Fluorometric and Colorimetric Procedures for Determining Residues of Benomyl

H. L. Pease and J. A. Gardiner

Methods for determining benomyl, methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, residue in plant and animal tissues and in soil involve isolation of the residue by extraction with an organic solvent, purification of the extract by a liquid-liquid partitioning procedure, conversion of the residue to 2-aminobenzimidazole, and final determination by direct fluorometric measurement or colorimetric analysis following bromination. Each method has a sensitivity of 0.1 p.p.m. based on a 50-gram sample. Average recoveries of about 87% have been obtained on all samples investigated, using both the fluorometric and colorimetric methods.

Sensitive analytical methods have been developed for determining methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate residue in plant and animal tissues and in soil. This compound, formerly du Pont fungicide 1991, has the approved common name of benomyl and is the active chemical ingredient of Benlate benomyl fungicide. It is a systematic fungicide having both preventive and curative properties as well as a mite ovicide effect. It is potentially useful at low dosage rates for control of a wide range of fungus diseases affecting fruit, vegetables, field crops, and ornamentals (Delp and Klopping, 1968).

Benomyl is extracted from the substrate with an organic solvent, and the extract is purified by a liquid-liquid partitioning process. The compound is then converted to 2-aminobenzimidazole, and the final determination made either by direct fluorometric measurement or by colorimetric analysis following bromination. The analytical methods are capable of determining benomyl and any degradation products hydrolyzable to 2-aminobenzimidazole (2AB). Each method has a sensitivity of about 0.1 p.p.m. based on a 50-gram sample and satisfactory recoveries have been demonstrated at this level on a variety of substrates. Average recoveries of about 87% have been obtained on all samples investigated, using both the fluorometric and colorimetric methods. Agreement has been satisfactory with both procedures when applied to field-treated samples.

EXPERIMENTAL

Apparatus and Reagents. The Aminco Bowman spectrophotofluorometer Model 4-8203 with off-axis ellipsoidal mirror condensing system was used for fluorometric analysis. The Beckman Model B spectrophotometer was used to make the colorimetric measurements. Centrifugation was carried out with an International Model BE 50 centrifuge equipped with 250-ml. bottles. Homogenization and extractions were conducted using an electric blender such as an Osterizer disintegrator or a Waring Blendor. The reference sample of benomyl was obtained from the Industrial and Biochemicals Department, Agrichemical Sales Division, E. I. du Pont de Nemours & Co., Inc., Wilmington, Del. The solvents used were distilled-In-glass, purchased from Burdick and Jackson Laboratories, Inc., Muskegan, Mich. Spectrograde methanol was used for the fluorometric determinations.

Isolation. Weigh 50 grams of a representative sample into a 1-quart Osterizer jar, add 150 ml. of ethyl acetate, cover, and blend at high speed for 5 to 10 minutes. Transfer the blended sample to a 250-ml. centrifuge bottle, using several small volumes of ethyl acetate for wash. Centrifuge at 2000 r.p.m. for 10 to 15 minutes and carefully decant the ethyl acetate extract through a cotton plug into a 400-ml. beaker. Rinse the Osterizer jar with 100 ml. of ethyl acetate, add this wash to the substrate remaining in the centrifuge bottle, stopper, and shake vigorously for 2 to 3 minutes. Centrifuge this as before and combine the ethyl acetate extracts. Repeat the extraction procedure once more, using another 100 ml. of ethyl acetate.

Add 25 ml. of 0.1*N* HCl to the combined organic solvent extracts. Place on a steam bath in a well ventilated hood and evaporate the ethyl acetate. Reduce the volume to 10 to 15 ml. To hasten the procedure, the acid may be added after the second ethyl acetate extraction and evaporation of the solvent began, with the third extract added when these steps are completed.

Carefully wash down the sides of the beaker with small volumes of hot water and transfer to a 125-ml. separatory funnel. Total volume should now be about 30 ml. After cooling to room temperature, add 50 ml. of *n*-hexane to the separatory funnel, shake 2 minutes, and allow the phases to separate. Centrifuge, if necessary, to obtain a clean separation. Discard the hexane layer. Repeat the hexane wash two more times, each time using additional 50-ml. portions of solvent. Discard the hexane after each wash.

