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Note

Quantitative routine determination of thiabendazole by fluorimetric evaluation of thin-layer chromatograms

H. OTTENEDER

Landesuntersuchungsamt für das Gesundheitswesen Nordbayern, Fachbereich Chemie, Aussenstelle Regensburg, Regensburg (G.F.R.)

and

U. HEZEL

Abteilung für physikalisch-chemische Analyse, Carl Zeiss, Oberkochen (G.F.R.)

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Thiabendazole [2-(4-thiazolyl)benzimidazole; TBA] is used in banana and citrus fruit cultivation as a systemic fungicide and is commercially available under the names "Mertect" and "Tecto". Another, more important, application of this substance is for treating the rind of bananas or citrus fruits to protect them from decay during storage and transport. As TBA becomes increasingly used in the producer countries, it has been included in the regulations for preservatives of the EC Council of April 17th, 1971, which limit the TBA content of citrus fruits to 6 ppm and of bananas to 4 ppm; these regulations have been in force in Germany since March 28th, 1972. In the U.S.A., there has been an upper limit of 6 ppm of TBA for citrus fruits since 1971.

The active substance can be determined spectrophotometrically[1] with the aid of a colour reaction, or by gas chromatography[2,3]; it can also be determined by measuring the UV absorption or the fluorescence intensity of a TBA extract purified either by specific extraction steps[3,4] or by thin-layer chromatography (TLC)[5,6].

After TLC separation of the extracted components, TBA can also be determined directly on the chromatogram by reflectance–absorption photometry[7] with measurement at 302 nm or fluorimetrically[8] at 355 nm, with excitation at 313 nm (Hg line). Compared with elution and subsequent photometry, direct evaluation on the chromatogram has the advantages that it saves time and requires less substance per spot.

EXPERIMENTAL

Measuring methods

The chromatograms can be directly evaluated by reflectance, because TLC spots absorb radiation of a wavelength characteristic of the applied substance and reflect less radiation than the non-absorbing background. This diminution of reflectance by absorption in the visible and UV regions can be used for quantitative evaluation by scanning the spots with a slit adapted to the spot width. Monochromatic

radiation impinges vertically on to the sample, and a photomultiplier measures the intensity of the diffusely reflected radiation at 45°. The recording is an indication of the distribution of the substance on the chromatogram, and the area between the curve of reflectance *versus* locus and the baseline is a measure of the amount of substance in the spot. When the square of this area is plotted against the amount of substance, the result will generally be a straight line.

In a previous paper[7], the reflectance method was preferred because there is no photochemical decomposition during measurement. However, absorption measurements in reflectance and in transmission are greatly affected by the shape of the spot, which in turn depends on the relative amounts of the fruit and rind extracts (see Table I); comparison between pure TBA and TBA in extracts, therefore, requires an empirical correction method.

Instead of UV absorption, the fluorescence of TLC spots can be used for their direct quantitative determination, the fluorescence of the sample being excited by radiation from a high-energy source. With low concentrations the measured fluorescence intensity is directly proportional to the amount of substance[9]. The measured fluorescence is thus independent of the spot shape, and the sensitivity of the fluorimetric measurement is superior by two orders of magnitude to that of absorption measurements in reflectance. These advantages, which are of paramount importance for routine work, have led to new investigations in view of TBA determinations with the fluorescence method (see ref. 8). As will be shown, photochemical decomposition of TBA during irradiation can be kept negligibly small by selecting suitable measuring conditions. The measuring systems used with the Zeiss Chromatogram Spectrophotometer for the work described here are shown diagrammatically in Fig. 1.

In system A, exciting radiation at 313 nm from a medium-pressure mercury source with filter M313 impinges on the sample at an angle of 45° (in the direction of the arrow) and reaches the monochromator together with the emitted radiation of wavelength 355 nm, which is specific for TBA. The emitted radiation is isolated within a narrow band before it reaches the detector. This system has very high spectral sensitivity and is thus advantageous whenever fluorescing satellite substances cannot be entirely separated chromatographically.

