Determination of Benomyl Residues in Soils and Plant Tissues by High-Speed Cation Exchange Liquid Chromatography

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Benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] residues in soils and plant tissues may be determined by procedures using high-speed cation exchange liquid chromatography (lc). The method for soils is based on an acidic methanol extraction of the residues, which converts residual benomyl to MBC (methyl 2-benzimidazolecarbamate). After a liquid–liquid partitioning cleanup, the total benomyl and/or MBC residues are measured as MBC by high-speed liquid chromatography. Any 2-AB (2-aminobenzimidazole) present in the original sample is also extracted and simultaneously determined as a separate lc peak. Recoveries of benomyl, MBC, and 2-AB from various types of soils average 92, 88, and 71%, respectively. The lower limit of sensitivity of the method is 0.05 ppm for each of these components. The method for plant tissues is based on ethyl acetate extraction of the residue followed by liquid–liquid partitioning cleanup and liquid chromatographic measurement. Recoveries and limit of sensitivity of this method for plant tissues were essentially that found for soils.

Analytical methods have previously been described for determining residues of benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate], the active ingredient in Du Pont's Benlate benomyl fungicide, in plant and animal tissues, and in soil (Pease and Gardiner, 1969; Pease and Holt, 1971). These procedures involve the quantitative conversion of benomyl to methyl 2-benzimidazolecarbamate (MBC) and then to 2-aminobenzimidazole (2-AB) by a two-stage acid–base hydrolysis procedure. Ethyl acetate is used as the initial extraction solvent in these methods. Final determination is made by direct fluorometric measurement or a colorimetric analysis following bromination. Therefore with these procedures, benomyl, its principal degradative product, MBC, and any degradation products hydrolyzable to 2-AB are measured as a composite value.

This paper describes methods for determining benomyl residues in soils and plant tissues by high-speed cation exchange liquid chromatography (lc). A liquid chromatographic procedure has recently been described for simultaneously determining residues of benomyl and/or MBC and its metabolites, methyl 5-hydroxy-2-benzimidazolecarbamate and methyl 4-hydroxy-2-benzimidazolecarbamate, in cow milk, urine, feces, and tissues (Kirkland, 1973).

Use of high-speed lc for the final measurement of benomyl residues offers an advantage in both the sensitivity and accuracy of data. In addition, the procedures herein described distinguish between the total benomyl and/or MBC residues and free 2-AB, a minor metabolite, in a single analysis.

METHOD

Reagents. Distilled-in-glass ethyl acetate was obtained from Burdick & Jackson Laboratories, Muskegan, Mich. Purified samples of benomyl, methyl 2-benzimidazolecarbamate, and 2-aminobenzimidazole were supplied by the Agrichemical Sales Division, Biochemicals Department, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.

Apparatus. Liquid chromatography was carried out with Du Pont Model 830 liquid chromatograph equipped with an ultraviolet photometric detector operating at 254 nm. A 1 m, 2.1 mm i.d. column of Zipax SCX strong cation exchange packing (Part No. 830950402, Instrument Products Division, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) was used.

Isolation of Residues from Soils. Place a 50-g representative sample in a 1-l. round-bottomed flask. Add 300 ml of 83% methanol–17% 1 *N* HCl solution and several Hengar granule boiling chips. With a water-cooled condenser attached in position, reflux this mixture for 4 hr using a heating mantle. Cool the solution to room temperature and withdraw 200 ml of the aqueous phase using a 100-ml syringe. Filter the solution through a 1-in. cotton plug contained in a 3-in. funnel into a 600-ml beaker. Concentrate the combined soil extract to about 20 ml by allowing it to stand in the rear of a well-ventilated hood overnight at room temperature, or by use of a rotary vacuum evaporator. Add 10 ml of 6.5 *N* NaOH and transfer the solution to a 250-ml separatory funnel. Carefully wash down the beaker walls with small volumes of hot distilled water and transfer these washes to the separatory funnel. Total volume in the separatory funnel should now be about 50 ml. Add 50 ml of chloroform, shake for 1 min, allow the phases to separate, and discard the chloroform layer.

Extract the aqueous phase with four 75-ml portions of ethyl acetate using 2–3 min vigorous shaking periods for each extraction. Centrifuge these mixtures for complete separation of the layers, if required, and withdraw the upper ethyl acetate layer using a 100-ml syringe. Filter this extract through a bed of anhydrous Na_2SO_4 into a 400-ml beaker. Concentrate the dried ethyl acetate extract at room temperature in the rear of a well-ventilated hood or with the use of a rotary vacuum evaporator to a volume of about 20 ml.

