3.0 MATERIALS

Equivalent equipment and materials may be substituted unless otherwise specified; note any specifications in the following descriptions before making substitutions. The equivalency/suitability of any substitution should be verified with acceptable control and fortification recovery data.

3.1 EQUIPMENT

Standards Preparation: Mettler AE 160 series, 4-place analytical balance (Mettler Instrument Corp., Hightstown, N.J.); Pyrex® Class A glass 10-, 50-, and 100-mL volumetric flasks (VWR, Bridgeport, N.J.); Syringes, glass, high-performance, Luer-Lok® tip, 250-µL and 1000-µL capacities (Model Nos. 1725 and 1001, respectively [Hamilton Company, Reno, Nev.]); Syringe needles, #22 x 2", blunt (perpendicular) tip with Luer® hub, Cat. No. 72-15 (Alltech Associates, Deerfield, Ill.).

Sample Extraction: Covered in References 1-3.

LC/MS/MS Analysis (supplies): Millipore "HV" Durapore® polyvinylidene fluoride membrane filters, 47-mm diameter, 0.45-µm pores, Cat. No. HVLP 047 00 (Millipore Corp., Bedford, Mass.); Filter apparatus, all-glass, Cat. No. XX15 04700 (Millipore Corp.); Filter units (disposable), Millipore Millex®-HV13, 13-mm diameter, 0.45-µm pores, Durapore® polyvinylidene fluoride membrane, Cat No. SJHV 013 NS, (Millipore Corp.); Syringes, Fortuna® disposable, sterilized, 2.5-mL capacity, all-polypropylene/polyethylene, Cat. No. Z11685-8 (Aldrich Chemical Company, Milwaukee, Wisc.); Vials, Waters autosampler, 1-mL capacity, clear glass with caps, Cat. No. WAT025054 (Waters Corporation, Milford, Mass.).

LC/MS/MS Analysis (instrumentation):

Liquid Chromatograph (Waters Corporation, Milford, Mass.).
Waters 616 HPLC System, consisting of Model 616 Pump (Ser. No. MXSKM3052M) and Model 600S Controller (Ser. No. SXSKM0105M)
Waters Temperature Control Module (Ser. No. 1837).
Waters Column Oven Heater Module (Ser. No. CEM002354)
Waters Model 717 plus Autosampler (Ser. No. MXSKM2944M)

HPLC Columns (Mac-Mod Analytical, Inc., Chadds Ford, Pa.)
DO NOT SUBSTITUTE ANALYTICAL COLUMNS WITHOUT EVALUATING EQUIVALENCY
Zorbax® C8 Guard Cartridge, Part No. 820674-906
followed by
Zorbax® Rx-C8 Analytical Column, 4.6 mm x 25 cm, Part No. 880967-901

Mass Spectrometer (Finnigan MAT Corporation, San Jose, Calif.)
Finnigan Model TSQ7000 Mass Spectrometer (Ser. No. TS 010025)
Finnigan MAT Thermospray LC Interface Module, Model TSP-2 (Ser. No. 01011001)
Digital DEC3000 Model 300LX Computer (Ser. No. NI51000X10)
ICIS Software (Version 8.2.1)

Other Instrumentation
Mettler PB602, 2-place, toploading analytical balance (Mettler Instrument Corp., Hightstown, N.J.); Fisher Accumet® 15 pH meter (Fisher Scientific, Pittsburgh, Pa.)

3.2 *Reagents and Standards*

Reagents: *Substitutions of equivalent reagents should only be made if equivalency/suitability has been verified.* Water, Milli-Q™ Type I deionized, distilled water system (Millipore Corporation, Bedford, Mass.); methanol, OmniSolv® distilled, Product #MX0488-1 (EM Science, Gibbstown, N.J.); acetic acid (glacial), 'Baker Analyzed'®, HPLC Reagent grade, Cat. #9515-03 (J. T. Baker); ammonium acetate, 'Baker Analyzed'®, HPLC Reagent grade, Cat. #0599-08 (J. T. Baker).

Standards: Cymoxanil, DPX-T3217-101, 99.9% pure (DuPont Agricultural Products, Wilmington, Del.).

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 PRINCIPLES OF ANALYTICAL METHODS

4.1.1 Sample Extraction

Sample extractions are performed according to procedures detailed in **References 1-3.**

4.1.2 Extract Purification

SPE clean ups of sample extracts are described in **References 1-3.** The only additional purification required is preanalysis filtration (0.45 µm) of an aliquot of the final analysis solution, to prevent introduction of particulates into the HPLC system.

