A simple device for making a concave concentration gradient for general use in column chromatography

ALM, WILLIAMS AND TISELIUS[1] pioneered the use of concentration gradients for the separation of solutes on chromatographic columns. They deduced on the basis of adsorption isotherms that sharpest peaks and optimal separation would generally be obtained by the use of a gradient in which the concentration of the de-sorbing solvent increases in more than linear proportion, *i.e.* one whose plot of concentration ordinate against volume is concave. Such concave gradients have been widely used, and may be made by a variety of methods[2-9], some simple and some requiring complex apparatus. As WREN[9] points out, many of these methods assume equality of density between the dilute and concentrated solvents; he alone takes account of the more usual circumstance where one solvent is denser than the other, and he has improved the theory to take this into account. The device here to be described is simple to assemble, and has characteristics which do not alter for a given pair of vessels whatever solvents may be used. General accounts of gradients elution systems have often been given[3,4,6,10], but the special features of this system made it seem worthwhile to describe it.

The device consists of a conical reservoir standing on its base, filled with concentrated solvent, and feeding through a floating siphon[11] a stirred cylindrical mixing
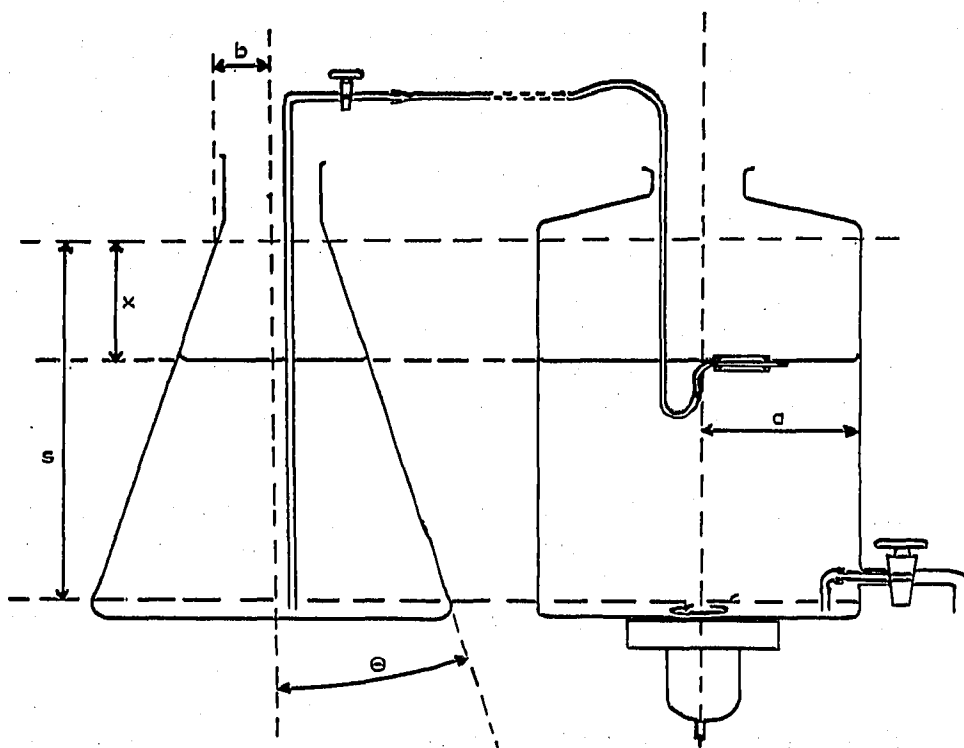

Fig. 1. Apparatus for constructing a concave elution gradient. The conical flask contains concentrated eluent which siphons over into the stirred cylindrical aspirator as outflow proceeds through the tap. The siphon floats at the surface of the mixed solvent, thus compensating for density differences between the liquids; a measured maximum difference in levels of about 2 mm occurred in practice with flow rates of 250 ml per h with a siphon of 3 mm bore. Only a very small difference in levels is required for the siphon to flow. The dimensions are those referred to in the theoretical discussion.

