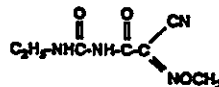


**I. INTRODUCTION****A. Scope**

Cymoxanil (DuPont Identification No. DPX-T3217) is the active ingredient in Curzate® Fungicide, a DuPont agrichemical used for control of select plant diseases in crops such as potatoes, principally in Europe, Latin America, and recently the United States. Its chemical structure and *Chemical Abstracts* name are as follows:



Cymoxanil (DPX-T3217)  
2-Cyano-N-[(ethylamino) carbonyl]-2-(methoxyimino)acetamide  
CAS Registry No. 57966-95-7

As a result of its use for disease control in crops, there is need for an analytical method to selectively detect residues of cymoxanil in water samples. This report describes a suitable method. The method has been applied to detect levels of cymoxanil at approximately 0.50 ppb or above in 100-g water samples.

Select physical properties (Reference 1) of cymoxanil are as follows:

Melting Point:	160-161°C
Solubility (25°C):	
Water	1 g/kg
Acetone	105 g/kg
Hexane	<1 g/kg

Stable at pH 2 to 7.3.

*Note:* Cymoxanil is chemically stable between a pH of 2.9 and 6.0 and optimally stable between a pH of approximately 5.0 and 6.0 (Reference 2).

**B. Principle**

One-hundred gram water samples are prefiltered through a 0.45-micron filter to remove suspended solids if necessary. The resulting solution is passed through a 500-mg carbon black cartridge where cymoxanil is retained. Cymoxanil residues are

eluted from the carbon black cartridge and the eluate is concentrated, exchanged into ethyl acetate solvent, and diluted with hexane. The resulting solution is further purified by passage through a 500-mg silica cartridge where cymoxanil is retained. After elution of cymoxanil from the silica cartridge and concentration, the sample is exchanged into acetonitrile, diluted with aqueous 10 mM  $\text{KH}_2\text{PO}_4$  buffer (pH = 2.9), and analyzed by reverse-phase HPLC using either a 15-cm x 4.6-mm SB-C8 or SB-CN column. Cymoxanil is detected by UV absorption at 245 nm (see Figure 1 for a representative UV spectrum of cymoxanil). Cymoxanil is separated from matrix impurities such as fulvic and/or humic acids allowing detection at approximately 0.50 ppb (Limit of Detection or LOD).

## II. MATERIALS AND METHODS

### A. Equipment

Alternate equipment may be substituted for the following unless otherwise indicated. However, if substitutions are made, care must be taken to establish that method performance is equivalent.

#### *Sample Extraction and Work-up Equipment*

##### *Analytical Balances*

A Mettler AE 160 balance capable of weighing to  $\pm 0.01$  mg was used to weigh the analytical standards. A Mettler Model PE600 top-loading balance capable of weighing to  $\pm 0.01$  g was used for all other weighings.  
(Mettler Instrument Corporation, Princeton, N.J.)

##### *Centrifuge Tube*

Pyrex® Corning 8084, 50-mL capacity with standard taper #16 stopper, 28-mm OD x 151-mm length, VWR Catalog #Z1048-050 (VWR Scientific, Bridgeport, N.J.)

##### *Centrifuge Tube*

Kimble 45176, 13-mL capacity with flat head stopper, 17-mm OD x 130-mm length, VWR Catalog #21054-187 (VWR Scientific, Bridgeport, N.J.)

*Mobile Phase Filters and Vacuum Filter Apparatus*

0.45- $\mu$ m pore, Type HA low water extractable TF filter, Millipore Catalog #FIATF 047 00. This filter is used to filter 10-mM aqueous  $\text{KH}_2\text{PO}_4$  buffer (pH = 2.9) and any water samples that contain suspended matter prior to analysis.

0.5- $\mu$ m pore, Type FH Fluoropore membrane filter, Millipore Catalog #FHUP 047 00. This filter is used to filter the organic mobile phase.

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 #XX1004705. This apparatus was used to filter all mobile phase solvents and solutions.  
(Millipore, Inc., Milford, MA).

*Syringes*

2.5-mL disposable plastic syringes, Part #Z11685-8  
(Aldrich Chemical Co., Milwaukee, WI)

Hamilton syringes were used to prepare standards and transfer fortification solutions.  
(Hamilton Company, Reno, NV).

*pH Meter*

Fisher Scientific Accumet™ pH Meter Model 915  
(Fisher Scientific Co., Pittsburgh, PA).

