Journal of Chromatography, 192 (1980) 19–36

CHROM. 12,571

GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

XII. OPTIMIZATION OF CONDITIONS FOR GRADIENT ELUTION

PAVEL JANDERA and JAROSLAV CHURÁČEK
Department of Analytical Chemistry, University of Chemical Technology, Pardubice (Czechoslovakia)
(First received February 26th, 1979; revised manuscript received July 20th, 1979)

SUMMARY

Different possibilities for the optimization of gradient elution conditions are discussed and compared. An approach is developed that permits calculations of the optimal initial concentration and slope of the gradient for the separation of mixtures containing compounds with known relationships of capacity ratio *versus* composition of the mobile phase. The approach was used for the selection of optimal conditions in the reversed-phase gradient elution chromatography of barbiturates and substituted uracils. The agreement between the experimental and expected chromatographic data is compared. A general approximate method is suggested for the prediction of the slope of the gradient in reversed-phase chromatography.

INTRODUCTION

Gradient elution is widely accepted as a highly efficient technique for adjusting adequately the retention of sample compounds during elution[1]. The conditions for gradient elution are usually selected by a trial-and-error method. In many instances it would be useful, however, if we were able to calculate the optimal gradient elution conditions for a given separation problem from the properties of the chromatographic system and compounds to be separated, without performing a number of preliminary experiments. Such a rational choice of the optimal gradient elution profile for a required separation is not possible without a good understanding of the influence of the profile of the concentration gradient on retention characteristics such as retention volume, peak width and resolution. This aspect has been treated quantitatively in Part XI[2] and elsewhere[3].

A few papers have been published in which the positions of the maxima and bandwidths in gradient elution chromatography were correlated with the profile of the concentration gradient in special instances[4–8], but such approaches cannot be used as the basis for the optimization of gradient elution chromatography because of the lack of general applicability and the complexity of the resulting equations.

Snyder and co-workers[9,10] developed the concept of so-called "linear solvent strength" gradients, which are relatively simple to understand and treat. This concept

was recently elaborated by this group and a method for the optimization of these gradients has been suggested[3,11,12].

In this paper another approach to gradient optimization is suggested and compared with the treatment of Snyder and co-workers.

OPTIMIZATION OF RESOLUTION IN GRADIENT ELUTION CHROMATOGRAPHY: COMPARISON OF DIFFERENT APPROACHES

Optimization of the chromatographic process means finding adequate conditions so as to obtain a required resolution of sample compounds in as short a time as possible. To meet this aim in gradient elution chromatography, the components (solvents) from which the gradient is formed and the profile of the gradient should be judiciously chosen. Most practical separation problems can be solved by using no more than two solvents of different elution strengths. After an appropriate choice of the weaker (a) and the stronger (b) solvent, the profile of the gradient should be selected, *i.e.*, the shape (curvature) and the slope (B) of the gradient and the starting concentration (A) of solvent a in the mobile phase. The optimization of the gradient profile requires different treatments if the sample compounds are known and if there is information available about their chromatographic behaviour under isocratic conditions, or if an essentially unknown sample is to be separated.

Approach according to Snyder and co-workers

Snyder and co-workers[3,11,12] suggested an optimization approach for "linear solvent strength" gradients in reversed-phase chromatography. In "linear solvent strength" gradients the logarithms of the capacity ratios of sample compounds, k_i', decrease linearly with time according to[9,10]

$$\log k_i' = \log k_a - b(t/t_0) \tag{1}$$

where k_a is k_i' in the mobile phase at the beginning of gradient elution, t_0 is the column dead time and b characterizes the slope of the gradient but depends also on the behaviour of sample compound i in a given chromatographic system (for a more detailed discussion, see refs. 3, 9 and 10). The concentration profile of a "linear solvent strength" gradient together with the character of the relationship between k_i' and the composition (isocratic) of the mobile phase (such as described by eqn. 2a or 2b in further discussion below) determine the shape of the concentration gradient. In reversed-phase chromatography, where eqn. 2b usually applies well, this means a linear concentration gradient (linear change of concentration of solvent b in the eluent with time)[11]. With certain simplifying assumptions (*e.g.*, k_a for all compounds very large, *i.e.*, $A \to 0$; constant separation factors for sample compounds during the gradient), a simplified equation for resolution in gradient elution chromatography as a function of b in eqn. 1 could be derived[3,10,11].

