636 in corn forage and grain. Quantitation is achieved at levels as low as 0.05 ppm with recoveries of 85 to 95 percent based on a 10-gram sample.

B. Introduction

Two HPLCs are enlisted for the determination of DPX-E9636 residues in corn forage and grain. The first of the two HPLCs is identified as the "Clean-Up Chromatograph" and removes most sample coextractives. The second HPLC, the "Analytical Chromatograph," separates the remaining sample components, allowing quantitation of DPX-E9636. Alternatively, a single instrument may be used for both functions by cleaning a batch of samples and then changing the column and eluent for their analysis. Only slight modification to an existing HPLC is required to perform this method. This two-system approach will be referred to as the "Split Method" of analysis (as contrasted with the "Automated Method" described in Appendix 2). The Split Method is suggested for enforcement purposes, where only a few samples require analysis.

This method has been designed for increased simplicity and productivity by eliminating manually intensive operations such as liquid/liquid extraction, evaporation and solid phase

extraction clean-up. Concentration of analyte and clean-up of sample matrix are performed by the chromatographs. This approach is successful due to the acid-base character of DPX-E9636. Retention of DPX-E9636 in these reverse-phase HPLC systems is dependent on the degree of the molecule's protonation, which in turn is affected by the HPLC eluent's pH. Retention versus eluent pH is illustrated in Figure 8 of Appendix 1. The structure of the compound is shown below:



DPX-E9636

N-((4,6-Dimethoxypyrimidin-2yl)aminocarbonyl)-3-(ethylsulfonyl)-2-pyridinesulfonamide
(A common name is not yet available)

In the clean-up step, two mL of Sample Extract is injected onto a Zorbax® Phenyl column in an aqueous eluent of 44% methanol buffered at pH 3.5. DPX-E9636, which is predominately uncharged in the pH 3 Sample Extraction Solution (25% methanol), concentrates at the head of the column during sample injection. As chromatography continues, DPX-E9636 is separated from a large number of polar compounds which could interfere in the subsequent analytical chromatographic step.

The column effluent which would contain the DPX-E9636 is collected from the Clean-Up Chromatograph. This fraction is further acidified with concentrated phosphoric acid and diluted to a known volume with water (to cause the protonated DPX-E9636 to concentrate at the head of the column in the Analytical Chromatograph). Two mL of this solution is injected into the Analytical Chromatograph with an eluent at higher pH (6.50) and lower methanol concentration (22%) and a Zorbax® R_X™ column Since the DPX-E9636 is ionized at this pH it has a reduced

affinity for the column. As a result, it is separated from the sample components collected from the Clean-Up HPLC, which are now comparatively less polar. DPX-E9636 is detected in both systems by a UV detector at 254 nm. This allows DPX-E9636 quantitation to 0.05 ppm on the Analytical Chromatograph.

II. MATERIALS/METHODS

A. Equipment

Equivalent equipment may be substituted in all cases. For minimum HPLC instrumentation requirements see Section I, Modifications or Potential Problems.

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

Centrifuge Rotors - Du Pont models HS4 and SS34 (Du Pont Instruments)

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

Centrifuge Bottles - 250 mL, polypropylene, IEC® Maxiforce® #2050, VWR #21018-037 (VWR Scientific, Bridgeport, NJ)

Centrifuge Tubes - Sepcor® 29 mm x 103 mm, polypropylene, VWR #21007-303 (VWR Scientific)

Food Processor - Hobart chopper model 84145 or 84186 (Hobart Corp., Troy, OH)

Mill - Sunbeam Oskar Food Processor (Sunbeam Appliance Company, Chicago, IL)

pH Meter - Beckman model PHI® 11 (Beckman Instruments, Inc., Fullerton, CA)

Narrow-Range pH Paper - EM® ColorpHast® Indicator Strips, Narrow range 2.5 - 4.5, VWR #EM-9581-3 (VWR Scientific)

Filter - Millipore® 47 mm Type GS, 0.22 µ pore size filter #GSWP 047 00, and glass filter holder and flask #XX15 047 00 (Millipore Inc., Milford, MA)

Liquid Chromatographs

Clean-Up Chromatograph:

Pump: Kratos Spectroflow model 400 with high efficiency filter (ABI Analytical, Foster City, CA)

Detector: Kratos Spectroflow model 783G with 12-µL flowcell (ABI Analytical)

Recorder: Hewlett Packard model HP7132A strip chart recorder (Hewlett Packard Co., Mt. View, CA)

HPLC column: Du Pont Zorbax® Phenyl 4.0 mm x 80 mm, 5 micron Reliance® cartridge, 3-pack, #820662-942 and column end-fittings #820669-001 (MAC-MOD Analytical Inc., Chadds Ford, PA)

Column oven: Waters model WAT038039 (Millipore Inc.)

Eluent Selection Valve: Rheodyne model 5011P low pressure six- position rotary valve (Rheodyne Inc., Cotati, CA)

Injection and Backflush Valves: Rheodyne model 7000P 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, ~~3-way valve #38-082 (Rainin Instrument Co , Inc.,~~ Woburn, MA)

Analytical Chromatograph:

Pump: Hitachi model 655A-11 pump and L-5000 Gradient Controller (EM® Science, Cherry Hill, NJ)

Detector: Kratos Spectroflow model 783G with 12-µL flowcell (ABI Analytical)

Recorder: Hewlett Packard model HP7132A strip chart recorder (Hewlett Packard Co)

HPLC column: Du Pont Zorbax® R_x^{TM} 4.6 mm x 250 mm, 5 micron analytical column, #880967-901 (MAC-MOD Analytical)

Column oven: Hitachi model 655A-52 (EM® Science)

Injection Valve: Rheodyne model 7000P high pressure six-port, two-position valve (Rheodyne Inc.)

B. Reagents and Standards

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, NJ)

K_2HPO_4 - "Baker Analyzed"® Reagent #3252-01 (J.T. Baker Chemical Co., Phillipsburg, NJ)

KH_2PO_4 - EM® low absorbance grade #PX 1566-2 (EM® Science)

H_3PO_4 - "Baker Analyzed"® Reagent #0260-02 (J.T. Baker Chemical Co.)

DPX-E9636 - DPX-E9636 Reference Standard (Agricultural Products Department, E. I. du Pont de Nemours and Company, Inc., P. O. Box 80402, Wilmington, DE 19880-0402)

C. Preparation of Solutions

1M KH_2PO_4:

Dissolve 136 g of KH_2PO_4 in approximately 800 mL of water and dilute to 1 L. Filter through a Millipore® Type GS 0.22-micron filter.

1M K_2HPO_4:

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

Sample Extraction Solution, 25% methanol/75% pH 7 buffer:

Mix 150 mL of 1M K_2HPO_4 with 1350 mL of water; adjust the pH of this solution (as measured by a calibrated pH meter) to 7.0 by addition of concentrated phosphoric acid (2-2.5 mL). Add 500 mL of methanol and mix. Final pH measures about 7.5.

Eluents:

Eluent A; 44% methanol, pH 3.5:

Add 10 mL of 1M KH_2PO_4 to a 1000-mL graduate cylinder and dilute to 560 mL with water. Add 440 mL of methanol and mix. Adjust the pH of this solution to 3.50 (as measured by a calibrated pH meter) by addition of concentrated phosphoric acid. Sparge briefly (about 5 minutes) with helium to degas; further sparging is unnecessary and may change the methanol concentration due to evaporation.

Eluent B; 22% methanol, pH 6.5:

Add 10 mL of 1M K_2HPO_4 to a 1000-mL graduate cylinder and dilute to 780 mL with water. Add 220 mL of methanol and mix. Adjust the pH of this solution (as measured by a calibrated pH meter) to 6.50 with concentrated phosphoric acid. Sparge briefly with helium.

Eluent C, 90% methanol/10% water

Add 100 mL of water to 900 mL of methanol and mix. Sparge briefly with helium

Standards:

Stock Standard Solution:

Accurately weigh 0.0200 g of DPX-E9636 and dissolve in 100 mL of acetonitrile to make a 200 µg/mL stock standard. Make an intermediate dilution from the stock standard to 5.0 µg/mL in acetonitrile; this will be used for fortification of samples and preparation of chromatographic standards. These standard solutions should be stable up to 6 months if stored at 4° C or over a year when stored at -20° C.

Chromatographic Standard Solutions:

Prepare chromatographic standards at 0.050, 0.025, 0.010 and 0.005 µg/mL by dilution of the 5.0 µg/mL Stock Standard Solution with Sample Extraction Solution. The concentration of acetonitrile in these final dilutions is kept at or below 2% to keep the total amount of organic modifier in the sample solution sufficiently low. Keep all chromatographic standards at 4° C when not in use. These standards should be stable for a week if they are not acidified Before injection onto the chromatograph the standard solution's pH must be adjusted to between 2.5 and 3.5 (as determined by narrow-range pH paper) with concentrated phosphoric acid Once acidified, the stability of the standard is greatly reduced and has a useful lifetime (<10% degradation) of about 8 hours if kept at 0° C See Table 3 (page 29) for DPX-E9636 stability in acidic solution at various temperature.

D. Analytical Procedure

1 Preparation of Sample

Frozen forage and fodder samples should be cut into 2-4 inch pieces using a floral cutter or knife and then chopped in a Hobart chopper with dry ice. Grain should be removed from the ears and ground frozen in a mill. The dry ice is allowed to evaporate, and samples are stored at -20° C until sampled for analysis.

2. Fortification of Samples

Thaw prepared untreated corn samples and place 10 grams in a 250-mL centrifuge bottle. For a 0 100 ppm fortification add 0 200 mL of the 5 0 µg/mL DPX-E9636 standard in acetonitrile to the above sample. Fortify over the range of expected levels of DPX-E9636 in the field samples and at the quantitation limit (0 05 ppm) of this analytical method, generally 0.050 ppm to 0.500 ppm. Evaporate the acetonitrile under a stream of nitrogen for about 15 minutes.

While making fortifications, it is convenient to prepare the chromatographic standards from the same stock standard solution while making the fortifications by adding the same volume of stock standard to a 100-mL volumetric flask for dilution to volume with Sample Extraction Solution (see Preparation of Solutions section).

3. Extraction

a) Thaw and accurately weigh 10 grams (+/- 0.1) prepared sample into a 250-mL centrifuge bottle. Add 100 (+/- 2) mL of Sample Extraction Solution and mix. Let soak for about 15 minutes. Grind with a Tissumizer® for 1 minute at about 60% of maximum output (at higher output mixture foams excessively). Wait 5 minutes and repeat the Tissumizing-rest procedure two more times.

b) Centrifuge the sample-buffer mixture in the Sorvall® RC-5C centrifuge (HS-4 rotor) for 10 minutes at 7000 rpm (brake on, refrigeration optional). If using an alternate speed, centrifuge until most particulates have separated from the Extraction Solution

c) Remove 20-40 mL of supernatant with a glass syringe and large bore needle and place in a 40-mL Sepcor® tube. In preparation for lowering the pH, cool the sample below 4° C by placing the tubes in a freezer for at least an hour; this will greatly improve DPX-E9636 stability once the sample is acidified. The analysis may be interrupted at this point if the sample is stable for at least two days at this pH (7.5) and temperature (-20° C).

d) Acidify samples by addition of concentrated phosphoric acid until a pH of 2.5 to 3.5 is reached (as determined by pH paper; generally 150-250 µL acid). Immediately return samples to the freezer for an additional 10 to 15 minutes, this allows any precipitate to form before centrifugation and further cools the sample, minimizing DPX-E9636 decomposition.

e) Centrifuge in the Sorvall® RC-5C centrifuge (SS-34 rotor) for 10 minutes at 20,000 rpm (2-5° C, brake on). If a refrigerated centrifuge is not available, cool the sample longer before acidification and minimize the time the sample is at room temperature.

f) Decant the supernatant for injection onto the HPLC, being careful to avoid particulates. This supernatant will be referred to as Sample Extract. Keep the Sample Extract at a temperature between 0° C and -20° C.

4. Chromatography

Sample Extract is ready for injection onto the Clean-Up Chromatograph. DPX-E9636 standards in Sample Extraction Solution require acidification with concentrated phosphoric acid (to pH of 2.5 to 3.5) before chromatography. Samples and standards should be kept cold (0° to -20° C) until chromatographed.

General chromatographic operating 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 which allows the injection of 2-mL samples. The chromatograph has provisions for reversing the eluent flow through the column and selecting an alternate eluent for cleaning the column. The system also allows collection of column effluent during the prescribed time "window" when the DPX-E9636 would elute. The Clean-Up Chromatograph is configured as shown in Figure 4 (page 33), the chromatographic conditions are summarized in Table 4 (page 32) The sequence of events - sample loop in line, eluent collection, and backflushing - can easily be performed by manual activation of the valves Alternatively, these valves are controlled by a

Kratos Spectroflow detector program (see Detector Programming, page 35 and Table 5, page 36). Control through the detector demands less operator time and attention.

Sample Extract clean-up is carried out on a Zorbax® Phenyl column with an aqueous eluent of 44% methanol buffered at pH 3.5. A DPX-E9636 standard solution is first chromatographed to determine the analyte's retention time; from this time suitable times may be set to collect the DPX-E9636 as it elutes from the phenyl column. Two minutes prior to the analyte's retention time, a stream-switching valve following the detector is actuated so eluent may be collected. Collection continues for 5 minutes, resulting in 6.5 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-E9636 decomposition in the acidic chromatographic eluent. After collection one drop (about 30 µL) of concentrated phosphoric acid is added to further reduce the pH, and the contents of the flask brought to volume (10 mL) with water to ensure that the DPX-E9636 will concentrate at the head of the analytical column during subsequent injection. This results in a five-fold dilution of the DPX-E9636 in the original sample since only 2 mL of Sample Extract was injected onto the Clean-Up Chromatograph. Addition of water and acid is necessary to ensure that DPX-E9636 will concentrate on the column of the Analytical Chromatograph.

After eluent collection, the Zorbax® Phenyl column is backflushed with 90% methanol/10% water (eluent C) for 10 minutes by switching the back-flush valve (Figure 4, page 33) and selecting the appropriate eluent. After 10 minutes, the system is returned to its original state and the column allowed to reequilibrate with eluent A (44% methanol, pH 3.5) for about 10 minutes.

The Analytical Chromatograph consists of a pump with a valve to select between two eluents, an injection valve with a 2-mL sample loop, a column oven and UV detector.

