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Note

Quantitation of benomyl and its metabolites by thin-layer densitometry

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Benomyl [methyl 1-(butylcarbamoyl)-2-benzimidazolecarbamate] is the active ingredient in the important fungicide DuPont Benlate benomyl. MBC (methyl 2-benzimidazolecarbamate) is the principal degradation product of benomyl, while 2-AB (2-aminobenzimidazole) has been shown to be a minor degradation product in plants. Benomyl[1–4], MBC[3–6], and 2-AB[1,3] have been detected as quenched spots on thin layers containing fluorescent indicator, while benomyl has also been observed as a naturally fluorescent spot before and after heat treatment of the layer[7]. 2-AB was detected as a yellow-brown spot after exposure of the layer to bromine[8]. Radioscanning, radioautography and liquid scintillation counting have been used to detect benomyl and its degradation products[9] and bioautography for visual estimation of benomyl and MBC zones[10].

Several different approaches have been taken for the analysis of residues of benomyl, MBC, and 2-AB. Composite benomyl–MBC residues were quantitatively determined as MBC by UV spectrophotometry at 287 nm after elution from a thin layer[5,6]. Benomyl and/or MBC residues were hydrolyzed to 2-AB and detected as such by thin-layer chromatography (TLC)[8] or quantitated by direct fluorimetry in methanol solution at 335 nm[11,12]. Benomyl was converted to MBC and determined by fluorimetry in solution as this species[13]. A high-speed cation-exchange liquid chromatographic (LC) method was described for determining total benomyl and/or MBC residues as MBC and free 2-AB metabolite separately[14].

In view of these diverse reports and the absence of previous studies of densitometry, the separation, detection, and quantitation of benomyl, MBC, and 2-AB by fluorescence and fluorescence quenching on thin layers of silica gel were studied.

EXPERIMENTAL

Standards of benomyl, MBC, and 2-AB were supplied by H. L. Pease of the Biochemicals Department, E. I. DuPont (Wilmington, Del., U.S.A.). Benomyl standard was dissolved in ethyl acetate at a concentration of 1 μg/μl and MBC and 2-AB standards were dissolved in methanol at 0.5 μg/μl. Secondary solutions were prepared by dilution as needed.

Kontes (Vineland, N.J., U.S.A.) Chromaflex precoated TLC plates containing a 250-μm layer of silica gel including fluorescent indicator (silica gel GF_{254}) were used as received for studies of UV absorption (fluorescence quenching). Eastman-

Kodak (Rochester, N.Y., U.S.A.) Chromagram plastic-backed silica gel layers (No. 6061) were used to study fluorescence after purification by an overnight ascending development with trichloromethane–methanol (1:1) and drying in a hood for several hours. These layers were found to allow the most sensitive quantitation of fluorescent spots in an earlier study[15].

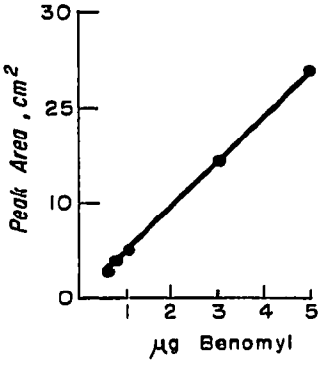
Initial zones were applied by repeated spotting from 1-μl Drummond Microcap micropipets and development was carried out in solvent-saturated, paper-lined rectangular glass TLC tanks (N-tanks). After air drying for 20 min, developed layers were inspected under 254 and 360 nm UV hand lamps.

Densitometry of quenched zones was made using the Kontes Chromaflex fiber optics scanner with the short-wave UV source. The TLC plate was placed with the adsorbent down onto the cleaned quartz cover plate and the pair of plates positioned inside the instrument so that scanning of transmitted light was across the direction of chromatographic development. Negative peaks result from scanning quenched spots. The methods described before[15] for proper use of the Kontes densitometer in the fluorescent mode were followed.

The scanner was connected to a Bausch and Lomb VOM 6 recorder operated on the 2.5-mV range. Peak areas were measured by height $\times$ width at half height. The scanner attenuation was initially set to give an approximately full scale peak with the highest concentration spot and was lowered as needed to record the spots of smaller concentration. The minimum quantitation level was that amount which gave a peak just distinguishable from the background noise. This paper is the first report, to our knowledge, of the use of the Kontes scanner for fluorescence quenching.

