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Study Title

**ANALYTICAL METHOD FOR THE DETERMINATION OF
DPX-JE874 AND CYMOXANIL RESIDUES IN VARIOUS
MATRICES**

Data Requirement

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STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

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

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GOOD LABORATORY PRACTICE STATEMENT

The EPA Good Laboratory Practice (GLP) requirements as specified in 40 CFR Part 160 are not applicable to analytical methods development. The methods validation work contained in this report was performed in GLP compliance; however, there was no protocol and no conduct audit.

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SUMMARY

Cymoxanil (DPX-T3217) is the active ingredient in Curzate® Fungicide. DPX-JE874 is an experimental fungicide currently undergoing field evaluation by DuPont. These two fungicides may be combined and sold in the United States for control of select plant diseases in crops such as potatoes or tomatoes. An enforcement method is needed to selectively detect and quantitate both active ingredients either alone or in combination. This report describes a method that uses a common extraction procedure. Extracts are split and purified separately. DPX-JE874 is quantitated by GC; cymoxanil is quantitated by HPLC. The assay was developed jointly at Morse Laboratories (SOP #Meth-86) and DuPont. Extraction efficiency of incorporated ¹⁴C-labeled cymoxanil residues is currently being evaluated and will be reported separately as part of plant metabolism reports. The presence of cymoxanil residues can be independently confirmed using alternate chromatography conditions described in this method. The Limit of Quantitation of the method is 0.02 ppm and 0.05 ppm for DPX-JE874 and cymoxanil, respectively.

MORSE LABORATORIES, INC.

SOP# Meth-86Revision #2 Date 11/95DETERMINATION OF DPX-JE874 AND CYMOXANIL RESIDUES IN VARIOUS MATRICES

- Reasons for Revision:**
1. To better describe preparation of DPX-JE874 standard solutions.
 2. To include as Appendix IV, a discussion on the silylation of glassware.

Method References

1. Morse Laboratories, Inc. SOP #Meth-85, original, "Determination of DPX-JE874 Residues in Various Matrices"
2. Morse Laboratories, Inc. SOP #Meth-76, Revision #2, "Determination of Cymoxanil in Plant Matrices"
3. DuPont Report No. AMR 3060-94, "Analytical Method for the Determination of Residues of Cymoxanil in Grapes and Tomatoes Using Column Switching Liquid Chromatography"

Principle

Water is added to all samples, regardless of the moisture content. Acetonitrile is added and the sample is extracted with the acetonitrile/water mixture. The water is salted out. Separate aliquots of acetonitrile extract, one for each analyte, are then processed through additional analyte-specific cleanup procedures. Briefly, both aliquots undergo a hexane partition to remove fats, oils, and other non-polar coextracted interferences. The DPX-JE874 fraction is further cleaned up by Florisil column chromatography, then detected and quantified by gas chromatography using N/P detection. The limit of quantitation is 0.02 ppm. The cymoxanil fraction is further cleaned up by solid phase extraction (SPE) purifications, then detected and quantified by high pressure liquid chromatography using UV detection. The limit of quantitation is 0.05 ppm.

Note: During the conduct of this analysis, equivalent apparatus, solvents, glassware, or techniques (such as sample concentration) may be substituted for those specified in this method, except where otherwise noted.

Reagents

Acetonitrile:	HPLC grade solvent
Dichloromethane:	Pesticide residue quality
Ethyl acetate:	HPLC grade solvent
Ethyl ether AR:	Mallinckrodt, <u>ACS</u> grade w/ preservative, Baxter catalog no. 0853-500*NY. <u>Do not substitute.</u>
Hexane:	HPLC grade solvent
Methanol:	HPLC grade solvent
Water:	HPLC grade (for reagents and columns)
Water:	Deionized (for extraction)
Florisil:	PR Grade, 60/100 mesh (U.S. Silica)
Phosphoric Acid (H_3PO_4):	Analytical reagent grade, 85%
Potassium Phosphate Monobasic, Crystal (KH_2PO_4):	Analytical reagent grade
Sodium chloride:	Reagent grade
Sodium Sulfate:	Granular, anhydrous reagent (Mallinckrodt, 8024)
Filter paper:	VWR #417, 18.5 cm, VWR catalog no. 28313-126
Glass wool	
Peroxide test strips:	EM Quant, VWR catalog no. EM-10011-1
Dimethyldichlorosilane:	Supelco, Catalog No. 3-3009

Primary standards

JE874 (DPX-JE874): Analytical standard grade JE874, DPX-JE874, available from DuPont Agricultural Products, Global Technology Division (E. I. du Pont de Nemours and Company, Wilmington, DE)

Cymoxanil (DPX-T3217): Analytical standard grade cymoxanil, DPX-T3217, available from DuPont Agricultural Products, Global Technology Division (E. I. du Pont de Nemours and Company, Wilmington, DE)

Preparation of Standard Solutions**A. DPX-JE874 analytical standards:****1. Stock solution**

Using an analytical balance and a weighing boat, weigh 25.0 mg (corrected for purity) of analytical standard grade DPX-JE874. Transfer the standard to a 25 mL volumetric flask (using small amounts of ethyl acetate). Add additional ethyl acetate to approximately 15 mL and swirl the volumetric to dissolve the solid. When it is in solution dilute to the mark (25.0 mL) with ethyl acetate. The final concentration is 1000 $\mu\text{g/mL}$. Store at 1-8 °C.

2. Fortification (Spiking) Solutions

100 $\mu\text{g/mL}$: Transfer 2.5 mL of 1000 $\mu\text{g/mL}$ standard in ethyl acetate to a 25 mL volumetric flask. Dilute to volume with ethyl acetate. Mix well. Store at 1-8 °C.

10 $\mu\text{g/mL}$: Transfer 2.5 mL of 100 $\mu\text{g/mL}$ standard in ethyl acetate to a 25 mL volumetric flask. Dilute to volume with ethyl acetate. Mix well. Store at 1-8 °C.

1 $\mu\text{g/mL}$: Transfer 250 μL of 100 $\mu\text{g/mL}$ standard in ethyl acetate to a 25 mL volumetric flask. Dilute to volume with ethyl acetate. Mix well. Store at 1-8 °C.

3. GC (Calibration) Standard Solutions:

Typically the following concentrations are prepared:

1.6 $\mu\text{g/mL}$: Transfer 400 μL of 100 $\mu\text{g/mL}$ standard in ethyl acetate to a 25 mL volumetric flask. Dilute to volume with ethyl acetate and mix well.

0.8 $\mu\text{g/mL}$: Transfer 200 μL of 100 $\mu\text{g/mL}$ standard in ethyl acetate to a 25 mL volumetric flask. Dilute to volume with ethyl acetate and mix well.

0.4 $\mu\text{g/mL}$: Transfer 100 μL of 100 $\mu\text{g/mL}$ standard in ethyl acetate to a 25 mL volumetric flask. Dilute to volume with ethyl acetate and mix well.

0.12 $\mu\text{g/mL}$: Transfer 300 μL of 10 $\mu\text{g/mL}$ standard in ethyl acetate to a 25 mL volumetric flask. Dilute to volume with ethyl acetate and mix well.

All calibration standard solutions are to be stored at 1-8 °C when not in use.

B. Cymoxanil analytical standards:

1. Stock Solution

Using an analytical balance and a weighing boat, weigh approximately 10.0 mg of analytical standard grade cymoxanil (DPX-T3217). Record the exact weight of cymoxanil. Transfer the standard to a 100 mL volumetric flask (using small amounts of acetonitrile). Add additional acetonitrile to approximately 60 mL and swirl the volumetric to dissolve the solid. When it is in solution, dilute to the mark (100.0 mL) with acetonitrile. The final concentration is approximately 100 $\mu\text{g/mL}$. Cymoxanil is stable in solution for at least 2 months when stored at 1-8 °C when not in use.

2. Fortification (Spiking) Solutions

Note: Solutions used to fortify samples are prepared in ethyl acetate whereas HPLC standards are prepared in 82% 10 mM KH_2PO_4 , pH = 2.9/18% methanol (v/v). The solvent composition for the HPLC standards is selected to bring the solvent strength to a range suitable for HPLC analysis. Ethyl acetate is selected for sample fortification to facilitate solvent evaporation after matrix fortification.

10 $\mu\text{g}/\text{mL}$: Transfer 2.5 mL of 100 $\mu\text{g}/\text{mL}$ standard in acetonitrile to a 25-mL volumetric flask. Dilute to volume with **ethyl acetate**. Mix well. This solution is stable for 2 months when stored at 1-8 °C.

