Determination of Methyl 2-Benzimidazolecarbamate in Soil by Competitive Inhibition Enzyme Immunoassay

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Soil was analyzed for the fungicide or fungicide degradation product methyl 2-benzimidazolecarbamate (MBC) or carbendazim by employing commercially available polyclonal enzyme immunoassay (EIA) kits. MBC extraction from soil was done either by an overnight shaking with methanol–water (80:20) or by a 10-min 2 M ammonium chloride–ethanol shake followed by partitioning into methylene chloride. Intraassay and interassay percent coefficients of variation (% CVs) ranged from 2.2 to 13 for the standards and from 7 to 41 for the samples. A total of 101 soil samples obtained from Maine, Florida, and Switzerland were analyzed for MBC using EIA and liquid chromatography (LC). The correlation coefficients were 0.998 (tube EIA vs LC), 0.966 (plate EIA vs LC) and 0.946 (tube EIA vs plate EIA) when MBC concentrations were higher than 10 ppb. Detectable MBC concentrations ranged from 1 to 4778 ng/g. Detection limits were 2 ppb for the tube immunoassay, 1 ppb for the plate immunoasay, and 3 ppb for the HPLC. However, limit of quantitation was set at 10 ppb for all techniques.

INTRODUCTION

Methyl 2-benzimidazolecarbamate (MBC) is a degradation product of the fungicide benomyl, and in Europe it is known as carbendazim, which is registered there as a fungicide. Benomyl is used extensively in the United States, while MBC or carbendazim is widely used in Europe, and both are employed as preharvest fungicides for fruits and vegetables. Benomyl breaks down to MBC not only in fruits and vegetables but also in soil and even in organic solvents. Thus, analytical methods for the analysis of benomyl because of its instability have focused on the determination of MBC or carbendazim, with this value being used to ascertain the amount of benomyl (Austin et al., 1975).

Because of the recent concerns about the toxicity of benomyl/MBC (Winter, 1993; Anonymous, 1987), the longevity of MBC in soil (Solel et al., 1979; Baude et al., 1974), and the fact that MBC is systemic, there is a concern and need to determine the amount of MBC in soil.

Previous MBC residue methods including both chromatographic and immunoassay techniques have primarily been concerned with the analysis of fruits and vegetables (Chiba and Veres, 1980; Zweig and Gao, 1983; Gilvydis and Walters, 1990; Newsome and Collins, 1987; Newsome and Sheilds, 1981; Bushway et al., 1990, 1991, 1992; Bushway, 1992). There have been a few chromatographic soil procedures (Kirkland et al., 1973; Austin et al., 1975; Austin and Briggs, 1976) and one magnetic particle-based immunoassay soil method by Itak et al. (1993) that appeared in print during the review process of our manuscript. However, Itak et al.'s technique is different from the one described here.

This paper describes a rapid, inexpensive, and sensitive EIA procedure for the quantitation of MBC in soil. Such a technique should be very useful for monitoring soil MBC concentrations.

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Table 1. Properties of the Soils Used for All Studies

soil site	pH	% OM[a]	CEC[b]	% sand	% silt	% clay	texture
central Maine	7.7	10.9	12.6	63	27	10	sandy loam
central Florida	6.1	1.2	8.4	92	4	4	sand
north Switzerland	7.5	6.9	13.7	56	29	15	sandy loam
west Maine	5.9	2.1	2.9	84	10	6	loamy sand
central Maine	5.9	0.9	1.4	90	6	4	sand

[a] OM, organic matter. [b] CEC, cation-exchange capacity.

MATERIALS AND METHODS

Materials. All reagents pertaining to the preparation of immunogens for raising antisera to MBC were previously described (Bushway et al., 1990). MBC pesticide standard (99%) was obtained from the U.S. Environmental Protection Agency, Research Triangle Park, NC. All solvents were of HPLC grade and were purchased from EM Science (Gibbstown, NJ). Phosphate salts, ammonium chloride, sodium sulfate, and ammonium hydroxide were bought from VWR (Boston, MA). Skim milk or nonfat dry milk was obtained from local supermarkets (Bangor, ME).

Soil samples were obtained from Maine, Florida, and Switzerland and were from land that was known to have been treated with bennomyl and carbendazim. The soil was sent to the Department of Food Science, University of Maine. Upon arrival they were air-dried under a hood followed by storage in plastic containers at room temperature. Properties of the soils are given in Table 1.

