DETERMINATION OF BENOMYL AND 2-(4-THIAZOLYL)BENZIMIDAZOLE IN PLANT TISSUES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY USING FLUORIMETRIC DETECTION

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SUMMARY

Methods for determining benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate] residues in plant tissues involve isolation of the residue by extraction with ethyl acetate, hydrolysis of the extracted residue to 2-aminobenzimidazole and final determination by high-performance liquid chromatography using a fluorimeter. 2-(4-Thiazolyl)benzimidazole is also determined by a similar procedure without hydrolysis. The sensitivities of these methods are 0.02 ppm for benomyl and 0.001 ppm for 2-(4-thiazolyl)benzimidazole. Recoveries of these compounds from various plant tissues were 90.5–102.9 %. No interference with these methods from the plant tissues tested was found.

INTRODUCTION

Benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazole carbamate (MBBC)] and 2-(4-thiazolyl)benzimidazole (TBZ) are systemic fungicides that have preventive and curative properties and also a mite ovicide effect. Analytical procedures for MBBC[1,2] and TBZ[3] involving solvent extraction followed by spectrophotometric or fluorimetric methods have been described. The detection and identification of MBBC and other benzimidazole fungicides by thin-layer chromatography (TLC) have also been investigated[4–6]. Most of the methods for MBBC are based on the formation of 2-aminobenzimidazole (2-AB) by alkaline hydrolysis of MBBC following direct fluorimetic measurement, or colorimetric analysis after bromination[1,2]. TBZ is also determined by a fluorimetric procedure based on the fluorescence itself in acidic medium[3]. Fluorimetry is known to be about 100 times more sensitive than colorimetry, but fluorimetric methods for MBBC and TBZ have the disadvantages of a complicated procedure and the existence of some interferences.

Recently, Kirkland and co-workers[7,8] reported the high-performance liquid chromatography of MBBC residues in soil and plant tissues. This method is superior to the former fluorimetric and colorimetric methods because MBBC, methyl 2-benzimidazolecarbamate and 2-AB can be determined simultaneously as separate peaks. However, the crude extract had to be cleaned up by a liquid–liquid partitioning pro-

cedure in order to eliminate interfering compounds because of the use of a UV monitor for determination.

In this paper, a method for determining MBBC and TBZ residues in plant tissues by means of a high-performance liquid chromatograph equipped with a fluorimeter is described.

EXPERIMENTAL

Apparatus

A Hitachi Model 634 high-speed liquid chromatograph, a Hitachi Model 204 fluorescence spectrophotometer equipped with a xenon lamp, a Hitachi variable-wavelength spectrophotometric detector, a Waring blender, a Sakuma Model 45 CFS centrifuge and a Kyowaseimitsu Milton Roy Model KSU pump were used.

Materials

Analytical standards of MBBC, methyl 2-benzimidazole carbamate (MBC), 2-AB and TBZ were obtained from Sankyo Pharmaceutical Co. (Tokyo, Japan). Ethyl acetate, n-hexane and methanol were of reagent grade and were purified by re-distillation before use. Hydrochloric acid, sodium hydroxide, potassium hydroxide and glacial acetic acid were also of reagent grade.

Reagent solution

The reagent solutions used were 0.1 N hydrochloric acid, 6.5 N sodium hydroxide, 0.03 N potassium hydroxide–methanol and 0.1 and 5.0% (v/v) acetic acid–methanol.

Standard solutions

Standard solutions of 2-AB and TBZ were prepared by dissolving the analytical standard compounds in methanol at concentrations of 100, 50, 40, 20, 10, 5 and 4 μg/ml.

