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INCREMENTAL GRADIENT ELUTION

SOME FACTORS THAT AFFECT RESOLUTION AND ANALYSIS TIME

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SUMMARY

When using incremental gradient elution with a given column, the volume of the mixing vessel, the solvent period, the column dead volume and the flow-rate condition the resolution and analysis time for the separation of any given mixture.

These factors affect the chromatographic performance due to the fact that they condition both the concentration profile of the solvent entering the column, the solute dispersion and the effective k' values of the solute peaks as they are eluted.

This paper describes results obtained from the investigation of the effect of these parameters and from these results suggests values for the variables that will afford a compromise between adequate resolution and acceptable analysis time. Examples of some separations using these chosen conditions are included.

INTRODUCTION

The apparatus necessary for incremental gradient elution development has been described[1] and a series of twelve solvents chosen on a rational basis for use with the technique has been published[2].

However, the effective use of incremental gradient elution can only be achieved if the correct solvent concentration profile is fed to the column to provide broad chromatographic scope and to allow a mixture containing any and all solute types to be separated.

The factors affecting the solvent concentration that enters the column are the mixing vessel volume, the solvent flow period and the column flow-rate. In this paper the influence of these parameters on resolution and resolution per unit time is experimentally determined for a column of given dimensions using silica gel (Bio-Sil A) as the stationary phase. The conditions are optimized both with respect to maximum resolution and maximum resolution per unit time and the effect of column flow-rate is also examined.

THEORETICAL CONSIDERATIONS

It has been shown[3] that the function that conditions the concentration profile

of the solvents leaving the mixing chamber is of the form $e^{-Qt/V}$, where Q = the flow-rate into and out of the mixing chamber (ml/min); t = the period of flow (min); and V = the volume of the dilution vessel (ml).

The function Qt/V will be given the symbol β and thus it will be the value of β that decides not only the concentration of a specific solvent after a time t but also how the concentration changes during the time t. Now for a given value of β the variables Q, t and V can take a wide variety of values. It follows that, as Q will govern the column efficiency and the product Qt will provide a limiting value for k' at which any solute is eluted by a particular solvent, then the possibility of there being optimum values for both Q and Qt must also be examined as well as the determination of the optimum value for β. As chromatography is used for separation purposes, maximum resolution and maximum resolution per unit time are taken as criteria for deciding the optimum values of β and the product Qt.

To simplify the procedure, t was maintained constant for all solvents, *i.e.* the time program for the gradient system was linear. It is possible that there may be an optimum value of β for each solvent but as the polarity difference between solvents is approximately constant[2], it is likely that the same value of β will be optimum for all solvents and this was assumed to apply for the purpose of this study. As the object of the gradient elution procedure is to cope with mixtures having components of diverse polarities, the resolution between pairs of components eluted over the complete polarity range had to be determined for different values of the variables being opti-

