Cover Sheet for

ENVIRONMENTAL CHEMISTRY METHOD

Pestcide Name: Cymoxanil

MRID #: 441807-49

Matrix: Soil

Analysis: HPLC/UV

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Analytical Method for the Determination of Residues of Cymoxanil in Soil using Liquid Chromatography

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1.0 Summary

This report describes the analytical method to determine cymoxanil residues in soil. Samples were extracted with a solution of acetone and 100 mM ammonium acetate buffer. The cymoxanil residues are concentrated and separated from the remaining aqueous solution using C18 SPE. Cymoxanil residues are analyzed by reverse-phase HPLC using a C18 Column with UV detection at 245 nm.

Cymoxanil recoveries ranged from 76 to 110% with an average of 91 ± 10% (Standard Deviation). The sample sets were conducted on three different soils with varying parameters such as pH, % organic matter and sand, silt, and clay compositions. An extraction efficiency experiment was conducted with ^{14}C-labeled cymoxanil. Recoveries were comparable to those discussed above.

The limit of quantitation for cymoxanil in soil was determined to be 0.05 µg/g and the limit of detection 0.02 µg/g for a 20-g sample. Cymoxanil residues were confirmed by LC/MS.

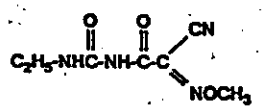
2.0 Introduction

2.1 *Background*

Cymoxanil (DuPont Identification No. DPX-T3217) is the active ingredient in Curzate® Fungicide, a DuPont agrochemical 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:

ABBREVIATIONS PAGE

CFR	Code of Federal Regulations
GLP	Good Laboratory Practices
HPLC	High Pressure Liquid Chromatography
LC/MS	Liquid Chromatography/Mass Spectrometry
LOD	Limit of Detection
LOQ	Limit of Quantitation
mL	Mills
mM	milli-molar
nm	Nanometer
OM	Organic Matter
ppm	Parts per million
QA	Quality Assurance
RIC	Reconstructed Ion Chromatogram
SPE	Solid Phase Extraction



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

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.

2.2 *Objective*

As a result of its use for disease control in crops, there is a need for an analytical method to selectively detect residues of cymoxanil in soil. This report describes a suitable method. The method has been applied to detect levels of cymoxanil at approximately 0.02 ppm or above in 20-g soil samples. The limit of quantitation for the method has been established at 0.05 ppm.

2.3 *Principle of Method*

Twenty-gram soil samples are weighed into centrifuge bottles and extracted two times with 50 mL of acetone and 10 mL of 100 mM ammonium acetate buffer (pH 4.5). The acetone is then stripped away from the extract under vacuum. The cymoxanil residues are concentrated and separate from the remaining aqueous solution using C18 SPE.

Cymoxanil residues are eluted from the C18 SPE with a 20/80 ethyl acetate/hexane solution. The eluate is concentrated, exchanged into acetonitrile, concentrated again, and diluted with 10 mM ammonium acetate buffer (pH 4.5) to a 70/30 buffer/acetonitrile solution.

Cymoxanil residues are analyzed by reverse-phase HPLC using C18 Column (25-cm x 4.6-mm). Cymoxanil is detected by UV absorption at 245 nm (see Figure 1 for a representative UV spectrum of cymoxanil).

3.0 MATERIALS

3.1 *Equipment*

Equivalent equipment or materials may be substituted. Substitutions should be documented in the study records. Substitutions must give equivalent performance as documented by acceptable control and fortification data.

Sample Extraction and Work-Up Equipment

Balances- Mettler Model AE 160 and PM600 (Mettler Instrument Corporation, Princeton, NJ).

Centrifuge Bottles- 250 mL with wide mouth and sealing cap, Catalog No. 21010-590 (VWR Scientific, Bridgeport, NJ).

Centrifuge Tube- 13-mL capacity, Catalog No. 21054-187 (VWR Scientific, Bridgeport, NJ).