Quantitatively transfer the aqueous phase to a 150-ml. beaker using small volumes of water as rinse; total volume is now about 40 ml. Add 15 ml. of 6.5*N* NaOH, cover, and boil gently on a hot plate for 15 minutes. Use a glass stirring rod to prevent bumping and to assure more even boiling. Wash the cover glass, stirring rod, and side of beaker while hot with small volumes of hot water. Carefully transfer to a 250-ml. separatory funnel, using several small volumes of water to assure quantitative transfer.

Cool to room temperature and extract the aqueous

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

hydrolyzate with four 75-ml. portions of ethyl acetate, using 2- to 3-minute shaking periods for each extraction (ethyl acetate neutralizes the basic solution to pH 8 to 11). After a separation of the layers (centrifuge if necessary), filter the ethyl acetate through cotton into a 400-ml. beaker. Concentrate the solvent to about 50 ml. by evaporation at room temperature in a well ventilated hood.

Transfer the concentrated extract to a 125-ml. separatory funnel. Rinse the beaker thoroughly with small volumes of ethyl acetate. Continue the transfer using 10 ml. of 0.1*N* HCl. Shake for 2 minutes and allow the phases to separate. Drain the aqueous phase into a second 125-ml. separatory funnel. Check the extract with pHydrion paper to verify that it is acidic. If necessary, add 1 ml. of 1*N* HCl to the separatory funnel to ensure an acidic aqueous phase at this point. Extract the ethyl acetate with a second 10-ml. portion of 0.1*N* HCl, combining the acid phase in the 125-ml. separatory funnel.

Add 2 ml. of 6.5*N* NaOH to the combined acid extract, check with pHydrion paper to verify that the solution is now strongly basic, and extract with four 50-ml. portions of water-saturated ethyl acetate. Each extraction should consist of 2 to 3 minutes of vigorous shaking. After a complete separation of the phases, filter the ethyl acetate extract through cotton into a 250-ml. beaker. Concentrate the solvent at room temperature to about 20 ml., using a well ventilated hood. Transfer the concentrated ethyl acetate quantitatively to a 50-ml. beaker and continue to evaporate the solvent to dryness. Final determination of the resulting residue should proceed promptly. The residue may be analyzed by either a fluorometric or a bromination-colorimetric method. Both detection methods can be used conveniently only if the sample is split at this point.

Fluorometric Analysis. Immediately dissolve the residue in a methanolic solution containing 10% of aqueous 0.1*N* NaOH. Transfer this quantitatively, using several washes of the methanolic solution, into a 10-ml. volumetric flask, dilute to volume with the methanolic solution, and mix well. Determine the fluorescence of the methanol solution at 335 mμ, using an Aminco Bowman spectrophotofluorometer with an excitation wavelength of 285 mμ. Compare the reading with a previously prepared 2-aminobenzimidazole calibration curve and calculate the amount of benomyl in parts per million as follows:

$$\text{P.p.m. benomyl} = \frac{(\mu\text{g. 2AB})\ (2.18)}{(Q)\ (R)\ (S.W.)}$$

where Q = quenching factor, R = recovery factor, and $S.W.$ = sample weight in grams.

Determine the quenching factor at least once on a control sample from each series of sample analyzed by adding a known amount of 2AB to the untreated control extract and comparing the relative intensity obtained with the value obtained on a standard solution of the same concentration. Factors can vary as much as about 20% relative. Good sample cleanup is therefore required in order to minimize errors caused by this variable quenching factor. The self-quenching of 2AB at higher concentrations is compensated for by use of the calibration curve. It may be necessary to correct the results for minor positive blanks obtained on certain substrates.

Prepare the calibration curve by reading the relative intensities of standard solutions of 2AB, prepared in the methanol-NaOH solution, at concentrations of 0.2, 0.5, 1.0, 2.0, 3.0, and 5.0 μg. per ml. Plot the relative intensity obtained *vs.* micrograms of 2AB per 10 ml. of solution. Check the calibration curve with each series of samples analyzed.