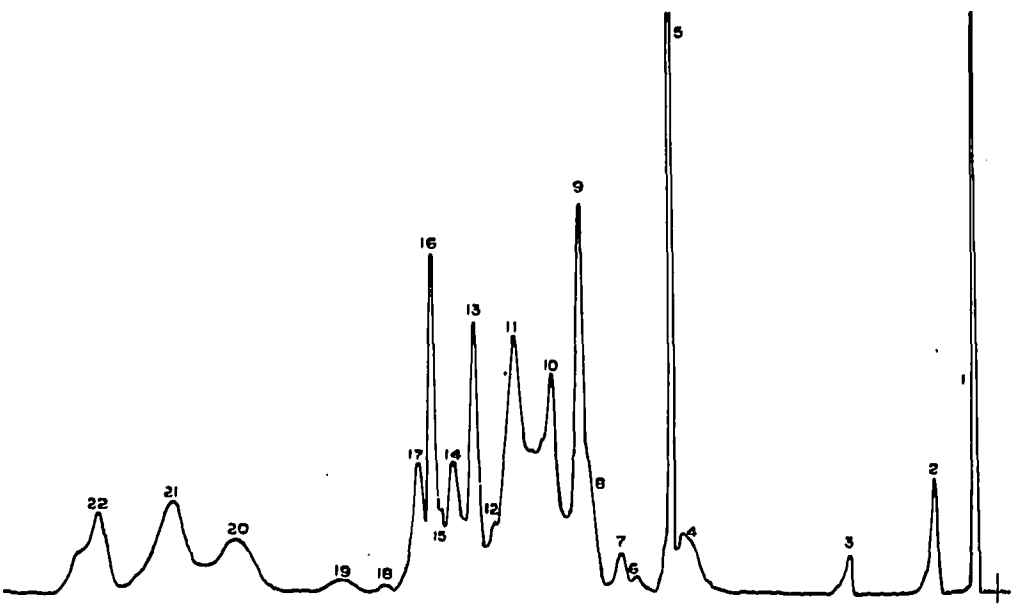


Fig. 1. An example of a chromatogram of a 22-component mixture used for program optimization. 1 = Squalane, 2 = anthracene, 3 = methyl stearate, 4 = benzophenone, 5 = *o*-chloroaniline, 6 = *p*-nitroaniline, 7 = *p*-dinitrobenzene, 8 = *p*-nitrophenol, 9 = dihydrocholesterol, 10 = catechol, 11 = phenacetin, 12 = adenine, 13 = phenolphthalein, 14 = ethyl 1,2-dihydro-2-ethoxy-1-quinoline-carboxylate (EEDQ), 15 = quinine, 16 = acetylsalicylic acid, 17 = benzoic acid, 18 = *tert.*-Boc (butoxycarbonyl) leucine, 19 = *tert.*-Boc glycine, 20 = alanine, 21 = citric acid, 22 = glucose.

mized. A mixture containing 22 different solutes was employed for this purpose and an example of a chromatogram used for the optimization procedure is shown in Fig. 1. The pairs of peaks chosen for measurement were 6–7, 13–14, 16–17 and 20–21. The resolutions of these pairs of solutes were measured under the following conditions:

(1) At different values of β for fixed values of Q and t by varying V (see Table I).
(2) At constant values of β and Q but varying t and V (see Table II).
(3) At constant values of β, the product Qt and V but varying Q and t (see Table III).

TABLE I

CONDITIONS FOR DETERMINING OPTIMUM VALUE OF β

Column length, 50 cm; I.D., 4.6 mm; dead volume, 6 ml; packing, Bio-Sil A silica gel 22–44 μm; flow-rate, 2.0 ml/min; column reconditioned at a flow-rate of 2.5 ml/min for 15 min per reconditioning solvent; solvent period, 8 min per solvent; dilution volume, 18 ml; mixing vessel constant, $\beta = 0.85$; detector sensitivity, 3.2×10^{-11} A f.s.d.; wire speed, $\times 2 = 5$ cm/sec; evaporation temperature, 150°; sample size, 8 μl of *ca.* 50% solution of sample; chart speed, 4 in./h; total analysis time, *ca.* 90 min.

β	Q (ml/min)	t (min)	V (ml)
0.36	1.2	15	50
0.40	1.2	15	45
0.50	1.2	15	36
0.60	1.2	15	30
0.66	1.2	15	27
0.75	1.2	15	24
1.00	1.2	15	18
1.50	1.2	15	12
2.00	1.2	15	9

TABLE II

CONDITIONS FOR DETERMINING OPTIMUM VALUE OF Qt

For conditions, see Table I.

β	Q (ml/min)	t (min)	Qt (ml)	V (ml)
0.85	1.2	5	6.0	7.1
0.85	1.2	8	9.6	11.3
0.85	1.2	10	12.0	14.1
0.85	1.2	13	15.6	18.3
0.85	1.2	15	18.0	21.2
0.85	1.2	17	20.4	24.0
0.85	1.2	20	24.0	28.2

Results from the first series of experiments permitted the determination of the optimum value of β (Table I). The second series of experiments provided an optimum value of Qt, the total volume of each solvent employed for the previously determined optimum value of β (Table II). Finally, the last series of experiments provided information on the effect of flow-rate on resolution when optimum values of β and Qt were employed (Table III).

TABLE III

CONDITIONS FOR DETERMINING EFFECT OF FLOW-RATE, Q

Conditions, see Table I.

