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Determination of Benomyl by High-Performance Liquid Chromatography/Mass Spectrometry/Selected Ion Monitoring

Chao-Hong Liu, Gregory C. Mattern, Xiaobing Yu, and Joseph D. Rosen*

Department of Food Science, Cook College, Rutgers University, New Brunswick, New Jersey 08903

Methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate (benomyl) was analyzed by reversed-phase high-performance liquid chromatography/mass spectrometry/selected ion monitoring (HPLC/MS/SIM) using 2-aminobenzimidazole (2-AB) as internal standard. Benomyl was quantitatively converted to methyl 2-benzimidazolecarbamate (carbendazim) by acid hydrolysis, and the latter was recovered by partitioning into ethyl acetate. The minimum detectable level in apples, peaches, and tomatoes was 0.025 ppm. Recoveries of benomyl fortified at 0.1 ppm were in the range 85–110% in all three commodities. Twenty-five samples each of tomatoes, peaches, and apples were analyzed. Four apple samples contained levels of 0.15–0.59 ppm benomyl, while eighteen of the peach samples analyzed had levels of 0.25–3.48 ppm. Trace levels of benomyl were also found in three tomato samples and one apple sample.

Benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] is a fungicide that is widely used for the control of a variety of plant diseases. Benomyl has proven to be a difficult analytical target because it readily decomposes in many common organic solvents, as well as in water (Calmon and Sayag, 1976). Because of this instability, residues of benomyl in crops are determined by acid hydrolysis of benomyl to the stable compound, carbendazim, which is then analyzed by HPLC on strong cation-exchange or reversed-phase (Bardalaye and Wheeler, 1985) columns.

We have adapted these methods to the determination of benomyl in peaches, apples, and tomatoes by reversed-phase HPLC/MS.

MATERIALS AND METHODS

Chemicals. Benomyl and carbendazim reference standards were donated by the EPA (Research Triangle Park, NC). 2-Aminobenzimidazole (2-AB) was purchased from Alfa Products, (Danvers, MA). Acetonitrile, ammonium acetate, ethyl acetate, and hexane were high-purity HPLC grade (Fisher Scientific, Springfield, NJ). ACS-grade anhydrous sodium sul-

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fate, hydrochloric acid, and sodium hydroxide were also purchased from Fischer Scientific.

Samples. Twenty-five samples of tomatoes (thirteen New Jersey grown, seven Mexican, one Israeli, four Florida), apples (thirteen New Jersey, nine New York, one Pennsylvania, one Vermont, one California) and peaches (thirteen New Jersey, seven South Carolina, five Georgia) were collected by the New Jersey Department of Health from New Jersey farms and supermarket distribution centers.

Instrumentation. A Kratos Spectraflow 400 liquid chromatograph (Kratos, Ramsey, NJ) interfaced to a Vestec Model 201 thermospray LC/MS (Vestec Corp., Houston, TX) and controlled by a Teknivent Vector/One data system (Teknivent Corp., St. Louis, MO) on a Compac Deskpro 286 personal computer was used. The HPLC column was a 25 cm × 4.6 mm (i.d.) Whatman Partisil 5 ODS-3 (Whatman, Clifton, NJ) with a phase particle size of 5 μm. The mobile phase was 85% acetonitrile/15% 0.1 M ammonium acetate buffer at a flow rate of 1 mL/min. The UV detector was set at 280 nm. The mass spectrometer was operated in the positive-ion filament mode, and selected ion monitoring of m/z 192 (carbendazim) and m/z 134 (2-AB) was performed. Scan time was 0.5 s, and sweep width was 0.1 amu. The vaporizer tip temperature was held at 190 °C.