10 $\mu\text{g}/\text{mL}$: Transfer 2.5 mL of 100 $\mu\text{g}/\text{mL}$ standard in acetonitrile to a 25-mL volumetric flask. Dilute to volume with **acetonitrile**. Mix well. This solution is stable for 2 months when stored at 1-8 °C. **This standard is to be used for SPE cartridge trials only.**

1 $\mu\text{g}/\text{mL}$: Transfer 250 μl of 100 $\mu\text{g}/\text{mL}$ standard in acetonitrile to a 25-mL volumetric flask. Dilute to volume with ethyl acetate. Mix well. This solution is stable for 1 month when stored at 1-8 °C.

3. HPLC Chromatographic Standard Solutions

Note: For optimal chemical stability, solutions of cymoxanil should be maintained at pH 2.9 to 5.5.

Prepare a 1 $\mu\text{g}/\text{mL}$ HPLC intermediate solution by placing the required volume of the 100 $\mu\text{g}/\text{mL}$ acetonitrile standard (approximately 1.0 mL) into a 100-mL volumetric flask. Add sufficient methanol to the flask (approximately 17 mL) using a 25-mL graduated cylinder so that the volume of acetonitrile plus methanol equals 18.0 mL. Sopicate and swirl the flask to ensure complete solution of cymoxanil. Dilute to the mark (100.0 mL) with 10 mM KH_2PO_4 buffer, pH = 2.9. This standard solution is stable for approximately 1 month when stored at 1-8 °C when not in use.

Working HPLC standards are prepared in 82% 10 mM KH_2PO_4 buffer, pH = 2.9/18% methanol (v/v) by quantitatively diluting appropriate volumes of the 1 $\mu\text{g}/\text{mL}$ intermediate standard. If 10-mL volumetric flasks are used for the following suggested concentrations, the volumes required to prepare the 0.10, 0.05, 0.025, and 0.01 $\mu\text{g}/\text{mL}$ working standards for the analysis are:

Desired Standard Concentration ($\mu\text{g}/\text{mL}$)	Volume of 1 $\mu\text{g}/\text{mL}$ Working Standard Required in a 10-mL Volumetric (μL)
0.10	1000
0.05	500
0.025	250
0.01	100

These working standards are stable for approximately 2 weeks if stored at 1-8 °C when not in use.

Preparation of Reagents and Reagent Solutions

A. DPX-JE874 Analysis

1. Florisil: activate by placing in an oven at approximately 130 °C for a minimum of 16 hours. Keep in the oven until just before use.
2. 10% ethyl ether in hexane: add 25 mL of ethyl ether to 225 mL hexane, mix. Prepare daily. Discard any excess.

Note: Prior to using ethyl ether check peroxide level; check daily.

3. 20% ethyl acetate in hexane: add 100 mL of ethyl acetate to 400 mL hexane, mix. Prepare this solution weekly.

B. Cymoxanil Analysis

1. 10 mM Potassium Phosphate Monobasic, pH = 2.9

Dissolve 2.74 g of potassium phosphate monobasic (KH_2PO_4) in 2.0 liters of HPLC grade water in a beaker. Adjust the pH to 2.9 by dropwise addition of concentrated 85% phosphoric acid (approximately 18 drops). Filter the solution through a 0.45 μm filter prior to use. Prepare fresh buffer every two weeks to avoid formation of sediment and bacterial growth.

Note: The weight of potassium phosphate monobasic must be increased from the figure shown above if water of hydration is present in the salt used for buffer preparation.

2. 90% Dichloromethane/10% Methanol (v/v)

To a 500-mL graduated cylinder, add 450 mL of dichloromethane. Measure 50 mL of methanol in a second graduated cylinder and add this to the 500-mL graduated cylinder. Mix well. Do not adjust the final volume to 500 mL. Prepare this solution weekly.

3. 90% Hexane/10% Ethyl Acetate (v/v)

To a 500-mL graduated cylinder, add 450 mL of hexane. Measure 50 mL of ethyl acetate in a second graduated cylinder and add this to the 500-mL graduated cylinder. Mix well. Do not adjust the final volume to 500 mL. Prepare this solution weekly.

4. 80% Hexane/20% Ethyl Acetate (v/v)

To a 500 mL graduated cylinder, add 400 mL of hexane. Measure 100 mL of ethyl acetate in a second graduated cylinder and add this to the 500 mL graduated cylinder. Mix well. Do not adjust the final volume to 500 mL. Prepare this solution weekly.

5. 60% Hexane/40% Ethyl Acetate/Methanol (v/v)

To a 500-mL graduated cylinder, add 300 mL of hexane. Measure 200 mL of ethyl acetate in a second graduated cylinder and add this to the 500-mL graduated cylinder. Add 4.5 mL of methanol to the graduate.

Mix well. Do not adjust the volume of the final solution. Prepare this solution weekly.

6. 82% 10 mM KH_2PO_4 , pH = 2.9/18% Methanol (v/v)

To a 500-mL graduated cylinder, add 410 mL of 10 mM potassium phosphate monobasic buffer, pH = 2.9. Measure 90 mL of methanol in a second graduated cylinder and add this to the 500-mL graduated cylinder. Do not adjust the final volume to 500 milliliters. Prepare this solution every two weeks.

7. HPLC Eluents

Primary Column:

Eluent A:

(Mobile Phase) 83% of 10 mM KH_2PO_4 buffer (pH 2.9), 17% Acetonitrile:

Using appropriate graduated cylinders, transfer 830 mL of 10 mM KH_2PO_4 buffer (pH 2.9) and 170 mL of acetonitrile into a reservoir. Mix well.

Eluent B:

(Rinse Solution) 50% 10 mM KH_2PO_4 buffer (pH 2.9), 50% Acetonitrile:

Using appropriate graduated cylinders, transfer 500 mL of 10 mM KH_2PO_4 buffer (pH 2.9) and 500 mL of acetonitrile into a reservoir. Mix well.

Eluent C: 50% HPLC Grade Water/50% Acetonitrile (v/v)

Optional Column #1:

Eluent A:

(Mobile Phase) 80% of 10 mM KH_2PO_4 buffer (pH 2.9), 20% Methanol:

Using appropriate graduated cylinders, transfer 800 mL of 10 mM KH_2PO_4 buffer (pH 2.9) and 200 mL of methanol into a reservoir. Mix well.

Eluent B:
(Rinse Solution) 50% 10 mM KH_2PO_4 buffer (pH 2.9), 50% Methanol:
Using appropriate graduated cylinders, transfer 500 mL of 10 mM KH_2PO_4 buffer (pH 2.9) and 500 mL of methanol into a reservoir. Mix well.

Eluent C: 50% HPLC Grade Water/50% Methanol (v/v)

Optional Column #2:

Eluent A:
(Mobile Phase) 70% of 10 mM KH_2PO_4 buffer (pH 2.9), 30% Methanol: Using appropriate graduated cylinders, transfer 700 mL of 10 mM KH_2PO_4 buffer (pH 2.9) and 300 mL of methanol into a reservoir. Mix well.

Eluent B:
(Rinse Solution) 50% 10 mM KH_2PO_4 buffer (pH 2.9), 50% Methanol: Using appropriate graduated cylinders, transfer 500 mL of 10 mM KH_2PO_4 buffer (pH 2.9) and 500 mL of methanol into a reservoir. Mix well.

Eluent C: 50% HPLC grade water/50% methanol (v/v)

All eluents must be thoroughly degassed daily. If low-pressure mixing HPLC is used for sample analysis, mobile phases should be sparged continuously to ensure that air does not diffuse into the HPLC solvents. While not needed for the analytical method, it is useful to prepare Eluent C (regarding the primary and both optional columns) for rinsing the buffer from the analytical HPLC column whenever sample analysis is discontinued.

Column Switching:

Mobile Phase A: 100% Methanol/Acetonitrile (50/50, v/v)

Mobile Phase B: 100% 10 mM KH_2PO_4 buffer, pH 2.9

Mobile Phase C: 50% Acetonitrile/50% HPLC Grade Water (v/v)

Apparatus and Equipment

Assorted laboratory glassware, graduated cylinders, short stem glass funnels, pipets, volumetric flasks, evaporating flasks, microliter syringes.

Analytical Balances: Mettler AE 160 balance capable of weighing to ± 0.1 mg to weigh the analytical standards.

Mettler Model PE1600 top-loading balance capable of weighing to ± 0.01 g for all other weighings.

Centrifuge Tubes: Pyrex 50-mL graduated with screw cap closure (silylated)

Pyrex 15-mL graduated with screw cap closure (silylated)

Chromatographic column: 11 mm i.d. \times 300 mm with 250 mL reservoir

Solid-Phase Extraction

Columns: SAX Bond Elut[®] Extraction Column, Part #1225-6013, 6 cc/1-gram strong anion exchange sorbent (Varian, Inc., Harbor City, CA)

Supelclean[™] Envi[™]-Carb SPE Tubes, Catalog #5-7094, 6-mL/500-mg carbon black sorbent. **Do not substitute.** (Supelco, Inc., Bellefonte, PA)

Silica Bond Elut[®] Extraction Column, Part #1210-2037, 3-cc/500-mg silica sorbent. **Do not substitute.** (Varian, Inc., Harbor City, CA)

Filter Funnel: Kimax[®] Kimble 58 Short Stem Chemical Funnel, 63-mm stem, 55 mm top, Catalog #30205-068 (VWR Scientific)

Graduated mixing cylinders with glass stoppers: 500 mL, 250 mL

EDP Electronic Pipet with suitable tips to prepare HPLC standards
(Rainin, Ridgefield, N.J.)