Wrist Action Shaker, Model 75 (Burrell Corp., Pittsburgh, PA)

Solvent Evaporation

RapidVap™ Evaporation System Model 7900 (Labconco Corp., Kansas City, MO).

Sample Tube- 600 mL, Catalog No. 79065 (Labconco Corp., Kansas City, MO).

N-Evap Model III Laboratory Sample Evaporator (Organomation Associates, South Berlin, MA).

Solid-Phase Extraction

C18 Mega Bond Elute® Extraction Column, 6-cc/1.0-g absorbent, Catalog No. 1225-6001-2037 (Varian, Inc., Harbor City, CA).

15-mL Reservoir, Catalog No. 1213-1010 (Varian, Inc., Harbor City, CA).

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

HPLC System

Hewlett-Packard Series II 1090 Liquid Chromatograph DAD Series II Detector, Vectra MX/2 Windows Chem Station (Hewlett-Packard, Wilmington, DE).

Symmetry C18, 4.6 X 250 mm Column, Catalog #WAT05275 (Waters, Milford, MA).

3.2 *Reagents and Standards*

Equivalent reagents may be substituted.

Acetone- (OmniSolv, Residue Grade) Catalog # AX0116-1 (EM Science, Gibbstown, NJ).

Acetonitrile- (OmniSolv, HPLC Grade) Catalog # AX0142-1 (EM Science, Gibbstown, NJ).

Hexane- (OmniSolv, Residue Grade) Catalog # HX0296-1 (EM Science, Gibbstown, NJ).

Ethyl Acetate- (OmniSolv, Residue Grade) Catalog # AX0241-1 (EM Science, Gibbstown, NJ)

Acetic Acid- (Glacial) # AX0073-1 (EM Science, Gibbstown, NJ).

Methanol- (OmniSolv, HPLC Grade) Catalog # AX0142-1 (EM Science, Gibbstown, NJ).

De-ionized Water- Millipore Ultra Pure Water System.

Ammonium Acetate- HPLC Grade, Catalog # JT0599-8 (J.T.Baker, Phillipsburg, NJ).

Analytical Standard- Cymoxanil, T3217-101, 99.9% Purity (DuPont Agricultural Products, Wilmington, DE).

3.3 *Safety and Health*

No unusually hazardous materials are used in this method. All appropriate material safety data sheets should be read and followed, and proper personal protective equipment should be used.

4.0 METHODS

4.1 *Principle of the Analytical Method*

Soil samples are weighed into centrifuge bottles and extracted two times with a mixture of 50 mL of acetone and 10 mL of 100 mM ammonium acetate buffer (pH 4.5). It is important to maintain the pH between 4 and 5. Cymoxanil is not stable at pH ranges above 7 or less then 3. Acetone is evaporated from the extract under vacuum of approximately 0.2 bar.

After evaporation, the cymoxanil residues are concentrated and separated from the remaining aqueous solution using C18 SPE. It is important to remove the acetone or the cymoxanil will not be retained on the C18 SPE. After the samples are applied, the SPE

column is washed with hexane and water. Cymoxanil residues are eluted from the C18 SPE with a 20/80 ethyl acetate/hexane solution.

The eluent is concentrated to approximately 1 mL under a stream of nitrogen. The sample volume is brought up to 5 mL with acetonitrile, concentrated to 1.5 mL and diluted with 3.5 mL of 10 mM ammonium acetate buffer (pH 4.5) to form 5 mL of a 70/30 buffer/acetonitrile solution. For consistent peak shape, it is important to maintain the 70/30 buffer acetonitrile composition. Sample is filtered through 0.45-µm filter and placed in a 2-mL sample vial for HPLC analysis.

Cymoxanil residues are analyzed by reverse-phase HPLC using a Waters Symmetry-C18 (25-cm x 4.6-mm). The HPLC run is isocratic using a mobile phase which consists of a 70/30 H_2O/acetonitrile. Cymoxanil is detected by UV absorption at 245 nm (see Figure 1 for a representative UV spectrum of cymoxanil).