Quantitatively transfer the concentrated extract to a 30-ml beaker using ethyl acetate washes and continue the evaporation until about 3–5 ml volume is obtained. Add 1 ml of 0.1 *N* H_3PO_4 and continue to evaporate until all of the ethyl acetate is removed. Quantitatively transfer the aqueous solution to a 2-ml volumetric flask, using a drawn-out dropper and several small volumes of 0.1 *N* H_3PO_4 wash. Dilute the flask to volume with 0.1 *N* H_3PO_4 and mix. If the extract must be held for later analysis, it should be stored in a freezer.

Measurement of Isolated Residues. Quantitative determinations of benomyl/MBC and 2-AB after extraction and cleanup are conducted by high-speed cation exchange chromatography. The lc instrument with the Zipax SCX strong cation exchange column is first equilibrated using the following conditions: column temperature, 60°; mobile phase, 0.025 *N* tetramethylammonium nitrate–0.025 *N*

E. I. du Pont de Nemours & Co., Inc., Biochemicals Department, Experimental Station, Wilmington, Delaware 19898.

HNO_3; carrier flow rate, 0.5 ml/min; inlet pressure, 300 psig; uv detector, 0.02 absorbance, full-scale; chart speed, 0.1 in./min. Under these conditions the retention times of MBC and 2-AB are approximately 18 and 22 min, respectively.

Calculations are based on calibration factors obtained for MBC and 2-AB by dividing the concentration of known MBC and 2-AB standard solutions (micrograms/milliliters) by the peak heights (mm) found for these materials. The calibration factors are obtained by chromatographing 100-μl aliquots of 5 or 10 μg/ml solutions of MBC and 2-AB in 0.1 N H_3PO_4. A plot of peak height *vs.* various concentrations of these components is linear; therefore, a single calibration point in the form of a calibration factor is satisfactory for the calibration. Calibration factors should be determined daily or for each batch of extracts to be analyzed. Calculate the amount of benomyl or 2-AB in ppm as follows:

$$\text{ppm benomyl} = \frac{(\text{total } \mu\text{g MBC found})\ (1.53)\ (AF)}{(R)\qquad(SW)}$$

$$\text{ppm 2-AB} = \frac{(\text{total } \mu\text{g 2-AB found})\ (AF)}{(R)\qquad(SW)}$$

where R = recovery factor, AF = aliquot factor = 300/200, and SW = sample weight in grams. The 2-AB values may be converted to ppm benomyl by multiplying by 2.18.

RESULTS AND DISCUSSION

In the procedure for soils, benomyl is quantitatively converted to methyl 2-benzimidazolecarbamate (MBC) by acid hydrolysis. Therefore, benomyl and its principal degradative product, MBC, are measured as MBC as a composite value. Another degradative product, 2-AB, is isolated in the extractive procedure and simultaneously determined as a separate peak in the liquid chromatographic measurement. The acidic methanol extraction system used in the determination of benomyl-MBC and 2-AB residues in soils effectively removes and isolates these residues. This has been confirmed by studies involving the exposure of soils in the field treated with [2-^{14}C]benomyl (Baude *et al.*, 1973). The procedure described in this paper is preferred for the determination of benomyl residues in soils since higher sensitivity and significantly improved extraction efficiencies are obtained compared to the method previously described (Pease and Gardiner, 1969).

The liquid chromatographic method for soils provides a highly selective and sensitive measurement of benomyl residues. This selectivity is largely a result of the high efficiency, high-speed cation exchange column used in this method. Figure 1 shows a chromatogram of a mixture of MBC and 2-AB standards obtained by the recommended chromatographic procedure. Figure 2 shows chromatograms of extracts from control soil and soil fortified with benomyl and 2-AB. Recoveries of benomyl, MBC, and 2-AB by this new procedure averaged 92, 88, and 71%, respectively, for these materials on three different types of soils, as shown by the data in Tables I–III. Recoveries of 2-AB are subject to more variation than benomyl and MBC, probably as a result of the increased difficulty in extracting this more polar compound from the substrate, and the possibility for reaction of the free amino group on this molecule with components also extracted from the substrate.