vessel, which is of similar height to the cone, and stands beside it (Fig. 1). The chromatography column is fed from the base of the cylinder. Standard "Pyrex" glassware was used (J. A. Jobling, Ltd., Sunderland, England), conical flasks and aspirator bottles being available with dimensions suitable for a wide range of assembly sizes and volumes. The siphon consists of a flexible tube about 1–3 mm bore depending upon the scale of the assembly. One end of the tube may conveniently be attached to a glass tube which dips to the base of the conical reservoir; the other end is made to float at the surface of the liquid in the mixing vessel. Suitable floats may be made simply in several ways (Fig. 2). Narrow tubing of sufficient flexibility is obtainable from Portland Plastics Ltd., Hythe, Kent, England (type 4) or from Precision Rubbers Ltd., Bagworth, Leicester, England (grade B. 502).
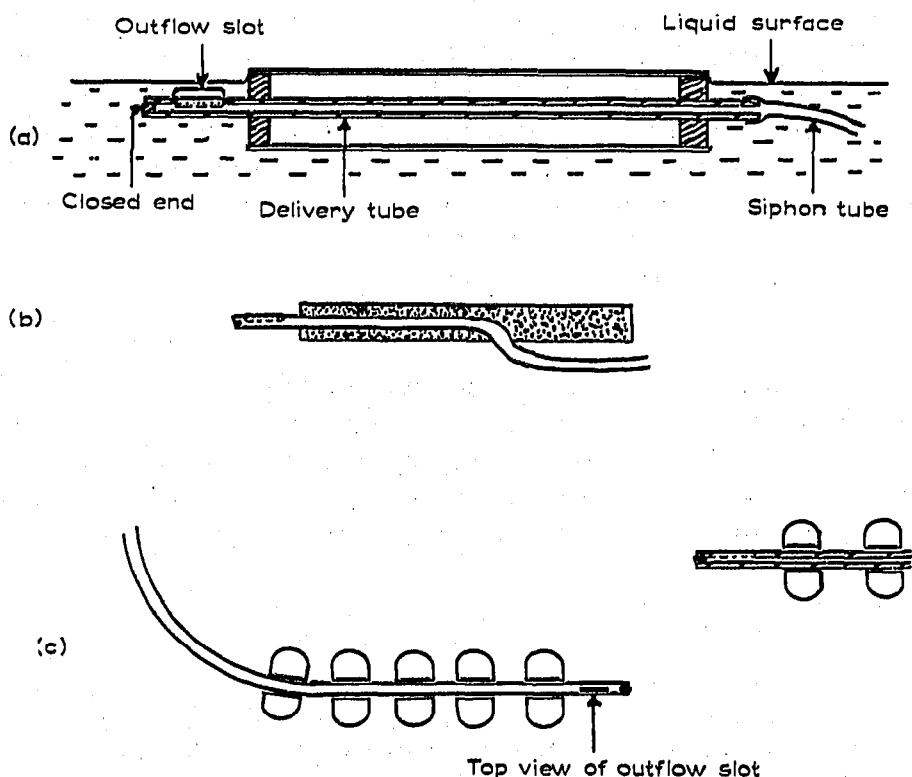

Fig. 2. Construction of floating siphon. The delivery tube may be made from polythene tubing, approximately 2.5 mm bore, 3.5 mm O.D. The flexible siphon tube is attached to the proximal end. The distal end, through which the concentrated eluent is delivered, has proved to function best if the eluent flows from a slot about 3 mm long, 0.5 mm wide cut along the top of the tube. The open bore is then plugged. This device results in an upward outflow which prevents blockage by air bubbles. Floats: (a) The delivery tube is surrounded by about 6 cm of thin-walled polythene tubing of 10 mm I.D., its ends sealed with rubber rings cut from tubing of 10 mm O.D., 3 mm bore. (b) A thin disc of expanded polymer or cork, about 5 × 0.5 cm through the thickness of which the delivery tube is threaded. (c) Hollow glass beads, advantageous for organic solvents.

Automatic adjustment of the levels of the two liquids is now ensured, however disparate their respective densities. Providing the rate of efflux to the column does not exceed the capabilities of the siphon tube, the system will reproduce the same gradient with any two liquids. The characteristics of the gradient may be determined by a trial run using water in the cylinder and either acid or a coloured substance in the cone, sampling at several stages of the measured effluent.

Details of technique

Vessels of such a volume are chosen that the total volume of the system is used for the chromatogram. The narrowest part of the cone is mounted level with the top of the cylinder, and the vessels are filled to this level, which is then marked on each vessel for future reference. In a similar way, the lowest convenient level of the system is marked on each vessel using a spirit level. The gradient will comprise the combined volume in both vessels between the initial and final levels.