Chromatographic conditions are listed in Table 4 (page 32). The eluent obtained from the Clean-Up Chromatograph is injected on a Zorbax® R_x™ column with eluent B, one of lower methanol concentration (22%) and higher pH (6.5) than used in the Clean-Up HPLC. After the DPX-E9636 has eluted, the column is forward-flushed with 90% methanol/10% water (eluent C) for 4 to 5 minutes following each sample run to remove any highly retained sample components. This flush is not necessary after chromatography of a standard solution. The system is allowed to reequilibrate with eluent B, requiring less than 10 minutes.

2. Operating Conditions

a) See Table 4 (page 32) for a list of chromatographic operating conditions for both the Clean-Up and Analytical Chromatographs. All Sample Extracts and acidified standard solutions are at a temperature of 0° C to 4° C when loaded into the sample loops in order to minimize DPX-E9636 degradation. Samples and standards should be the same temperature to avoid error in calculation of DPX-E9636 concentrations

b) Determination of eluent collection times (to be done daily).
Equilibrate the Clean-Up Chromatograph with eluent A. If a new Zorbax® Phenyl column is to be used, first condition it by injecting Sample Extract, running about 30 minutes with eluent A, backflushing the column with 90% methanol/10% water (eluent C), and reequilibrating with eluent A. Determine the retention time for DPX-E9636 standard (approximately 20 to 22 minutes). Eluent collection should begin 2 minutes prior to the DPX-E9636 retention time and should end 3 minutes after, to collect 6.5 mL of eluent. It is important that the peak width at baseline for a 0.01 µg/mL standard be no greater than 3.5 minutes to ensure quantitative collection of the analyte. If greater, a new column should be substituted.

3. Calibration Procedure

a) Prepare chromatographic standards by diluting the 5.0 µg/mL stock standard with Sample Extraction Solution as specified in the Reagents and Standards section. Make 3 or 4 standards at and above the quantitation limit and which cover the expected range of DPX-E9636 in the analyses; 0.050, 0.010 and 0.005 µg/mL standards are typically run on the Analytical Chromatograph. A five-fold dilution of the 0.005 µg/mL DPX-E9636 standard solution with Extraction Solution should also be prepared for chromatography on the Analytical HPLC to ensure adequate detector response at the quantitation limit for a corn sample (0.05 ppm).

b) A 0.010 µg/mL DPX-E9636 standard is typically run on the Clean-Up HPLC at the start of the day to determine the eluent collection time and judge column performance (see Operating Conditions above). The eluent collection window is set to begin 2 minutes prior to and end 3 minutes after the DPX-E9636 retention time. A second standard may be run at the end of the day if column degradation is suspected (as evidenced by low recoveries for fortified control samples).

c) Otherwise, chromatographic standards are run on the Analytical Chromatograph only (they do not pass through the Clean-Up HPLC).

d) Chromatograph each acidified standard solution and measure the DPX-E9636 peak height or area, recording pertinent detector and recorder attenuations DPX-E9636 has a retention time of approximately 20 minutes on the Analytical HPLC. A plot of the peak height or area versus standard concentration should be linear and pass through the origin (see Figure 1, page 28)

4. Determination of DPX-E9636 in Sample

a) Clean Sample Extracts by injecting 2 mL in the Clean-Up Chromatograph (a minimum volume of 5 mL is required to flush and fill the sample loop). Collect eluent in a chilled 10-mL volumetric flask at the appropriate time (determined daily, see Calibration section, step B). Acidify this with one drop (approximately 30 µL) of concentrated phosphoric acid and dilute to volume with water. Keep this sample at or below 4° C at all times to reduce DPX-E9636 degradation.

b) Inject 2 mL of the cleaned Sample Extract from above onto the analytical column and chromatograph (a minimum volume of 5 mL is required to flush and fill the sample loop). Identify the presence or absence of DPX-E9636 by the retention time determined in the calibration runs. Measure the DPX-E9636 peak, if present, in the same manner used for calibration.

c) Calculate the concentration of DPX-E9636 in each sample using the equation described under Method of Calculation (page 20).

F. Interferences

Several residue methods (References 7, 8) have been developed using the same technique, and we have found them to be very specific and relatively free of significant interference We expect this method will be free of interference from most pesticides and impurities in reagents, sample matrices and on glassware. IN-70941 and IN-70942, the most commonly found soil and plant metabolites have been shown not to interfere with the DPX-E9636 peak.

If a significant interference is apparent, the pH of eluent B may be adjusted slightly to selectively move the DPX-E9636 peak relative to the interference. A pH drop of 0.25 units will increase the DPX-E9636 retention time by approximately 2 minutes.

G. Confirmatory Techniques

The presence of DPX-E9636 may be confirmed by substituting a Zorbax® Phenyl 250 mm x 4.6 mm column for the Zorbax® R_X™ column in the Analytical Chromatograph.

The acid/base character of DPX-E9636 may also be used for confirmation. Changing the pH of Eluent B from 6.5 to a lower value such as 5.5 will selectively change the retention time of DPX-E9636. At lower pHs the methanol concentration may need to be increased to maintain a reasonable retention time.

H. Time Required for Analysis

Sample processing is relatively rapid, typically four to eight sample extracts can be prepared in about two hours. With this split approach, sample clean-up requires about 40 minutes, analysis about 30 minutes. When two chromatographs are used the two functions can be staggered so that the required time is effectively 40 minutes per sample. If only one chromatograph is used for both tasks, throughput is roughly halved. It is suggested a batch of samples be processed through the Clean-Up HPLC on one day, stored overnight at -20° 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, page 35). Alternatively, valve actuation may be done manually with acceptable precision.

Appropriate substitutions may be made for the components of the chromatograph. The Clean-Up Chromatograph requires a pump capable of selecting between two eluents, a column oven, and UV detector. Comparable equipment can be configured for this role by the addition of two high-pressure switching valves for sample injection and column back-flushing. Similarly, most any HPLC capable of selecting between two eluents and injecting a 2-mL sample volume can be used as the Analytical HPLC. If equipment is limited, it is possible to use one instrument and reconfigure the Clean-Up HPLC for operation as the Analytical Chromatograph. Following clean-up, the samples may be stored at -20° C overnight to allow time for the 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 A with eluent B and a change in columns from a Zorbax® Phenyl to an analytical Zorbax® R_x™ column. Cleaned samples may be analyzed the second day.

If resolution is insufficient on the Zorbax® R_x™ column, eluent B may be adjusted by changing pH and/or methanol concentration. The change in pH should selectively move the DPX-E9636 peak relative to an interference; the methanol concentration may then need to be adjusted to obtain desired retention times.

Sample Extracts are typically not filtered prior to injection onto the Clean-Up Chromatograph. It is unnecessary as particulates will be removed from the head of the column during the backflush step. If samples are filtered, slight loss of analyte may be seen (as a loss in recovery) due to binding of the DPX-E9636 to the filter. Filtering is optional as it also allows additional opportunity for contamination of the sample.

J. Method of Calculation

1. Calibration Factor (CF)

The calibration factor (CF) is the ratio of detector response (height or area) to the DPX-E9636 concentration. Measure the peak height or peak area for each standard (normalize for a given attenuation if required); divide this value by the standard's concentration (in units of µg/mL). A standard should be run after every 3 to 4 samples. Use the average CF from the standard runs preceding and following a group of samples to determine DPX-E9636 concentration in those samples.

CF = (peak height or area) / (DPX-E9636 concentration [µg/mL] in standard)*

2. Analyte in the Sample

ppm DPX-E9636 in sample = [M x D x (100+B)] / [SW x CF]

M = Height or area of the DPX-E9636 peak. Height or area is in the same units and normalized to the same scale as that used for CF calculation above

D = Dilution factor.
D = 5 for samples processed through the Clean-Up HPLC.

B = Volume of water contributed by the sample (mL).
B = % water by weight x SW x 1 mL/g water

SW = Sample weight in grams (10 g)

CF = Calculation Factor described above

* Peak heights are measured with the detector and recorder at set attenuations. If these parameters are changed, heights must be normalized to one set of conditions before CF values and analyte concentrations are calculated.

III. RESULTS/DISCUSSION

A. Accuracy

Recoveries from duplicate control samples at four fortification levels ranging from 0.500 ppm to 0.050 ppm and tested over two days were conducted to assess the accuracy of the analytical method. DPX-E9636 recoveries from eight fortified control forage samples averaged 94%. Recoveries averaged 89% for eight control corn grain samples (Reference 1; see Table 1, page 26). Extraction efficiency of DPX-E9636 from field treated samples was estimated by the analysis of corn plants treated with radiolabeled DPX-E9636. The extraction efficiency is estimated to be between 89% and 93% (Reference 3; see Table 2, page 27).

The accuracy of this method is enhanced by elimination of many manual sample preparation steps such as liquid/liquid extractions, evaporations and solid phase extraction. All can result in lowered recoveries and are potential sources of contamination from glassware.

A major source of error in this method is decomposition of DPX-E9636 in the acidified Sample Extraction Solution. Decomposition can be minimized by keeping acidified samples and standards below 4° C and by and by minimizing the time between sample clean-up and analysis. (See Table 3, page 29, for stability vs. temperature data.)

Recovery may also be compromised if the eluent collection window is too narrow relative to the baseline width of the DPX-E9636 peak. The collection time cannot be lengthened significantly because of the need to allow for dilution of the collected fraction before injection on the Analytical Chromatograph. The phenyl column on the Clean-Up Chromatograph should be evaluated daily or after every 8-12 samples to ensure adequate peak shape and size for complete analyte collection.

Slight error can be attributed to variable water content of samples. Calculations contain a provision for sample water content, but this is only estimated for the sample type (forage or grain). The effect of this estimation is slight however; placing the water content of a corn forage sample at 60% when it is actually 50% would generate an analysis which is only 1% too high.

B. Precision

The standard deviations (sample standard deviation, σn-1) of recoveries were 5% and 1% for eight analysis of each sample matrix (see Table 1, page 26). DPX-E9636 concentrations ranged from 0.500 ppm to 0.050 ppm. The range of recovery for the above samples was 86% to 99% for the forage samples and 87% to 91% for the grain samples. Decomposition of the analyte is most likely the main contributor to the variability of the results, although variability is within acceptable limits. Since many manual operations have been eliminated, the rest of the method is inherently very precise.

C. Limits of Detection and Quantitation

The limit of quantitation is determined by the signal to noise ratio of the detector and the magnitude of coeluting peaks. When using a sensitive detector such as the Kratos 783G the limit of quantitation is 0.050 ppm or lower. The DPX-E9636 peak height for a sample containing 0 050 ppm is greater than 0.1 mAU (milliAbsorbance Units). The noise of a good detector should be 0.01 mAU or less. This gives a signal to noise ratio of greater than 10 which should be adequate for quantitation. Chromatograms of 0.050 ppm DPX-E9636 fortified samples can be seen in Appendix 1

D. Ruggedness Testing

The method uses reverse-phase liquid chromatography with UV detection; both are well understood and known to be stable and reliable. The eluent collection time window is large (5 minutes) relative to the DPX-E9636 peak (2.5-3.5 minutes). This allows for dead volume in the

tubing of the chromatograph, as well as variability in DPX-E9636 retention time and non-ideal analyte peak shape on the phenyl column (band broadening or peak tailing).

E. Limitations - Instability of DPX-E9636 in Acid Solution

DPX-E9636 is unstable in acidic solutions at room temperature. Figure 2 (page 30) shows the rate of decomposition for DPX-E9636 in Sample Extraction Solution which has been adjusted to lower pHs by the addition of phosphoric acid. The solutions are relatively stable at pH 8.0 and 6.5. At pH 2.5 and 3.5, well below the pK_a of DPX-E9636, the solutions decompose quickly and at similar rates The decomposition is very temperature dependent We have determined the rate of decomposition of DPX-E9636 at 0° C, 15° C, and 35° C. From this data we have predicted the rate of decomposition at other temperatures. See Table 3 and Figure 3 (pages 29 and 31) for more information on the relationship between DPX-E9636 decomposition rate and temperature.

IV. CONCLUSION

This analytical method is suitable for the determination of DPX-E9636 in corn forage and grain at levels down to 0.05 ppm. The equipment and technique used in this method are generally applicable to many if not most commercial sulfonylurea herbicides.

This method has been designed to be run on commercial HPLC equipment with minor modifications. It is most useful for enforcement purposes or other applications where there is a limited number of samples to analyze. When a large number of samples require analysis, a more automated approach, detailed in Appendix 2, is suggested. The Automated Method requires significantly more set-up time and more elaborate instrumentation, but provides a far higher throughput and less operator attention. The two approaches, the Split Method vs. the Automated Method, are compared in Appendix 3

V. CERTIFICATION

ANALYTICAL METHOD FOR THE QUANTITATION OF DPX-E9636 IN CORN (FORAGE AND GRAIN)

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: J. H. Larochelle — Date: 8/10/89
Study Director

Approved by: R. D. Collins — Date: 8/10/89
Research Supervisor

Date Study Initiated.	August 12, 1988
Date Study Completed.	May 1, 1989
Notebook Reference:	NB# E52647
Storage Location of Records, and Final Report:	E. I du Pont de Nemours & Company, Inc. Agricultural Products Department Experimental Station and/or Records Management Center P. O. Box 80402 Wilmington, Delaware 19880-0402
Sponsor:	E. I du Pont de Nemours & Company, Inc. Agricultural Products Department Research and Development Division Experimental Station P. O. Box 80402 Wilmington, Delaware 19880-0402

VI. TABLES/FIGURES

TABLE 1

Recovery Data for Validation of Method of Analysis for DPX-E9636

Sample Matrix	DPX-E9636 Fortification Level (ppm)	DPX-E9636 Recovered (ppm)	Percent Recovery	Average Percent Recovery	Std. Dev. (%)
forage	0.500	0.483	97 %	94 %	5 %
	0.500	0.478	96 %		
	0.250	0 247	99 %		
	0.250	0 244	98 %		
	0.100	0.093	93 %		
	0.100	0 093	93 %		
	0 050	0 044	88 %		
	0.050	0 043	86 %		
grain	0.500	0 444	89 %	89 %	1 %
	0 500	0 452	90 %		
	0 250	0 218	87 %		
	0.250	0 219	88 %		
	0.100	0.088	88 %		
	0 100	0 091	91 %		
	0 050	0 045	89 %		
	0.050	0 043	87 %		