*Ultrasonic Bath*

Branson Model 3200 Ultrasonic Bath  
(VWR Scientific, Bridgeport, N.J.).

*Mixer*

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

*Graduated Cylinders*

Kimax® 10, 25, 50, 100, 250, 500, and 1000-mL graduated cylinders, Catalog #24713-053, #24713-075, #24713-097, #24713-111, #24713-144, #24713-166, and #24713-188, respectively  
(VWR Scientific, Bridgeport, N.J.).

*Volumetric Flasks*

Pyrex® 10, 50, and 100 mL volumetric flasks, Catalog #29619-201, #29619-233, and #29619-234 (VWR Scientific, Bridgeport, N.J.).

*Beakers*

Pyrex® 250 mL Double Scale Griffin beaker, Catalog #13912-207 (VWR Scientific, Bridgeport, N.J.).

*Sampling Bottles*

Amber glass Boston round bottles, I-Chem, Superfund-Analyzed 1000 mL (32 oz), case of 12, Catalog #IR349-1000 (VWR Scientific, Bridgeport, N.J.).

*HPLC Sample Filters*

Milllex®-HV13, 0.45 µm, 13-mm Duropore filter units, Catalog #SJHV013MS (Millipore, Inc., Bedford, MA).

*Pipets*

Disposable Pasteur Pipets, Borosilicate Glass, 9-inch length, Catalog #14673-043 (VWR Scientific, Bridgeport, N.J.).

Solid-Phase Extraction Columns and Filtration Collection Apparatus

*Solid-Phase Extraction Columns*

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

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

*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 flowrate for each SPE column can be individually controlled. Vacuum must be adjustable to properly control solvent flow through reservoirs stacked on top of carbon

*black or silica cartridges. The manifold used for this method must have this capability.*

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

75-mL Reservoirs, Part #1213-1012  
(Varian, Inc., Harbor City, CA).

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

*Evaporator*

N-Evap Model III Laboratory Sample Evaporator attached to a  
nitrogen source  
(Organomation Associates, South Berlin, MA).

*Liquid Chromatograph*

Waters 600E Pump and Controller  
(Waters Division of Millipore, Inc., Milford, MA).

Waters WISP 712 Autoinjector equipped with a 2.0-mL syringe and  
cooling module  
(Waters)

Waters Temperature Control Module  
(Waters)

Waters Column Oven  
(Waters)

Applied Biosystems 783A Programmable Absorbance Detector  
(Applied Biosystems, Inc., Foster City, CA).

Hewlett-Packard HP3396 Series II Integrator  
(Hewlett-Packard, Wilmington, DE).

*HPLC Column*

*Pre-Column*

DuPont Zorbax® SB-C8 4.0- x 12.5-mm, 5- $\mu$  Reliance Cartridge  
Guard Column, Part #820674.915

or

DuPont Zorbax® SB-CN 4.0- x 12.5-mm, 5- $\mu$  Reliance Cartridge  
Guard Column, Part #820674.916

Column end-fittings, Part #820529.901  
(Mac-Mod Analytical Inc., Chadds Ford, PA).

*Columns*

DuPont Zorbax® SB-C8 4.6 mm x 15 cm, 5- $\mu$  Analytical Column,  
Part #883975.906

or

DuPont Zorbax® SB-CN 4.6 mm x 15 cm, 5- $\mu$  Analytical Column,  
Part #883975.905

(Mac-Mod Analytical Inc., Chadds Ford, PA).

*Note:* Only the above columns have been shown to provide satisfactory separation for this analysis. Similar columns might be substituted but satisfactory performance would have to be experimentally established and validated. These columns have been specifically engineered to withstand acidic buffers below pH 3.0. Each precolumn (SB-C8 or SB-CN) was used with its corresponding analytical column. Zorbax® RX-C8 and SB-C8 columns are identical products.

*Autosampler Vials*

Waters 4-mL Vials, Part #72710. If necessary, low-volume glass units and springs can be used, Part #72704  
(Millipore, Milford, MA).

*B. Reagents and Standards*

Equivalent reagents may be substituted based on local availability. If substitutions are made, care should be taken to establish that impurities are not introduced that interfere with cymoxanil based on HPLC analysis of final reagents such as solvents or buffer solutions needed for analysis.

*HPLC Grade Water*

Deionized water passed through a Milli-Q® Plus Water Purification System, Catalog #ZD60115UV  
(Millipore, Bedford, MA).

*Dichloromethane*

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

Warning - Dichloromethane is a suspected carcinogen. Use this solvent in a fume hood.