Evaporators: N-Evap Laboratory Sample Evaporator attached to a
nitrogen source (Organomation Associates, South Berlin,
MA)

Rotary evaporator with Dewar condenser and constant
temperature water bath (i.e., Buchler)

Extraction Apparatus: Visiprep™ Solid-Phase Extraction Vacuum Manifold,
Catalog #5-7030M. (Supelco, Inc., Bellefonte, PA)

Note:

This vacuum manifold is equipped with individual flow
control valves so that the vacuum to each SPE column
can be individually controlled. Vacuum must be
adjustable to properly control solvent flow through the
stacked SAX and carbon black cartridges.

15-mL Reservoirs, Part #1213-1010
(Varian, Inc., Harbor City, CA)

Adapters for column connection, package of 10, Product
Number 7122-00 (J.T. Baker, Phillipsburg, NJ)

Pint and quart extraction jars

HPLC Sample Filter: Millex®-HV13, 0.45 μm , 13-mm Duropore filter units,
Catalog #SJHV013NS (Millipore, Inc., Bedford, MA)

Mobile Phase Filter: 0.45- μm pore, Type HA cellulose acetate and nitrate
filter, Millipore Catalog #HAFT 047 00. This filter is
used to filter 10 mM aqueous KH_2PO_4 buffer (pH = 2.9).

Vacuum Filter Apparatus: Millipore vacuum filter apparatus consisting of a glass
filter holder #XX1004700, a ground glass base with
stopper #XX1004702, a funnel cover #XX2504754, and
a 1-L filter flask #XX104705. This apparatus is used to
filter all mobile phase solvents and solutions. (Millipore,
Inc., Milford, MA).

Erlenmeyer flasks with ground glass stoppers:	500 mL
Separatory funnels:	500 mL, 250 mL, 125 mL
High speed homogenizer:	(i.e., PowrPulse homogenizer)
Mixer:	Vortex Genie 2 (VWR Scientific)
Wrist action shaker:	(i.e., Burrell wrist action shaker)
Syringes:	2.5 mL, plastic, disposable (Aldrich Chemical Co., Milwaukee, WI)
Ultrasonic Bath:	Branson Model 2210 Ultrasonic Bath (VWR Scientific)

Extraction (Use part A, B, C, or D depending on type of sample matrix)

- Notes:**
- This analytical method requires that centrifuge tubes used for concentration of cymoxanil matrix samples be silylated before use. See Appendix IV for silylating procedure.
 - Cymoxanil has a tendency to adsorb onto matrix impurities present during the extraction and purification portion of this analysis. As a result, cymoxanil extracts cannot be taken to dryness or erratic results will be obtained. All cymoxanil solutions must be exchanged into various solvents during each sample cleanup step described in this method.

A. Applicable to dry and low moisture, oily and non-oily crops and related matrices (i.e., potato flakes, wheat grain)

1. Weigh 20.0 g of sample into a pint extraction jar. As applicable, fortify spikes at this point. Add 100 mL deionized (DI) water and let sample soak for approximately 10 minutes.
2. Add 200 mL of acetonitrile and homogenize at a medium speed for 3 minutes. Allow matrix to settle for approximately 1-2 minutes. Decant extract through 18.5 cm VWR #417 filter paper in a glass funnel into a 500 mL mixing cylinder containing 50 g NaCl. Allow most of the extract to pass through the filter paper, then add remaining contents of the extraction jar.

Note: Fluting the filter paper will increase speed of filtration.

3. When filtering is complete, shake the mixing cylinder for 1 minute. It is important to invert the cylinder when mixing to dissolve the salt into the water, thus causing the water and acetonitrile to separate. After complete separation of layers, let the cylinder sit for an additional 15 to 30 minutes to allow solids in the acetonitrile layer to settle out. (Solids in matrices such as potatoes and cucumbers may settle out in approximately 15 minutes, fresh tomatoes may take approximately 30 minutes.)
4. For *Cymoxanil* analysis, take a 1.2 g (12 mL) aliquot from the upper acetonitrile layer and place into a silylated 50 mL centrifuge tube. Proceed with hexane partition.
5. For *DPX-JE874* analysis, take a 10.0 g (100 mL) aliquot of the upper acetonitrile layer and place into a 500 mL separatory funnel. Proceed with hexane partition.

B. Applicable to unusual dry and low moisture, oily and non-oily crops and related matrices (i.e., wheat straw, raisins)

1. Weigh 20.0 g of sample into a quart extraction jar. As applicable, fortify spikes at this point. Add 100 mL DI water and soak for approximately 10 minutes. Add 300 mL acetonitrile and homogenize for 3 minutes at a medium speed.
2. Allow matrix to settle for approximately 1-2 minutes. Decant extract through an 18.5 cm VWR #417 filter paper in a glass funnel into a 500 mL mixing cylinder containing 50 g NaCl. Allow most of the extract to pass through the filter paper, then add remaining contents of the extraction jar.

Note: Fluting the filter paper will increase speed of filtering.

3. When filtering is complete, shake the mixing cylinder for 1 minute. It is important to invert the cylinder when mixing to dissolve the salt into the water, thus causing the water and acetonitrile to separate. After complete separation of layers, let the cylinder sit for an additional 15 to 30 minutes to allow solids in the acetonitrile layer to settle out.

4. For *Cymoxanil* analysis, take a 1.2 g (18 mL) aliquot of the upper acetonitrile layer and place into a silylated 50 mL centrifuge tube. Proceed with hexane partition.
5. For *DPX-JE874* analysis, take a 10.0 g (150 mL) aliquot of the upper acetonitrile layer and place into a 500 mL separatory funnel. Proceed with hexane partition.

C. Applicable to high and medium moisture, oily and non-oily crops and related matrices (i.e. ~~potato~~ tomato)

1. Weigh 20.0 g of sample into a pint extraction jar. As applicable, fortify spikes at this point. Add 60 mL DI water and let soak for approximately 10 minutes. Add 120 mL acetonitrile and homogenize for 3 minutes at a medium speed.
2. Allow matrix to settle for approximately 1-2 minutes. Decant extract through an 18.5 cm VWR #417 filter paper in a glass funnel into a 250 mL mixing cylinder containing 30 g NaCl. Allow most of the extract to pass through the filter paper, then add remaining contents of the extraction jar.

Note: Fluting the filter paper will increase speed of filtering.

3. When filtering is complete, shake the mixing cylinder for 1 minute. It is important to invert the cylinder when mixing to dissolve the salt into the water, thus causing the water and acetonitrile to separate. After complete separation of layers, let mixture sit for an additional 15 to 30 minutes to allow solids in the acetonitrile layer to settle out.
4. ~~For *Cymoxanil* analysis~~, take a 1.2 g (7.2 mL) aliquot of the upper acetonitrile layer and place into a silylated 50 mL centrifuge tube. Proceed with hexane partition.
5. For *DPX-JE874* analysis, take a 10.0 g (60 mL) aliquot of the upper acetonitrile layer and place into a 250 mL separatory funnel. Proceed with hexane partition.

D. Applicable to soil

1. Weigh 50.0 g of sample into a 500 mL Erlenmeyer flask which has a ground glass stopper. As applicable, fortify spikes at this point. Add 50 mL DI water and let soak for approximately 10 minutes. Add 100 mL acetonitrile, stopper and secure stopper with tape (i.e., masking tape, box tape). Shake on a wrist action shaker for 1 hour at a speed which agitates all samples adequately, usually low speed, without splashing sample into the neck of the flask.
2. Allow matrix to settle for approximately 1-2 minutes. Decant extract through an 18.5 cm VWR #417 filter paper in a glass funnel into a 250 mL mixing cylinder containing 30 g NaCl. Allow most of the solvent to pass through the filter paper, then add remaining contents of the flask.

Note: Fluting the filter paper will increase speed of filtration.

3. When filtering is complete, shake the mixing cylinder for 1 minute. It is important to invert the cylinder when mixing to dissolve the salt into the water, thus causing the water and acetonitrile to separate. After complete separation of layers, let mixture sit for an additional 15 to 30 minutes to allow solids in the acetonitrile layer to settle out.
4. For Cymoxanil analysis, take a 1.2 g (2.4 mL) aliquot of the upper acetonitrile layer and place into a silylated 15 mL centrifuge tube. Proceed with hexane partition.
5. For DPX-JE874 analysis, take a 10.0 g (20 mL) aliquot of the upper acetonitrile layer and place into a 125 mL separatory funnel. Proceed with hexane partition.