Colorimetric Analysis. Immediately add 10 ml. of saturated bromine water to the residue contained in the 50-ml. beaker, cover, and place on a steam bath for 30 minutes. Remove cover and allow to remain on the steam bath until the excess bromine is completely eliminated, as judged by absence of a characteristic odor. Carefully transfer to a 125-ml. separatory funnel, using a plastic spatula and several small portions of hot distilled water to obtain quantitative transfer. Cool and dilute to about 30 ml. with distilled water. Continue the transfer by rinsing the beaker with 25 ml. of water-saturated ethyl acetate, add this to the aqueous phase in the separatory funnel, shake vigorously for 3 to 4 minutes, and allow the phases to separate. Filter the ethyl acetate through cotton into a 50-ml. beaker. Repeat the wash and extraction using a second 25-ml. portion of water-saturated ethyl acetate. Combine the extracts and concentrate to about 1 ml. Transfer the concentrated extract to a 2-ml. volumetric flask, using a dropper with a fine tip. Wash the beaker with several small portions of ethyl acetate, dilute to volume, and mix thoroughly.

Measure the absorbance at 445 mμ using semimicro quartz cells having a 10-mm. light path. Use ethyl acetate as the reference solution. Determine micrograms of 2AB from a calibration curve previously prepared following the described bromination and extraction procedure. Calculate the amount of benomyl in parts per million as follows:

$$\text{P.p.m. benomyl} = \frac{(\mu\text{g. 2AB})\ (2.18)}{(R)\ (S.W.)}$$

where R = recovery factor and $S.W.$ = sample weight in grams.

Prepare the standard solutions of 2AB used for calibration purposes in ethyl acetate and take aliquots representing 5, 10, 20, 30, and 50 μg. for bromination. Evaporate the solvent to dryness before adding the saturated bromine water and continue the procedure. Plot absorbance *vs.* micrograms to give the calibration curve used for subsequent determinations.

RESULTS AND DISCUSSION

Benomyl can be converted quantitatively to 2-aminobenzimidazole. To ensure complete conversion of the residue to 2-aminobenzimidazole, it is necessary to treat the extracted residue with dilute acid prior to basic hydrolysis by adding 0.1*N* HCl to the original ethyl acetate extracts just prior to reducing the volume by

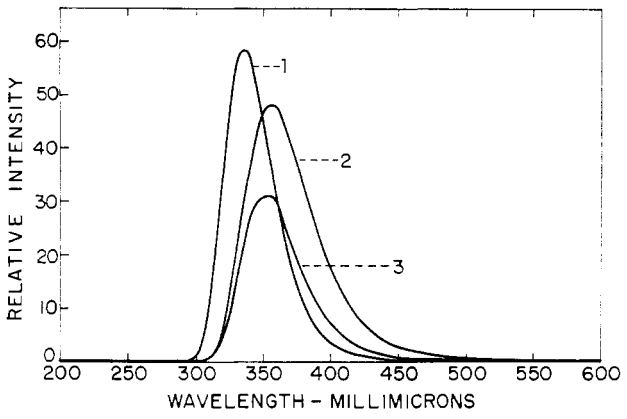

Figure 1. Fluorescence curves for hydrolysis products

1. Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate
2. 2-(4-Thiazolyl)benzimidazole
3. 2-(2-Furyl)benzimidazole

evaporation on a steam bath and cleanup with hexane.

2-Aminobenzimidazole has not been detected as a substituent in naturally occurring material, and thus this method has a high degree of selectivity. Hydrolysis products of two fungicidal benzimidazoles fluoresce at wavelengths similar to that of 2AB (Figure 1). Thus, bromination is recommended when treatment with these fungicides is suspected, because neither produces colored bromination products under these conditions.

The applicability of each procedure has been demonstrated on a variety of substrates, and the results compare favorably. Recovery of benomyl added to untreated control samples averaged about 91% over the range of 0.10 to 2.1 p.p.m. for the fluorometric procedure and about 86% for the colorimetric procedure (Table I). Recoveries on orange peel and edible pulp using the colorimetric procedure were somewhat lower, averaging 70% over the range of 0.10 p.p.m. on edible pulp to 8.5 p.p.m. on orange peel.

Recoveries were conducted by adding known amounts of compound to the untreated control contained in the Osterizer jar. The standard solutions used to fortify the untreated samples were prepared in ethyl acetate and used immediately. Analyses were carried out as described above. To judge further the adequacy of ethyl acetate extraction, experiments were also conducted on both benomyl-fortified samples and field-treated samples using an acidic methanol extraction. Recovery of the compound was comparable; however, appreciably more interfering material was extracted, requiring more elaborate cleanup techniques.