Liquid Chromatography System. The HPLC consisted of a Waters 510 pump (Waters Associates, Milford, MA), a Valco pneumatic injector (VICI Instruments, Houston, TX) containing a 10-μL loop, a Waters 470 fluorescence detector, and a Hewlett-Packard 3396A integrator.

EIA Kits. Benomyl/MBC immunoassay tube and plate kits were purchased from Millipore Corp. (Bedford, MA).

Methods. *Extraction Procedures.* One soil extraction technique was modified from the thiabendazole method of Cayley and Lord (1980). A 5-g air-dried soil sample was added to a 50-mL polypropylene centrifuge tube followed by 2 mL of 10% phosphoric acid. This mixture was allowed to stand for 10 min before 5 mL of absolute ethanol, 15 mL of 2.0 M ammonium chloride (pH 9.5) (concentrated ammonium hydroxide was used to get pH 9.5), 20 mL of methylene chloride, and 5 stainless steel ball bearings (5-mm diameter) were added. Samples were

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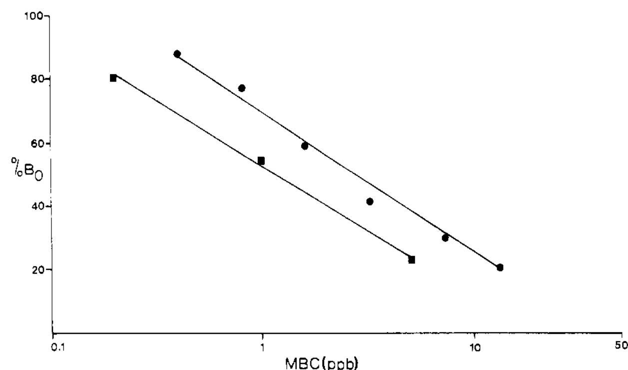

Figure 1. Standard curve for MBC tube EIA using skim milk as the diluent (●) and using water as the diluent (■).

Table 2. Concentrations of MBC Determined in Soil (Nanograms per Gram) by Using Two Different Extraction Methods with Methanol and Methylene Chloride[a]

soil	MeOH extract (ppb)	$MeCl_2$ extract (ppb)
1	4	25
2	21	32
3	3	13
4	7	49
5	5	21
6	8	61
7	15	32
8	71	226
9	734	4565
10	1384	2240

[a] Soil samples were analyzed by tube immunoassay.

Table 3. Reproducibility of the MBC Tube Immunoassay for Standards

MBC standard (ppb)	% CV (intraassay)[a]	% CV (interassay)[b]
0.4	2.4	4.5
0.8	5.7	8.4
1.6	7.1	4.9
3.2	7.6	6.4
6.4	11	8.2
13	13	7.5

[a] Percent coefficients of variation based on six determinations in 1 day. [b] Percent coefficients of variation based on six determinations performed on 6 different days.

Table 4. Reproducibility of the MBC Plate Immunoassay for Standards

MBC standard (ppb)	% CV (intraassay)[a]	% CV (interassay)[b]
0.2	3.4	3.1
1.0	3.3	2.2
5.0	3.9	5.2

[a] Percent coefficients of variation based on three determinations in 1 day. [b] Percent coefficients of variation based on six determinations performed on 6 different days.

vigorously shaken for 10 min and then centrifuged at 5000*g* for 10 min. The bottom layer of methylene chloride was removed with a Pasteur pipet and placed into a 20-mL glass scintillation vial containing approximately 0.2 g of sodium sulfate. A 10-mL aliquot of methylene chloride was removed and placed into another 20-mL glass scintillation vial before being dried under air. The residue was dissolved in 1 mL of HPLC MBC mobile phase (500 mL of water–260 mL of acetonitrile–70 mL of methanol–0.1 mL of monoethanolamine) and sonicated prior to centrifuging in a 1.5-mL polypropylene centrifuge tube at 5000*g* for 5 min.

The other MBC soil extraction employed a mixture of 80/20 methanol–water. Five grams of dried soil was weighed into a 20-mL plastic vial, followed by 10 mL of 80/20 methanol–water. This mixture was vortexed vigorously for 2 min and then shaken overnight on an orbital shaker. Once shaken, the samples were allowed to set for 5 min before centrifuging at 5000*g* for 5 min.

