Study Title

ANALYTICAL METHOD FOR THE DETERMINATION OF RESIDUES OF CYMOXANIL IN GRAPES AND TOMATOES USING COLUMN SWITCHING LIQUID CHROMATOGRAPHY

Data Requirement

U.S. EPA Pesticide Assessment Guidelines
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GOOD LABORATORY PRACTICE STATEMENT

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

Sponsor:

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Edward C. Nathan and Sidney J. Hill

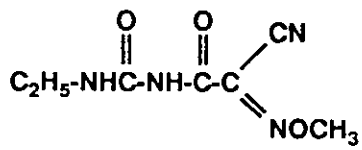
Reason for Revision

To include two lines of text at the top of page 27 which were available electronically, but due to a printer problem did not print.

I. Introduction

A. Scope

Cymoxanil (DPX-T3217) is the active ingredient in Curzate® Fungicide, a DuPont agrichemical used for control of select plant diseases in crops such as grapes and tomatoes principally in Europe. 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, there is need for an analytical method to selectively detect and enforce import tolerances in commodities such as grapes, tomatoes, and tomato products.

This report describes an analytical method which can be used for cymoxanil analysis. The method has been applied to quantitate cymoxanil at levels of 0.05 ppm or above in wine grapes, tomatoes, and tomato paste. Individual crops or commodities are extracted with ethyl acetate. An aliquot is removed, concentrated, reconstituted in acetone/water and purified by passing the solution through a stacked strong anion exchange (SAX) and carbon black cartridge. After elution, partially purified extracts are passed through a silica solid phase

extraction (SPE) cartridge, concentrated, and analyzed by reverse-phase HPLC using UV detection.

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.

B. Principle

Six-gram samples of grape or tomato products are weighed into a 250-mL centrifuge bottle and ground under controlled conditions with a Tissumizer® in contact with ethyl acetate. After two ethyl acetate extractions, extracts are combined and a homogeneous aliquot removed, concentrated, and exchanged into acetone. The solution is diluted with water and passed through a 1-gram SAX cartridge stacked on top of a 500-mg carbon black cartridge. Cymoxanil passes through the SAX cartridge and is retained on the carbon black cartridge. After elution from the carbon black cartridge, the eluate is concentrated, exchanged into ethyl acetate solvent, and diluted with hexane to achieve a final solvent composition of 90% hexane/10% ethyl acetate (v/v). The solution is further purified by passage through a 500-mg silica cartridge.

After elution and concentration, the sample is exchanged into methanol, 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-CN column alone or a SB-CN column followed by a 15 cm x 4.6 mm SB-C18 column (column switching HPLC). When column switching is used the SB-CN column provides additional cleanup for samples that require it. The entire sample is then transferred (switched) to a SB-C18 column where the analytical separation occurs. In both cases, 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 allowing quantitation at 0.05 ppm (Limit of Quantitation or LOQ) in grapes, tomatoes, and tomato products. The overall average recovery (± standard deviation) for fortified samples of grapes and tomatoes was 94.9% ± 7.5% with a relative standard deviation of 7.9%.

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 was used to weigh the analytical standards. A Mettler Model PE600 top-loading balance was used for all other weighings.
(Mettler Instrument Corporation, Princeton, N.J.).

Centrifuge

DuPont Sorvall® Model RC-3B
(DuPont Medical Products, Wilmington, Del.).

Centrifuge Rotor

DuPont Sorvall® Model H2000B (low speed) rotor
(DuPont Medical Products, Wilmington, Del.).

Centrifuge Bottles

250-mL, polypropylene, IEC Maxiforce® #2050, VWR Catalog #21018-037
(VWR Scientific, Bridgeport, 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.).

Homogenizer

Tekmar® SKT Tissumizer® Model SDT-1810 equipped with a Model STD-182 EN shaft
(Tekmar Co., Cincinnati, OH).

Wrist Action Shaker

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

Mobile Phase Filters and Vacuum Filter Apparatus

0.45-µm pore, Type HA filter, Millipore Catalog #HATF 047 00. This filter is used to filter 10-mM aqueous KH_2PO_4 buffer (pH = 2.9).

0.5-µm pore, Type FH 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.).

Erlenmeyer Flasks

Pyrex® 250-mL Erlenmeyer flasks, Catalog #29411-029
(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.).

HPLC Sample Filters

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

Funnel

Kimax® Kimble 58 Short Stem Chemical Funnel, 63-mm stem, 55-mm top, Catalog #30205-068
(VWR Scientific, Bridgeport, N.J.).

Solid-Phase Extraction Columns and Filtration Collection Apparatus

Solid-Phase Extraction Columns

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

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
(Varian, Inc., Harbor City, CA).

Extraction Apparatus

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

15-mL Reservoirs, Part #1213-1010 and adapters, Part #1213-1001
(Varian, Inc., Harbor City, CA).

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-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).

Column 1

DuPont Zorbax® SB-CN 4.6 x 150 m, 5-µ Analytical Column, Part #883975.905
(Mac-Mod Analytical Inc., Chadds Ford, PA).

Column 2

DuPont Zorbax® SB-C18 4.6- x 150-m, 5-µ Analytical Column, Part #883975.902
(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

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.).

Acetone

EM Acetone, GR-grade solvent, Catalog #AX0120D-30 (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.).

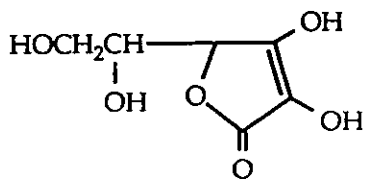
Phosphoric Acid (H_3PO_4)

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

Hydrochloric Acid (HCl)

Hydrochloric Acid, 36.5-38.070, Baker Analyzed Reagent Catalog #9535-01
(J. T. Baker, Phillipsburg, N.J.).

L-Ascorbic Acid



Fisher Certified ACS Reagent, 99.9%, A-61
(Fisher Scientific Co., Fair Lawn, N.J.).

Cymoxanil (DPX-T3217)

Analytical standard grade IN-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 drops). Filter the solution through a 0.45-µm filter prior to use. Prepare fresh buffer at least weekly to avoid formation of sediment and bacterial growth.

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

Aqueous Ascorbic Acid (10 g/liter), pH = 2.0

Dissolve 10.0 g of L-ascorbic acid ($C_6H_8O_6$) in 1.0 liter of HPLC grade water in a beaker. Use a magnetic stirrer to assist solution.

Acidify the resulting solution to pH = 2.0 with dilute hydrochloric acid. Prepare this solution weekly. Discard if yellowing is observed. Store out of contact with light. This can be accomplished by using an amber container or wrapping the bottle containing the solution with aluminum foil.

50% Methanol/50% Acetonitrile (v/v)

To a 1000-mL graduated cylinder, add 500 mL of methanol. 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 1 liter. Prepare this solution weekly.

Note: *50% methanol/50% acetonitrile (v/v) is used to improve cymoxanil peak shape during HPLC analysis.*

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.

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

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

84% 10 mm KH_2PO_4, pH = 2.9/16% Methanol(v/v)

To a 500-mL graduated cylinder, add 420 mL of potassium dihydrogen phosphate buffer, pH = 2.9. Measure 80 mL of methanol in a second graduated cylinder and add this to the 500-mL graduated cylinder. Do not adjust the final volume to 500 milliliters. Prepare this solution weekly.

HPLC Eluents

Eluent A: 100% 50% Methanol/50% Acetonitrile (v/v)
Eluent B: 100% 10 mM KH_2PO_4 buffer, pH = 2.9

Mobile phase must be thoroughly degassed daily. If low-pressure mixing HPLC is used for sample analysis, mobile phases should be sparged continuously 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 columns when needed.

Standards

Use Class A volumetric flasks when preparing all standard solutions.

Cymoxanil Standard Stock Solution

Using an analytical balance and weighing boat, weigh approximately 10.0 mg of analytical standard grade cymoxanil (DPX-T3217) into a 100-mL volumetric flask. Record the 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 (100.00 mL) with acetonitrile. The final concentration is approximately 100 µg/mL. Cymoxanil is stable in the solution for at least 2 months when stored at 4°C when not in use.

Intermediate Standard Solutions

Note: Solutions used to fortify crop matrices are prepared in ethyl acetate whereas HPLC standards are prepared in 84% 10 mM KH_2PO_4, pH = 2.9/16% methanol (v/v). The solvent composition for the HPLC standards is selected to bring the solvent strength to the required range for HPLC analysis. Ethyl acetate is selected for sample fortification to assist solvent evaporation after matrix fortification.

