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COLUMN SEPARATIONS DESIGNED PRECISELY WITH THE AID OF THIN-LAYER CHROMATOGRAPHY

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SUMMARY

The use of thin-layer silica gel as a column adsorbent has been investigated. It was found that excellent separations of a plant wax could be obtained with quantitative recovery. Further, by the prior use of thin-layer chromatography on the same adsorbent it was possible to predict the course of the separation knowing only the elution volume of the first peak and the point at which solvents were changed. Thus thin-layer chromatography may be used to design column separations.

INTRODUCTION

The components of plant waxes have been isolated by column chromatography on Florisil, preparative thin-layer chromatography (TLC) on silica gel, or a combination of these two methods[1,2]. Florisil does not separate adequately the least polar components. Preparative TLC is suitable only for small amounts of wax and up to 40 % of some components can be lost, apparently by irreversible binding to the dried thin-layer. Since excellent resolution of plant wax components can be obtained by TLC on silica gel[3] it seemed that the use of this material in columns could be profitably investigated. In this paper it is shown that the properties of silica gel are the same in thin-layers and columns.

EXPERIMENTAL

Materials and methods

A diagram of the apparatus is given in Fig. 1.

Boiling Tube. Before entering the column the solvent is passed through a boiling tube to remove air which might otherwise come out of solution in the low-pressure side of the pump. The tube, which is internally ground to prevent "bumping", is coiled with "Eureka" resistance wire and heated by the circuit shown in Fig. 1.

Pump. Since thin-layer silica gel has a very small particle size (10–40 μ) the flow rate of solvents is restricted. However, a reasonable and constant flow rate (approx. 0.2 ml/min) can be attained with a pump (Beckman Model 746).

Pressure equalising U-tube. The pump used has a piston action and thus the

pressure equalising U-tube (Fig. 1a) is introduced between the pump and the column to ensure constant head pressure on the column.

Fittings. The use of stainless steel swagelock fittings with teflon ferrules (Duff & McIntosh, Sydney, Ltd.) and teflon tubing (Polypenco TFE, AWG 20; The Polymer