Table 5. Reproducibility of the MBC Plate and Tube Immunoassay for Soils

		% CV (intraassay)[a]		% CV (interassay)[b]	
soil	MBC (ppb)	plate	tube	plate	tube
1	8.8	23	18	27	27
2	3.0			24	41
3	8.0	16	15		
4	2.4			17	36
5	22			21	37
6	45			34	35
7	37	29	23	18	30
8	16	9	35	28	38
9	138	14	10	8	12
10	183	17	16	15	24
11	1970			24	21
12	63	16	36	17	15
13	48	7	11	30	23

[a] Percent coefficients of variation based on six determinations in 1 day for the tube assay and the same for the plate assay except for the following soil tube assays: soil 1, $n = 5$; soil 9, $n = 5$; soil 12, $n = 4$. [b] Percent coefficients of variation based on six determinations on 6 different days with the following exceptions: tube assay $n = 5$, soil 6; $n = 3$, soil 11; $n = 5$, soil 12; plate assay $n = 5$, soil; $n = 3$, soil 11.

Table 6. Accuracy of MBC Immunoassay for Soil

		MBC (ppb)			
soil	N[a]	added	found	mean rec (%)	% CV
1	9	5	5.53	111	19
2	10	10	12.3	123	19
3	10	100	95.2	95	23
4	9	1000	956	96	18

[a] N = number of different spiked samples analyzed. These soils were comprised of the samples in Table 1. Soils were analyzed by plate and tube immunoassay to obtain the percent recovery value.

A 100-μL aliquot was removed from the supernatant and added to 0.9 mL of protein diluent [1% bovine serum albumin (BSA) and 0.05% Tween 20 in phosphate buffer (pH 7.2)].

Preparation of Standards. A stock solution of MBC was prepared at a concentration of 0.34 mg/mL in 90/10 methanol–acetonitrile. From the stock standard an intermediate solution of 0.68 μg/mL in mobile phase was made. Separate working standards were prepared for the tube EIA, plate EIA, and HPLC by making serial dilutions from the intermediate standard. Working standards of 0.4, 0.8, 1.6, 3.2, 6.4, and 13 ng/mL were used for the tube EIA; 0.2, 1.0, and 5.0 ng/mL for the plate EIA; and 34, 68, and 136 ng/mL for HPLC. [The linearity has been previously established as 0.25–500 ng injected (Bushway et al., 1991).] The final diluent for these standards was skim milk (tube EIA), water (plate EIA), and mobile phase (HPLC).

Liquid Chromatography Conditions. Operating conditions were as follows: injection volume, 10 μL; flow rate, 1.0 mL/min; column, Ultracarb 30 ODS, stainless steel, 15 cm × 4.6 mm i.d. (Phenomenex, Torrance, CA); mobile phase, 500 mL of water–260 mL of acetonitrile–70 mL of methanol–0.1 mL of monoethanolamine; excitation wavelength, 286 nm; emission wavelength, 305 nm; attenuation, 8; gain, 100; filter, 1.5 s.

Tube EIA Method for Soil Analysis of MBC. Standards and samples were analyzed by adding 160 μL to coated tubes from the tube kit, followed by 160 μL of enzyme conjugate (up to eight samples with two controls can be prepared simultaneously). Samples were first diluted 1/10 in skim milk before being added to the tubes. The tubes were incubated for 10 min at room temperature and then rinsed four times with water to remove unreacted sample and enzyme conjugate. A 320-μL mixture (1:1) of substrate–chromogen was added to each rinsed tube and incubated for 5 min before 1 drop of 2.5 N sulfuric acid was added to stop the reaction (color changes from blue to yellow).

Each tube was read at 450 nm using a tube reader (Millipore Corp.), or alternatively a conventional spectrophotometer set at 450 nm can be used. The % B_0 values were calculated from the readings.

Plate EIA Method for Soil Analysis of MBC. Standards and samples were analyzed by adding 100 μL to each well of a coated

