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GRAFT THIN-LAYER CHROMATOGRAPHY

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SUMMARY

Graft thin-layer chromatography, a novel multiplate system with layers of the same or of different adsorbents for isolation of the components of natural and synthetic mixtures on a preparative scale, is described. Advantages are simplicity, economy, rapidity and efficiency of recovery. In this method, two plates are grafted together and clamped in a lapjoint fashion with the edges of their adsorbent layers in intimate contact so that a component of a chromatogram developed on the first plate can be transferred onto the second plate without the usual scraping of bands, eluting and reloading. The technique is illustrated by the separation of mixtures of dinitrophenylhydrazone derivatives of aliphatic and aromatic aldehydes and ketones derived from degradation of polyene antibiotics, of streptovaricin complex, and of a phytoalexin mixture.

INTRODUCTION

Thin-layer chromatography (TLC)[1–4], coupled with modern instrumentation, is a powerful and versatile research tool used routinely for analysis, quantitation, and separation, its success arising from its low cost, simplicity, sensitivity and selectivity. However, particularly on a preparative scale, TLC is time-consuming and recoveries are low.

While some mixtures can be separated in a single TLC development on an analytical scale, others require multiple development, which can be performed in the same direction with the same solvent (programmed multiple development)[5] or with a different solvent, or can be carried out in a different direction (two-dimensional TLC), usually at right angles so that the spots obtained in the first development are at the bottom in the second. The two-dimensional procedure, however, is not applicable to preparative TLC, where the sample is applied as a streak or a series of spots (rather than as a single spot) at the bottom of the plate.

Until now, use of a multiple solvent system to resolve a particular band by preparative TLC has involved isolating the material from one plate and reloading it onto a second plate before applying a new solvent system. We report here a method by which many of these transfer steps are eliminated and recovery of materials is improved. The technique is particularly useful for preparative TLC and is the equivalent of two-dimensional preparative TLC.

MATERIALS

Commercial TLC plates

If commercially available pre-coated plates (20 × 20 cm, 1 to 2 mm thick) are to be used, they are modified as described under Methods to conform to the specifications in Fig. 1 for plates 1 and 2. Modified pre-coated silica gel G plates 1 mm thick (Analtech, Newark, Del., U.S.A.) were used with the colored streptovaricin complex[6,7] and with the dinitrophenylhydrazone derivatives of mixtures of ketones and aldehydes obtained in the reaction of polyene antibiotics with base[8,9]. Modified pre-coated silica gel G plates 2 mm thick (Brinkmann, Westbury, N.Y., U.S.A.) were used with fluorescent indicator UV 254 with the phytoalexin extract[10].

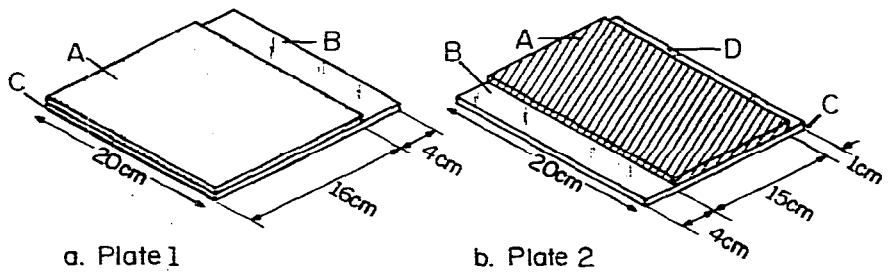
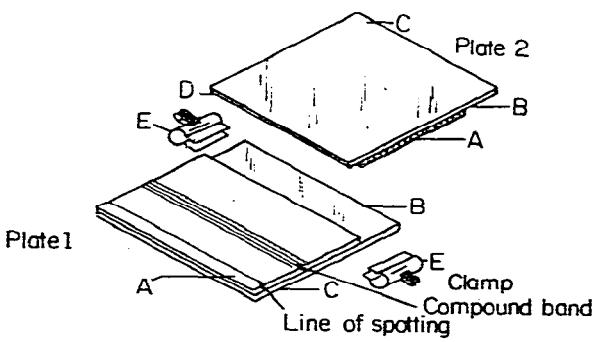


Fig. 1. Plates used for graft TLC. A = adsorbent coating layer; B and D = uncoated areas of glass plate, C.

