CHROMATOGRAPHY OF ORGANIC COMPOUNDS

II. INVERTED DRY-COLUMN CHROMATOGRAPHY*, **

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(Received May 3rd, 1966)

The introduction of thin-layer chromatography (TLC)[1-4] has greatly aided column chromatography by providing it with an efficient and quick monitoring method. Furthermore, separations obtainable by TLC are far superior to those attainable by column chromatography and this has led to the development of preparative layer chromatography[4-8] and the necessary equipment is now available commercially. However, preparative layer chromatography is, generally speaking, rather tedious for quantities larger than a few hundred milligrams and the recoveries of air-sensitive compounds (*e.g.* terpenoids) are, in general, not satisfactory owing to the exposure of the compounds in the thin layers to atmospheric oxygen. Attempts have also been made to approximate the conditions obtained in TLC in column chromatography[9,10], but the reported methods have found no general acceptance, because of several limitations. We now describe a method which we have now used for some time on a variety of mixtures, both of natural and synthetic origin, and find that it can faithfully duplicate the TLC results on the column. The method is efficient, quick and does not require any special equipment. We have termed this method "inverted dry-column" chromatography***.

EXPERIMENTAL

Thin-layer chromatography

First of all, TLC of the mixture is studied and the solvent system, the adsorbent and its activity are selected so as to give as good a separation as possible, while keeping the R_F values within the range 0.2 to 0.7. If the mixture is colourless, then the position of one of the five azobenzenes (used for the standardisation of the adsorbent[11]) which has an R_F value, under the same conditions, at least 0.2–0.3 units higher (or lower in case of fast-moving components, *e.g.* hydrocarbons) is also determined on the same chromatogram. The solvent front is allowed to move 10–15 cm from the start.

Preparation of the column

The column is prepared in a glass-tube which is flared at one end only (Fig. 1). Depending on the sample size and ΔR_F values of its components, suitable tube di-

* Communication No. 912, National Chemical Laboratory, Poona 8, India.
** Part I, *J. Chromatog.*, 12 (1963) 189.
*** We prefer this term to "ascending column" chromatography.