Cleanup Procedures

A. Hexane Partition

1. To the acetonitrile extract obtained from either steps 4 or 5 from the preceding extraction schemes, add the following quantity of hexane depending on the extraction method (A, B, C, or D) used:

<u>Method</u>	Analyte <u>DPX-JE874</u>	Analyte <u>Cymoxanil</u>
A	100 mL	12 mL
B	150 mL	18 mL
C	60 mL	7.2 mL
D	20 mL	2.4 mL

Note: The acetonitrile extracts for cymoxanil analysis (both before and after hexane partition) are stable for approximately five days if stored at 1-8 °C.

- Shake for 1 minute. Allow layers to separate.
- Discard the upper hexane layer and repeat steps 1 and 2 above.

Note: For cymoxanil analyses, the hexane layer in the centrifuge tube is drawn off with a disposable glass pipet.

- Place the acetonitrile layer for DPX-JE874 analysis into an appropriate evaporating flask, either 125 mL or 250 mL. Leave the acetonitrile layer for cymoxanil analysis in the centrifuge tube.
- Proceed with ~~Florisil column cleanup~~ for the DPX-JE874 aliquot and solid phase extraction (SPE) purifications for the cymoxanil aliquot.

Note: Analysis for the DPX-JE874 aliquot may be interrupted at this point for overnight storage at 1-8 °C.

B. Florisil column cleanup (applicable to DPX-JE874 analysis only)

- Evaporate the DPX-JE874 extract from step 4 of "Hexane Partition" to approximately 1-2 mL on a rotary evaporator at ≤ 35 °C. Blow remaining solvent dry with N₂ and add 5 mL of 10% ethyl ether in hexane. Sonicate the flask for approximately 30 seconds to dislodge solids.

Notes:

- A properly dried extract should not look moist or sticky.
- Check peroxide level of ethyl ether daily, before use.

2. Prepare a glass chromatography column with a plug of glass wool at the bottom. Add in order: 1 cm of sodium sulfate, 5 cm of activated Florisil (directly from oven), and 1 cm of sodium sulfate. Pre-wet the column with 15 mL of hexane and drain to the top of the column bed. Discard eluate. Add the extract from step 1 above. Adjust flow rate to ≤ 4 mL/min.

Note: Prepare Florisil columns just prior to use so that the sorbent does not deactivate.

3. Rinse the flask twice with 2 mL of 10% ethyl ether in hexane and add to the column. Drain rinses to the top of the column bed, then add an additional 15 mL of 10% ethyl ether in hexane. Drain to the top of the column bed. Discard washes.
4. Elute DPX-JE874 with 50 mL of 20% ethyl acetate in hexane, collecting the eluate in a 125 mL evaporating flask.

Analysis may be interrupted at this point for overnight storage at approximately 1-8 °C in a covered vessel.

5. Evaporate the eluate on a rotary evaporator at ≤ 35 °C to approximately 1-2 mL. Blow dry with N₂, then add approximately 2 mL of ethyl acetate. Transfer to a test tube calibrated at 1.0 mL. Rinse the flask twice with approximately 1 mL ethyl acetate, transferring each rinse to the test tube.
6. Concentrate the extract to 1.0 mL on an N-Evap at ≤ 35 °C. The sample is ready for GC analysis. Final concentration is 1.0 mL = 10.0 g.

C. Solid Phase Extraction (SPE) Purifications: (applicable to cymoxanil analysis only)

- Notes:**
- SPE cartridges may not be uniformly packed as received from the manufacturer. To ensure that channels do not exist in the column packing, tap all cartridges firmly on a lab bench for at least 20 seconds before the columns are used. If applicable, push down upper column frit (against packing) if sorbent settles from tapping.

- For maximum convenience, conditioning and eluting solvent combinations such as 90% dichloromethane/10% methanol (v/v) can be prepared in advance.
- Check or calibrate the SPE cartridges prior to use in order to ensure optimum method performance. In general, check one cartridge per box even though there may be more than one box per lot number. This assessment should be conducted well in advance of needing the columns for sample analysis. Recovery of >90% is desired to ensure that a box of cartridges is suitable for use. The analyses are conducted on a reagent spike basis. Note: When spiking the reagents with analytical standard, inject the standard into, not onto, the solvent going to the cartridge.

See Appendix I for detailed instructions on assessment of all SPE cartridges.

1. Concentrate the cymoxanil extract from step 4 of "Hexane Partition" to 2.0 ± 0.1 mL at 40°C using the N-Evap. (At start of evaporation, keep N-Evap needles positioned high with high nitrogen flow so surface of solvent "rocks" without splashing. As solvent evaporates, adjust needle closer to solvent and reduce nitrogen flow.)

Add 11 mL HPLC water to the extract. Vortex mix for at least 30 seconds and sonicate for at least 5 minutes. (Do not let solvent touch centrifuge cap when vortex mixing.)

The sample extract (at 13 mL) is now ready for the first SPE cleanup.

Note: Cymoxanil is most stable at pH 4.5 to 5.5 in water. For troubleshooting purposes, check the pH of the water used for dilution and adjust if necessary.

2. Condition a 1-gram SAX cartridge by passing 5 mL of methanol and then 15 mL of HPLC grade water through the cartridge. Do not let the cartridge go to dryness after conditioning. (Stop elution when water reaches top of frit.)

3. Condition a 500-mg Envi™-Carb tube by passing 5.0 mL of 90% dichloromethane/10% methanol (v/v) through the cartridge. Next pass 2.0 mL of methanol through the cartridge and then 15 mL of HPLC water. After conditioning, do not let the cartridge go to dryness. (Stop dichloromethane/methanol elution approximately 5 mm from top of frit; stop water elution approximately 10 mm from top of frit.)
4. Stack the SAX cartridge on top of the Envi™-Carb tube using an adapter and place the stacked cartridges on an SPE manifold. A 15-mL reservoir can be placed on the SAX cartridge to help load all solutions and solvents.

- Notes:
- When stacking the two cartridges it is necessary to have the adapters connected very tightly. This will ensure that the bottom column does not go dry.
 - Before applying the vacuum, it may be necessary to "prompt" the washes through the columns with a pipet bulb fitted with a stopper.

Pass the solution from step 1 above through the stacked cartridges at a flow rate that does not exceed 180 drops per minute and allow the solvent to pass through the SAX cartridge until the liquid level is just above the frit of the SAX cartridge.

Wash the 50-mL centrifuge tube with an additional 5-mL portion of HPLC water. Sonicate the centrifuge tube for at least one minute after the water addition and vortex mix the tube to ensure solution of any residue remaining in the tube. Pass the wash through the SAX cartridge and take the SAX cartridge to dryness but do not allow the Envi™-Carb tube to go to dryness. Cymoxanil passes through the SAX cartridge and is retained on the Envi™-Carb tube. Remove and discard the SAX cartridge.

5. Wash the Envi™-Carb tube by passing the following solvents through the cartridge in the order listed:

5 mL of HPLC water. After water passes through the cartridge, pull vacuum through the cartridge for 1 minute to remove as much water as possible from the cartridge.

1 mL of methanol. Slowly pass this solvent through the cartridge. Once solvent passes through the cartridge, pull air through the cartridge for 1 minute to remove as much solvent from the cartridge as possible.

Note: Methanol is used to remove water from the Envi™-Carb tube prior to eluting cymoxanil.

6. Using gravity flow, elute the Envi™-Carb tube by passing 10 mL of 90% dichloromethane/10% methanol (v/v) through the tube. The vacuum may be turned on, then off, to initiate the flow.

Collect eluate in a silylated 15-cc centrifuge tube.

Note: The centrifuge tubes used in this step cool well below room temperature during collection of the 90% dichloromethane/10% methanol(v/v) fraction. These tubes should be allowed to warm to room temperature before they are placed in an N-Evap bath. Otherwise the glass centrifuge tubes may crack. Due to evaporation, the final solvent volume in the silylated 15-cc centrifuge tube is less than 10 mL.

Particles of carbon black may collect in some samples during elution. These are removed during subsequent silica cartridge cleanup. **The analysis can be interrupted at this point if desired.** Samples are stable for at least 24 hours if stored at approximately 1-8 °C in closed tubes.

7. Concentrate the dichloromethane/methanol solvent to 0.5 ± 0.1 mL with nitrogen using an N-Evap at 40°C. Add 1.5 ± 0.1 mL of ethyl acetate to the tube and concentrate the solvent to 0.5 ± 0.1 mL with nitrogen on an N-Evap at 40°C. Add 1.5 mL of ethyl acetate and again concentrate the solvent to exactly 1.0 ± 0.1 mL using nitrogen and an N-Evap at 40°C. Dilute the contents of the tube to exactly 10.0 mL with hexane. After about 4 mL of hexane have been added, shake the tube to mix the solvents. After hexane addition is complete, vortex mix the contents of the tube for at least 30 seconds.

