A SIMPLE, GENERAL METHOD FOR GRADIENT ELUTION USING ELUENTS OF UNEQUAL DENSITY

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INTRODUCTION

Generally when complex mixtures of compounds have to be fractionated by column chromatography it is advantageous to elute with a continuous concentration gradient[1]. This makes the fractionation automatic and easily reproducible, and avoids the formation of spurious peaks that occurs when discontinuous gradients are used. It reduces tailing and improves resolution, moreover, if the type of gradient used is well chosen. (Gradients may be classified[2,3] as linear, convex, concave, or compound.) Despite these advantages continuous gradient elution is not yet widely used for natural lipid mixtures. One reason why this is so appears to be a misconception, based upon reports about convex gradients, that all continuous gradients inevitably resolve lipids less well than discontinuous gradients[2]. Another reason is the paucity of apparatus producing linear and concave gradients with eluents of unequal density[4].

In the method of LAKSHMANAN AND LIEBERMAN[5,6] the column is fed from a stirred reservoir which initially contains the less polar eluent, and to which the more polar eluent is added at a controlled rate; the addition may be made by means of a motor-driven syringe[7]. Linear[8] and concave[9] gradients may also be produced by means of metering pumps. These methods all require a constancy of flow rate through the column that is often difficult to achieve.

Apparatus of the type first described by PARR[10] is probably the simplest and most versatile available for producing continuous gradients. It consists (Fig. 1) of

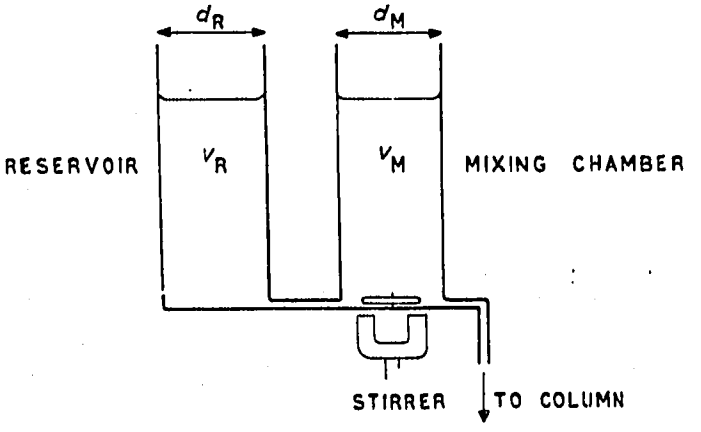

Fig. 1. Parr's apparatus[10].

two vertical, parallel-walled vessels, open to the atmosphere and joined below by a narrow tube. With efficient stirring and slow delivery the system may be considered always at hydrostatic equilibrium. Then the concentration, c, of the more polar eluent in the mixing chamber (and being delivered to the column) when a volume v has been delivered is given by the equation:

$$c = c_R - (c_R - c_M) \cdot \left(1 - \frac{v}{v_R + v_M}\right)^{v_R/v_M} \quad (1)$$

where v_R and v_M are the volumes initially present in the reservoir and mixing chamber respectively, and c_R and c_M the initial concentrations of the more powerful eluent. Equation (1) is known[11-13] to apply to liquids of equal density that do not change in volume when mixed. Since $v_R/v_M = d_R^2/d_M^2$ linear gradients are predicted when $d_R = d_M$ (internal diameter of reservoir = internal diameter of mixing chamber), convex when $d_R > d_M$, and concave when $d_R < d_M$. Convexity and concavity are slightly greater after the half-way marks [$v = (v_R + v_M)/2$] than before[3,11,12].

The above predictions fail for liquids of unequal density. For example, if $d_R = d_M$ one volume of chloroform in the mixing chamber requires nearly two volumes of methanol in the reservoir to establish hydrostatic equilibrium: then a convex, not a linear gradient is produced. The hydrostatic problem can be solved[14,15] by using motor-driven syringes in place of open vessels, but no such apparatus has been described that is suitable for organic solvents. (Large PTFE pistons[16] would be necessary.)

