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Note

Thin-layer chromatographic measurement of low activities of tritiated substances mixed with non-radioactive quenching plant pigments

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Tritium (^3H) is frequently used as a tracer in radiochromatography of samples of biological origin, although difficulties arise in its determination owing to the low electron energies (E_{max} = 18.5 keV) involved. For radiocarbon (^{14}C), the electron energies are larger by a factor of *ca.* 10, and the problems of detection are greatly reduced. The standard method of film detection of low activities of ^{14}C in thin-layer chromatography (TLC) is predominantly an autoradiographic process entailing the direct interaction of the ^{14}C β-particles with the emulsion[1], and is several thousand-fold less sensitive for ^3H. To enhance the speed and the sensitivity of ^3H film detection, scintillators must be incorporated into the TLC plate, or applied to the plate after chromatographic development, which in turn produces an image in the photographic emulsion (fluorography)[1–3]; a sensitivity as low as 1 nCi ^3H per cm^2 per day can be obtained, but it varies strongly with the experimental conditions[1]. The TLC spots are scraped off and suspended in a scintillation solution, and the ^3H radioactivity is measured using a liquid scintillation spectrometer[4–6]. These methods involve the detection of luminescence induced by β-particles, and can thus be hampered by quenching. During TLC analysis of samples from plant extracts, spots of coloured plant pigments, especially the chlorophylls, can partly or completely quench the radioactivity of the spots of the tritiated compounds under study (the pesticides and their metabolites, for example). We have developed a sensitive and reliable method for detection of tritiated substances on TLC plates when their spots are mixed with those of such pigments. The TLC of the tritiated fungicide triforine [*NN'*-bis-(1-formamido-2,2,2-trichloroethyl)piperazine, uniformly ^3H-labelled in the piperazine ring] is given as an example.

MATERIALS AND METHODS

^3H-Triforine (105 μCi/mg) was received from Cela Merck (Ingelheim am Rhein, G.F.R.), and stored as a powder until use. TLC was carried out with activated (105°, 24 h) DC-Plastikfolien Kieselgel 60 F_{254} (Merck, Darmstadt, G.F.R.) divided into strips (2 × 20 cm/0.25 mm). ^3H Measurements were made at 8° in polyethylene counting vials (Packard) with a liquid scintillation counter (Packard, Model Tri-Carb 2425) in scintillant I (60 g naphthalene, 4 g PPO, 200 mg POPOP, 100 ml methanol,

20 ml ethylene glycol, dioxane to 1 l) or II (7 g PPO, 0.6 g POPOP, toluene to 1 l), prepared with scintillation grade products from Packard (Downers Grove, Ill., U.S.A.). The other chemicals were of analytical grade from Merck.

Standard solutions of ^{3}H-triforine in methanol or benzene were prepared, and counted (100 μl) in scintillant II. Absolute activities and counting efficiencies were determined by using internal standards of ^{3}H-toluene (Packard), the sample giving a homogeneous solution with the scintillant. A mixture of plant pigments was obtained by the extraction of barley leaves with chloroform and concentrated in a rotatory vacuum evaporator[7]. The total chlorophyll content of the concentrate was measured by visible absorption spectrophotometry ($\lambda = 652$ nm) of an aliquot dissolved in 80% aqueous acetone[8,9]. Aliquots of the concentrate were added to solutions of ^{3}H-triforine, giving standard solutions of mixtures of ^{3}H-triforine and chlorophylls (and other plant pigments), which were spotted (20 μl) on the TLC strip. This was developed for 16 cm with ethyl acetate, the R_F of triforine being 0.67. The dried chromatogram was cut into 12 equal segments, the radioactivities of which were measured separately, and then summed. The distribution of the radioactivity along the chromatogram was thus determined. Several methods were tried for the determination of the radioactivity on the TLC strip.

Method 1. The TLC segment was put directly into the counting vial containing scintillant II (10 ml).

Method 2. The TLC segment was scraped off. The powder was transferred quantitatively into the counting vial containing scintillant II (10 ml), and the whole was shaken for 1 min.

Method 3. The TLC segment was scraped off. The powder was transferred into the counting vial containing 0.2 ml Cl_2–water (*ca.* 5 g Cl_2 per l water) and shaken for 2 h after which scintillant I (10 ml) was added.

Method 4. The TLC segment was scraped off. The powder was transferred into the counting vial containing 0.1 ml Br_2–water (saturated solution) and shaken for 2 h, after which scintillant I (10 ml) was added.

Method 5. The TLC segment was put into the counting vial containing a 10 g% solution (1.2 ml) of benzoyl peroxide in toluene. After irradiation for 2 h under intense light, scintillant II (10 ml) was added.

Method 6. The TLC segment was scraped off. The powder was treated in a sealed tube (105°, 17 h) with propan-2-ol (0.3 ml) and 30 vol% hydrogen peroxide (0.3 ml). The contents of the cooled sealed tube were transferred quantitatively into scintillant I (10 ml).

The absolute activity (as well as the amount of chlorophylls) spotted on the TLC strip was always accurately known. By comparing this with the sum of the measured activities of the segments, obtained after chromatography and application of one of the methods outlined above, we measured the total efficiency of the method. If the solution studied was spotted on a TLC segment which was not chromatographically developed, the radioactivity measurement efficiency was similar to that observed after chromatography. All six methods gave similar results. All samples were counted at least four times with 10,000 counts collected in the counting channel, and the counting was repeated several hours later to check the absence of disturbing luminescence after an adequate time of dark adaptation. The background count rates varied from 10 to 18 cpm.