4.2 Analytical Procedure

4.2.1 Glassware and Equipment Cleaning Procedures

The effectiveness of any cleaning procedure used should be demonstrated by preparation and analysis of reagent blanks. In general, all reusable glass- and plasticware should be washed in hot tap water with laboratory grade, non-phosphate detergent, rinsed several times with tap water, rinsed several times with deionized water, rinsed once with acetone, and allowed to fully dry before use. Care should be taken to avoid working with high levels of the analyte being monitored in the same laboratory where samples are being extracted and analyzed.

4.2.2 Preparation and Stability of Reagent Solutions

1 M ammonium acetate buffer - dissolve 77 g in approximately 1000 mL of de-ionized water. Monitor the pH of the solution, with a pH meter and add conc. acetic acid to reach a pH of 4.5 ± 0.1. The solution is stored in a refrigerator at approximately 4°C and is stable for 6 months.

100 mM ammonium acetate buffer - Dilute 100 mL of the 1 M to 1.0 L. Store in refrigerator at 4°C. Solution should be prepared weekly.

10 mM ammonium acetate buffer - Dilute 10 mL of the 1 M to 1.0 L. Store in refrigerator at 4°C. Solution should be prepared weekly.

20/80 ethyl acetate/hexane- Mix 20 mL of ethyl acetate with 80 mL of hexane. Prepare fresh weekly.

All of the volumes described in this section are approximate. Volumes used should be within 10% of the stated value.

4.2.3 Stock Standard Preparation and Stability

Approximately 10 mg of cymoxanil analytical standard are accurately weighed on an analytical balance. Record the weight to the nearest 0.0001 g. Dilute to 100 mL with acetonitrile in a volumetric flask. The concentration of the stock solution is 100 µg/mL. The stock standard solution is kept in a refrigerator at 4°C and is stable for six months.

4.2.4 Fortification Standard Preparation and Stability

A 10-µg/mL cymoxanil standard is prepared by diluting 10 mL of the stock standard to 100 mL in a volumetric flask with acetonitrile. The fortification standard solution is kept in a refrigerator at 4°C and is stable for one month.

4.2.5 Chromatographic Standard Preparation and Stability

Chromatographic standards ranging from 0.10 to 2.0 µg/mL are prepared by diluting the 10-µg/mL fortification standard in a 10.0-mL volumetric flask. Maintain the 70/30 10 mM ammonium acetate buffer (pH 4.5)/acetonitrile composition by adding the volumes of acetonitrile and 10 mM ammonium acetate buffer (pH 4.5) shown below. The chromatographic standards are stored in a refrigerator at 4°C and are stable for one week.

Standard Conc. (µg/mL)	µL added	Volume of acetonitrile (mL)	Volume of NH_4AC (mL)
0.1	100	2.9	7.0
0.5	500	2.5	7.0
1.0	1000	2.0	7.0
2.0	2000	1.0	7.0

4.2.6 Source (& Characterization) of Samples

Three different soil types were used to evaluate the method and their sources and summary of their characterizations are listed below:

	Elkton, MD	Madera, CA	SD
Texture	Silt Loam	Loam	Silty Clay Loam
% Sand	6.9	39.2	17.2
% Silt	78.3	44.0	52.0
% Clay	14.8	16.8	30.8
% OM	3.1	1.5	5.2
pH	6.3	7.8	5.7
Sample ID	68344	89745	1602-120

Drummer soil type from South Dakota was available in the soil bank at the DuPont Experimental Station. It's high clay and organic matter content make it a good soil to test out a residue method. Soil from the CA and MD sites was available from the cymoxanil soil dissipation study, AMR 3401-95.

4.2.7 Storage & Preparation of Samples

Samples were homogenized with a Hobart mixer and stored frozen until analysis.