Development tank

A special glass tank (45 × 28 × 10 cm) closed by a cover glass is used for development of the grafted plates, which are held together firmly by clamps (Figs. 2–4).



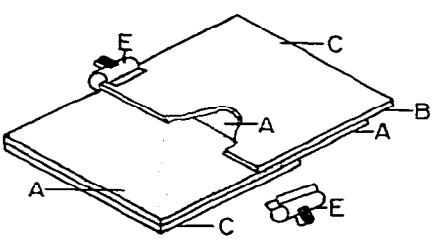


Fig. 2. Placement of plate 2 on plate 1. A–D, as in Fig. 1; E = clamp.

Fig. 3. Relative positions of plate 1 and plate 2 after grafting A–E as in Figs. 1 and 2.

Solvents

Analytical-reagent grade solvents (Mallinckrodt, St. Louis, Mo., U.S.A.) were used.

Samples

Streptovaricin complex[6,7] (U-7750, Lot 11560-10) was supplied by Upjohn (Kalamazoo, Mich., U.S.A.), the phytoalexin extract from soybean hypocotyls[10] by

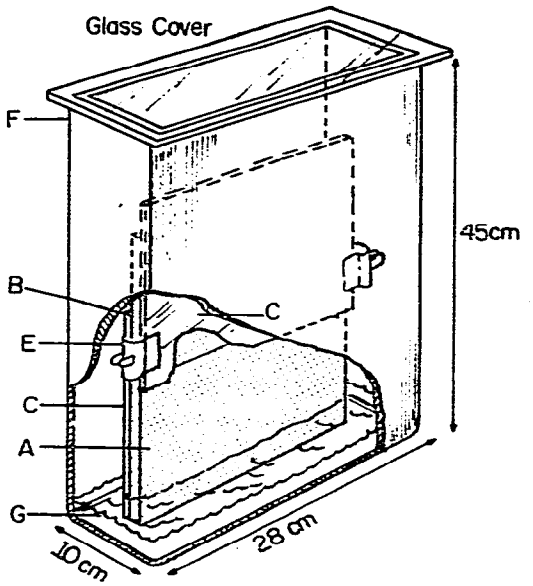


Fig. 4. Development of a grafted plate in a special glass tank. A–E as in Figs. 1 and 2; F = special glass chamber; G = solvent.

Professor J. D. Paxton (Department of Plant Pathology, University of Illinois, Urbana, Ill., U.S.A.). Mixtures of dinitrophenylhydrazone derivatives were obtained in the course of work on polyene antibiotics[8,9] in this laboratory.

METHODS

Preparation of plates

Plate 1 is prepared by clamping a glass plate (4 × 20 cm) onto a plate (20 × 20 cm) to mask an area (4 × 20 cm) from the adsorbent coating. A slurry of the required adsorbent in distilled water is then applied either by hand or by a commercial applicator to give the desired thickness (0.1–2 mm). After drying at room temperature (*ca.* 30–60 min), the protective plate is removed and the coated plate is activated at 110° for 90 min. If it has been stored at room temperature, a plate is activated at 110° for 1 h.

Plate 2 is prepared from a glass plate (20 × 20 cm) by masking it with a plate (4 × 20 cm) at one end and a plate (1 × 20 cm) at the opposite end. A slurry of adsorbent, either the same as or different from that coated on plate 1, as suits the mixture to be separated, is applied and plates are dried as above.

Alternatively, any glass-supported, commercially pre-coated plate can be modified by removing some adsorbent to leave areas coated as specified in Fig. 1a and b for plates 1 and 2, respectively.