Isolation of residues from plant tissues

MBBC. Weigh 50 g of plant tissues in a Waring blender, add 150 ml of ethyl acetate and blend at high speed for 5–10 min. Centrifuge the blended mixture at 2000 rpm for complete separation of the layers. Carefully withdraw the upper ethyl acetate layer and filter it through a filter-paper into a 500-ml round-bottomed evaporation flask. Repeat the extraction with two additional 100-ml portions of ethyl acetate. Evaporate the combined ethyl acetate extract to about 20 ml under reduced pressure below 40° and add 25 ml of 0.1 N hydrochloric acid to it, followed by evaporation until the ethyl acetate is completely removed. Quantitatively transfer the aqueous acid solution into a 100-ml separating funnel and wash it with three 50-ml portions of n-hexane, discarding the n-hexane layer after phase separation. Transfer the acidic aqueous phase quantitatively into a 100-ml beaker and boil it gently for 15 min on a hot-plate after addition of 15 ml of 6.5 N sodium hydroxide solution. Transfer the alkaline hydrolyzed solution into a 100-ml separating funnel and extract 2-AB with four 40-ml portions of ethyl acetate. Filter each extract through a bed of anhy-

drous sodium sulphate. Concentrate the combined ethyl acetate extract to about 2 ml under reduced pressure, and then evaporate it to dryness under a stream of nitrogen. Dissolve the dried residue in 1.0 ml of methanol, and assay 2-AB by the liquid chromatographic method described below.

TBZ. Residues of TBZ in plant tissues are extracted and cleaned up as described for MBBC but without the alkaline hydrolysis step. After washing the acidic layer with *n*-hexane, add 15 ml of 6.5 N sodium hydroxide solution to the acidic layer, check with a pH test-paper to verify that the solution is now strongly basic, and extract TBZ with four 40-ml portions of ethyl acetate. Evaporate the combined extract to dryness, dissolve the residue in 1.0 ml of methanol and assay TBZ by the liquid chromatographic method described below.

Liquid chromatographic analysis of 2-AB and TBZ

2-AB. The liquid chromatograph, with a Hitachi Gel No. 3010 (particle size 20–23 μm) column (500 $\times$ 2.1 mm), is first equilibrated using the following conditions: column temperature, 45°; mobile phase, 0.1% (v/v) acetic acid–methanol; carrier flow-rate, 1.2 ml/min; inlet pressure, 30 kg/cm^2; UV monitor, 0.16 absorbance full-scale; fluorimeter, sensitivity selector 1 $\times$ 8; chart speed, 2.5 or 5.0 mm/min; 0.03 N potassium hydroxide–methanol is pumped at the flow-rate of 1.6 ml/min. The effluent is monitored at an excitation wavelength of 285 nm and an emission wavelength of 315 nm with the fluorimeter and at 277 nm with the UV monitor. A flow diagram is shown in Fig. 1.
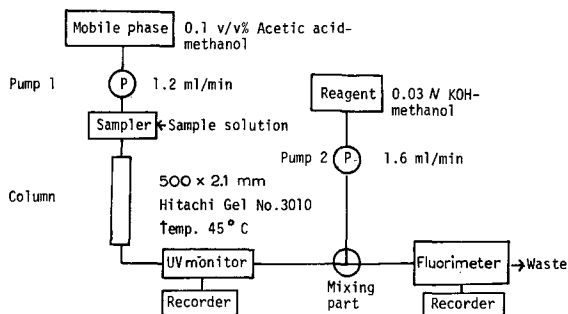

Fig. 1. Flow diagram for 2-AB using high-performance liquid chromatography with a spectrofluorimeter.

TBZ. A column (500 $\times$ 2.1 mm) packed with Hitachi Gel No. 3010–CH$_2$OH (particle size 20–23 μm) with 5.0% (v/v) acetic acid–methanol as the mobile phase are used, and a reagent pump (pump 2 in Fig. 1) and the mixing part are removed. All other conditions were identical to those for 2-AB. The effluent is monitored at an excitation wavelength of 305 nm and an emission wavelength of 355 nm.