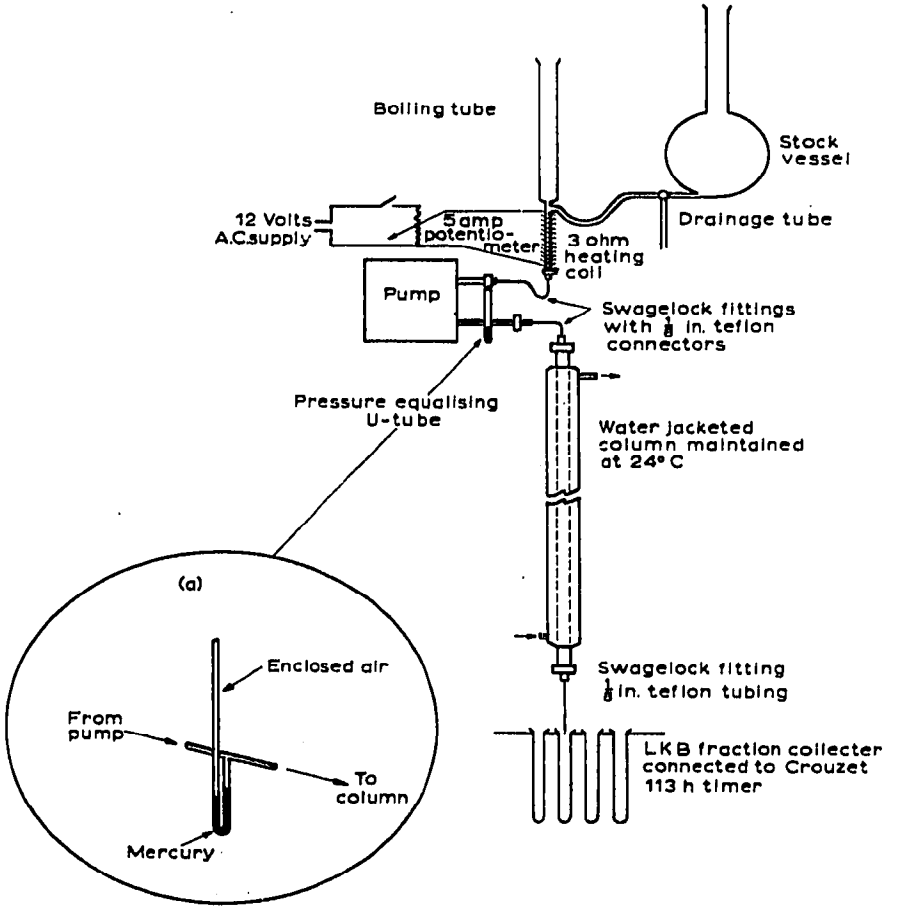

Fig. 1. Diagram of apparatus.

Corporation of Pennsylvania) provide flexible connections free from leaks. The swagelock fitting on the base of the column provides a seat for a filter paper pad to support the adsorbent and the narrow bore teflon tubing on the outlet reduces evaporation of the solvent thus eliminating deposition of solutes on the column tip.

Column. The column was 140 cm long with an I.D. of 1.3 cm. As considerable amounts of heat can be released by the adsorption of some solutes and solvents the column was water jacketed and maintained at constant temperature of 24° using a Colora Ultra-thermostat.

Adsorbent preparation. Five 20 × 20 cm glass plates were spread with silica gel (Merck, Kieselgel H) to a depth of 1 mm using Shandon equipment. These were activated by heating for 30 min at 110° and then washed with carbon tetrachloride by ascending chromatography to remove organic impurities. Following the removal of the top 1 in. of silica gel the plates were air-dried and reactivated. The remaining silica gel was then weighed.

TCL of standards. Thin-layer plates 0.3 mm thick were prepared from the same

batch of silica gel as that used to prepare the column adsorbent. These were then activated and washed in exactly the same way as the plates used for adsorbent preparation. Samples of gl_6 wax and its components (4 μl of a 1 % solution in $CHCl_3$) were then spotted onto the plates. One plate was run, to a pre-marked line, in each of the following solvents: carbon tetrachloride, benzene and ethyl acetate. The spots were detected as previously described[1] and the R_F value of each component determined.

Packing the column. Sufficient carbon tetrachloride was added to the silica gel to make a thin slurry which was then boiled to remove dissolved air. Aliquots were packed in the column under pressure from the pump. The flow rate at the tip of the column was determined after packing was complete.

Sample preparation. Wax was extracted from a non-glaucous mutant of kale (*Brassica oleracea*) known as gl_6, by dipping the leaves in boiling petrol ether (60–80°) for 20 sec. The solid wax was obtained following filtration and evaporation of the petrol ether under oxygen-free nitrogen. This wax contains fatty acids and although these can be eluted from silica gel by acetic acid, this procedure was not adopted because acetic acid attacks the swagelock fittings. Therefore, prior to chromatography on silica gel the wax was chromatographed on a Florisil column (60–100 mesh, acetone washed to remove an oily residue and reactivated). All components of the wax except the fatty acids were eluted with ethyl acetate. This eluent was dried, reweighed and less than 0.1 % of the adsorbent weight dissolved in a minimum volume of carbon tetrachloride and added to the silica gel column. The apparatus was then filled with boiled carbon tetrachloride and the elution commenced. The fatty acids were eluted from the Florisil column by 4 % acetic acid in ether[1].

Solvents. All the chromatography solvents were distilled and dried before use. Solvents were changed at the appropriate times by removing all the old solvent from the head of the column, rinsing the apparatus and refilling with the new solvent.

Collection of fractions. An LKB fraction collector connected to a Crouzet 113H timer set at 50.5 min was used.