β	Q (ml/min)	t (min)	Qt (ml)	V (ml)
0.85	0.50	30	15	17.3
0.85	0.79	19	15	17.3
0.85	1.00	15	15	17.3
0.85	1.25	12	15	17.3
0.85	1.50	10	15	17.3
0.85	1.67	9	15	17.3
0.85	2.14	8	15	17.3
0.85	2.50	6	15	17.3

EXPERIMENTAL

The apparatus used has been previously described[1] but for the present set of experiments the Milton Roy, Model Milroyal D was replaced by a Waters Model 6000. As the Waters pump is virtually pulse-free, the pulse dampener used in the original apparatus was also eliminated. The components employed in the standard mixture used for optimization purposes are shown in the legend to Fig. 1. A 10% solution of these substances was made up in a solvent containing equal volumes of all the solvents used in the incremental gradient elution procedure.

The solvents used have been previously described, but a list showing their composition together with the column reconditioning solvents is shown in Table IV.

TABLE IV

SOLVENTS USED FOR INCREMENTAL GRADIENT ELUTION

Solvent	Composition
Development solvents	
1	100% *n*-heptane
2	100% carbon tetrachloride
3	58% v/v carbon tetrachloride, 42% v/v chloroform
4	36% v/v carbon tetrachloride, 26% v/v chloroform, 38% v/v 1,2-dichloroethane
5	20% v/v carbon tetrachloride, 14% v/v chloroform, 21% v/v 1,2-dichloroethane, 45% v/v 2-nitropropane
6	14% v/v carbon tetrachloride, 11% v/v chloroform, 14% v/v 1,2-dichloroethane, 33% v/v 2-nitropropane, 28% v/v nitromethane
7	36% v/v nitromethane, 64% v/v propyl acetate
8	100% methyl acetate
9	100% acetone
10	100% ethyl alcohol
11	100% methyl alcohol
12	100% water
Reconditioning solvents	
1	ethyl alcohol
2	acetone
3	ethyl acetate
4	1,2-dichloroethane
5	*n*-heptane

In all cases charges of 10 μl were employed using on-column injection. The column was reconditioned after each experiment by pumping six column volumes of each of the reconditioning solvents shown in Table IV through it.

From the chromatograms obtained, the resolution between the chosen pairs of peaks was calculated and a measure of the resolution taken as the ratio of the distance between the peak maxima to the average of the respective peak widths. In cases where resolution was poor, peak widths were measured by geometric construction. Curves of β plotted against resolution are shown in Fig. 2. In routine chromatographic analysis, although resolution is a pre-requisite, the time taken to effect the separation is also important and thus the resolution per unit time is of interest. A measure of the resolution per unit time was taken as the ratio of the resolution (determined in the manner above) to the average retention time of each pair of peaks. Resolution per unit time determined in this manner is shown plotted against the value of β in Fig. 3. In Fig. 4 the resolution obtained at the optimum value of β for the maximum resolution per unit time is shown plotted against the total volume of each solvent employed, *Qt*. *Qt* is measured in column dead volumes and thus represents the maximum k' value at which any solute can be eluted by a given solvent. Finally, in Fig. 5 the effect of changes in column flow-rate is shown as curves plotting resolution against flow-rate for the mixture being separated at the optimum values of β and *Qt* that provide the maximum resolution per unit time.

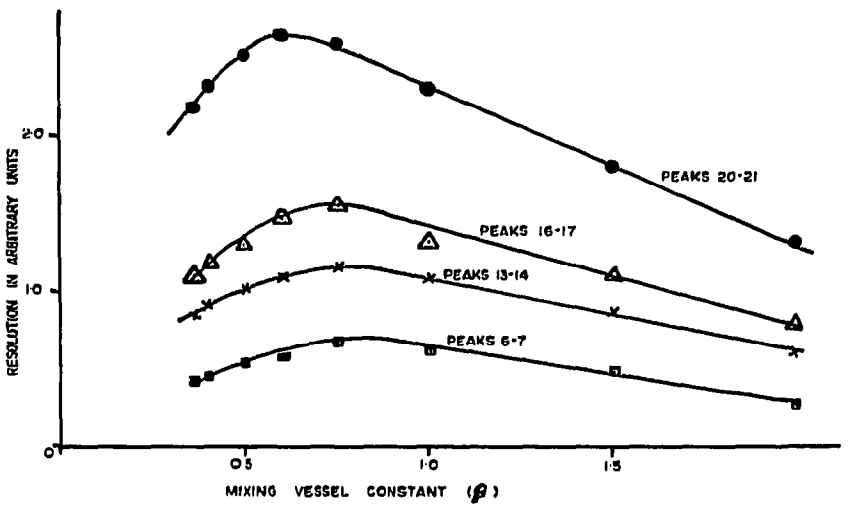


Fig. 2. Graph of resolution against mixing vessel constant, β.