It is an advantage to de-gas the concentrated solvent at a suction pump to avoid the blockage by air bubbles of the siphon tube. The siphon tube is then filled, the stopcock permitting easy handling of the filled siphon, and the float inserted into the mixing vessel whose liquid vessel is adjusted, and the elution may commence. With 2 mm bore siphon tubing a flow rate of 300 ml per h has been attained without overloading the siphon delivery. If desired the apparatus may be operated at increased pressure by equal application to both vessels of the required amount. The system has proved successful in chromatography of nucleotides and crude protein extracts, giving sharp peaks without tailing. By varying the initial and final levels subtle variations of the gradient are produced, while inverting the cone, or varying the shape of either vessel allows an astonishing variety of gradient forms to be produced. A full account of these with their complete theoretical analysis is being prepared for submission to a mathematical journal.

Theory

This type of system is complicated to analyse, since as outflow proceeds a varying proportion of concentrated eluent is drawn into the mixing vessel, whose contents are progressively diminishing in volume. An elementary analysis by the "mass-conservation" principle has been made. The function which defines effluent concentration C in terms of the concentration B of the eluent in the reservoir is:

$$\frac{C}{B} = 1 - \exp\left[\frac{\tan^2\theta}{a^2}\left\{\left(\frac{b}{\tan\theta} + s\right)^2 \log\frac{s-x}{s} + \frac{x^2}{2} + xs + \frac{2bx}{\tan\theta}\right\}\right] \tag{1}$$

where

C = concentration of effluent when the surface is x cm below the initial level, defining the initial contents of the mixing vessel as having concentration zero;

s = total depth from initial to final liquid levels;

θ = cone angle;

a = radius of cylinder;

b = radius of cone at initial solvent level (see Fig. 1).

Expression (1) gives the relation between effluent concentration and the distance x travelled by the liquid level; there is no simple relationship between the volume of flow and concentration. Volume of flow V is related to x by the expression:

$$V = \frac{\pi x}{3}\{3a^2 + 3b^2 + 3bx\tan\theta + x^2\tan^2\theta\} \tag{2}$$

Values of C/B from expression (1) were computed for 150 values of x in a system of measured dimensions. The curve of x against V was constructed manually from

expression (2) and hence the volumes of flow corresponding to each value of x could be read off, and the concentration gradient for the particular system was drawn.

The experimental gradient was determined by titration in a system of hydrochloric acid and water. Good agreement between theory and experiment was obtained Fig. 3).
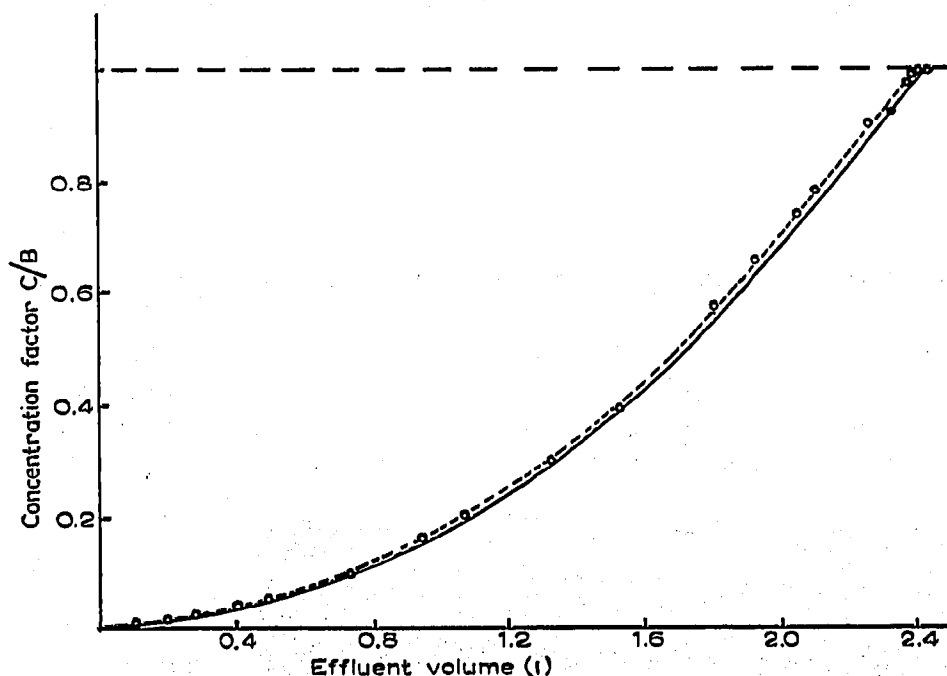

Fig. 3. Theoretical and experimental gradients. Solid line: curve computed using the exponential function (1). Broken line with points: curve as determined by titration of acid appearing in effluent during gradient formation in the following system: conical reservoir, 1.027 N HCl, nominal size ot cone 1 l; mixing chamber, nominal size 2 l, initially filled with water. Total volume of gradient (*i.e.* volume between initial and final liquid levels): 2454 ml.