METHOD

It has now been found that when eluents of unequal density are used in apparatus of the type shown in Fig. 1 the gradients produced are described well by an empirically modified equation,

$$c = c_R - (c_R - c_M) \cdot \left(1 - \frac{v}{v_R + v_M}\right)^{\rho_M d_R^2/\rho_R d_M^2} \quad (2)$$

where ρ_R is the density of the liquid in the reservoir and ρ_M that of the liquid initially present in the mixing chamber. The gradients are:

linear when $\rho_M d_R^2 = \rho_R d_M^2$,

convex when $\rho_M d_R^2 > \rho_R d_M^2$,

and *concave* when $\rho_M d_R^2 < \rho_R d_M^2$.

Gradients can be designed and prepared quite simply in the following way.

(i) Choose the initial and final concentrations (c_M, c_R) of the more powerful eluent.

(ii) Decide upon convexity, concavity, or linearity by choosing the concentration (c_H) at the half-way mark.

(iii) Calculate the ratio of the diameters from the derived equation:

$$\frac{d_R}{d_M} = \sqrt{\frac{\rho_R}{\rho_M} \cdot \frac{-1}{\log 2} \cdot \log\left(\frac{c_R - c_H}{c_R - c_M}\right)} \quad (3)$$

This is done by means of a nomogram (Fig. 2).

(iv) Choose approximate volumes (v_R, v_M).

(v) Select two reasonably cylindrical vessels that will contain these volumes and fulfil the diameter ratio.

(vi) Connect the vessels and proceed as indicated below.

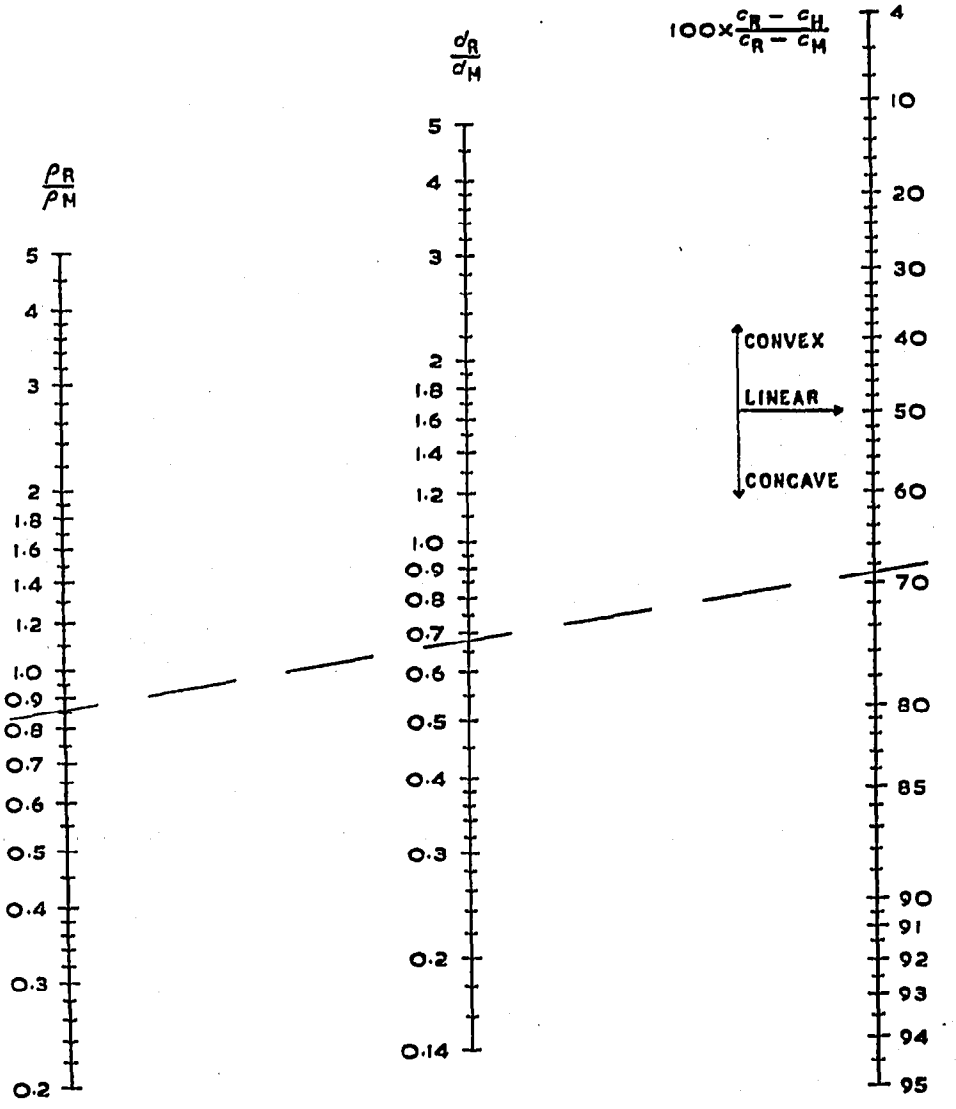

Fig. 2. Nomogram for eqn. (3). The dashed line illustrates the computation of the diameter ratio for a concave gradient, 0–30 % methanol in chloroform, $c_H \doteqdot 10\,\%$.