DISCUSSION

From Fig. 2 it is seen that the optimum value of β varies with the positions (polarity) of the eluted solute which also corresponds to the polarity of the eluting solvent. The optimum value of β for maximum resolution between the late eluted (more polar) solutes occurs at about 0.6, whereas, for solutes eluted early in the chromatogram (non-polar), the optimum value is about 0.85. A compromise value for a mixture containing a wide range of solute types would be about 0.70.

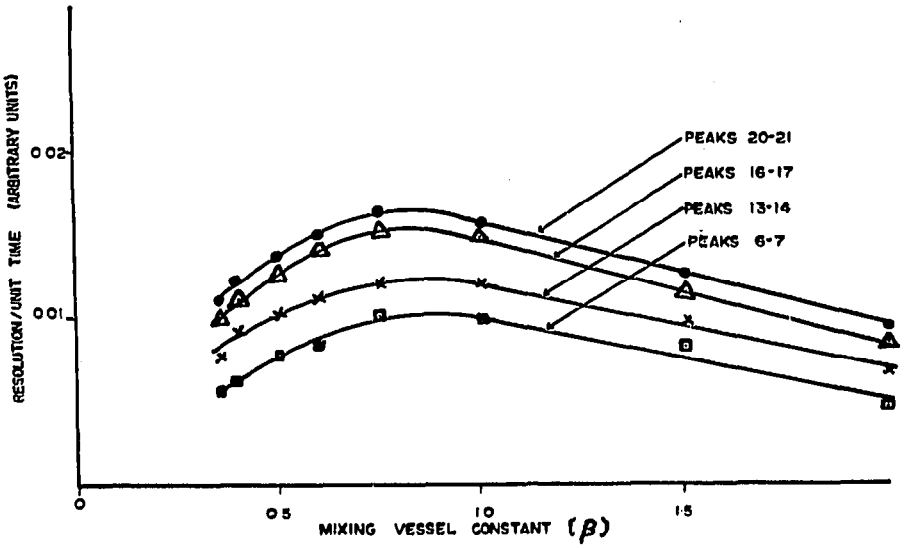


Fig. 3. Graph of resolution per unit time against mixing vessel constant, β.

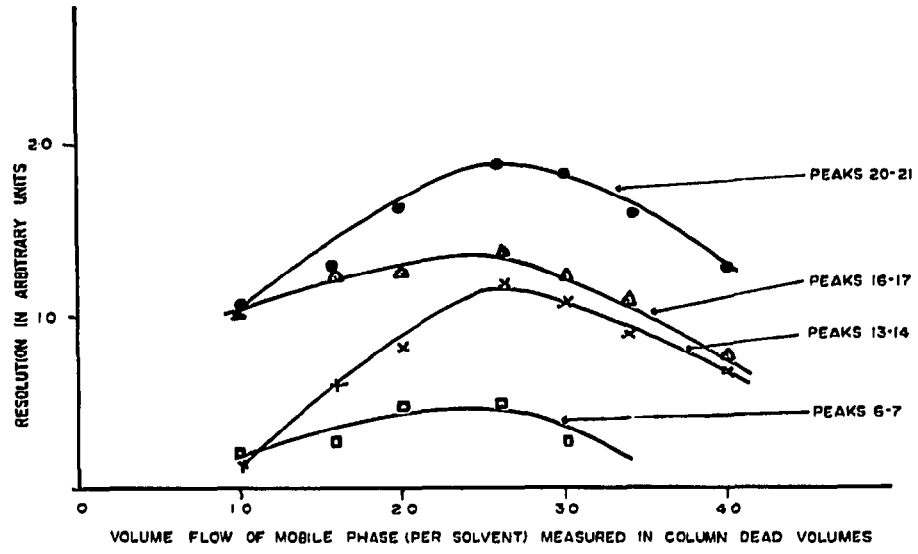


Fig. 4. Graph of resolution against volume flow of mobile phase measured in column dead volumes.