Conditions Flow rate, 0.21 ml/min; weight of activated adsorbent, 60.4 g; fatty acid free sample weight, 51.3 mg, timer setting, 50.5 min; volume of fractions, 10.6 ml; volume of solvents, carbon tetrachloride 693 ml, benzene 288 ml, ethyl acetate 291 ml.

Analysis of fractions. The test tubes were placed in a 50° water bath and their contents evaporated to dryness under a stream of oxygen-free nitrogen. When evaporation was complete each tube was visually inspected for wax. An appropriate volume of chloroform (visually estimated, 0.1–5 ml) was added to each tube and the tube warmed to dissolve the wax. Using Microcap pipettes (Drummond Scientific Co.) 4 μl from each tube were applied to a Silica Gel G plate. The plates were developed and the spots detected and then the intensity of each spot was estimated on an arbitrary, approximately linear scale (1, 2 or 3). Tubes containing the same components were pooled and chloroform solutions of each component forced through a millipore filter, in a Sweeney syringe, to remove silica gel particles. Following drying under vacuum overnight, each component was weighed. The scale given on the ordinate of Fig. 2 was arrived at for each tube by the following formula:

$$\frac{\text{vol. } CHCl_3 \times \text{spot intensity}}{\Sigma(\text{vol. } CHCl_3 \times \text{spot intensity})} \times \text{total mass of component}$$

where the summation is over all tubes containing the component in question.

THEORETICAL CONSIDERATIONS

JOHNSON[5] derives the following three equations of general applicability to chromatographic systems:
For a complete column (*i.e.* a thin-layer plate):

$$R_F = \frac{B}{B + 1}$$

For a flowing column:

$$\frac{V}{v} = \frac{B + 1}{B}$$

and

$$B = K \cdot \frac{v}{w}$$

Where
B = effective distribution coefficient;
K = distribution coefficient;
V = volume of solvent required to elute peak;
v = volume of mobile phase;
w = weight of adsorbent.

Since the adsorbents for the column and the plates were prepared in the same way we may assume that the values of K are the same.
Thus for a column:

$$B_c = K\rho_c$$

where, by definition:

$$\rho_c = \frac{v_c}{w_c}$$

and for a plate:

$$B_p = K\rho_p$$

where, by definition:

$$\rho_p = \frac{v_p}{w_p}$$

$$\therefore R_F = \frac{K\rho_p}{K\rho_p + 1}$$

and

$$\frac{v}{V} = \frac{K\rho_c}{K\rho_c + 1}$$

$$\therefore K = \frac{R_F}{\rho_p - R_F\rho_p} = \frac{v}{V\rho_c - v\rho_c}$$

$$\therefore V = \frac{v(R_F\rho_c - R_F\rho_p + \rho_p)}{R_F\rho_c} \qquad (1)$$

The experimental data gained to date indicates that under the conditions used:

$$\rho_p = \rho_c$$

In which case eqn. 1 reduces to:

$$V = \frac{v}{R_F} \tag{2}$$

Thus, knowing the elution volume of the first peak and the R_F of components on thin-layers one can calculate the elution volume of all other peaks eluted by the first solvent. However, it is advisable to check that $\rho_p = \rho_c$. This can be done using the experimentally determined elution volume of the second peak:
From above

$$R_{F_1} = \frac{K_1\rho_p}{K_1\rho_p + 1} \qquad R_{F_2} = \frac{K_2\rho_p}{K_2\rho_p + 1}$$

$$\therefore \frac{K_1}{K_2} = \frac{R_{F_1}(1 - R_{F_2})}{R_{F_2}(1 - R_{F_1})}$$

where the subscripts 1 and 2 refer to components 1 and 2
and

$$V_1 = \frac{v(K_1\rho_c + 1)}{K_1\rho_c}, \qquad V_2 = \frac{v(K_2\rho_c + 1)}{K_2\rho_c}$$

$$\therefore \frac{K_1}{K_2} = \frac{V_2 - v}{V_1 - v}$$

$$\therefore \frac{V_2 - v}{V_1 - v} = \frac{R_{F_1}(1 - R_{F_2})}{R_{F_2}(1 - R_{F_1})}$$