4.1.3 LC/MS Analysis

An HPLC separation coupled to a mass spectrometer (via a thermospray interface) is the basis for detection and quantitation of cymoxanil residues. The MS is operated in the positive, selected ion monitoring (SIM) mode, set to monitor ions having m/z of 199 and 216, which correspond to the protonated molecular ion of cymoxanil (MW = 198) and an ammonium adduct (i.e., cymoxanil + NH_4^+), respectively. Quantitation is based on the Reconstructed Ion Chromatogram (RIC).

4.2 ANALYTICAL PROCEDURE

4.2.1 Glassware and Equipment Cleaning Procedures

Disposable labware are generally used in this method. Reusable labware, including volumetric flasks for standard solutions, are cleaned by washing with a laboratory-grade detergent followed by tap-water rinses (3) and distilled water rinses (3). A final acetone rinse may be used to remove the residual water and promote drying.

4.2.2 Preparation and Stability of Reagent Solutions

Prepare an aqueous 0.10 M ammonium acetate solution by weighing 7.71±0.02 g of the reagent (CH_3COONH_4, FW = 77.08 g) in a plastic weighboat, transferring to 1 L of Milli-Q® water (already contained in a 1-L beaker), and thoroughly mixing (magnetic stirring). Adjust the pH

to 4.5 ± 0.1 with glacial acetic acid (magnetic stirring). Transfer this solution to a suitable container for storage at ambient temperature. Replace solution monthly (≤30 days), or sooner if turbidity is observed. Prior to use as an HPLC mobile phase component, this solution must be filtered through 47-mm diam. filters (0.45-µm pore size).

4.2.3 Stock Solutions Preparation

If possible, use standards with a purity greater than 95%. Prepare a 100±2-µg/mL stock standard solution by diluting 10.0±0.20 mg of cymoxanil to volume with methanol, using a 100-mL volumetric flask.

Determine sample weights to 3 significant figures. The analytical balance must provide a weight precision to at least 3 significant figures.

Clearly label as a stock solution with date prepared, analyte, and concentration. Store this stock solution under refrigeration (4±2°C). Replace stock solutions at 6 months, or sooner if chromatography indicates significant degradation.

4.2.4 Fortification Solutions Preparation

Prepare fortification solutions according to procedures given in **References 1-3.**

4.2.5 Calibration Solutions Preparation

Prepare a 1.00-µg/mL intermediate standard (cymoxanil) by transferring 1.00 mL of the 100-µg/mL stock solution (use a syringe or equivalent) to a 100-mL volumetric flask and diluting to volume with methanol.

Prepare calibration solutions at concentrations over a range of 0.00500-0.100 µg/mL from dilutions of the 1.00-µg/mL intermediate standard. The dilution solvent is 20% methanol / 80% 0.10 M ammonium acetate (pH 4.5) (v/v). A minimum of 4 standard concentrations should be prepared over this range including the lower and upper limits. Use the following table as a guide for standards preparation.

1.0 µg/mL Standard Aliquot (mL)	Volumetric Size (mL)	Final Concentration (µg/mL)*
0.0500	10.0	0.00500
0.100	10.0	0.0100
0.200	10.0	0.0200
0.500	10.0	0.0500
1.00	10.0	0.100

Clearly label calibration solutions with date prepared, analytes, and concentration. Store calibration solutions in a refrigerator (4±2°C) and replace weekly (≤7 days).

CAUTION: Glassware is a source of possible contamination if not cleaned properly. Rinse flasks 2-3 times with 50% methanol / 50% water prior to preparing standard solutions.

4.2.6 *Source of Samples*

Soil samples (see Table 1 for characterization information) were obtained from two sources:

- Madera, Calif. (Sample Barcode No. S00089759, DuPont Study No. AMR 3401-95)
- Environmental Studies Soil Bank, DuPont Agricultural Products, Experimental Station, Wilmington, Del. ("Drummer" Soil)

The "Drummer" soil has a high clay and % organic matter content, making it an especially good soil for residue-method testing.

Water was obtained (28-Nov-95) from the Brandywine Creek (near the DuPont Experimental Station, Wilmington, Del.).

Untreated potatoes (tubers) were purchased locally (supermarket), homogenized (ground with dry ice), and stored frozen 19-Nov-94.

