THE TRANSFER OF CHROMATOPLATE RESOLUTIONS TO LARGE SCALE SEPARATIONS ON ADSORPTION COLUMNS

A NEW APPARATUS FOR PRODUCING THIN LAYERS

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With the advent of the technique of thin-layer chromatography[1], it has now become possible to chromatograph substances in a very short time and to obtain, occasionally, separations which cannot be obtained using paper chromatography. If it is required that a large scale separation be carried out, it is possible to prepare "preparative plates"; this becomes less feasible when gram quantities are being considered. It is also not satisfactory when two substances are not resolved completely; in some cases this may be overcome by "over-running" the plate[2] but this technique requires a much longer time and more elaborate apparatus. An approach to a satisfactory separation may be made from another direction by carrying out a column chromatogram under the identical conditions which occur on the plate. The experimental work cited below illustrates a procedure which can be used. It becomes necessary, when utilizing this procedure, to decide beforehand by a plate chromatogram, whether a satisfactory resolution of two substances will occur or not on the column. A criterion r is here introduced which can be used as a measure of the expected resolution:

$$r = \frac{a}{b + 0.1\,a}$$

where $a = R_F$ of the fast moving substance A;
$b = R_F$ of the slow moving substance B.

When r is greater than unity, a resolution will occur on the column; when smaller than unity, either the substances will not be resolved or mixtures of varying concentrations will be obtained.

It has been found in our laboratory that the various commercial apparatus available for preparing thin layers of adsorbent on a plate*, are dependent upon the thickness of the plate and its uniformity. In one instance, the plates should, optimally, be of identical thickness. This has led to problems in the matching of plates and in the preparation of uniform layers. An apparatus is here described which is simple, cheap and easy to make and which yields layers whose thicknesses are independent of the plate thickness or uniformity.

* Desaga apparatus DS 200/0.3 and Appareil de coulée, Modèle J. E.

EXPERIMENTAL

Purpose

It had been ascertained, using paper chromatography, that a mixture of 524 mg of material from *Pachycarpus concolor*[3] contained possibly three cardenolides, namely, digitoxigenin (I), 3-*epi*-digitoxigenin (II) and xysmalogenin (III) (see Fig. 1) and it

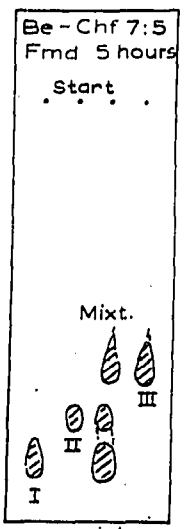

Fig. 1. Paper chromatogram of the mixture. (System: Be–Chf 7:5/Fmd.)

was necessary that II be isolated for identification purposes. I and III had previously been isolated. It had also earlier been ascertained that, through a scheme of preparative papers, non-crystallizable II was obtained (perhaps due to some contaminant); this precluded the use of a normal partition column. It was observed that on the plate

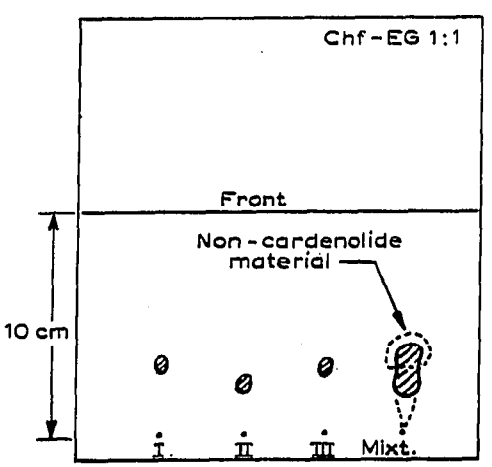

Fig. 2. Thin layer chromatogram of the mixture on Kieselgel G. (System: Chf–EG 1:1.)

a non-cardenolide material ran ahead of II (Fig. 2); this suggested that a separation scheme similar to a plate might be of some value. The value of r in this instance, taking I and III as A and II as B, was found to be 1.21; according to this criterion a separation should occur.