Using a syringe, prepare a 1-µg/mL fortification working standard by transferring the required volume of the 100-µg/mL cymoxanil acetonitrile standard (approximately 1.00 mL) to a 100-mL volumetric flask. Dilute to the mark (100.00 mL) with ethyl acetate. 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 methanol to the flask (approximately 15 mL) using a 25 mL graduated cylinder so that the volume of acetonitrile plus methanol equals 16.0 mL. Sonicate and swirl the flask to ensure complete solution of cymoxanil. Dilute to the mark (100.00 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 84% 10 mM KH_2PO_4 buffer, pH = 2.9/16% methanol (v/v) by quantitatively diluting appropriate volumes of the 1-µg/mL intermediate standard. If 10-mL volumetric flasks are used for these dilutions, the following volumes are required to prepare the 0.02, 0.04, 0.10, and 0.13-µg/mL working standards required for the analysis.

Desired Standard Concentration (µg/mL)	Volume of 1-µg/mL Working Standard Required in a 10-mL Volumetric (µL)
0.13	1300
0.10	1000
0.04	400
0.02	200

These working standards are stable for approximately 2 weeks if stored at 4°C when not in use, but they should be prepared weekly to insure best calibration of the assay.

Fortification Standard Solutions

The 1-µg/mL intermediate fortifying standard can be used directly to fortify crop matrix samples. Alternatively further dilutions of the 1-µg/mL fortifying standard can be prepared by further dilution with ethyl acetate if desired. The ethyl acetate fortification standard solution is 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 84% 10 mM KH_2PO_4 buffer, pH = 2.9/16% methanol (v/v). This ensures best analytical results.

D. *Analytical Procedure*

Note: *This analytical method requires that centrifuge tubes used for concentration of cymoxanil matrix samples be silated before use. Appendix 1 outlines the reagents, their source, and the procedure utilized. This assay has been demonstrated for analysis of grape, tomato fruit and tomato paste samples.*

Special Note: ***Cymoxanil in grape and tomato extracts has a tendency to adsorb on matrix impurities present during the extraction and purification portion of***

this analysis. As a result, cymoxanil, extracts can not be taken to dryness or erratic results will be obtained. All cymoxanil solutions must be exchanged into various solvents during each sample cleanup step described in this method.

1. *Preparation of Sample*

Frozen samples of grapes or tomatoes are ground to uniform composition in a Quaker City or similar mill with dry ice. The ground samples are stored in a freezer at 4°C until analyzed. Dry ice sublimes during storage. Samples such as tomato paste can be stored in a refrigerator and used as is for analysis. All solid samples should be weighed in a ground homogenous state with care taken to obtain material representative of the entire sample mass at the time of sampling. Samples should be brought to room temperature before weighing. Weigh 6.0 ± 0.1 g samples into a 250-mL centrifuge bottle. Fortify the sample with the appropriate amount of cymoxanil if necessary and allow ethyl acetate to evaporate for 15 minutes.

2. *Extraction*

a. Add 100 mL of ethyl acetate to the 250-mL centrifuge bottle containing the sample to be analyzed, swirl until the sample is completely wetted and let the sample stand for at least 2 minutes. Grind the sample thoroughly with a Tissumizer® for at least 2 minutes at a voltage setting at least 60% of full scale (Variac setting of 60 out of a maximum setting of 100).

b. Centrifuge the slurry at 2500 rpm (H2000B rotor) for 15 minutes. Solids pelletize on the bottom of the centrifuge bottle.

c. Place a small piece of glass wool in the stem of a short-stem funnel and place the funnel on top of a 250-mL graduated cylinder. Slowly pour the liquid contents of the centrifuge bottle into the funnel taking care not to transfer any residual solids to the funnel. If solids are transferred to the graduate, they can introduce matrix impurities that will interfere with the analysis. Wash the Tissumizer® probe with a few milliliters of ethyl acetate to clean the probe if necessary. Grind the sample a second time with 100 mL of ethyl acetate for at least 2 minutes at a Variac setting of 60. Centrifuge the sample at 2500 rpm for 15 minutes and carefully pour the liquid extract into the funnel taking care not to transfer

solids to the funnel. When filtration is complete, ***record the final volume of the extract***.

Note: Tomato paste samples are ground with a Tissumizer® as described above during the first ethyl acetate extraction but during the second extraction the centrifuge bottle should be filled with 100 mL of ethyl acetate and the centrifuge bottle shaken on a wrist action shaker for 2 minutes. Residual tomato paste gets into the Tissumizer® probe causing the probe to seize if samples are ground a second time. If tomato paste compacts on the bottom of the centrifuge bottle after centrifugation, it can be loosened with a spatula prior to shaking on the wrist action shaker.

Pour the contents of the graduate into a 250-mL Erlenmeyer flask and swirl the contents of the flask well to ensure they are mixed to uniform concentration. Using a 50-mL graduated cylinder measure 40 mL of extract and transfer this to a silanized 50-cc centrifuge tube. Alternatively, 40 mL of extract may be transferred by pipet to a silanized centrifuge tube directly from the graduate but, if this is done, the graduated cylinder must be inverted several times using a stopper or a piece of dental dam to retain the liquid in the graduate to ensure the contents of the graduate are mixed to uniform concentration before sampling.

e. Place the 50-mL centrifuge tube in an N-Evap and concentrate the ethyl acetate to 1.0 ml using nitrogen at 40°C. Add 5.0 mL of acetone to the tube and vortex mix the contents for at least 30 seconds. Concentrate the solvent to 0.50 ± 0.10 mL under nitrogen at 40°C using an N-Evap.

Note: If it is judged faster or more convenient, the 40-mL aliquot can be transferred to a single-neck, pear-shaped 200-mL flask and concentrated on a rotary evaporator or concentrated on a Turbo-Vap evaporator but if this is done, the sample must not be taken to dryness. The concentrated extract should be transferred to a silanized 50-cc centrifuge tube with sufficient ethyl acetate to ensure quantitative transfer and then concentrated to 1.0 mL prior to addition of acetone so cymoxanil can subsequently be quantitatively redissolved.

f. Add 12 mL of Milli-Q® water to the centrifuge tube and vortex mix the contents of the tube for at least 30 seconds. Sonicate the tube for at least 5 minutes. The sample is ready for the first cartridge purification.

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 and stacked cartridges are used with adapters, it may be necessary to loosen the adapters to allow solvent to drip onto the head of SPE columns before vacuum is applied. This allows more uniform control of solvent flow through the SPE columns. 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 1 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 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 ensure consistent performance of each lot of SPE cartridges. Elution conditions can be adjusted if necessary to optimize recovery depending on the performance of a particular lot of cartridges and the nature of samples being analyzed. A careful check of elution conditions is particularly important if fortified recoveries decrease during use of this method.***

a. Condition a 1-gram SAX cartridge by passing 5 mL of methanol and then 15 mL of Milli-Q® water through the cartridge. Do not let the cartridge go to dryness after conditioning.

b. 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 aqueous ascorbic acid, pH = 2.0. After conditioning, do not let the cartridge go to dryness.

c. Stack the SAX cartridge on top of the Envi™-Carb tube using an adapter and place the stacked cartridges on an SPE manifold. Pass the solution from Step 2f through the stacked cartridges at a flow rate that does not exceed 180 drops per minute and allow the SAX cartridge to go to dryness. A 15-mL reservoir can be used to help load all solutions and solvents. After the sample has passed through the SAX cartridge, wash the 50-mL centrifuge tube with an additional 5-mL portion of Milli-Q® water. Sonicate the centrifuge tube for at least one minute after the water addition and vortex mix the tube to ensure solution of any residue remaining in the tube. Pass the wash through the SAX cartridge and take the SAX cartridge to dryness. Remove and discard the SAX cartridge..

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

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

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

   6 mL of 90% methylene chloride/10% methanol (v/v). Collect this fraction in a 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.*

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.

e. Concentrate the 90% methylene chloride/10% methanol solvent to 0.5 mL with nitrogen using an N-Evap at 40°C. Add 5.0 mL of ethyl acetate to the tube and concentrate the solvent to 0.5 mL with nitrogen on an N-Evap at 40°C. Add 5.0 mL of ethyl acetate and again concentrate the solvent to exactly 1.0 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.

f. 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 to condition the cartridge.

After conditioning, pass the solution from Step 3e 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.

8-mL 60% hexane/40% ethyl acetate /methanol (v/v). Collect this last fraction in a silanized 13-cc centrifuge tube and concentrate the solvent to 0.5 mL under nitrogen at 40°C using an N-Evap.

Add 5.0 mL of methanol to the centrifuge tube and concentrate the solvent to 0.5 mL. Add 5.0 mL of methanol and again concentrate the solvent to exactly 0.5 mL.