Table 7. Comparison of the Tube and Plate Immunoassays and HPLC for the Determination of MBC in Soils[a]

soil	tube EIA MBC (ppb)	plate EIA MBC (ppb)	HPLC MBC (ppb)	soil	tube EIA MBC (ppb)	plate EIA MBC (ppb)	HPLC MBC (ppb)
1	4565	–[b]	4778	52	9	10	11
2	2240	1182	2100	53	35	30	31
3	304	278	275	54	38	30	26
4	55	138	59	55	372	365	371
5	20	11	15	56	27	24	27
6	240	172	196	57	18	19	18
7	37	28	31	58	56	61	61
8	34	29	34	59	2	1	3
9	2	2	9	60	5	26	3
10	2	1	8	61	280	176	226
11	29	22	36	62	37	35	33
12	ND[c]	ND	ND	63	4	3	5
13	7	7	8	64	29	26	23
14	7	6	8	65	29	23	24
15	7	6	11	66	11	12	12
16	5	5	11	67	35	43	32
17	3	1	9	68	80	–	86
18	2	1	4	69	11	12	13
19	5	3	8	70	7	7	9
20	ND	ND	ND	71	ND	ND	ND
21	3	2	12	72	38	35	41
22	3	3	7	73	180	156	116
23	26	46	32	74	90	105	112
24	3	3	11	75	52	54	62
25	376	326	354	76	9	12	15
26	700	711	824	77	13	15	13
27	296	248	289	78	72	63	74
28	5	3	4	79	62	–	81
29	620	554	621	80	620	720	770
30	3	2	6	81	800	649	706
31	64	71	80	82	19	23	15
32	3	2	6	83	20	21	13
33	17	13	12	84	68	81	75
34	26	19	25	85	84	86	75
35	35	34	32	86	272	228	243
36	14	13	14	87	300	224	243
37	43	42	49	88	ND	ND	ND
38	20	18	21	90	ND	ND	ND
39	5	5	6	90	26	20	13
40	30	–	22	91	10	13	11
41	26	22	18	92	66	62	–
42	31	26	29	93	272	–	198
43	21	20	20	94	280	238	–
44	17	12	16	95	32	–	22
45	35	28	31	96	16	–	12
46	31	28	24	97	28	–	24
47	10	10	11	98	26	–	21
48	22	18	20	99	52	–	54
49	64	15	61	100	35	–	26
50	21	17	24	101	5	–	7
51	30	29	32				

[a] Correlation coefficients = tube vs plate, 0.946 (62 samples 10 ppb or greater); tube vs HPLC, 0.998 (71 samples 10 ppb or greater); and plate vs HPLC, 0.966 (60 samples 10 ppb or greater). [b] –, samples were not analyzed by that method. [c] ND, none detected at detection limits of 2 ppb for the tube immunoassay, 1 ppb for the plate immunoassay, and 3 ppb for the HPLC.

microtiter plate followed by 100 μL of enzyme conjugate (up to 96 samples and controls can be run simultaneously). Samples were first diluted 1/10 in water prior to being added to the wells. The plate was incubated for 60 min at room temperature and then rinsed four times with water to remove unreacted sample and enzyme conjugate. A 160-μL mixture (1:1) of substrate–chromogen was added to each rinsed well and incubated for 30 min before 1 drop of 2.5 N sulfuric acid was added to stop the reaction (color changes from blue to yellow).

Each plate was read at 450 nm by employing a plate reader (Millipore Corp.). The % B_0 values were calculated from the readings.

Recovery Study. Soil was fortified with MBC at the following concentrations: 5, 10, 100, and 1000 ng/g. This study was used to determine the accuracy of the immunoassay and the efficiency of the MBC extraction technique. Soil types used are shown in Table 1.

Reproducibility Study. Several soil samples were analyzed several times on the same day and different days to determine the intraassay and interassay variation of the EIA methods.

RESULTS AND DISCUSSION

All of the samples with high concentrations of MBC were diluted to appropriate concentrations so that they would fit within the linearity ranges of the standard curves prepared for the three methods used. For example, standard curves for both the tube and plate EIA are shown in Figure 1. As can be seen, the working range of the tube EIA is from 0.4 to 13 ng/mL, while with the plate EIA it is 0.2–5 ng/mL. Thus, the plate assay is more sensitive. For samples having a concentration greater than 13 ng/mL for the tubes or 5 ng/mL for the plates (indicated by % $B_0 < 20$), a simple serial dilution must be made using either a solution of 90/10 skim milk–HPLC mobile phase (for tubes) or distilled water (for plates). HPLC mobile phase was used to dilute the chromatographic samples.

Detections limits based on standard deviations were set at 2 parts per billion (ppb or ng/g) for the tube immu-

noassay, 1 ppb for the plate immunoassay, and 3 ppb for the HPLC. However, the limit of quantitation (again based on standard deviations) was set at 10 ppb for all techniques.

