Cover Sheet for

ENVIRONMENTAL CHEMISTRY METHOD

Pestcide Name: Cymoxanil

MRID #: 441807-51

Matrix: Water

Analysis: HPLC/UV

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TRADE SECRET

Study Title

ANALYTICAL METHOD FOR THE DETERMINATION OF CYMOXANIL IN WATER USING LIQUID CHROMATOGRAPHY

Data Requirement

U.S. EPA Pesticide Assessment Guidelines
Subdivision O, 174-1

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Performing Laboratory

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GOOD LABORATORY PRACTICE STATEMENT

The U.S. EPA Good Laboratory Practice Regulations (40 CFR Part 160) are not applicable to method development. The data generated for this report was performed in a GLP-compliant lab with appropriate SOP's by trained GLP-compliant personnel. However, no protocol was written and no auditing performed. The data and final report will be archived in the DuPont Agricultural Product's archives.

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15-Sep-1995
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DuPont Registration Representative

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Table of Contents

TABLE OF CONTENTS (CONTINUED)

Analytical Method for the Determination of Cymoxanil in Water Using Liquid Chromatography

Edward C. Nathan and Sidney J. Hill

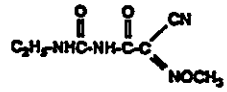
Summary

This report describes a method which can be used for analysis of cymoxanil in water samples. It quantitates cymoxanil at a level of approximately 2.0 ppb or above in 100-g water samples and detects cymoxanil residues at a level of approximately 0.5 ppb in 100-g samples of water. Water samples are passed through a preconditioned carbon black cartridge where cymoxanil is retained. After elution, partially purified samples are extracted onto a silica solid phase extraction (SPE) cartridge, eluted, concentrated, and analyzed by reverse-phase HPLC using UV detection.

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 KH_2PO_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). Using the method, the overall average recovery (± standard deviation) of cymoxanil from four different types of fortified water samples representing 60 fortified samples was 98% ± 10.8% with a relative standard deviation of 11.1%. Cymoxanil residues can be analyzed using a SB-C8 column and confirmed using a SB-CN column.

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 ±0.01 mg was used to weigh the analytical standards. A Mettler Model PE600 top-loading balance capable of weighing to ±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 #21048-050
(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-µm pore, Type HA low water extractable TF filter, Millipore Catalog #HATF 047 00. This filter is used to filter 10-mM aqueous KH_2PO_4 buffer (pH = 2.9) and any water samples that contain suspended matter prior to analysis.

0.5-µm 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

Millex®-HV13, 0.45 µm, 13-mm Durapore 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 values 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-µ Reliance Cartridge Guard Column, Part #820674.915
or
DuPont Zorbax® SB-CN 4.0- x 12.5-mm, 5-µ 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-µ Analytical Column, Part #883975.906
or
DuPont Zorbax® SB-CN 4.6 mm x 15 cm, 5-µ 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®, HPLC-grade solvent, Catalog #EX0241-1
(EM Science, Gibbstown, N.J.).

Hexane

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

Methanol

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

Potassium Phosphate Monobasic, Crystal (KH_2PO_4)

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

Phosphoric Acid (H_3PO_4)

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-T3217)

Analytical standard grade cymoxanil, DPX-T3217, 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_2PO_4) in 2.0 liters of Milli-Q® 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-µm 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 KH_2PO_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 KH_2PO_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 KH_2PO_4, pH = 2.9/18% Acetonitrile (v/v)
Eluent B: 50% 10 mM KH_2PO_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-T3217) 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 KH_2PO_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 KH_2PO_4 buffer, pH = 2.9/18% acetonitrile (v/v) by quantitatively diluting appropriate volumes of the 1-µg/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-µg/mL working standards needed for the analysis.

Desired Standard Concentration (µg/mL)	Volume of 1-µg/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 µg/mL (the high standard). This was done by placing 0.12 mL of the 100-µg/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 KH_2PO_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-µg/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-µg/mL intermediate standard is prepared in 82% 10 mM KH_2PO_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

52

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 manufactures 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 cymoxanil.

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 ± 0.1 mL with nitrogen using an N-Evap at 40°C. Add 2.0 ± 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 2.0 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.

