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High-Performance Liquid Chromatographic Method for Simultaneous Determination of Benomyl and Carbendazim in Aqueous Media

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A simple reversed-phase HPLC method has been developed for the individual determination of benomyl and carbendazim in aqueous media. Benomyl is quantitatively converted to 3-butyl-2,4-dioxo-*s*-triazino[1,2-*a*]benzimidazole (STB) with NaOH at pH 13 and determined as STB, while carbendazim, present in the sample, is unaffected by this alkaline treatment and determined as carbendazim. Each of two HPLC systems used was run isocratically at a flow rate of 0.8–1.5 mL/min with a UV detector at 280 nm. Two types of 15 cm × 4.6 mm reversed-phase columns (ODS, ODS-II) were used with mobile-phase mixtures consisting of acetonitrile, water, and pH 7 buffer. The minimum detectable limit in solution was 0.03–0.05 μg/mL for STB and carbendazim with a 50-μL injection.

Benomyl, methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate, is a widely used systemic fungicide, but analytical methods for this compound are not yet well developed. Although many papers have been published regarding the analysis of benomyl, the majority of methods published in the past are to determine carbendazim (methyl 2-benzimidazolecarbamate, well-known as MBC), benomyl's most common degradation compound (Figure 1). The major reason for this approach is due to many technical difficulties that are associated with the specific behavior of benomyl in different solvents.

As analytical methods, high-performance liquid chromatographaic (HPLC) methods in which benomyl is determined as carbendazim seem to be very popular (Austin and Briggs, 1976; Kirkland, 1973; Kirkland et al., 1973). In one particular case, carbendazim, present as a natural degradation compound in a sample, was converted to and determined as benomyl; in this case the benomyl, which existed in the sample in its intact form from the beginning, was also determined as benomyl (Zweig and Gao, 1983). Results obtained with either of these conversion methods, however, do not represent the biological activity of samples. Carbendazim, which is deliberately produced from benomyl during the analytical procedure, cannot be distinguished from carbendazim, which was present in the sample as a natural degradation compound of benomyl. Similarly, benomyl, converted from carbendazim, cannot be distinguished from benomyl which was present in its intact form from the beginning.

Although the above kind of approach is not generally acceptable in pesticide residue methodologies, in the case of benomyl, this approach was well accepted in the past, mainly because carbendazim is also fungitoxic and because analysis of intact benomyl is difficult. The fungitoxicity of carbendazim, however, is markedly different from that of benomyl (Hall, 1980; Koller et al., 1982). Accordingly, the total quantity expressed as carbendazim or benomyl cannot be used to assess the toxicity or biological activity of fungicides present in a test sample.

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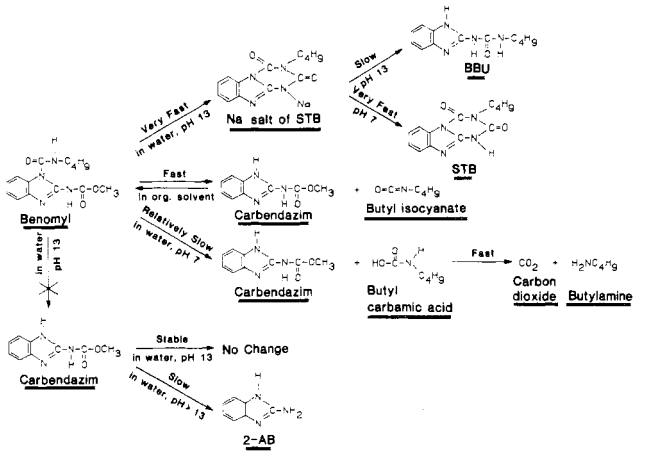

Figure 1. Degradation pathways of benomyl under different conditions at room temperature.

On the basis of the above principle, a great deal of effort has been made by Chiba and his co-workers to determine benomyl and carbendazim individually. A spectrophotometric method has been developed (Chiba, 1977) to elucidate the degradation of benomyl in organic solvents, and the method has been refined to determine both benomyl and carbendazim in WP formulations (Chiba, 1979). For individual determination of residual benomyl and carbendazim in plants, an HPLC method has been developed by the same workers (Chiba and Veres, 1980). In all the above cases, benomyl and carbendazim are determined in organic solvents. A reversed-phase HPLC method to individually analayze the two compounds has been needed for many years because of its ease of operation and wider applicability.