Sample Preparation. Benomyl was extracted and hydrolyzed by a procedure based on the method of Bardalaye and Wheeler (1985) with some modification. Fifteen pounds of the cored or pitted crop were chopped and mixed. One-hundred-gram samples were extracted with 150 mL of 1:1 methanol/ethyl acetate with a Waring blender for 1 min at low speed. The cake was washed with an additional 150-mL portion of the extracting solvent after filtration under reduced pressure through glass fiber filter paper (Fisher Scientific). The filtrate was transferred to a 1-L evaporation flask containing 2.5 mL of 1 M hydrochloric acid. This solution was concentrated to ca. 50 mL by rotary evaporation at 40 °C. The concentrate was extracted with 10 mL of hexane, and the hexane layer was discarded. The acidic solution was adjusted to pH 7.5–8.0 with 1 M sodium hydroxide. Carbendazim was recovered from this basic aqueous phase by extracting with three 100-mL portions of ethyl acetate. The combined extracts were dried by passing through anhydrous sodium sulfate supported by glass wool in a large funnel. The sodium sulfate was washed with an additional 50 mL of ethyl acetate. The dried ethyl acetate extract was then concentrated to 3–5 mL by rotary evaporation at 35 °C and then quantitatively transferred to a graduated centrifuged tube. The solution was then evaporated to near-dryness under a gentle stream of nitrogen. Ten microliters of 3 mg/mL 2-AB in chloroform was added as internal standard, and the volume was adjusted to 1.0 mL with 1:1 methanol/acetonitrile. The solution was filtered through a 0.45-μm HPLC syringe filter, and 20 μL of the filtrate was analyzed by HPLC/MS/SIM.

Preparation of Calibration Curve. A stock solution of carbendazim was prepared by dissolving 10 mg of carbendazim reference standard in methanol in a 100-mL volumetric flask. A series of standard solutions was prepared by adding various volumes of the stock solution into graduated tubes, adding 20 μL of 2-AB internal standard stock solution (3 mg/mL in chloroform), and diluting to the 2-mL mark with 1:1 methanol/acetonitrile. These standard solutions were made to contain 1, 2, 5, 10, 30, and 50 ng/μL of carbendazim and 30 ng/μL of 2-AB internal standard and were analyzed by HPLC/MS/SIM with duplicate injections for each concentration level. We monitored m/z 192 (carbendazim) and m/z 134 (2-AB), and the peak area ratios of m/z 192 over m/z 134 were plotted against the amounts of carbendazim in the standard solutions. A linear calibration curve with a regression coefficient of 0.992 was obtained. In the recovery studies and sample analyses, benomyl was calculated by multiplying the amount of carbendazim with the molecular weight factor, 290/191 (the molecular weight of benomyl is 290).

RESULTS AND DISCUSSION

The major problem associated with the analysis of benomyl is formation of carbendazim during analysis and the fact that some carbendazim is already present due to environmental degradation. Chiba and Veres (1980) attempted to overcome this problem by derivatizing carbendazim with *n*-propyl isocyanate and extracting benomyl from freeze-dried sample with chloroform at 1 °C, a rather difficult, inconvenient, and time-consuming procedure. Stringham and Teubert (1984) took advantage of the equilibrium between benomyl and its degradation products, carbendazim and *n*-butyl isocyanate, by extracting wettable powder formulations with acetonitrile containing 3% *n*-butyl isocyanate. This procedure prevented loss of benomyl but also converted any carbendazim present to benomyl. Chiba and Singh (1986) converted benomyl to 3-butyl-2,4-dioxo-*s*-triazino[1,2-*a*]benzimidazole (STB) at pH 13 (a procedure that has no effect on carbendazim) and determined both compounds simultaneously by reversed-phase HPLC with UV detection. This method was used for the analysis of benomyl and carbendazim in water but not in food where more sensitive procedures would be required. Unfortunately, STB gave a poor

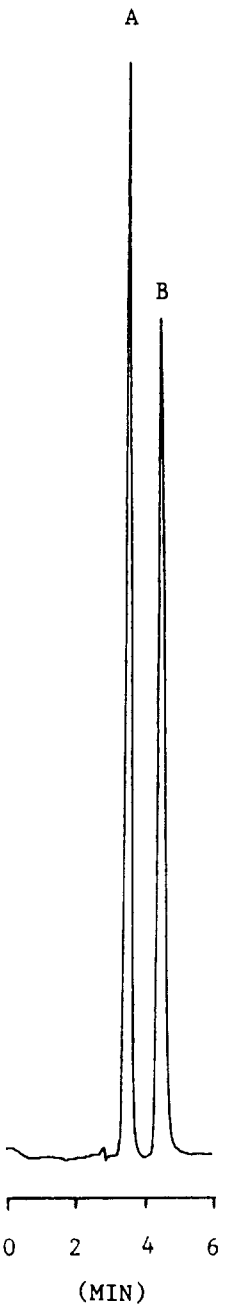

Figure 1. Chromatogram of carbendazim (A) and 2-AB (B), both at 30 ng/μL with 20-μL injection: integrator attenuation 2^7; detector range 0.02.