Sample application and development

Each sample mixture is applied with a disposable capillary pipette as a series of spots or with an automatic streaker at a distance of 2–3 cm from the coated end of plate 1, which is then developed in a glass chamber containing solvent to a depth of 8–10 mm and lined on three sides with filter paper. The tank is equilibrated for 30 min

and can be covered with black polythene or kept in the dark to avoid sample decomposition. Development is allowed to proceed until the solvent front reaches the desired height, then the adsorbent coating above the level of the band to be transferred is removed in a straight line with a sharp spatula. Plate 2 is then lapjoined and clamped, facing plate 1, so the adsorbent of plate 1 is in direct contact with the adsorbent of plate 2 (Figs. 2 and 3), *i.e.*, the two plates are grafted onto one another. Development proceeds in the same or in a different solvent in a special chamber (Fig. 4), lined with filter paper and equilibrated as above, until the band of interest has just moved onto plate 2. The two grafted plates are then removed from the development tank, separated and dried. This process can be repeated to transfer several bands from one plate to different plates or a particular band from several plates onto one plate.

Because some widening of the sample band takes place during transfer onto plate 2, a polar solvent in which the compound is very soluble is used to develop plate 2 to a height (3–5 cm) such that the transferred sample forms a fine, thin band. The plate is then dried, developed in a suitable solvent, and worked up in the usual way by scraping silica gel from the plate and extracting or by eluting by the wick technique[11]. Alternatively, the whole grafting process can be repeated as many times as necessary to effect a separation.

RESULTS AND DISCUSSION

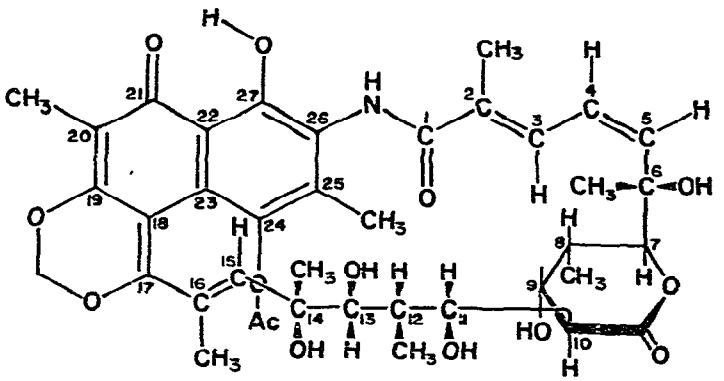
The method has been successfully used for separation of a wide variety of mixtures, which can be illustrated by the following examples.

Mixtures of dinitrophenylhydrazone derivatives of aromatic and aliphatic ketones derived by degradation of polyene antibiotics

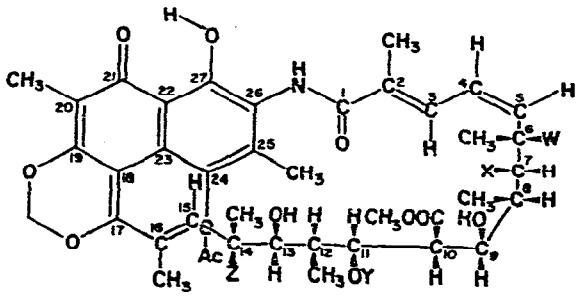
Base treatment of hamycin A (ref. 8) and partricins A and B (ref. 9) followed by steam distillation into an acidic solution of 2,4-dinitrophenylhydrazine yielded a mixture of dinitrophenylhydrazone derivatives of aliphatic ketones and aldehydes and aromatic ketones which were separated by graft TLC on 2 mm thick silica gel plates using chloroform. The fast-moving orange band was transferred quantitatively to another silica gel plate and was developed (5 cm) in chloroform–methanol (1:1) to straighten the band. The dried plate was finally developed in chloroform–benzene (1:1), which resolved the transferred band into two bands. These were separated by the usual method of scraping and eluting or by the wick technique[11] and identified as dinitrophenylhydrazone derivatives of acetone and acetaldehyde. The slower moving brick-red band from plate 1 was scraped off and extracted with chloroform–methanol (1:1). The dark brick-red compound isolated was identified as the dinitrophenylhydrazone derivative of *p*-aminoacetophenone.

