Notes on Methodology

Application of a nomogram to gradient elution chromatography*

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► Gradient elution has been found to be an extremely useful procedure for separating mixtures on chromatographic columns since it provides an infinite number of solvent compositions, each with a different ability to elute the adsorbed materials. Constant volume gradient elution procedures were recently described by Hirsch and Ahrens (1), who showed the effectiveness of this procedure in separating plasma lipids. Although the stepwise change of solvent is useful, and even desirable in some cases (2), it is difficult to use trial-and-error procedures to choose the best solvent compositions. Gradient elution can be used initially to survey mixtures by column chromatography and to identify the solvent compositions at which various compounds are eluted from the column. Once the relationship between the extent of elution of the compound and solvent composition has been determined, it would be possible to predict the proper solvent mixtures for use later in stepwise elution procedures. In order to calculate conveniently the many different solvent compositions produced in constant volume gradient elution systems, a general equation has been derived and represented in the form of a nomogram (Fig. 1).

Assume (1) Two-solvent system, A and B.
(2) Constant volume mixing chamber.

Let X = volume fraction of A leaving mixing chamber.
X_0 = initial volume fraction of A in mixing chamber.
X_R = volume fraction of A in reservoir.
Q = amount of A in mixing chamber.
F = volume that has flowed through the mixing chamber.
V = volume of mixing chamber.

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At any point in the elution, the change in Q will be

$$dQ = VdX$$

And $VdX = X_R dF - XdF = (X_R - X)dF$

Rearranging gives

$$\frac{dX}{X - X_R} = -\frac{dF}{V} \qquad (1)$$

If X_R is constant, integration of (1) gives

$$\ln(X - X_R) = -\frac{F}{V} + C \qquad (2)$$

$$\text{When } F = 0,\ C = \ln(X_0 - X_R) \qquad (3)$$

Therefore,

$$\ln\frac{X - X_R}{(X_0 - X_R)} = -\frac{F}{V}$$

or

$$(X - X_R) = (X_0 - X_R)\, e^{-F/V} \qquad (4)$$

A nomogram representing equation 4 can then be constructed, using the method described by Allcock and Jones (3).

Step 1. Two parallel lines are drawn 20 cm. apart to represent the loci of $(X_0 - X_R)$ and F/V. The locus of $(X_0 - X_R)$ is arbitrarily made 25 cm. long and is graduated linearly from 1.0 to 0.

Step 2. The following equation, $\Delta L_1 = 4e^{F/V} - 4.42$, where ΔL_1 is the linear distance in cm. between $F/V = 0.1$ and F/V, is used to calibrate the F/V locus. The point $F/V = 0.1$ should be directly opposite $(X_0 - X_R) = 1.0$.

Step 3. The third locus $(X - X_R)$ lies obliquely between the two parallel lines. A point lying on the $(X - X_R)$ locus is located by the intersection of two construction lines from corresponding values of F/V and $(X_0 - X_R)$ for any given value of $(X - X_R)$. Two such points determine the $(X - X_R)$ line.

Step 4. A line joining $(X_0 - X_R) = 1.0$ and $F/V = 0.1$ is graduated according to the following formula,

$$\Delta L_2 = \frac{125}{6.25 - \dfrac{1}{(X - X_R)}}$$

Projection lines, parallel to F/V and $(X_0 - X_R)$, are then drawn from these graduations to intersect the $(X - X_R)$ locus. These intersections represent the $(X - X_R)$ values used to calculate ΔL_2.

The separation of egg lecithin and lysolecithin by silicic acid chromatography provides an illustration of the usefulness of the nomogram shown in Figure 1.

The egg lecithin was purified by chromatography on alumina and the lysolecithin was prepared from this purified lecithin by the action of *Crotalus adamanteus* venom. A lipid sample containing 9 and 15 μmoles of lecithin and lysolecithin, respectively, was applied to a 5 g. silicic acid column (1 × 12 cm.) in 10 per cent methanol in chloroform. Either Mallinckrodt or Baker and Adams silicic acid (100 to 200 mesh) was used without any special activation. The column was washed with 16 ml. of 30 per cent methanol in chloroform, which removed a small amount of lecithin (about 0.05 μmoles of phosphorus per ml.). The gradient was started and the eluates (8 to 13 ml.) were collected in tubes on a fraction collector. The mixing chamber volume was 150 ml., while X_O and X_R for chloroform were 0.7 and 0.3, respectively. The solvent composition in each tube was then readily determined from the nomogram, knowing F/V for each tube. Straight lines connecting the point $X_O - X_R = 0.4$ with values of F/V intersect the value of $X - X_R$ related to F/V. In this example, $0.3 + (X - X_R)$ is the fraction of chloroform, and $0.7 - (X - X_R)$ is the fraction of methanol.