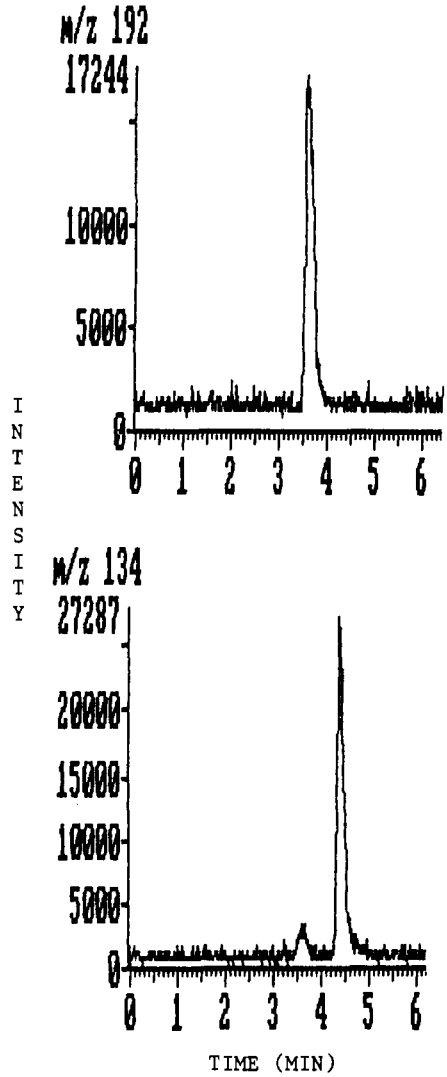

Figure 2. SIM chromatogram of a standard solution of carbendazim (upper trace) and 2-AB (lower trace) both at 30 ng/μL with 20-μL injection.

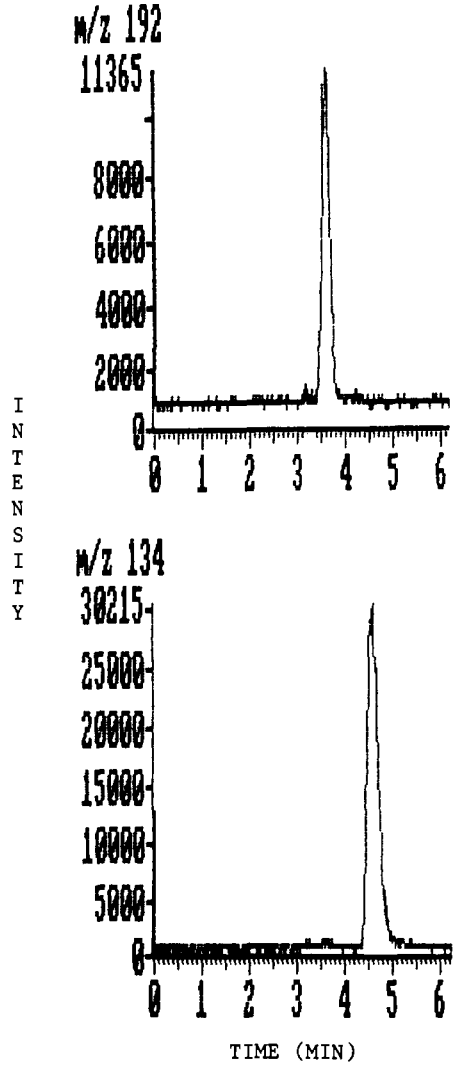

Figure 3. SIM chromatogram obtained from a peach sample spiked with a 0.1 ppm benomyl before extraction and to which internal standard was added to the concentrated extract.

detector response under our HPLC/MS conditions and was thermally unstable on a GC capillary column.