Because of the limited solubility of this class of compounds (E. I. du Pont de Nemours & Co., 1968), it is necessary to take extreme care in all transfer and solvent partitioning steps in order to obtain reliable and reproducible results. Studies conducted using 2-C^{14}-labeled benomyl show that losses resulted primarily in the residue transfer steps; however, vigorous shaking and complete phase separation during the solvent partitioning steps are very essential. These studies were conducted by adding tagged material to the untreated substrate in the Osterizer jar, and continuing the procedure as described above. Liquid scintillation counting analyses were made on aliquots taken from all extraction steps to check the extraction efficiencies, on washes of all beakers to check residue transfer efficiencies, and on the final extract to determine the over-all efficiency of the method. The results obtained on the final extract compared favorably with those obtained using the described methods.

It is also important to continue through the entire procedure without unnecessary delay. A series of four to six samples is a convenient number for one group of analyses. Extreme care must be exercised in handling these compounds. 2-Aminobenzimidazole oxidizes readily under certain conditions—for example, appreciable losses will occur when dilute solutions of 2AB prepared in ethyl acetate saturated with dilute acid are evaporated to dryness.

Additional cleanup steps during the extraction may be considered if needed for specific samples. For example, additional washes of basic or acidic aqueous solutions of the residue with hexane may be introduced at any time without appreciable losses; a chloroform

Table I. Recovery on Substrates Fortified with Benomyl

	Colorimetric Method				Fluorometric Method			
	Residue level, p.p.m.	No. of detns.	Recovery, % Av.	Recovery, % Range	Residue level, p.p.m.	No. of detns.	Recovery, % Av.	Recovery, % Range
Apples	0.10–1.0	3	86	80–92	0.10–1.0	3	93	90–96
Peaches	0.11–1.6	6	91	84–99	0.11–2.1	5	92	78–97
Apricots	0.10–1.0	3	79	70–90	0.10–1.0	3	84	79–92
Cherries	0.10–1.0	3	89	77–96	0.10–1.0	4	88	77–106
Grapes	0.10–1.0	6	78	56–100	0.10–1.0	6	92	77–110
Prunes	0.12–1.0	5	97	77–117	0.12–0.48	5	97	76–123
Orange peel	2.10–8.5	3	69	62–73	- - - - - - - - Complete quenching - - - - - - - -			
Orange pulp	0.10–0.42	3	71	67–75	- - - - - - - - Complete quenching - - - - - - - -			
Celery	0.10–1.8	5	86	72–100	0.10–1.8	5	90	84–94
Pecans	0.11–0.56	3	86	76–103	0.11–0.60	3	88	83–96
Lean muscle	0.20–1.0	3	84	64–98	0.20–1.0	3	85	83–87
Liver	0.20–1.0	3	88	80–98	0.20–1.0	3	93	87–98
Urine	0.20–2.0	4	86	73–98	0.20–2.0	4	91	82–110
Soil	0.20–2.0	4	75	62–87	0.20–2.0	4	91	82–98

Table II. Quenching Studies of Fluorometric Method

Sample	Degree of Quenching, %[a]	Sample	Degree of Quenching, %[a]
Apples	0	Liver	20
Grapes	0	Celery	21
Soil	0	Prunes	22
Lean muscle	15	Pecans	29
Apricots	16	Urine	33
Peaches	18	Orange peel	100
Cherries	18	Orange pulp	100

[a] Determined by adding a known quantity of 2-aminobenzimidazole to untreated control extract and comparing the relative intensity with that obtained on a standard solution of the same concentration.

wash of basic or acidic solutions can be used, but should not be undertaken until after the system has been fairly well cleaned up, because the partitioning characteristics may change in the presence of large amounts of green or waxy material; or an ethyl acetate cleanup wash from an acid medium may be used with minimal losses of residue. All precautions previously discussed should be observed with these washes.

Since extremely water-insoluble materials are encountered in this work, the cleanup of glassware is particularly important to avoid contamination from heavily treated samples. The procedure used included a vigorous soap solution treatment and water wash with hot water, followed by distilled water rinses, and finally a thorough wash with acetone.