*Acetonitrile*

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

*Ethyl Acetate*

EM Omni Solv<sup>®</sup>, HPLC-grade solvent, Catalog #EX0241-1  
(EM Science, Gibbstown, N.J.)

*Hexane*

EM Omni Solv<sup>®</sup>, HPLC-grade solvent, Catalog #HX0296-1  
(EM Science, Gibbstown, N.J.)

*Methanol*

EM Omni Solv<sup>®</sup>, HPLC-grade solvent, Catalog #MX0488-1  
(EM Science, Gibbstown, N.J.)

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

Baker Analyzed Reagent, Catalog #3246-05  
(J. T. Baker, Phillipsburg, N.J.)

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

Baker Analyzed Reagent, reagent grade, 85% phosphoric acid,  
Catalog #0260-01  
(J. T. Baker, Phillipsburg, N.J.)

*Citric Acid, Anhydrous Granular*

ACS Grade, 99.5% citric acid, Catalog #CX1723-3  
(EM Science, Gibbstown, N.J.)

*Cymoxanil (DPX-13217)*

Analytical standard grade cymoxanil, DPX-13217, Lot #54, 99.9%  
pure, available from DuPont Agricultural Products, Global  
Technology Division  
(E. I. du Pont de Nemours and Company, Wilmington, DE).

**C. Preparation of Solutions**

**10-mM Potassium Dihydrogen Phosphate, pH = 2.9**

Dissolve 2.74 g of potassium dihydrogen phosphate (KH<sub>2</sub>PO<sub>4</sub>) in  
2.0 liters of Milli-Q<sup>®</sup> water in a beaker. Use a magnetic stirrer to  
assist solution of the salt. Adjust the pH to 2.9 by dropwise  
addition of conc. 85% phosphoric acid (approximately 6 to  
18 drops). Filter the solution through a 0.45- $\mu$ m filter prior to use.  
Prepare fresh buffer every two weeks to avoid formation of  
sediment and bacterial growth.

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

**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. Do not adjust the final volume to 500 mL. Prepare this solution weekly.

**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. Do not adjust the final volume to 500 mL. Prepare this solution weekly.

**40% Hexane/60% Ethyl Acetate/Methanol (v/v)**

To a 500-mL graduated cylinder, add 200 mL of hexane. Measure 300 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. Do not adjust the volume of the final solution. Prepare this solution weekly.

**82% 10 mM  $\text{KH}_2\text{PO}_4$ , pH = 2.9/18% Acetonitrile (v/v)**

To a 1000-mL graduated cylinder, add 820 mL of 10 mM potassium dihydrogen phosphate buffer, pH = 2.9. Measure 180 mL of acetonitrile in a second graduated cylinder and add this to the 1000-mL graduated cylinder. Do not adjust the final volume to 1000 milliliters. Prepare this solution every two weeks.

**50% 10 mM  $\text{KH}_2\text{PO}_4$ , pH = 2.9/50% Acetonitrile (v/v)**

To a 1000-mL graduated cylinder, add 500 mL of 10 mM potassium dihydrogen phosphate buffer, pH = 2.9. Measure 500 mL of acetonitrile in a second graduated cylinder and add this to the 1000-mL graduated cylinder. Do not adjust the final volume to 1000 milliliters. Prepare this solution every two weeks.

**10 mM Citric Acid**

Dissolve 1.92 g of anhydrous citric acid in 100 mL of Milli-Q® water in a beaker using a magnetic stirrer. Transfer the solution to a bottle for storage. The pH of the final solution was 2.23.



**HPLC Eluents**

Eluent A: 82% 10 mM  $\text{KH}_2\text{PO}_4$ , pH = 2.9/18% Acetonitrile (v/v)  
Eluent B: 50% 10 mM  $\text{KH}_2\text{PO}_4$  buffer, pH = 2.9/50% Acetonitrile (v/v)

Mobile phases must be premixed to properly control cymoxanil retention time and thoroughly degassed daily. If low-pressure mixing HPLC is used for sample analysis, mobile phases should be sparged at least 50% of the time required for sample analysis to insure air does not diffuse into the HPLC solvents. While not needed for the analytical method, it is useful to prepare Eluent C: 50% Milli-Q® water/50% acetonitrile (v/v) and have degassed solvent available for cleaning buffer from the analytical HPLC column whenever sample analysis is discontinued.

**Standards**

Use Class A volumetric flasks when preparing all standard solutions.