Our analytical method is based on the chemistry used by several other groups of investigators, i.e., conversion of benomyl to carbendazim with subsequent determination of the latter (Bardalaye and Wheeler, 1985; Spittler et al., 1984; Zweig and Gao, 1983; Kirkland et al., 1973). To the best of our knowledge, methodology based on this chemistry is currently used by the Food and Drug Administration. This method may overestimate the actual amount of benomyl present for two reasons: First, carbendazim, itself, while not registered for use in the United States is used elsewhere and may be present in imported food. Second, and far more troublesome, carbendazim is also a known metabolite (Soeda et al., 1972; Cano et al., 1987) of thiophanate-methyl, a fungicide not as widely used as benomyl but registered for use on some fruits and vegetables. Using a variety of published and unpublished data for the years between 1979 and 1986, the National Research Council (1987) estimated the use of benomyl and thiophanate-methyl as 2 000 000 and 30 000 lb of AI/year, respectively.

For the major objective of our study, i.e., to assess the carcinogenic potential of some heavily consumed commodities, and from a toxicological point of view, the source of the carbendazim is academic because both fungicides have essentially the same carcinogenic activity (Wiles, 1987). For regulatory purposes, however, the limitations inherent in this method present a serious problem.

With this proviso in mind, the modified method we have described for the analysis of benomyl as carbendazim is fast, accurate, and sensitive while giving a higher degree of confirmation of identity than methods based on UV detection alone. Reversed-phase HPLC allows for elution of carbendazim and 2-AB in less than 5 min as compared to retention times of approximately 18 min (Kirkland et al., 1973; Spittler et al., 1984) for HPLC methods using a strong cation-exchange column. The specificity of the mass spectrometer detection system allows us to chromatograph rapidly because none of the peach, apple, or tomato coextracts give $(M + H)^+$ or $(M + NH_4)^+$ ions at m/z 192 and 134. Thermospray HPLC/MS gives chemical ionization type mass spectra. Fragmentation to numerous ions, such as occurs in electron-impact mass spectrometry, is minimized.

2-Aminobenzimidazole was chosen as an internal standard because it has a structure similar to benomyl and carbendazim and because it has a retention time close to that of carbendazim. Although 2-AB is a suspected decomposition product of benomyl and/or carbendazim, this decomposition occurs only at pH > 13 (Chiba and Singh, 1986), and there has never been evidence for the presence of 2-AB residues in plant matrices (Spittler et al., 1984; Bardalaye and Wheeler, 1985). To further assure