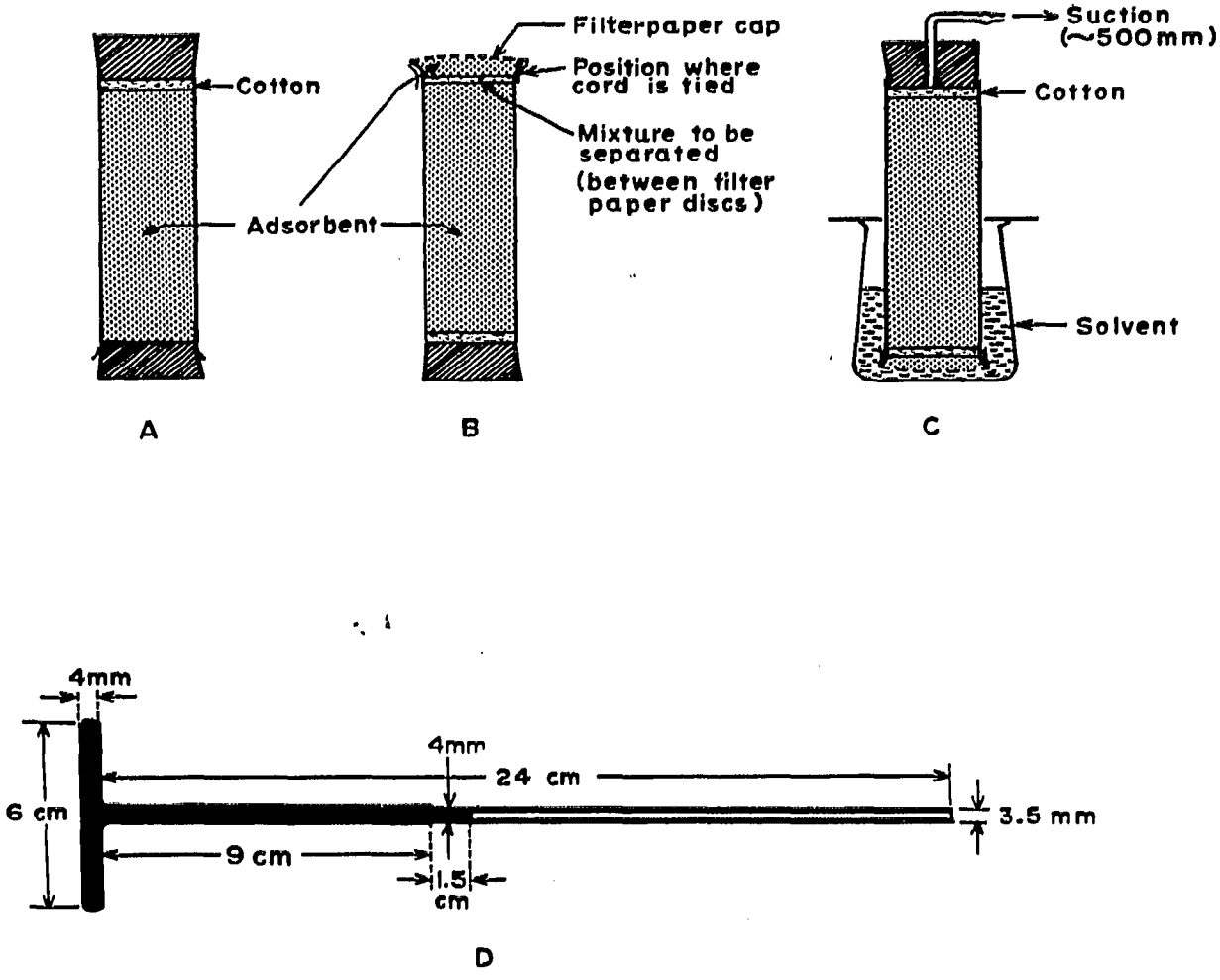


Fig. 1. Set-up for inverted dry-column chromatography. A = Packed column; B = column after loading the mixture, ready for development; C = column arranged for development; D = scoopula.

mensions are selected; Table I gives the recommended sizes. The flared end of the tube is closed by a rubber stopper and the selected adsorbent (of the same activity as that used for TLC) is introduced into approximately half the length of the column. The open end is then closed with another rubber stopper and the lower flared end is gently tapped on the surface of a table till the adsorbent does not settle any further. The process is repeated till the column is filled to within about 0.5 cm of the lower end of the top stopper; this stopper is now removed and a suitably-sized cotton pad placed on top of the adsorbent and the stopper replaced so that there is no air-gap between the stopper and the cotton pad (Fig. 1A). This operation normally takes about 40–50 min. We find it convenient to pack several sizes of column and keep them ready for use, as and when required.

We have carried out most of our separations on silica gel and find it essential to use silica gel of TLC grade (without binder; —250 mesh). The activity of the silica gel layer on the TLC plate was determined by preparing several plates (10 × 15 cm, 0.5 mm layer) and activating them under identical conditions. The layers of a couple of plates were rapidly scraped off and the material packed in a column and its activity determined by the procedure of HERNANDEZ *et al.*[12]. We find that if the plates,

TABLE I

RECOMMENDED SIZES OF COLUMNS

No.	*Weight of silica gel** (g)	*Column size 25 cm × I.D.** (cm)	*Weight of mixture*** (g)
1	25	× 1.5	0.1
2	75	× 2.5	0.3
3	125	× 3.3	0.5
4	250	× 4.7	1.0
5	500	× 6.6	2.0
6	1000	× 9.4	4.0

* Bulk density = 0.70 g/ml.
** When $\Delta R_F \sim 0.1$.

after drying at room temperature ($\sim 25°$) for 10–12 h, are activated at 110° for 1 h, the activity is IIA*. For the examples given below we used TLC plates activated as above and hence for column packing, silica gel of grade IIA was employed.

The ratio of the mixture to the adsorbent is dependent on the R_F of the contiguous components as determined by TLC. When the ΔR_F is of the order of 0.1, then a ratio of 1:250 (mixture:silica gel) has been found to work well. When the components are well separated, the amount of adsorbent can be cut down to one-half or even less.