In regards to the use of lc for plant tissue analysis, it has been determined that the total extraction procedure used for soils is not applicable. The acidic methanol extracts contain many interfering materials from plant sub-

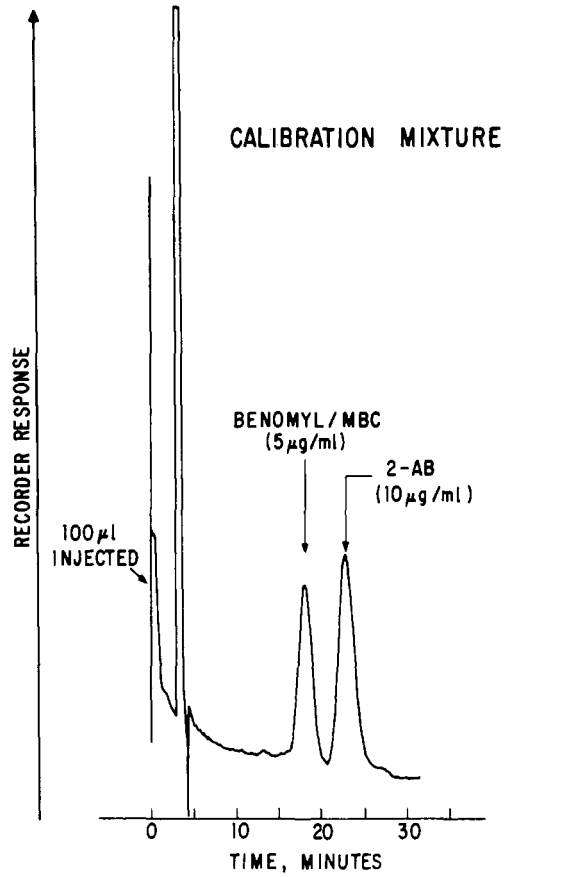

Figure 1. Liquid chromatogram of calibration mixture. Sample, 100 μl of 5 ppm of MBC (methyl 2-benzimidazolecarbamate), 10 ppm of 2-AB (2-aminobenzimidazole) in 0.1 N H_3PO_4; column, 1 m × 2.1 mm i.d. Zipax SCX strong cation resin; carrier, 0.025 N tetramethylammonium nitrate–0.025 N HNO_3; carrier flow rate, 0.5 ml/min; pressure, 300 psig; column temperature, 60°; uv detector, 0.02 absorbance, full-scale.

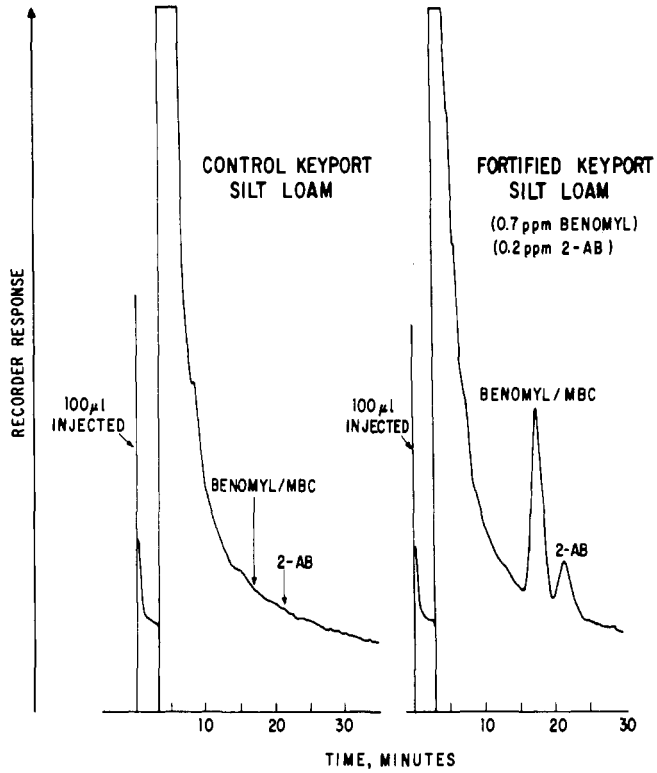

Figure 2. Analysis of soil extracts. Samples, 100 μl of extract. Conditions same as for Figure 1.

Table I. Recoveries from Keyport Silt Loam (Delaware) (50-g Samples)

Fortification level, ppm			% recovery		
Benomyl	MBC	2-AB	Benomyl[a]	MBC	2-AB
	0.05			72	
0.05		0.05	108		72
0.1			76		
0.1		0.1	102		60
0.1		0.1	120		48
0.2		0.4	87		42
0.2		0.4	96		42
0.5		1.0	100		78
0.1		0.1	112		72
1.0		1.0	75		66
	2.0			64	
	6.0			80	
		4.0			54
		20			61
0.6		0.6	83		57
2.0		2.0	107		80
10		10	80		45
4.0[b]			91		
10[b]			101		
20[b]			73		
	10			91	
	20			91	
0.7		0.2	83		90
		Avg	93%	80%	62%