**Cymoxanil Standard Stock Solution**

Using an analytical balance and a weighing boat, weigh approximately 10.0 mg of analytical standard grade cymoxanil (DPX-13217) into a 100-mL volumetric flask. Record the exact weight of cymoxanil. Add approximately 60 mL of acetonitrile 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 µg/mL. Cymoxanil is stable in solution for at least 2 months when stored at 4°C when not in use.

**Intermediate Standard Solutions**

Using a syringe, prepare 1-µg/mL and 0.1-µg/mL fortification working standards by transferring the required volume of the 100-µg/mL or 1.0-µg/mL cymoxanil acetonitrile standard (approximately 1.00 mL from each stock solution) to a 100-mL volumetric flask. Dilute to the mark (100.0 mL) with acetonitrile. The 100-, 1.0-, and 0.1-µg/mL standards are used to fortify water samples over the concentration range required for the method.

Prepare a 1-µg/mL HPLC standard working solution by placing the required volume of the 100-µg/mL acetonitrile standard (approximately 1.0 mL) in a 100-mL volumetric flask. Add sufficient acetonitrile to the flask (approximately 17 mL) using a 25-mL graduated cylinder so that the volume of acetonitrile plus the volume of the standard equals 18.0 mL. Sonicate and swirl the flask to ensure complete solution of cymoxanil. Dilute to the mark

(100.0 mL) with 10 mM  $\text{KH}_2\text{PO}_4$  buffer, pH = 2.9. Both these standard solutions are stable for approximately 1 month when stored at 4°C when not in use.

#### HPLC Chromatographic Standard Solutions

Working HPLC standards are prepared in 82% 10 mM  $\text{KH}_2\text{PO}_4$  buffer, pH = 2.9/18% acetonitrile (v/v) by quantitatively diluting appropriate volumes of the 1- $\mu\text{g}/\text{mL}$  HPLC standard. If 10-mL volumetric flasks are used for these dilutions, the following volumes are required to prepare the 0.033, 0.12, 0.40, 0.67, and 0.90- $\mu\text{g}/\text{mL}$  working standards needed for the analysis.

Desired Standard Concentration ( $\mu\text{g}/\text{mL}$ )	Volume of 1- $\mu\text{g}/\text{mL}$ Working Standard Required in a 10-mL Volumetric (mL)
0.033	0.33
0.12	1.20
0.40	4.00
0.67	6.70
0.90	9.00

A sixth HPLC chromatographic standard solution was also prepared at a concentration of 1.20  $\mu\text{g}/\text{mL}$  (the high standard). This was done by placing 0.12 mL of the 100- $\mu\text{g}/\text{mL}$  cymoxanil standard in acetonitrile in a 10-mL volumetric, adding 1.68 mL of acetonitrile with a syringe and diluting the solution to the mark with 10 mM  $\text{KH}_2\text{PO}_4$  buffer, pH = 2.9.

These working standards are stable for approximately 2 weeks if stored at 4°C when not in use.

#### Fortification Standard Solutions

The 100-, 1.0-, and 0.1- $\mu\text{g}/\text{mL}$  standards in acetonitrile were used to fortify water samples (see p. 19). The volume used to fortify 100-g water samples should not exceed 5.0 mL. The acetonitrile fortification standard solutions are stable for at least 2 months if stored at 4°C when not in use but new solutions should be prepared whenever a new 1- $\mu\text{g}/\text{mL}$  intermediate standard is prepared in 82% 10 mM  $\text{KH}_2\text{PO}_4$  buffer, pH = 2.9/18% acetonitrile (v/v). This ensures best calibration of the method.

**D. Analytical Procedure**

**Note:** This analytical method requires that glassware such as centrifuge tubes used for concentration of cymoxanil samples be silanized before use. Appendix I outlines reagents, their source, and a representative procedure. Alternate silating procedures can be used but recoveries equivalent to those described in this report must be obtained.

There is potential for contamination of glassware with cymoxanil because of the low level of analyte detected by this method. SPE vacuum manifold control valves should be cleaned with reagent-grade acetone after use. N-Evap luer needles should also be cleaned with reagent-grade acetone after they have been used to concentrate samples. Glassware that comes in contact with cymoxanil such as beakers and centrifuge tubes can be cleaned with solutions of laboratory soap, but they must be rinsed well with tap water, Milli-Q® water and reagent-grade acetone afterward. After cleaning, centrifuge tubes should be filled with HPLC-grade methanol and sonicated for 30 minutes. Glassware can be allowed to air dry after organic solvent washes are discarded. Beakers can be sonicated with HPLC-grade methanol as well if contamination is observed during use of this method.