8. Condition a 500-mg silica SPE cartridge by passing 10 mL hexane through the cartridge at a flow rate not to exceed 180 drops/minute. Force hexane through with a pipet bulb/stopper assembly to saturate column, then use vacuum to pull remaining hexane to top of frit. Do not let the cartridge go to dryness once it is conditioned.
9. After conditioning, pass the solution from step 7 (SPE Purification) through the silica cartridge at a flow rate not to exceed 180 drops/minute. Wash the tube with 2 mL of 90% hexane/10% ethyl acetate (v/v) and add this to the silica cartridge to ensure quantitative transfer of the sample to the cartridge. Elute the cartridge at a flow rate not exceeding 180 drops per minute. Discard this wash.
10. Wash the column with 10 mL 90% hexane/10% ethyl acetate (v/v). Discard this wash.
11. Wash the column with 10 mL 80% hexane/20% ethyl acetate (v/v). Discard this wash.
12. Using gravity flow, elute the analyte with 12 mL 60% hexane/40% ethyl acetate/methanol (v/v). The vacuum may be turned on, then off, to initiate flow. Collect this last fraction in a silylated 15-cc centrifuge tube. Take the silica cartridge to dryness. The volume of solvent collected may be below 12 mL as result of evaporation. Concentrate the solvent to 0.5 ± 0.1 mL under nitrogen at 40°C using an N-Evap.
13. Add 1.5 mL of methanol to the centrifuge tube and concentrate the solvent to 0.5 ± 0.1 mL. Add 1.5 mL of methanol and again concentrate the solvent to exactly 0.5 mL.
14. Dilute the sample to exactly 3.0 mL with 10 mM KH_2PO_4 buffer, pH = 2.9. Vortex mix the sample for at least 30 seconds. Sonicate the sample for at least 2 minutes, and vortex mix the sample for at least 30 seconds.

Note: The analysis can be interrupted at this point if desired. Samples are stable for at least one week if stored at approximately 4°C.

Filter the solution through a 0.45-mm, Millex®-HV13 filter using a disposable syringe into an autosampler vial. The sample is ready for HPLC analysis. Final concentration is 1.0 mL = 0.40 g

Instrumental Analysis**A. DPX-JE874, GC Analysis:**

Note: The column and conditions have been shown to be satisfactory for the matrices being analyzed. However, depending on the resolution required during actual chromatography, additional columns may have to be used. The column temperatures and flow rates listed are typical conditions for this analysis. Specific conditions used will be noted on each chromatographic run and will not otherwise be documented.

Instrument: Hewlett-Packard HP5890A gas chromatograph, equipped with an N/P detector

Primary column:

Column: 15m × 0.53 mm i.d. fused silica column crosslinked with 1.5 μm film thickness DB-5ms, (manufacturer: J&W Scientific)

Temperatures: Column: initial: 260 °C hold 1 minute
rate: 3 °C/minute
final: 275 °C hold 1 minute

Detector: 280 °C
Injector: 280 °C

Carrier Gas and Flow Rate: Helium at 25 mL/min., 10 mL/min. makeup

Injection Volume: 2 μL

Retention Time: approximately 4 minutes

Optional column:

Column: 15m × 0.53 mm i.d. fused silica column coated with 1.0 μm film thickness Rtx-200, (manufacturer: Restek)

Primary Column: (suitable for matrices such as potato, potato processed commodities, tomato, tomato processed commodities, cucumber, lettuce and bell pepper)

Column: 25 cm × ¼" o.d. × 4.6 mm i.d. Dupont Zorbax SB-Phenyl, 5 μ Particle Size

Pre-column: 4 mm × 12.5 mm Dupont Zorbax SB-CN, 5 μ Reliance Cartridge Guard Column with Mac-Mod Column End-Fittings

HPLC Conditions:

UV Wavelength: 245 nm

Column Oven Temperature: 40.0 °C

Injection Volume: 100 μL

Initial Flow Rate: 1.0 mL/min.

Solvent A: 83% 10mM KH₂PO₄ buffer, pH 2.9 + 17% Acetonitrile

Solvent B: 50% 10 mM KH₂PO₄ buffer, pH 2.9 + 50% Acetonitrile

Solvent C: 50% HPLC water/50% Acetonitrile(v/v)

Integrator Chart Speed: 0.50 cm/min.

Typical Solvent Program:

Time (min.)	% A 83% 10 mM KH ₂ PO ₄ buffer pH 2.9 + 17% <u>Acetonitrile</u>	% B 50% 10 mM KH ₂ PO ₄ buffer pH 2.9 + 50% <u>Acetonitrile</u>	Flow (mL/min.)
0-24.9	100	0	1.0
25.0-29.9	0	100	2.0
30.0-44.9	100	0	2.0
45.0-50.0	100	0	1.0

Note: The changes in solvent strength in this program are instantaneous step gradients. There is no time lag in the solvent composition changes.

Optional Column #1: (suitable for matrices such as wheat grain)

Column: 25 cm × ¼" o.d. × 4.6 mm i.d. Dupont Zorbax SB-CN, 5 μ Particle Size

Pre-column: 4 mm × 12.5 mm Dupont Zorbax SB-CN, 5 μ Reliance Cartridge Guard Column with Mac-Mod Column End-Fittings

HPLC Conditions:

UV Wavelength: 245 nm

Column Oven Temperature: 40.0 °C

Injection Volume: 100 μL

Initial Flow Rate: 0.7 mL/min.

Solvent A: 80% 10mM KH₂PO₄ buffer, pH 2.9 + 20% Methanol

Solvent B: 50% 10mM KH₂PO₄ buffer, pH 2.9 + 50% Methanol

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Solvent C: 50% HPLC water/50%
Methanol (v/v)

Integrator Chart Speed: 0.50 cm/min.

Typical Solvent Program:

Time (min.)	% A 80% 10 mM KH ₂ PO ₄ buffer pH 2.9 + 20% <u>Methanol</u>	% B 50% 10 mM KH ₂ PO ₄ buffer pH 2.9 + 50% <u>Methanol</u>	Flow (mL/min.)
0-24.9	100	0	0.7
25.0-32.9	0	100	2.0
33.0-48.9	100	0	2.0
49.0-55.0	100	0	0.7

Note: The changes in solvent strength in this program are instantaneous step gradients. There is no time lag in the solvent composition changes.

Optional Column #2:

Column: 25 cm × 1/4" o.d. × 4.6 mm i.d. Dupont Zorbax RX-C8,
5 μ Particle Size

Pre-column: 4 mm × 12.5 mm Dupont Zorbax SB-C8, 5 μ Reliance
Cartridge Guard Column with Mac-Mod Column End
Fittings

HPLC Conditions:

UV Wavelength: 245 nm

Column Oven Temperature: 40.0 °C

Injection Volume: 100 μL

Initial Flow Rate: 0.7 mL/min.

Solvent A: 70% 10 mM KH₂PO₄ buffer,
pH 2.9 + 30% Methanol

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Solvent B: 50% 10 mM KH_2PO_4 buffer,
pH 2.9 + 50% Methanol

Solvent C: 50% HPLC water/50%
Methanol (v/v)

Integrator Chart Speed: 0.5 cm/min.

Typical Solvent Program:

Time (min.)	% A 70% 10 mM KH_2PO_4 buffer pH 2.9 + 30% <u>Methanol</u>	% B 50% 10 mM KH_2PO_4 buffer pH 2.9 + 50% <u>Methanol</u>	Flow (mL/min)
0-29.9	100	0	0.7
30.0-37.9	0	100	2.0
38.0-52.9	100	0	2.0
53.0-59.0	100	0	0.7

Note: The changes in solvent strength in this program are instantaneous step gradients. There is no time lag in the solvent composition changes.