Application of the mixture

After packing the column as above, the column is clamped with the flared end upwards. The stopper is carefully removed; slight cracking in one or two parts of the upper portion of the adsorbent column often occurs while removing the stopper, but usually the column settles back when the stopper has been completely removed, if not, gentle tapping suffices to close up the cracks in the column. The glass column at the open end is now freed of any adsorbent to a depth of 1.0–1.5 cm and the adsorbent surface is levelled and gently pressed down with a suitably-sized small cork. A filter paper disc (of diameter just smaller than the internal diameter of the glass tube) is placed on the surface and is gently pressed down level. The sample mixture, either absorbed on filter paper discs or on the same adsorbent (see below), is uniformly introduced and covered with another filter paper disc; this is then gently pressed down with the same cork. Adsorbent is next added, levelled and gently pressed, and the process repeated until the adsorbent layer is flush with the upper rim of the glass column. A little more adsorbent is added, levelled and covered with a filter paper disc having a diameter double that of the column. The filter paper is carefully and smoothly folded downward and secured in place by tying with a strong cotton cord (which has been washed with water, acetone and alcohol) against the rim (Fig. 1B). It is important to see that there is no air-gap left between the adsorbent and the filter-paper cap. The column is now ready for development.

The absorption of the mixture on filter paper discs or adsorbent, as mentioned

* An attempt has been made to describe the activity of TLC plates in terms of the R_F values of the standard azobenzenes, but the results were not satisfactory because of the considerable dependence of these values on the thickness of the layers; *cf.* HERMANEK *et al.*[13].

above, is carried out as described below. For colourless compounds 5–10 mg of the selected azobenzene is also incorporated in the mixture for every 100 g of adsorbent.

(*i*) *On filter paper discs* (*for quantities up to 500 mg*). A small watch-glass is supported on a petri-dish (diameter smaller than that of watch glass). Two capillary tubes (m.p. capillary tubes with both ends sealed) are placed parallel to each other, across the watch glass. A filter paper disc (of diameter *just* smaller than that of the internal diameter of the glass column) is supported on these capillaries. The mixture, dissolved in a suitable low-boiling solvent, is applied to the filter paper disc with a dropper, so that the paper gets uniformly coated; another filter paper disc is now placed over the earlier disc and the mixture applied as before. More filter paper discs are placed, one by one, and the process is repeated till all the mixture has been transferred to the filter paper discs. The pile of treated discs is then transferred to the column. A pair of forceps is ideal for handling the discs.

(*ii*) *On the adsorbent* (*for quantities larger than* 0.5 *g*). The mixture is dissolved in a suitable solvent and adsorbent (twice the weight of the mixture) is added and the whole taken to dryness under suction with swirling at ~ 35°. The product is a free-flowing mixture which is transferred to the column.

Development

The column is clamped upright with the filter paper capped end downward. The rubber stopper is replaced by another one with a connection for vacuum. The column is lowered into the solvent system, determined earlier for TLC, and contained in a narrow tall beaker, so that about 5 cm length of the column is immersed. The top is connected to a water-pump, preadjusted for ~ 500 mm pressure (Fig. 1C). The open end of the beaker is closed with a split-cover placed over it. The development is allowed to take place to a height of 20 cm from the point at which the mixture was loaded, and takes ~ 1.5 to 2.5 h. After the development is over, the column is withdrawn from the solvent and after a couple of minutes disconnected from the vacuum source. The stopper with the vacuum connection is removed and replaced by the previous rubber stopper.

Location of zones

For coloured compounds location of zones offers no difficulty. For colourless compounds the following general method has worked very well.

From the R_{dye} values[14] of the compounds, as determined on the TLC plate, the approximate positions of the components on the column are computed from the position of the same azobenzene, which has been earlier incorporated in the mixture. The filter paper cap is now removed and the packing (adsorbent and filter paper discs) dug out upto ~ 3 cm from the centre (computed) of the slowest-moving component. A rod-spatula serves very well for this operation. A sharp sampling device, which we call a "scoopula"* and which is described below, is now inserted at the periphery of the adsorbent column against the glass surface to a depth of 5–8 cm and then carefully withdrawn, when one obtains a similar length of sample (~ 3 mm wide and ~ 2 mm at the deepest point). Without removing it from the scoopula, the sample (after the solvent has been allowed to evaporate) is exposed to iodine vapours or sprayed with a suitable reagent (see below) and the exact position of the zones

* From *scoop* + spat*ula*.

located. By matching this against the scooped area, the zones are marked on the column and suitable cylindrical sections dug out. If necessary, the remaining length of the column is now sampled, marked in the same way, and dug out. The separated components are now extracted from the adsorbent obtained from the respective zones and worked up as usual.