Reference 1

TABLE 2

Extraction Efficiency Data

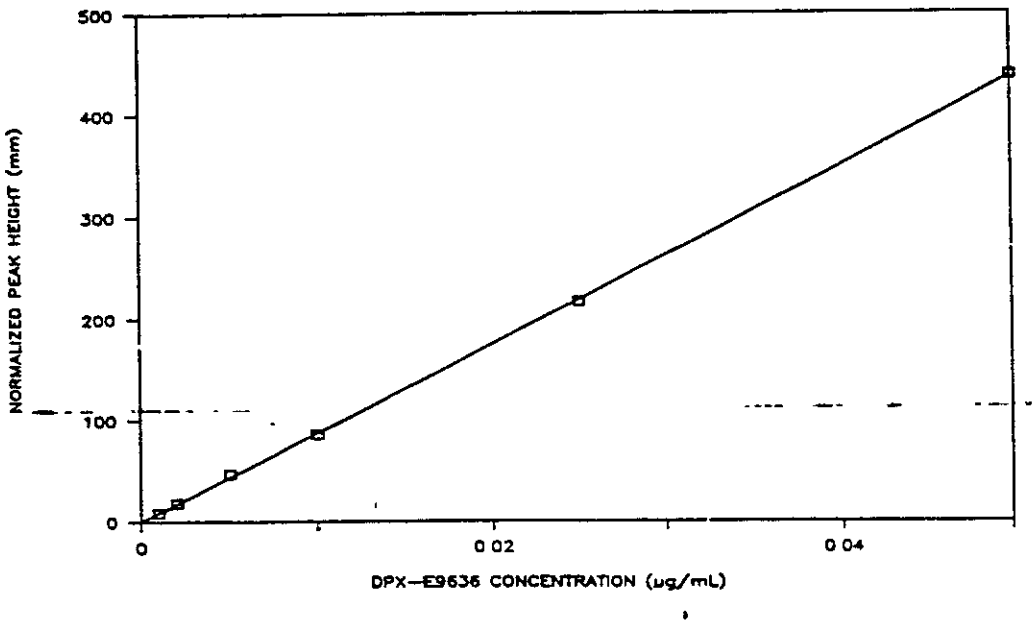
Sample	Quantity* Extracted dpm	Quantity Bound dpm	Percent Extracted	Percent Bound
Day 0 - Pyridine label corn forage	$3.80e^6$	$3.39e^5$	91.8 %	8.2 %
Day 0 - Pyridine label	$5\ 32e^6$	$4\ 76e^5$	91.7 %	8.2 %
Day 8 - Pyridine label	$2.62e^6$	$2.02e^5$	92.9 %	7.2 %
Day 8 - Pyridine label	$2.44e^6$	$2.92e^5$	89.4 %	10.7 %

* Extraction solution is 25% methanol, 75% 0.1 M Potassium Phosphate pH 7.0, that was used in this analytical method.

Reference 3.

FIGURE 1

Calibration Curve from Validation of Split Method of Analysis for DPX-E9636



Reference 4

TABLE 3

Decomposition of DPX-E9636 in Acid Solution

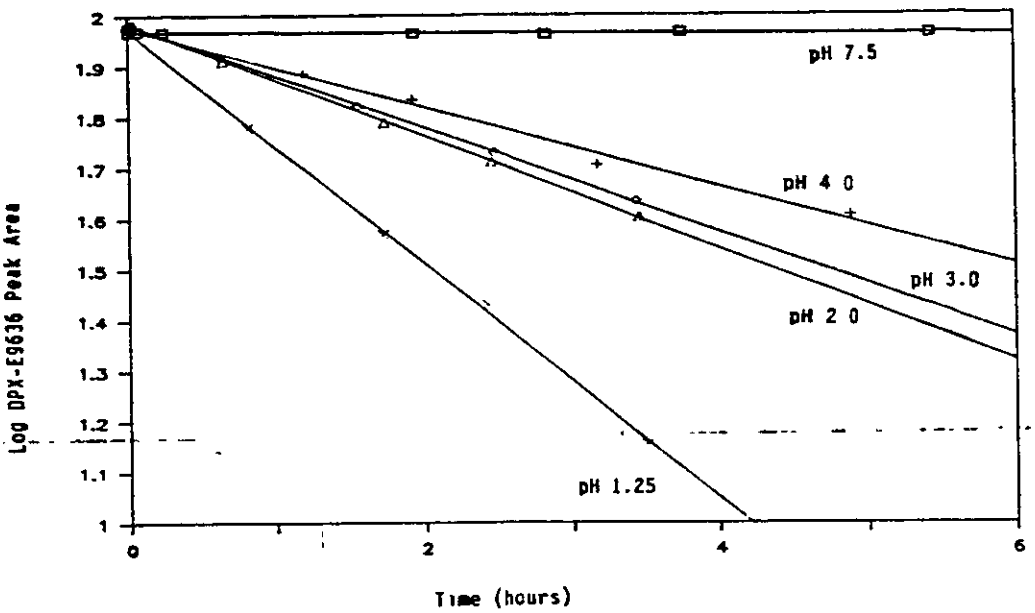
Temperature (° C)	Decomposition (% loss/hr)	Time for 10% DPX-E9636 Decomposition
30	25	0.3 hours
20	11	0.9 hours
10	4	2.6 hours
0	1.2	8 hours
-10	0.4	1 day
-20	0.10	1 week

The data in the above table was calculated based on kinetic data obtained at 35, 15, and 0° C.

Reference 5

FIGURE 2

Log DPX-E9636 Peak Area vs. Time and pH

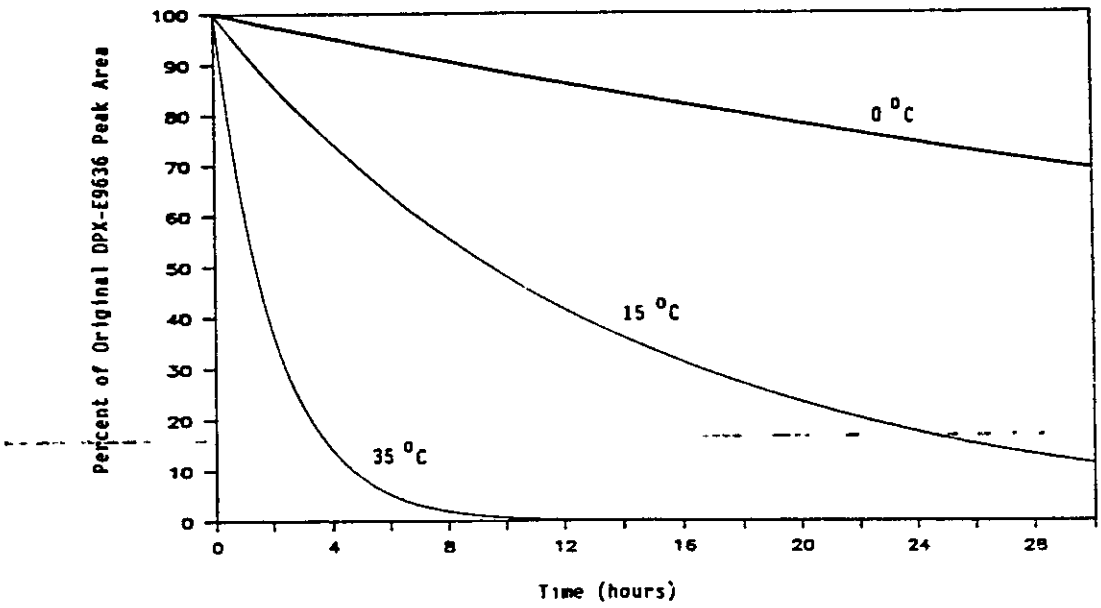


Note: All solutions are 25 % methanol, 0.1 M Potassium Phosphate except for pH 1.25, this is dilute phosphoric acid, 0 % methanol. All solutions stored at room temperature.

Reference 6

FIGURE 3

DPX-E9636 Assay vs. Time and Temperature



DPX-E9636 Standard Solutions are in acidified Sample Extraction Solution (acidified to pH 2-3 with concentrated H_3PO_4).

Reference 5

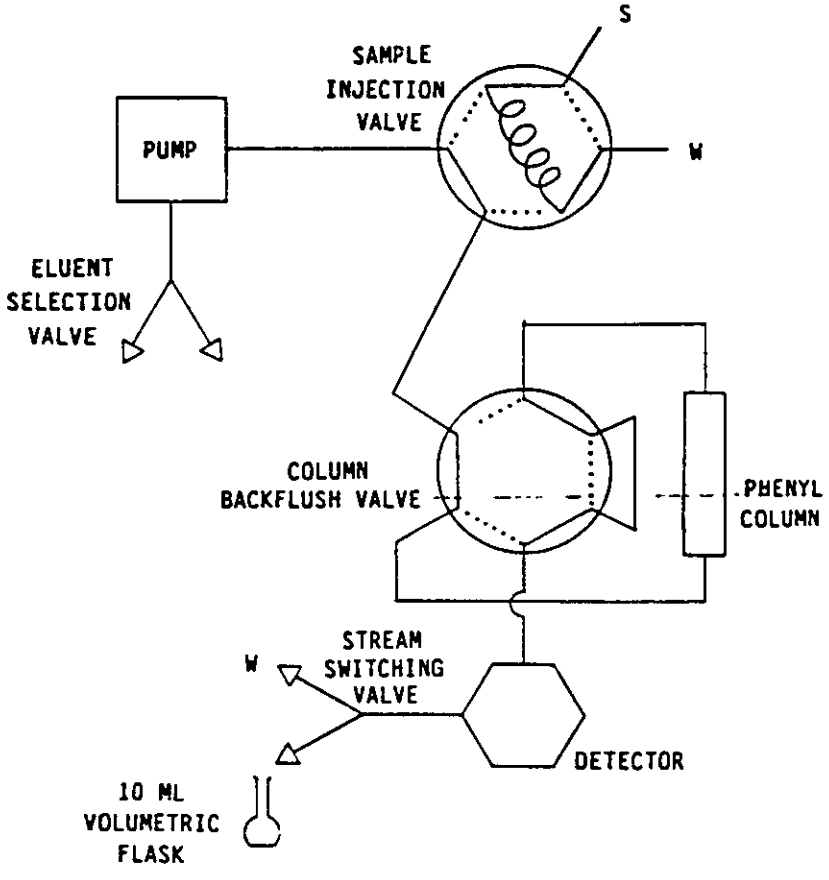
TABLE 4

Summary of Chromatographic Conditions

Clean-Up Chromatograph	
Column:	Du Pont Zorbax® Phenyl, 4.0 mm x 80 mm 5 micron Reliance® Cartridge
Column Temperature:	35° C
Eluent Flow Rate:	1.3 mL/min
Eluents:	A - 44% methanol, pH 3.5 C - 90% methanol/10% water
Sample Volume:	2.0 mL
Detector Settings.	Wavelength - 254 nm Sensitivity - 0 010 AUFS
Recorder Settings:	Chart Speed - 2.5 mm/min. Attenuation - 10 mV
Analytical Chromatograph	
Column:	Du Pont Zorbax® R_x™, 4.6 mm x 25 mm 5 micron analytical
Column Temperature:	35° C
Eluent Flow Rate:	1.3 mL/min.
Eluents:	B - 22% methanol, pH 6.50 C - 90% methanol/10% water
Sample Volume:	2.0 mL
Detector Settings·	Wavelength - 254 nm Sensitivity - 0 010 or 0.005 AUFS
Recorder Settings:	Chart Speed - 2.5 mm/min. Attenuation - 10 mV

FIGURE 4

Schematic of Clean-Up Chromatograph



S = Sample Injection Port

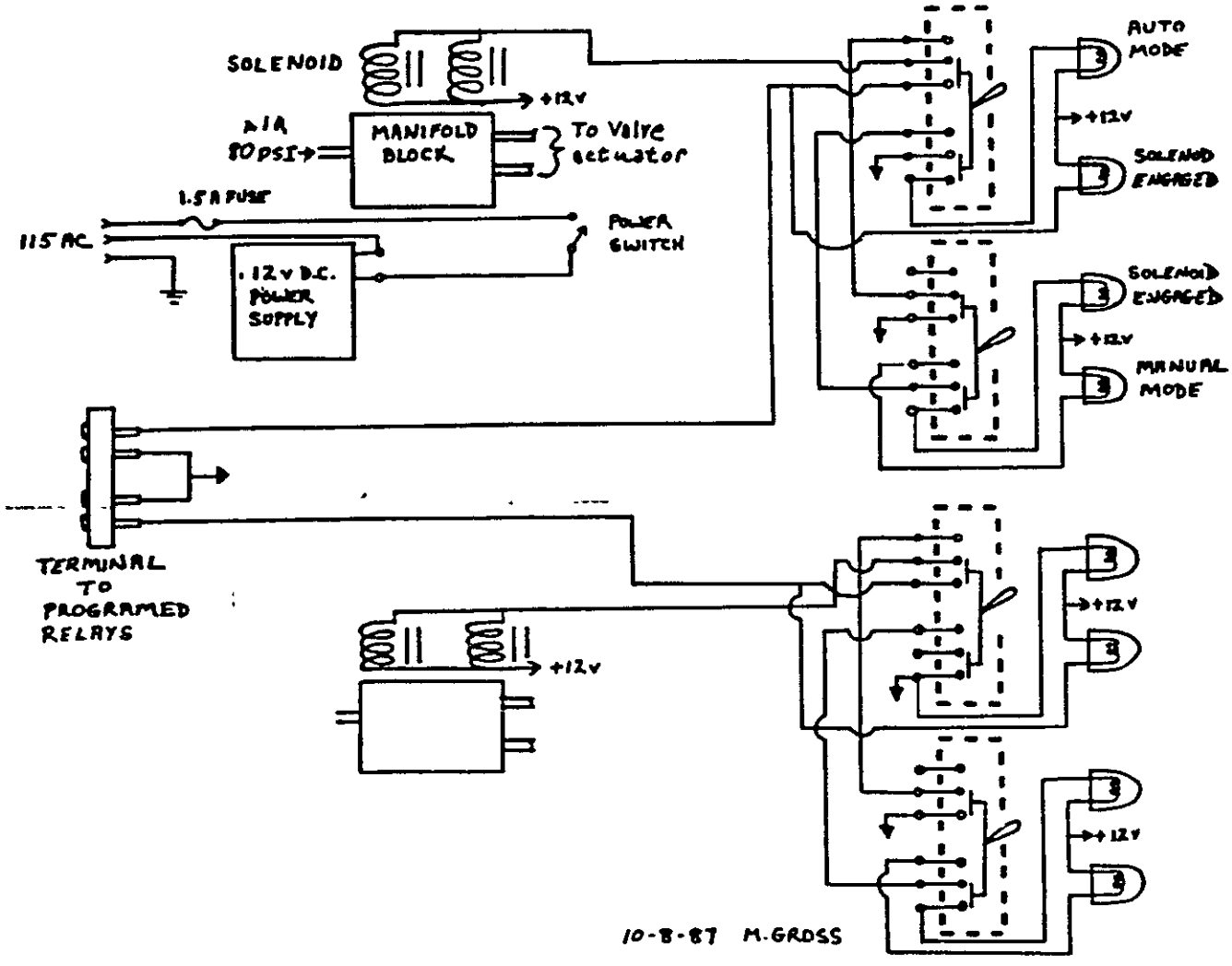
W = Waste

····· Valve On (Clockwise)

—— Valve Off (Counterclockwise)

FIGURE 5

Schematic of Pneumatic Valve-Switching Stations



Key to Detector Programming

The valves used in the Clean-Up Chromatograph are controlled by the Kratos Spectroflow detector program which follows. The program controls four internal relays which each switch a 12-volt power supply to activate solenoid air valves which, in turn, activate the chromatographic valves. Relay 1 controls the Sample Injection Valve, relay 2, the Column Backflush Valve, relay 3, the Eluent Selection Valve, and relay 4 controls the Stream Switching Valve. (Refer to Figure 4, page 33 for instrument configuration.) "Pneumatic valve-switching stations" may be inserted to allow optional manual override of the detector. The schematic for these stations is attached as Figure 5.