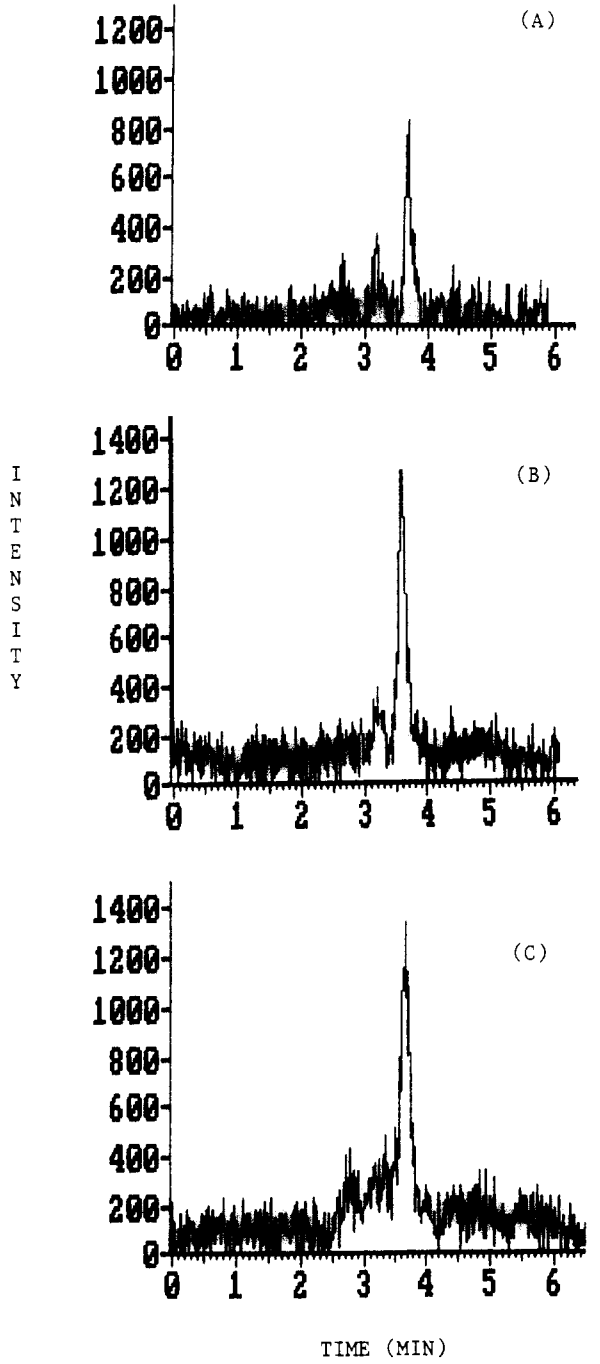

Figure 4. SIM chromatograms (monitored at m/z 192) for peaches (A), apples (B), and tomatoes (C), each spiked with 0.05 ppm benomyl before extraction.

ourselves that 2-AB could not arise from our methods, we analyzed apples spiked with 4 ppm benomyl and carried it through our procedure without adding 2-AB just before analysis. No 2-AB was detected.

Figure 1 shows the chromatogram obtained from the reversed-phase HPLC of carbendazim and the added internal standard 2-aminobenzimidazole (2-AB). Figure 2 depicts selected ion monitoring chromatograms obtained simultaneously by monitoring the M + 1 ions of carbendazim (m/z 192) and 2-AB (m/z 134). The selected ion monitoring chromatogram for peaches spiked with 0.1 ppm benomyl is shown in Figure 3. Similar chromatograms (not shown) were obtained from apples and tomatoes spiked with 0.1 ppm benomyl. UV detection of this sample under our HPLC conditions results in an unuseable chromatogram. Sensitivity declines sharply below 0.1 ppm, but reasonable chromatograms are still observed at 0.05 ppm (Figure 4). The limit of detection was estimated at about 0.025 ppm. Tomatoes spiked with 1.0 ppm benomyl (the lowest tolerance level for any food) gave recoveries of 108.8, 102.9, 91.6, 90.3, and 75.7% (average 93.9%, coefficient of variation 13.6%). At the 0.1 ppm level recoveries of benomyl were 105.0, 100.3, and 85.6% (average 97.0%, coefficient of variation 10.4%) in apples; 110.3, 94.4, and 84.9% (average 96.5%, coefficient of variation 13.3%) in peaches; and 96.4, 92.4, and 92.3% (average 93.7%, coefficient of variation 2.5%) in tomatoes.

Using the method discussed, we analyzed 25 samples each of tomatoes, apples, and peaches. Although results are discussed in terms of benomyl, it should be remembered that whole or part contributions to the concentration of the actual analyte, carbendazim, may have risen from sources other than benomyl (vide supra). Three of the tomato samples (Israel, Mexico, Florida) contained benomyl at trace (ca. 0.025 ppm) levels. Two samples of apples grown in New York contained 0.56 and 0.59 ppm benomyl while another sample of New York grown apples contained benomyl at trace levels. Two of the New Jersey grown apple samples contained benomyl at 0.15 and 0.16 ppm. Benomyl was most frequently found in peaches (18 positives out of 25 samples). Seven of the New Jersey grown peaches had benomyl residues of 3.48, 0.66, 0.31, 0.29, 0.28, 0.28, and 0.25 ppm, while five contained no detectable residues. Six South Carolina grown peaches had benomyl residues of 2.08, 1.97, 1.79, 1.02, 0.67, and 0.42 ppm, and one sample had no detectable residue. All of the Georgia peaches were positive (2.17, 2.04, 0.93, 0.88, 0.62 ppm). None of the samples we analyzed contained residue levels above tolerance (peaches, 15 ppm; apples, 7 ppm; tomatoes, 5 ppm).