RESULTS AND DISCUSSION

Calibration curves are shown in Figs. 1–4 for benomyl, MBC, and 2-AB after chromatographic development on silica gel GF_{254} with a solvent which provides compact zones and carries the compound near the middle of the plate, in which area quantitation is more consistent[16]. Solvents chosen were methanol–benzene (7:93) for



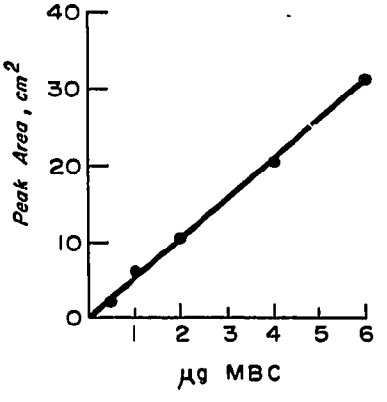
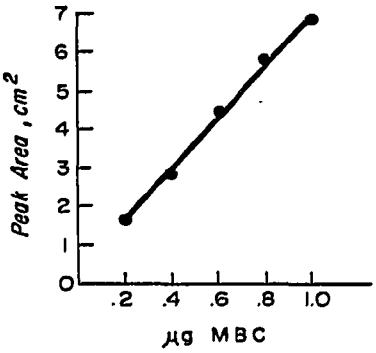


Fig. 1. Calibration curve for serial amounts of benomyl separated by TLC and quantitated by spectrodensitometry of fluorescence quenching.

Fig. 2. Calibration curve for 0–6 μg of MBC on silica gel GF_{254} scanning fluorescence quenching.



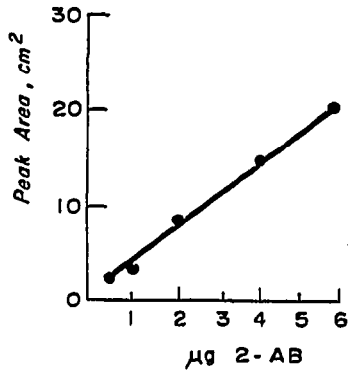


Fig. 3. Calibration curve for 0–1 μg of MBC on silica gel GF_{254} scanning fluorescence quenching.
Fig. 4. Calibration curve for 0–6 μg of 2-AB on silica gel GF_{254} scanning fluorescence quenching.

benomyl ($R_F \approx 0.6$), methanol–benzene (15:85) for MBC ($R_F \approx 0.5$), and methanol–benzene (1:1) for 2-AB ($R_F \approx 0.5$). Separation of the three compounds can be easily accomplished by development with methanol–benzene (15:85) solvent in the order benomyl ($R_F \approx 0.9$), MBC (0.5), and 2-AB (0.1).

Calibration curves were linear in all cases over the ranges shown in Figs. 1–4. Studies at higher levels indicated the MBC and 2-AB curves were linear up to at least 9 μg, but there was a break in the benomyl curve with a lower slope beyond 5 μg.

The visual detection limit for benomyl by quenching was 600 ng, the same as the lower limit of quantitation by the Kontes scanner. Other brands of fluorescent silicagel layers were tested, but none was superior to the Kontes Chromaflex plates. Benomyl was detectable visually on non-fluorescent silica gel down to 1 μg as a bright fluorescent spot on a blue background under 254-nm UV light. The scanner, however, did not detect this fluorescence even at a level as high as 10 μg. It has been noted before[15] that the instrument may not detect fluorescent spots easily seen by the eye.

Despite determination of MBC by fluorimetry in solution[13], the compound did not fluoresce on silica layers at levels up to 9 μg. This may be due to differences in fluorescence characteristics of the compound in solution and adsorbed on silica gel, or to the lack of a monochromator in the hand lamp or scanner to enable selection of the optimum excitation wavelength. The visual and instrumental detection levels of MBC were both 200 ng by quenching.

2-AB was detected at 500-ng levels by quenching (visual and instrumental), but fluorescence was not noted up to 10 μg. Since 2-AB is fluorimetrically measured in methanolic sodium hydroxide solution[17], attempts were made, without success, to induce fluorescence by spraying the chromatogram with this solution. Also unsuccessful was spraying with fluorescamine, a reagent which yields fluorescent spots with aliphatic amines[18] and many aromatic amines but not with any amine-substituted heterocyclic compounds so far tested[15].

Although any of the three compounds could be quantitated by scanning of quenched zones (Figs. 1–4), necessary extraction–purification procedures usually preclude measurement of benomyl residues as such since MBC will continually form from

benomyl during lengthy work-up steps while in solution at low levels[9]. It is therefore proposed that *in situ* TLC quantitation of MBC and/or 2-AB be carried out after application of established extraction–purification procedures, the exact nature of which will depend on the sample substrate. These include isolation methods for benomyl and/or MBC residues in food crops[5,13] or plants and soils[19] as MBC; benomyl plus any MBC and/or 2-AB in plant and animal tissues and soil as 2-AB[11,12]; or benomyl and/or MBC in soils and plant tissues as MBC, while 2-AB residues are also measured directly as a separate zone[14].

Any of these approaches can be utilized since MBC or 2-AB can be quantitated with about equal sensitivity, and the two compounds are well separated by TLC. After establishment of calibration curves under local conditions, an "external standard" should be included on each plate containing actual samples to correct results (which may be slightly variable from run to run) back to the curve. This correction can be made by multiplication of the area of the sample zone times A_1/A_2, where A_2 is the peak area of the external standard and A_1 is the peak area for the same amount of compound from the original calibration curve. The quantitative TLC determination is selective, sensitive, rapid, and simple, and offers an attractive alternative to read-out by gas chromatography, high-speed LC, or spectrophotometry for residues of benomyl and its metabolites.

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