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. Relays 1 through 4 actuate the following:

Relay 1 - Sample Injection Valve; 1 = valve counterclockwise for sample injection.
Relay 2 - Column Backflush Valve; 1 = valve counterclockwise for column backflush
Relay 3 - Eluent Selection Valve. We used a 6-position valve to select between two eluents. Eluent A is supplied at position 2 and eluent C at position 3. Program steps 8 through 17 are required to reset the valve to position 2 after the column is cleaned. (This valve is rotated clockwise one step at a time by a ratchet mechanism. Therefore to advance the valve one position the actuator must be reset before the next actuation). Any low pressure valve able to select between two eluent may be substituted.
Relay 4 - Stream Switching Valve; 1 = eluent stream switched to allow for collection into flask.

Status: Abbreviations for status of instrument at each step.

L = Sample Injection Valve in load position.
I = Sample Injection Valve in inject position.
F = Backflush Valve in backflush position.
C = Stream Switching Valve in collect position.
1-6 = Eluent Selection Valve position.

TABLE 5

Detector Program #5 for the Clean-Up Chromatograph

STEP	TIME	RELAY 1 2 3 4	STATUS	EVENT
0	0.00	0 0 0 0	L . 2 .	Start - Eluent position = 2 Sample loop in load position
1	1.00	1 0 0 0	I . 2 .	Sample loop in inject position
2	8.00	0 0 0 0	L . 2 .	Reset valve to load position
3	*	0 0 0 1	. . 2 C	Begin eluent collection
4	*	0 0 0 0	. . 2 .	End eluent collection
5	25.00	0 0 1 0	. . 3 .	Eluent position = 3
6	25.04	0 0 0 0	. . 3 .	Reset valve 1
7	25.10	0 1 0 0	. F 3 .	Reverse flow for backflush
8	36.00	0 0 1 0	. . 4 .	End backflush Eluent position = 4
9	36.04	0 0 0 0	. . 4 .	Reset valve 1
10	36.06	0 0 1 0	. . 5 .	Eluent position = 5
11	36.10	0 0 0 0	. . 5 .	Reset valve 1
12	36.14	0 0 1 0	. . 6 .	Eluent position = 6
13	36.18	0 0 0 0	. . 6 .	Reset valve 1
14	36.20	0 0 1 0	. . 1	Eluent position = 1
15	36.24	0 0 0 0	. . 1 .	Reset valve 1
16	36.28	0 0 1 0	. . 2 .	Eluent position = 2
17	36.38	0 0 0 0	. . 2 .	Reset valve 1

* Step 3: DPX-E9636 retention time minus 2 minutes
Step 4: DPX-E9636 retention time plus 3 minutes

VII. REFERENCES

1. P. G. Rossi, "Validation - DPX-E9636 Determination in Corn, Split Method," E. I. du Pont de Nemours & Company, Inc. Laboratory Notebook #E52647, pages 32 through 50 .

2. L. J. Major, "Validation - DPX-E9636 Determination in Corn, Automated Method," E. I. du Pont de Nemours & Company, Inc. Laboratory Notebook #E52647, pages 2 through 15 and 22 through 25.

3. A. M. Brown, "Extraction Efficiency Experiments Using C^{14}-Treated Corn," E. I. du Pont de Nemours & Company, Inc. Laboratory Notebook #E58268, pages 123 through 140.

4. L. J. Major, P. G Rossi, "Calibration Curve for DPX-E9636 Split Method Validation," E. I. du Pont de Nemours & Company, Inc. Laboratory Notebook #E52647, pages 41 and 42.

5. L. J. Major, "DPX-E9636 Stability vs. Temperature," E. I. du Pont de Nemours & Company, Inc. Laboratory Notebook #E52680, pages 115 and 116

6. L. J. Major, "DPX-E9636 Stability vs. pH at Room Temperature," E. I. du Pont de Nemours & Company, Inc. Notebook #E52680, pages 106 and 107.

7. J. H. Larochelle, L. J Major, R. D. McFetridge, AMR-1260-88, entitled "Analytical Method for the Quantitation of DPX-V9360 in Corn (Forage, Fodder, Silage, and Grain)."

8. J. H. Larochelle, L. J. Major, AMR-1242-88, entitled "Analysis of DPX-L5300 in Wheat Grain and Wheat Straw by Liquid Chromatography."

9. L. J. Major, "DPX-E9636 Retention vs. Eluent pH, "E. I. du Pont de Nemours & Company, Inc. Laboratory Notebook #E52680, pages 101 and 102.

10. L. J. Major, "Chromatography of A7273 in Soil," E. I. du Pont de Nemours & Company, Inc. Notebook #E52680, pages 12 through 20.

11. L. J. Major, P. G. Rossi, "DPX-E9636 Correlation Study - Forage and Grain," E. I. du Pont de Nemours and Company, Inc. Laboratory Notebook #E52647, pages 52 through 68.

12. L. J. Major, "DPX-E9636 Magnitude of Residues, Rochelle, IL," E. I. du Pont de Nemours, Inc. Laboratory Notebook #E52647, page 16.

13 P. G. Rossi, "DPX-E9636 Analysis of Corn Grain Samples," E. I. du Pont de Nemours & Company, Inc. Laboratory Notebook #E58311, page 89.

VIII. APPENDIX 1

Representative Chromatograms

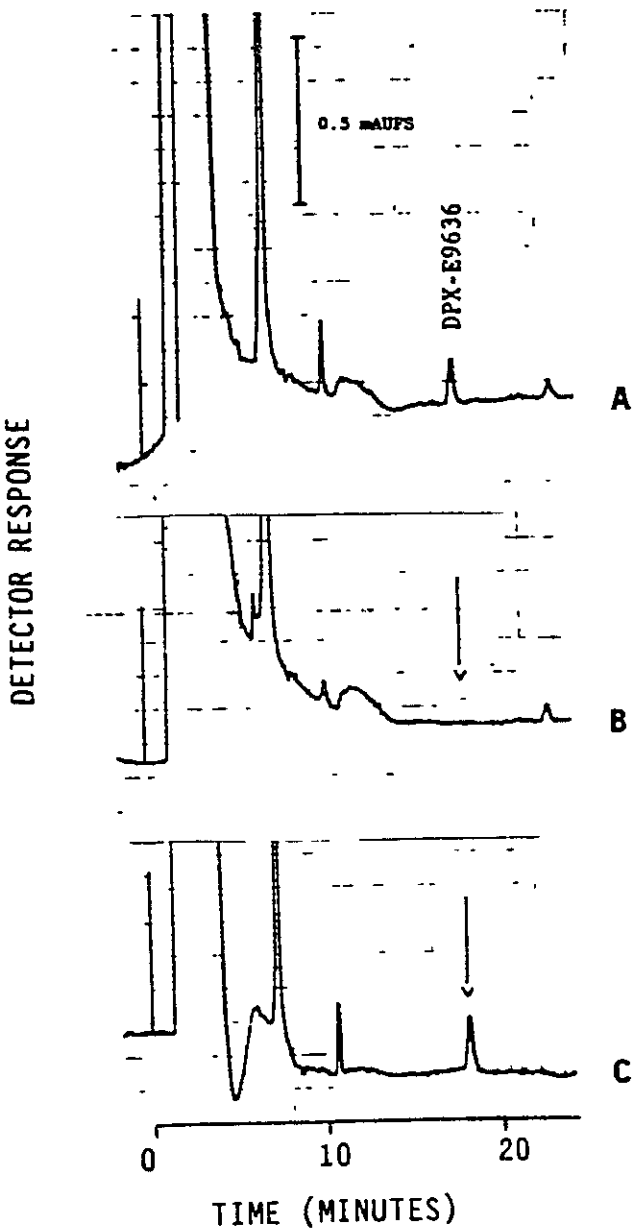
Figure 6 - Chromatograms of Corn Forage
Figure 7 - Chromatograms of Corn Grain

Other

Figure 8 - K' of DPX-E9636 vs. Eluent pH

FIGURE 6

Analytical Chromatograms of Corn Forage



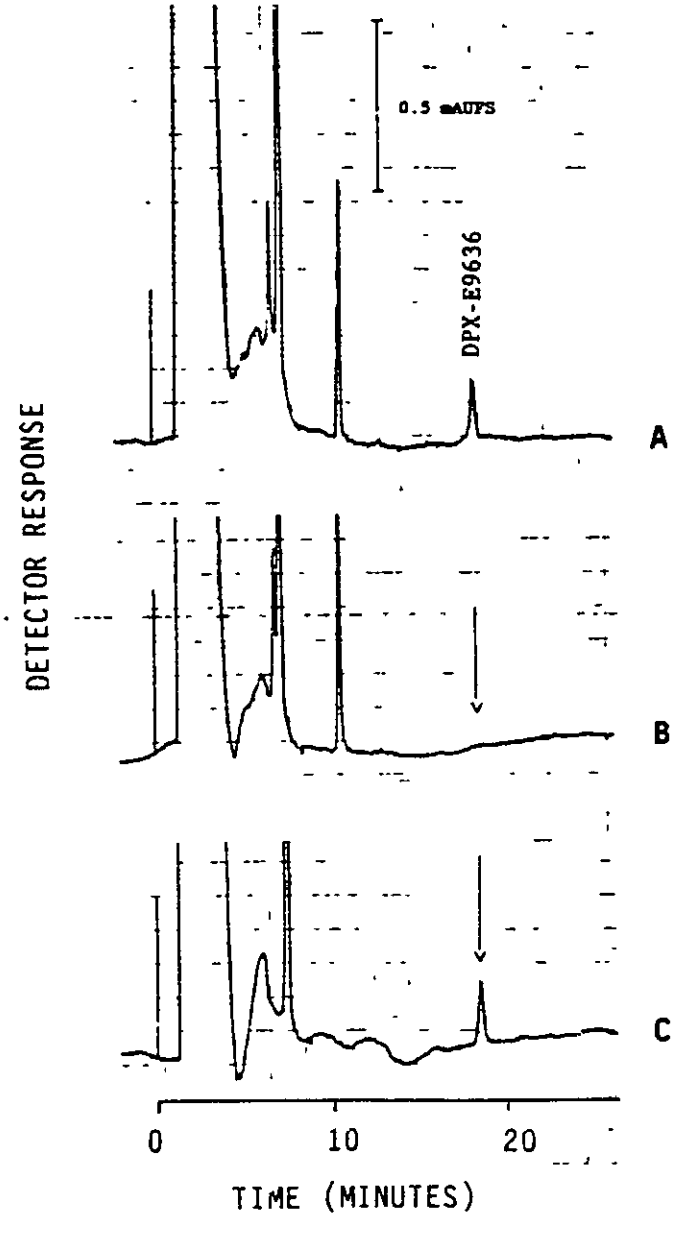
A) 0.050 ppm DPX-E9636 fortified control corn forage

B) control corn forage

C) 0.001 ug/mL DPX-E9636 standard

FIGURE 7

Analytical Chromatograms of Corn Grain



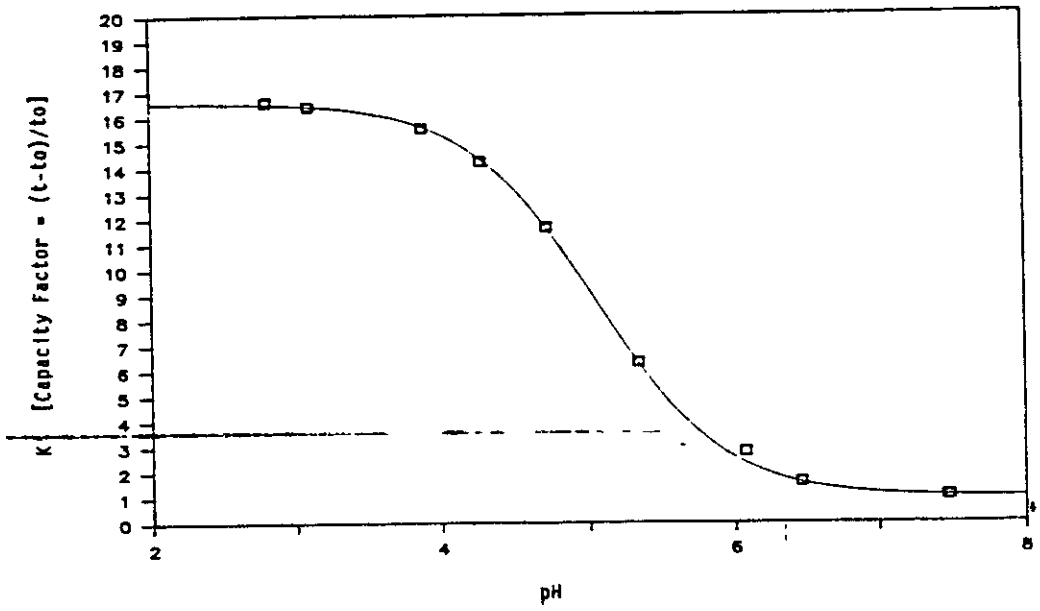
A) 0 050 ppm DPX-E9636 fortified control corn grain

B) control corn grain

C) 0 001 ug/mL DPX-E9636 standard

FIGURE 8

K' of DPX-E9636 vs. Eluent pH



Reference 9

IX. APPENDIX 2

AUTOMATED METHOD OF ANALYSIS FOR THE QUANTITATION OF DPX-E9636 IN CORN FORAGE AND GRAIN

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(Continued)

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I. SUMMARY/INTRODUCTION

A. Summary

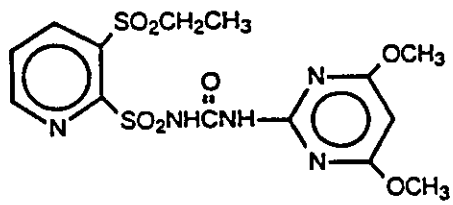
This method uses a high performance liquid chromatograph (HPLC) with eluent and column switching and UV detection at 254 nm for the determination of DPX-E9636 residues in corn forage and grain. It is fully automated to allow unattended analysis. Sample clean-up is achieved through reverse-phase chromatography enlisting eluent-switching. Column-switching provides the resolution required for quantitation of DPX-E9636. This approach has been successfully applied to several sulfonylurea herbicides in many matrices. The method described herein provides a means to quantitate DPX-E9636 in corn forage and grain at levels as low as 0.05 ppm based on a 10-gram sample.