4. *HPLC Analysis*

a. Dilute the sample from Step 3f 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 24 hours 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:	30 or 40 µL depending on detector response.
Initial Flow Rate:	1.00 mL/min
Initial Flow Path:	Zorbax® SB-CN to detector (Valve Position 1, see Figure 2)
Mobile Phase A:	100% Methanol/Acetonitrile (50/50,v/v)
Mobile Phase B:	100% 10 mM KH_2PO_4 buffer, pH = 2.9
Mobile Phase C:	50% Acetonitrile/50% Milli-Q® Water (v/v)
Helium Sparge Rate:	100%
Integrator Chart Speed:	0.50 cm/min
Integrator Attenuation (2^n)	n = 0

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

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

methanol/acetonitrile (50/50, v/v), 70% 10 mM KH_2PO_4 buffer pH-2.9 (30% A/70% B) through Column I and Column II. After equilibration, the switching valve is rotated to the inject position to bring Column I on line (Column II off-line) and Column I is equilibrated for at least 1 hour at a flow rate of 1.0 mL/minute to a solvent composition of 18% methanol/acetonitrile (50/50, v/v)/82% 10 mM KH_2PO_4 buffer, pH = 2.9 (18% A/82% B). The flowrate can be increased to 2.0 mL/minute to shorten the equilibration time required for Column I if desired. Column I is left on-line with solvent passing to the detector at the start of the analysis. A diagram of the column and column-switching valve configuration is shown in Figures 2 and 3. The Zorbax® SB-CN and Zorbax® SB-C18 analytical columns are described in the HPLC Column section of this report.

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

TYPICAL SOLVENT PROGRAM

Time (Minutes)	Flow Rate (mL/min)	% A Methanol/Acetonitrile (50/50, v/v)	% B 10 mm KH_2PO_4 Buffer, pH = 2.9
Initial	1.00	18	82
12.00	2.00	50	50
22.00	2.00	30	70
32.00	1.00	30	70
49.00	2.00	18	82
59.00	1.00	18	82
63.00	0.50	18	82

TYPICAL VALVE SWITCHING PROGRAM

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

The autosampler is programmed to inject samples every 62 minutes.

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

During analysis of a set of fortified or control samples (a sample set), it is desirable but not mandatory that samples be ordered on an autosampler from low to high cymoxanil concentration. Treated samples of unknown concentration included in the sample set should be randomly ordered on the basis of expected concentration but intermixed with standards so that a standard is analyzed after every 2 or 3 treated samples. Standard concentrations should bracket the cymoxanil concentrations found in treated samples. A higher concentration standard can be included as a first sample in a following sample set or the unbracketed sample reanalyzed if necessary to achieve bracketing standard response. A cymoxanil standard should be the first and last sample analyzed in a sample set. In addition, an unfortified matrix sample and at least one fortified matrix sample should be included in the set along with four cymoxanil standards at concentrations of 0.02 (LOQ fortification), 0.04, 0.10, and 0.13 ppm. If higher cymoxanil residues are seen during analysis of a particular sample matrix, the range of standard concentrations will need to be adjusted.

Before a set of chromatographic standards are used to quantitate the amount of cymoxanil in treated or fortified samples, they should be analyzed by HPLC to ensure they have been properly prepared and provide linear response over the concentration range of interest. A minimum four-point calibration curve should be prepared. Calibration curves generated with newly

prepared standards should produce similar response (slope) and y intercept from week to week.

The following discussion describes the column-switching routine used for sample analysis in more detail. For reviews of column-switching HPLC, see References 2 and 3.

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

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

The switching valve times (the transfer window or cut window) must be determined before analysis of each set of samples. The retention time and width of the cymoxanil peak is determined by injecting the highest concentration cymoxanil standard to be included in the sample set (a 0.13-µg/mL standard) three times on Column I only. Prior to injection, the HPLC system must be equilibrated and the temperature of the HPLC column stabilized at 40.0°C. Calculate the mean retention time of the three

standard injections and accurately measure the baseline width of the cymoxanil peaks in centimeters with a ruler. Divide the peak width by the integrator chart speed expressed in cm/minute to determine the peak width in minutes. The first valve switching time is then calculated as:

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

The second valve switching time is calculated as:

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

The figure of 0.20 minute is used to assure that the cymoxanil collection window is set conservatively enough to collect the entire analyte peak. The retention times of the cymoxanil injections on Column I must not differ by more than a relative standard deviation of 1.5% (RSD = Std. Dev./mean x 100) in order for analysis to proceed.

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

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

Peak Width:	0.50 cm
Chart Speed:	0.50 cm/minute
Peak Width (Minutes):	0.50 cm/0.50 cm/min = 1.0 minute

1.0 minute/2 + 0.20 minute = 0.70 minute (half the peak window width).

8.57 minutes - 0.70 minute = 7.87 minutes (lower window setting)
8.57 minutes + 0.70 minute = 9.27 minutes (upper window setting)

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

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

Tomato paste samples require column switching during sample analysis but it was possible to analyze grape and tomato fruit samples directly on the SB-CN column without the need for column switching. This shortens the analysis time for these samples significantly. The following representative solvent and autosampler program were entered into the Waters 600E controller.

TYPICAL SOLVENT PROGRAM

Time (Minutes)	Flow Rate (mL/min)	% A Methanol/ Acetonitrile (50/50, v/v)	% B 10 mM K_2HPO_4 Buffer, pH = 2.9
Initial	1.00	18	82
15.00	2.00	50	50
25.00	2.00	18	82
35.00	1.00	18	82
36.00	0.50	18	82

TYPICAL AUTOSAMPLER PROGRAM

Step	First Vial	Last Vial	# of Inj	Inj Vol µL	Run Time
1	1	12	1	40	13.00
2	13	13	1	40	37.00

Note: *The solvent program has been arranged to wash the HPLC SB-CN column with strong solvent when the analysis of a set of samples has been completed(analysis of sample 13 in the above example, injection of solvent only). This shortens the total analysis time vs. washing the column with strong solvent after injecting each sample. While the method has been tested with a number of different grape matrices and different lots of Zorbax® SB-CN columns, it has not been possible to inject enough different grape or tomato 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-CN columns or guard columns. Peak broadening and the appearance of interferences in the chromatograms of standards is an indication that the HPLC column may need replacement.*

Special Note: ***Sufficient resolution has been obtained with 15-cm analytical HPLC columns to allow quantitation of cymoxanil residues in grapes and tomato fruit samples. While an effort has been made to look at different grape samples, matrix peaks might appear that interfere with cymoxanil. If this happens, a 25-cm analytical column should be used to resolve interferences and if necessary, the column should be washed after each injection with strong solvent (50% methanol/50% acetonitrile (v/v)/50% 10 mM KH_2PO_4 buffer, pH = 2.9 [50% A/50% B]). The column is then reequilibrated to starting conditions (18% A/82% B). If interferences are still not resolved, column-switching HPLC can be used for sample analysis. In addition, the transfer or cut window can be reduced from 1.4 minutes to as little as 0.4 minute (heart-cutting analysis) if necessary. Note if heart-cutting analysis is employed, the internal diameter of the 10-cm tubing used as the By-pass Loop in Figure 3 should be reduced from 0.01-inch ID to 0.005-inch ID and the Relative Standard***

Deviation of standards injected prior to analysis of a set of samples should not exceed 1.0%. HPLC injection volumes will also need to be increased to achieve proper sensitivity. Tomato (fruit) samples have been successfully analyzed using the column-switching procedure described in this report without the need to use heart-cutting.

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 during analysis of each set of fortified or treated 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 ppm of cymoxanil found in each sample.

$$\text{ppm Cymoxanil Found} = \text{Least Squares Concentration } (\mu g/mL) \times \frac{\text{Injection Volume of Standard } (\mu L)}{\text{Injection Volume of Sample } (\mu L)} \times \frac{\text{Final Sample Volume (mL)}}{\text{Sample Weight (g)}} \times \text{Aliquot Factor}$$

The Aliquot Factor was calculated for each sample by dividing the volume of extract measured in Step 2c of the Analytical Procedure by the aliquot volume used for each sample (40 mL). The % recovery for *fortified* control samples were calculated according to the following equation correcting for interferences, if any, seen in unfortified controls.

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

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

Example Calculations

The linear regression equation f(x) = Mx + B defined by analysis of cymoxanil standards analyzed during analysis of fortified red wine grapes (see Figure 4) is $f(x) = 6.495 \times 10^{2}x + 4.095 \times 10^{-1}$.

Substituting a peak height value of 24 mm observed for red grape sample #8 (a 0.10-ppm fortification) and solving for x as follows:

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

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

$$x = \frac{f(x) - 4.095 \times 10^{-1}}{6.495 \times 10^{2}}$$

$$x = \frac{24 - 4.095 \times 10^{-1}}{6.495 \times 10^{2}}$$

gives a cymoxanil Least Squares Concentration of 0.0363 µg/mL.