Column Switching: (this technique to be used when the primary or 2 optional columns fail to produce a suitable interference-free window around the analyte's retention time)

Column 1: 15 cm \times 1/4" o.d. \times 4.6 mm i.d. DuPont Zorbax SB-CN,
5 μ Particle size

Column 2: 15 cm \times 1/4" o.d. \times 4.6 mm i.d. DuPont Zorbax SB-C18,
5 μ Particle size

Pre-Column: 4 mm \times 12.5 mm DuPont Zorbax SB-CN, 5 μ Reliance
Cartridge Guard Column with Mac-Mod Column End-
Fittings

HPLC Conditions:

UV Wavelength: 245 nm

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Column Oven Temperature:	40.0 °C
Injection Volume:	30 or 40 μ L depending on detector response.
Initial Flow Rate:	1.00 mL/min.
Initial Flow Path:	Zorbax® SB-CN to detector (Valve Position 1, see Appendix II, Figure 1)
Mobile Phase A:	100% Methanol/Acetonitrile (50/50, v/v)
Mobile Phase B:	100% 10 mM KH_2PO_4 buffer, pH = 2.9
Mobile Phase C:	50% Acetonitrile/50% HPLC Grade Water (v/v)
Helium Sparge Rate:	100%
Integrator Chart Speed:	0.50 cm/min.
Integrator Attenuation (2^n)	n = 0

Note: *When doing trace analysis, integrators may have difficulty setting baselines when multiple peaks are present in a chromatogram. If erroneous responses (peak heights) are obtained during analysis, manually measured peak heights should be used to calculate analytical results.*

Newly installed Zorbax® SB-CN columns must be equilibrated for at least 2 hours at a flow rate of at least 1.00 mL/min. to a solvent composition of 18% methanol/acetonitrile (50/50, v/v), 82% 10 mM KH_2PO_4 buffer pH 2.9 (18% A/82% B). Zorbax® SB-C18 columns are equilibrated at a flow rate of at least 1.00 mL/min. for at least 2 hours to a solvent composition of 30% methanol/acetonitrile (50/50, v/v), 70% 10 mM KH_2PO_4 buffer pH 2.9 (30% A/70% B). The column oven must be stabilized at 40 °C. With both Column I (the Zorbax® SB-CN column) and Column II (the Zorbax® SB-C18 column) on line (valve in the load position), this can be achieved by pumping 30% methanol/acetonitrile (50/50, v/v), 70% 10 mM KH_2PO_4 buffer pH 2.9 (30% A/70% B) through Column I and II. After equilibration, the

switching valve is rotated to the inject position to bring Column I on line (Column II off-line) and Column I is equilibrated for at least 1 hour at a flow rate of 1.0 mL/minute to a solvent composition of 18% methanol/acetonitrile (50/50, v/v)/82% 10 mM KH₂PO₄ buffer, pH = 2.9 (18% A/82% B). The flow rate can be increased to 2.0 mL/minute to shorten the equilibration time required for Column I if desired. Column I is left on-line with solvent passing to the detector at the start of the analysis. A diagram of the column and column-switching valve configuration is shown in Appendix II Figures 1 and 2. The Zorbax® SB-CN and Zorbax® SB-C18 analytical columns are described in the HPLC Column section of this report.

The following representative solvent and valve-switching programs are entered into the Waters 600E controller.

TYPICAL SOLVENT PROGRAM

<u>Time</u> <u>(Minutes)</u>	<u>Flow Rate</u> <u>(mL/min.)</u>	<u>% A</u> <u>Methanol/</u> <u>Acetonitrile</u> <u>(50/50, v/v)</u>	<u>%B</u> <u>10 mM KH₂PO₄</u> <u>buffer, pH = 2.9</u>
Initial	1.00	18	82
12.00	2.00	50	50
22.00	2.00	30	70
32.00	1.00	30	70
49.00	2.00	18	82
59.00	1.00	18	82
63.00	0.50	18	82

TYPICAL VALVE SWITCHING PROGRAM

<u>Time</u>	<u>Event</u>	<u>Action</u>	<u>Switching</u> <u>Valve Position</u>	<u>Column</u> <u>1 = SB-CN,</u> <u>2 = SB-C18</u>
Initial	Alarm	Off	Inject	1
7.87	S2	Pulse	Load	1 + 2
9.27	S1	Pulse	Inject	1
34.00	S2	Pulse	Load	1 + 2
47.00	S1	Pulse	Inject	1

The autosampler is programmed to inject samples every 62 minutes.

Note: *The solvent program has been arranged to wash Column I only with strong solvent. The sample cleanup portion of the analysis produces samples that are clean enough that Column II (the analytical column) does not require cleanup after analysis of each sample. This shortens the analysis time required for each sample. While the method has been tested with a number of different sample matrices, it has not been possible to inject large numbers of samples to determine if interferences eventually buildup on the second column to the point that column cleaning becomes necessary. Accordingly, it may be necessary to wash the second column with strong solvent and reequilibrate it to typical starting conditions (30% A/70% B) after some number of sample analyses. Alternatively, during each analysis, Column II could be washed with strong solvent (50% methanol/50% acetonitrile (v/v)/50% 10 mM KH_2PO_4 buffer, pH = 2.9 [50% A/50% B]) after cymoxanil elutes from Column II (approximately 42 minutes), and reequilibrated to starting conditions (30% A/70% B). Based on experience with the method, it has not proved necessary to do this.*

See Appendix III for a discussion which describes the column-switching routine used for sample analysis in more detail.

Analysis

Prepare a four-point standard curve by injecting constant volumes of the cymoxanil standard solutions. Inject a constant volume of the sample extract into the instrument. Sample responses not bracketed by the standard curve require dilution and reinjection. Inject a standard after every 4-5 sample injections.

Calculations

Calculations for instrumental analysis are conducted using a validated software application to create a standard curve based on linear regression. These regression functions are used to calculate a best fit line (from a set of standard concentrations in $\mu\text{g/mL}$ versus peak height response) and to determine concentrations of the analyte found during sample analysis from the calculated best fit line.

The equation used for least squares fit is:

$$y = mx + b$$

where,

y = peak height response

x = $\mu\text{g/mL}$ found for peak of interest

m = slope

b = y-intercept

A. DPX-JE874

1. The amount of DPX-JE874 (in ppm) found in the sample is calculated according to the following equation:

$$\text{ppm DPX-JE874 found} = \frac{\mu\text{g/mL DPX-JE874} \times \text{mL solv.} \times \text{mL final vol.} \times \text{GC dil. fact.}}{\text{sample wt. (g)} \times \text{mL aliquot}}$$

where:

$\mu\text{g/mL DPX-JE874}$ is calculated by linear regression based on peak height response.

mL solvent = extraction solvent (acetonitrile) volume

mL aliquot = volume of sample extract taken through hexane partition

sample weight (g) = amount of sample extracted

mL final volume = volume of final extract submitted to GC

GC dil. fact. = the magnitude of dilution required to bracket the response of the sample within the standard curve responses. When the sample requires no dilution, the GC dilution factor = 1

2. The percent recovery for fortified control samples is calculated as follows:

$$\% \text{ Recovery} = \frac{\text{ppm found in fortified control} - \text{ppm found in control}}{\text{ppm added}} \times 100$$

B. Cymoxanil

1. The amount of cymoxanil (in ppm) found in the sample is calculated according to the following equation:

$$\text{ppm cymoxanil found} = \frac{\mu\text{g/mL cymoxanil} \times \text{mL solvent} \times \text{mL final volume} \times \text{HPLC dil. fact.}}{\text{sample weight (g)} \times \text{mL aliquot}}$$

where:

$\mu\text{g/mL}$ cymoxanil is calculated by linear regression based on peak height response

mL solvent = extraction solvent (acetonitrile) volume

mL aliquot = volume of sample extract taken through hexane partition

sample weight (g) = amount of sample extracted

mL final volume = volume of final extract submitted to HPLC

HPLC dil. fact. = the magnitude of dilution required to bracket the response of the sample within the standard curve responses. When the sample requires no dilution, the HPLC dilution factor = 1

2. The percent recovery for fortified control samples is calculated as follows:

$$\% \text{ Recovery} = \frac{\text{ppm found in fortified control} - \text{ppm found in the control}}{\text{ppm added}} \times 100$$

Note: Interference in control samples should not exceed 15 to 20% of the height of the cymoxanil peak at the proposed tolerance fortification of 0.10 ppm.

APPENDIX I

Quality Control for SPE Cartridges

SAX Cartridges

Add 25 μL of 100 $\mu\text{g}/\text{mL}$ cymoxanil standard (in acetonitrile) into 2.0 mL of acetonitrile in a 50 mL silylated centrifuge tube. Add 11.0 mL HPLC grade water. Vortex 30 seconds, sonicate 5 minutes. Condition SAX cartridge with 5 mL methanol, then 15 mL HPLC grade water. Do not let column go dry. Using gravity flow, pass fortified solution through cartridge, collecting in a 25 mL volumetric flask. Rinse centrifuge tube with 5 mL HPLC grade water; sonicate 1 minute, vortex 30 seconds. Add rinse to cartridge before elution is complete. Allow cartridge to go to dryness. Bring final volume to 25 mL, mix and submit to HPLC. Final cymoxanil concentration is 0.1 $\mu\text{g}/\text{mL}$.