A typical arrangement for a concave gradient of methanol in chloroform is shown in Fig. 3. The reservoir is a 2 l measuring cylinder and the mixing chamber a Winchester bottle. Connecting the mixing chamber to the reservoir and to the column are siphon tubes (K, L), which are made of capillary glass (I.D. 2 mm) and have looped side pieces (S, T) closed with PTFE-barrelled stopcocks or screw clips on plastic sleeves.

The *starting procedure* is as follows.

(i) Place the liquids in R and M, including a 10 % excess over v_R in R to allow for minor errors in measurement and computation.

(ii) Close S and T.

(iii) Start the siphon through L, by applying a vacuum line to the lower end, and connect it to the column.

(iv) Start the stirrer.

(v) Start the siphon through K by connecting the vacuum line to S and opening the stopcock briefly.

(vi) Raise R to cause visible flow into M, and then lower it to the minimum height necessary for flow to continue.

(vii) Close R and M with tight cotton-wool plugs and aluminium foil.

(viii) Remove any air bubbles that rise in L from the column by applying the vacuum line through T.

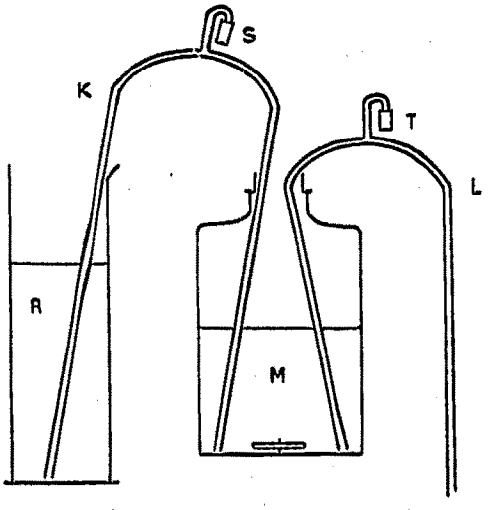

Fig. 3. Apparatus producing a continuous, concave gradient of methanol in chloroform.

The arrangement shown in Fig. 3 is suitable whenever the less polar eluent is the denser. But when it is the lighter an upward-screw mechanical stirrer or (preferably) an upward impelling vibratory mixer should be used. The lighter liquid in M may render the siphon useless by rising in currents in K, despite the opposing flow from R. This difficulty can be overcome by directing the outlet from K upwards: some alternative arrangements for connecting the vessels are shown in Fig. 4. The tubes must always be arranged so that L cannot take in unmixed liquid.

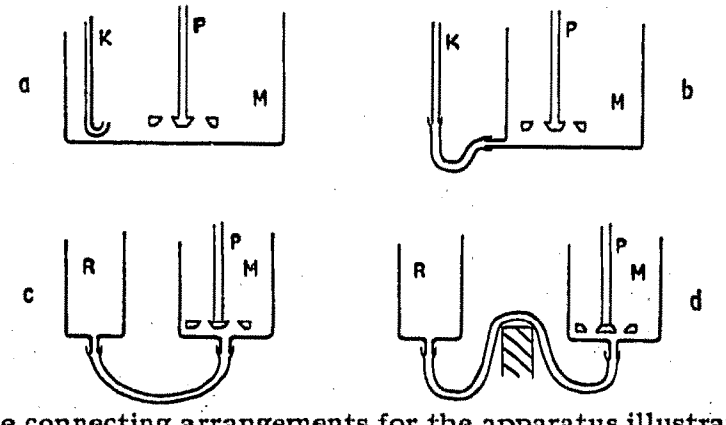

Fig. 4. Some alternative connecting arrangements for the apparatus illustrated in Fig. 3: (a), (b), and (c) are suitable when the more polar eluent is the denser, and (d) when it is the less dense. Flexible connections are made with narrow PTFE tubing. P is a vibratory mixer.