For analysis of red wine grape samples 30-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 six-gram sample was extracted to give a final extract volume of 210 mL and a 40-mL aliquot was carried through the analytical work-up (Aliquot Factor = 210/40). Substituting these values in the equation for ppm cymoxanil found gives:

$$\text{ppm Cymoxanil Found} = 0.0363\ \mu g/mL \times \frac{30\ \mu L}{30\ \mu L} \times \frac{3\ mL}{6\ g} \times \frac{210\ mL}{40\ mL}$$

ppm Cymoxanil Found = 0.0953 µg/g or 0.0953 ppm

Red grape sample #8 was fortified with 0.10 ppm of cymoxanil. No interference was observed in the control sample. The Percent Recovery was calculated as:

$$\%\ \text{Recovery} = \frac{0.0953 - 0}{0.10} \times 100 = 95.3\%\ \text{Recovery}$$

F. *Results and Discussion*

1. Recovery Results

Samples of red and white wine grapes, tomato fruit, and tomato paste were each fortified with cymoxanil at 0.05, 0.10, and 0.30 ppm and analyzed by HPLC using the method described in this report. Detector response was linear over the range of

standards analyzed during the analysis of each matrix. For a representative standard curve generated during analysis of red wine grape samples, see Figure 4. White and red grape and tomato fruit samples were sufficiently clean after sample workup that they could be analyzed directly using a SB-CN HPLC column. Column switching was unnecessary for these samples. However, tomato paste samples had to be analyzed using column switching HPLC. Tables 1 and 2 summarize recovery data for grape and tomato samples, respectively. Recoveries were acceptable over the fortification range examined (0.05 ppm, the LOQ; 0.10 ppm, 1X the proposed tolerance; and 0.30 ppm, 3X the proposed tolerance). The average recovery (± the standard deviation) for all fortified samples at all fortification levels for all sample matrices was 94.9% ± 7.5%. The Relative Standard Deviation was 7.9%. Recoveries for all samples ranged from a low of 76.3% to a high of 112.4%.

2. *Sample Chromatograms*

Appendix II shows typical chromatograms for unfortified control samples of red and white grapes, tomatoes, and tomato paste. Also shown are typical chromatograms for a 0.10-ppm cymoxanil fortification in each of these matrices. This fortification corresponds to the tolerance level proposed for each matrix. In all cases, no interfering peaks were observed in the unfortified controls.

3. *Determination of the LOQ*

Data collected during validation were examined to estimate the Limit of Quantitation (LOQ) for cymoxanil residues in grapes and tomatoes. 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 4). Examination of the chromatograms of unfortified grape and tomato samples shows that a 0.05-ppm fortification produces a signal approximately 10X that of unfortified controls. Acceptable recoveries were also obtained. As a result, the analytical method can achieve an LOQ of 0.05 ppm in grapes and tomato samples.

4. *Extraction Efficiency*

The metabolism of cymoxanil has been investigated in grapes treated under field conditions with eight applications of

[2-^{14}C]labeled cymoxanil (DuPont Report No. AMR 2181-91, submitted as part of the tolerance petition for cymoxanil, Reference 5). An exaggerated application rate of 210 g ai/ha was used for each treatment. Eighteen days after the last (eighth) application approximately 2 ppm of total carbon 14 expressed as cymoxanil equivalents remained in the grapes. Approximately 5% of this total was extractable into ethyl acetate, 65% was extractable into water, and 31% was unextractable. Characterization showed parent cymoxanil was present in the ethyl acetate extract only. The level declined from 0.23 ppm immediately after the last field application to 0.05-ppm eighteen days after the last (eight) treatment. The minimum label proposed PHI for treatment of grapes is 7 days.

Characterization of ethyl acetate extracts from grapes harvested 10 days after the eighth cymoxanil field treatment showed 0.11 ppm of residue was present (4.6% of the 2.4 ppm of total ^{14}C residue determined by combustion analysis). Of this, cymoxanil comprised 0.10 ppm or 91% of the total. Characterization of an aqueous extract of the same grape sample showed 1.55 ppm of residue (65% of the total) was distributed among several natural products-glycine 23.0%, amino acids 5.6%, acetic acid 9.2%, fatty acids 2.0%, polycarboxylic acids 6.3%, sugars 14.4%, and lignin materials 4.0%. No intact cymoxanil was found.

Because ethyl acetate extraction was shown to recover intact cymoxanil with high efficiency from grapes containing ^{14}C-incorporated residues during a period in which these residues declined rapidly, ethyl acetate extraction was adopted for use in this analytical method.

5. *Interferences/Method Ruggedness*

Examination of chromatograms of all sample matrices indicated that interferences are no higher than baseline noise at the retention time corresponding to cymoxanil. A single workup is used to prepare all matrices for analysis. HPLC conditions were varied depending on the matrix analyzed. The method provides a consistent approach for analysis of grape and tomato matrices.

6. *Time for Sample Preparation*

At least three matrix 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 several people who contributed to completion of this work. Chuck Powley provided references to his work combining SAX and C-18 SPE cartridge purification. Sheldon Sumpter and Brock Peterson provided a copy of their AMR describing work with column-switching HPLC. Richard Trubey helped us analyze problems encountered during various stages of method development. Frank Behmke handled sample extraction and preparation. Without his help, this report would have been significantly delayed. Miriam Carr and Carol Ashman typed and prepared the final report. Their expert help made this project much easier to complete.

III. CERTIFICATION

ANALYTICAL METHOD FOR THE DETERMINATION OF RESIDUES OF CYMOXANIL IN GRAPES AND TOMATOES USING COLUMN SWITCHING LIQUID CHROMATOGRAPHY

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

Revision No. 1 by:

Edward C. Nathan
Study Director

14-Nov-1995
Date

Approved by:

Sidney S. Goldberg
Research Supervisor

14-Nov-95
Date

Date Study Completed:

Original Report: October 6, 1994
Revision No. 1: November 14, 1995

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TABLE I
RECOVERIES FOR FORTIFIED RED GRAPE SAMPLES

Sample Description	PPM Found	% Recovery
Control Red Grapes	none	--
Control Red Grapes	none	--
0.050-ppm Fortification	0.046	92.8
0.050-ppm Fortification	0.051	101.3
0.10-ppm Fortification	0.095	95.3
0.10-ppm Fortification	0.095	95.3
0.30-ppm Fortification	0.281	93.8
0.30-ppm Fortification	0.281	93.8

Mean = 95.4
Standard Deviation = 3.1%
% RSD = 3.2
n = 6

RECOVERIES FOR FORTIFIED WHITE GRAPES SAMPLES

Sample Description	PPM Found	% Recovery
Control White Grapes	none	--
Control White Grapes	none	--
0.050-ppm Fortification	0.049	99.3
0.050-ppm Fortification	0.042	85.5
0.10-ppm Fortification	0.090	90.0
0.10-ppm Fortification	0.090	90.4
0.30-ppm Fortification	0.228	76.3
0.30-ppm Fortification	0.279	93.2

Mean = 89.1
Standard Deviation = 7.7%
% RSD = 8.7
n = 6

TABLE II
RECOVERIES FOR FORTIFIED TOMATO FRUIT SAMPLES

Sample Description	PPM Found	% Recovery
Control Tomatoes	none	--
Control Tomatoes	none	--
0.050-ppm Fortification	0.048	97.7
0.050-ppm Fortification	0.048	96.4
0.10-ppm Fortification	0.087	87.2
0.10-ppm Fortification	0.090	90.7
0.30-ppm Fortification	0.286	95.6
0.30-ppm Fortification	0.286	85.5

Mean = 92.2
Standard Deviation = 5.1%
% RSD = 5.6
n = 6

RECOVERIES FOR FORTIFIED TOMATO PASTE SAMPLES

Sample Description	PPM Found	% Recovery
Control Tomato Paste	none	--
Control Tomato Paste	none	--
0.050-ppm Fortification	0.052	105
0.050-ppm Fortification	0.056	112.4
0.10-ppm Fortification	0.102	101.6
0.10-ppm Fortification	0.094	94.1
0.30-ppm Fortification	0.306	102.2
0.30-ppm Fortification	0.306	102.2

