Journal of Chromatography, 209 (1981) 117–120
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

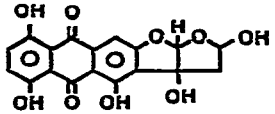
CHROM. 13,648

Note

Determination of dothistromin by quantitative reversed-phase thin-layer chromatography

R. A. FRANICH
Forest Research Institute, Private Bag, Rotorua (New Zealand)
(Received January 13th, 1981)

Dothistromin (1) is the major metabolite produced by the pine needle pathogen *Dothistroma pini* (Hulb.) grown in culture[1–3], and the sole metabolite identified in extracts of diseased pine foliage. During studies on the effect of dothistromin on pine needle tissue, large numbers of samples were required to be analysed for the quantity of dothistromin injected into pine needles, for residual dothistromin after various physiological treatments of seedlings or detached pine needles, and also for dothistromin in natural disease lesions[‡].



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Previously, dothistromin has been routinely analysed in this laboratory by adsorption thin-layer chromatography (TLC) and fluorescence densitometry (silica gel GF, developed in ethyl acetate–dichloromethane (1:1) containing *ca.* 4% formic acid to suppress dothistromin ionisation; dothistromin R_F 0.5). Ethyl acetate solutions of dothistromin exhibit a fluorescence excitation maximum at 470 nm and an emission maximum at 550 nm. When dothistromin is adsorbed on to a silica gel G TLC plate, the excitation maximum appears at 436 nm (Fig. 1). This method has been satisfactory for analysis of dothistromin extracted from fungal cultures, bioassays, and from dead, brown pine needles, but did not give reliable quantitative analyses of dothistromin extracted from green pine needles. Extraction of green pine needles containing very small amounts of dothistromin also released comparatively large amounts of chlorophylls and phenols. These on adsorption TLC migrated to R_F 0.7–0.9 but with variable streaking, which appeared as a faint blue fluorescence when examined at 366 nm, and quenched silica gel 254 nm fluorescence in addition to causing variable quenching of dothistromin fluorescence, making the analysis unusable for green tissue extracts.

This problem has been overcome by using a cheap, simple reversed-phase TLC system described in this paper.

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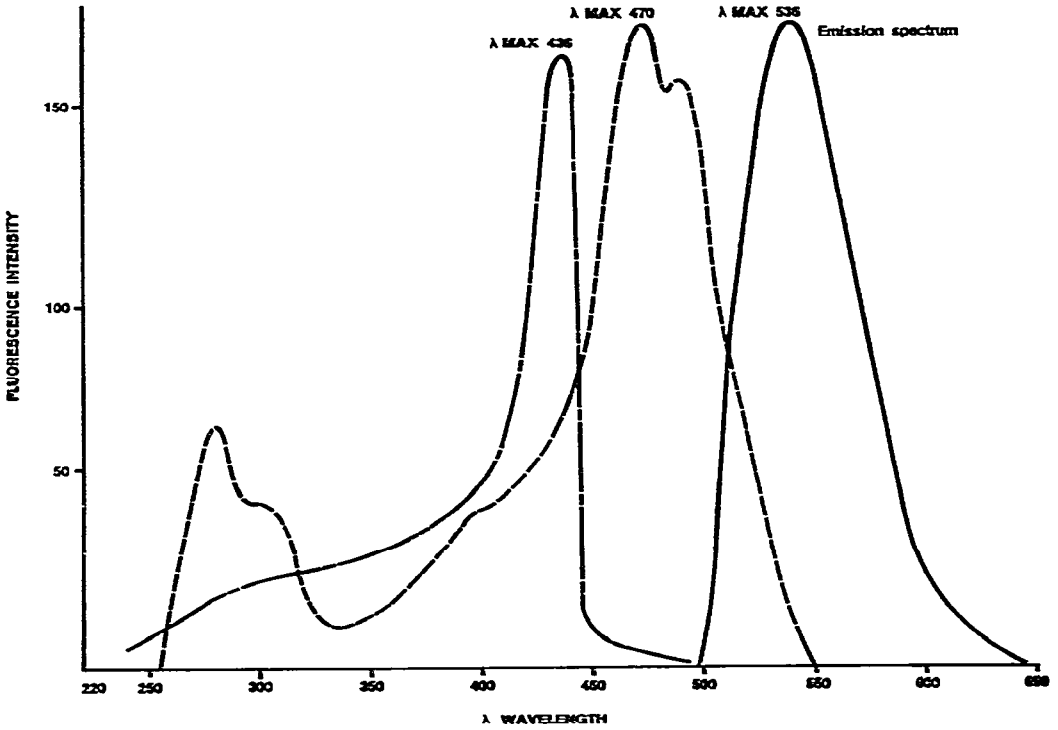