Presented in this paper is a method to determine low concentrations of benomyl and carbendazim in aqueous media by HPLC. It has been reported that benomyl is converted to 3-butyl-2,4-dioxo-*s*-triazino[1,2-*a*]benzimidazole (STB) under alkaline conditions (Calmon and Sayag, 1976; White et al., 1973). This basic finding was utilized to develop an analytical method in which benomyl is converted to and determined as STB, while carbendazim, present in the sample and unaffected by the alkaline treatment, is determined as carbendazim (Figure 1).

EXPERIMENTAL SECTION

Materials and Reagents. *Benomyl.* Analytical grade benomyl (99+%), obtained from E. I. du Pont de Nemours and Co., Inc., and Benlate 50% WP, purchased locally, were used.

Carbendazim. Analytical standard was obtained from E. I. du Pont de Nemours and Co., Inc.

STB. STB was obtained from Benlate 50% WP by the alkaline treatment of benomyl and purified as reported previously (Singh and Chiba, 1985).

Solvents. Acetonitrile and methanol used were HPLC grade from Caledon Laboratories Ltd., Georgetown, Ontario, Canada.

Preparation of Buffer Solutions. *For Mobile-Phase Mixtures.* Solutions of Na_2HPO_4 and KH_2PO_4 were prepared at 0.067 M individually, and the two were mixed at 3:2 (v/v) so that pH of the prepared buffer was 6.98 ± 0.05 (buffer A).

For Preparation of Samples. Solutions of Na_2HPO_4 and KH_2PO_4 were prepared at 1.20 M individually, and the two were mixed at 3:2 (v/v) so that the pH of the prepared buffer was 6.98 ± 0.05 (buffer B).

Preparation of Standard Solutions. A stock solution of carbendazim was prepared in methanol, and STB was prepared in acetonitrile, both at 100 μg/mL. The STB standard was used as standard for benomyl determination; 10.0 μg/mL of benomyl is stoichiometrically equivalent to 8.9 μg/mL of STB.

A mixed standard solution was prepared from the above stock solutions to contain 5 μg/mL of STB and carbendazim as follows. Five milliliters each of STB and carbendazim stock solutions were taken into a 100-mL volumetric flask, and then 5 mL of methanol, 60 mL of water, 5 mL each of 2 N NaOH, 2 N HNO_3, and buffer B were added in the order as stated. Finally, the flask was made up to volume with water.

Lower concentrations of mixed standard solutions containing STB and carbendazim were prepared as follows. First, lower concentrations of individual stock solutions were prepared at the concentrations of 60, 40, 20, 10, 5, 2, 1, and 0.4 μg/mL by diluting the initial 100 μg/mL stock solution of STB with acetonitrile and that of carbendazim with methanol individually. Second, 5 mL of each stock solution thus prepared was taken in a 100-mL volumetric flask, and then 5 mL of methanol and other ingredients were added as above to have 3, 2, 1, 0.5, 0.25, 0.1, 0.05, and 0.02 μg/mL standards, respecitvely.

Both STB and carbendazim in solutions thus prepared are stable for an extended period at room temperature. However, it should be noted that the solvent composition will change gradually by frequently opening the volumetric flask because it consists of acetonitrile, methanol, and water. The change in the solvent composition will reflect on the detector response.

HPLC. *Apparatus.* A Perkin-Elmer Series 3 HPLC equipped with a Rheodyne syringe loop-type injector and a Perkin-Elmer LC-55-S detector and a Spectra Physics SP-8000 HPLC equipped with a Valco loop-type autoinjector and a Spectra Physics SP-8300 UV detector were used. The wavelength used was 280 nm with both detectors, and injection volumes were 10–50 μL. The columns used were 15 cm × 4.6 mm Regis HiChrom reversible reversed-phase columns packed with 5-μm Spherisorb; two columns were ODS (C-18) and a third was ODS-II. The theoretical numbers of plates of these columns with dimethylphthalurea (test sample) was in the range of (66–71) × 10^3/m. A 5 cm × 4.6 mm precolumn, dry packed with Co:Pell ODS 25–37 μm (Whatman), was used ahead of each of the above analytical columns.