4.2.8 Sample Fortification Procedure

Samples are fortified with the 10-µg/mL cymoxanil standard. A syringe was used to add volumes of 100 to 500 µL of the standard to the 20-g soil sample. Wait 15 minutes after fortification has been made to allow solvent to evaporate.

4.2.9 Analyte Extraction Procedure

1) Weigh 20 ± 0.2 g soil into 250-mL centrifuge bottle. Fortify if necessary.
2) Add 10-mL 100 mM acetate buffer and 50-mL acetone.
3) Shake at high speed for 10 minutes using a wrist-action shaker.
4) Sonicate for 10 minutes.
5) Centrifuge 15 minutes at 10,000 RPM. Decant into Rapidvap sample tube.
   (Repeat Steps 2-5 and decant into same Rapidvap tube).
6) Concentrate for 90 minutes (reduce vol. to 10-15 mL) on Rapidvap (Block temp 40°C, 0.2-bar pressure).
7) Sonicate Rapidvap tube for 1 minute.

4.2.10 Analyte Purification Procedure

1) Attach a 15-mL reservoir to the C18 solid phase extraction column, SPE (6 cc/1 g Mega Bond Elut).
   Condition C18 columns with 10 mL of CH_3OH then 10 mL of 100 mM acetate buffer (pH 4.5). Do not allow C18 SPE to go to dryness once column is conditioned. Discard solution.
2) Transfer extract from Step 7 of Section 4.2.9 to reservoir; apply vacuum so that ~3 mL/min flow of extract is achieved.

Rinse rotovap tube with an additional 15 mL of 100-mM buffer and elute through C18 SPE column.

3) Wash C18 SPE with 12 mL of H_2O followed by 12 mL of hexane. Discard liquid from manifold. Place 13-mL centrifuge tubes in manifold to collect eluent.
4) Elute with 6 mL of 20/80 ethyl acetate/hexane solution. (Procedure may be stopped here and resumed next day.)
5) Concentrate to approximately 1 mL under nitrogen using N-EVAP at 40°C.
6) Add acetonitrile and adjust volume to 5 mL.
7) Blow down to ~1.5 mL on N-EVAP.
8) Bring up to 5 mL with 10 mM acetate buffer (pH 4.5).
9) Use a disposable syringe and filter through a 0.45-µm (13-mm filter unit) into HPLC vials. Samples are now ready for HPLC analysis.

4.3 *Instrumentation*

4.3.1 *Description*

Method validation data reported in this study were generated on a Hewlett-Packard Series II 1090 liquid chromatograph. The mobile phase consists of a 70/30 mixture of H_2O and acetonitrile. The column used for the analysis is a Waters Symmetry C18 (25-cm x 4.6-mm). Cymoxanil is detected by UV absorption at 245 nm.

4.3.2 *Operating Conditions*

Column:	Waters Symmetry-C18 (25-cm x 4.6-mm).
Oven Temp.:	40°C
Mobile Phase:	
Reservoir A -	H_2O
Reservoir B -	acetonitrile
Injection Volume:	50 µL
Detection:	UV at 245 nm
Flow Rate:	1.0 mL/min

4.3.3 *Calibration Procedures*

Prepare at least four chromatographic standards of cymoxanil intended to bracket the levels found in the samples and fortified samples. Preparation of these standards is described in *Section 4.2.5* of this report.

4.3.4 Sample Analysis

- A sample set consists of at least one control and one fortified control in addition to the treated samples (approximately 20% of the samples in a set should be fortifications).
- Fortifications should cover the anticipated range of residues, and should be made at the LOQ and at least two other levels with the same acceptance criteria as discussed in the LOQ section.
- The first and last injection during a sequence should be standards as well as a standard after every third or fourth sample.
- Dilute samples that fall outside the range of standards and reinject with fresh standards.
- Keep sample extracts stored after preparation for analysis in a refrigerator at approximately 3°C. Acceptable recoveries for accompanying fortified samples validate the storage interval.
- Except during analysis, store all soil samples in freezer at approximately 15°C.