From which

$$v = \frac{V_2 - V_1 \cdot \dfrac{R_{F_1}(1 - R_{F_2})}{R_{F_2}(1 - R_{F_1})}}{1 - \dfrac{R_{F_1}(1 - R_{F_2})}{R_{F_2}(1 - R_{F_1})}} \tag{3}$$

If v calculated by eqn. 3 does not turn out to be equal to the value obtained by eqn. 2, ρ_p/ρ_c can be calulated from eqn. 1 using the value for v obtained from eqn. 3.

The elution volumes of components eluted by the second and third solvents can be calculated from the equations derived below.

Let a be the volume through which a component has been displaced when the second solvent is applied to the top of the column. Let b be the total displacement volume of this component when the solvent front of the second solvent overtakes it. Then, since R_F is the displacement volume of the solute divided by the displacement volume of the solvent front,

$$\frac{b - a}{b} = R_{F_1}$$

where the subscript refers to the first solvent.

$$\therefore b = \frac{a}{1 - R_{F_I}}$$

Also,

$$\frac{a}{V_I} = R_{F_I}$$

where V_I is total volume of solvent I added to the column.

$$\therefore b = \frac{R_{F_I} V_I}{1 - R_{F_I}}$$

The elution volume in solvent II can now be found since after solvent II overtakes the component, the remainder of the column is equivalent to a short column loaded at the top with the component and with solvent II as eluent. Thus, the effective column volume in solvent II is:

$$v - \frac{R_{F_I} V_I}{1 - R_{F_I}}$$

From eqn. 2 the elution volume for this partial column is:

$$\frac{1}{R_{F_{II}}} \left(v - \frac{V_I R_{F_I}}{1 - R_{F_I}} \right)$$

thus the total elution volume is:

$$V_I + \frac{R_{F_I} V_I}{1 - R_{F_I}} + \frac{1}{R_{F_{II}}} \left(v - \frac{V_I R_{F_I}}{1 - R_{F_I}} \right) \tag{4}$$

By an extension of this line of reasoning it can be shown that the elution volume of a component eluted by solvent III is:

$$V_I + \frac{R_{F_I} V_I}{1 - R_{F_I}} + V_{II} + \frac{R_{F_{II}} V_{II}}{1 - R_{F_{II}}} + \frac{1}{R_{F_{III}}} \left[v - \left(\frac{R_{F_I} V_I}{1 - R_{F_I}} + \frac{R_{F_{II}} V_{II}}{1 - R_{F_{II}}} \right) \right] \tag{5}$$

Note that the derivations of eqns. 4 and 5 assume that $\rho_p = \rho_c$. If this is not

TABLE I

R_F VALUES FROM THIN-LAYER PLATES

Compound	*Solvent*		
	Carbon tetrachloride	*Benzene*[a]	*Ethyl acetate*[a]
Hydrocarbons	0.82	—	—
Esters	0.37	—	—
Ketones	0.26	—	—
Aldehydes	0.23	—	—
Secondary alcohols	0.078	0.34	—
Unknown	0.054	0.27	—
Primary alcohols	0.024	0.12	0.65

[a] Values not listed are not required for this experiment.

the case the R_F values obtained from the thin-layers will have to be modified using eqn. 1 to obtain an R_F value that can be applied to the column.

RESULTS AND CALCULATIONS

Table I gives the R_F values obtained from the thin-layer plates. An elution diagram for the column separation is given in Fig. 2.