Mobile Phase. Mobile-phase mixtures used were CH_3CN–H_2O–buffer A. Of these, the most suitable mobile-phase mixtures were 40:45:15% (v/v) with ODS columns and 23:72:5% (v/v) with an ODS-II column. Each mobile phase was run isocratically at a flow rate of 0.8–1.5 mL/min.

Methods. *Alkaline Treatment of Sample, Adjustment of pH, and HPLC Analysis.* To a 100-mL volumetric flask were added 70 mL of aqueous sample and 5 mL of 2 N NaOH, and the mixture was shaken on a wrist action shaker for 20 min. To this solution were added 5 mL each of 2 N HNO_3 and buffer B. The pH of the solution at this stage should be 6.98 ± 0.05. Finally, 5 mL of acetonitrile was added and flask made up to volume with methanol. The solution thus prepared was injected into the HPLC system under above conditions and determination made by comparing peak height or area count with that of a mixed standard.

Actual Analysis of Samples. Water samples that contained commercial Benlate 50% WP at concentration levels of 50.0, 6.5, and 0.60 μg (a.i.)/mL were prepared. These concentrations are within the concentration range expected in common water samples and are substantially

Table I. Retention Times of STB and Carbendazim, with Different Mobile-Phase Solvents under Different Experimental Conditions

mobile phase: CH_3CN–H_2O–buffer A (v/v)	retentn time, min		operating condns		
	STB	carbendazim	HPLC	column	flow rate, mL/min
23:72:5	6.1	3.2	Perkin-Elmer	ODS-II	1.5[a]
25:70:5	2.6	2.2	Perkin-Elmer	ODS-II	1.5
35:55:10	5.2	7.0	Spectra Physics	ODS[b]	1.0
40:45:15	3.0	3.9	Spectra Physics	ODS[b]	1.0[c]
40:45:15	3.8	4.6	Perkin-Elmer	ODS[B]	0.8
45:40:15	2.7	3.6	Perkin-Elmer	ODS[b]	1.0
50:40:10	2.6	3.4	Perkin-Elmer	ODS[b]	0.8
60:30:10	2.4	3.2	Perkin-Elmer	ODS[b]	0.8

[a] See Figure 2 for chromatogram. [b] Same packing material, but different columns. [c] See Figure 3 for chromatogram.

Figure 2. Chromatogram of carbendazim and STB, both at 5.0 μg/mL with 50-μL injection: column, 15 cm × 4.6 mm Regis ODS-II; mobile phase, CH_3CN–H_2O–buffer A (23:72:5).

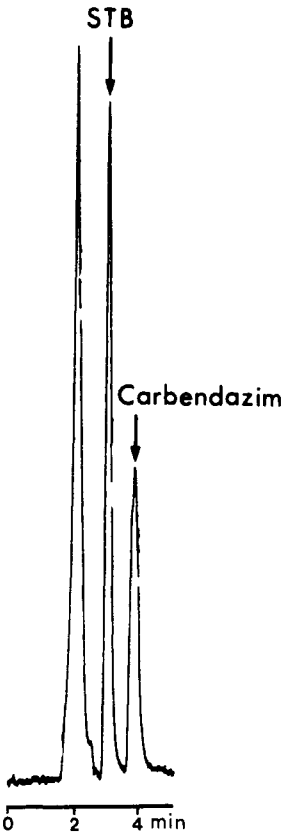

Figure 3. Chromatogram of STB and carbendazim, both at 5.0 μg/mL with 50-μL injection: column, 15 cm × 4.6 mm Regis ODS; mobile phase, CH_3CN–H_2O–buffer A (40:45:15).

lower than those being used for commercial spray applications at 90–190 μg/mL. Sample suspensions were prepared at each of the above concentrations with a formulated product of Benlate 50% WP. The suspensions were treated with alkali 1 h after the initial preparation of suspensions and further processed for quantitative determination as described. Sample suspensions were freshly prepared three more times on different days, and analayses were made accordingly.