B. Introduction

Through eluent and column switching, one chromatograph is able to accomplish both sample clean-up and analysis for the quantitation of DPX-E9636 in corn. Sample clean-up and concentration are achieved by reverse-phase chromatography on a Zorbax® phenyl column with eluents of varied pH and methanol concentration; analysis follows on an analytical Zorbax® R_x™ column. This approach will be referred to as the Automated Method of analysis. Instrument programming allows for unattended, overnight operation, and high sample throughput.

This method has been designed for increased simplicity and productivity by eliminating manually intensive operations such as liquid/liquid extraction, evaporation and solid phase extraction clean-up Concentration of analyte and clean-up of sample matrix are performed by the chromatographs. This approach is successful due to the acid-base character of DPX-E9636. Retention of DPX-E9636 in this reverse-phase HPLC system is dependent on the degree of the

molecule's protonation, which in turn is affected by the HPLC eluent's pH. Retention versus eluent pH is illustrated in Figure 8. The structure of the compound is shown below:



DPX-E9636

N-((4,6-Dimethoxypyrimidin-2-yl)aminocarbonyl)-3-(ethylsulfonyl)- 2-pyridinesulfonamide
(A common name is not yet available)

Two mL of Sample Extract is pumped onto a Zorbax® Phenyl column and chromatographed in an aqueous eluent of 44% methanol buffered at pH 3.5. DPX-E9636, which is predominately uncharged in the pH 3 Sample Extraction Solution (25% methanol), concentrates at the head of the column during sample injection. As chromatography continues, DPX-E9636 is separated from a large number of polar compounds which could interfere in the subsequent analytical step. At a fixed time before the analyte would normally elute, the eluent is switched to one of lower methanol concentration (11%) and higher pH (7 5); this eliminates most potential interferences which would have eluted with the analyte in the first eluent, as they become highly retained by the column. The DPX-E9636 continues to elute close to its original rate despite the lowered methanol concentration because it is anionic at this pH and very weakly retained by the phenyl column. Shortly before the analyte would elute on the phenyl column, the eluent containing the DPX-E9636 peak is switched to a Zorbax® R_x™column for resolution from the remaining sample components. Chromatography is carried out in an aqueous eluent of 22% methanol at pH 6.5. The DPX-E9636 is detected with a UV detector at 254 nm.

II. MATERIALS/METHODS

A. Equipment

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

Centrifuge Rotors - Du Pont models HS4 and SS34 (Du Pont Instruments)

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

Centrifuge Bottles - 250-mL, polypropylene, IEC® Maxiforce® #2050, VWR #21018-037 (VWR Scientific, Bridgeport, NJ)

Centrifuge Tubes - Sepcor® 29 x 103 mm, polypropylene, VWR # 21007-303 (VWR Scientific)

Food Processor - Hobart chopper model 84145 or 84186 (Hobart Corp., Troy, OH)

Mill - Sunbeam Oskar Food Processor (Sunbeam Appliance Company, Chicago, IL)

pH Meter - Beckman model PHI® 11 (Beckman Instruments, Inc., Fullerton, CA)

Narrow Range pH Paper - EM® ColorpHast® Indicator Strips, Narrow range, 2.5 - 4.5, VWR #EM-9581-3 (VWR Scientific)

Filter - Millipore® 47 mm Type GS, 0.22 µ pore size filter #GSWP 047 00, and glass filter holder and flask #XX15 047 00 (Millipore Inc., Milford, MA)

Liquid Chromatograph -

Pumps: Pump 1 - Waters model 510 with high pressure dampening filter (Millipore Inc.)

Pump 2 - Kratos Spectroflow model 400 with high efficiency filter (ABI Analytical, Foster City, CA)

Detector: Kratos Spectroflow model 783G with 12-µL flowcell and gradient control option (ABI Analytical)

Data collector: Hewlett Packard 7994A HPLC Chemstation and HP 1090L HPLC with A-D convertor (Hewlett Packard Co., Mt. View CA)

HPLC columns: Column 1 - Du Pont Zorbax® Phenyl, 4.0 mm x 80 mm 5 micron Reliance® cartridge 3-pack, #820662-942 and column end-fittings, #820669-001 (MAC-MOD Analytical Inc., Chadds Ford, PA)

Column 2 - Du Pont Zorbax® R_x® 4.6 mm x 250 mm, 5 micron analytical column, #880967-901 (MAC-MOD Analytical)

Column oven: Waters model WAT038039 (Millipore Inc.)

Valves: Valve 1 - Rheodyne model 5011P low pressure six-position rotary valve (Rheodyne Inc., Cotati, CA)

Valve 2 - Valco model ECSD12P-HC high pressure 12-position rotary valve with electric actuator (Valco Instruments Co. Inc., Houston, TX)

Valves 3-4 - Rheodyne model 7000P high pressure six-port two-position valve (Rheodyne Inc.)

Solenoid Air Valves: Rheodyne model 7163 set for four-way operation (Rheodyne Inc.)

B. Reagents and Standards

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

Methanol - EM® Omnisolv® (EM® Science, Cherry Hill, NJ) #MX0488-1

Acetonitrile - Fisher, HPLC-grade (Fisher Scientific, Fair Lawn, NJ) #NA1648

K_2HPO_4 - "Baker Analyzed"® Reagent (J.T. Baker Chemical Co , Phillipsburg, NJ) #3252-01

KH_2PO_4 - EM® low absorbance grade (EM® Science) #PX 1566-2

H_3PO_4 - "Baker Analyzed"® Reagent (J.T. Baker Chemical Co.) #0260-02

DPX-E9636 - DPX-E9636 Reference Standard (Agricultural Products Department, E. I. du Pont de Nemours and Company, Inc., P O. Box 80402, Wilmington, DE 19880-0402)

C. Preparation of Solutions

1M KH_2PO_4:

Dissolve 136 g of KH_2PO_4 in 800 mL of water and dilute to 1 L. Filter through a Millipore® Type GS 0.22 micron filter.

1M K_2HPO_4:

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

Sample Extraction Solution, 25% methanol/75% pH 7 buffer:

Mix 150 mL of 1M K_2HPO_4 with 1350 mL of water; adjust the pH of this solution (calibrated pH meter) to 7.0 by addition of concentrated phosphoric acid (2-2.5 mL). Add 500 mL of methanol and mix. Final pH measures about 7.5.

Eluents:

Eluent 1; 44% methanol, pH 3.5:

Add 10 mL of 1M KH_2PO_4 to a 1000-mL graduated cylinder and dilute to 560 mL with water. Add 440 mL of methanol and mix. Adjust the pH of this solution to 3.50 (calibrated pH meter) by addition of concentrated phosphoric acid. Sparge briefly (about 5 minutes) with helium to degas; further sparging may change methanol concentration due to evaporation.

Note: There is no Eluent 2 because position 2 of the eluent selector valve (valve 1 of Figure 10) is used to select the sample.

Eluent 3; Eluent 3 is the same as eluent 1
Eluents 1 and 3 are the same but supplied at positions 1 and 3 of valve 1.

Eluent 4; 11% methanol, pH 7.5

Add 10 mL of 1M K_2HPO_4 to 1000-mL graduate cylinder and dilute to 890 mL with water. Add 110 mL of methanol and mix. Adjust the pH of this solution (calibrated pH meter) to 7.50 with concentrated phosphoric acid Sparge briefly with helium.

Eluent 5; 22% methanol, pH 6.5

Add 10 mL of 1M K_2HPO_4 to a 1000-mL graduate cylinder and dilute to 780 mL with water. Add 220 mL of methanol and mix. Adjust the pH of this solution (calibrated pH meter) to 6 50 with concentrated phosphoric acid. Sparge briefly with helium.

Eluents 6 and 7; 90% methanol/10% water

Add 100 mL of water to 900 mL of methanol and mix. Sparge briefly with helium

Standards:

Stock Standard Solution

Accurately weigh 0.0200 g of DPX-E9636 and dissolve in 100 mL of acetonitrile to make a 200 µg/mL stock standard. Make an intermediate dilution from the stock standard to 5.0 µg/mL in acetonitrile; this will be used for fortification of samples and preparation of chromatographic standards. These standard solutions should be stable up to 6 months if stored at (4° C) or over a year when stored at (-20° C)

Chromatographic Standard Solutions

Prepare chromatographic standards at 0.050, 0.025, 0.010 and 0 005 µg/mL in Sample Extraction Solution from the 5.0 µg/mL stock standard solution. The concentration of acetonitrile in these final dilutions is kept at or below 2%. Keep all chromatographic standards a 4° C when not in use These standards should be stable for a week if they are not acidified. Before injection onto the chromatograph the standard solution's pH must be adjusted to between 2 5 and 3 5 (as determined with narrow range pH paper) with concentrated phosphoric acid. Once acidified, the stability of the standard is greatly reduced and has a useful lifetime (<10% degradation) of about 8 hours if kept at

0° C. See Table 3 (page 29) for DPX-E9636 stability in acidic solution at various temperatures.

D. Analytical Procedure

1. Preparation of Sample

Frozen forage and fodder samples should be cut into 2-4 inch pieces using a floral cutter or knife and then chopped in a Hobart chopper with dry ice. Grain should be removed from the ears and ground frozen in a mill. The dry ice is allowed to evaporate, and samples are stored at -20° C until sampled for analysis.

2. Fortification

Thaw prepared, untreated corn sample and place 10 grams of untreated corn sample in a 250-mL centrifuge bottle. For a 0.100 ppm fortification add 0.200 mL of the 5.0 µg/mL DPX-E9636 standard solution in acetonitrile to the above sample. Fortify over the range of expected levels of DPX-E9636 in the samples and at the quantitation limit (0.050 ppm) of this analytical method, generally 0.050 to 0.500 ppm. Evaporate the acetonitrile under a stream of nitrogen for about 15 minutes. While making fortifications, it is convenient to prepare the corresponding chromatographic standards from the same stock standard solution by adding the same volume of stock standard to a 100-mL volumetric flask for dilution to volume with Sample Extraction Solution (see Preparation of Solutions section).

3. Extraction

a) Thaw and accurately weigh 10 (+/-0 1) grams chopped corn sample into a 250-mL centrifuge bottle. Add 100 (+/- 2) mL of Sample Extraction Solution and mix. Let soak for about 15 minutes. Grind with a Tissumizer® for 1 minute at about 60% of maximum output (at higher output the sample-buffer mixture foams excessively) Wait 5 minutes and repeat the Tissumizing-rest procedure two more times.

b) Centrifuge the sample-buffer mixture in the Sorvall® RC-5C centrifuge (HS-4 rotor) for 10 minutes at 7000 rpm (brake on, refrigeration optional). If using an alternate speed, centrifuge until most particulates have separated from the Extraction Solution.

c) Remove 20-40 mL of the supernatant with a glass syringe and large bore needle and place in a 40-mL Sepcor® tube. In preparation for lowering the pH, cool the sample to at least 4° C by placing the tubes in a freezer for at least an hour; this will greatly improve DPX-E9636 stability once the sample is acidified. The analysis may be interrupted at this point as the sample is stable for at least two days at this pH (7.5) and temperature (-20° C).

d) Acidify samples by addition of concentrated phosphoric acid until a pH of 2.5 to 3.5 is reached (as determined by pH paper; generally 150-250 µL of acid). Immediately return samples to the freezer for an additional 10 to 15 minutes; this allows any precipitate to form before centrifugation and further cools the sample, minimizing DPX-E9636 decomposition.

e) Centrifuge in the Sorvall® RC-5C centrifuge (SS-34 rotor) for 10 minutes at 20,000 rpm (2-5° C, brake on). If a refrigerated centrifuge is not available, cool the sample longer before acidification and minimize the time the sample is at room temperature.

f) Decant the supernatant for injection onto the HPLC, being careful to avoid particulates. This supernatant will be referred to as Sample Extract. Keep the Sample Extract at a temperature between 0° C and -20° C.

4. Chromatography

Sample Extract is ready for injection onto the chromatograph. DPX-E9636 standards in Sample Extraction Solution require acidification with concentrated phosphoric acid (to pH of 2.5

to 3.5) before chromatography. Samples and standards should be kept cold (0° to -20° C) until chromatographed.

Chromatographic operating conditions and instrumentation are described in the following section.

E. Instrumentation

1. Description

The configuration of the chromatographic equipment is shown in Figure 10 (page 67). Valve 1 allows for eluent selection, valve 2 for sample selection and valves 3 and 4 for introduction of columns 1 and 2 respectively into the HPLC system. The chromatographic conditions are shown on Table 7 (page 66).

The valves shown in Figure 7 are controlled by Kratos Spectroflow detector programs. The detector programs are listed in Tables 8 to 10 (pages 70 - 72). In addition to the valves, the detector controls the flow of eluent 7 through pump 2 using gradient flow programs listed in Table 11.