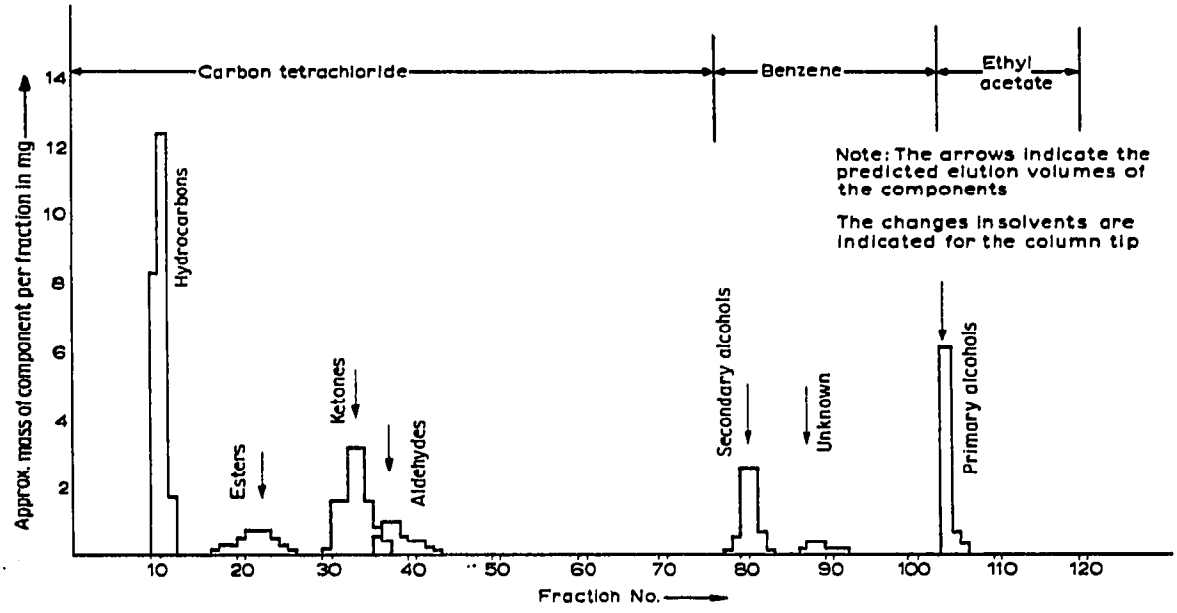

Fig. 2. Column separation of gl_6 wax on thin-layer silica gel.

Calculations

Elution volume of hydrocarbons (see Fig. 2): 10½ tubes = 111 ml. From eqn. 2, $v = 111 \times 0.82 = 91$ ml. Elution volume of esters: end of tube 22 = 254 ml. Checking v from eqn. 3:

$$v = \frac{254 - \frac{111 \times 0.82 \times 0.63}{0.37 \times 0.18}}{1 - \frac{0.82 \times 0.63}{0.37 \times 0.18}} = 91 \text{ ml.}$$

Thus, $\rho_p = \rho_c$.

Using eqn. 2 the elution volumes of esters, ketones and aldehydes can be calculated. These values are given in Table II. The elution volumes of the secondary alcohols and the unknown were calculated using eqn. 4, and the elution volume of primary alcohols by eqn. 5. These values are also given in Table II. The experimental values determined from Fig. 2 have been included for comparative purposes.

DISCUSSION

The experimental and calculated values given in Table II are in good agreement, the maximum error being about 1 %. Since the experimental values were found by

attempting to estimate the position of the peaks in 10.6 ml fractions, a better agreement could hardly be expected. This emphasises that the assumptions underlying the calculations are correct. Thus K, the distribution coefficient, is not dependent on the chromatographic system and in the column K is not affected by the passage of previous solvents.

TABLE II

COMPARISON OF CALCULATED AND EXPERIMENTAL VALUES FOR ELUTION VOLUMES

Component	*Elution volume (ml)*	
	Calculated	*Experimental*
Hydrocarbons	—	111
Esters	246	254
Ketones	350	350
Aldehydes	396	392
Secondary alcohols	847	848
Unknown	924	934
Primary alcohols	1091	1098

The separation illustrated in Fig. 2 took nearly four days to complete. Although this may be considered a disadvantage, it is not serious since once the procedure is commenced it requires little maintenance. All that has to be done is to observe the elution volume of the first peak so that the elution volumes of the other peaks can be calculated. By calculation, one can then find which times for solvent changes can be used which still allow satisfactory separations. In general a solvent should be changed just after the elution of a component with an R_F of about 0.25 on TLC in that solvent. If an accurate determination of the complete elution of such a peak is required so that the optimal time for the change of solvents is known, the R_F value for the trailing edge of the spot on TLC should be measured and substituted in either eqn. 2 or eqn. 4.