Two different extraction procedures were tried. The first was a methanol–water procedure that involved overnight shaking, and the other was an acid soaking with extraction in a strong base and a partition step into methylene chloride. The results of a ministudy with 10 soil samples are shown in Table 2. In all instances the base extraction was more efficient. However, if just spiked samples were employed, the methanol extraction would have been shown to be adequate since the recoveries were 80–90%. This points out the importance of working with aged soil samples vs spikes.

With any analytical technique, precision within and between days is crucial. Consistency results of each type of immunoassay for standards and soil samples are illustrated in Tables 3–5. Reproducibility data for standards are given in Tables 3 and 4. Intraassay percent coefficients of variation (% CVs) ranged from 2.4 to 13 (tubes, Table 3) and from 3.3 to 3.9 (plates, Table 4), while interassay % CVs varied from 4.5 to 8.4 (tubes, Table 3) and from 2.2 to 5.2 (plates Table 4).

Sample reproducibility is depicted in Table 5 for both types of EIA. Analysis was done on actual soil samples containing 2.4–1970 ng/g MBC. Tube intraassay % CVs ranged from 10 to 36 with most 23 or less, while the plate values were from 7 to 29 with the majority 17 or less. Interassay % CVs for the tube assay varied from 12 to 41 with most 35 or below, and those for the plate EIA ranged from 8 to 34 with the majority lower than 28.

The plate assays for both standards and samples had lower intra- and interassay % CVs than the tube EIA, indicating the plates are more precise. However, the tubes are field adaptable and quicker. Futhermore, the difference between the % CVs for the plate and tube assays are not all that great. Much of the inconsistency is interpreted to be due to the nonhomogeneity of the soil samples. It is very difficult to obtain a homogeneous soil sample.

Recovery studies were also performed on soil samples containing no detectable levels of MBC. Results are given in Table 6, which includes tube and plate data together. Spiking levels varied from 5 to 1000 ng/g, and recoveries ranged from 96 to 123% (mean recovery 106%). Thus, the accuracy was acceptable. Reproducibility was also adequate in this study. Percent CVs ranged from 18 to 23. Futhermore, these lower % CVs for the spiked soils further support the fact that the higher % CVs for samples were partly caused by nonhomogeneity.

Cross-reactivity of the benomyl antibody has been extensively discussed in a previous paper (Bushway et al., 1992). Like most antibodies, it does show some cross-reactivity but nothing that would appear to affect the quantitation of MBC in soil since, besides equally reacting with benomyl and MBC, the only other real reactive pesticide is thiabendazole. Thiabendazole is applied as a postharvest treatment on fruits and vegetables but not to soil.

A correlation study between the two immunoassay formats and HPLC was conducted on 101 soil samples from Maine, Florida, and Switzerland (Table 7). The soils analyzed from these areas were comprised of sand, sandy loam, and loamy sand (Table 1). Of these 101 samples, 5 were shown to contain no detectable levels of MBC by all three assays, while the other 96 soils demonstrated detectable amounts of MBC. The positive soils ranged in concentrations of 1–4778 ng/g depending upon the assay employed, with most below 36 ng/g. These data demonstrate that for the most part MBC is present at very low levels in these types of soil.

Several correlations were made with the positive data. For example, the correlation between tube EIA and HPLC was 0.998 for samples greater than or equal to 10 ppb, while the correlation was 0.457 for samples lower than 10 ppb. Plate EIA and HPLC correlation was 0.966 for samples equal to or greater than 10 ppb, while the correlation was 0.318 for samples between 1 and 9 ppb. Plate EIA and tube EIA correlation was 0.946 for soil ≥10 ppb and 0.929 for samples from 1 to 9 ppb. These correlations indicate that all methods agree well for soils containing 10 ppb or greater MBC, while samples lower than 10 ppb of MBC were poor. Even though one can use these methods to detect MBC lower than 10 ppb, the best accuracy will be obtained with soils containing ≥10 ppb of MBC. All of these correlations were close to each other and near 1, indicating that the methods were in good agreement. Thus, it appears that matrix effects are nonexistent with this extraction procedure.

In conclusion, the immunoassay procedures compare well with the HPLC method for determination of MBC in soil and the EIA should be very useful for monitoring soils for MBC residues. Furthermore, the methanol extraction procedure, although it is not quantitative, would be suitable for field site work to detect the presence of MBC since a simple serial dilution could be made on the extract before the EIA is performed.

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