The steps shown below describe the events which occur during chromatography. They are consistent with step numbers in Detector Program #4 (Table 8, page 70). Although specific to our particular instrument, these events and their timing can be adapted for similar equipment.

STEP	EVENT
1	Initial condition eluent 1. Eluents are selected by valve 1, but alternately may be switched by a pump equipped with a proportioning valve. This would eliminate the need for valve 1 for eluent selection

LOADING THE SAMPLE

2 Valve 1 is stepped to position 2 to select the sample; valve 3 is rotated counter clockwise to bypass column 1. This allows for flushing of the pump and tubing with the sample.

3 The pneumatic actuator to valve 1 is reset. (This valve is rotated clockwise one step at a time by a ratchet mechanism. Therefore to advance the valve one position the actuator must be reset before the next actuation.)

4 Valve 3 is rotated clockwise to position 2. This directs the flow of sample onto column 1. The time between steps 4 and 5 and the flow rate control the volume of sample "injected" onto the column.

5 Valve 1 is stepped to eluent 3 (the same as eluent 1 but identified as 3 to be consistent with the valve port used); valve 3 is rotated counter clockwise to bypass column 1, flushing sample from the pump and solvent lines.

Alternatively, the sample may be introduced to the column via an autosampler, eliminating the need for sample introduction by valve 1 and sample selection by valve 2 and, consequently, the steps above.

6 Valve 3 is rotated clockwise. This returns column 1 to the system and chromatography with eluent 3 is initiated

7 This step occurs at time t-E4, defined as the time of eluent 4 introduction. It is determined experimentally at the start of each day (see Calibration section). Valve 1 is stepped to eluent 4, which contains less methanol but is at a higher pH, at a fixed time before DPX-E9636 would have eluted in eluent 3. DPX-E9636 elutes from the column close to its original rate while potential interferences are retained.

8 Reset pneumatic actuator to valve 1.

9 This step marks the start of the column-switching window and is defined as time T-B; it is determined experimentally at the start of each day (see Calibration section) Valve 4 is rotated counterclockwise, putting column 2 in series with column 1 for DPX-E9636 transfer. This allows a second stage of chromatography to separate DPX-E9636 from interferences coeluting from column 1. Column 2 chromatography is performed on the anionic form of DPX-E9636. A Zorbax® R_X™ column was chosen for column 2 because it has a larger K' for the anionic form of DPX-E9636 than column 1 (phenyl column) This results in refocussing of the analyte on column 2.

10 T-E is the end of the column-switching window. It occurs 6 minutes after T-B, having allowed 7 8 mL of column 1 effluent to transfer to column 2. Valve 3 is rotated counter clockwise, ending transfer of DPX-E9636 from column 1 to 2. Rotation of the valve also puts column 1 in series with pump 2 for backflushing with eluent 7. At some time near T-E the detector's gradient program is used to start flow through pump 2. (It is important that pump 2 not be running during steps 2 through 5 since it will

remove the sample.) Eluent 5 is selected by valve 1; it is slightly stronger than eluent 4 to adjust for the analyte's larger K' on the Zorbax® R_x™ column and provide the desired retention.

The detector may be autozeroed a few minutes before the DPX-E9636 is expected to elute to ensure the chromatogram will be on-scale of the recording device.

CLEAN-UP AND REEQUILIBRATION

11 Reset the air actuator to valve 1. Select the next sample by stepping valve 2 to the next position.

12 Eluent 6 is selected to clean column 2.

13 Reset air actuator to valve 1.

14 Eluent 1 is selected to place a weak eluent in column 2.

15 Reset air actuator to valve 1

16 Valve 3 is rotated clockwise to end back-flushing and place column 1 back in the main system. Valve 4 is rotated clockwise to switch column 2 out of the system. Shortly after, pump 2 flow is stopped by the same gradient program referred to in step 10. Column 1 is allowed to equilibrate with eluent 1 for 7 minutes before the next run is initiated.

2 Operating Conditions:

a) See Table 7 (page 66) for a list of chromatographic operating conditions. Samples and standards are injected onto the HPLC at their storage temperature of below 4° C to minimize DPX-E9636 degradation Samples and standards should be the same temperature upon injection to avoid error in calculation of DPX-E9636 concentrations

b) Determination of t-E4 (to be done daily) Equilibrate column 1 with eluent 1. If a new Zorbax® Phenyl column is to be used, first condition it by injecting Sample Extract, running at least 20 minutes with eluent 1, and then backflushing the column with 90% methanol/10% water (eluent 7) prior to equilibration with eluent 1 (for about 10 minutes) Inject a 0 05 µg/mL standard solution and determine the DPX-E9636 retention time (about 20 minutes after chromatography begins, see program step 3).

t-E4, the time at which eluent 4 is introduced, is this retention time minus 9 minutes. This time, specific to our particular instrument, allows about 6 minutes between the eluent 4 solvent front and the analyte peak on the chromatogram. The actual time must be determined experimentally for each instrument (see discussion in Section I, Modifications or Potential Problems).

c) Determination of column switching times (to be done daily): Set the t-E4 time and repeat chromatography of the standard on column 1 only with eluents 1 and 4. Determine the new retention time for DPX-E9636 (approximately the same as before). Subtract 2 5 minutes from this time, this is the beginning of the column-switching window, T-B. The column-switching window ends at T-E, 6 minutes after the window's start, or 3 5 minutes after the DPX-E9636 retention time. It is important that the peak width at baseline for a 0 05 µg/mL standard be no greater than 4 minutes to ensure quantitative transfer of the analyte to the second column.

3. Calibration Procedure:

a) Prepare chromatographic standards by diluting the 5.0 µg/mL stock standard with Sample Extraction Solution as specified in the Reagents and Standards section. Make 3 or 4 standards at and above the quantitation limit which cover the expected range of DPX-E9636 in the analysis, 0 050, 0 010 and 0 005 µg/mL standards are typically run.

b) Acidify each of the above standards to a pH of 2 5 to 3 5 prior to injection by adding concentrated phosphoric acid dropwise and checking the pH with narrow range pH paper. (A minimum of 8 mL of standard solution is required for line-flushing and "injection" of 2 mL onto the column)

c) Chromatograph each acidified standard solution DPX-E9636 has a retention time of approximately 40 minutes after chromatography begins Measure the DPX-E9636

peak height or area. The columns need not be cleaned after the injection of a standard. A plot of the peak area or height vs. concentration should be linear and pass through the origin (see Figure 9, page 65).

4. Determination of DPX-E9636 in Sample:

a) Chromatograph the Sample Extract. (A minimum of 8 mL sample is required for line-flushing and "injection" of 2 mL onto the column.)

b) Identify the presence or absence of DPX-E9636 based on its retention time determined in the calibration runs. Measure the DPX-E9636 peak, if present, in the same manner as used for calibration.

c) Calculate the concentration of DPX-E9636 in each sample using the equation described under Methods of Calculation.

F. Interferences

Several residue methods (References 7, 10) have been developed using the same technique, and we have found them to be very specific and relatively free of significant interference. We expect this method will be free of interference from almost all other pesticides and impurities found in reagents, sample matrices and on glassware. IN-70941 and IN-70942, the most commonly found soil and plant metabolites have been shown not to interfere with the DPX-E9363 peak

If a significant interference is apparent, the pH of eluent 5 may be adjusted slightly to selectively move the DPX-E9636 peak relative to the interference. A pH drop of 0.25 units will increase the DPX-E9636 retention time by approximately 2 minutes.

G. Confirmatory Techniques

The presence of DPX-E9636 may be confirmed by substituting a Zorbax® Phenyl 4 6 x 250 mm column for the Zorbax® R_x™ column in the chromatograph.

The acid/base character of DPX-E9636 may also be used for confirmation. Changing the pH of eluent 5 from 6.50 to a higher value such as 7.2 will selectively change the retention time of DPX-E9636. (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 maintain a reasonable retention time.

H. Time Required for Analysis

Sample processing is relatively rapid; typically four to eight sample extracts can be prepared in about two hours. Time for chromatography is 60-70 minutes per sample or standard, but may range from 40 to 80 with alternate equipment. This does not include the time for determination of t-E4 (switch from eluent 3 to eluent 4) and column-switching times; this requires an additional hour at the beginning of each day

I. Modifications or Potential Problems

When using alternate equipment, the actual arrival time of a new eluent to the column will depend on the dead volume between the eluent-select valve and the column Thus differences in pump head and tubing volumes for different chromatographs will change the time for eluent arrival to the column. The t-E4 time may have to be adjusted for the particular chromatograph. It should be selected such that the DPX-E9636 peak is sufficiently resolved from the solvent front on the chromatogram, and coeluting sample components will not prove to interfere with the DPX-E9636 peak on the second column. Additional time may also be required for cleaning and reequilibration of column 2 to allow for longer eluent arrival times.

If an autosampler is inserted into the HPLC system between the pump and column in lieu of sample introduction through the pump, pre-column volume may be further increased, affecting eluent switching times still more. Sample extracts are typically not filtered prior to

injection onto the chromatograph. It is generally unnecessary as particulates will be removed from the head of the phenyl column during the backflush step. However, use of an autosampler might require sample filtration so as not to damage this component. If samples are filtered, slight loss of analyte may be seen (as a loss in recovery) due to binding of DPX-E9636 to the filter. If this is suspected, acidified standard solutions might be similarly filtered, or filters might be preconditioned with excess Sample Extract.

The column-switching time window, as defined by T-B and T-E, is set to last 6 minutes and is skewed relative to the DPX-E9636 peak (retention time minus 2.5 minutes to retention time plus 3.5 minutes). Shorter or longer column-switching windows may be possible depending on the instrument and column used. The window length must be adequate to encompass the analyte peak and also should allow for peak drift and column degradation (as evidenced by peak tailing) on the first column.

If resolution is insufficient on the Zorbax® R_x™ column, eluent 5 may be altered by changing its pH and/or methanol concentration. A change in pH should selectively move the DPX-E9636 peak relative to sample matrix components; the methanol concentration may then need to be adjusted to achieve targeted retention times.

J. Method of Calculation

1. Calibration Factor (CF)

The calibration factor (CF) is the ratio of detector response (peak height or height) to the DPX-E9636 concentration. Measure the peak height or peak area for each standard (normalize for a given recorder attenuation if required), and divide this value by the DPX-E9636 concentration (in units of µg/mL). A standard should be run after every 3 to 4 samples. Use the average CF from the standard runs preceding and following a group of samples to determine DPX-E9636 concentrations in those samples

CF = (peak height or area) / (DPX-E9636 concentration [µg/mL] in standard)

2. Concentration of Analyte in the Sample

ppm DPX-E9636 in sample = [M x (100+B)] / [SW x CF]

M = Height or area of the DPX-E9636 peak. Height or area is in the same units as that used for CF calculation above.

B = Volume of water contributed by the sample (mL).
B = % water by weight x SW x 1 mL/g water

SW = Sample weight in grams (10 g)

CF = Calculation Factor described above

III. RESULTS/DISCUSSION

A. Accuracy

Recoveries from duplicate control samples at four fortification levels ranging from 0.500 ppm to 0.050 ppm and tested over two days were conducted to assess the accuracy of the analytical method. DPX-E9636 recoveries from eight fortified control forage samples averaged 86%. Recoveries averaged 90% for eight control corn grain samples (See Table 6, page 64). Extraction efficiency of DPX-E9636 from field treated samples was estimated by the analysis of corn plants treated with radiolabeled DPX-E9636. The extraction efficiency is estimated to be between 89% and 93% (Reference 3; see Table 2, page 27).

The accuracy of this method is enhanced by elimination of many manual sample preparation steps such as liquid/liquid extractions, evaporations and cartridge chromatography All can result in loss of recoveries and are potential sources of contamination from glassware.

A major source of error in this method is decomposition of DPX-E9636 in the sample or standard after addition of acid in preparation for HPLC injection. Decomposition can be minimized by keeping the acidified samples and standards on ice or in a refrigerated autosampler, if

available, and by minimizing the time between sample clean-up and analysis (See Table 3, page 29 for stability vs. temperature data)

Recovery may also be compromised if the column-switching time window is too narrow relative to the baseline width of the DPX-E9636 peak. Time windows may be increased slightly to accommodate this, but this increases the potential for interferences. The phenyl column should be evaluated daily or after every 8-12 samples by chromatography of a standard solution (as in determination of t-E4; see Operating Conditions, Section B) to ensure adequate peak shape and size. A 0.05 µg/mL DPX-E9636 standard should produce a peak no wider than 4.0 minutes at its baseline to ensure complete transfer to the Zorbax® R_X™ column.

Slight error can be attributed to variable water content of the samples. Calculations contain a provision for sample water content, but this is only estimated for the sample type (forage or grain). The effect of this estimation is slight however; placing the water content of a corn forage sample at 60% when it is actually 50% would generate an analysis which is only 1% too high.

B. Precision

The standard deviation (sample standard deviation, σn-1) of recoveries were 2% and 4% for eight analysis of each sample matrix (see Table 6, page 64). DPX-E9636 concentrations ranged from 0.500 to 0.050 ppm The range of recovery for the above samples was 80% to 92% for the forage samples and 87% to 93% for the grain samples. Decomposition of the analyte is most likely the main contributor to the variability of the results, although variability is within acceptable levels. Since many manual operations have been eliminated, the rest of the method is inherently very precise.

C. Limits of Detection and Quantitation

The limit of quantitation is determined by the signal to noise ratio of the detector and the magnitude of coeluting peaks When using a sensitive detector such as the Kratos 783G, the limit

of quantitation is 0.05 ppm or lower. The peak height for a sample containing 0.050 ppm is about 0.2 milliAbsorbance Units (mAU). The noise of a good detector should be 0.01 mAU or less. This gives a signal to noise ratio greater than 10 which is adequate for quantitation. Chromatograms of 0.050 ppm DPX-E9636 fortified samples can be seen in Figures 12 and 13 (pages 74 and 75).

D. Ruggedness Testing

This method uses reverse-phase liquid chromatography with UV detection; both are well understood and known to be stable and reliable. The column-switching time window is large (6 minutes) relative to the baseline peak width of the analyte (less than 4 minutes). This allows for dead volume in the tubing of the chromatograph, as well as variability in DPX-E9636 retention time and non-ideal analyte peak shape on the first column (band broadening or peak tailing).