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 ± 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 ± 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 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. Filter the solution through a 0.45-mm, 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_2PO_4, pH = 2.9/18% acetonitrile
Solvent B:	50% 10 mM KH_2PO_4 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 (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. 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_2PO_4 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 18% Acetonitrile/ 82% 10 mM KH_2PO_4, pH = 2.9	% B 50% 10 mM K_2HPO_4 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 Zorbax® 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 µ, 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 18% Acetonitrile/ 82% 10 mM KH_2PO_4, pH = 2.9	% B 50% 10 mM K_2HPO_4 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.

ppb Cymoxanil Found = Least Squares Concentration (µg/mL) x

$$\frac{\text{Final Sample Volume (mL)}}{\text{Sample Weight (g)}} \times$$

HPLC Dilution Factor x 1000 ng/µg

55

Equal volumes of standards and samples were injected during analysis (typically 20 µL). 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.

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

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 µg/mL.

For analysis of water samples 20-µL 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:

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

ppm Cymoxanil Found = 2.97 ng/g or 2.97 ppb

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.

F. *Results and Discussion*

1. *Recovery Results*

Samples of Brandywine River (4 replicate determinations), Delaware River (4 replicate determinations), Wilmington, DE, tap water, and Waverly, FL, groundwater were each fortified with cymoxanil at 1.0, 3.33, 10, 25, 100, and 500 ppb and analyzed by HPLC using the method described in this report. Detector response of standards was linear over the range of concentrations utilized for sample analysis (LOD to 10X the LOQ, approximately 33X). For a representative standard curve obtained during analysis of Delaware River water (Replicate 2), see Figure 2. All samples were sufficiently clean after sample workup that they could be analyzed directly using an SB-C8 or SB-CN analytical HPLC column. The SB-CN column serves as an alternate column for analysis/resolution of sample impurities and can be used to confirm the presence of cymoxanil. Table I summarizes recovery data for all water samples. Recoveries were acceptable over the fortification range examined (1.0 ppb to 500 ppb). 100- and 500-µg/mL fortifications were diluted within the range of the assay prior to analysis (dilution factors of 4.0 and 20.0, respectively). The average recovery (± the Standard Deviation) for all fortified samples at all fortification levels for all samples analyzed on the SB-C8 column was 98% ± 10.8%. The Relative Standard Deviation was 11.1%. Information on recoveries, standard deviations, and relative standard deviations by fortification level is contained in Table I.

2. *Sample Chromatograms*

Appendix II shows typical chromatograms for both columns for unfortified control samples of water from each water source. Also shown are typical chromatograms for standards and 1.0-, 3.33-, 25-, and 500-ppb cymoxanil fortifications in each type of water sample. The 1.0-ppb fortification corresponds to the lowest fortification

level used during method validation. In all cases, sufficient resolution was achieved; small interference and/or contamination peaks were observed in the unfortified controls for the Waverly, FL, groundwater sample, but this could be resolved using a SB-CN column.

3. *Determination of the LOD and LOQ*

Data collected during validation were examined to estimate the Limit of Quantitation (LOQ) for cymoxanil residues in water. By definition, the LOQ is the analyte concentration that produces a signal 10X the baseline noise of an unfortified control sample measured at the retention time corresponding to cymoxanil. This approach has been promoted by FDA (Reference 3). The average baseline noise (peak to valley) measured with a ruler in millimeters for all (ten) unfortified water controls analyzed on the SB-C8 column was calculated at the retention time corresponding to that of cymoxanil (2.2 mm). Peak heights 3X and 10X this value were calculated. A response curve was prepared by plotting the averaged peak height in millimeters for the 0-, 1.0-, 3.33-, and 10.0-ppb fortifications grouped by fortification level vs. the cymoxanil fortification level (see Figure 3). The curve was used to determine the fortification levels in ppb to which the 3X and 10X peak heights corresponded. These fortification levels were the experimentally determined LOD and LOQ for the method and were found to be 0.50 and 2.0 ppb, respectively. Acceptable recoveries were obtained for all samples. The analytical method can achieve an LOD of at least of 0.50 ppb.

4. *Interferences/Method Ruggedness*

Examination of chromatograms of all unfortified water samples indicated that interferences and/or contamination at the retention time corresponding to that of cymoxanil are not present or are very small. Alternate columns were able to resolve observed peaks. Alternate columns can also be used to resolve peaks from other crop protection chemicals if necessary. A single workup was used to prepare samples for analysis. It was not necessary to vary HPLC conditions for the analysis of water samples on either HPLC column.