Fig. 1. Fluorescence excitation and emission spectra for dothistromin. ———, Adsorbed on to silica gel G; — — —, in ethyl acetate.

EXPERIMENTAL

Solvents were laboratory-reagent grade. TLC plates were prepared by spreading silica gel GF_{254} (Woelm, Eschwege, G.F.R.; 25 g) slurried in distilled water (55 ml) on five 20 × 20 cm glass plates. Layer thickness 0.25 mm. Plates were allowed to air-dry at 20°C for 12 h. Reversed-phase TLC plates were prepared by developing the silica-gel GF plates once in a solution of 5% liquid paraffin (BDH, Poole, Great Britain; infrared-spectroscopy grade) in hexane, and then air-drying[5]. The solvent system for reversed-phase TLC was methanol–water (2:1) containing *ca.* 4% formic acid. Vapour phase saturation was not used. Both solvent developments were in the same direction.

Dothistromin standards in ethyl acetate were prepared in the range 1–100 mg/l. Natural *Dothistroma* lesions and dothistromin-induced lesions in pine needles were excised (*ca.* 5 mm needle length; 1.8 mg fresh wt. tissue) and cut into *ca.* 0.5-mm pieces with fine scissors directly into 25-ml vials. Ethyl acetate (1 ml) and formic acid (50 μl) were added, the vials capped, and kept at 20°C for 12 h. The light green solutions were mechanically separated from tissue fragments and transferred quantitatively (Pasteur pipette) into 1-ml tapered vials and evaporated in a current of air at 20°C. The residue was dissolved in ethyl acetate (25 μl).

Samples (25 μl) and standards (10 μl) were applied to the reversed-phase TLC plates using a liquid-tight stepper-motor-driven 50-μl microsyringe.

After development the TLC plates were allowed to air-dry completely and analysed using a Vitatron TLD 100 densitometer with the following parameters: Hg light source, primary interference line filter λ 436 nm, secondary blocking filter $\lambda >$ 500 nm, slit 1 mm, oscillation 14 mm, scan speed 10 mm/min, photomultiplier sensitivity 100. The raw signal was minimally corrected for positive baseline drift (1.5% full scale/h) and baseline noise (2% full scale) (Kontes Corrector K-495100). Peaks were integrated (Hewlett-Packard 3371 B integrator).

RESULTS AND DISCUSSION

When extracts of dothistromin-treated pine needles were chromatographed using reversed-phase TLC as above, dothistromin migrated to R_F 0.6. The solvent front was allowed to travel 10 cm from the point of sample application. Chlorophylls remained at the origin, identifiable by their deep red fluorescence when irradiated at 436 nm and examined through a 500-nm blocking filter. Blue-fluorescent compounds appeared close to the chlorophylls at $R_F < 0.1$, and also close to the solvent front at $R_F > 0.9$. No fluorescence was detectable from R_F 0.2–0.9 in chromatograms of green, healthy, pine needle extract. Despite the samples and standards being applied to the reversed-phase TLC plate in ethyl acetate (which must initially also dissolve the paraffin coating on the silica gel), no adsorption of dothistromin on to the silica gel at the application site was observed, since after chromatography of 100 ng of dothistromin, no fluorescence was detectable at the origin. Dothistromin migrated on reversed-phase TLC as a compact spot, and a typical response from 50 ng is shown in Fig. 2.

