A HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE DETERMINATION OF BENOMYL (AS MBC), MBC, AND 2-AB RESIDUES IN SOIL

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INTRODUCTION

Scope

This report describes an analytical method for determining residues of benomyl and its major metabolites in soil, MBC (carbendazim) and 2-amino-benzimidizole (2-AB). Benomyl is the active ingredient in Du Pont's Benlate® Fungicide. The chemical names and structures of benomyl, MBC, and 2-AB are given in Figure 1.

Analytical methods have been described previously for determining residues of benomyl in soil [Kirkland et al. (1) and Pease and Gardiner (2)]. This method has been developed because the earlier methods yielded low, erratic recoveries for the determination of 2-AB.

Soil from Illinois, North Dakota, California, Florida, Louisiana, and Arkansas were examined during method development. Characteristics of these soils are presented in Table I.

PRINCIPLES

A high-performance liquid chromatographic (HPLC) method with ultraviolet (UV) detection is used to measure residues of total benomyl (as MBC), MBC, and 2-AB in soil. The method involves extracting benomyl and its metabolites, MBC and 2-AB, from soil in a mixture of acetonitrile/ammonium hydroxide, removing the solvent by rotary evaporation, and dissolving the residue in mobile phase. Some soil samples require additional cleanup during the extraction step. The analyte of interest is then determined by HPLC.

The method hydrolyzes any residual benomyl to MBC. Residues are then quantified as MBC by reference to a standard curve and converted to benomyl equivalents using the molecular weight factor of 1.53. Free 2-AB present in the sample is simultaneously determined as a separate peak and quantified using a standard curve. The lower limit of detection (LOD) is 10 ppb for each of these components. The limit of quantitation (LOQ) is 50 ppb for each component.

Method development was conducted at E. I. du Pont de Nemours and Company and Morse Laboratories. The recovery study included approximately 400 soil samples from eight test sites; more than ten operators conducted recoveries during method validation. Fortifications ranged from 0.01 ppm (10 ppb) to 500 ppm. The average recovery of benomyl (as MBC) in soil was 84% with a standard deviation of 12% (272 samples); the average recovery of MBC in soil was 84% with a standard deviation of 7% (36 samples), and the average recovery of 2-AB in soil was 83% with a standard deviation of 14% (86 samples). Tables II-IV show fortification levels and individual recoveries.

SAFETY AND HANDLING

Consult MSDS prior to making stock solution.

MATERIALS AND METHODS

The following sections include a list of suggested equipment and reagents and recommendations for preparing stock solutions and standards. During all analyses, equivalent apparatus, solvents, glassware, or techniques (such as sample concentration) may be substituted for those specified in the method.

Equipment

1. Tekmar Tissuemizer®, Model #SDT182EN (Tekmar Co., Cincinnati, Ohio).
2. Sorvall® Centrifuge, Model #RC3B (Sorvall® Instruments, Du Pont Co., Wilmington, Del.).

3. Brinkmann Rotovapor RE11 rotary evaporator (Brinkmann Instruments, Inc., Westbury, N.Y.).
4. Hewlett Packard Liquid Chromatograph, Model 1090 (Hewlett Packard, Avondale Division, Avondale, Penn.) equipped with a diode array detector.
5. Spectra 100-2 UV Detector with Isochrom Pump or equivalent instrument.

Reagents and Solutions

All reagents are analytical-grade, HPLC-grade or better.

1. Reagent-grade ammonium hydroxide (J. T. Baker, Inc., Phillipsburg, N.J.).
2. Glass-distilled acetonitrile, Omnisolv (EM Science, Div. of EM Industries, Inc., Gibbstown, N.J.).
3. Milli-Q® water (Millipore Corp., Bedford, Mass.).

Standards

The 2-AB standard (compound B572), supplied by Kodak Chemical Co., Rochester, N. Y., had a chemical purity of approximately 95%. Du Pont Agricultural Products, E.I. Du Pont de Nemours and Co., Wilmington, Del., supplied the benomyl and MBC standards (approximately 99% chemical purity).

Stock Solutions and Fortifications

Prepare the benomyl and 2-AB stock solutions (100 µg/mL) by accurately weighing out 10.0 mg of either benomyl or 2-AB into a 100-mL volumetric flask. Dissolve the compound in acetonitrile and bring the flask to volume with the same solvent. To prepare the MBC stock solution (100 µg/mL), accurately weigh out 10.0 mg of MBC into a 100-mL volumetric flask. Bring to volume with 0.1 N phosphoric acid. Prepare a 10-µg/mL solution for each analyte by taking a 5-mL

aliquot of the 100-µg/mL stock solution and bringing to volume in a 50-mL volumetric flask. These stock solutions are used for preparing the standard dilutions.