Mean = 102.9
Standard Deviation = 5.9%
% RSD = 5.7
n = 6

FIGURE 1
UV SPECTRUM OF CYMOXANIL

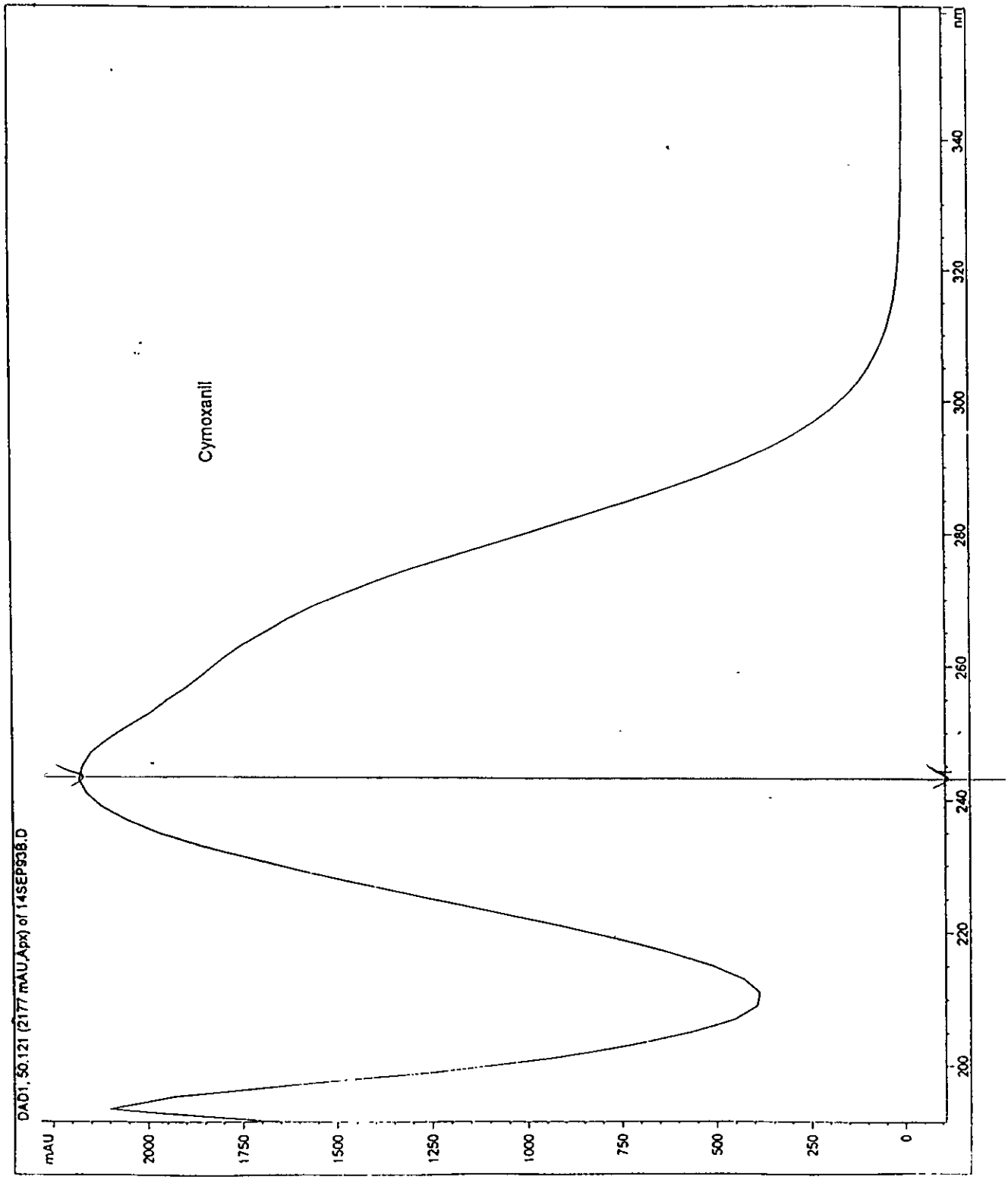
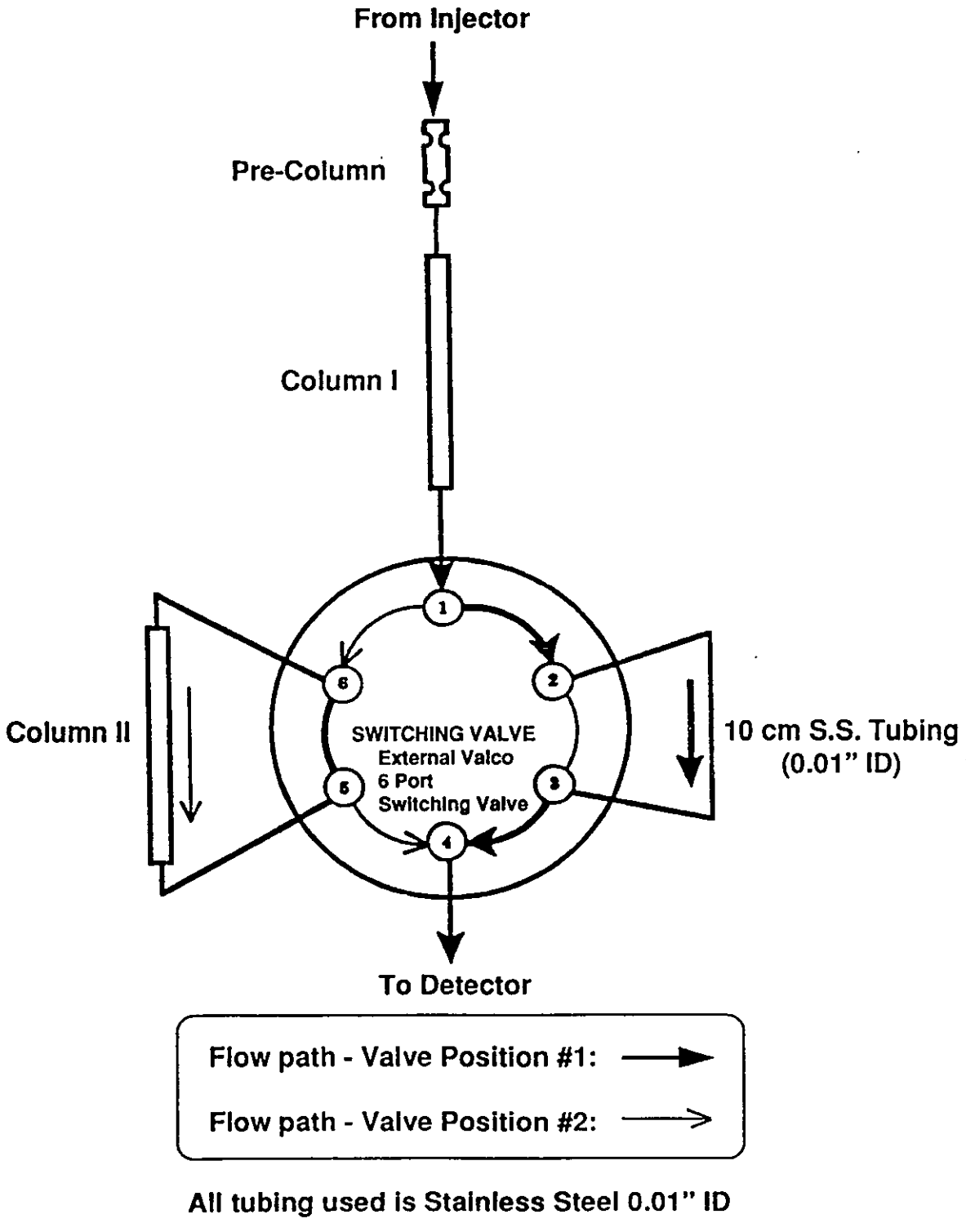