**Special Note:** Cymoxanil has a tendency to adsorb on glass. 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 the preparation of each sample cleanup for final analysis.

**1. Storage and Preparation of Water Sample**

Water samples were collected in 1000-mL amber glass I-Chem bottles, capped, and labeled to describe the source of the water. To help insure the chemical stability of cymoxanil, the pH of each water sample used for method validation was measured after sample collection and if the pH exceeded 6.0, an appropriate volume of 10 mM aqueous citric acid was added to adjust the pH below 6.0. The initial pH values of each water sample used in this study are listed below along with the final pH after addition of aqueous citric acid. Where acidification was necessary, 1.0 mL of 10 mM aqueous citric acid was sufficient to adjust the final pH. All samples were stored in a 4°C refrigerator until fortified for analysis.

Water Sample Source	pH before Acidification	pH after Acidification
Brandywine River Wilmington, DE	6.97	5.92
Delaware River New Castle, DE	6.58	5.82
Wilmington, DE Tap water	6.68	5.89
Waverly System P-3 Water Waverly, FL	4.84	not acidified

Aqueous citric acid solution up to a maximum of 10 mL/liter of water may be used for acidification. pH adjustment of water samples thought to contain cymoxanil should occur as soon as practically possible after collection. Cymoxanil is stable in water between a pH of 6.0 to 2.9 and optimally stable between a pH of approximately 5.0 to 6.0 (half-life >300 days at pH = 5.0 and 6.0 at 15°C, half-life >200 days at pH = 0.1 at 15°C, Reference 2). After collection and acidification, all collected water samples should be stored at 4°C as quickly as possible. If water samples are frozen, care must be taken to insure bottles do not crack or break. This is most easily done by insuring sample bottles are not over-filled when collected but the best course of action is not to freeze water samples in glass but use plastic bottles if samples have to be frozen. All samples should be warmed to room temperature and thoroughly mixed before analysis.

#### 2. Water Fortification Procedure

All water samples were shaken to insure samples, particularly those containing suspended matter, were homogeneous. One hundred grams of each sample were weighed into a clean 250-mL beaker. Each sample was fortified with the appropriate volume of an acetonitrile cymoxanil standard. The cymoxanil standard concentration used for fortification was chosen so that the volume of acetonitrile added to water samples was 5.0 mL or less. For the validation samples described in this report, the following fortification volumes were used:

Acetonitrile Standard Conc (µg/mL)	Volume of Acetonitrile Standard (mL)	Weight of Water Sample (g)	Final Fortification Level (ppb)
0.10	1.00	100	1.00
1.0	0.333	100	3.33
1.0	1.00	100	10.00
1.0	2.50	100	25.00
100	0.10	100	100.00
100	0.50	100	500.00

After fortification, all samples were swirled to mix the contents to homogeneity and each sample was carefully inspected by eye to determine if it contained suspended matter. Any samples observed to contain suspended solids (groundwater samples from Waverly, Florida) were filtered through a 0.45-µ, 47-mm HATF 04700 filter prior to SPE extraction. Filtration was necessary to insure solids did not obstruct carbon black cartridges during cymoxanil extraction.

### 3. Solid-Phase Extraction Purification

**Note:** 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. When solvent reservoirs are used with adapters, it may be necessary to loosen the adapter connecting the reservoir to the cartridge and allow solvent to drip onto the head of cartridge before vacuum is applied. This allows more uniform control of solvent flow through the SPE column. For maximum convenience, conditioning and eluting solvent combinations such as 90% methylene chloride/10% methanol (v/v) can be prepared in advance (see the Preparation of Solutions section of this report).

**Special Note:** The SPE cartridge and column elution conditions described below have been carefully developed and shown to give high recoveries of cymoxanil in control experiments in which 0.1 µg and 50 µg of cymoxanil was dissolved in appropriate solvents (see the experimental description below) and passed through the SPE cartridges as described in this method. Recovery was checked against the response

*of cymoxanil standards by HPLC. While every effort has been made to develop robust conditions, there can be no guarantee that similar SPE cartridges from other manufacturers or all cartridge lots from the same cartridge manufacturer will always give high recoveries under these precise conditions. Accordingly, to ensure optimum method performance, it is essential that recoveries be checked with cymoxanil standards to positively establish proper performance of each lot of SPE cartridges prior to use of this method. Elution conditions can be adjusted if necessary to optimize recovery depending on the performance of a particular lot of cartridges and the nature of water samples being analyzed. A careful check of elution conditions is particularly important if fortified recoveries decrease during use of this method. Particular attention should be paid to silica cartridges. Envi™-Carb and silica cartridges must be calibrated separately.*