To prepare a standard curve, dilute appropriate quantities of either the MBC or 2-AB stock solution with mobile phase to achieve the desired concentration. To fortify control samples, combine 20 mL of either the benomyl, MBC, or 2-AB stock solution at the appropriate concentration with 20 g of control soil. Standards should cover the expected concentration range. Refrigerate standards and stock solutions at 4°C when not in use. The standard solutions should be stable for at least two weeks if stored at 4°C.

Sample Preprocessing

Segment soil cores and transfer to sample containers. Break up soil into uniform particles, mix well, and sieve to remove rocks and organic materials. After sieving, weigh samples for extraction and moisture determination. Fine soils containing a large amount of organic debris may have to be screened first through a large-mesh screen and again through a smaller-mesh screen. Guidelines for screening soil samples are shown below:

SOIL	MESH	OPENING SIZE
0-10 cm samples	No. 45 mesh screen	355 mm opening, U. S. standard
10-20 cm samples and lower	No. 12 mesh screen	1.651 mm opening, U. S. standard
damp soil	No. 8 mesh screen	2.362 mm opening, U. S. standard

Analytical Procedure

The steps involved in the analytical procedure are outlined in the following sections. Steps marked with an asterisk (*) indicate a suitable stopping point as the solution is stable overnight.

Preparation of Extraction Solution. Prepare fresh extraction solvent (10% NH_4OH in acetonitrile) for sample extraction by mixing a 90:10 solution of HPLC-grade acetonitrile and reagent-grade ammonium hydroxide. This mixture should be used within two hours.

Soil Extraction and Fortification. Weigh 20 grams of soil into a 250-mL centrifuge bottle for each analysis. (Note: For recovery studies, fortify control samples at this point with the desired concentration of either benomyl, MBC, or 2-AB. Freshly fortified samples are analyzed concurrently with other soil samples.) Add 50 mL of the extraction solution to the centrifuge bottle and tissuemize at 50% power for 30 seconds. Allow the contents to settle and then decant the extract into an appropriate centrifuge bottle. Repeat the extraction with fresh solvent three more times.* Centrifuge the combined extracts for 10 minutes at 3000 rpm.*

Filtering and Transfer. Decant clear extract into a 500-mL evaporating flask.* If necessary, use #541 Whatman filter paper to obtain a clear extract; rinse the filter before filtration with ± 20 mL of pure acetonitrile. Rotary evaporate the combined extract to between 2 to 10 mL (do not take to dryness) at a bath temperature of <40°C.* Unless sample requires additional cleanup following extraction, continue with the steps outlined below.

Transfer the concentrated extract to a properly labeled and calibrated test tube containing ± 0.5 mL pH 3.5 sodium phosphate buffer. Use no more than 3 mL of acetonitrile for rinsings and transfers. Evaporate the acetonitrile over a

<40°C water bath to less than 1 mL (again, do not take to dryness) and adjust the final volume to 2 mL with mobile phase. Mix well using a vortex mixer; the sample is now ready for HPLC analysis. (1 mL = 10 g of sample.)

Sample Cleanup. Some samples may require additional cleanup prior to HPLC analysis. If this is necessary, follow the procedures outlined in this section. Add ± 5 mL of 0.1 N H_3PO_4 to the concentrated extract and rotovap *gently* to remove the acetonitrile. A steam bath can be used for additional evaporation to insure complete removal of acetonitrile. Transfer the acidic, aqueous extract to a 50-mL centrifuge tube using additional 0.1 N H_3PO_4 rinses to bring the final volume to approximately 10 mL. Insure the acidity of the aqueous extract with wide-range pH paper (pH 1-12). The pH should be about 3.5.

Treat acidic extract with 10-mL rinses of nanograde hexane (up to four times). Decant and discard all hexane washes (top layer). Centrifuge if necessary. (Note: Use each 10-mL hexane portion to rinse original sample flasks.) Remove all traces of residual hexane using the steambath or a gentle stream of nitrogen.*

(Note: MBC and 2-AB are unstable in basic solution at this stage. Work with one sample at a time from this point on. Ethyl acetate extractions have to be completed as quickly as possible.)

After hexane clean-up, make the acidic extract which is in the centrifuge tube basic (pH 11 - 12.5) using 6 N NaOH. Return the base adjusted extract to the original sample flask to dissolve any matrix residue not soluble in the acid phase. Return the basic extract to the centrifuge tube, and extract with 10 mL of water-saturated, HPLC-grade ethyl acetate (four to five times).