ENVITTM-Carb Tubes

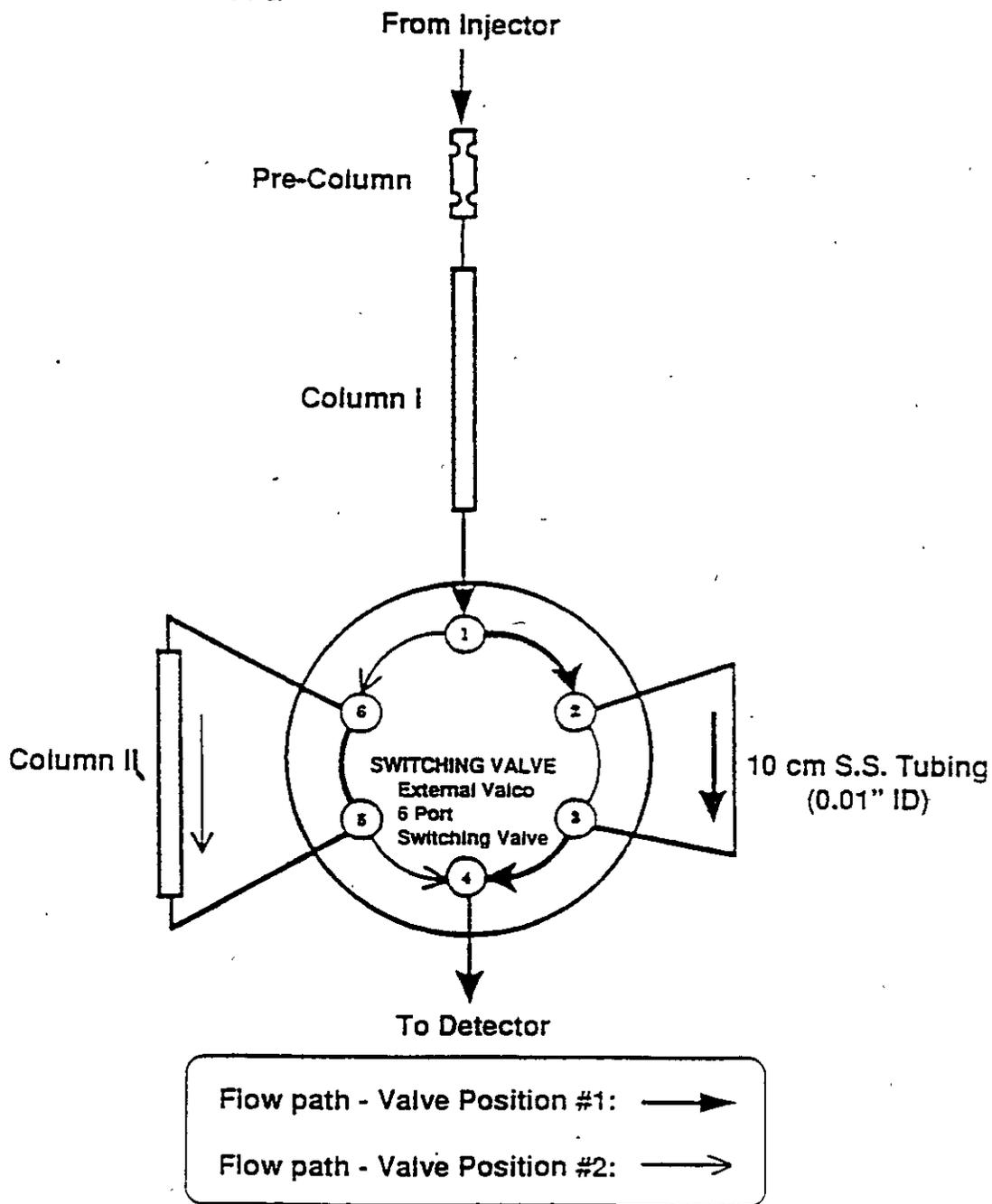
Add 30 μL of 10 $\mu\text{g}/\text{mL}$ cymoxanil standard (in acetonitrile) into 2.0 mL of acetonitrile in a 50 mL silylated centrifuge tube. Add 11.0 mL HPLC grade water. Vortex 30 seconds, sonicate 5 minutes. Condition ENVITTM-Carb tube with 5 mL 90% dichloromethane/10% methanol, then 2 mL methanol, and finally 15 mL HPLC grade water. Do not let cartridge go dry. Pass fortified solution through the tube. Rinse the centrifuge tube with 5 mL HPLC water. Sonicate 1 minute, vortex 30 seconds and add to solution passing through the tube. Stop when solution is just above column frit. Wash tube with 5 mL HPLC water, go to dryness and pull air through tube for 1 minute. Add 1 mL methanol, slowly pass through column and go to dryness. Pull air through tube for 1 minute. Elute by gravity with 10 mL 90% dichloromethane/10% methanol into a 15 mL silylated centrifuge tube. Concentrate eluate to 0.5 ± 0.1 mL on N-evap at 40 °C. Follow steps 13 and 14 of solid phase extraction (SPE) purifications. Final cymoxanil concentration is 0.1 $\mu\text{g}/\text{mL}$.

Silica Cartridges

Add 30 μL of 10 $\mu\text{g}/\text{mL}$ cymoxanil standard (in ethyl acetate) into 9.0 mL hexane and 970 μL ethyl acetate in a 15 mL silylated centrifuge tube. (This makes a 90% hexane/10% ethyl acetate final solution.) Vortex mix 30 seconds. Condition column with 10 mL hexane. Saturate the column by forcing solvent through it with the column manifold open for the cartridge. Do not let column go dry. Pass fortified solution through the cartridge. Rinse centrifuge tube with 2 mL 90% hexane/10% ethyl acetate and add to cartridge. Discard this wash. Follow steps 10 through 14 of solid phase extraction (SPE) purifications. Final cymoxanil concentration is 0.1 $\mu\text{g}/\text{mL}$.