4.4 Calculations

4.4.1 Methods

The concentration of cymoxanil (µg/g) was determined by obtaining cymoxanil concentration (µg/mL) in final extract from the linear regression standard curve and applying appropriate weighing factors as shown below:

ppm cymoxanil (µg/g) = [(Pk Ht)(slope) + y-intercept] (µg/mL)
X [final volume (mL)/Sample Wt (g)]

% Recovery = (ppm Cymoxanil/Fortification level) X 100

4.4.2 Examples

Data sheet for example calculation (Sample C) is on page 35. Substituting into the linear equation of the standard curve a peak height of 2.639 corresponds to a cymoxanil concentration of :

ppm Cymoxanil = [(2.639IU)(0.1449µg/mL IU)+ 0.008287µg/mL]
= 0.3906 µg/mL X (5.0 mL/20 g)
= 0.0977 µg/g

% Recovery = (0.0977 µg/g)/(0.10 µg/g) X 100
= 98%

% Recoveries are rounded to two significant figures.

5.0 Results and Discussion

5.1 *Method Validation Results*

5.1.1 *Detector Response*

A typical chromatogram of a cymoxanil standard is shown in Figure 2. A calibration curve of concentration (µg/mL) versus peak height for cymoxanil is shown in Figure 3. The standard curve displays good linearity with a correlation coefficient (R^2) of 0.9988 and a near zero intercept 0.008287 µg/mL.

During the study, the correlation coefficients ranged from 0.9988 to 0.9999. The tested linear range of the calibration curve was 0.10 to 2.0 µg/mL.

5.1.2 *Controls*

Chromatograms of controls are displayed in Figures 4, 5, and 6. The Drummer soil and soil from Madera both show trace levels of an interference peak at the same retention time as cymoxanil. The levels of the interference peak in the control samples are at least 5 times lower then the limit of quantitation and will be discussed further in Section 5.1.5 and 5.4.3.

5.1.3 *Recoveries (Accuracy & Precision)*

Samples were fortified at three levels, 0.050, 0.10, and 0.50 g/g. Average recoveries are listed below:

Fortification µg/g	Recovery Range %	Average ± SD %
0.050	73-110	91 ± 12 (n=7)
0.10	76-98	92 ± 8 (n=7)
0.50	80-100	92 (n=2)

Individual values are listed in Table 1. All recoveries are in the acceptable range. Representative chromatograms of the 0.050 fortifications are shown in Figures 7, 8, and 9. Data Summary Sheets are displayed in Appendix I.

5.1.4 Extraction Efficiency

The solution used for extraction, 50-mL acetone, and 10 mL of 100 mM ammonium acetate (pH 4.5), was effective at removing cymoxanil residues from soil. Based on the metabolism study (Reference 2), the half-life of cymoxanil was less than 24 hours. Four replicate samples were spiked with the ^{14}C-labeled cymoxanil, two were analyzed immediately and two were aged for 24 hours. Based on the half-life from the aerobic metabolism study, 24 hours should be an adequate interval for cymoxanil residues to be incorporated into the soil.

The samples were extracted as outlined in Section 4.2.9, with the exception that the samples from Madera, CA, acetonitrile were used instead of acetone. At both sites, approximately 100% of the ^{14}C-labeled cymoxanil was recovered for the freshly fortified samples. Approximately 90% of the radioactivity was obtained from the aged samples from Madera, CA, and 50% from Elkton, MD. This data is consistent with the half-life found in the soil dissipation study (Reference 3).

5.1.5 Limit of Quantitation and Detection

Control samples from two of the three soil types had an interference peak (Figures 4 and 5). Based on an interfering peak of 0.01 µg/g in the control, the limit of quantitation was established at five times this level of 0.05 µg/g, rather than using the signal-to-noise ratio of the control sample. Liquid chromatography with a mass spectrometer detector demonstrated that the interference was not cymoxanil (see Section 5.4.3).