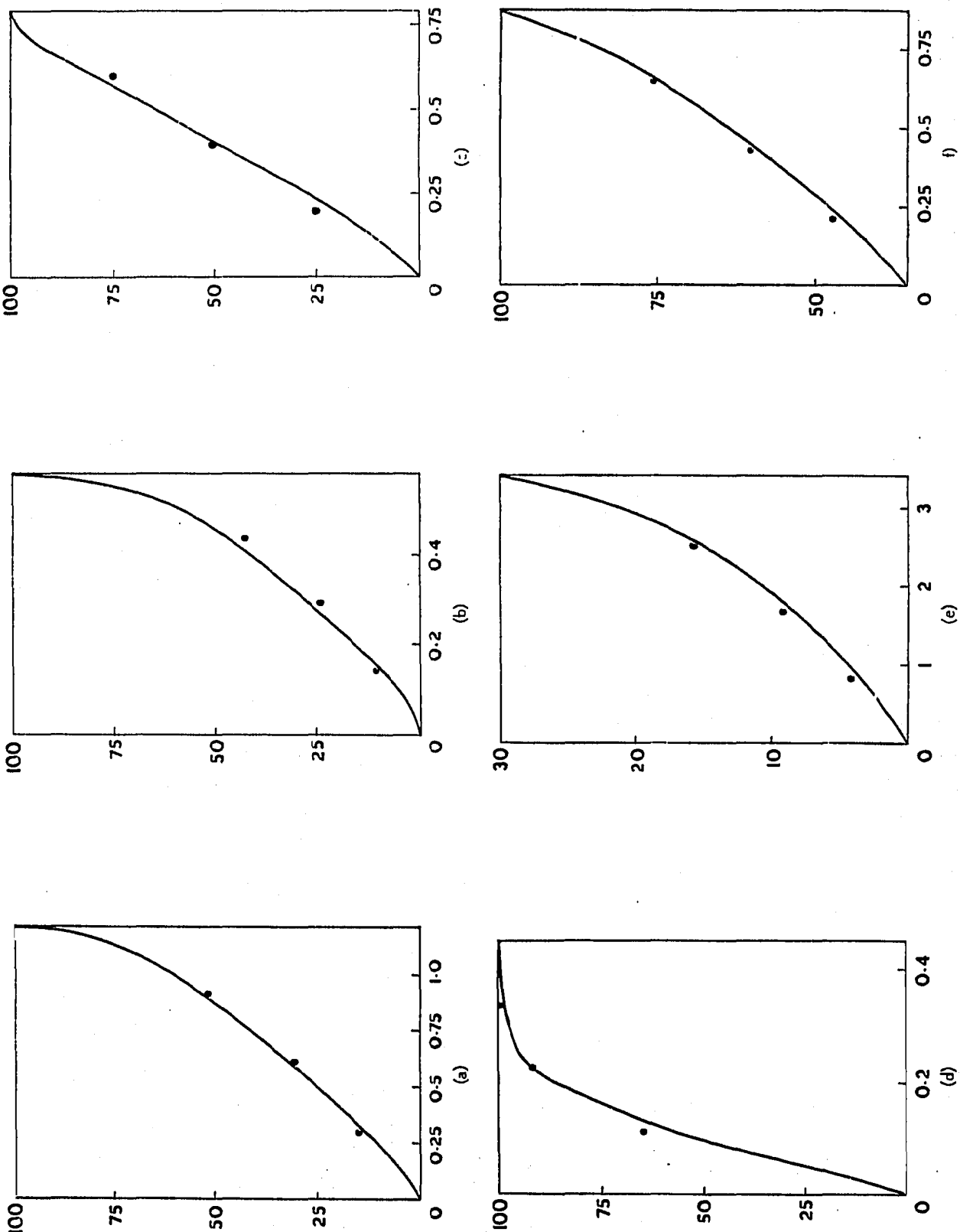

Fig. 5. Gradients produced by the method described. Ordinates: % (v/v) of more powerful eluent. Abscissae: volume (l) delivered to column. Points (●) calculated from d_R, d_M, ρ_R, ρ_M. Gradient (b) was determined ($D_{600\ m\mu}$) as Sudan Black, which was dissolved (10 μg/ml) in the ethanol; the remainder were determined by refractive index measurements. (a) Concave, 0–100 % diethyl ether in petroleum ether. Reservoir: 16 oz. reagent bottle (d_R = 75 mm). Mixing chamber: Mallinckrodt silicic acid bottle (d_M = 100 mm). (b) Concave, 0–100 % ethanol in diethyl ether. Reservoir: wash bottle (d_R = 50 mm). Mixing chamber: reagent bottle (d_M = 75 mm). (c) Linear, 0–100 % chloroform in petroleum ether. Reservoir: Winchester bottle (d_R = 121 mm). Mixing chamber: reagent bottle (d_M = 80 mm). (d) Convex, 0–100 % chloroform in petroleum ether. Reservoir: 1 l bottle (d_R = 96 mm). Mixing chamber: chromatographic column tube (d_M = 34 mm). (e) Concave, 0–30 % methanol in chloroform. (Diameter ratio computed in Fig. 2 and apparatus illustrated in Fig. 3.) Reservoir: 2 l measuring cylinder (d_R = 79 mm). Mixing chamber: Winchester bottle (d_M = 116 mm). (f) Concave, 35–100 % methanol in chloroform. Reservoir: wash bottle (d_R = 64 mm). Mixing chamber: 1 l bottle (d_M = 96 mm).

RESULTS AND DISCUSSION

A selection of gradients that have been produced by this method and measured is shown in Fig. 5. Clearly the method is applicable for linear, convex, and concave gradients, and whether the more or the less polar eluent is the denser. Since the basis of the calculations is empirical and since the glassware used did not have strictly regular