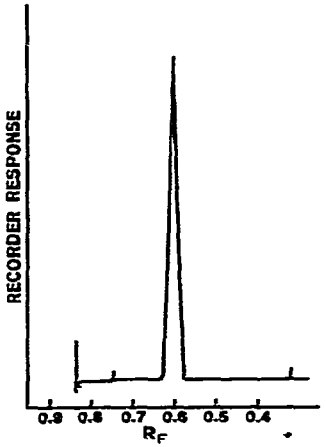

Fig. 2. Reversed-phase thin-layer chromatogram of 50 ng dothistromin.

The weight–response log–log plot for dothistromin standards is shown in Fig. 3, and is linear over the range 10–150 ng. This is distinct from the weight–response curve determined for adsorption TLC, where linearity extended over the range 10–700 ng. It is apparent that self-quenching of the dothistromin spot where quantities are greater than 200 ng is due to its slower lateral diffusion rate on reversed-phase TLC compared with adsorption TLC. The small linear range is a disadvantage of the

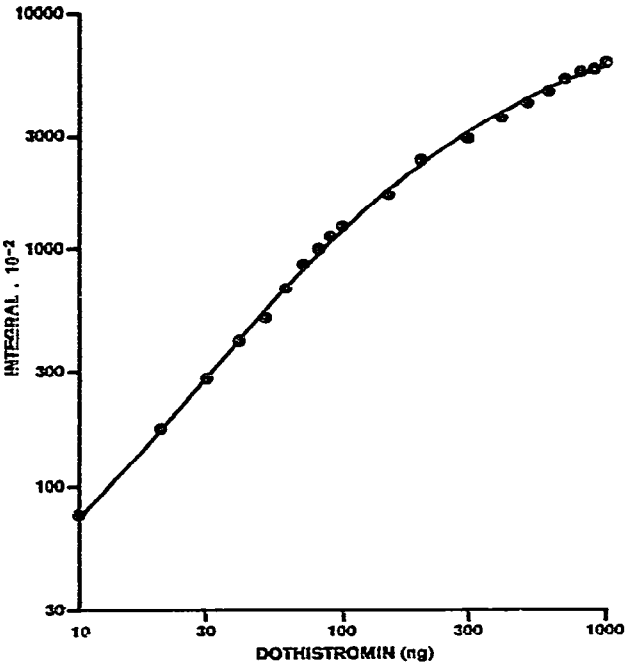

Fig. 3. Weight–response plot for dothistromin standards.

reversed-phase system. Precision of the analysis was determined by chromatographing ten 100-ng samples of dothistromin, and analysing by fluorescence densitometry. The mean integral ± standard deviation was equal to 100 ± 10.6 ng, *i.e.*, a 10% error can be expected for the determination.

For determination of dothistromin in pine needle extracts, three standards (10, 50, 100 ng) and nine samples are applied to each TLC plate. Time for sample application, chromatography, and analysis is 2 h.

In addition to providing a simple, rapid analysis for small quantities of dothistromin extracted from pine needles, the reversed-phase TLC described may also be useful for examination of foliage polyphenols and phytoalexins where interference from chlorophylls may be a problem[4].

REFERENCES

1 C. Bassett, Margaret Buchanan, R. T. Gallagher and R. Hodges, *Chem. Ind. (London)*, (1970) 1659.
2 R. T. Gallaher and R. Hodges, *Aust. J. Chem.*, 25 (1972) 2399.
3 A. V. Danks and R. Hodges, *Aust. J. Chem.*, 27 (1974) 1603.
4 L. Shain and R. A. Franich, unpublished results.
5 E. Stahl, in E. Stahl (Editor), *Thin-Layer Chromatography —A Laboratory Handbook*. Academic Press, New York, 1965, p. 37.