RESULTS AND DISCUSSION

HPLC and Sample Preparation. A typical chromatogram of STB and carbendazim using an ODS-II column is shown in Figure 2. The mobile phase used was a mixture of acetonitrile, water, and buffer (23:72:5 v/v).

With an ODS column, the elution order of STB and carbendazim was reversed (Figure 3). Retention times of STB and carbendazim are listed in Table I.

The resolution of STB and carbendazim is influenced not only by the composition of mobile phase but also by the composition of sample solutions prepared for HPLC injection. Factors to be considered are percentages of organic solvents (acetonitrile and methanol), pH, and buffer concentrations. This is why standard and sample solutions were prepared as described in order to minimize any possible errors that might occur if these solutions do not have the same constituents.

The effect of composition of mobile phase on the retention times of STB and carbendazim was significant. The retention time of both compounds became shorter when the percentage of acetonitrile in the mobile phase was increased and the percentage of buffer solution was decreased. The use of buffer solution is essential to obtain good resolution and good reproducibility; this effect was more significant with STB than with carbendazim. The composition of sample solution must be maintained constant as suggested because, as pH increases, the retention time becomes shorter, and the peak height decreases as buffer concentrations increase. Further details of this kind of solvent effect will be published elsewhere.

Benomyl can be detected qualitatively in its intact form by a simple RP-HPLC method, with an appropriate mobile phase as reported previously (Singh and Chiba, 1985). It is difficult, however, to perform quantitative analysis of

benomyl per se because it is exceptionally difficult to prepare accurate concentrations of benomyl standards in aqueous media that are suitable for HPLC analyses. The reason for this is because its solubility in water is low (Singh and Chiba, 1985) and it is difficult to dissolve a known quantity of benomyl. In addition, benomyl, once dissolved in water, decomposes slowly, and the purity of benomyl is hard to assess.

Benomyl can be dissolved in organic solvents, but it decomposes rapidly at room temperature (Chiba and Cherniak, 1978). The decomposition can be slowed down by using a low temperature (e.g. 1 °C) (Chiba, 1979), but only a few laboratories have the access to the low-temperature operation. The breakdown can be slowed down also by the use of a stabilizer, *n*-butyl isocyanate (BIC) in organic solvents (Chiba, 1977), but the stabilizer does not work well in aqueous systems.

The kinetics of conversion of benomyl to STB have been reported by Calmon and Sayag (1976); the rate of conversion is substantially faster at pH >12. While this conversion is in progress, the STB further degrades to BBU (1-(2-benzimidazolyl)-3-*n*-butylurea; Figure 1), but this degradation is substantially slower than the conversion of benomyl to STB. At pH 13 our kinetic study proved that benomyl completely converted to STB in 8 min, but the formation of BBU was undetectable for a period of 30 min under the above condition. Accordingly, a reaction time of 20 min was employed in this method to standardize the procedure. After the reaction was completed, the solution was adjusted to pH 7, which is most suited to reversed-phase columns. Even when a small quantity of undissolved benomyl (i.e., Benlate 50% WP) was present at a level of 50 μg/mL, the conversion was completed within 10 min and STB thus formed was dissolved quite well at pH 13. (Strictly speaking, pH is supposedly slightly higher than 13 under the condition, because $[OH^-]$ at the time of conversion is 0.133 M.)

The linearity of the response through the origin is very good at least up to 5 μg/mL with both compounds at pH 7.0. If final concentrations in the solution prepared for HPLC analysis are substantially higher than 5 μg/mL, it is recommended that the final solution be appropriately diluted with the following solvent mixture: 70 parts of water, 5 parts each of 2 N NaOH, 2 N HNO_3, buffer B, and acetonitrile, and 10 parts of methanol. In contrast, the lowest concentration detectable in aqueous samples (judged by the response equal to twice the noise level) is 0.03–0.05 μg/mL for both compounds. Actual concentrations of benomyl and carbendazim can be calculated by applying a dilution factor, because in this method 70 mL of sample solution is adjusted to 100 mL for the final HPLC analysis. Gradient systems can also be utilized successfully, but with the choice of appropriate mobile-phase mixtures the isocratic system works well as demonstrated; it is faster, simpler, and more accurate.