FIGURE 2
PLUMBING DIAGRAM FOR COLUMN SWITCHING



All tubing used is Stainless Steel 0.01" ID

FIGURE 3
VALVE POSITIONS FOR COLUMN SWITCHING

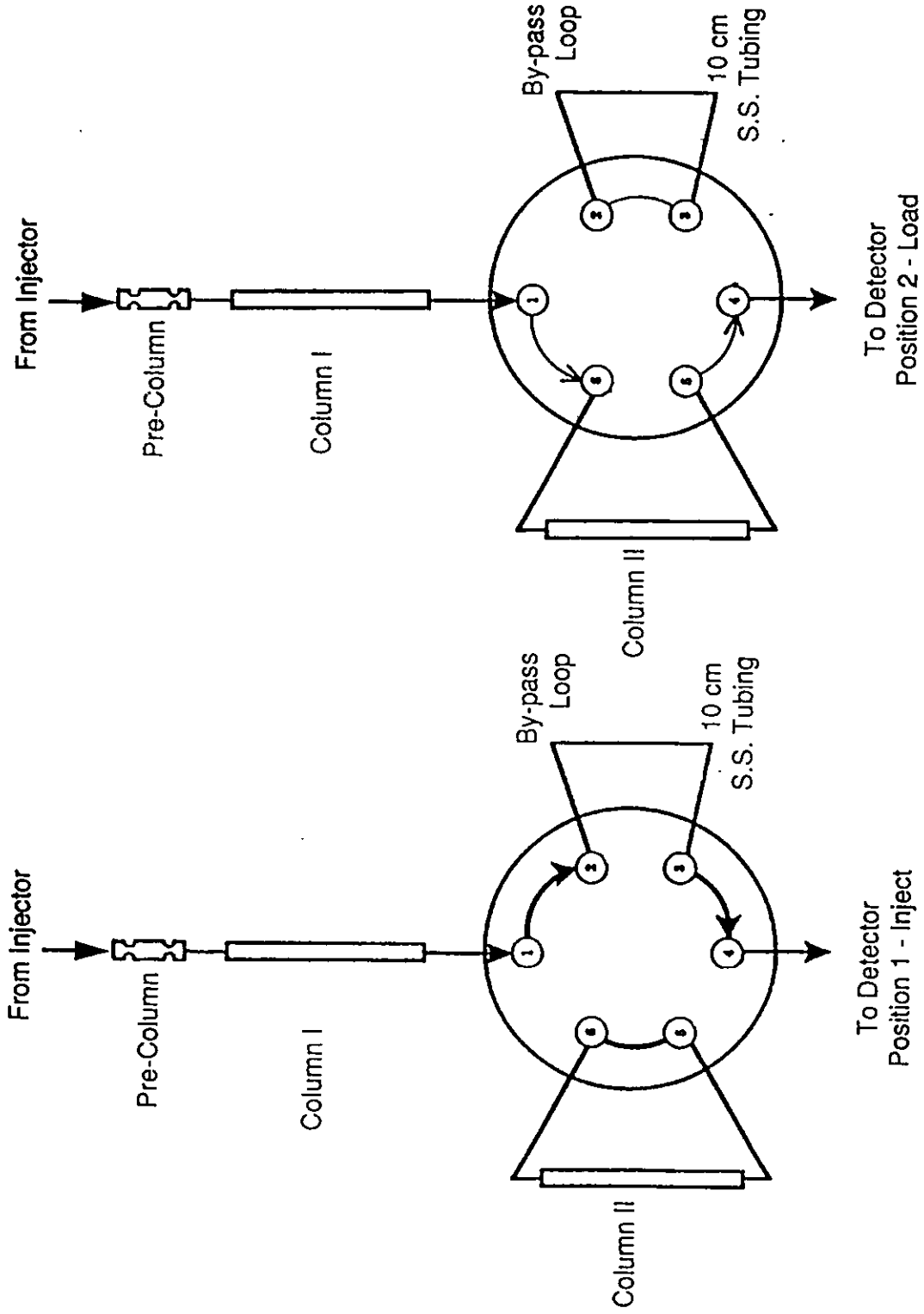
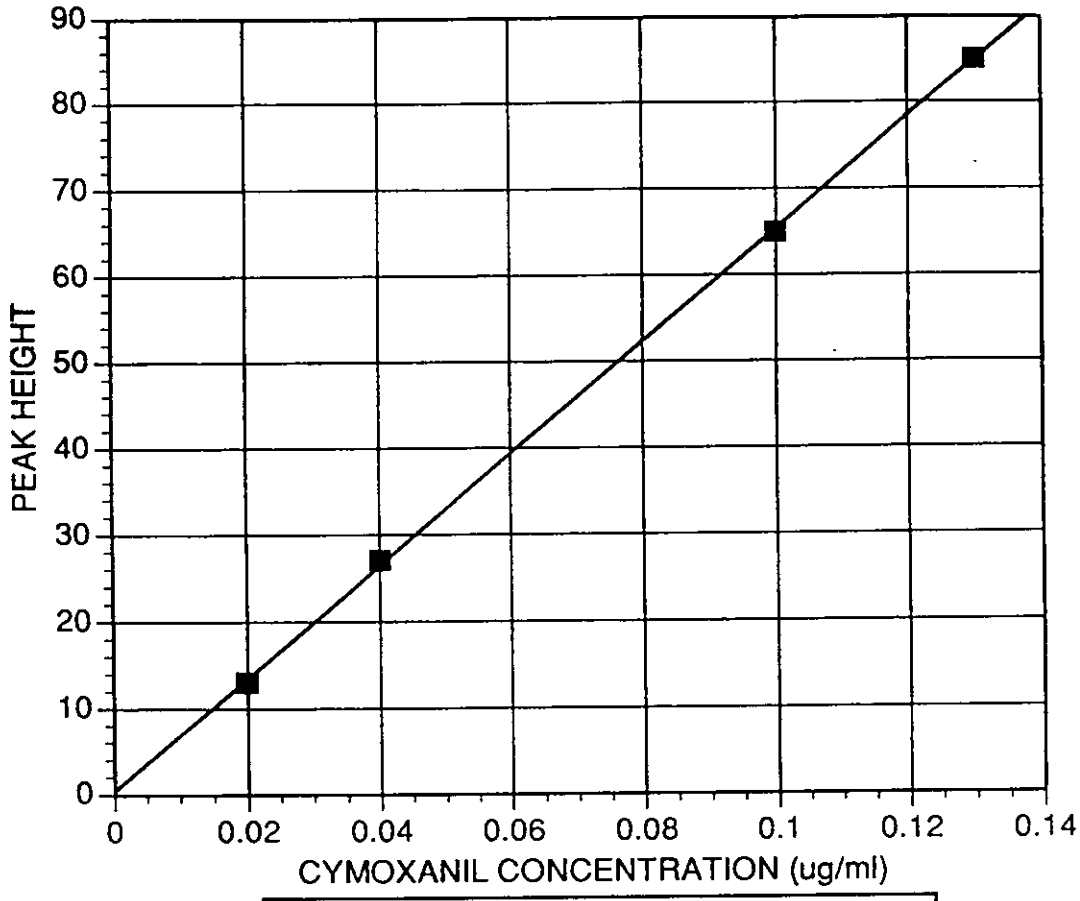


FIGURE 4
STANDARD CURVE - ANALYSIS OF RED WINE GRAPE SAMPLES - SEPTEMBER 7, 1994



SAMPLE #	T3217 CONC (ug/ml)	PEAK HT
1	0.02	13
5	0.04	27
9	0.1	65
12	0.13	85

ANALYSIS OF T3217 STANDARDS ANALYZED DURING ANALYSIS OF RED WINE GRAPES SAMPLES ON SEPTEMBER 7, 1994

f(x) = 6.495238E+2*x + 4.095238E-1 R^2 = 9.997936E-1

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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.

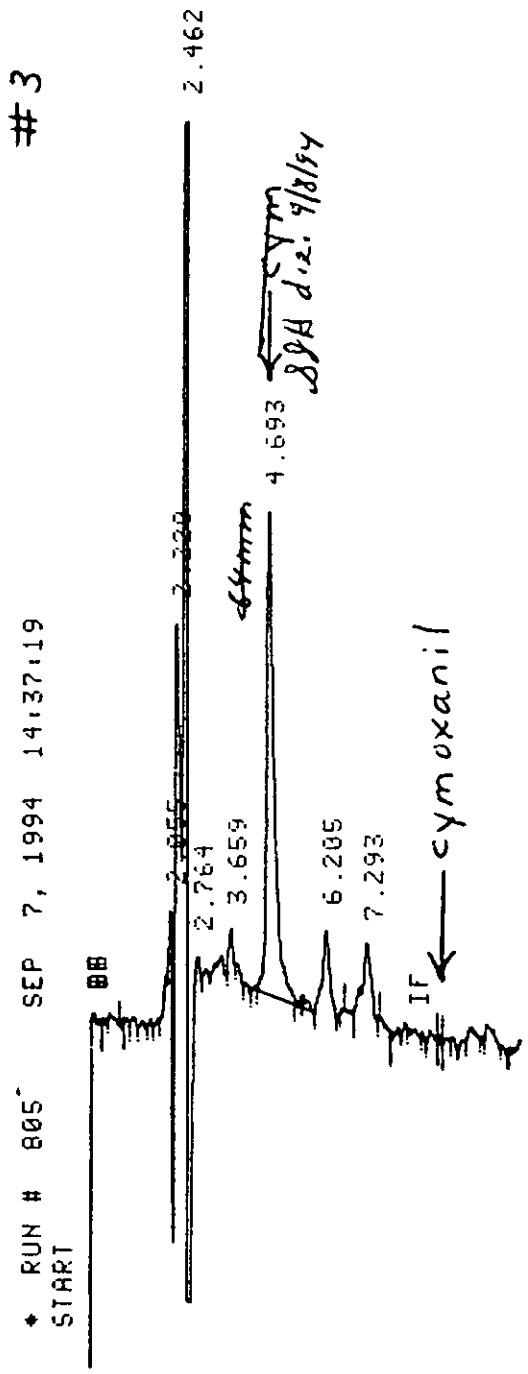
- Let the solution stand for 1 or 2 minutes, pour the solution back into a storage bottle.
- Rinse glassware with toluene; discard wash as waste.
- Rinse glassware with methanol; discard wash as waste.
- Rinse glassware with water; discard wash as waste.
- Rinse glassware with methanol; discard wash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