In practice, 50 or 25 g of sample are taken for the assay, the final volume of extracted sample is made up to 1.0 ml and then 50 μl of the solution are chromatographed under the conditions described above. If the final solution exhibits turbidity, it should first be filtered through a glass filter. The amount of 2-AB or TBZ is determined by comparing the peak height or peak area with the working curve for 2-AB or

TBZ, and the residual amount of MBBC or TBZ in parts per million can be calculated as follows:

$$\text{MBBC (ppm)} = \frac{(\mu\text{g 2-AB}) \cdot 2.18 \cdot 1000}{W \cdot V \cdot R}$$

$$\text{TBZ (ppm)} = \frac{(\mu\text{g TBZ}) \cdot 1000}{W \cdot V \cdot R}$$

where W = weight of sample in grams, V = volume of the final sample solution injected in microlitres, R = recovery factor, 2.18 = conversion factor from 2-AB to MBBC and 1000 = volume of the final sample solution in microlitres.

RESULTS AND DISCUSSION

The columns packed with porous polystyrene polymer, either Hitachi Gel No. 3010 or and 3010–CH$_2$OH (particle size 20–23 μm) (the latter is a porous polymer containing CH$_2$OH groups), gave effective results. The following parameters were examined in order to determine the optimal conditions for the determination of MBBC or TBZ residues in plant tissues.

Selection of mobile phase

Of the many solvent systems tested, the most suitable was found to be a mixture of acetic acid and methanol. Small variations in the acetic acid concentration gave improved separations and peak heights. The peak heights of 2-AB and TBZ became constant above 0.1 and 5 % (v/v), respectively.

Effect of the concentration of potassium hydroxide–methanol as an fluorescent reagent

2-AB gave no fluorescence in neutral and acidic media, but was highly fluorescent in alkaline media. The effluent from the column should therefore be mixed with an alkaline reagent. As shown in the flow diagram (Fig. 1), the effluent stream was mixed with potassium hydroxide–methanol solution pumped at the rate of 1.6 ml/min. The peak height of 2-AB reached a maximum at 0.03 N potassium hydroxide–methanol after a sharp increase, and then a slight decrease was observed as the concentration of potassium hydroxide increased. With TBZ, an alkaline solution was not needed as it was highly fluorescent in acidic media.

Working curves and sensitivities

Each 5-μl portion of the standard solutions was injected by using a Hamilton syringe. The working curves were prepared by use of the peak heights or peak areas, as shown in Fig. 2. The relationships between the amounts of 2-AB or TBZ and the peak heights or peak areas were linear from 0.02 to 0.8 μg of 2-AB and from 0.005 to 0.5 μg of TBZ. The working curve obtained by using the UV monitor is also presented in Fig. 2 for comparison. The smallest amounts that could be determined were 20 ng of 2-AB and 5 ng of TBZ by the proposed fluorimetric method, and 25 ng of 2-AB using the UV monitor. Therefore, sensitivities of these methods are 0.02 ppm for MBBC and 0.001 ppm for TBZ when 50 g of sample are taken, the final volume of sample is made up exactly to 1.0 ml and 50 μl are chromatographed.
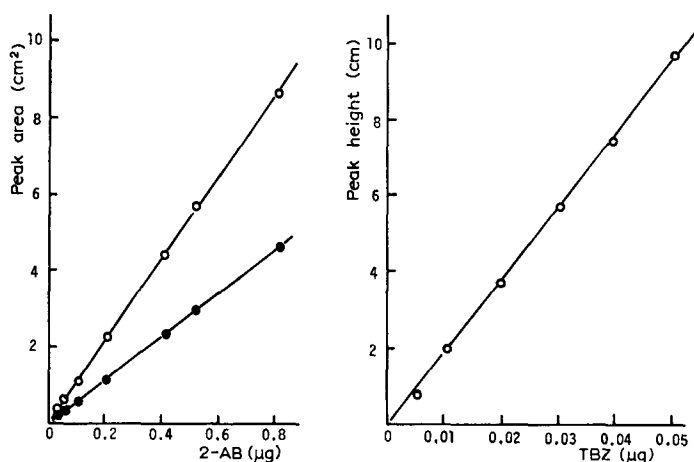
Fig. 2. Working curves for 2-AB and TBZ. ○, Fluorimetry, sensitivity 1 × 8; ●, UV detection, 0.16 a.u.f.s.