- a. Condition a 500-mg Envi™-Carb tube by passing 5.0 mL of 90% methylene chloride/10% methanol (v/v) through the cartridge. Next pass 2.0 mL of methanol through the cartridge and then 15 mL of Milli-Q® water. After conditioning, do not let the cartridge go to dryness.
- b. Place a 75-mL reservoir on top of the Envi™-Carb tube using an adapter and place the stacked reservoir and cartridge on an SPE manifold. Pass 100-g water samples from Step 2 through the carbon black cartridges at a flow rate that does not exceed 10 mL per minute.

Wash the 250-mL beaker with 5 mL of Milli-Q® water and pass the wash through the 75-mL reservoir and Envi™-Carb tube. Cymoxanil is retained on the Envi™-Carb tube. Remove and discard the 75-mL reservoir.

- c. After water passes through the cartridge, completely open the flow control valve on the vacuum manifold and pull vacuum through the cartridge for 1 minute to remove as much water as possible.

Pass 1 mL of methanol dropwise through the cartridge. Once solvent passes through the cartridge, completely open the flow control valve on the vacuum manifold and pull air through the cartridge for 1 minute to remove as much solvent from the cartridge as possible.

**Special Note:** Methanol is used to remove additional water from the Envi™-Carb cartridge prior to eluting cymocanil.

Elute the Envi™-Carb tube by passing the following solvents through the tube in the order listed:

1 mL of methanol.

10 mL of 90% methylene chloride/10% methanol (v/v).

Collect both solvents in the same silanized 13-cc centrifuge tube.

**Note:** The centrifuge tubes used in this step cool well below room temperature during collection of the 90% methylene chloride/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 13-cc centrifuge tube is less than 11 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 4°C in stoppered tubes in a refrigerator.

d. Concentrate the methylene chloride/methanol solvent to  $0.5 \pm 0.1$  mL with nitrogen using an N-Evap at 40°C. Add  $2.0 \pm 0.1$  mL of ethyl acetate to the tube and concentrate the solvent to  $0.5 \pm 0.1$  mL with nitrogen on an N-Evap at 40°C. Add 2.0 mL of ethyl acetate and again concentrate the solvent to exactly  $1.0 \pm 0.1$  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.

e. Condition a 500-mg silica SPE cartridge by passing the following solvents through the cartridge in the order listed at a flow rate not to exceed 180 drops/minute. Do not let the cartridge go to dryness once it is conditioned.

10-mL hexane. This solvent is used to condition the cartridge.

After conditioning, pass the solution from Step 3.d. 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 with the following solvents in the order listed taking care that the flow rate does not exceed 180 drops per minute.

10-mL 90% hexane/10% ethyl acetate (v/v). Discard this fraction.

12-mL 40% hexane/60% ethyl acetate/methanol (v/v). Collect this last fraction in a silanized 13-cc centrifuge tube. Take the silica cartridge to dryness. The volume of solvent collected is below 12-mL, as result of evaporation. Concentrate the solvent to  $0.5 \pm 0.1$  mL under nitrogen at 40°C using an N-Evap.

**Note:** If interferences appear in the chromatogram that cannot be resolved chromatographically, an additional wash step of 10 mL of 80% hexane/20% ethyl acetate can be added after washing the silica cartridge with 10 mL of 90% hexane/10% ethyl acetate. In addition, cymoxanil can be eluted from the silica cartridge with 60% hexane/40% ethyl acetate (no added methanol).

If these changes are used, cartridges should be recalibrated to insure these changes give proper recovery. Other variations in elution conditions such as elution of cymoxanil with 12 mL of 60% hexane/40% ethyl acetate (v/v) can also be tried but these must be shown to be effective in cartridge calibration trials.

Add 1.5 mL of acetonitrile to the centrifuge tube and concentrate the solvent to  $0.5 \pm 0.1$  mL. Add 1.5 mL of acetonitrile and again concentrate the solvent to exactly 0.5 mL.

#### 4. HPLC Analysis

- a. Dilute the sample from Step 3.e. to exactly 3.0 mL with 10 mM  $\text{KH}_2\text{PO}_4$  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. Filter the solution through a 0.45- $\mu\text{m}$ , Millex®-HV13 filter using a 2.5-mL disposable syringe into a 4-mL autosampler vial. The sample is ready for HPLC analysis.