In system B, the arrangement used for reflectance measurements is shown; this system is best suited for absorption photometry (which is routine in food-chemical laboratories). It can easily be converted into the fluorescence system (C) without additional wiring, simply by exchanging the light source and incorporating a suitable filter.

In fluorescence system C, the monochromator isolates the 313-nm line from the radiation of the mercury source, and exciting radiation is eliminated from the emission spectrum of TBA by means of barrier filter FL39, which does not transmit at wavelengths below 390 nm. A high proportion of the TBA emission (which has its maximum at 355 nm), however, is also absorbed, and this reduces the sensitivity of the method. The sensitivity may be increased by incorporating a Schott WG3 barrier filter (thickness 2 mm; absorption edge at 340 nm); the wide-band filter M365 included in the basic equipment offers at least equal sensitivity. This filter eliminates sufficient exciting radiation from the emission, has good transmittance for the TBA emission radiation and cuts off interfering fluorescence of wavelengths longer than 400 nm. Apart from its easy conversion into the reflectance mode and *vice versa*, fluorescence

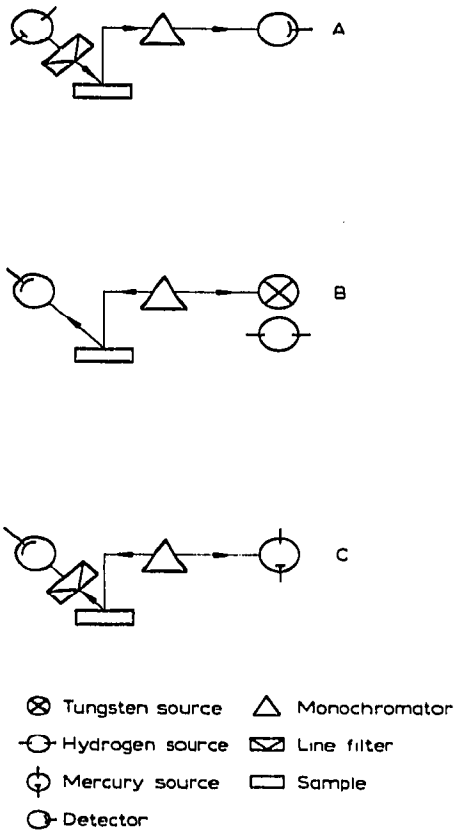

Fig. 1. Diagrams showing different measuring assemblies of the Chromatogram Spectrophotometer: A, assembly for fluorescence measurement with M313 filter; B, assembly for reflectance measurement; C, assembly for fluorescence measurement with M365 filter.

assembly C has the advantage that only a small amount of radiation impinges on the sample. As will be described in detail under *Thin-layer chromatography*, interference-free evaluation requires narrow-banded (and thus specific) emission; this requirement is optimally fulfilled by systems A and C (with filter M365).

Measurement results

Photochemical decomposition of TBA. If the TBA spot on a developed chromatogram with 8 μg of sample applied is exposed to exciting radiation of 313 nm in fluorescence system A (excitation at 313 nm; emission at 355 nm; iris; scanning speed 0; recorder speed 30 mm/min), the fluorescence intensity decreases by 14% per min. This can produce faulty results, because during the focusing and adjustment of the exciting radiation to the sample spots they are exposed to different strains from radiation. Thereby very different substance losses can be caused among the spots. This effect is minimized by using smaller amounts of sample; with 0.6 μg of sample, the fluorescence loss is only 1% per min.

Influence of rind substances on results in fluorescence and reflectance. Table I

TABLE I

MEASURED RESULTS FOR ZONES CONTAINING 250 ng OF TBA AND DIFFERENT AMOUNTS OF RIND EXTRACTS

Weight of fruit (g)	*Measured results*	
	Fluorescence area (cm^2)	*Reflectance area[2] (cm^1)*
0	14.0	226
70	13.9	188
140	13.6	189
210	13.8	180
280	14.7	170
350	14.0	135

shows a comparison of results for pure TBA solution and TBA solutions containing increasing amounts of rind extracts, determined on the chromatogram in unit areas or squares of unit areas. For the fluorescence mode, the measured value corresponding to the amount of substance in the spot is almost constant. For reflectance measurements, the measured result decreases as the content of the spot increases, resulting in a falsely low result for the TBA content. As already mentioned, the reflectance assembly gives useful results only when the allowance for the rind substances is made in the calibration. As this method requires greater outlay and has certain experimental difficulties, fluorescence evaluation of chromatograms is now preferred.