5. *Time for Sample Preparation*

At least seven water samples can be prepared for analysis by a single analyst in an 8-hour day. Using an autosampler, HPLC analyses can be conducted unattended overnight.

ACKNOWLEDGMENTS

We would like to express our deep appreciation to Carol Ashman, who typed and prepared the final report. Her expert help made this project much easier to complete.

57

III. Certification

Analytical Method for the Determination of Cymoxanil in Water Using Liquid Chromatography

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

Report by:

Edward C. Nathan
Study Director

15-Sep-1995
Date

Approved by:

Sidney S. Goldberg
Research Supervisor

15-September-95
Date

Date Study Initiated:

March 13, 1995

Date Study Completed:

September 15, 1995

Storage Location of Records, Specimens, and Final Report:

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and/or
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Wilmington, Delaware 19880

Sponsor:

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DuPont Agricultural Products
Global Technology Division
Experimental Station
Wilmington, Delaware 19880-0402

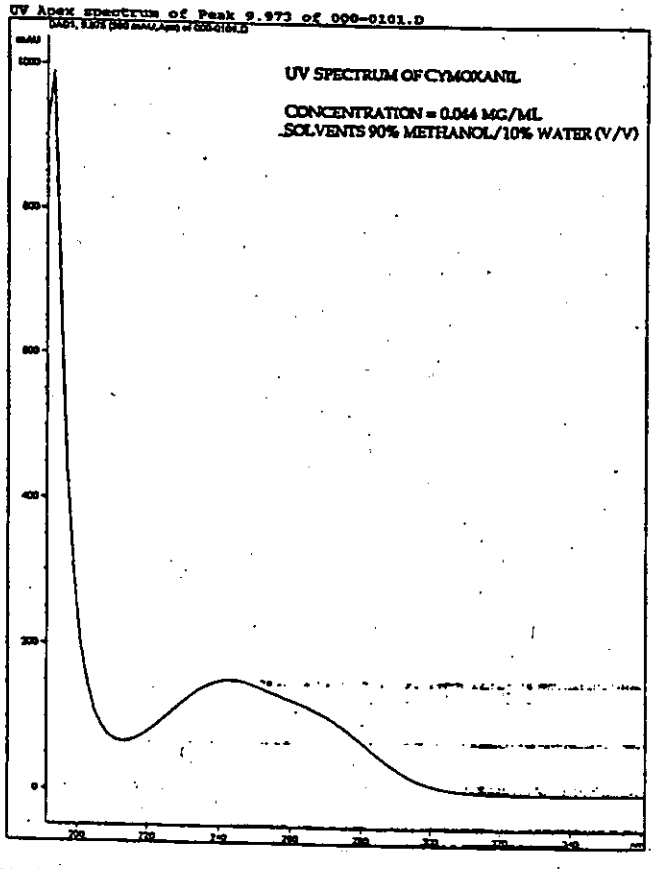
TABLE I
PERCENT RECOVERIES FOR FORTIFIED WATER SAMPLES

Water Sample Source	Unfortified Control	1-PPB Fortification	2.53-PPB Fortification	10-PPB Fortification	25-PPB Fortification	100-PPB Fortification	500-PPB Fortification
Brandywine River Replicate 1	0	114	113	103	103	112	118
Replicate 2	0	120	110	93	96	95	101
Replicate 3	0	95	84	106	78	93	101
Replicate 4	0	93	90	90	90	96	95
Delaware River Replicate 1	0	119	109	100	98	110	101
Replicate 2	0	85	89	86	97	95	104
Replicate 3	0	88	84	77	86	100	108
Replicate 4	0	92	97	100	89	115	99
Wilmington, DE Tap Water	0	97	94	85	88	70	100
Waverly, FL Groundwater	0	100	101	95	99	125*	
Mean by Fortification Level		100	97	94	92	101	103
Standard Deviation		13.3	10.5	9.1	7.9	14.3	6.4
Relative Standard Deviation		13.3	10.8	9.7	8.5	14.1	6.2
Overall Mean	98						
Overall Standard Deviation	10.8						
Overall Relative Standard Deviation n = 60	11.1						

* Average of four determinations (127, 117, 117, and 117%)