E. Limitations - Instability of DPX-E9636 in Acid Solution

DPX-E9636 is unstable in acidic solutions at room temperature. Figure 2 (page 30) shows the rate of decomposition for DPX-E9636 in Sample Extraction Solution which has been adjusted to lower pHs by the addition of phosphonic acid. The solutions are relatively stable at pH 8.0 and 6.5. At pH 2.5 and 3.5, well below the pK_a of DPX-E9636, the solutions decompose quickly and at similar rates. The decomposition is very temperature dependent. We have determined the rate of decomposition of DPX-E9636 at 0° C, 15° C, and 35° C. From this data we have predicted the rate of decomposition at other temperatures. See Table 3 and Figure 3 (pages 29 and 31) for more information on the relationship between DPX-E9636 decomposition rate and temperature. When the Automated System is employed for unattended analysis, samples must be kept chilled by an ice bath or some other means.

IV. CONCLUSION

This analytical method is suitable for the quantitation of DPX-E9636 in corn forage and grain at levels down to 0.05 ppm. The equipment and technique used in this method are generally applicable to many if not most commercial sulfonylurea herbicides.

This method has been designed to be run on commercial HPLC equipment with modifications. It demands a greater instrument and set-up time investment than the less automated Split Method of analysis, and hence is most appropriate where a large number of samples require analysis. Automation allows unattended analysis of over a dozen samples or standards overnight.

V. TABLES/FIGURES

A. Method Validation

Table 6 - Recovery Data for Method Validation
Figure 9 - Calibration Curve for Validation

B. Instrument Configuration and Operation

Table 7 - Summary of Chromatographic Conditions
Figure 10 - Schematic of Chromatograph
Figure 11 - Schematic of Pneumatic Valve-Switching Stations

Detector Programming

Table 8 - Detector Program #4
Table 9 - Detector Program #2
Table 10 - Detector Program #6
Table 11 - Gradient Programs #2,4,6

C. Representative Chromatograms

Figure 12 - Chromatograms of Corn Forage
Figure 13 - Chromatograms of Corn Grain

TABLE 6

Recovery Data for Method Validation

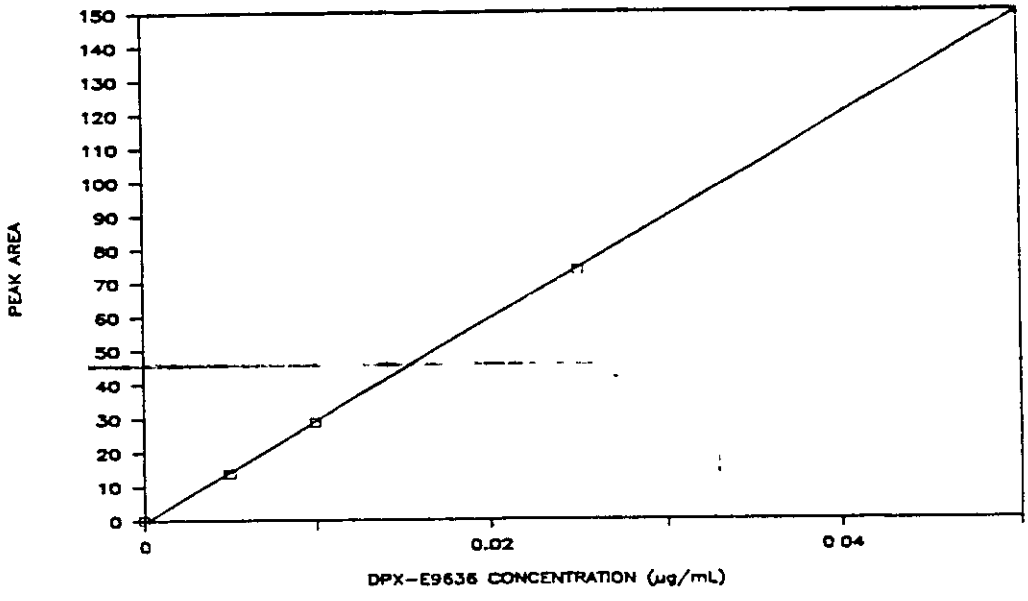
Sample Matrix	DPX-E9636 Fortification Level (ppm)	DPX-E9636 Recovered (ppm)	Percent Recovery	Average Percent Recovery	Std. Dev. (%)
forage	0.500	0.430	86 %	86 %	4 %
forage	0.500	0.450	90 %		
forage	0.250	0.222	89 %		
forage	0.250	0.215	86 %		
forage	0.100	0.083	83 %		
forage	0.100	0.083	83 %		
forage	0.050	0 046	92 %		
forage	0.050	0.040	80 %		
grain	0.500	0 466	93 %	90 %	2 %
grain	0.500	0.452	90 %		
grain	0.250	0.227	91 %		
grain	0.250	0.227	91 %		
grain	0.100	0.089	89 %		
grain	0.100	0.089	89 %		
grain	0 050	0 044	87 %		
grain	0.050	0.043	87 %		

Control materials were fortified in duplicate at four DPX-E9636 levels.

Reference 2

FIGURE 9

Calibration Curve fromValidation of Automated Method of Analysis for DPX-E9636



Reference 2

TABLE 7

Summary of Chromatographic Conditions

Column:	1	Du Pont Zorbax® Phenyl 4.0 mm x 80 mm 5 micron Reliance® Cartridge
	2	Du Pont Zorbax® R_x™ 4.6 mm x 250 mm 5 micron analytical
Column Temperature:		35° C
Eluent Flow Rate:		1 3 mL/min.
Eluent: *	1,3	44% methanol, pH 3 5
	4	11% methanol, pH 7 5
	5	22% methanol, pH 6.5
	~~6,7~~	90% methanol/10% water
Sample Volume:		2.0 mL
Detector:	Wavelength	254 nm
	Sensitivity	0 02 AUFS

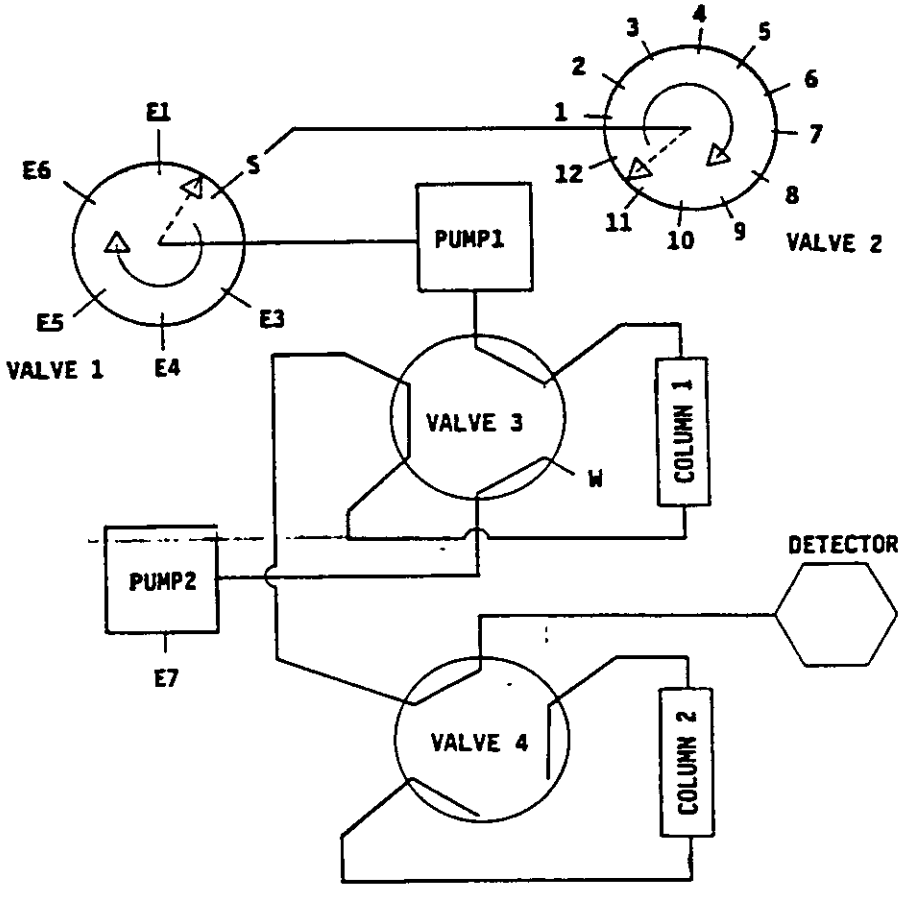
The HP 1090M HPLC initiates the detector programs through event 4, a fused 100 V DC rated contact closure.

The HP Chemstation collects detector output through an analog-digital converter.

* Eluent number corresponds to the position of the eluent selector valve (valve 1). Eluents 1 and 3 are the same, but supplied at positions 1 and 3 of valve 1.

FIGURE 10

Schematic of Chromatograph



W = Waste

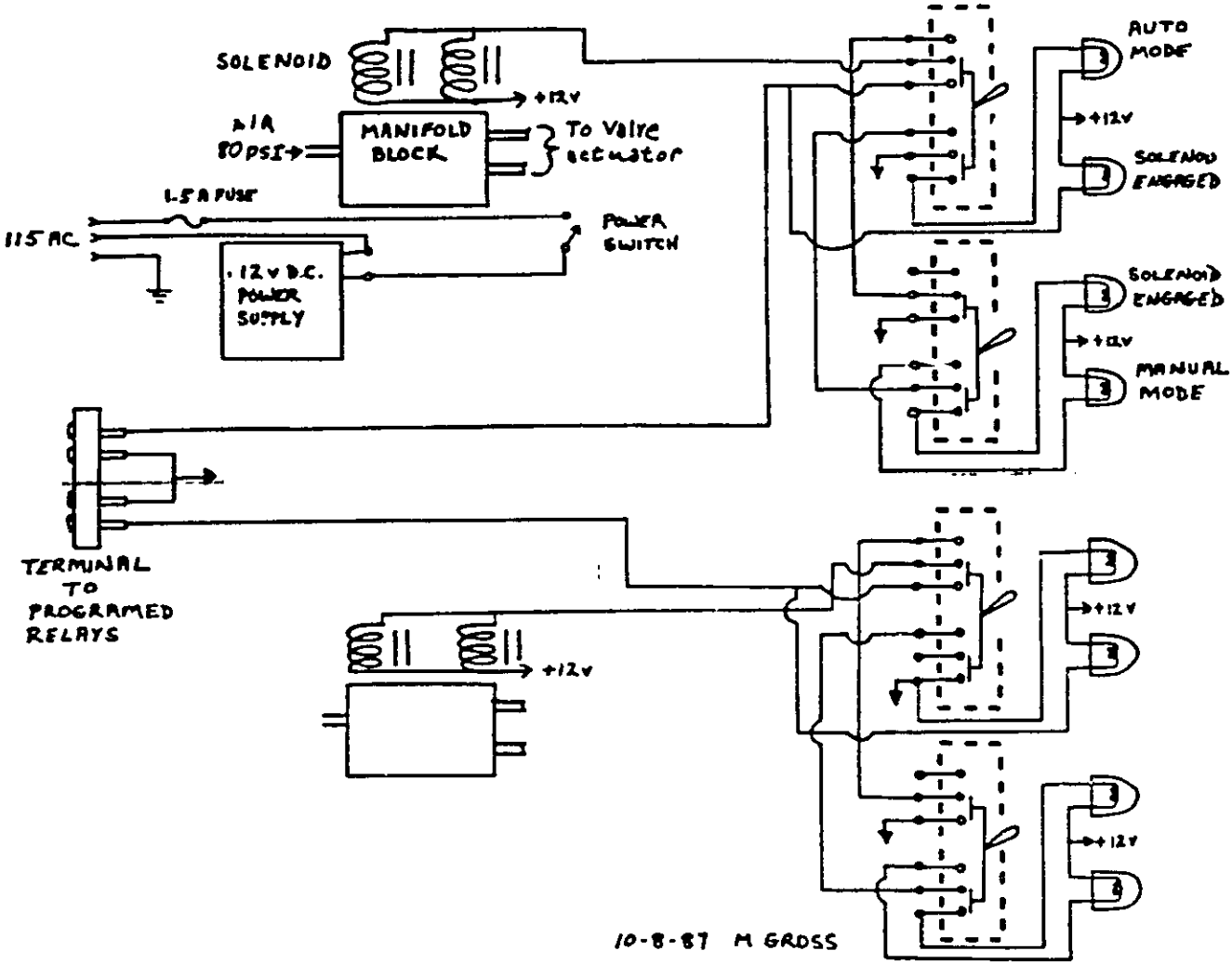
S = Sample Injection Port

——— Valve Clockwise

····· Valve Counterclockwise

FIGURE 11

Schematic of Pneumatic Valve-Switching Stations



KEY TO DETECTOR PROGRAMMING

The valves shown in Figure 10 are controlled by Kratos spectroflow detector programs. These programs control four internal relays, three of which switch a 12-volt power supply to activate solenoid air valves which, in turn, activate chromatographic valves 1, 3 and 4 Relay 1 controls backflushing of column 1; relay 2, addition or removal of column 2; relay 3, eluent selection, and relay 4, sample selection from valve 2 (electrically actuated). (Refer to Figure 10, page 67 for instrument configuration.) "Pneumatic valve-switching stations" may be inserted to allow optional manual override of the detector. The schematic for these stations is attached as Figure 11.

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. Relays 1 through 4 actuate the following:

Relay 1 - Valve 3; 1 = valve counterclockwise for column 1 backflushing by pump 2.

Relay 2 - Valve 4; 1 = valve counterclockwise to put column 2 in line with pump 1.

Relay 3 - Valve 1; 1 = valve steps to next eluent position, 0 = valve resets Valve 1 is rotated clockwise by a ratchet mechanism. Thus, the actuator must be reset before the valve position can advance.

Relay 4 - Valve 2; 1 = valve steps to next position to select next sample

Status: Abbreviations for status of instrument at each step. The column order corresponds to the order of relays in the preceding column.

C1 = Column 1 in line with pump 1.

C2 = Column 2 in line with pump 1.