**Note:** The analysis can be interrupted at this point if desired. Samples are stable for at least 1 week if stored at approximately 4°C.

The HPLC system has been described in the **MATERIALS AND METHODS** section of this report. The liquid chromatograph is set as follows for cymoxanil analysis.



UV Wavelength: 245 nm  
Column Oven Temperature: 40.0°C  
Injection Volume: 20 µL depending on detector response  
Initial Flow Rate: 1.00 mL/min  
Flow Path: Zorbax® SB-C8 to detector  
Solvent A: 82% 10 mM KH<sub>2</sub>PO<sub>4</sub>,  
pH = 2.9/18% acetonitrile  
Solvent B: 50% 10 mM KH<sub>2</sub>PO<sub>4</sub> buffer,  
pH = 2.9/50% acetonitrile  
Solvent C: 50% Acetonitrile/50% Milli-Q®  
Water (v/v)  
Helium Sparge Rate: At least 50%  
Integrator Chart Speed: 0.50 cm/min  
Integrator Attenuation (Z<sup>m</sup>) 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. Samples containing more than approximately 25 µg of cymoxanil will require dilution to keep peaks on scale. This can be done using a 50-cm centrifuge tube.*

Newly installed Zorbax® SB-C8 columns must be equilibrated for at least 2 hours at a flow rate of at least 1.00 mL/min using a solvent composition of 18% acetonitrile, 82% 10 mM KH<sub>2</sub>PO<sub>4</sub> buffer pH-2.9 (100% A). The column oven must be stabilized at 40°C.

The following representative solvent and autosampler program were entered into the Waters 600E controller.

## TYPICAL SOLVENT PROGRAM

Time (Minutes)	Flow Rate (mL/min)	% A	% B
		18% Acetonitrile/ 82% 10 mM KH <sub>2</sub> PO <sub>4</sub> pH = 2.9	50% 10 mM K <sub>2</sub> HPO <sub>4</sub> Buffer, pH = 2.9/50% acetonitrile
Initial	1.00	100	0
17.00	2.00	0	100
32.00	2.00	100	0
52.00	1.00	100	0
56.00*	0.50	100	0

\* The flow rate change at 56.00 minutes conserves solvent after sample set analysis is complete.

## TYPICAL AUTOSAMPLER PROGRAM

Step	First Vial	Last Vial	# of Inj	Inj Vol μL	Run Time
1	1	13	1	20	55.00
2	14	14	1	20	55.00*

\* Injection 14 was acetonitrile. A single injection of solvent was used to allow the solvent program to clean the column at the conclusion of the sample set.

**Note:** The changes in solvent strength in this program are instantaneous step gradients. There is no time lag in the solvent composition changes. This solvent program washes the HPLC SB-C8 column with strong solvent (50/50) after analysis of each sample in a sample set. While the method has been tested with a number of different water samples and different lots of Zarbar® SB-C8 columns, it has not been possible to inject enough different water samples to determine exactly when interferences might build-up on the column to the point that it must be cleaned or replaced. Based on work to date it has not been necessary to replace SB-C8 columns or guard columns. Peak broadening, poor peak shape, or the appearance of interferences in the chromatograms of standards are an indication that the pre-column or HPLC column may need replacement.

**Special Note:** Sufficient resolution has been obtained with 15-cm analytical SB-C8 or SB-CN HPLC columns to allow quantitation of cymoxanil residues in water samples. While an effort has been made to look at water samples from different sources, matrix peaks might appear in some samples that interfere with

*cymoxanil. If this happens, a 4.6 mm x 25 cm, 5  $\mu$ , SB-C8, SB-CN or SB-Phenyl analytical column should be examined to resolve interferences. Additional options include adjusting solvent strength or using methanol in place of acetonitrile as the organic eluting solvent. The following representative solvent program (instantaneous step gradients) has been used with a 15-cm SB-CN analytical HPLC column. Cymoxanil residues can be analyzed using an SB-C8 column and confined using an SB-CN column using the same mobile phase composition.*

Time (Minutes)	Flow Rate (mL/min)	% A	% B
		18% Acetonitrile/ 82% 10 mM KH <sub>2</sub> PO <sub>4</sub> , pH = 2.9	50% 10 mM K <sub>2</sub> HPO <sub>4</sub> Buffer, pH = 2.9/ 50% acetonitrile
Initial	1.00	100	0
11.00	2.00	0	100
26.00	2.00	100	0
46.00	1.00	100	0
50.00*	0.50	100	0

\* The flowrate change at 50.00 minutes conserves solvent after sample set analysis is complete.