Streptovaricin complex

Separation of individual streptovaricins from the intensely yellow, light-sensitive streptovaricin complex is extraordinarily challenging because the many components differ only slightly in their structures (Fig. 5, Table I, streptovaricins A–E, F_G, G, J and K)[12] and decompose readily. Previous separations[13] involved column chromatography followed by preparative TLC; however, scraping the bands, eluting, and reloading were time-consuming and allowed the sensitive compounds to decom-



STREPTOVARICIN F_G



STREPTOVARICIN	W	X	Y	Z
A	OH	OH	Ac	OH
B	H	OH	Ac	OH
C	H	OH	H	OH
D	H	OH	H	H
E	H	O=	H	OH
G	OH	OH	H	OH
J	H	OAc	H	OH
K	OH	OAc	H	OH

Fig. 5. Structures of individual streptovaricins[12].

pose, leading to poor recoveries. We have successfully applied the graft TLC technique to solve this problem.

Analytical thin-layer plates were used to define the best adsorbents, layer thickness and solvent systems for the separations.Alumina, silica gel and cellulose plates (0.25–2 mm), both manually prepared and commercial, were tested with various solvent systems [1, chloroform–methanol (98:2); 2, ethyl acetate–diethyl ether (2:1); 3, benzene–dioxane (1:1); 4, benzene–acetone (7:3); 5, chloroform–methanol (05:5); 6, chloroform–methanol (92:8); 7, ethyl acetate–cyclohexane (2:1)].

TABLE I

CHARACTERIZATION OF STREPTOVARICINS

Streptovaricin	R_F*,**	*Color***	*Band No. in Fig. 6*
A	0.27	golden yellow	V
B	0.42	dark yellow	IV
C	0.55	golden yellow	III
D	0.59	dull yellow	III
E	0.65	golden yellow	I
F_G	0.10	yellow	VI
G	0.42	red-yellow	IV
J	0.60	orange-yellow	II

* Silica gel, 20 × 20 cm, 0.25 mm; chloroform–methanol (95:5); solvent front, 15 cm.
** R_F of authentic material is identical.
*** Color of authentic material is identical.

The best overall separation of the streptovaricin complex was achieved using two 1 mm thick pre-coated 20 × 20 cm silica gel plates (Analtech), each developed in solvent system 5 (Fig. 6) and then transferring bands from the two plates to a single plate. The results of the separation under those conditions are given below.