From a practical point of view the maximum resolution per unit time is more important, and curves relating resolution per unit time and β are shown in Fig. 3. Here it is seen that the change in optimum value of β with solute polarity is very slight and that a value for β of 0.85 will be close to the optimum for all solutes.

The effect of the total volume of each solvent used on the resolution when employing an optimum value of 0.85 for β is shown in Fig. 4. It is seen that the shapes

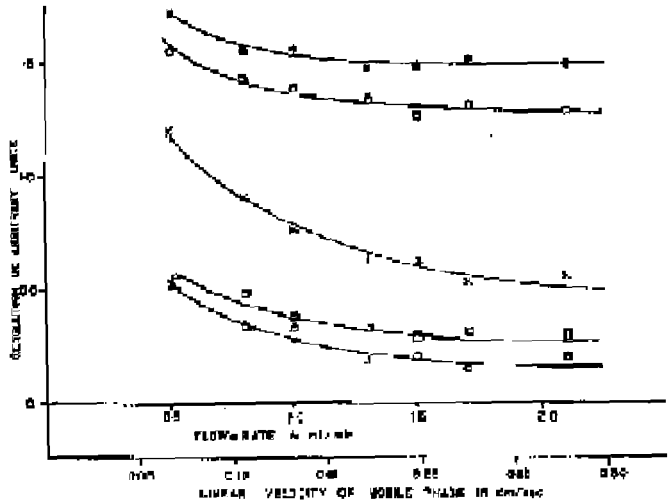


Fig. 5. Graph of resolution against column flow-rate obtained under optimum gradient conditions.

of the curves vary considerably with the polarity of the solute eluted and thus its position on the chromatogram. However, the optimum solvent volume is closely similar for all solutes. From the curves in Fig. 4 an optimum value for solvent volume was taken as 2.5 column dead volumes per solvent. This value is coincident with the optimum k' value suggested by Grushka and Guiochon[4] which is the value of k' that provides maximum resolution under isocratic development.

Employing an optimum value for β of 0.85 and for the product, Qt, of 2.5 column dead volumes, the effect of flow-rate on resolution is shown in Fig. 5. It is seen, as one would expect, that there is no optimum flow-rate within the range of flow-rates employed and that generally the lower the flow-rate the greater the resolution. This merely reflects the effect of the shape of the HETP curve and shows that the efficiency of the column increases with decreasing linear mobile phase velocity. For a value of $\beta = 0.85$, an optimum value for the solvent volume of 2.5 (column dead volumes), and a flow-rate of 1 ml/min, the composition of the solvent entering the column can be calculated using the equations previously given[4].

In Fig. 6 the mobile phase composition expressed as % v/v is shown for the complete gradient program. It is interesting to note that throughout the major part of the gradient elution procedure the eluting mobile phase is a complex mixture of a number of different solvents.

From a theoretical point of view the optimum conditions for columns of other dimensions should be predictable from the ratio of the respective column volumes.

The concentration of solvent ($n + 1$) in solvent (n) entering column 1 has been shown to be[3]

$$X_0 (1 - e^{-Q_1 t / V_1})$$

where Q_1 and V_1 are the flow-rate and mixing vessel volume for column 1, respectively, and X_0 is the concentration in mass per unit volume originally in the vessel.