[a] Added and calculated as benomyl, measured as MBC. [b] Two-hundred-gram soil samples were spiked and wet-ballmilled for 16 hr as a thin slurry in 10% methanol–H_2O. After a 3–4 day air-drying period, 50-g subsamples were taken for analysis.

strates. However, final extracts from either of the two earlier isolation procedures (Pease and Gardiner, 1969; Pease and Holt, 1971) can be measured by lc rather than the usual fluorimetric readout. These earlier isolation procedures both utilize an initial ethyl acetate extraction, which minimizes interference from plant tissue, and both methods utilize hydrolysis steps to convert all residues to 2-AB, which is measured by fluorimetry. The lc method is different only in the final measurement of the 2-AB; therefore, recoveries are equivalent. Of course for the lc measurement, final extracts should be redissolved in 2.0 ml of 0.1 *N* H_3PO_4 prior to injection into the liquid chromatograph.

Residue laboratories may also wish to consider yet another alternative for analyzing benomyl residues in crop tissues. This approach utilizes ethyl acetate extraction and lc readout, but eliminates the need for converting all residues to 2-AB. Instead, benomyl and/or MBC residues

Table II. Recoveries from Leon Immokalee Fine Sand (Florida) (50-g Samples)

Fortification level, ppm			% recovery		
Benomyl	MBC	2-AB	Benomyl[a]	MBC	2-AB
	0.05			120	
0.05		0.05	128		72
0.1		0.1	94		60
0.08		0.5	115		100
1.0		1.0	72		90
8.0			83		
80			70		
		Avg	94%		81%

[a] Added and calculated as benomyl, measured as MBC.

Table III. Recoveries from Cecil Loamy Sand (North Carolina) (50-g Samples)

Fortification level, ppm			% recovery		
Benomyl	MBC	2-AB	Benomyl[a]	MBC	2-AB
0.05[b]			61		
		0.1			130
		4.0			61
0.1		0.1	130		90
0.15[b]			70		
0.25[b]			106		
0.75[b]			71		
1.0		1.0	92		78
0.3			87		
	0.2			85	
1.0			100		
	1.0			100	
		0.5			80
		2.0			70
4.0[b]			80		
2.0[b]			82		
	2.0			94	
		Avg	88%	93%	85%

[a] Added and calculated as benomyl, measured as MBC. [b] Two-hundred-gram soil samples were spiked and wet-ballmilled for 16 hr as a thin slurry in 10% methanol–H_2O. After a 3–4 day air-drying period, 50-g subsamples were taken for analysis.

are determined by lc as MBC, while 2-AB residues are measured directly. After extraction of the crop (25-g samples) with ethyl acetate, as described by Pease and Gardiner (1969), the original method is followed down to the last full paragraph on page 267, just after the addition of 15 ml of 6.5 *N* NaOH but before the basic extracts are boiled on a hot plate for 15 min. Instead, after addition of the 6.5 *N* NaOH, the mixture is *not* heated, but immediately processed through the subsequent extraction into ethyl acetate. In this way, conversion of MBC to 2-AB is eliminated. At the end of the subsequent steps of the published procedure and prior to "Fluorometric Analysis," concentrate the final ethyl acetate extract to 3–5 ml volume and add 1 ml of 0.1 *N* H_3PO_4. Continue to evaporate until all the ethyl acetate is removed. Transfer the final solution quantitatively to a 2.0-ml volumetric flask and

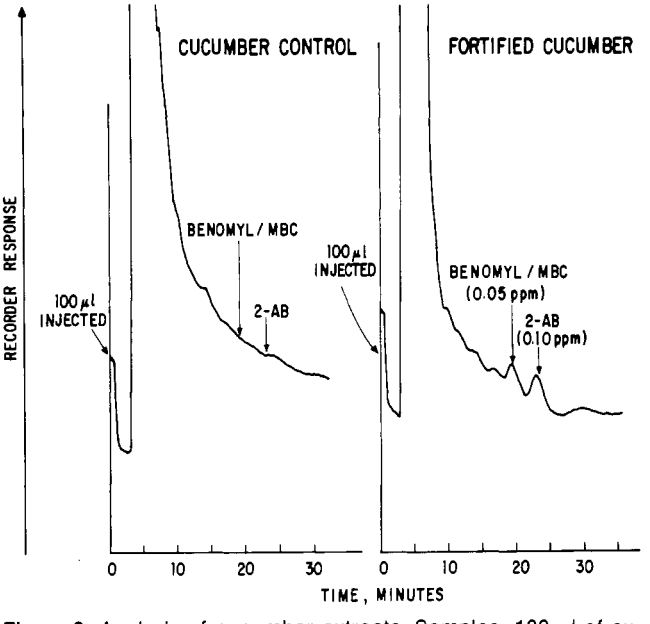

Figure 3. Analysis of cucumber extracts. Samples, 100 µl of extract. Conditions same as for Figure 1.