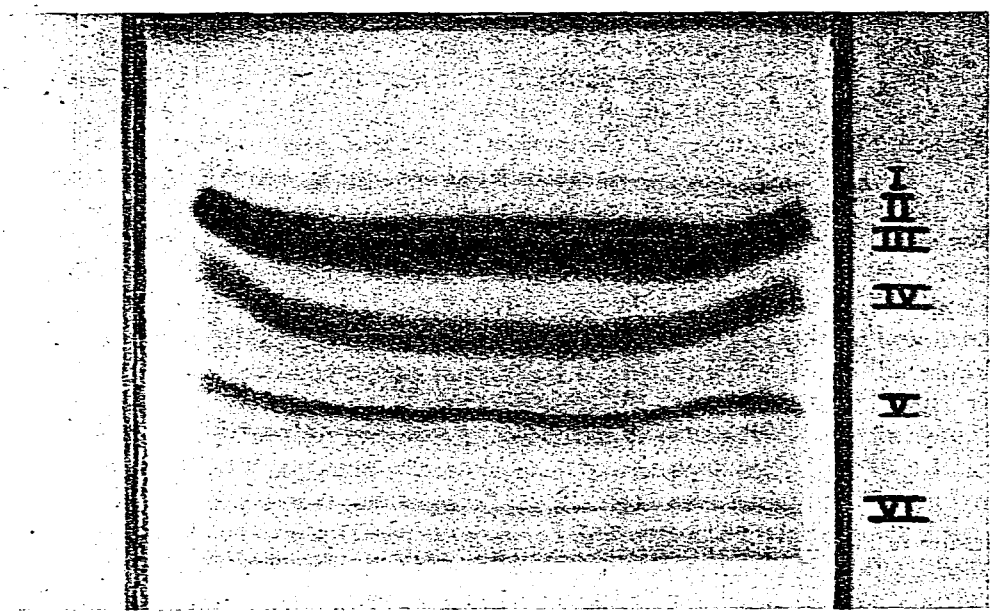


Fig. 6. Chromatogram showing separation of bands I–VI containing components of streptovaricin complex. Plate: silica gel (Analtech), 20 × 20 cm, 1 mm thick; solvent system: chloroform–methanol (95:5); solvent front: 17 cm × 2.

Streptovaricin complex (100 mg) was applied to plate 1 by the spotting technique. The developed chromatogram (Fig. 6) showed some unmoved material and 6 well-resolved bands (I–VI, 3 major and 3 minor) on plate 1, which was then ready for grafting to plate 2.

Band I was transferred from two such plates onto plate 2 and straightened with solvent system 6 (1 cm). After drying under nitrogen, development in solvent system 5 or other solvents showed band I to contain a single component, identified as streptovaricin E (Fig. 5, Table I).

Band II was similarly transferred from two plates, further developed with solvent system 5, and shown to contain streptovaricin J (Fig. 5, Table I).

Band III was transferred from two plates and developed with solvent system 5 followed by solvent system 4 to give two bands, each of which was transferred to a fresh plate for a third development using solvent system 4. The fast-moving, minor component was characterized as streptovaricin D, the slower, major component as streptovaricin C (Fig. 5, Table I).

Band IV, transferred as above (Fig. 7) from two plates to one, was the most difficult to resolve, but multiple development (four times) with solvent system 7 finally gave two distinct bands (Fig. 8, IVa and IVb) separated by 0.5 cm, R_F 0.185 and 0.274 for IVa and IVb, respectively. Graft TLC showed each of these to be pure, the

