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1 A. CARISANO AND F. CARRÀ, *Ind. Conserve (Parma)*, 38 (1963) 28.
2 W. M. LAMKIN AND C. W. GEHRKE, *Anal. Chem.*, 37 (1965) 383.
3 *Analytical Methods for the Soup Industry*, Association of Swiss Soup Manufacturers, Berne, 1961, 1/5.
4 A. CARISANO AND F. CARRÀ, *Biochem. J.*, 81 (1961) 98.

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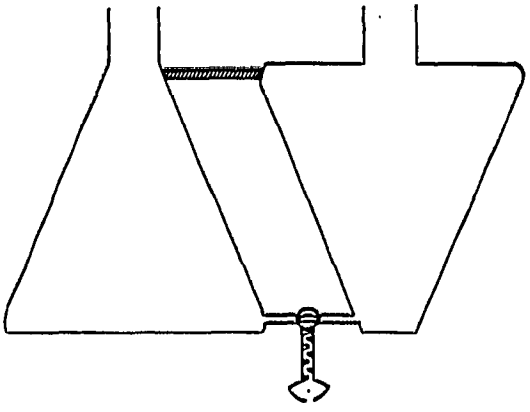
A simple device for making a gradient for chromatography

A number of methods to produce a gradient for use in column chromatography have been described[1–4]. This paper describes the theory and application of a simple inexpensive apparatus* for producing such a gradient. Two similar conical chambers, such as 1000 ml Erlenmeyer flasks, one inverted and one upright, side by side, are joined by glass tubing with a three-way stopcock at their lowest point, from which they feed a chromatography column (see Fig. 1). A gradient may be produced by filling these chambers with solutions of different compositions. When these chambers are full the major contribution to the column will come from the inverted chamber but as they empty this contribution will lessen and the contribution of the upright chamber will increase. The ratio of the contributions of these chambers at any one time is equal to the ratio of the squares of their radii at the level of the top of the solutions at that time. Although the gradient produced by such an arrangement is not quite linear but is slightly sigmoid, its approach to linearity is sufficient for most purposes (see Fig. 2).

The characteristics of each cone of which the two flasks are frustums (segments of cones not including the apices) should be known to properly plan the gradient. The $\Delta r/\Delta h$, where r is the radius of the cone and h is the height of the cone, is needed and is found by R/H, where R is the radius of the base of the cone and H is the total height of the cone. The following method will give R and H.

The lowest portion of the apparatus that is conical should be established by filling the flasks to this level while freely connected to each other and this level well marked and measured from the top or bottom of each flasks as a point of reference. A note of caution should be added at this point. The shape of the flasks, by producing an optical illusion and by the optical effect of the glass, makes the level in the two flasks appear to be different even though the levels are the same. This effect

* Obtainable from Tudor Scientific Glass Co., Belvedere, S.C.



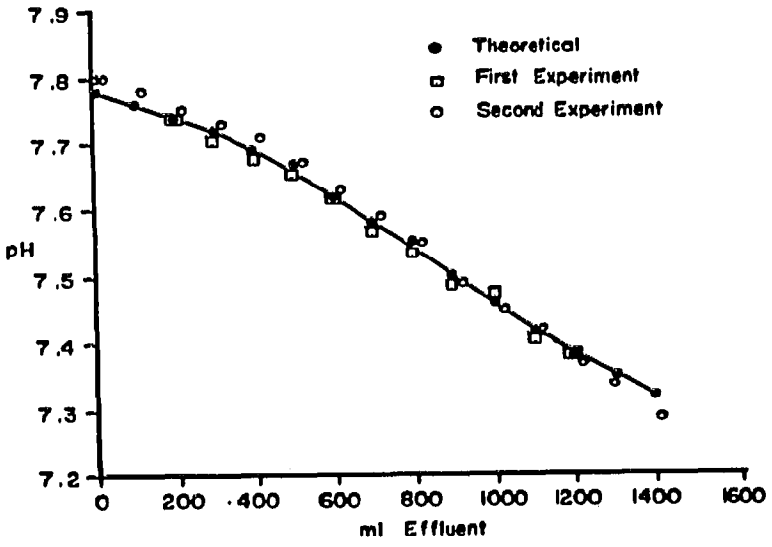


Fig. 1. A reciprocal cone apparatus for making a chromatography gradient.

Fig. 2. Graph of the pH gradient made by the reciprocal cone apparatus using Tris-HCl buffers, 0.05 *M*.

can be quite strong and must be guarded against by checking the levels when there is free connection between the flasks. After closing the flasks to each other, aliquots of water are added to each flask and the rise in water level measured. This is done at least twice but it is felt advisable to obtain more values if possible. These additional values allow additional calculations which can be used to check the consistency of the values obtained and can give some idea of whether or not the $\Delta r/\Delta h$ is constant throughout the flask, as it should be.

v, the volume of a frustum, is found as follows:

$$v = 1/3\,\pi h(r_1^2 + r_1 r_2 + r_2^2) \tag{1}$$

In this equation v is volume, h is height of the frustum, r_1 is the radius of one of the planes of the frustum and r_2 is the radius of the other plane of the frustum. Frequently, one of these will be R and will be presented as such. From this equation, eqn. 2 is derived.

$$R^2 = \frac{\frac{3v}{\pi h}}{3 - \frac{3h}{H} + \frac{h^2}{H^2}} \tag{2}$$

If two frustums have R in common, R^2 is the same for both and the right hand side of eqn. 2 of one frustum equals the right hand side of eqn. 2 of the other frustum and such an equation contains only one unknown, H, which can be found by solving the equation. Then using this value, H, eqn. 2 can be solved for R; thus $\Delta r/\Delta h$ can be calculated.