Table IV. Recovery Studies on Plant Tissues by lc Measurement

Plant tissue	Benomyl[a]				MBC				2-AB			
	Residue level, ppm	No. of det'ns	Recovery, % Avg	Recovery, % Range	Residue level, ppm	No. of det'ns	Recovery, % Avg	Recovery, % Range	Residue level, ppm	No. of det'ns	Recovery, % Avg	Recovery, % Range
Peaches	0.05–2.2	6	100	83–121								
Apricots	0.10–1.3	4	87	76–102								
Cherries	0.20–2.2	4	86	71–100								
Grapes	0.10–0.66	3	67	65–70								
Orange peel	0.22–1.3	3	73	61–91								
Orange pulp	0.11–0.66	5	84	69–100								
Celery	0.10–1.9	9	83	68–92								
Pecans	0.10–0.42	3	83	81–84								
Apples	0.10–1.0	5	76	72–80								
Cucumbers	0.20–1.0	3	83	80–86								
Cucumbers	0.05–0.20[b]	2	74	64–84					0.05–0.10	2	63	61–64
Cantaloupe	0.20–1.0	3	86	96–91								
Squash	0.10–1.0	4	85	75–92								
Corn kernel					0.40–2.0	3	99	92–110	0.20–0.40	3	53	40–72
Corn foliage					0.40–0.80	4	89	65–100	0.20–0.80	4	51	40–64
Carrot roots	0.05–0.20[b]	2	92	88–95					0.05–0.20	2	62	60–64
Snap beans	0.10–0.20[b]	2	93	88–98					0.05–0.10	2	76	64–88
Sugar beet roots	0.10–0.20[b]	2	88	80–95					0.05–0.10	2	56	56
Sugar beet foliage	0.20–0.50[b]	2	88	83–92					0.10–0.20	2	57	56–58

[a] Added and calculated as benomyl, measured as MBC. [b] 50-g samples.

dilute to volume with 0.1 N H_3PO_4. If the extract must be held for later analysis, it should be stored in a freezer.

Recoveries of added benomyl have been obtained on various crop substrates using this modified method coupled with an lc measurement (Table IV) and are equivalent to those reported by other workers (Pease and Gardiner, 1969; Pease and Holt, 1971; White and Kilgore, 1972). Some additional recovery data for MBC and 2-AB are also shown in Table IV. The data indicate that a wide range of crop substrates can be satisfactorily analyzed by an lc method if desired. In a typical illustration, Figure 3 shows liquid chromatograms of control and fortified cucumber extracts.

Because of the unique selectivity afforded by the combination of the extraction procedure and an lc measurement, interferences with an lc procedure for residues of benomyl and its degradation products are less likely than with the fluorimetric and colorimetric methods. For instance, the high selectivity of a very similar lc method has been confirmed by a study involving the measurement of benomyl and its metabolites in cow milk, tissues, urine, and feces (Kirkland, 1973).

The high-speed strong cation exchange liquid chromatographic packing used in these studies consists of Zipax-controlled surface porosity support coated with a thin film of a polyfluorinated aliphatic sulfonic acid (Kirkland, 1969, 1970). This strong sulfonic acid substituted polymer is highly stable chemically because of its polyfluorinated character and is a stronger acid than the conventional sulfonic acid ion exchangers. Columns of this material have been in continuous use for more than 18 months for the determination of benomyl residues without change in chromatographic characteristics. However, if samples containing undissolved solids are injected into the column, the packing can become so contaminated that it will not perform the desired analysis. Therefore, samples containing insoluble matter should be filtered before they are chromatographed. Filtration can be conveniently carried out by use of a 0.5 micro-Millipore filter held in a "Swinney" adapter (Millipore Corporation, Bedford, Mass.). If the characteristics of a column change because of contamination by a heavy retention of soluble impurities, it can be conveniently regenerated by pumping 1 M HNO_3 through it for 2 hr at 60° at a rate of about 1 ml/min. After reequilibration with a carrier, the column should return to its normal operating condition; if not, the column should then be replaced.

These studies have demonstrated the usefulness of modern, high-efficiency liquid chromatography for conducting sensitive and selective analyses of pesticide residues that cannot be analyzed by gas chromatographic procedures because of the lack of volatility or instability at high temperatures (for a complete discussion of modern lc, see Kirkland, 1971).

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