#### E. Method of Calculation

Sample concentrations are calculated by substituting peak heights observed for each fortified or treated sample into the linear least square equation  $f(x) = Mx + B$  developed from analysis of standards analyzed along with each set of fortified samples. A correlation coefficient of at least 0.99 was observed for the linear least squares equation for each set of cymoxanil standards analyzed during validation of this method. The following equation was then used to calculate ppb of cymoxanil found in each sample.

$$\text{ppb Cymoxanil Found} = \text{Least Squares Concentration } (\mu\text{g/mL}) \times \frac{\text{Final Sample Volume (mL)}}{\text{Sample Weight (g)}} \times \text{HPLC Dilution Factor} \times 1000 \text{ ng}/\mu\text{g}$$

Equal volumes of standards and samples were injected during analysis (typically 20  $\mu$ L). The HPLC Dilution Factor allows dilution of samples into the proper concentration range for analysis. The % recovery for *fortified* control samples was calculated according to the following equation correcting for interferences, if any, seen in unfortified controls.

$$\% \text{ Recovery} = \frac{\text{ppb Cymoxanil Found} - \text{ppb in Unfortified Control}}{\text{ppb Cymoxanil Fortification}} \times 100$$

*Note: Interference in control samples should not exceed 15 to 20% of the height of the cymoxanil peak at the proposed detection level of approximately 0.50 ppb.*

#### Example Calculations

The linear regression equation  $f(x) = Mx + B$  defined by analysis of cymoxanil standards analyzed during analysis of fortified Delaware River water (Replicate 2, see Figure 2) is  $f(x) = (1.67 \times 10^4)x + 89.34$ . Substituting a peak height value of 1738 observed for sample #5 (a 3.33-ppm fortification) and solving for  $x$  as follows:

$$f(x) = Mx + B$$

$$x = \frac{f(x) - B}{M}$$

$$x = \frac{f(x) - 89.34}{1.67 \times 10^4}$$

$$x = \frac{(1738 - 89.34)}{1.67 \times 10^4}$$

gives a cymoxanil Least Squares Concentration of 0.099  $\mu$ g/mL.

For analysis of water samples 20- $\mu$ L injections of standards and sample were used and the sample was diluted to 3 mL prior to analysis (Final Sample Volume = 3.0 mL). A 100-gram water sample was extracted. The HPLC Dilution Factor was 1.0. Substituting these values in the equation for ppb cymoxanil found gives:

$$\text{ppb Cymoxanil Found} = 0.099 \mu\text{g/mL} \times \frac{3 \text{ mL}}{100 \text{ g}} \times 1.0 \times 1000 \text{ ng}/\mu\text{g}$$

$$\text{ppm Cymoxanil Found} = 2.97 \text{ ng/g or } 2.97 \text{ ppb}$$

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Delaware water sample #5 from Replicate 2 was fortified with 3.33 ppb of cymoxanil. No interference was observed in the control sample. The Percent Recovery was calculated as:

$$\% \text{ Recovery} = \frac{2.97 - 0}{3.33} \times 100 = 89\% \text{ Recovery}$$

A dilution factor of 4.0 and 20.0, respectively, was used for analysis of 100- and 500-ppb water fortifications in this study.

**APPENDIX I  
PROCEDURE FOR SILANIZING GLASSWARE**

**Solution**

Mix 3760 mL of toluene and 240 mL of dichlorodimethylsilane.

Fill clean glass centrifuge tubes with silanizing solution.

- Let the solution stand for 1 or 2 minutes, pour the solution back into a storage bottle.
- Rinse glassware with toluene; discard wash as waste.
- Rinse glassware with methanol; discard wash as waste.
- Rinse glassware with water; discard wash as waste.
- Rinse glassware with methanol; discard wash as waste.
- Rinse glassware with acetone; discard wash as waste.
- Rinse glassware with 50/50 (v/v) methanol/2-propanol; discard wash as waste.

Let glassware dry. It is convenient to mark the glassware with tape to indicate it has been silanized. Clean all glassware well and resilanize after 3 weeks of use.

Dichlorodimethylsilane (No. 83410) can be purchased from Pierce Chemical Company, Rockford, Ill. HPLC grade Toluene (No. 9351-03) is purchased from J. T. Baker, Inc., Phillipsburg, N.J.