

GRADIENT ELUTION IN COLUMN CHROMATOGRAPHY

THE USE OF CONTINUOUS LINEAR GRADIENTS TO APPROXIMATE CURVED GRADIENTS BY MEANS OF LAYERED SOLUTIONS*

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The theory of gradient elution and methods to establish many different shapes of gradients have been described^{1,2}. One of the simplest devices for establishing gradients is composed of two cylindrical containers connected at the base (Fig. 1).

As illustrated, the cylinders contain solutions of concentrations A and B . The left-hand cylinder is the mixing chamber, from which the eluant flows into the column. If the cross-sectional areas of the cylinders $A_2 > A_1$, the shape of the gradient will be convex upwards, if $A_1 > A_2$, concave upwards, and if $A_1 = A_2$, linear, with the initial concentration of eluant = A and final concentration = B . These relationships are given in the following equation²:

$$E = B - (B - A) (1 - V)^{A_1/A_2} \quad (1)$$

where:

E = concentration of eluant,

B = concentration of solution in right-hand cylinder,

A = concentration of solution in left-hand cylinder,

V = fraction of *total* original volume eluted from apparatus,

A_2 = cross-sectional area of right-hand cylinder,

A_1 = cross-sectional area of left-hand cylinder.

When compounds of a similar nature are to be separated on a column using a linear gradient, emergence at the bottom of the column is often of an exponential type, *i.e.* initial eluate peaks are quite close together and final eluates are increasingly far apart. It often becomes necessary to arrange an exponential gradient to achieve a linear separation. If, in such a separation, several peaks appear close together, a change in the shape or slope of the elution curve becomes necessary: a change that will affect all of the eluted peaks in a way that does not necessarily represent an improvement.

At present, an automatic device is available which will effect changes in one portion of an elution curve without unduly distorting the remainder³. Such devices are relatively complex and expensive. The following will illustrate a somewhat

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simpler means of producing the same effect. This method takes advantage of certain facts:

(A) When $A_2 = A_1$ in eqn. 1, the exponential term disappears, making calculations quite simple.

(B) It is possible to layer solutions of different density one on top of the other in order of decreasing density.

(C) When such layers are established with sharp interfaces, the boundaries and density differences act as diffusion barriers.

(D) It is possible to establish differences by varying the concentration of the non-interfering substance X, and once a series of layers is established, to vary the concentration of the eluting substance Y (or of several substances), independently.

Thus, by using cylinders of identical cross-sectional area and establishing a series of density layers, one may elute continuously, following a series of continuous linear gradients.

EXPERIMENTAL

Density differences were accomplished by making solutions of sucrose (9%, 6% and 3%). The concentration of eluting substance was approximated by methylene blue in sucrose of different optical densities.

Layers were established in cylinder B as follows: 400 ml of 9% sucrose were poured into cylinder B. A cylindrical polyethylene float, of $\frac{1}{4}$ in. less internal diameter than cylinder B and 1 in. thick, was connected to a separatory funnel by means of flexible tubing, perforated just above the float. 400 ml of 6% sucrose were added to the separatory funnel, and then allowed to flow slowly into cylinder B. When the addition was complete, a length of tubing was lowered to the 300 ml marker, and fluid was aspirated until a glassy interface could be seen. This process was then repeated with the top layer. Cylinder A was filled to the same depth as B, the two cylinders were connected together, the magnetic mixer was started under A, and the resulting "eluant" was led into a Packard fraction collector set to collect fractions of approximately 10 ml, using a drop counter.

Fractions were collected in 18×150 mm culture tubes, and the optical density at $650 m\mu$ was read directly on the tube in a Coleman Junior spectrophotometer.

RESULTS

The calculated and observed results of three experiments are shown in Tables I-III.

CONCLUSIONS

In general we believe that the theoretical approach to this problem is verified by the experiments performed. Two types of deviations can be observed:

(1) Scatter in the optical density readings. This we believe to be due to the scatter in total diameter and wall thickness of the culture tubes used.

(2) Approach to, but not reaching calculated limits. This deviation we believe to be due to the large hold up volume of the rubber tubing used to connect the two

TABLE I

EXPERIMENT 1

Left-hand cylinder: 900 ml distilled water, optical density (OD) 0.

<i>Right-hand cylinder</i>	<i>Slope (OD)</i>	
	<i>Calculated*</i>	<i>Observed</i>
Layer 1: 300 ml 9% sucrose, OD 0.60	0-0.20**	0-0.20
Layer 2: 300 ml 6% sucrose, OD 0.23	0.20-0.22	0.20-0.23
Layer 3: 300 ml 3% sucrose, OD 0.84	0.22-0.84	0.23-0.77

* Sample calculation: $E = 0.60 - (0.60-0) (1-1/3)$
 $0.60 - 0.40$
 $0.20.$

** First linear slope of 600 ml.

TABLE II

EXPERIMENT 2

Left-hand cylinder: 900 ml distilled water, OD 0.

<i>Right-hand cylinder</i>	<i>Slope (OD)</i>	
	<i>Calculated</i>	<i>Observed</i>
Layer 1: 300 ml 9% sucrose, OD 2.40	0-0.80	0-0.71
Layer 2: 300 ml 6% sucrose, OD 0.95	0.80-0.90	0.71-0.80
Layer 3: 300 ml 3% sucrose, OD 0.90	0.90-0.90	0.80-0.80

TABLE III

EXPERIMENT 3

Left-hand cylinder: 900 ml distilled water, OD 0.