4.2.7 *Storage and Preprocessing of Samples*

This information is available in **References 1-3.**

4.2.8 *Sample Fortification Procedure*

This information is available in **References 1-3.**

* equivalent to ppm

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4.2.9 Analyte Extraction Procedures

This information is available in **References 1-3.**

4.2.10 Analyte Purification Procedure

SPE clean-up procedures are available in **References 1-3.** Prior to instrumental analysis, an approximately 1-mL aliquot from each final sample solution is filtered into an autosampler vial using a 2.5-mL plastic syringe equipped with a 0.45-µm filter (Millex®-HV13).

4.3 Instrumentation

4.3.1 Description

This method uses an LC/MS system for determination of cymoxanil residues in a wide variety of sample matrix types. Mass spectrometry is the most widely accepted technique for confirmation of residue concentrations.

The LC system components are listed in ***Equipment***. An isocratic, reversed-phase separation using a Zorbax® Rx-C8 analytical column is performed.

HPLC requirements for an LC/MS system are more stringent than for LC/UV; the chromatograph should have minimal pressure fluctuations, as this has a major effect on mass spectrometer baseline noise.

A low dead-volume, 0.5-µm pore filter is critical in the tubing leading to the MS interface to prevent exposure of the MS interface to particulates. (Finnigan MAT supplies an appropriate in-line filter for their MS system.)

In addition to being a useful mobile phase buffer, ammonium acetate is necessary to provide a proton source as part of the ionization process occurring in the MS interface (in a thermospray interface, droplet desolvation and analyte ionization occur fairly simultaneously following introduction of LC effluent — in the form of a hot aerosol — into the MS source region **(References 4 and 5)**).

The MS system is equipped with a high-pressure switching valve, between the LC and the MS source, to allow convenient diversion of LC effluent to waste during time periods irrelevant to analyte quantitation. **This feature is essential** in residue methods such that early eluting sample materials are prevented from fouling the MS source (especially over the course of numerous analyses).

The mass spectrometer is a triple-quadrupole design equipped with a thermospray source (see Section *4.1.3*). However, a single-quadrupole instrument would be equivalent for this method (only one quadrupole — Quad 1 — was involved in mass selectivity).

A typical full-scan spectrum is shown in Figure 2. The base peak (greatest abundance) at m/z 216 corresponds to the ammonium adduct (cymoxanil + NH_4^+). The other relevant peak (m/z 199) corresponds to the protonated molecular ion.

For quantitation of cymoxanil residues, the mass spectrometer was operated in the Selected Ion Monitoring (SIM) mode, such that Quad 1* selected both relevant ions (m/z 199,216). Quantitation was based on the RIC.

A low-level chromatographic standard (cymoxanil) should be analyzed prior to the start of analyses to establish suitability of selected MS parameters. In our work, the electron multiplier voltage was adjusted such that the peak area for a 0.100-µg/mL standard (100 µL injection) yielded at least 10^6 counts for cymoxanil peak area (sum of both ions — same as RIC).

The typical linear range for calibration standards was 0.00500–0.100 µg/mL (see Figure 3).

* Quads 2 and 3 were not involved in mass selectivity (they were operated to pass all masses through).

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4.3.2 LC/MS Operating Conditions

Operating Conditions (HPLC):

Column Temp: 40°C
Injection Vol: 100 µL

Mobile phase: 40% methanol /
60% 0.10 M ammonium acetate, pH 4.5
(pH adjusted with glacial acetic acid)

Flow Rate: 1.00 mL/min

Operating Conditions (MS):

(Reference: Conditions were recorded 28-Aug-1996, in instrument records; these would be considered typical.)
Vaporizer Temp: 90°C
Aerosol Temp: 103°C
Source Block Temp.: 200°C
Repeller: 100 V
Electron multiplier voltage: 1600 V

SIM Mode (Q1MS), m/z 199.0, 216.0 (0.50 sec/mass [1 scan/sec])

4.3.3 Calibration Procedures

Instrumentation calibration is based on average response factors (defined in Section *4.4.1*) calculated as the ratio of detector response (chromatographic peak area) to concentration of standard injected (all injections should be same volume [100 µL suggested]).

If the relative standard deviation for an individual group of 3 standards is less than 20%, then the method is considered to be operating correctly (otherwise, appropriate corrective action, such as instrument maintenance or trouble-shooting, should be taken). Standards at 3 concentration levels (minimum) should be used to verify this criterion at the beginning of a particular day's analysis set as well as throughout the day's run.

The successful detection (S/N > 5:1) of the lowest calibration standard is an additional criterion to be used to verify proper tuning and

calibration of the instrument. Again, this criterion should be evaluated (and appropriate action taken, if needed) prior to running samples.