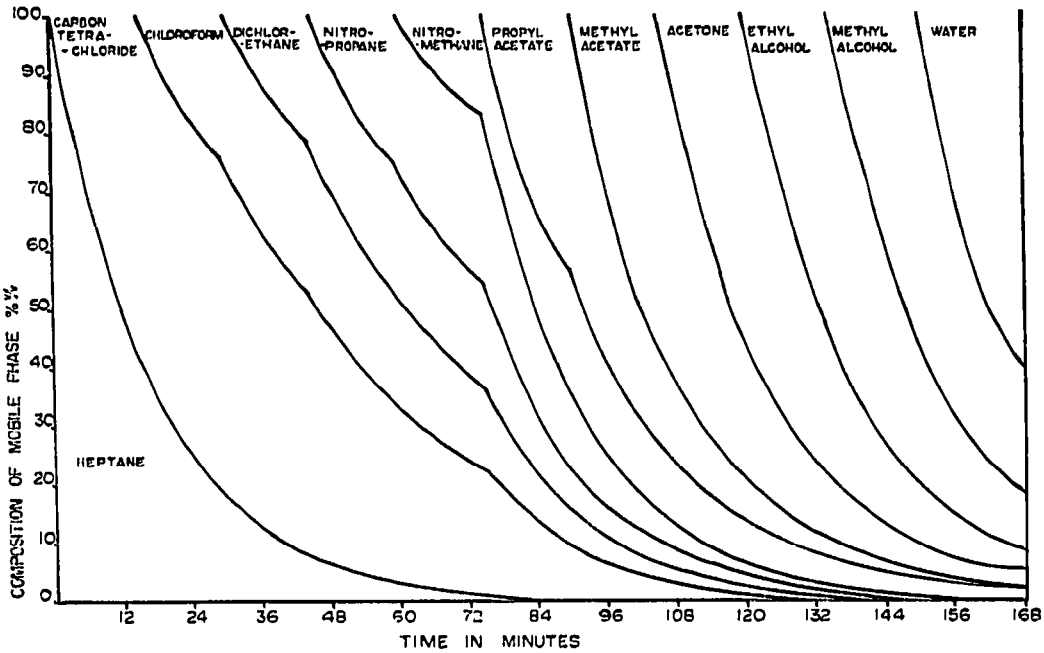


Fig. 6. Composition of mobile phase entering column under optimized incremental gradient elution conditions. $\beta = 0.85$; $t = 17$ min; $Q = 1$ ml/min; $k' = 2.5$; and $D = 6$ ml.

Now neglecting interstitial dead volumes, if solvent $(n + 1)$ breaks through the column after time t'_1, its concentration will be:

$$X_0 (1 - e^{-Q_1 t'_1/V_1})$$

The solvent $(n + 1)$ flowing into the column between $t = 0$ and $t = t'_1$ has been adsorbed by the adsorbent and provides the deactivation effect. Now if $m_{1(n+1)}$ is the mass of solvent $(n + 1)$ required for deactivation, then for column 1:

$$m_{1\,(n+1)} = X_0 \int_0^{t'_1} (1 - e^{-Q_1 t/V_1})\, Q_1 dt =$$

$$= X_0 Q_1 \left[t + V_1/Q_1 e^{-Q_1 t/V_1}\right]_0^{t'_1} =$$

$$= X_0 V_1 \left[Q_1 t'_1/V_1 - (1 - e^{-Q_1 t'_1/V_1})\right]$$

and for column 2:

$$m_{2\,(n+1)} = X_0 V_2 \left[Q_2 t'_2/V_2 - (1 - e^{-Q_2 t'_2/V_2})\right]$$

Now assuming under optimum conditions the concentration of solute $(n + 1)$

in the eluent leaving the two columns after times t'_1 and t'_2 must be the same, then:

$$X_0 (1 - e^{-Q_1 t'_1/V_1}) = X_0 (1 - e^{-Q_2 t'_2/V_2})$$

Further, if the columns are packed in a similar manner

$$\frac{m_{1\,(n+1)}}{m_{2\,(n+1)}} = \frac{\pi r_1^2 l_1}{\pi r^2 l_2} = \frac{V_1}{V_2} = \frac{D_1}{D_2} \tag{1}$$

where r_1, r_2, l_1, l_2, and D_1, D_2 are the radii, lengths and dead volumes of the two columns, respectively.

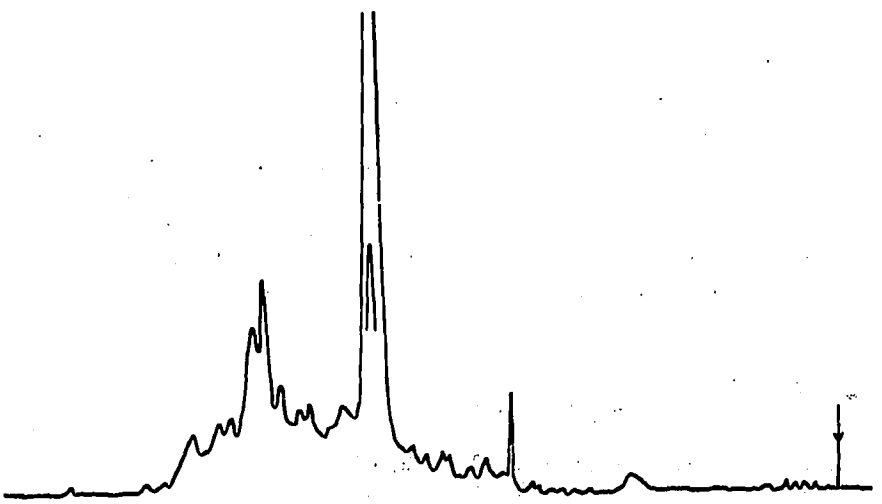
If it is assumed that an optimum k' value for each column is 2.5 D then