Pipet upper ethyl acetate layer into a 125-mL evaporating flask through a layer of Na_2SO_4. Rinse Na_2SO_4 with ± 5 mL ethyl acetate. Rotary evaporate

combined ethyl acetate extracts using *gentle* pressure (or use steambath) until volume is approximately 2 mL.

Transfer extract to a properly labeled and calibrated test tube containing ± 0.5 mL 0.1 N H_3PO_4. Use no more than 3 mL ethyl acetate for rinsings and transfers. Remove ethyl acetate using a water bath at <40°C. Adjust the final volume of the extract to 2 mL with 0.1 N H_3PO_4. Mix well using the vortex mixer and analyze using HPLC.*

HPLC Procedure. Bring the sample residue to volume (2 mL) with mobile phase. Use a 5-mL syringe to filter 2 mL of the sample through a 0.45-µm Gelman Acrodisc® filter assembly (CR #4219) into a 2-mL autosampler vial. Seal the vial using a crimper (use Teflon® caps). Sample is now ready for analysis. The following is a list of instruments used for this method development work. The instrument conditions represent nominal values. Any instrument with at least a dual pumping system and UV detector capable of achieving the following conditions should be acceptable.

Instrument :	Spectra Physics 100-2 UV Detector with IsoChrom Pump or equivalent instrument
Column:	Zorbax® CN (25 cm x 4.6 mm), 5.0 micron particle size
Column Temperature:	Ambient
UV Wavelength	280 nm
Mobile Phase:	8% acetonitrile in 0.05 M sodium dihydrogen phosphate at pH 3.5
Flow Rate:	1 mL/min

Standard Curves. Inject suitable concentrations of the appropriate standards into the HPLC to construct a standard curve based on peak height (in mm) of standards. Peaks below 100 mm are measured to the nearest 0.5 mm.

Peaks above 100 mm are measured to the nearest millimeter. Area measurements can be substituted if acceptable precision is obtained.

Methods of Calculation. Determine residues of benomyl (as MBC), MBC, and 2-AB in soil samples by comparing the chromatographic peak height of the analyte with the corresponding peak height for standards of known concentration. The amount of MBC present in the sample is calculated using the following equation:

1) $$\text{ppm MBC} = \frac{\text{ng MBC}}{\text{mg sample}}$$

where,

ppm MBC	=	parts per million of MBC in the sample
ng MBC	=	ng MBC from the standard curve based on peak height response of the sample extract
mg sample	=	mg sample extract injected into the HPLC

Benomyl equivalents are calculated using the following equation:

2) $$\text{ppm benomyl equivalents} = \frac{\text{ng MBC x 1.53}}{\text{mg sample}}$$

where,

ppm benomyl equivalents	=	parts per million of benomyl in the sample
ng MBC	=	ng MBC from the standard curve based on peak height response of the sample extract
mg sample	=	mg sample extract injected into the HPLC
1.53	=	conversion factor from MBC to benomyl, $\left(\frac{\text{Molecular Weight of benomyl}}{\text{Molecular Weight of MBC}}\right)$

The following equation is used to calculate the levels of 2-AB:

3) $$\text{ppm 2-AB} = \frac{\text{ng 2-AB}}{\text{mg sample}}$$

where,

ppm 2-AB	=	parts per million of 2-AB in the sample
ng 2-AB	=	ng 2-AB from the standard curve based on peak height response of the sample extract
mg sample	=	mg sample extract injected into the HPLC

The percent recovery for fortified samples is calculated as follows:

$$\% \text{ Recovery} = \frac{[\text{ppm analyte found in spike}] - [\text{ppm analyte found in control}]}{\text{fortification level}} \times 100$$

The percent moisture present in the samples can be calculated as follows:

4) $$\% \text{ Moisture} = \frac{\text{gm initial weight} - \text{gm final weight}}{\text{gm sample weight}} \times 100$$

where,

gm initial weight	=	weight of aluminum pan plus sample in grams before drying
gm final weight	=	weight of aluminum pan plus sample after drying in grams
gm sample weight	=	weight of sample before drying (10.0 grams)

Calibration Procedures. Prepare several standard solutions in mobile phase at concentrations that span the concentration ranges expected for the analyte in the sample solutions. Analyze the standards frequently during a series of sample analyses to provide data for standard curves. If the analyte peak height for a sample falls outside the working range of the standard curve, quantitatively dilute the sample, then reinject the diluted extract to keep the detector response within the working range of the standard curve.

To determine recovery efficiency, prepare and analyze at least one control sample and three spiked samples (control samples fortified with known quantities

of the analytes) before daily sample extraction. Fortification levels should approximate the residue concentration of the samples being analyzed.