The open flared end of the column should always be kept closed with a rubber stopper, which is removed only when sampling or digging operations are being carried out; the solvent retained by the adsorbent is essential for the sampling or digging operations.

In the case where the zones containing the components overlap somewhat, one or two extra cuts in the column of adsorbent are made in the overlapping region. Test portions from the various zones that have been dug out are now extracted (in a test tube with a small amount of solvent) and the purity of the extracts checked by TLC. Suitable mixing of the scooped materials is done, if necessary, before the final extractions and work-up.

If a number of columns (even with a different diameter) are prepared with the same adsorbent and used to separate a given mixture, then after the zones of the separated components have been located on one column by the scoopula method, zones on the other columns (developed under identical conditions) can be directly marked in terms of distances from the place at which the mixture was applied.

The scoopula, which is made of stainless steel consists of a rod portion, which serves as a handle and an open tubular part which receives the sample. The dimensions are shown in Fig. 1D and this size is very suitable for column size 3 (Table I) or larger; for smaller columns, the width of the tubular portion should be reduced to half. The edges of the tubular part are sharpened, as and when necessary.

For the visualisation of the zones in the scoopula, exposure to iodine vapour[15],

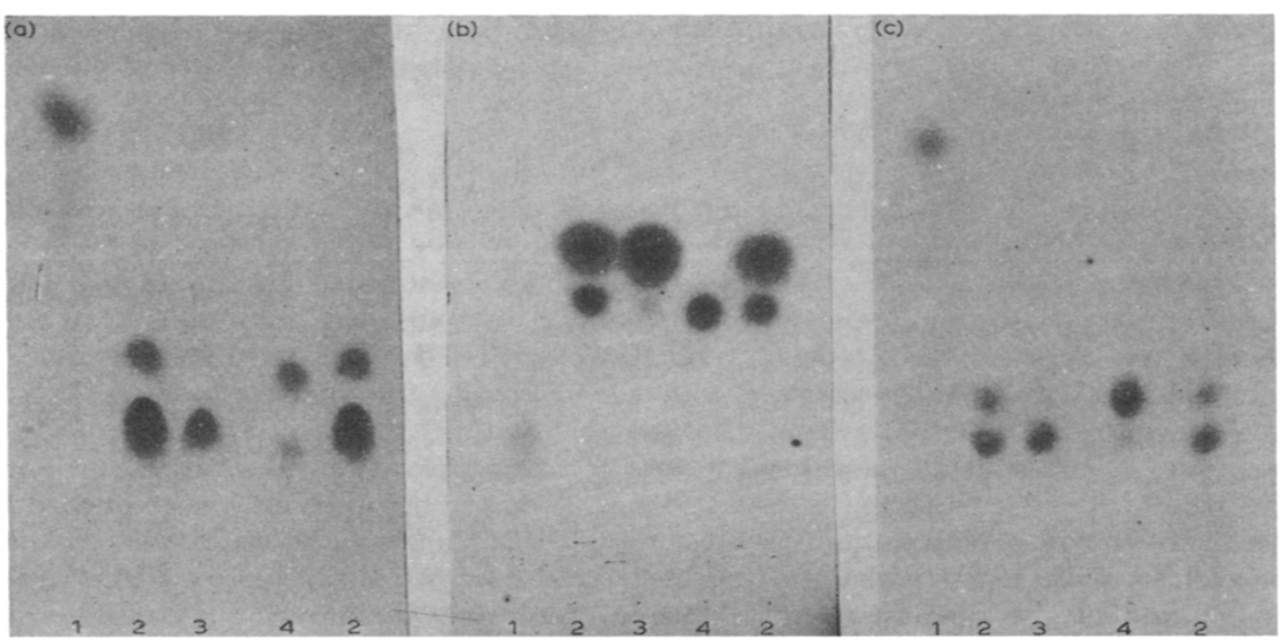


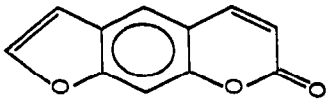
Fig. 2. Thin-layer chromatography of components after separation by the inverted column technique. Solvent system: (a) 5% EtOAc in C_6H_6; (b) petroleum ether; (c) 2% EtOAc in C_6H_6. Solvent front: 15 cm. Temperature: 26°. Visualisation: (a) iodine vapours; (b) picric acid; (c) iodine vapours. 1 = Azobenzene; 2 = mixture; 3 = fraction 1; 4 = fraction 2.

followed by exposure to air, serves very well in most cases. When visualisation is done with a specific spray reagent*, one must spray only lightly and select reagents which are not unduly corrosive; it is also preferable to reduce the concentration of the active components in the spray reagent. We find a weaker phosphoric acid–vanillin reagent[16] (0.5 g of vanillin in a mixture of 60 c.c. of water and 30 c.c. of 85 % phosphoric acid) very useful for terpenoids and related compounds, in general.