RESULTS AND DISCUSSION

The radioactivity of a solution of pure ^{3}H-triforine in toluene was measured in scintillant II. The counting efficiency was 44 $\pm$ 0.5%, and independent of the activity of the sample between $2 \cdot 10^3$ and $2 \cdot 10^5$ dpm. When a solution of pure ^{3}H-triforine was spotted on a TLC strip, and the radioactivity of the developed chromatogram was measured, the efficiency of counting was the same with both methods 1 and 2 (Fig. 1).

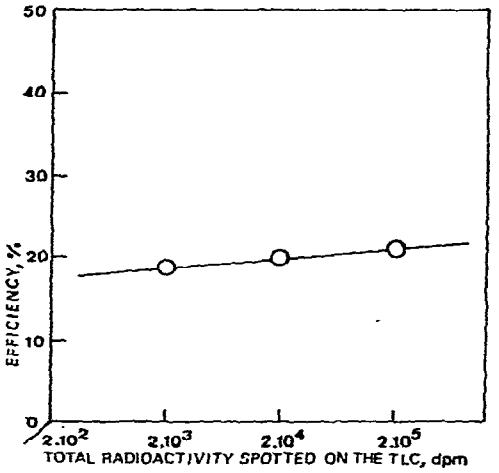

Fig. 1. Efficiency of pure ^{3}H-triforine radioactivity measurement after TLC and use of methods 1 and 2.

Standard solutions of mixtures of ^{3}H-triforine and chlorophylls (and other coloured plant pigments) were spotted on TLC strips, which were developed with ethyl acetate. The spot of triforine was mixed with those of plant pigments. The counting efficiency was low and similar with methods 1 and 2 (no bleaching) (Fig. 2). Similar results were obtained when there was no chromatographic development. There was no reliable relationship between efficiency and channels ratio. The efficiency was so poor that low activity (less than $2 \cdot 10^4$ dpm) of ^{3}H-triforine could not be usefully distinguished on developed TLC strips. Moreover, the counting solutions were green and luminescent so that, sometimes, as much 4 h elapsed before counting was possible.

When the same assays with mixtures of ^{3}H-triforine and plant pigments were performed using the bleaching methods 3 and 4 poor results were obtained, the counting efficiencies being less than 10%. Somewhat better results were obtained with the bleaching method 5 (Fig. 3); however, it was inefficient as the amounts of chlorophylls spotted on the TLC strip were usually *ca.* 30 μg. Similar efficiencies were obtained when there was no chromatographic development.

TLC assays with mixtures of ^{3}H-triforine (spotted activities between $2 \cdot 10^3$ and $2 \cdot 10^5$ dpm) and chlorophylls (spotted amounts of 0–150 μg) were performed using the bleaching method 6. The counting efficiencies after chromatographic development (or without development) were constant (22 $\pm$ 0.5%), and correspond to the counting

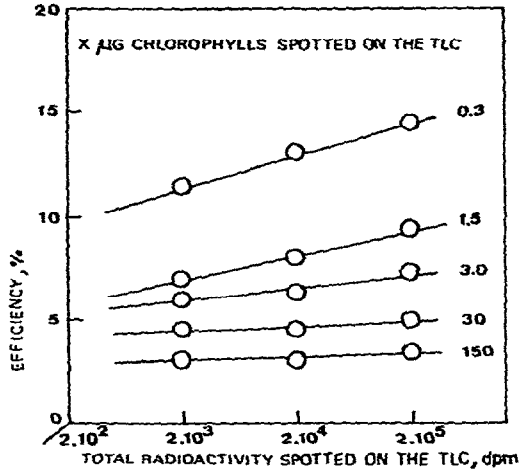

Fig. 2. Solutions of mixtures of ^{3}H-triforine and chlorophylls were spotted on TLC strips and, after chromatographic development, the efficiencies of radioactivity measurement by methods 1 and 2 (no bleaching) were recorded.

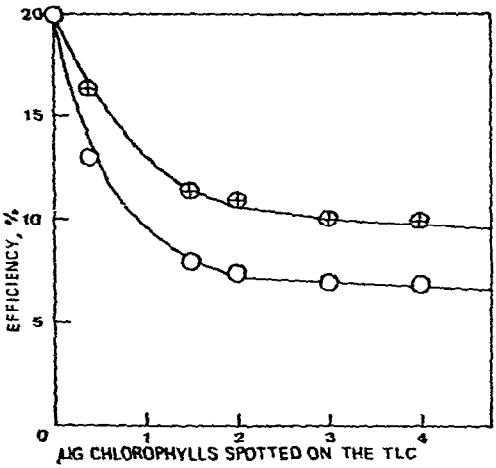

Fig. 3. Solutions of mixtures of ^{3}H-triforine and chlorophylls were spotted on TLC strips, the tritiated spotted activity being constant at $2 \cdot 10^4$ dpm. After chromatographic development, the efficiencies of radioactivity measurement by methods 1, 2 (no bleaching; similar results for both: ○), and 5 (⊕) were recorded.

efficiency of ^{3}H-toluene in scintillant I (10 ml) to which the pure bleaching solvents were added (0.3 ml of 30 vol% hydrogen peroxide, and 0.3 ml isopropanol). Counting could be performed immediately, as there was no interfering luminescence. Independently of the spotted amount of chlorophylls, ^{3}H-triforine activities as low as $2 \cdot 10^3$ dpm gave very clear spots after TLC development. This method is thus useful for the TLC measurement of low tritiated activities mixed with quenching coloured plant pigments. It also enables the measurement of the distribution of the radioactivity along the chromatogram, as the counting efficiency is constant and inde-

pendent of the presence of coloured spots mixed with the radioactive one. The limiting factor, although not concerned with the measurement method, was the amount of spotted chlorophylls. Indeed, above 50 μg chlorophylls, the TLC was oversaturated. This problem could be solved by using preparative TLC.

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