geometry, the closeness of the calculated points to the observed curves in Fig. 5 is gratifying; and for most column chromatographic work it is fully adequate. For quantitative work with micro columns the use of tubes of accurately measured dimensions, instead of bottles etc., would probably be justified.

It is interesting that BILLIMORIA *et al.*[17] have already proved that a linear gradient of methanol in chloroform was produced when the condition $\rho_M d_R^2 = \rho_R d_M^2$ was satisfied. BADER AND MORGAN[18] recently found that equation (2) predicted very well a concave gradient of methanol in chloroform. However, they did not apparently realize its more general implications. KOČENT[10] has already proposed nomograms for gradient elution but their application to eluents of unequal density would be very laborious. His equation differs from equation (2) and predicts less accurately, seemingly[20] because he neglected a density term in the development.

The concave gradients shown in Fig. 5 are all more concave after the half-way mark than before. This situation can be reversed if the cylindrical reservoir is replaced by an upright conical flask. Such gradients, like that shown in Fig. 6 (see also ref. 4),

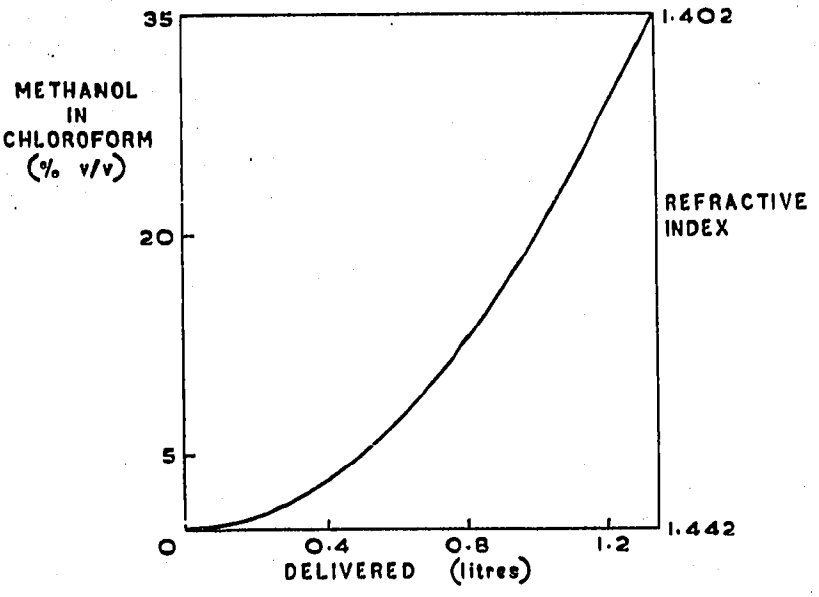

Fig. 6. Concave gradient, 0–35% methanol in chloroform. Reservoir: 0.5 l *conical* flask (maximum d_R = 95 mm). Mixing chamber: 1 l cylindrical bottle (d_M = 96 mm).

are needed when the mixture to be chromatographed contains a preponderance of compounds that are eluted by low concentrations of the more polar eluent[21]. In other circumstances upright or inverted conical flasks may find use as reservoirs or as mixing chambers.