<i>Right-hand cylinder</i>	<i>Slope (OD)</i>	
	<i>Calculated</i>	<i>Observed</i>
Layer 1: 300 ml 9% sucrose, OD 1.04	0-0.34	0-0.33
Layer 2: 300 ml 6% sucrose, OD 0	0.34-0.21	0.33-0.24
Layer 3: 300 ml 3% sucrose, OD 1.02	0.21-1.02	0.24-0.95

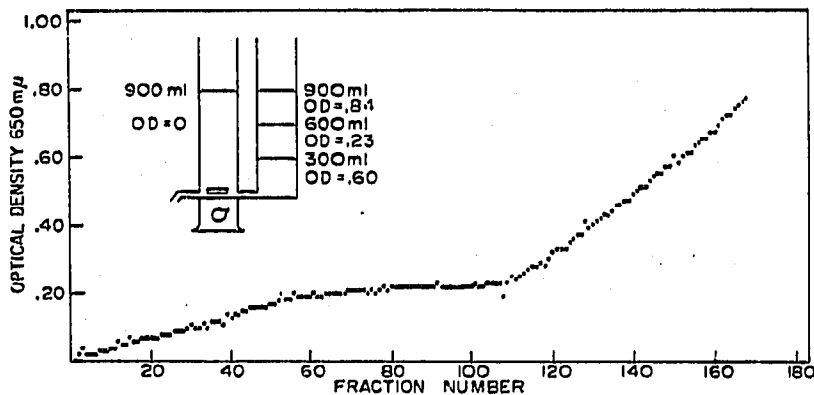
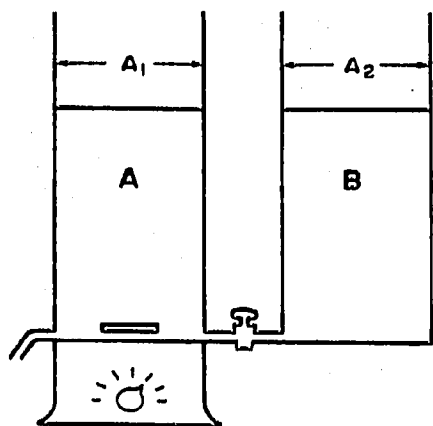


Fig. 1. Diagram of gradient elution apparatus.

Fig. 2. Experiment 1.

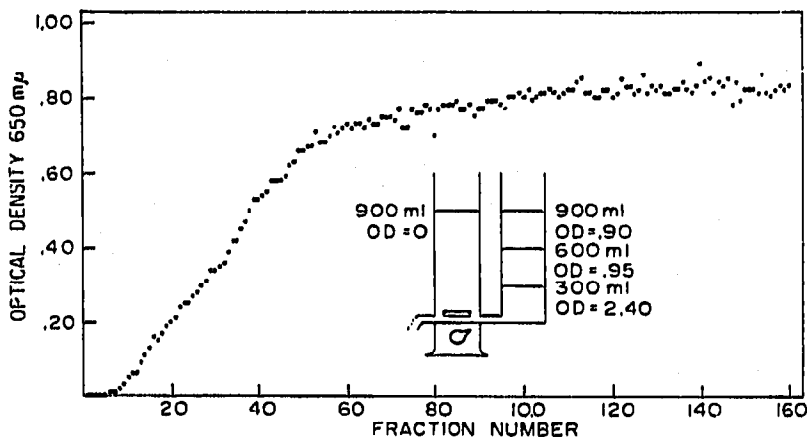


Fig. 3. Experiment 2.

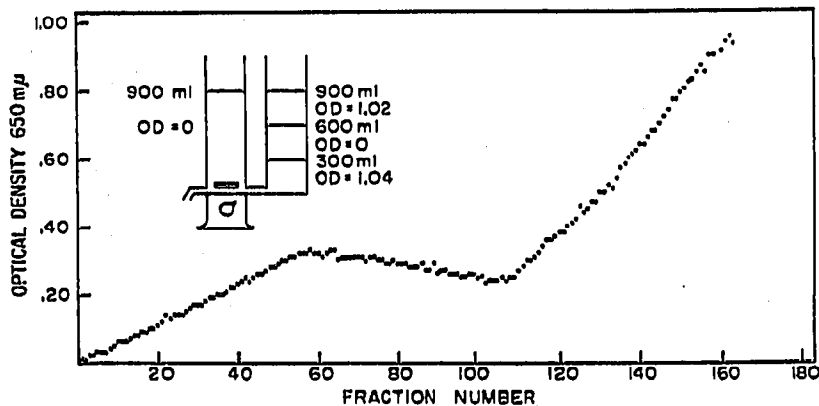


Fig. 4. Experiment 3.

cylinders and to connect the cylinder to the fraction collector, and to the fact that the exit tubes from the cylinders used were not on the bottom of the cylinders, so that the cylinders were not completely emptied.

It has been noted¹ that when solutions of unequal density are used in the two different cylinders, all slopes will be non-linear, since neither the heights, nor the flow rates are the same. However, we have noted very little difference in the heights of the columns of liquid and very little deviation from linearity in the slopes.

The most obvious use of the layering method is where organic solvents are involved, as in silicic acid chromatography. Density differences are often very large and can be utilized to advantage. Moreover, such solvents would tend to attack or erode the plastic containers used in automatic variable gradient machines.

SUMMARY

A method is described and tested experimentally to produce a series of continuous linear gradients for column chromatography. This effect is achieved by layering solutions of different densities on top of each other in a cylindrical glass gradient apparatus.

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