Reagents and materials

Chloroform (Chf), dried over calcium chloride and distilled twice; acetone (An), distilled from potassium permanganate and then once again; methanol (Me), distilled from conc. sulfuric acid, potassium hydroxide and distilled twice again; benzene (Be), prepared with conc. sulfuric acid, potassium hydroxide and distilled; ethyl acetate (EG), "Fluka"; formamide (Fmd), "Fluka"; Kieselgel G, "Merck" for thin-layer chromatography; Kieselgel, "Merck" 0.05 to 0.2 mm; trichloroacetic acid–chloramine T reagent (TCA–C)[4].

Apparatus

Thin-layer plates measuring 150 × 200 × 4 mm; chromatography column No. 2[5]; Martin packer; automatic fraction collector.

A thin-layer apparatus consisting of:

(a) stainless steel metal side strips, 0.5 mm in thickness, 20 cm long and which fit on the long side of the plate (see Fig. 3 for details);

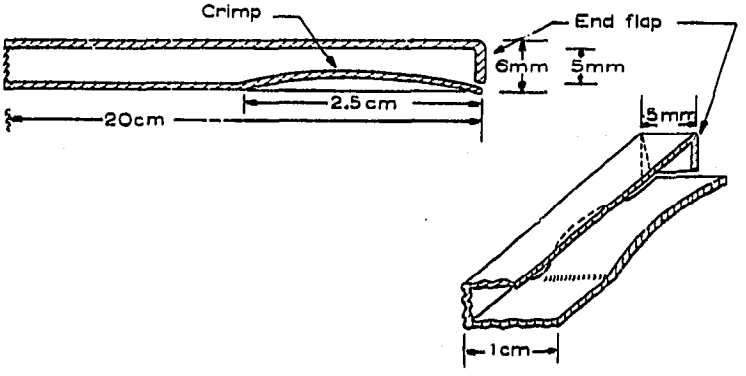

Fig. 3. Detail of side strips (right-hand side shown).

(b) a streaking rod, 20 cm long, 2 cm wide and 0.8 cm thick, the under-surface of which has been given a dull surface by grinding (see Fig. 4);

(c) a support for the plate of the dimensions shown (Fig. 4), made of wood or plastic upon which the plates are laid and which allows the "side strips" to float freely and to contact the upper surface of the glass plate evenly.

Preparation of the thin layer of adsorbent

1. The plates are cleaned thoroughly using successively, a soap, water and alcohol wash; the metal side strips are fitted as shown on the long side of the plate and the plate is laid on the support with the streaking rod lying on the strips, at right angles to them and at the end nearest the operator (see Fig. 4).

2. A mixture of Kieselgel G, one part by weight and Me–W 1:1, two parts by volume, is mixed thoroughly with shaking and stirring and 20 ml is poured in a line just in front of the streaker rod. The streaker rod is then pushed smoothly and with a medium speed, with light downward pressure towards and off the other end of the plate, thereby producing a uniform layer of the gel. (If desired, the rod, after being pushed to the end of the plate, may be returned once again over the layer to the starting position, further smoothing out the plate.)

3. The plate is removed to a level surface where it is allowed to air-dry for 15 min to a half-hour whereupon 1/2 cm of adsorbent is scraped from the edges and it is activated for one hour at 80 to 100°[6]. The side strips may be removed 3–5 min after the plate is streaked and used again. The plate is stored over calcium chloride prior to use.