Another characteristic that is needed is the equiradial level, *i.e.*, the level at which the radii of the two flasks are equal. Equation 3 will give this value.

$$R_{up} - h_{equ} \cdot \frac{R_{up}}{H_{up}} = r_{inv} + \frac{R_{inv}}{H_{inv}} \cdot h_{equ} \tag{3}$$

R_{up} is R of the upright flask, h_{equ} is the height of the equiradial level above the base of the upright flask, H_{up} is H of the upright flask, r_{inv} is the radius of the inverted flask at the R_{up} level, R_{inv} is R of the inverted flask and H_{inv} is H of the inverted flask. Knowing the height of the equiradial level, the radii at this level can be easily calculated.

Since the height of the usable frustums above and below the equiradial level can be easily calculated, the usable volumes above and below the equiradial level can be easily calculated. It is convenient to make a graph of the height above and below the equiradial level *versus* the total volume of the apparatus above and below the equiradial level. In this way the height for a particular volume can be quickly obtained.

Of the different possible uses for this device perhaps the most complex calculations are found in the use of this to make a pH gradient and these calculations will be given to illustrate the use of the apparatus. Since the graph of the gradient is slightly sigmoid shaped, for the nearest approach to a linear gradient the portions of the flasks nearest the equiradial level should be used if one is working around pK of the buffer. If it is desired to have a slightly flat portion of the gradient at the beginning or the end, then the gradient should be calculated to use the maximum or minimum volumes of the apparatus, respectively. Also, working above the pK of a buffer accentuates the slightly flat end of the gradient and working below the pK of a buffer accentuates the slightly flat beginning of the gradient (see Fig. 2).

The values needed to calculate a gradient are the beginning pH, the end pH, the buffer molarity and the volume of the gradient. If the $\Delta r/\Delta h$ of the two cones are similar then it is convenient to assign the midpoint of pH gradient to the equiradial level where both cones are contributing equally. From the graph of Δ total volume *versus* Δh above the equiradial level, the height of the beginning of the gradient is found and from this value the r^2's and thus the ratio of the contributions of the two flasks at the beginning of the gradient calculated (eqn. 2 will give r's which can be squared to give ratio of the contributions of the two flasks).

From the Henderson-Hasselbach equation, the ratio of the salt and acid or base needed at the beginning of the pH gradient and at the equiradial level (or at end of gradient) can be calculated. Since two known pH's with two known ratios of salt and acid or base are wanted, two equations like the following can be set up.

$$\text{Salt/acid or base/salt} = \frac{r_{up}^2(X_{up}) + r_{inv}^2(X_{inv})}{r_{up}^2(\text{molarity of buffer}-X_{up}) + r_{inv}^2(\text{molarity of buffer}-X_{inv})} \quad (4)$$

X_{up} is the molarity of the salt (with a salt–acid buffer) or base (with a base–salt buffer) in the upright flask and is an unknown value, and X_{inv} is the molarity of the salt (in a salt–acid buffer) or the base (in a base–salt buffer) in the inverted flasks, and r_{up} and r_{inv} are the radii at the top of the buffer in the upright and inverted flasks, respectively. These two equations can be solved to give the ratio of base/salt or salt/acid in each flask and thus the pH in these flasks can be easily calculated by the Henderson-Hasselbach equation.

Fig. 2 shows the results of two trial gradients produced by the apparatus described. 0.05 M Tris–HCl buffers—pK_a 8.08 (ref. 5)—were used and it can be seen that

there is good agreement between the values found and the expected values. There are several aspects that should be noted.

One aspect of importance is the size of the tube at the lower portions of the flasks. The size of this tube should depend upon the anticipated speed of drainage of the flasks. If the tube is small and the drainage of the flasks is rapid then there is a relatively large drop in pressure across this tube and since the buffer levels in the flasks are the same, and thus the pressure at the lower portions of the flasks are the same, the flow from the two flasks tends to be proportional to this pressure and thus equal, and not proportional to the radii at the top of the buffer level. With a small bore drainage tube, the flow has to be slow enough so that the pressure drop across the drainage tube approaches zero. On the other hand, if very slow flow rates are anticipated and very large bore drainage tubes are used, it is conceivable that diffusion in the drainage tube might cause problems. The author has encountered difficulty of the first type mentioned. Using 5 mm I.D. bore tubing for the T tube about 40 ml per hour could be drained with a good gradient.

As might be anticipated, adequate mixing of the buffers is necessary. The author has not encountered difficulty with mixing using only the nipple indentations to encourage mixing and small bore (1 mm I.D.) plastic tubing of 1.5–2.0 m length leading to the columns. However, inadequate mixing of the buffers is one area in which difficulty might be encountered.

From experience it has been found advisable when filling the flasks to fill them above the level desired and to open the stopcock and discard the excess buffer before starting the gradient. This allows the buffers to become level by hydrostatic pressure. It is exceedingly difficult to get them exactly level by measurement and if they are not exactly level the beginning of the gradient is not the proper pH.

Since all methods should have a convenient name, it is proposed to refer to this method and apparatus as the reciprocal cone method and apparatus, respectively.

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1 R. S. ALM, R. J. P. WILLIAMS AND A. TISELIUS, *Acta Chem. Scand.*, 6 (1952) 826.
2 R. M. BOCK AND N. S. LING, *Anal. Chem.*, 26 (1954) 1543.
3 J. C. HEGENAUER, K. D. TARTOF AND G. W. NACE, *Anal. Biochem.*, 13 (1965) 6.
4 E. A. PETERSON AND H. A. SOBER, *Anal. Chem.*, 31 (1959) 857.
5 J. T. EDSALL AND J. WYMAN, *Biophysical Chemistry*, Vol. 1, Academic Press, New York, 1958, p. 452.

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