The use of fluorometry for the analysis of pesticide residues is not unique. Moye and Winefordner (1965) reported on the phosphorescence of 52 pesticides. Giang *et al.* (1967) described a fluorometric method for determining terephthalic acid in chicken tissue. The residue method for the analysis of 2-(4-thiazolyl)benzimidazole, described by Tocco *et al.* (1965), is based on fluorometry. Although many aromatic hydrocarbons exhibit rather strong fluorescence, no significant positive interference was encountered with this procedure in any of the samples analyzed. As might be expected, however, a varying degree of quenching was encountered with the extracts of the various substrates (Table II). No quenching was observed with the soil, grape, and apple samples, whereas the final extracts of the orange peel and orange pulp were appreciably colored, and complete quenching was obtained.

The colorimetric procedure was applicable to all substrates analyzed, and the results compared favorably with the fluorometric procedure, but were not so consistent. This is in part due to handling difficulties in the transfer and extraction of the extremely insoluble brominated product.

The bromination product was examined by thin-layer chromatographic and mass spectrographic techniques. The product was separated into two components on a commercially available 250-micron silica gel GF TLC plate (Analtech, Inc., Wilmington, Del.) which contained 6% incorporated phosphor. The plate was developed for 10 cm. in a hexane–ethyl acetate–methanol developing system (10:10:1 v./v.). The R_f values of

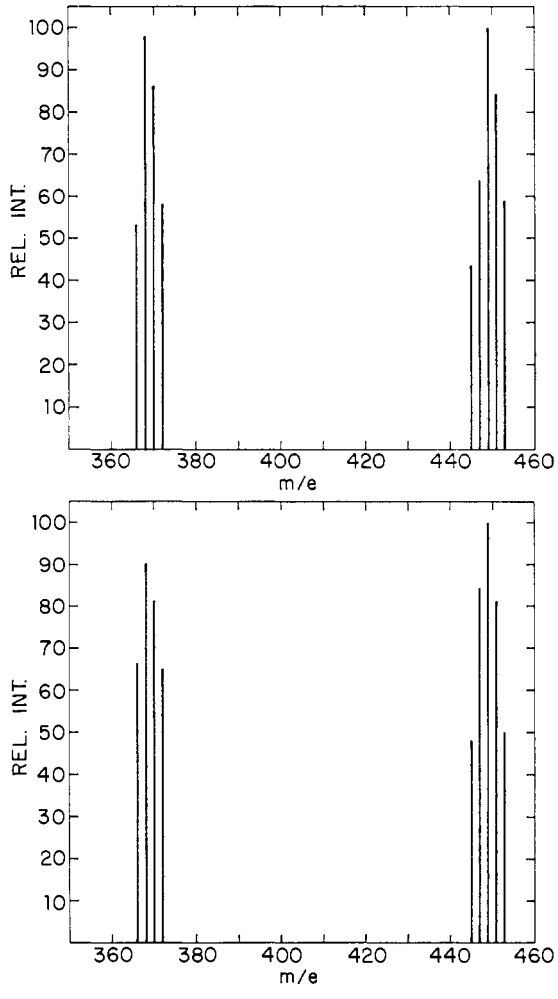

Figure 2. Mass spectrum of two isomers of tetrabromo-2-aminobenzimidazole

the two observed compounds were 0.55 and 0.75. The isolated materials were identified by mass spectrographic analyses on a Bendix Model 12-107 Time-of-Flight mass spectrometer as isomers of tetrabromo-2-aminobenzimidazole (Figure 2).

ACKNOWLEDGMENT

The authors thank D. C. Rakestraw for assistance with the fluorometric analyses and R. W. Reiser and R. C. Rhodes for help in determining the identity of the brominated product of 2-aminobenzimidazole.

LITERATURE CITED

Delp, C. J., Klopping, H. L., *Plant Disease Reptr.* **52,** 95 (1968).

Du Pont de Nemours & Co., E. I., Wilmington, Del., Industrial and Biochemicals Department, Agrichemical Sales Division, tech. information product bull., June 1968.

Giang, P. A., Schechter, M. S., Weissbecker, L., J. AGR. FOOD CHEM. **15,** 95 (1967).

Moye, H. A., Winefordner, J. D., J. AGR. FOOD CHEM. **13,** 516 (1965).

Tocco, D. J., Egerton, J. R., Bowers, W., Christensen, V. W., Rosenblum, C., *J. Pharmacol. Exptl. Therap.* **149,** 263 (1965).

Received for review October 23, 1968. Accepted January 14, 1969.