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Specific Enzymatic Microassays of α-Amylase and β-Amylase in Cereals

Yannick Sirou, Didier Lecommandeur, and Christiane Laurière*

Laboratoire de Physiologie des Organes Végétaux, CNRS, 4 ter Route des Gardes, F-92190 Meudon, France

Two colorimetric assays for specific determination in cereals of α- and β-amylase, respectively, were adapted for use in microtiter plates. The two micromethods allow easy and reliable determinations of α- and β-amylase in a large number of samples. Application is provided with K_m determination of barley, maize, sorghum, and rice α- and β-amylases, respectively.

Two methods using commercially available reagents have been recently developed for specific determination of α-amylase (McCleary and Sheehan, 1987) and β-amylase (Mathewson and Seabourn, 1983; Sopanen and Laurière, 1989) in cereals. Both are based on mixtures of maltosaccharide substrates and ancillary enzymes normally intended for α-amylase assay in human serum and urine. These substrates are chemically bonded to *p*-nitrophenol (PNP) through the reducing glucosyl group. Hydrolysis by the amylase, in conjunction with an α-glucosidase acting on short maltosaccharides, leads to PNP liberation, and *p*-nitrophenoxide formation is read at 405 nm.

The α-amylase assay substrate is *p*-nitrophenyl maltoheptaoside blocked at its nonreducing end (BPNPG7). β-Amylase can act only after the cleavage of BPNPG7 by α-amylase, in conjunction with α-glucosidase present in the reagent. This avoids any interference by β-amylase (McCleary and Sheehan, 1987) and renders the test fully specific for α-amylase.

The β-amylase assay substrate consists of a mixture of *p*-nitrophenyl maltopentoside (PNPG5) and *p*-nitrophenyl maltohexoside (PNPG6). Compared to β-amylase, cereal α-amylase cleaves these substrates very slowly (Mathewson and Seabourn, 1983), and it was shown on germinating barley, by selective inhibition studies, that α-amylase action on the test can be neglected (Sopanen and Laurière, 1989).

These assays were compared to the previous standard methods (Mathewson and Seabourn, 1983; McCleary and Sheehan, 1987). The new methods were found more specific and easily adaptable to microtitration. We report here the development of these two assays to a microplate format, maximizing the number of samples to be tested.

MATERIALS AND METHODS

An automatic BioTek enzyme immunoassay reader was used for absorbances in the microplate wells. The Enzyline PNP Unitaire kit used for α-amylase was from BioMérieux (Marcy l'Etoile, France). It is similar to the one used by McCleary and Sheehan (1987), except that the α-glucosidase relative amount is higher. The Testomar kit used for β-amylase was from Behring Diagnostics (La Jolla, CA). Chemicals were analytical grade from Serva (Heidelberg, FRG).

A barley malt (*Hordeum vulgare* L., cv. Conquest) was used for the assays developments. Grains of maize (*Zea mays* L., cv. Dea), barley (*Hordeum vulgare* L., cv. Menuet), sorghum (*Sorghum bicolor* L., cv. Oasis), and rice (*Oryza sativa* L., cv. Cigalon) germinated for 6 days were used for the determination of K_m values. Extracts were prepared by stirring 1 g of malt flour or germinated grains in 5 mL of 50 mM sodium malate, 50 mM NaCl, and 2 mM $CaCl_2$, adjusted to pH 5.2 (McCleary and Sheehan, 1987), for 30 min, centrifuging, and filtering the supernatant through 0.22-μm pore membranes (Millex GV, Millipore, Bedford, MA).

All determinations were made in triplicate. Linear regressions were computed and printed by means of the RS/1 graphic software (BBN Software Products Corp., Baltimore, MD).

α-Amylase Assay. The lyophilized substrate was dissolved in 150 mM malate, pH 5.0, to buffer it at pH 5.2, in the optimal pH range for cereal α-amylases activity.

For the standard assay, 1 mL of this buffer/vial was used, which gave concentrations in the test of 1.3 mmol/L for BPNPG7 and $\geq$15 U/mL for α-glucosidase. A 30-μL portion of Enzyline reagent was pipetted into individual wells of a microtiter 96-well plate (Greiner Labortechnik). A 30-μL portion of extract diluted in extraction buffer was added, and the reaction was allowed to proceed for 20 min at 30 °C. A 1% Tris solution (150 μL), was then added, the pH rise stopping the reaction and turning PNP to its phenoxide colored form. Absorbance at 405 nm was then read.

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