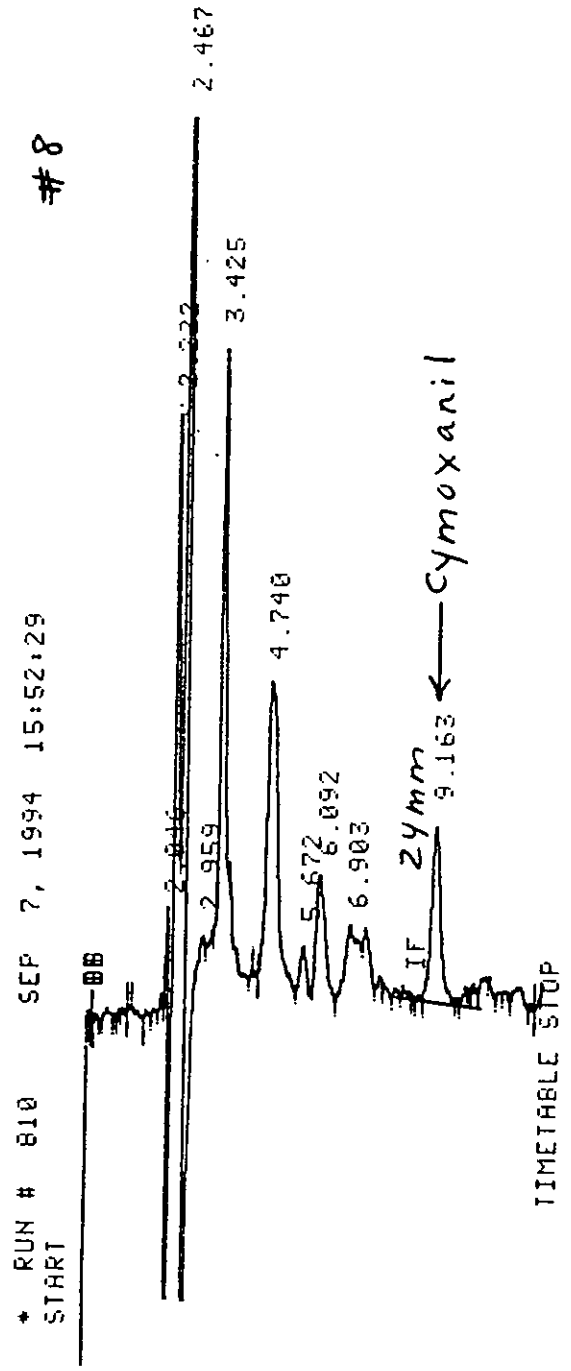
Dichlorodimethylsilane (No. 83410) can be purchased from Pierce Chemical Company, Rockford, Ill. HPLC grade Toluene (No. 9351-03) is purchased from J. T. Baker, Inc., Phillipsburg, N.J.

APPENDIX II
REPRESENTATIVE CHROMATOGRAMS

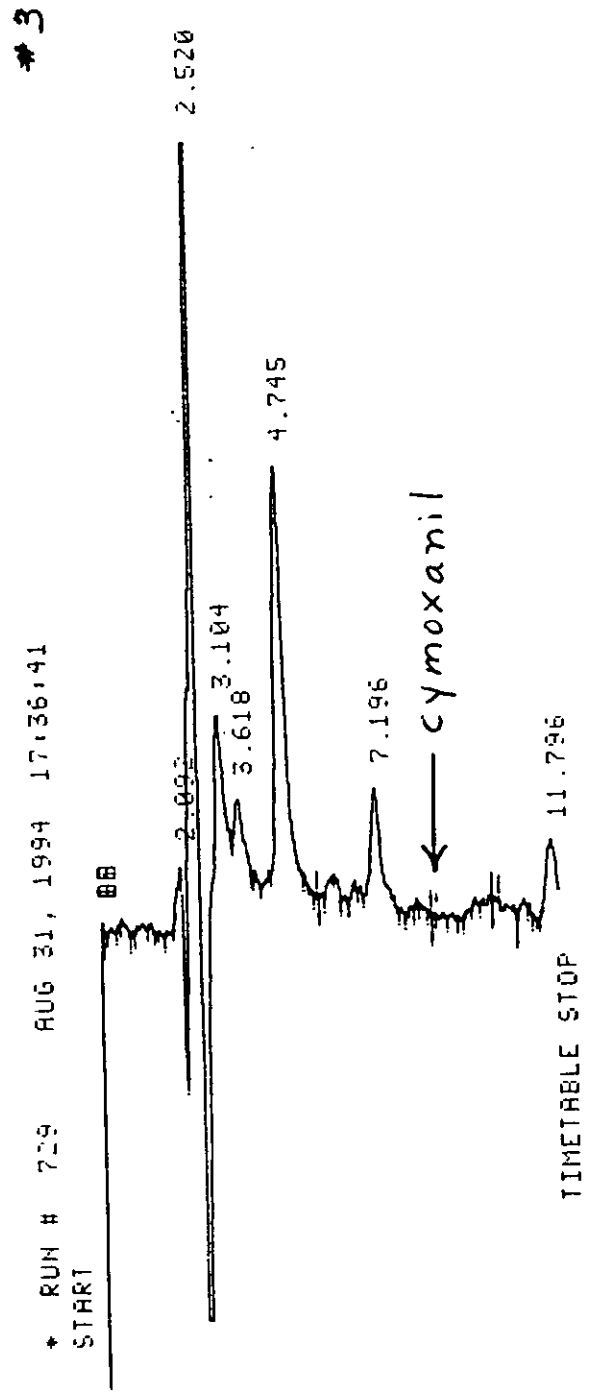
SAMPLE #3 INJECTION
RED WINE GRAPES - UNFORTIFIED