Thin-layer chromatography

The examination of numerous TBA-containing rind extracts of citrus fruits showed that a typical interfering substance was present in grapefruit. Depending on the separation on the TLC plates, the fluorescence curve of the TBA peak in grapefruit extracts showed a slope or satellite peak caused by fluorescent substances in the rind. The interfering substance was often completely overlaid by TBA. The fluorescence maximum of TBA that is overlaid by rind substances is no longer at 355 nm, but is displaced by 20–30 nm towards longer wavelengths; this led to recoveries considerably above 100% for TBA. The interfering substances could be well separated by developing with ethyl acetate–methyl ethyl ketone–formic acid–water (5:3:1:1), which eliminates falsely high results. Even the spectrally less specific measuring system C can then be used for extracts from all types of citrus fuit. As shown in Table II, there is no positive systematic error, although extraction losses must be ex-

TABLE II

RECOVERY OF TBA ADDED TO FRUITS

Fruit	*Number of samples*	*Recovery (%)*
Orange	6	93–100
Grapefruit	13	91–103
Lemon	3	90– 93
Banana	1	92

pected and these can exceed the scattering of the measurement method itself.

Accuracy

The standard deviation of the method, determined by the results of multiple determinations of different samples, is 2.67% (37 degrees of freedom); the individual values were determined for different TLC plates. For the discrete measuring value, this means a scattering of 5.42% ($P = 95\%$) and a confidence range for the average value of duplicate determinations is 2.61% ($P = 95\%$).

These values are considerably influenced by the application technique. We used 2-μl glass capillaries, together with a Merck DC applicator. Compared with the "micro-doser" application technique[7], this method yields starting spots of equal size and with a homogeneous distribution of TBA. The reproducibility of sample application as a partial error of the method shows a relative standard deviation of 1.9% for the capillary technique and 4.2% for the "micro-doser" (microlitre syringe with electrical drive). The above-mentioned results are obtainable only with capillary application.

The error of the results is also considerably influenced by the number of standard samples analyzed when plotting the calibration graphs. It has been recommended[10] that at least 5 points should be determined to establish the calibration line.

Apart from errors caused by the analysis method, fluctuations in the measured result can arise through inhomogeneous distribution of TBA on the individual fruits. For 12 fruit samples of 200–400 g from one batch, the TBA content ranged from 0.69 to 3.60 ppm (average 1.96 ppm); this corresponds with previous findings[11]. Owing to the lack of appropriate data, we cannot comment about the statistical distribution of the amounts of TBA. However, representative results cannot be obtained with the usual practice of extracting the rind of only 1 or 2 fruits, and we recommend that a 2.0–2.5 kg laboratory sample of individual fruits should be taken from different parts of a consignment.

Evaluation of measurements

Calibration is required for the quantitative evaluation of TLC plates, and this represents the relationship between the measured fluorescence emission and the concentration of fluorescent substance. With TBA, this relationship is not rectilinear, but it can be approximately represented as a branch of a parabola. According to Ebel and Herold[8], this phenomenon is due to overlapping of the excitation and fluorescence bands, which causes re-absorption. To determine the parameters of the compensation function, Ebel and Herold used a programmable desk calculator. In our experience, however, the relationship between fluorescence intensity and substance concentration can be represented as a straight line by using a logarithmic scale on both axes (see Fig. 2); this allows sufficiently exact evaluation without the need for a calculator.