By convention, the limit of detection is set three times lower than the LOQ at 0.02 µg/g. Fortifications below the LOQ were made on all three soil types at 0.01 and 0.02 µg/g; in each case, cymoxanil residues were detected.

5.2 *Timing*

Typically six to eight samples can be prepared in an eight-hour work day. The time required for the chromatographic run is approximately 15 minutes and these analyses may be performed by an auto-sampler unattended overnight.

5.3 *Modifications or Special Precautions*

To maintain recoveries, it is critical to remove all of the acetone from the extract during the concentration step. Otherwise cymoxanil will not be retained on the C18 bond elute column. Since different lots of bond elutes may have slight differences, it is important to profile each lot of C18 bond elutes using cymoxanil standards prior to the use of this method.

5.4 *Method Ruggedness*

5.4.1 *Stability*

The stability of reagents and standard solutions has been addressed in *Section* 4.2.2 though 4.2.5. Sample extracts should be analyzed as soon as possible but are stable for at least 48 hours if kept refrigerated at 4°C.

5.4.2 *Specificity/Potential Interference*

Cymoxanil may be applied in tank mixes with the following two DuPont products Manzate® (mancozeb) or DPX-JE874. Since mancozeb is insoluble in organic solvents, only ethylene thiourea (ETU) an impurity in the formulation was tested. Neither DPX-JE874, which was retained on the column , or ETU, which eluted at approximately 2.8 minutes, interfered with cymoxanil under the liquid chromatographic conditions used in this method.

Other commonly used fungicides were also tested for interferences: Ridomil® (metalaxyl) and Prowl® (pendimethalin) were retained on the column. Admire® (imidacloroprid) had a retention time of 6.0 minutes and Bravo® (chlorothalonil) had a retention time of 3.0 minutes. None of these products contained interferences with cymoxanil elutes at approximately 9 minutes under the liquid chromatographic conditions used in this method.

5.4.3 *Confirmatory Method*

LC-MS was used to confirm that the interference in the control sample 68355 was not cymoxanil. The MS was operated in the positive, selected ion monitoring mode set to monitor ions having m/z of 199 amu (protonated molecular ion and 216 amu (cymoxanil + ammonium ion).

Figure 10 shows a Reconstructed Ion Chromatogram, RIC, generated from the sum of the 199 and 216 amu. Both the cymoxanil standard and the field-treated sample contain a peak

corresponding to these masses at the retention time of cymoxanil. This provides positive confirmation of cymoxanil in these samples. The control sample, 68355, shows no such peak indicating that cymoxanil is not present.

6.0 Conclusions

This analytical method is suitable for the analysis of cymoxanil in soil with an LOQ of 0.05 µg/g and an LOD of 0.02 µg/g. The LOQ was set based on five times the level of an impurity in the control. LC/MS verified that this impurity was not cymoxanil.

Recoveries at the LOQ averaged 91 ± 12%. Extraction efficiency experiments yielded recoveries of approximately 100%. Interference testing showed that other commonly used fungicides do not interfere with the analysis of cymoxanil as outlined in this method.

7.0 Retention of Records

The raw data for this study and the final report are retained in the GLP Archives located at:

E. I. du Pont de Nemours and Company
DuPont Agricultural Products
Global Technology Division
Experimental Station
Wilmington, Delaware 19880-0402

8.0 References

1. The Pesticide Manual, 9th Edition, C.R. Worthing, Editor. The British Crop Protection Council, 1991; 206-207.

2. Boucher, C.R., "Aerobic Soil Metabolism of ^{14}C-Cymoxanil", DuPont Report No. AMR 3438-95, DuPont Agricultural Products, E. I. du Pont de Nemours and Company.

3. McClory, J.P. and Jones, W., "Field Dissipation of Cymoxanil Following Application of Curzate M-8 Fungicide", DuPont Report No. AMR 3595-95, DuPont Agricultural Products, E. I. du Pont de Nemours and Company.