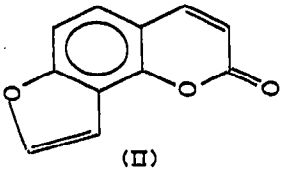
RESULTS

The results obtained on the application of the above method to three synthetic mixtures, are briefly given below. The mixtures contained two components and consisted of:

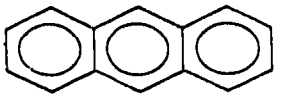
(a) psoralen (I) + isopsoralen (II),
(b) anthracene (III) + acenaphthene (IV),
(c) 5,6-cyclopentenoindanone (V) + 6,7-cyclopentenoindanone (VI).



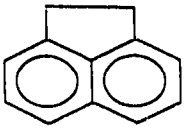
(I)



(II)



(III)



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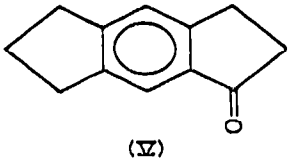
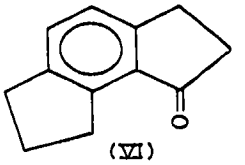


(V)



(VI)

Table II gives the necessary experimental data regarding their separation. As can be seen from the TLC (Fig. 2) of the separated fractions, and the other relevant data, collected in Table III, the results are quite good.

We have applied this method to a variety of multicomponent mixtures obtained during our studies on natural plant products and the results have been uniformly good. The application of this technique to olefine separations over $AgNO_3$–silica gel has several additional advantages and we propose to report these results in a future communication.

* For an extensive list of these reagents, see refs. 2 and 4.

TABLE II

INVERTED COLUMN CHROMATOGRAPHY OF SOME SYNTHETIC MIXTURES

Experimental data	*Mixture*		
	a	*b*	*c*
Weight of mixture taken	0.97 g	2.03 g	0.507 g
Ratio of components*	3:2	1:1	3:1
ΔR_F (TLC) of components	0.06	0.1	0.09
Weight of adsorbent**	250 g	500 g	125 g
Column dimensions (cm)	25 × 4.7	25 × 6.6	25 × 3.3
Method of loading the mixture	on adsorbent	on adsorbent	on filter paper
Azobenzene used	azobenzene	azobenzene	azobenzene
Solvent system	5% EtOAc in benzene	pet. ether (b.p. 40–60°)	2% EtOAc in benzene
Time taken for development	2.5 h	1.75 h	2.5 h
Location of zones	scoopula	scoopula	scoopula
Visualisation reagent	iodine	picric acid	iodine
No. of cuts made for extraction	two	two	two
Length of cuts*	2.6 cm; 2 cm	3 cm; 3 cm	4 cm; 2.3 cm
Total recovery (%)	95	95	97

* The first figure refers to the component with smaller R_F.
** Silica gel, TLC grade, activity IIA.

TABLE III

PURITY OF SEPARATED FRACTIONS

Component separated	*TLC purity (Fig. 2)*	*M.p.*		
		*Before crystallization**	*After one crystallization*	*Authentic sample*
Psoralen	Pure	150–153°	158–159°	160–161°
Isopsoralen	Very slight contamination	122–125°	136–137°	138–140°
Anthracene	Pure	211–213°	215–216°	215–216°
Acenaphthene	Very slight contamination	90–92°	95–96°	95–96°
Linear ketone (V)	Pure	78–80°	81–83°	82–83°
Angular ketone (VI)	Very slight contamination	67–73°	83–84°	83–84°

* The product obtained after extraction from adsorbent and solvent removal.

ACKNOWLEDGEMENT

The authors are grateful to Dr. R. C. PANDEY of this Laboratory for helpful suggestions.

SUMMARY

A column chromatographic method is described, which directly adopts the results of thin-layer chromatography. The method appears to have several advantages over preparative layer chromatography and conventional column chromatography.

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