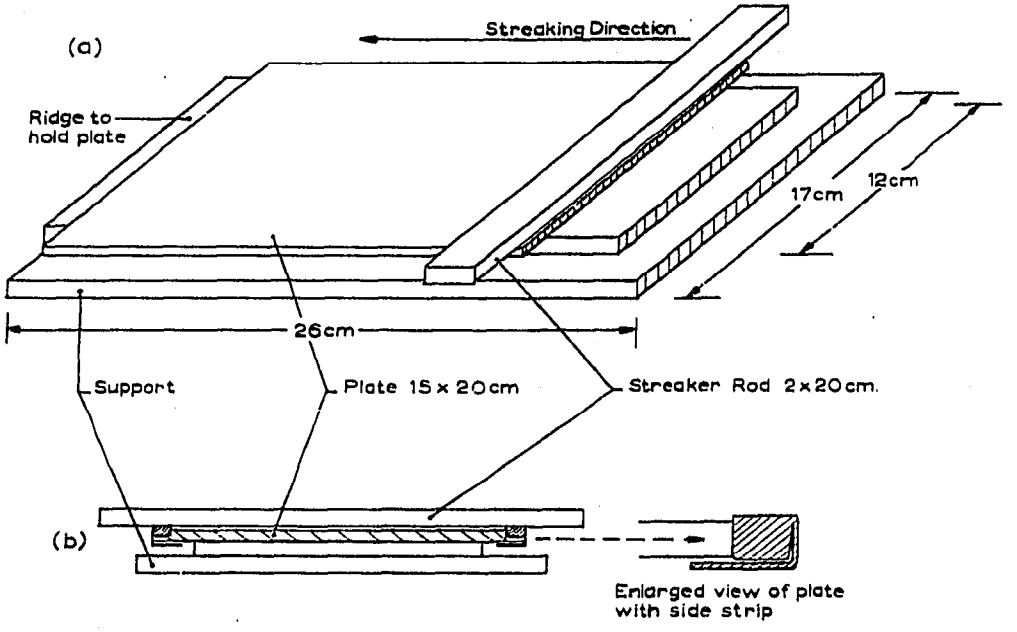

Fig. 4. (a) Thin layer apparatus (shown without side strips). (b) End view of thin layer apparatus (shown with side strips).

Development of the plate chromatogram

The plate chromatogram was developed in the normal fashion[1] using as solvent Chf–EG 1:1 and allowing the front to run 10 cm from the starting line. After development the plate was freed of solvent by air-drying, sprayed with TCA–C and heated for 5 min at 100°. The above-mentioned mixture was chromatographed in this manner and gave the following spots (see also Fig. 2):

non-cardenolide R_F 0.346 blue colour in visible light and brown in U.V.;
I and III R_F 0.330 yellow in visible and U.V.;
II R_F 0.240 yellow in visible and U.V.

The calculated r values were:

r (non-cardenolide/I + III) = 0.95;
r (I + III/II) = 1.21.

Column chromatography

The column was made by pouring 400 g of Kieselgel (0.05–0.2) slowly, to allow air to escape, into the No. 2 column which had been almost filled with the solvent, Chf–EG 1:1, and the gel, when air-free, was packed tightly with the aid of a Martin packer. The solvent was allowed to run off slowly during the packing. After preparation, a circular disc of filter paper was placed over the gel in the column to prevent any disturbances from the incoming solvent. 2 mg of Sudan III[7] in 2 ml of solvent was washed into the top in the normal fashion, following which 524 mg of the above-

mentioned mixture in *ca.* 3 ml of solvent was likewise washed in. The flow rate was adjusted to a rate of 20 ml per half-hour; fractions were collected at half-hour intervals. A total of 133 fractions were collected; the front (dye marker) appeared at the fifteenth fraction (*ca.* 300 ml). The results are tabulated in short form in Table I.

TABLE I

RESULTS OF THE COLUMN CHROMATOGRAM

Fraction No.	*Remarks*
1–14	no residue
15–21	dyestuff
21–43	17.0 mg non-cardenolide material
44–62	115.2 mg non-cardenolide material
63–84	143.3 mg mixture of I + III (Fraction 65 yielded crystalline I and Fractions 75–79 yielded crystalline III)
85	2.8 mg non-cardenolide material
86–103	63.5 mg pure II
104–113	20.4 mg mixture of II and IV?
114–133	20.9 mg non-cardenolide material
	Total: 383.1 mg

After the column was halted, it was noticed that a band of yellow material remained adsorbed at the top of the column. This was not eluted.

DISCUSSION

Chromatoplates

By using the above method for making thin-layer plates, the need for glass plates of uniform thickness is obviated since the streaker moves along a metal strip of constant thickness, thereby always yielding layers of identical thickness. At the present time, we are using strips made with metal of 0.5 mm thickness; this may be decreased if desired and thinner layers will result. During the plate making process, as described above, there is no need for rapid work; the Kieselgel G slurry will not set until it is streaked out over the plate (this is the purpose of the Me). It is suggested that between each plate, the streaker be washed and dried as some gel may adhere to it, partly dry, and thus interfere with perfect reproduction with the next plate. After the preparation of one or two plates, it will be found that the technique is very quickly learned. The author uses five pairs of strips; this number is sufficient to produce any quantity of plates. It will be noticed (Fig. 3) that the side strips are crimped along the lower part; this gives a tighter fit on the plate and they will not tend to slip off. The strips are so crimped at both ends. A flap at one end of each strip is also necessary so that the strips will not slide along the plate in the direction of travel of the streaker rod. This feature produces pairs of strips for each plate.

r value

For the substances separated here, r was greater than unity. A satisfactory separation was obtained and from this we may assume that it is a useful criterion*. It has further value in that it describes the resolution of two substances in any system, for example it is simple to say that r (II/I + III) = 1.21 (Chf–EG 1:1). A systematic survey of r values for two substances using various solvent systems then reveals which system, if any, would enable a separation to be obtained. It allows no room for "guesses".