Gradient elution as advocated in this paper has fully proved its value for silicic acid chromatography of washed total lipid extracts, although stepwise elution is

still often preferable[21] for rechromatography of isolated fractions. It must be emphasized that during gradient elution the concentration of the more powerful eluent in the effluent at any instant is often very much less than that in the eluent entering the top of a column. With certain reservations, the ultimate factor determining whether or not a compound is eluted is the concentration in the effluent. Thus the gradient shown in Fig. 6, which ended at 35 % methanol, did not elute lecithins from a column containing 45 g of silicic acid although these compounds can be eluted with 20 % methanol[2].

In adsorption chromatography the concentration in the effluent normally lags behind that in the eluent by more than the column volume because, in order to maintain adsorption equilibrium, the solid phase is continually attaching more of the more polar eluent. *Continuous* behaviour of this kind is unobjectionable, and may indeed be vital to separation processes. But if before gradient elution is begun the solid phase contains none of the more polar eluent, none will appear in the effluent until the whole column has reached equilibrium. (Adsorbents such as silicic acid and alumina are well known for their ability to remove polar solutes from non-polar solvents until they become saturated.) Then there will be a *discontinuous*, steep rise in concentration, very likely causing the elution of several compounds in a single, sharp peak. It is therefore a good practice to prepare columns using a 50:50 mixture of the eluents and wash them with 1 column volume of the less polar eluent before use.

SUMMARY

Problems of applying continuous gradient elution to the adsorption chromatography of lipids and other lipophilic compounds are discussed. A simple and versatile method for producing continuous concentration gradients from pairs of eluents of unequal density is described. The method makes use of a nomogram and common pieces of laboratory glassware, such as bottles, measuring cylinders, and chromatographic column tubes. It has been found satisfactory for linear, convex, and concave gradients whichever eluent is the denser.

REFERENCES

1 E. HEFTMANN, *Chromatography*, Reinhold, New York, 1961.
2 J. J. WREN, *J. Chromatog.*, 4 (1960) 173; *Chromatographic Reviews*, Vol. 3, Elsevier, Amsterdam, 1961, pp. 111, 177.
3 O. MIKEŠ, *Chem. Listy*, 54 (1960) 576.
4 J. J. WREN, *Nature*, 184 (1959) 816.
5 T. K. LAKSHMANAN AND S. LIEBERMAN, *Arch. Biochem. Biophys.*, 53 (1954) 258.
6 H. K. MITCHELL, M. GORDON AND F. A. HASKINS, *J. Biol. Chem.*, 180 (1949) 1071.
7 P. VESTERGAARD, *J. Chromatog.*, 3 (1960) 560.
8 D. F. H. WALLACH, J. SODERBERG AND L. BRICKER, *Cancer Res.*, 20 (1960) 397.
9 G. J. NELSON, *J. Lipid Res.*, 3 (1962) 256.
10 C. W. PARR, *Biochem. J.*, 56 (1954) xxvii.
11 R. M. BOCK AND N.-S. LING, *Anal. Chem.*, 26 (1954) 1543.
12 B. DRAKE, *Arkiv Kemi*, 8 (1955) 1.
13 P. LEBRETON, *Bull. Soc. Chim. France*, (1960) 2188.
14 R. M. BOCK, quoted by C. DE DUVE, J. BERTHET AND H. BEAUFAY, *Progr. Biophys. Biophys. Chem.*, 9 (1959) 325.
15 G. L. CHOULES, *Anal. Biochem.*, 3 (1962) 236.
16 A. C. ARCUS, *J. Chromatog.*, 3 (1960) 411.
17 J. D. BILLIMORIA, R. G. CURTIS AND N. F. MACLAGAN, *Biochem. J.*, 78 (1961) 185.
18 H. BADER AND H. E. MORGAN, *Biochim. Biophys. Acta*, 57 (1962) 562.
19 A. KOČENT, *J. Chromatog.*, 6 (1961) 324.
20 G. WHITE AND J. J. WREN, unpublished.
21 J. J. WREN, D. S. HOLUB, A. D. SZCZEPANOWSKA AND W. STERN, in preparation.