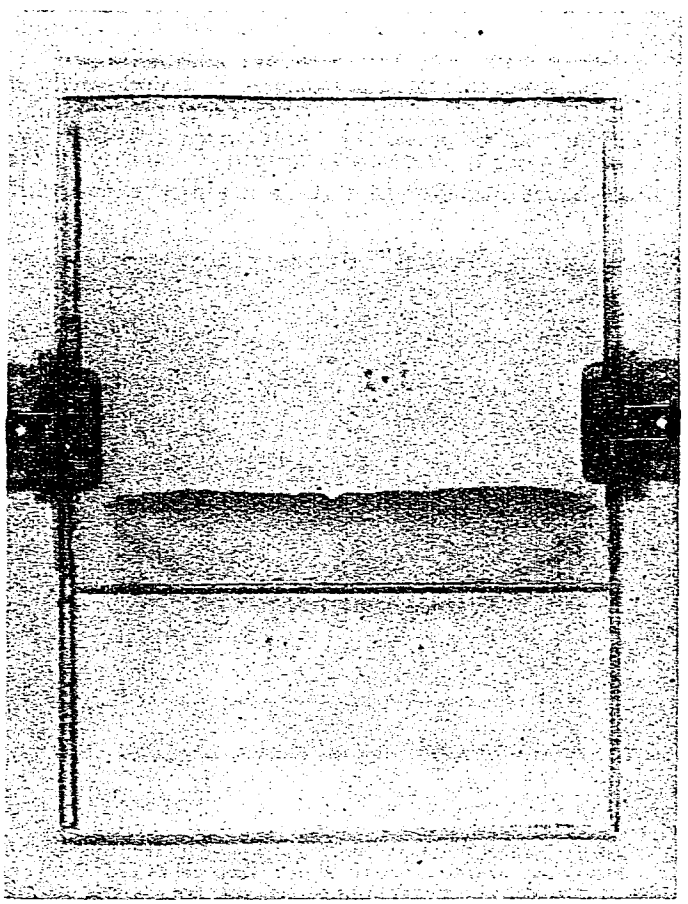


Fig. 7. Chromatogram showing transfer of band IV of streptovaricin complex (Fig. 6) to another silica gel plate. Solvent system: chloroform–methanol (95:5, multiple runs). The unusually short plate 1 arises from previous transfers of prior bands.

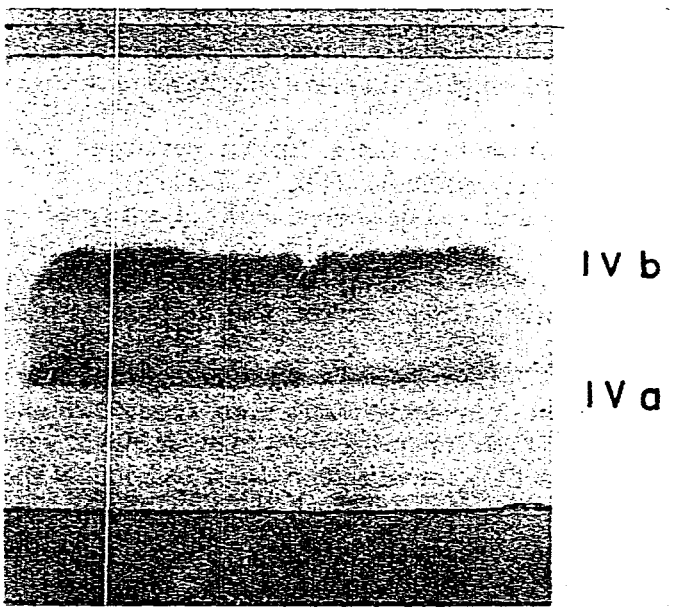


Fig. 8. Separation of band IV of streptovaricin complex into IVa and IVb. Plate: silica gel (Analtech) 15 × 20 cm, 1 mm thick; solvent system: ethyl acetate–cyclohexane (2:1); four-fold development of solvent front. IVa, red yellow band (identified as streptovaricin G); IVb, dark yellow band (identified as streptovaricin B).