The purity of analytical standard benomyl, marked as 99+% was found to be 98.0% and 2.0% carbendazim by this method. This result indicates that there is a possibility that a small percentage (less than 2%) of benomyl may be converted to carbendazim while essentially all the benomyl is converted to STB. Carbendazim, unaffected during the conversion period at pH 13, is determined as carbendazim. After the alkaline treatment, both STB and carbendazim are stable at pH 7 and were found to be unchanged over a period of at least 1 month.

Application of the Method for Actual Analysis. The method worked well as written for analysis of water samples containing variable concentrations of benomyl and carbendazim. Results of analyses of aqueous samples that were prepared with Benlate 50% WP are summarized in Table II. In some samples benomyl existed in solid form as suspension before conversion to STB, but total quantities of benomyl in water were determined easily. Modifications to the method for analysis of benomyl-formulated products and pathological samples will appear in future publications.

Table II. Analytical Results of Water Samples Containing Benomyl and Carbendazim at Three Concentrations

expected concn,[a] μg/mL	detected concn,[b] μg/mL		
	benomyl[c]	carbendazim[d]	total
50.0	40.8 (±4.08)[e]	9.9 (±1.78)	50.7
6.5	5.1 (±0.58)	1.2 (±0.25)	6.3
0.60	0.51 (±0.044)	0.13 (±0.092)	0.64

[a] A total concentration of benomyl plus carbendazim; the latter calculated as benomyl. Samples were diluted for analysis as described in the text. [b] Each value represents the average of four replicate analyses; the suspensions were prepared with Benlate 50% WP, which was taken from the same bag on four different days. [c] Determined as STB and calculated as benomyl. [d] Determined as carbendazim and calculated as benomyl. [e] Standard deviation.

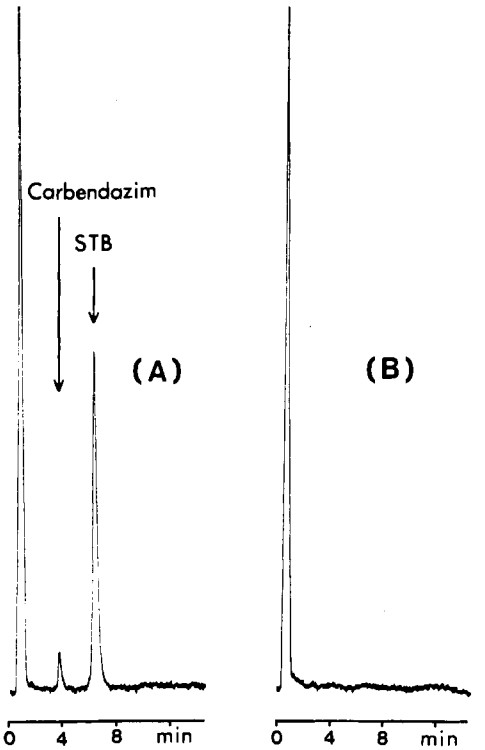

Figure 4. Chromatograms of pond water (A) with and (B) without the presence of benomyl (shown as STB) and carbendazim with 50-μL injection, respectively: column, 15 cm × 4.6 mm Regis ODS-II; mobile phase, CH_3CN–H_2O–buffer A (23:72:5). Concentrations as appeared were determined to be 3.30 μg/mL of STB (equivalent to 3.71 μg/mL of benomyl) and 0.52 μg/mL of carbendazim.

This method has been used to analyze environmental water samples also, without any interference. Chromatograms of a total of four samples (two samples each from streams and ponds) were no different from that of pure water. In Figure 4, chromatograms of water samples from one pond, with and without the presence of benomlyl (shown as STB) and carbendazim, are illustrated.

In conclusion, this method is simple and fast and has a wide range of application potential for analysis of benomyl and carbendazim in a variety of aqueous samples.