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An improved spray reagent for detecting lipids on thin-layer chromatograms

During the separations of lipids on thin-layer plates using silica gel a number of reagent sprays have been examined, both destructive (sulphuric acid, chromic acid, phosphoric acid, iodine) and non-destructive (Rhodamine B, 2',7'-dichlorofluorescein, 2',7'-dibromofluorescein, bromthymol blue, water). As the analysis of fatty acids of
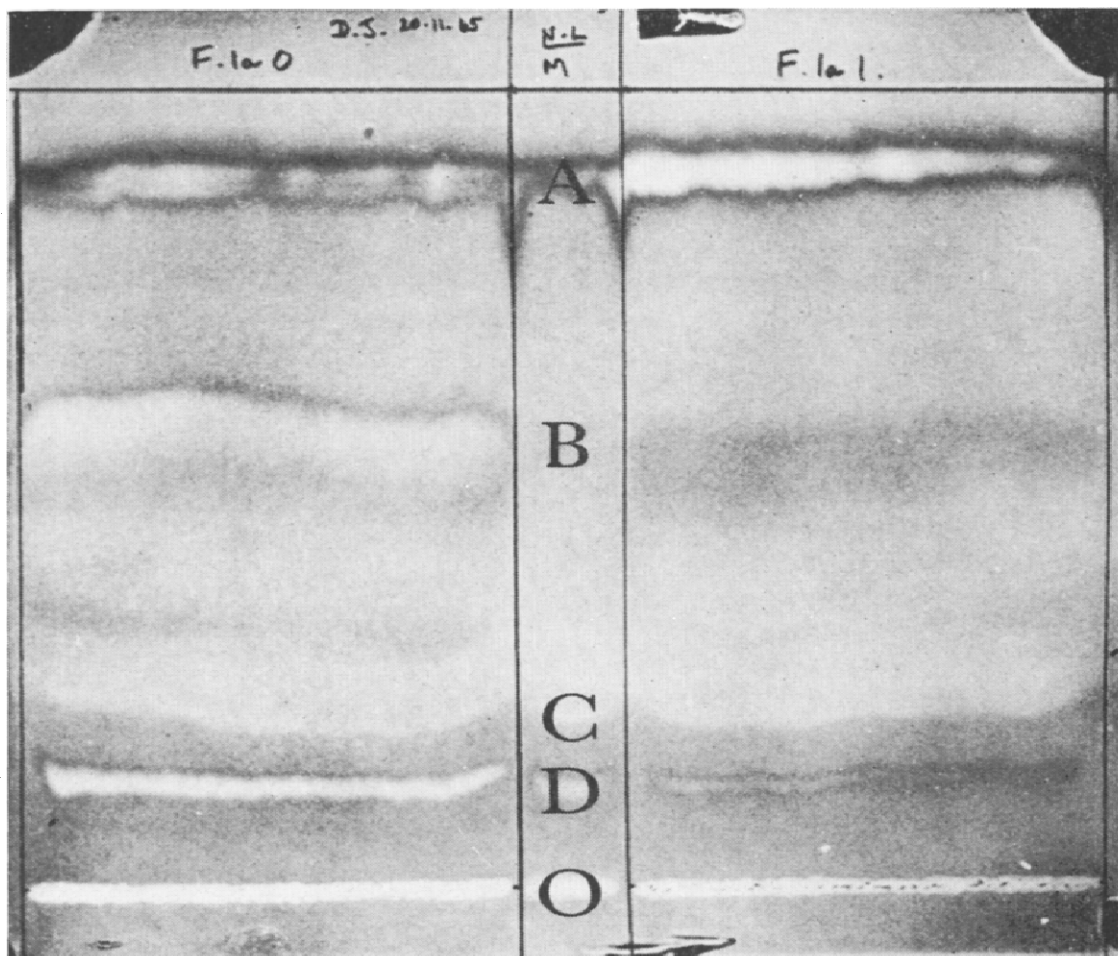

Fig. 1. Neutral lipids of chicken liver. A = Cholesterol esters; B = triglycerides; C = free fatty acids; D = cholesterol; O = origin (containing phospholipids).