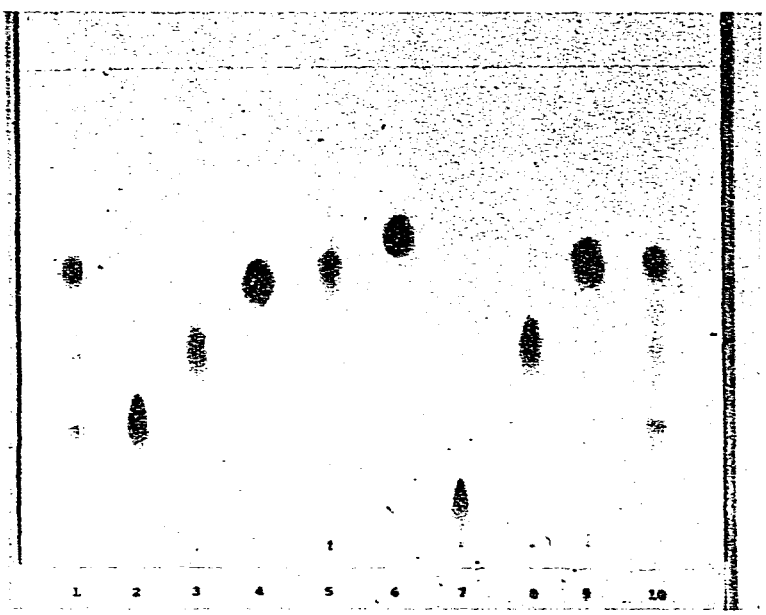


Fig. 9. Thin-layer chromatogram of various isolated streptovaricins. Plate: silica gel G (20 × 20 cm, 0.25 mm thick); solvent system: chloroform–methanol (95:5); solvent front: 15 cm; R_F values between brackets. 1 and 10, streptovaricin complex; 2, streptovaricin A (0.27); 3, streptovaricin B (0.42); 4, streptovaricin C (0.55); 5, streptovaricin D (0.59); 6, streptovaricin E (0.65); 7, streptovaricin F_G (0.10); 8, streptovaricin G (0.42); 9, streptovaricin J (0.60).

upper dark yellow band (IVb) being identified as streptovaricin B, the lower red-yellow band (IVa) as streptovaricin G (Fig. 5, Table I).

Bands V and VI were transferred to separate plates and developed with solvent system 5. Comparison of R_F values, together with mixture TLC (Table I), showed them to contain pure streptovaricins A and F_G, respectively.

R_F values of isolated streptovaricins on a 0.25 mm thick analytical plate (Fig. 9, Table I) using solvent system 5, illustrate that streptovaricins B and G have identical R_F values (0.42 under these conditions), while streptovaricin C is well resolved (R_F 0.55) from them.

Isolated individual streptovaricins were characterised by comparing their chromatographic behaviours with those of authentic compounds. Analytical plates were developed in the solvent system (system 5) used for preparative plates. Authentic streptovaricins and those isolated in this work were spotted on the same analytical plates; colors and R_F values were compared after development (Fig. 9, Table I).

Phytoalexin extract

A colorless extract of phytoalexin from soybean hypocotyls[10] was selected to demonstrate that graft TLC could be used to isolate colorless compounds. A solution of phytoalexin extract (100 mg) was spotted on two 2 mm thick modified preparative plates (plate 1) and developed in hexane–ethyl acetate–methanol (60:40:2). After all solvent had been scrupulously removed, the chromatogram showed four resolved bands under UV illumination: a light purple fluorescent band (band 1), a dark purple fluorescent band (band 2), a purple fluorescent band (band 3) and an unmoved golden