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Registry No. STB, 41136-38-3; H_2O, 7732-18-5; benomyl, 17804-35-2; carbendazin, 10605-21-7.

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Quantitative Analysis of 2-Acetyl-1-pyrroline in Rice

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A relatively simple practical method has been developed for the quantitative analysis of 2-acetyl-1-pyrroline in rice samples. The rice analysis method uses a steam distillation continuous-extraction isolation procedure with an acid-phase solvent extraction. This is followed by regeneration of the basic volatiles and capillary or packed column gas chromatography analysis. Testing of the method, with a bland rice variety containing known added concentrations of 2-acetyl-1-pyrroline, showed that the method was sufficiently accurate for the purpose.

INTRODUCTION

With both bland and aromatic rice types, as with most food crops, it is necessary to continually breed new varieties for disease or insect resistance and other environmental factors. In breeding new varieties, however, there is the danger of changing other subtle desirable features such as the flavor characteristics. A simple chemical analytical method for rice samples, to evaluate the flavor of the new varieties, could be very useful. Some of us had previously shown that 2-acetyl-1-pyrroline was an important flavor component of cooked rice particularly the aromatic (basmati type) rice varieties (Buttery et al., 1983).

The chemical analysis of 2-acetyl-1-pyrroline in rice by conventional methods is, however, difficult because of the presence of other interfering compounds and the instability of this compound. The present study was carried out to develop a relatively simple, practical method for analysis of this compound in rice.

EXPERIMENTAL SECTION

Materials. Authentic 2-acetyl-1-pyrroline was synthesized as outlined by some of us previously (Buttery et al., 1983). A standard solution of this compound containing 290 parts per million (ppm) of 2-acetyl-1-pyrroline was made in benzene solution. This was stored at −20 °C and seemed quite stable over several months.

Collidine (2,4,6-trimethylpyridine), used as an internal standard, was Eastman Organic Chemicals No. 4815. It was used as a water solution containing 30.0 ppm collidine. This seemed quite stable at room temperature.

Other reagents were of good-quality analytical reagent grade. Diethyl ether was freshly distilled and was protected by the addition of ca. 0.001% Ethyl antioxidant 330.

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Volatile-free water was obtained by boiling water in an open container removing about 10% of its volume. A volatile-free antifoam preparation was made by adding 20 mL of GE AF 60 Silicone antifoam emulsion to 600 mL of water in a wide-neck 1-L flask and concentrating the mixture to 200 mL by boiling.

All glassware was thoroughly cleaned and heated in an oven at 120 °C for several hours to remove all volatile contaminants. Aromatic rice variety samples were obtained from the International Rice Research Institute in the Philippines. Common rice samples were obtained from local markets.

Isolation of the Volatile Basic Fraction from Rice. Water (6 L) was added to a 12-L round-bottom flask containing a large efficient magnetic stirrer. The flask was supported by a 1300-W (two-circuit) 115-V heating mantle. While stirring, 200 g of rice was added gradually (so as not to interfere with the stirring motion). Antifoam solution (50 mL) and the internal standard (5.00 mL of the standard 30 ppm collidine solution) were then added. A Likens–Nickerson type steam distillation continuous-extraction head (cf. Kontes No. K-523010-0000; Nickerson and Likens, 1966) was attached to the neck of the 12-L flask. A 250-mL round-bottom flask, containing 80 mL of dilute sulfuric acid (80 mL of water + 2.0 mL of concentrated sulfuric acid) and 120 mL of diethyl ether with a magnetic stirrer, was attached to the solvent arm of the head. To heat the water in the 12-L flask to boiling, 100 V was applied to the upper mantle circuit and 80 V to the lower circuit. On boiling, these were changed to 80 V on the upper circuit and 70 V on the lower circuit. Care should be taken to prevent any "burning" of the rice on the bottom of the 12-L flask as this produces interfering compounds (alkylpyrazines). The solvent was refluxed (and stirred) vigorously (80 V on the solvent mantle). After 2 h of the steam distillation continuous extraction, the solvent flask was removed and the aqueous layer separated,