Solvent systems

The author would like to note here the approach used to a suitable choice of a solvent system for chromatoplates. It is assumed that when two solvents are mixed the resultant dielectric constant of the mixture bears a linear relationship to the dielectric constants of either pure solvent. With this assumption, solvent systems are prepared which possess the same dielectric constant. If two solvent systems, so prepared, yield different R_F values for like substances, we may conclude that the factor creating this difference lies in the different dipole character of either solvent. The author has observed that alcohols, as the adsorbed substance, move much faster when the solvent itself contains an alcohol. Perhaps a competition exists between the solvent and substance for active sites on the adsorbent; increased competition leads to de-sorption with a resultant higher R_F value. It has even been found that this competition is, in some respects, specific, for example, the R_F of a secondary alcohol decreases in the order Chf/i-PrOH > Chf/Me > Chf/An when all of the solvents have the same dielectric constant. It can also be observed that r is greater for two substances when a solvent system is used which does not interfere to a great extent with the adsorption of these substances (see Table II).

Column chromatography

The time required to effect a separation using this method is of two to three days duration and it may be carried out on any quantity of material. A separation may be expected if r is greater than unity even though the substances do not separate fully on the plate. Normally, the amount of silica gel required will be 500 to 1000 times the weight of material to be separated and the volume per fraction will be approximately 40 to 50 times in ml of the weight separated. If a wide column is used, the packing will, of course, have to be very uniform; the author deems it best to use preferably a longer column with a smaller cross-section. It is interesting to contrast this new

* To further substantiate this, two additional examples are given which have not yet been published. The author wishes to thank Dr. EK. WEISS for the permission to use the results given in Example 1 and Mr. R. BERTHOLD for those in Example 2.

Substance and weight	*Nature*	*r value*	*g SiO_2*	*Solvent*	*Flow ml/¼ h*	*Remarks*
1. A and B 564 mg	steroid-oximes (*syn-anti*)	A/B 1.13	250	Chf/i-Pr 7%	12	resolved
2. A, B and C 550 mg	genin-glycoside mixture	A/B 1.08 B/C 1.15	200	Bc/i-Pr 31%	15	resolved

method of column chromatography with the other method now commonly in use. The ratio of adsorbent to substance in the other method is 1:30–60 and the volume of eluting solvent collected per fraction is approximately 100 times the weight of sub-

TABLE II

r VALUES FOR DIGITOXIGENIN AND 3-EPI-DIGITOXIGENIN IN SOLVENT SYSTEMS OF EQUAL DIELECTRIC CONSTANT

System	*Diel. const.*	*r*	*Approx. movement*
Chf/BuOH 28%	5.8	1.06	fast
Chf/i-PrOH 7%	5.8	1.07	fast
Chf/PrOH 5%	5.8	1.13	slow
Chf/Me 5%	(6.4)	1.04	slow
Chf/An 4.5%	5.8	—	remains at origin
Chf/MEK 20%	(7.7)	1.11	slow
Chf/EG 56%	5.8	1.21	medium

stance. With this new method, much more adsorbent is used and the fractions taken are much smaller. This new method could be called a solid/liquid partition column; it approximates the ideal partition of a substance between two phases, a solid and a liquid. This method supplements other chromatographic procedures now in use. It is finding widespread application in our laboratories for separations in the steroid field.

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SUMMARY

In the present article the author describes a new, simple apparatus for making thin-layer plates which yields reproducible results regardless of the glass uniformity or thickness. A method of column chromatography is described whereby resolutions obtained on a chromatoplate may be transferred to the execution of large scale separations of the same substances. A criterion *r* for the resolution of two substances on a plate is suggested and its value illustrated with appropriate examples. A reference point for the preparation of solvent systems for thin-layer chromatography, namely, the dielectric constant, is discussed.

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