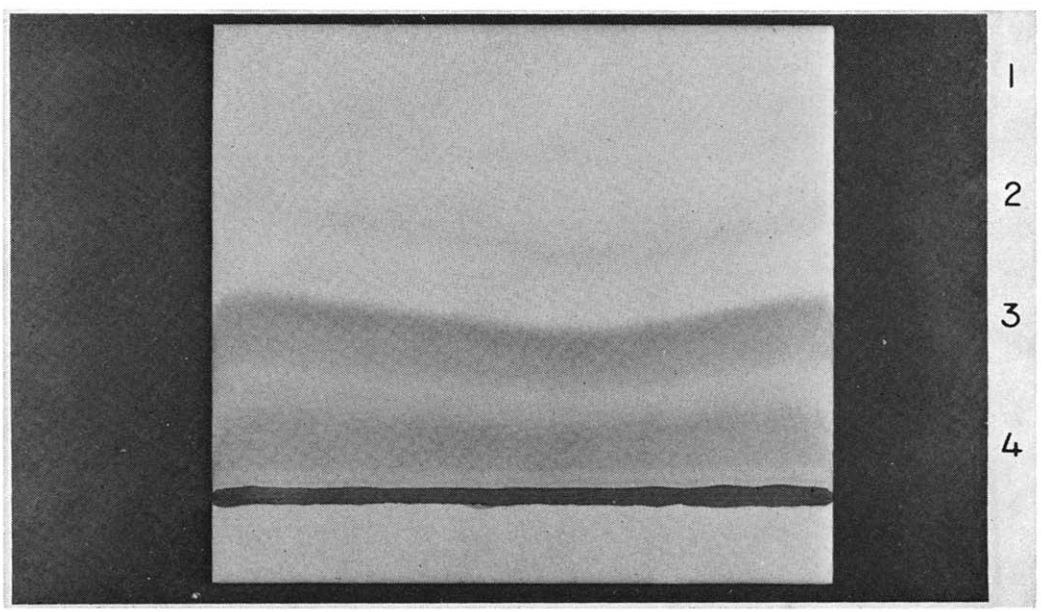


Fig. 10. Developed chromatogram of phytoalexin extract (photograph taken in long-wavelength UV light with filter). Plate: silica gel with fluorescent UV_{254} indicator (16 × 20 cm, 2 mm thick); solvent system: hexane–ethyl acetate–methanol (60:40:2). 1, Purple band; 2, dark purple band; 3, purple band; 4, golden band.

fluorescent band (band 4) (Fig. 10). Bands 1–4 (containing separated phytoalexins) were transferred and combined from each of the two plates onto separate plates using hexane–ethyl acetate–methanol (50:42:10), hexane–ethyl acetate–methanol (50:42:10), hexane–ethyl acetate–methanol (50:42:10), and hexane–ethyl acetate–methanol (2:60:40), respectively.

Golden band 4 was developed in *n*-butyl alcohol–acetic acid–water (4:1:5, upper layer) during 3–4 h, transferred using the same solvent system, eluted with methanol and concentrated to yield 60.8 mg of highly viscous light yellow material, which was precipitated from ether. Structural studies on the phytoalexins of bands 1–4 are in progress[14,15].

DISCUSSION

The graft TLC method is clearly very flexible with respect to possible coatings and layer dimensions (5 × 20 cm; 10 × 20 cm; 20 × 20 cm). It is adaptable to all commercial glass-supported adsorbents, whether silica gel, alumina, kieselguhr or cellulose, alone or in mixtures, although a preferred adsorbent is silica gel containing a small amount of calcium sulfate binder. Plastic-backed plates are unfortunately not suitable for this technique (Eastman-Kodak plastic strips).

The special graft thin-layer plates may be prepared to any specifications using commercial coating equipment. For a 20 × 20 cm plate, the uncoated band required for forming the lapjoint may vary from about 1 cm to about 10 cm, that is, from about 5% to about 50% of the area of the plate. Fig. 2 shows the manner of forming the lapjoint to form a stable, rigid assembly and to ensure that adsorbent layers A are in contact as shown in Fig. 3. It is important to shape the joining edges of the adsorbent accurately so that intimate contact is established along the entire width of the two plates. Careful grafting is necessary for even transfer of a band, but a correction may be made after transfer of the band is complete by developing the plate in a more polar solvent to such a height (3–5 cm) that the band is straightened and narrowed.

Certain supplementary precautions should be observed to get the maximum advantage from using graft TLC with unstable compounds. Plates should be dried in a stream of inert gas and protected during development by flushing the tank through a two-way valve before presaturating the atmosphere with solvent vapor. The chamber can also be refrigerated and protected from direct light.

To minimize the amount of solvent used, grafted plates can be developed in a trough in the special chamber, or twin troughs and two solvent systems can be used, one to equilibrate the chamber and the other to develop the plate. Alternatively, relative humidity can be controlled by using a sulfuric acid–water mixture in the spare trough.

CONCLUSION

Graft TLC allows the bands of a chromatogram developed on one plate to be transferred, in whole or in part, to an adjoining plate. If desired, the same band can be transferred from two or more plates to a single graft plate and then purified using another solvent system. In addition, intermediate bands can be transferred to separate plates one after the other. For some applications, different adsorbents on adjacent plates can be employed advantageously.

ACKNOWLEDGEMENT

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