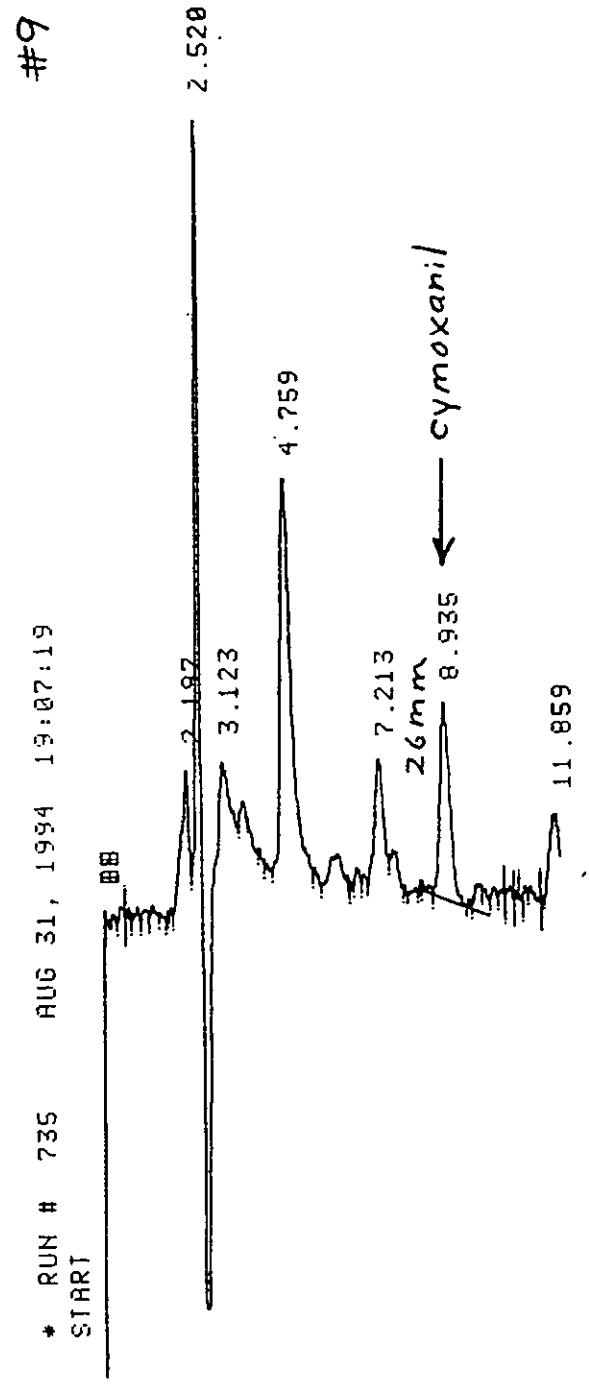
SAMPLE #8 INJECTION
RED WINE GRAPES FORTIFIED WITH
0.10 PPM OF CYMOXANIL



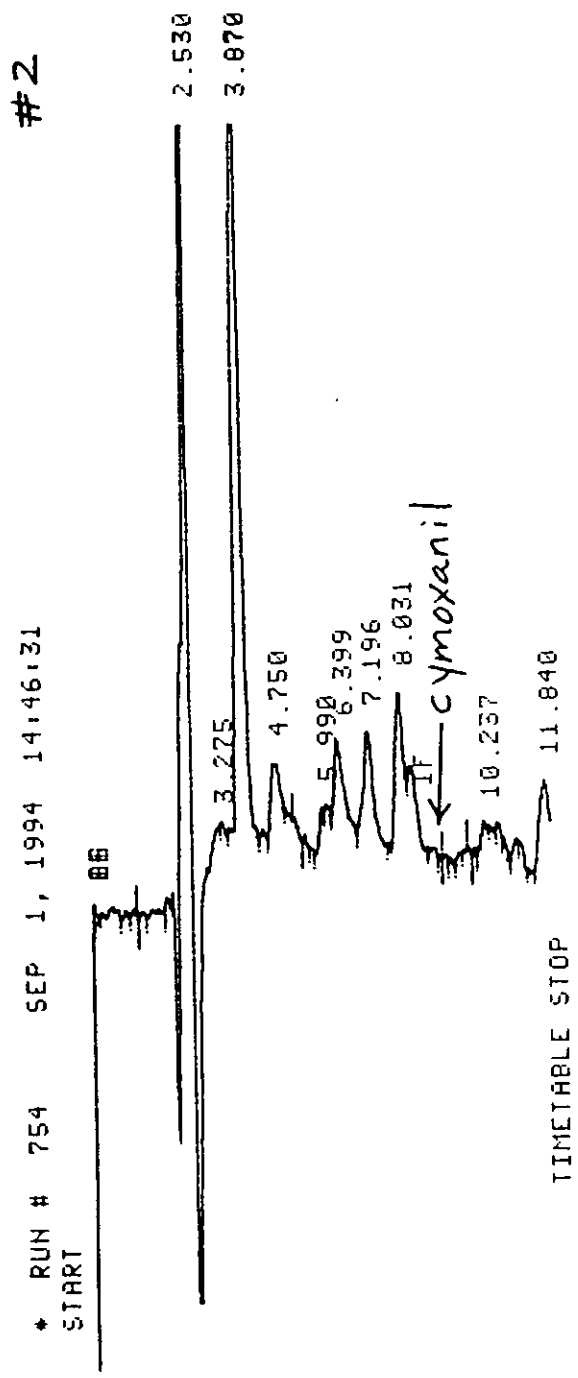
SAMPLE #3 INJECTION
WHITE WINE GRAPES - UNFORTIFIED



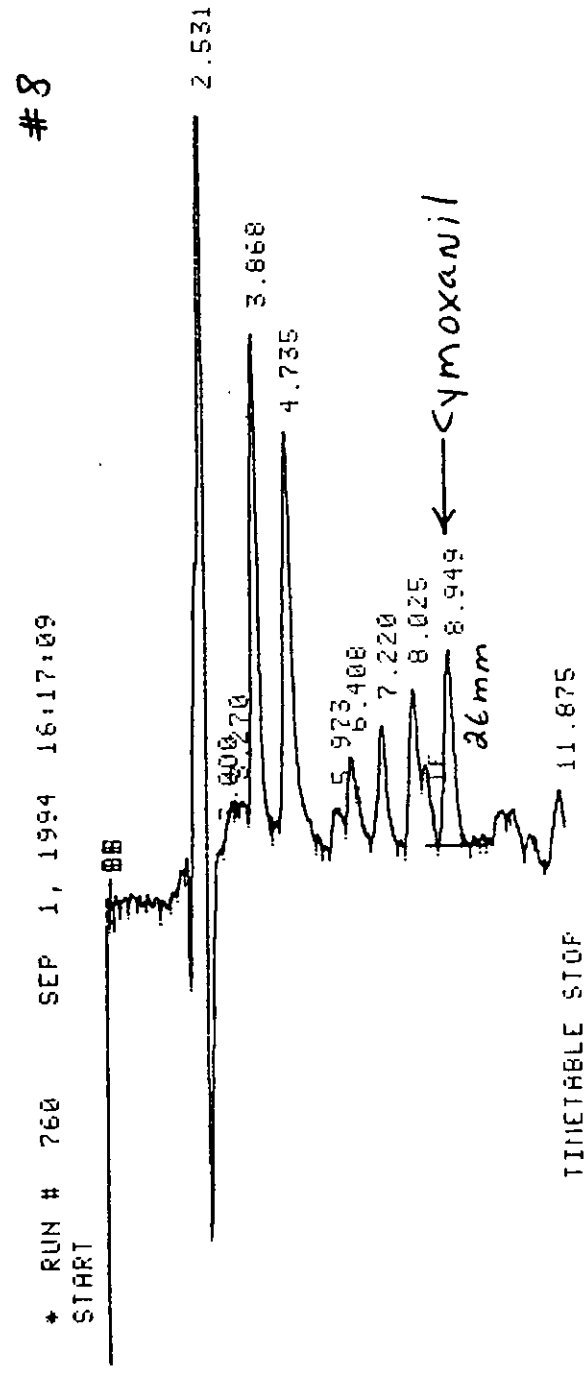
SAMPLE #9 INJECTION
WHITE WINE GRAPES FORTIFIED WITH
0.10 PPM OF CYMOXANIL



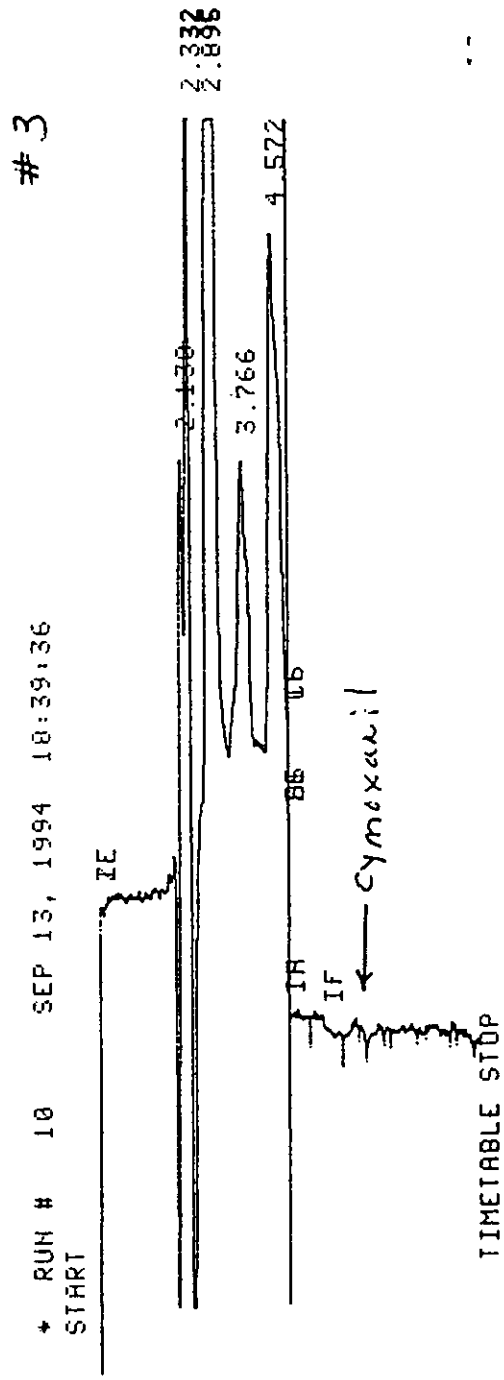
SAMPLE #2 INJECTION
TOMATOES - UNFORTIFIED



SAMPLE #8 INJECTION
TOMATOES FORTIFIED WITH
0.10 PPM OF CYMOXANIL



SAMPLE #3 INJECTION
TOMATO PASTE - UNFORTIFIED



SAMPLE #8 INJECTION
TOMATO PASTE FORTIFIED WITH
0.10 PPM OF CYMOXANIL