Quenching studies

It was known that a varying degree of quenching is encountered with extracts of various substances[1]. In particular, the final extracts of orange peel and pulp are appreciably coloured and complete quenching was obtained in the fluorimetric method reported by Pease and Gardiner[1]. Consequently, the quenching factor of each of the samples analyzed was determined by adding a known amount of 2-AB or TBZ to the untreated control extract and comparing the peak height obtained with that obtained from the standard solution under the same conditions. The results indicated that these factors ranged from 0.94 to 1.06. The quenching interferences, even with orange, seemed to be effectively separated by the procedures described and the quenching factor was therefore omitted from the calculations of the residual amounts.

Recovery and reproducibility

Some additional recovery and reproducibility data for MBBC and TBZ are shown in Table I. Recoveries of 90.5–102.9% for MBBC and 98.2% for TBZ were

TABLE I

RECOVERY AND QUENCHING FACTOR STUDIES ON PLANT TISSUES

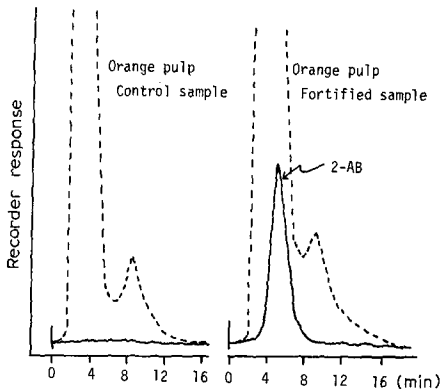| Compound	Plant tissue	Quenching factor	Average recovery (%)	No. of determinations	Range (%)
MBBC	Orange pulp	1.00	96.4	3	3.0
	Orange peel	0.96	90.5	2	2.1
	Tomato	1.03	99.0	3	9.9
	Cucumber	0.94	102.9	2	11.0
	Onion	0.98	98.3	4	9.7
	Peach	0.98	98.9	3	10.4
	Chestnut	1.00	98.2	7	3.3
TBZ	Orange pulp	1.06	98.2	7	5.3

Fig. 3. Chromatograms of orange pulp extracts. Operating conditions: Column, 500 × 2.1 mm, Hitachi Gel No. 3010 (particle size 20–23 μm); mobile phase, 0.1 % (v/v) acetic acid–methanol; column temperature, 45°; flow-rate, 1.2 ml/min; detector, fluorimeter and UV monitor; sample taken, 50 g; final volume, 1.0 ml; injected volume, 50 μl. Broken line, absorbance at 277 nm (0.16 a.u.f.s.); solid line, fluorescence intensity at excitation 285 nm and emission 315 nm (sensitivity: 1 × 8).

obtained. As a typical example, Fig. 3 shows liquid chromatograms of control and fortified orange extracts. The broken lines are the chromatograms measured with the UV monitor at 277 nm for 2-AB. Although the limit of determination for 2-AB obtained with the UV monitor was almost same as that obtained with the fluorimeter, the peak of 2-AB was overlapped by the strong band of UV-absorbing substances in orange extract and could not be detected. However, only the peak of 2-AB appeared on the chromatogram obtained with the fluorimeter, so 2-AB could therefore be determined quantitatively.

CONCLUSIONS

The advantage of high-performance liquid chromatography using a fluorimeter is its sensitivity and simplicity. Only the fluorescent compound is detected, no quenching was observed with any of the samples tested and the liquid–liquid partitioning clean-up operation is shortened. Therefore, the proposed methods for the determination of MBBC and TBZ residues in plants are suitable for routine analysis.

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