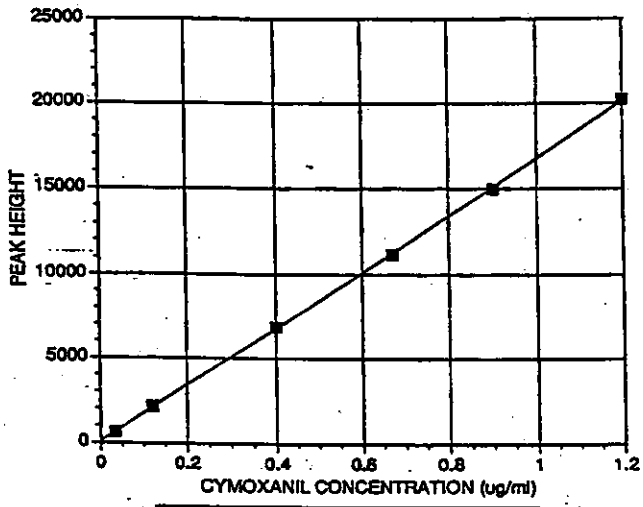
FIGURE 1
UV SPECTRUM OF CYMOXANIL

Print of window 39: UV Apex spectrum of Peak 9.973 of 000-0101.D Page 1 of 1



HP 1090 Win.system Friday, Feburary 10, 1995 7:51:28 AM by S_Swain

FIGURE 2
REPRESENTATIVE STANDARD CURVE - ANALYSIS OF DELAWARE RIVER WATER (REPLICATE 2)

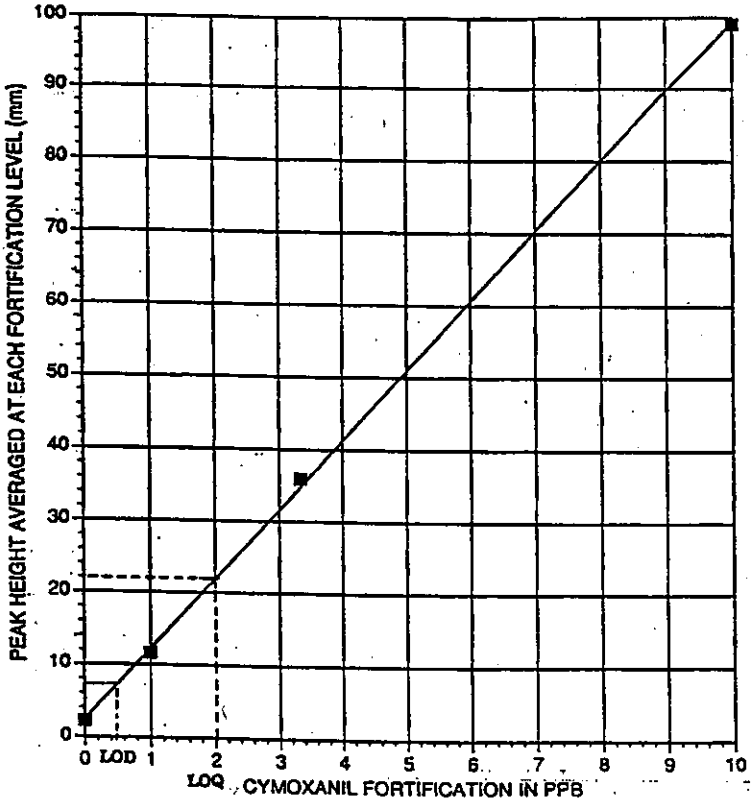


SAMPLE #	T3217 CONC (ug/ml)	PK HEIGHT
1	0.033	607
4	0.12	2127
7	0.4	6850
10	0.67	11186
12	0.9	14989
13	1.2	20193

ANALYSIS OF CYMOXANIL STANDARDS CONDUCTED DURING ANALYSIS OF FORTIFIED DELAWARE RIVER WATER SAMPLES ON JULY 31 AND AUGUST 1, 1995

f(x) = 1.667649E+4*x + 8.933527E+1 R^2 = 9.998723E-1

FIGURE 3
LOD AND LOQ DETERMINATION



GRAPHICAL DETERMINATION OF THE LIMIT OF DETECTION (LOD) AND LIMIT OF QUANTITATION (LOQ) FOR ANALYSIS OF WATER SAMPLES

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2. Horne, P., "Hydrolysis of [2-^{14}C]DPX-T3217. DuPont Report No. AMR 2018-91. DuPont Agricultural Products, E. I. du Pont de Nemours and Company, Wilmington, Del.
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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.