Fig. 2 shows that there was a slight overlap between the ketone and aldehyde peaks. The same overlap was found on TLC. Practically this is of little importance since the two components may be readily separated on a Florisil column after mild oxidation.

TABLE III

ANALYSIS OF gl_6 WAX

Components	*Mass (mg)*	*Adjusted mass (mg)*	*Composition (%)*
Hydrocarbons	22.2	22.2	39.7
Esters	3.7	3.7	6.6
Ketones	11.1	11.1	19.8
Aldehydes	2.5	2.5	4.5
Secondary alcohols	6.3	6.3	11.2
Unknown	1.4	1.4	2.5
Primary alcohols	7.0	4.1	7.3
Fatty acids	5.2	4.7	8.4
Total	59.4	56.0	100.0

Table III gives the percentage composition of gl_6 wax. It will be noted that the masses of the primary alcohols and fatty acids have been adjusted. The reasons for this are as follows. 56.0 mg of wax were applied to the Florisil column and 5.2 mg of fatty acids and 51.3 mg of other components were eluted from it, giving a total recovery of 56.5 mg. Florisil contains about 0.5 % Na_2SO_4 which can be eluted by acetic acid. The excess 0.5 mg recovered is thus assigned to Na_2SO_4 contaminating the fatty acid fraction. The mass of the primary alcohols was adjusted because although 51.3 mg were applied to the silica gel column a total of 54.2 mg was recovered. It was assumed for the purposes of calculating percentage composition that the excess 2.9 mg was due to a contaminant that was discovered in the primary alcohol fraction. This is a yellow oily substance and appears to be a contaminant on the silica gel. Thus the wash with carbon tetrachloride was inadequate. However, the problem can be overcome by washing with ethyl acetate instead of carbon tetrachloride.

Although precautions were taken to ensure that the resolved components were free of solvents and silica gel particles, it may be that part of the excess 2.9 mg assigned to the contaminant came from other sources. Nevertheless recovery from a silica gel column is quantitative; a fact that gives this procedure a decided advantage over preparative TLC.

It should be pointed out that it is not yet clear whether the procedure given here can be applied directly to solvent mixtures. This is because the column separates solvent mixtures as can be seen by applying a mixture of carbon tetrachloride and benzene and watching the boundary form. This also appears to occur on thin-layer plates, and thus accounts for the excellent separations given by solvent mixtures in TLC. The least polar component of the solvent mixture will move to the front and resolve the least polar solutes. The more polar components of the solvent will travel only a small distance up the plate and thus separate the more polar solutes at the bottom of the plate. A case in point is the hexane–ether–acetic acid mixture (70:30:2) used to analyse barley wax[6].

The results reported here show that there is no essential difference between TLC and column chromatography. Therefore one can design a column separation rapidly and quantitatively without actually packing a column. It seems pertinent to ask at this point why it is that column separations are so often inferior to thin-layer separations. Various authors (*e.g.* ref. 7) have emphasised the importance of a small particle size, but this is often sacrificed so that a high flow rate can be used. A small particle size gives an efficient use of the adsorbent because the void volume is small and the surface area is large. With difficult separations the efficient use of the adsorbent determines whether or not the separation can be made. If the separation is to be made, reduced flow rate must be accepted and inconvenience minimised by the use of automated equipment. Time saving faster flow rates can only be employed when the separation is easily made.

A future publication will give a more detailed analysis of gl_6 wax, and will examine the composition of the fractions isolated by the procedure reported in this paper.

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