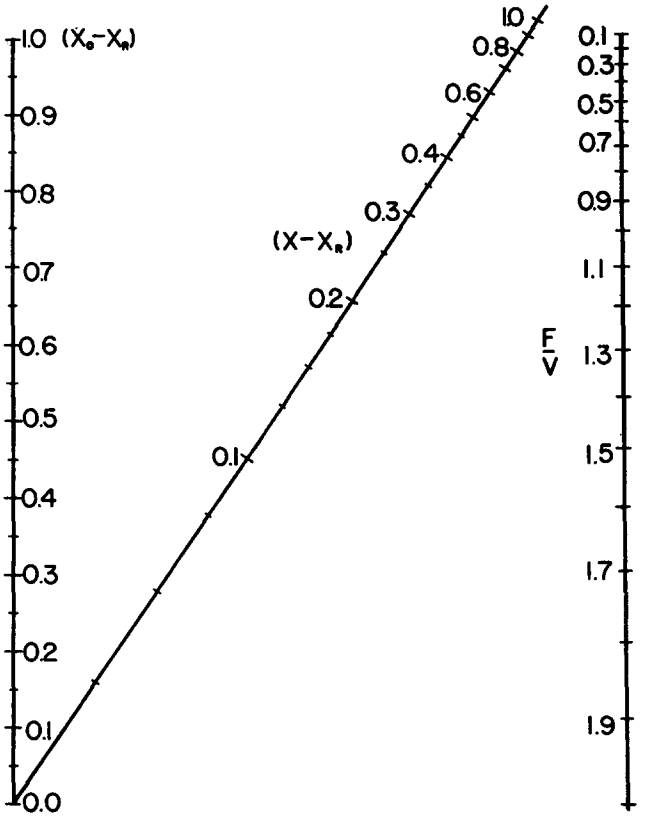

FIG. 1. Nomogram for determining the composition of solvent leaving the mixing chamber in constant volume gradient elution chromatography.

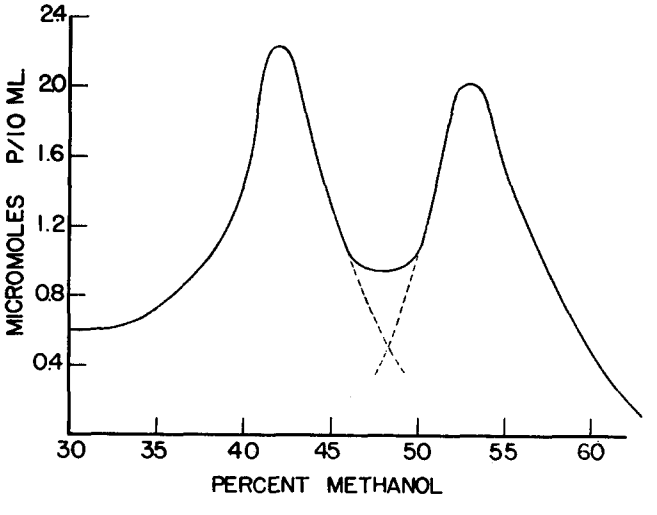

FIG. 2. Gradient elution of lecithin and lysolecithin from a silicic acid column by chloroform-methanol mixtures. The per cent methanol for each tube was calculated from the nomogram.

The total phosphorus concentration in each tube was determined after acid hydrolysis by the method of Fiske and Subbarow (4) and was plotted versus per cent methanol in Figure 2. The first peak is lecithin; the second is lysolecithin. The dashed lines represent the shape the peaks would assume if only one component were present.

The results from the gradient elution (Fig. 2) can now be used to develop a procedure for separating lecithin and lysolecithin using stepwise elution. Lecithin begins to move off the column very rapidly as the methanol concentration reaches 40 per cent, whereas lysolecithin is quite poorly eluted at this solvent composition. The lysolecithin, on the other hand, begins to move rapidly with 51 per cent methanol. A lipid mixture containing lecithin and lysolecithin may be resolved by adsorbing the lipid on the column and

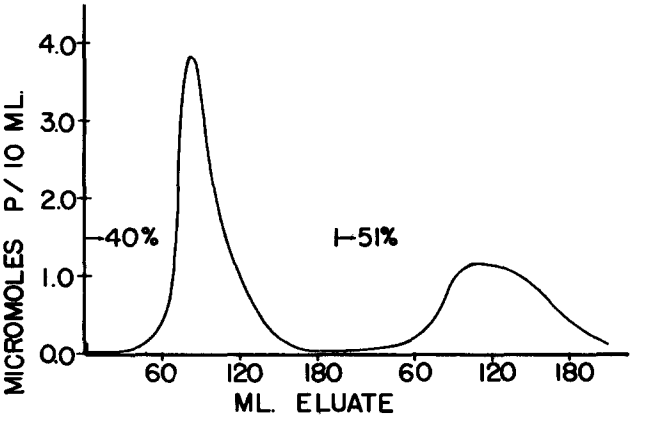

FIG. 3. Stepwise elution of lecithin and lysolecithin from a silicic acid column by chloroform-methanol mixtures.

washing with 40 per cent methanol in chloroform to elute the lecithin. After sufficient washing of the column with 40 per cent methanol, the lysolecithin may then be eluted with 51 per cent methanol in chloroform.

The stepwise elution of 16 μmoles of lecithin and 14 μmoles of lysolecithin is shown in Figure 3. In this experiment the lipids were added to a 5 g. silicic acid column in 10 per cent methanol in chloroform and eluted with 200 ml. of 40 per cent methanol and then 200 ml. of 51 per cent methanol.

In summary: A general equation describing the solvent composition flowing through the column in a constant volume gradient elution system has been developed and made into a nomogram. This nomogram is useful in quickly estimating solvent compositions and predicting procedures for separating components in a mixture by stepwise elution chromatography.

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