ANALYTICAL PROCEDURE

Extraction

Weigh and peel 2.0–2.5 kg of fruit, mix the chopped rinds well, and weigh them. Extract an amount of rind corresponding to 400 g of fruit for 3 h in a Soxhlet

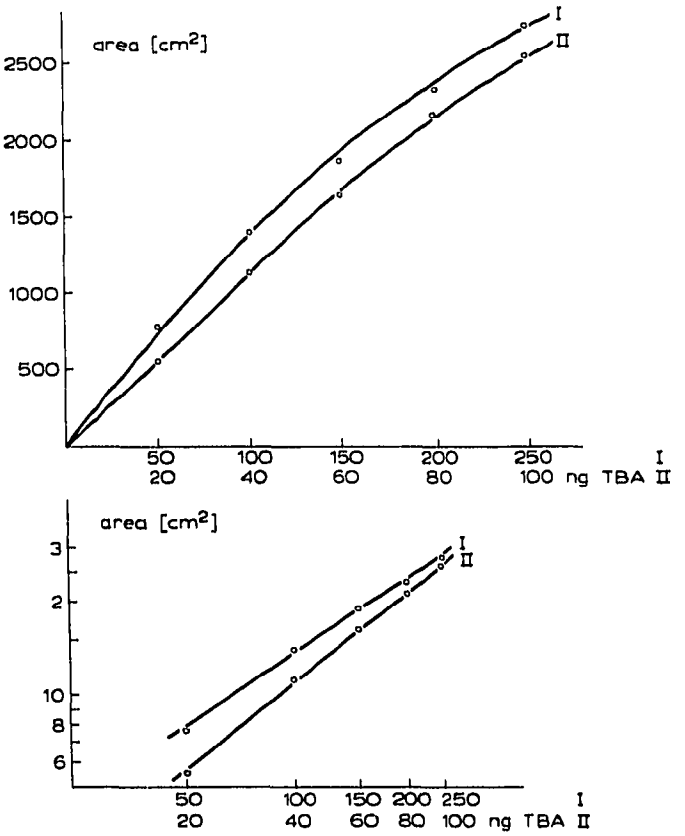

Fig. 2. Calibration graphs for fluorescence measurements plotted on linear and on logarithmic scales: I, 50–250 ng of TBA; II, 20–100 ng of TBA.

apparatus with dichloromethane, concentrate the extract in a rotary evaporator except for a few millilitres and make it up to 50 ml with ethanol in a measuring cylinder.

Chromatography and measurement

With the use of 2-μl capillary pipettes (Hirschmann, Eberstadt, G.F.R.) in combination with a Merck TLC applicator (catalogue No. 10226), apply 9 spots (4 of test solution and 5 of TBA standards) to a pre-coated silica gel G layer on glass or aluminium foil (Merck or Woelm 20 × 20 cm plates or foils are suitable). The spot centres should be about 1.8 cm apart. For the TBA standards, use solutions (in ethanol) containing 0.1–0.5 mg of TBA in 10 ml; this corresponds to 0.02–0.1 μg of TBA in a 2-μl application. Develop the chromatogram in a closed tank with ethyl acetate–methyl ethyl ketone–formic acid–water (5:3:1:1) for 90 min.

After development, evaluate the spots by using a Zeiss Chromatogram Spectrophotometer equipped for fluorescence measurement as in system A or C of Fig. 1. For both systems, the excitation wavelength is 313 nm. With system A, this is achieved by means of an M313 line filter, and a collective lens screwed in the box of the mercury source; with system C, a monochromator is used. Fluorescence is mea-

sured at 355 nm in system A by means of the monochromator; with system C, it is measured at 365 nm with use of an M365 filter. The slit width is 0.5 mm for system A and 2 mm for system C. For both systems, the slit height is 14 mm, the scanning speed is 20 cm/min, and the chart speed is 60 cm/min.

Calculation and evaluation

Determine the areas subtended by the fluorescence curves plotted against the locus of the TBA spots from the peak heights and the widths at half height by using a template for evaluation of Gaussian curves (Prof. Fricker, Messrs. Zeiss), and plot the peak areas (cm^2) against the TBA contents (ng) of the spots logarithmically.

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