1-6 = Eluent selection valve (valve 1) position

TABLE 8

Detector Program #4

Full chromatographic program, including eluent- and column-switching

STEP	TIME	RELAY 1 2 3 4	STATUS	EVENT
1	0.01	0 0 0 0	C1 . 1 .	Start, Valve 1 position = 1
2	1.33	1 0 1 0	. . 2 .	Column 1 off Valve 1 position = 2, flush line
3	1.43	1 0 0 0	. . 2 .	Reset valve 1
4	5.33	0 0 0 0	C1 . 2 .	Start sample loading onto column
5	6.87	1 0 1 0	. . 3 .	Column 1 off Valve 1 position = 3, flush line
6	10.00	0 0 0 0	C1 . 3 .	Column 1 on & reset valve 1
7	t-E4	0 0 1 0	C1 . 4 .	Valve 1 position = 4
8	23.00	0 0 0 0	C1 4 .	Reset valve 1
9	T-B	0 1 0 0	C1 C2 4 .	Add column 2
10	T-E	1 1 1 0	. C2 5 .	Valve 1 position = 5 Remove column 1, begin backflush
11	52.90	1 1 0 1	. C2 5 .	Reset valve 1 Move to next sample position
12	53.00	1 1 1 0	. C2 6 .	Valve 1 position = 6
13	55.00	1 1 0 0	. C2 6 .	Reset valve 1
14	55.05	1 1 1 0	. C2 1 .	Valve 1 position = 1 Equilibrate column 2
15	56.00	1 1 0 0	. C2 1 .	Reset valve 1
16	58.00	0 0 0 0	C1 . 1 .	Reset conditions: Remove column 2, add column 1 Equilibrate column 1

Total run time is 63 min., including reequilibration time.

TABLE 9

Detector Program #2

Chromatographic program for t-E4 determination: one column, one eluent

STEP	TIME	RELAY 1 2 3 4	STATUS	EVENT
1	0.01	0 0 0 0	C1 . 1 .	Start, Valve 1 position = 1
2	1.33	1 0 1 0	. . 2 .	Column 1 off Valve 1 position = 2, flush line
3	1.43	1 0 0 0	. . 2 .	Reset valve 1
4	5.33	0 0 0 0	C1 . 2 .	Start sample loading onto column
5	6.87	1 0 1 0	. . 3	Column 1 off Valve 1 position = 3, flush line
6	10.00	0 0 0 0	C1 . 3 .	Column 1 on & reset valve 1
7	44.00	1 0 1 0	. . 4 .	Column 1 off Valve 1 position = 4
8	44.10	1 0 0 0	. . 4 .	Reset valve 1
9	44.20	1 0 1 0	. . 5 .	Valve 1 position = 5
10	44.30	1 0 0 0	. . 5 .	Reset valve 1
11	44.40	1 0 1 0	. . 6 .	Valve 1 position = 6
12	44.50	1 0 0 0	. . 6 .	Reset valve 1
13	44.60	1 0 1 0	. . 1 .	Valve 1 position = 1
14	48.00	0 0 0 0	C1 . 1 .	Reset conditions: Reset valve 1, add column 1 Equilibrate column 1

Note: Program may be aborted after step 6 and valve 1 may be manually reset to position 1 after the DPX-E9636 peak has eluted.

TABLE 10

Detector Program #6

Chromatographic program for column-switching time determination: one column, two eluents

STEP	TIME	RELAY 1 2 3 4	STATUS	EVENT
1	0.01	0 0 0 0	C1 . 1 .	Start, Valve 1 position = 1
2	1.33	1 0 1 0	. 2 .	Column 1 off Valve 1 position = 2, flush line
3	1.43	1 0 0 0	. 2 .	Reset valve 1
4	5.33	0 0 0 0	C1 . 2 .	Start sample loading onto column
5	6.87	1 0 1 0	. . 3 .	Column 1 off Valve 1 position = 3, flush line
6	10.00	0 0 0 0	C1 . 3 .	Column 1 on & reset valve 1
7	t-E4	0 0 1 0	C1 . 4 .	Valve 1 position = 4
8	44 80	1 0 0 0	. 4 .	Column 1 off
9	45.00	1 0 1 0	C1 . 5 .	Valve 1 position = 5
10	45 10	1 0 0 0	C1 . 5 .	Reset valve 1
11	45.20	1 0 1 0	C1 . 6 .	Valve 1 position = 6
12	45.30	1 0 0 0	C1 . 6 .	Reset valve 1
13	45.40	1 0 1 0	C1 . 1 .	Valve 1 position = 1
14	45.50	0 0 0 0	C1 . 1 .	Reset conditions: Reset valve 1, add column 1 Equilibrate column 1

Note: Program may be aborted after step 6 and valve 1 may be manually reset to position 1 after the DPX-E9636 peak has eluted.

TABLE 11

Gradient Programs #2 & #6

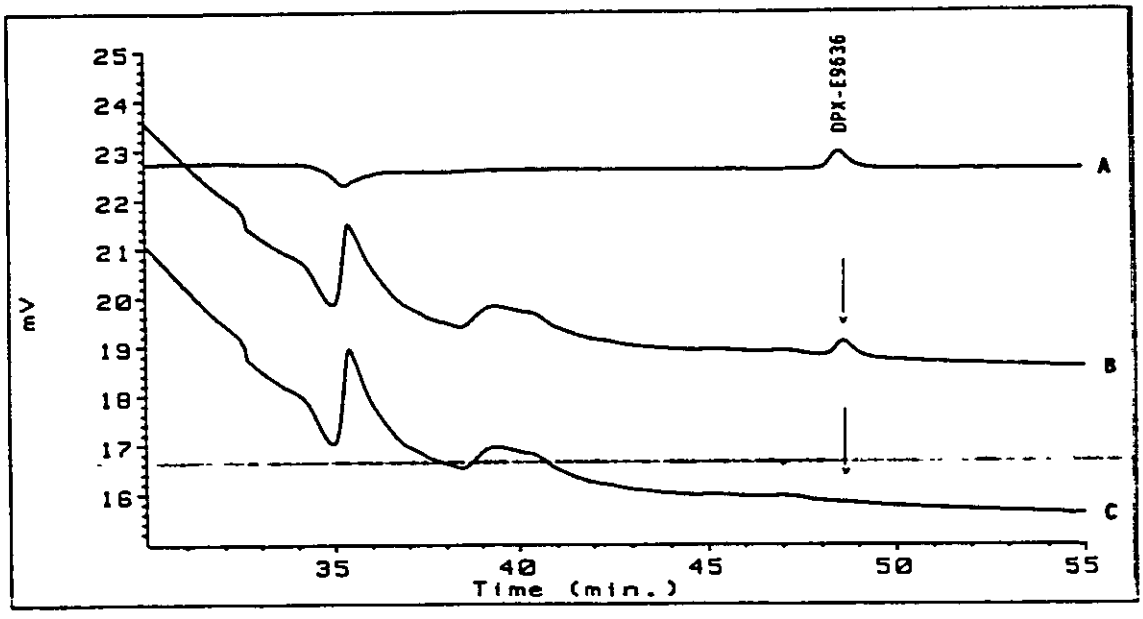
SEGMENT	START TIME (min.)	LENGTH (min.)	PUMP FLOW (mL/min.)
0	0	42	0
1	42	1	0
2	43	5	2
3	48	1	2
4	49	4	0
5	53	1	

Gradient Program #4

SEGMENT	START TIME (min.)	LENGTH (min.)	PUMP FLOW (mL/min.)
0	0	28	0
1	28	1	0
2	29	54	1
3	63	1	1
4	64	15	0
5	99		

FIGURE 12

Chromatograms of Corn Forage



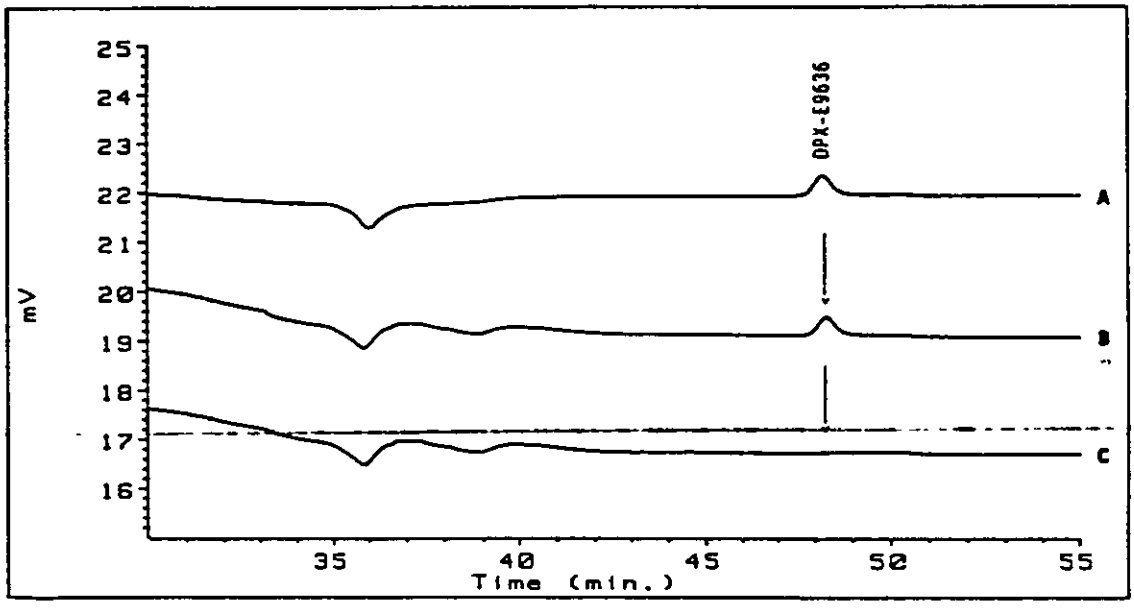
A) 5.0 ng/ml DPX-E9636 standard

B) 0.050 ppm DPX-E9636 fortified control corn forage

C) control corn forage

FIGURE 13

Chromatograms of Corn Grain



A) 5.0 ng/ml DPX-E9636 standard

B) 0.050 ppm DPX-E9636 fortified control corn grain

C) control corn grain

VI. APPENDIX 3

CORRELATION OF SPLIT AND AUTOMATED ANALYTICAL METHODS FOR THE QUANTITATION OF DPX-E9636 IN CORN (FORAGE AND GRAIN)

CORRELATION BETWEEN METHODS

A fully automated approach to DPX-E9636 analysis in corn was developed to increase sample throughout and decrease operator time. Effluent from the Zorbax® Phenyl column is directly transferred to the second, Zorbax® R_x™ , column instead of manual collection and reinjection onto a second chromatograph. This requires the addition of an intermediate chromatographic eluent which will allow the DPX-E9636 to reconcentrate on the second column (versus dilution with water and acidification performed in the Split Method of analysis). At a fixed time before DPX-E9636 elution from the phenyl column, eluent 4 (11% methanol at pH 7.5) replaces eluent 3 (44% methanol at pH 3.5). This not only allows concentration on the second column (due to the higher K' of DPX-E9636 on the Zorbax® R_x™ column), but also changes the coelutives which will be transferred to the second column. Unlike the Split System, DPX-E9636 elutes in a relatively high pH, low methanol eluent which has retarded chromatography of the less polar sample components which would have eluted with the analyte in the Split System. Although the same principle is used, chromatography on the analytical Zorbax® R_x™ column is different in the two approaches

The instrumentation requirements of the Automated System are more demanding. The system requires a greater number of components and more complicated plumbing. It must also be able to withstand the high back pressures (>5000 psi) which occur when the two columns are in series during eluent transfer. The minimum requirements of the Split System are one pump, two low-pressure 2-way valves, two high-pressure 6-port valves, a column oven and detector. The Automated Method requires two pumps, one low-pressure 6-port valve, one 12-port valve, two high-pressure 6-port valves and programming capability in addition to the column oven and detector.

The automated method also requires significantly greater set-up time. Many of the timed events are dependent on the exact configuration (tubing volumes, pump-head volumes) of

the instrument, and must be optimized for the particular configuration. Substitution of valve 2 (sample selection valve) with an autosampler will also greatly affect the chromatography. However, in the case where many samples need analysis, the time is well invested. Sample throughput is 2 to 3 times greater with the Automated Method, and requires only about a quarter of the operator time. The automated method has the added advantage of decreasing the opportunity for operator error and sample contamination.

Although validation of the two systems shows both are effective for analysis of corn samples and capable of DPX-E9636 quantitation to 0.05 ppm (References 1 and 2), a direct comparison of the two systems was performed. Field-treated corn samples, collected for use in AMR-1041-88 (Magnitude of Residues of DPX-E9636 Herbicide When Applied to Corn and Sampled at Various Stages) and AMR-1058-88 (Magnitude of Residues of DPX-E9636 Herbicide When Applied to Corn), were extracted and the extracts analyzed for DPX-E9636 on both the Split and Automated Systems (Reference 11). The results are listed in Table 12 (page 79). Chromatograms were characteristic of the representative chromatograms shown in Appendix 1 and 2. The differences in amount of DPX-E9636 detected are not significant. They range from 0% to 6% of the amount DPX-E9636 calculated, within the expected deviation for each of the two methods.

The Automated and Split Methods provide comparable results for the analysis of DPX-E9636 in corn. The Split Method is recommended for those applications where only a few samples require analysis. The system set-up time is much less, and sample throughput sufficient for a limited number of samples. In the case where many samples require analysis, the Automated approach is suggested. Although it requires more time to set-up, ultimate sample throughput and operator time savings will justify the investment.

TABLE 12

DPX-E9636 Residues in Corn Forage and Grain Samples Detected by Split and Automated Methods of Analysis

Sample I. D. *	Treatment Rate	PHI (days)	DPX-E9636 Detected Split Method (ppm)	DPX-E9636 Detected Automated Method (ppm)	Difference (ppm)	Percent Difference
Forage:						
S00036444	0.5	1	1.21	1.14	+0.068	5%
S00036445	1.0	1	1.75	1.64	+0.11	6%
S00036450	0.5	7	0.081	0 083	-0.002	2%
0.100 ppm fortification			0 089	0.085	+0 004	4%
0.050 ppm fortification			0.042	0 044	-0.002	4%
Grain:						
~~S00028081~~	~~0.5~~	~~86~~			~~both undetected~~	
S00028083	1.0	86	--	--	both undetected	
0 250 ppm fortification			0.23	0.23	0.00	0%
0 100 ppm fortification			0 092	0.092	0.000	0%
0 050 ppm fortification			0 043	0.044	-0 001	2%

*Forage samples were collected for AMR-1041-88, (Reference 12), from the Rochelle, IL field test location. Grain samples were collected for AMR-1058-88, (Reference 13), from the Madera, CA field test location.

Reference 11