4.3.4 Sample Analysis

A sample analysis run should start out with 3-4 (minimum) injections of standards (covering at least 3 calibration concentrations), for assessment of system suitability and performance (see previous section). Subsequently, no more than 4 samples should be injected between standards. The analysis run should conclude with at least one standard injection.

4.4 CALCULATIONS

4.4.1 Methods

The Finnigan data system was used to integrate, report, and print the relevant area counts for each chromatogram. The analyst examined each chromatogram (using the Finnigan program "CHRO" -- see Table 2 for typical integration parameters), manually reintegrated the peak(s) if necessary, then printed each chromatogram.

Area count responses (RIC) were manually entered into an EXCEL Spreadsheet table (Microsoft Corp., Version 5.0a). Spreadsheet formulas were used to complete the data analysis, using the Response Factor Method described below.

Response Factor Method

$$\text{Response Factor (RF)} = \frac{\text{Peak Area (counts)}}{\text{Standard Concentration (ppm)}}$$

Quantitation of analyte concentration in fortified or treated samples is performed based on the average response for standards preceding and following the samples, as follows:

$RF_{AVG} = [(RF_1) + (RF_2) + (RF_3)] / 3$, where

RF_1 = response factor of first standard,

RF_2 = response factor of second standard, and

RF_3 = response factor of third standard.

Normally, RF_1 and RF_2 will precede the sample and RF_3 will follow (other, similar bracketing schemes are also legitimate). These response factors (and also RF_{AVG}) were calculated by appropriate formulas contained in the EXCEL spreadsheet.

Analyte residue concentrations are calculated as follows:

$$\text{ppm Found} = \frac{(\text{Peak Area})(AF)(FV)(DF)}{(RF_{AVG})(SW)}$$

where

AF = aliquot factor,
FV = final volume of sample in mL,
DF = dilution factor (if additional sample dilution by the LC/MS analyst is required), and
SW = weight of sample in grams.

The percent recovery is calculated using the "ppm Found" and the fortification concentration (ppm) in the following formula:

$$\text{Percent Recovery} = \frac{\text{ppm Found} \times 100}{\text{Fortification Level}}$$

4.4.2 Examples

The concentration of cymoxanil found in white potato sample 1B fortified at 0.020 ppm is calculated as follows (see Data Sheet Number 093096 in Appendix 1):

Peak Area = 157,974 counts
Aliquot Factor = 99 mL/7.5 mL = 13.2 (a 7.5-mL aliquot was removed out of a 99-mL acetonitrile layer, during sample work up)
Final Volume = 3.00 mL
Dilution Factor = 1
RF_{AVG} = 17,743,397 counts/ppm
Sample Weight = 20 g

$$\text{ppm Found} = \frac{(157{,}974)(13.2)(3.00)(1)}{(17{,}743{,}397)(20)} = 0.0176$$

This value was reported as 0.018 ppm in Table 4.

The concentration of cymoxanil found in Brandywine water sample 2C fortified at 0.004 ppm is calculated as follows (see Data Sheet Number 091396 in Appendix 1):

Peak Area = 1,629,778 counts
Aliquot Factor = 1
Final Volume = 3.00 mL
Dilution Factor = 1
RF_{AVG} = 12,593,338 counts/ppm
Sample Weight = 100 g

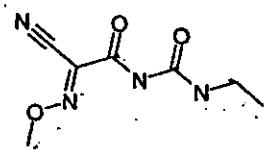
$$\text{ppm Found} = \frac{(1{,}629{,}778)(1)(3.00)(1)}{(12{,}593{,}338)(100)} = 0.00388$$

TABLE 2: TYPICAL INTEGRATION PARAMETERS

(Finnigan MAT "CHRO" software application)

Minimum peak width	9 or 10
Label noise factor	20 or 21
Baseline window	50
Multiplet resolution	10
Area tail extension	10
Area noise factor	20
Baseline subtraction	none
Smoothing	3 points

FIGURE 1: CHEMICAL STRUCTURES AND NAMES



Exact MW = 198.075

DuPont Code Number: DPX-T3217

Trivial Name: cymoxanil

CAS Chemical Name: 2-cyano-N-[(ethylamino)carbonyl]-2-(methoxyimino)acetamide

CAS Registry No.: 57966-95-7

IUPAC Chemical Name: 1-(2-cyano-2-methoxyiminoacetyl)-3-ethylurea