$$\frac{Q_2 t_2}{Q_1 t_1} = \frac{D_2}{D_1} \tag{2}$$

where t_2 and t_1 are the total solvent periods for column 2 and 1, respectively. Then from eqns. 1 and 2 it is seen that the optimum values for β for the two columns are the same and adjustments have only to be made for the product Qt and the volume V_1, the ratio Qt/V remaining constant at 0.85. This is in general agreement with the work of Stolyhwo and Privett[5].

This theory has been found applicable to columns up to 50 cm in length. However, applying it to columns of 100 cm in length it was found that the optimum value of β could not be predicted in this way and if the conditions suggested by eqns. 1 and 2 are met, very poor resolution is realized. The reason for this discrepancy between theory and practice is presently being examined.

A likely explanation for this effect is that the increased solvent volume that must be used for the longer column results in retarded peaks being eluted slowly along the column at an effective high k' value. This means that the peaks are eluted for significant periods under non-linear adsorption isotherm conditions with resulting band dispersion and tailing. If this asymmetric band dispersion is allowed to occur to any great extent then the peak sharpening process that results from the introduction of solvents of increasing polarity is not sufficient to produce symmetrical narrow bands. Conversely, if the solvent volume used for the longer column is reduced to prevent this



effect, then the optimum value of Qt is not used and thus little advantage is obtained in using the longer column.

The optimum value for Qt conditions the maximum k' value at which any solute will be eluted by a given solvent. As this value for Qt for a given column will vary with the adsorptive capacity of the adsorbent then change of adsorbent will require changes in the optimum value of Qt employed. Theoretically, if the adsorptive capacity of any adsorbent is known relative to Bio-Sil A, then the optimum value for Qt could be calculated. However, this relationship has not been verified and is presently under investigation.

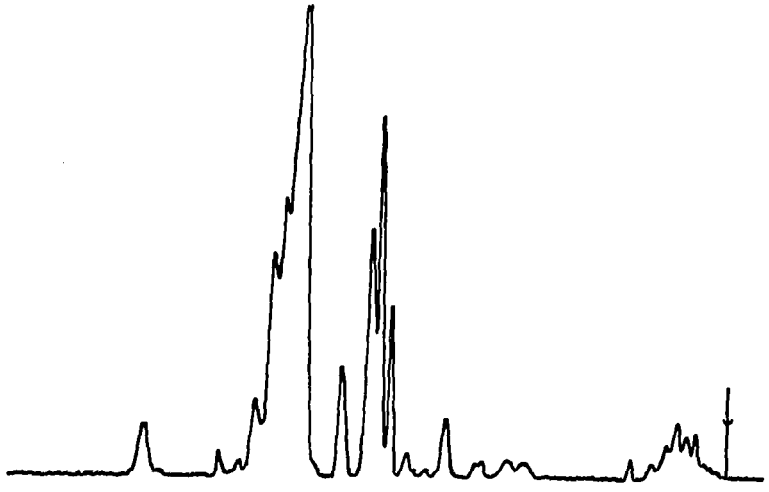


Fig. 8. Chromatogram of tocopherol oil distillate by incremental gradient elution.

Using the optimum conditions for maximum resolution per unit time, as described in Table I, samples of oxidized cholesterol and tocopherol oil distillate were chromatographed. The chromatograms obtained are shown in Figs. 7 and 8. It is seen that an effective and useful separation is immediately obtained that illustrates the range of polarity of the solutes present in the mixture together with an indication of its complexity.

CONCLUSION

There are optimum conditions for incremental gradient elution that must be employed to provide the maximum resolution or maximum resolution per unit time. The conditions will be satisfactory for the immediate separation of any type of solute mixture that is amenable to liquid–solid chromatography. The optimum conditions will vary with the column dimensions and the adsorptive capacity of the adsorbent. At present a method for predicting optimum conditions for columns of different geometries or packed with different adsorbents has not been established.

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