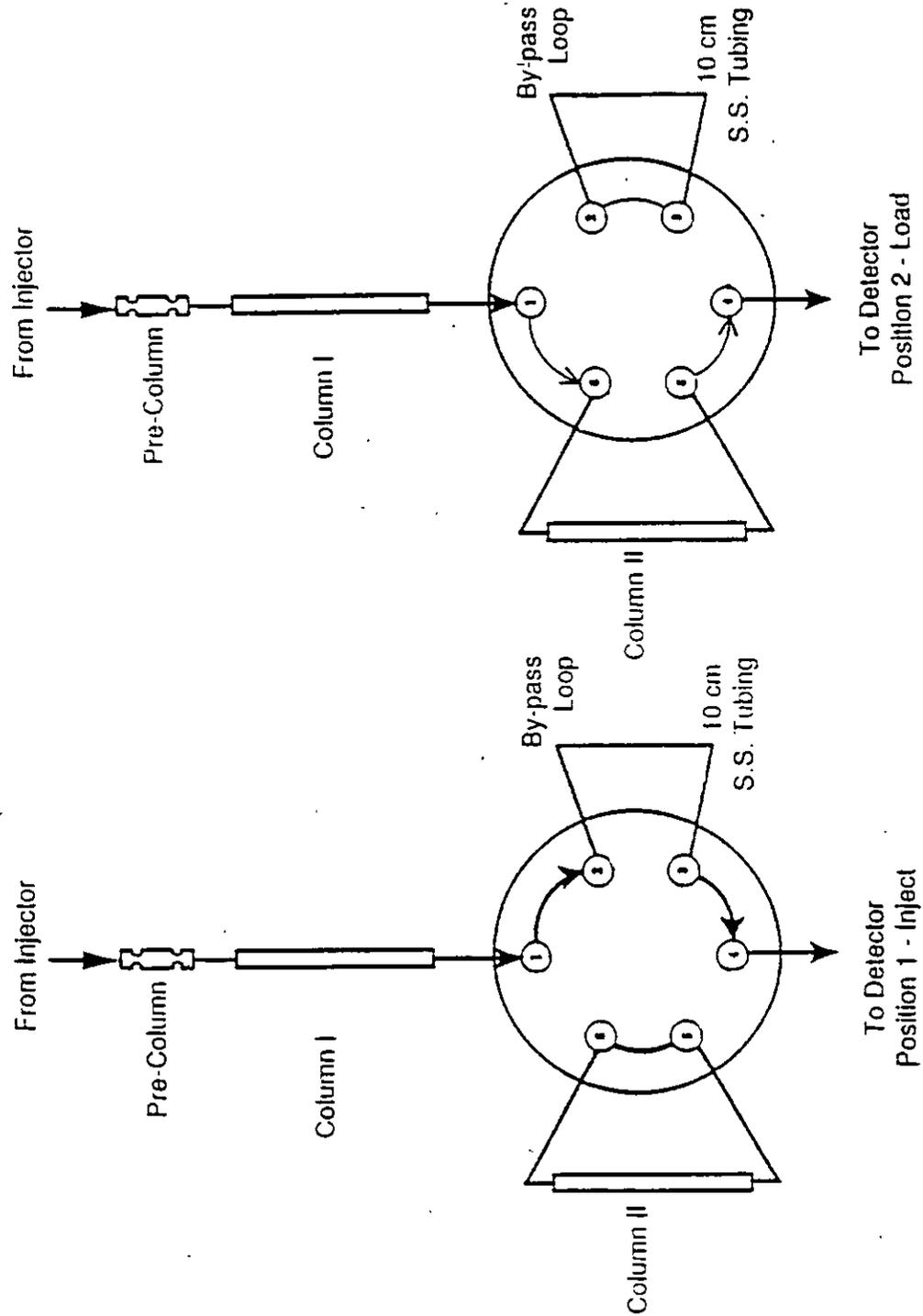
APPENDIX II

FIGURE 1
PLUMBING DIAGRAM FOR COLUMN SWITCHING



All tubing used is Stainless Steel 0.01" ID

FIGURE 2
VALVE POSITIONS FOR COLUMN SWITCHING



APPENDIX III

Referring to Appendix II Figure 2, the analysis is begun with the switching valve in position 1, the inject position. In this position, flow passes through the pre-column and SB-CN column (Column I), through the switching valve (valve position 1), through the bypass loop (valve positions 2 and 3), and then to the detector (valve position 4). At 7.87 minutes (the sample cut window, see further discussion below), the switching valve is rotated to position 2 and cymoxanil is transferred to Column II, the SB-C18 column. In position 2, the load position, the solvent flows through the pre-column and through the SB-CN column (Column I), through the switching valve (valve position 1), through the SB-C18 column (Column II, valve positions 6 and 5) and then to the detector (valve position 4). After the cymoxanil peak transfer is complete (9.27 minutes), the switching valve is rotated to position 1. At 12.00 minutes, Column I is cleaned with strong solvent and 22.00 minutes equilibrated to a solvent composition of 30% A/70% B. the solvent composition on Column II, in preparation for elution of cymoxanil. At 32.00 minutes, the flow rate is reduced to 1.00 mL/minute and at 34.00 minutes, the switching valve is rotated to position 2 bringing both Columns I and II on-line. Elution of cymoxanil begins in 30% A/70% B.

After elution of the cymoxanil peak (approximately 42 minutes), the switching valve is rotated to position 1 (47.00 minutes) and Column I is equilibrated to starting conditions (18% A/82% B). At 59 minutes, the flow rate on Column I is adjusted to 1.0 mL/minute. The next sample is injected at minute 62.00 or the autosampler program ends after the last sample is injected at minute 63.00, at which time the solvent flow rate is reduced to 0.5 mL/minute to conserve solvent while maintaining the HPLC equipment in operation for additional analyses.

The switching valve times (the transfer window or cut window) must be determined before analysis of each set of samples. The retention time and width of the cymoxanil peak is determined by injecting the highest concentration cymoxanil standard to be included in the sample set (a 0.13 $\mu\text{g}/\text{mL}$ standard) three times on Column I only. Prior to injection, the HPLC system must be equilibrated and the temperature of the HPLC column stabilized at 40.0 °C. Calculate the mean retention time of the three standard injections and accurately measure the baseline width of the cymoxanil peaks in centimeters with a ruler. Divide the peak width by the integrator chart speed expressed in cm/minute to determine the peak width in minutes. The first valve switching time is then calculated as:

$$\text{Average retention time (min.)} = \left[\frac{\text{peak width (min.)}}{2} + 0.20 \text{ (min.)} \right]$$

The second valve switching time is calculated as:

$$\text{Average retention time (min.)} + \left[\frac{\text{peak width (min.)}}{2} + 0.20 \text{ (min.)} \right]$$

The figure of 0.20 minute is used to assure that the cymoxanil collection window is set conservatively enough to collect the entire analyte peak but may be widened to insure peak collection if the cymoxanil retention time drifts during analysis of a set of samples. Premixing solvents to the composition required for analysis reduces changes in the retention time. If interferences appear in the chromatograms, solvent compositions on both columns should be varied to improve resolution. Decreasing solvent strength on the C-18 column from 30% organic is particularly effective. The retention times of the cymoxanil injections on Column I must not differ by more than a relative standard deviation of 1.5% ($\text{RSD} = \text{Std. Dev.}/\text{mean} \times 100$) in order for analysis to proceed.

Example calculations further demonstrating the procedure for determining the cymoxanil transfer window follow:

Retention Time for Three Cymoxanil Injections (Minutes):	8.515 8.622 8.585
Mean Cymoxanil Retention Time (Minutes):	$25.722/3 = 8.574$ minutes
Standard Deviation (Minutes $\sigma_{[N-1]}$):	0.0543
Relative Standard Deviation (RSD):	$\frac{0.0543}{8.574} \times 100 = 0.63\%$

Peak Width:	0.50 cm
Chart Speed:	0.50 cm/minute
Peak Width (Minutes):	$0.50 \text{ cm}/0.50 \text{ cm/min} = 1.0 \text{ min}$

$1.0 \text{ minute}/2 + 0.20 \text{ minute} = 0.70 \text{ minute}$ (half the peak window width).

$8.57 \text{ minutes} - 0.70 \text{ minute} = 7.87 \text{ minutes}$ (lower window setting)

$8.57 \text{ minutes} + 0.70 \text{ minute} = 9.27 \text{ minutes}$ (upper window setting)

These upper lower window settings are programmed into the 600E valve switching program.

When the mean retention time is being determined for standards eluting from Column I, the shape of the cymoxanil peak should be observed. If the peak shape is asymmetric (fronts or tails), the pre-column cartridge may need to be replaced, or the pre-column fittings may need to be tightened, and/or the analytical column may need to be cleaned or replaced before further analysis can be conducted. When analytical columns are installed or replaced, they should be flushed with 20-column volumes (approximately 30 mL) of 50% methanol/50% acetonitrile (v/v)/50% Milli-Q[®] water at a flow rate of 2.00 mL/minute before they are attached to the UV detector. After flushing, the columns are attached to the detector and one hundred percent 50% methanol/50% acetonitrile (v/v) is passed through the columns at a flow rate of 1 mL/minute until the baseline is stable. The columns should then be equilibrated to the solvent compositions required for analysis as described above. When the pre-column is replaced, it is purged with 100% methanol/acetonitrile (50/50, v/v) for 5 minutes at a flow rate of 2 mL/minute with the column disconnected from the analytical columns. The pre-column is then equilibrated to 18% A/82% B for 5 minutes at a flow rate of 2 mL/minute, solvent flow is stopped and the pre-column is connected to Column I (see Appendix II Figure 1).

APPENDIX IV

Silylation of Glassware

Purpose:

Silylation is a process used to chemically treat glassware in order to prevent or minimize binding of analyte residues to the glass surface.

Caution: **DO NOT ALLOW DIMETHYLDICHLOROSILANE TO COME IN CONTACT WITH WATER. CHLORINE GAS AND HYDROGEN CHLORIDE GAS WILL BE PRODUCED.**

THIS PROCEDURE MUST BE DONE UNDER A FUME HOOD. THE TECHNICIAN MUST WEAR HEAVY LATEX GLOVES.

Procedure:

1. Prepare 100 mL of a 5% (v/v) solution of dimethyldichlorosilane (DMDCS) in hexane.

To a glass stoppered glass container (approximately 200 mL volume) add 95 mL hexane. Slowly add 5 mL DMDCS. Stopper and invert to mix.

Larger volumes can be prepared using the proportions discussed above, however attempt to prepare amounts that will be nearly totally used to avoid disposal of excess solution.

2. Pour a small amount of the DMDCS solution into the glassware to be treated. Rotate the glassware to thoroughly coat the inside surfaces. Pour excess solution into the next piece of glassware to be treated.
3. Allow the treated glassware to dry (approximately 20 minutes). Rinse with deionized water, then acetone. Again allow to dry.
4. Glassware is now ready for use.

- Note:
- Any glassware that is cleaned with a brush after it has been silylanized, must be resilylanized.
 - Store pure DMDCS at room temperature.

- 5% solutions of DMDCS in hexane are stable for 5 days when stored well-stoppered at room temperature.

SOP Prepared by: Deirdre DeMario
Gary L. Westberg

APPROVED BY: Gary L. Westberg
DATE: 11/27/95

CERTIFICATION

**ANALYTICAL METHOD FOR THE DETERMINATION OF
DPX-JE874 AND CYMOXANIL RESIDUES IN VARIOUS
MATRICES**

We, the undersigned, declare that this report provides an accurate record of the procedures and results.

Report by:

Edward C. Nathan
Edward C. Nathan
Study Director

04-Dec-1995
Date

Approved by:

Sidney S. Goldberg
Sidney S. Goldberg
Research Supervisor

4-Dec-95
Date

Date Study Completed:

December 4, 1995

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Global Technology Division
Experimental Station
Wilmington, Delaware 19880-0402

EPA ADDENDUM

ACLB #B98-12

TMV for Cymoxanil in/on Potatoes

1) In the calculations the method mentions that the analyst should subtract the response of the control samples from the amount found in the samples. This is not permitted under enforcement guidelines as enforcement laboratories do not have control samples available. This should be omitted from the calculation table.

2) While mention is made in the method that "equivalent" sources could be substituted for the original. ACLB tried substituting an equivalent column and had poor success in separating some interferences. It is highly recommended that the analyst use only the Zorbax brand phenyl or cyano columns.