As in isocratic elution chromatography, where maximal resolution per time unit can be achieved at certain values of the capacity ratios of sample compounds, maximal resolution in a given separation time in gradient elution chromatography is obtained for a fixed value of b ($b \approx 0.2$; see detailed discussion in refs. 3, 11 and 12).

The optimization approach according to Snyder and co-workers[11,12] suggests

gradients using this value of b, from which the slope B of the linear concentration gradient can be calculated, provided that n in eqn. 2b is approximately constant in a given reversed-phase chromatographic system and its value can be estimated, which appears to be a realistic assumption in practice. The initial concentration of solvent b is $A = 0$.

This optimization approach can be applied generally in reversed-phase chromatography, but it cannot give the best utilization of the analysis time or the resolution required for each individual separation problem. Therefore, Snyder and coworkers[11,12] recommend the following "fine tuning" of the separation conditions using a trial-and-error method, for which they provide several hints. These should be tried subsequently in the following order:

(a) increase in the initial concentration, A, of solvent b (if sample compounds are eluted too late);

(b) variation in the parameter b in eqn. 1 to obtain a better resolution;

(c) increase in N by decreasing the flow-rate or by increasing the column length, if the resolution is still insufficient;

(d) changing the organic solvent b if the selectivity is too low or if the sample compounds are very strongly retained.

Present optimization approach

The optimization approach presented here allows the direct calculation of the best profile of the gradient necessary to achieve the separation of a mixture of known compounds in as short a time as possible with a gradient formed from two given solvents a and b. Thus, "tailor-made" gradients for each separation are calculated, in which the solvent strength does not necessarily change linearly. This approach can be used for both reversed-phase and normal adsorption and ion-exchange systems, but the relationships between the capacity ratios, k', of sample compounds and the concentration (c) of the stronger solvent b in the mobile phase under isocratic conditions must be known, in addition to the respective constants of this equation. In principle, different k' *versus* c functions may be used, but we shall restrict ourselves here to the two simplest and evidently most useful relationships:

$$k' = k_0' c^{-n} \tag{2a}$$

and

$$k' = k_0' 10^{-cn} \tag{2b}$$

where k_0' and n are experimental constants of the sample compound and system used.

Eqn. 2a can be used in many normal adsorption and ion-exchange systems, while eqn. 2b is suitable for reversed-phase chromatography. It is not the purpose of this paper to argue about the validity of and deviations from these equations; for a comprehensive discussion, see refs. 3, 11 and 13–19.

In our optimization approach it is assumed that the efficiency (plate number, N) of the column used does not depend significantly on the composition of the binary mobile phase, which seems to be a reasonable assumption in most situations. The optimization of separation by controlling N via the column length or flow-rate can be achieved by analogy with isocratic elution and is not considered in the present approach, where a fixed value of N is assumed (given column dimensions and flow-rate).

It is further assumed that there is no concentration change in the mobile phase caused by the column (solvent demixing is negligible) or by the geometry of the instrument.

Finally, it is assumed that the gradient-generating device used is capable of mixing two different liquids so as to produce a concentration gradient according to any mathematical function of concentration *versus* time. It is desirable that the concentration gradient be defined by a gradient function that should be simple and applicable to a wide variety of gradient profiles. The following gradient function is compatible with these requirements and has proved useful in practice[16]:

$$c = (A^{\frac{1}{\varkappa}} + BV)^{\varkappa} \tag{3}$$

where V is the volume of the mobile phase delivered by the gradient-generating device from the beginning of the gradient and A, B and $\varkappa$ are adjustable parameters of the gradient function; A denotes the initial concentration of the stronger eluting agent in the binary mobile phase at the beginning of the gradient, B is the gradient slope and $\varkappa$ characterizes the shape (curvature) of the gradient profile. Other forms of gradient function can be also used for the optimization approach.

Using eqns. 2a, 2b and 3, relationships for important retention characteristics in gradient elution chromatography (retention volume, peak width, resolution) were derived[16,17] and the influence of A, B and $\varkappa$ (eqn. 3) on these characteristics was determined[2]. The optimization approach suggested here is based on the conclusions from this previous work.

Resolution in gradient elution chromatography

Let us now consider the gradient elution separation of a two-component sample mixture. To achieve the resolution, R_S, required, the parameters A, B and $\varkappa$ in eqn. 3 can be calculated from the appropriate equation for R_S, by analogy with the approach for the selection of the optimal composition of the mobile phase necessary to obtain the resolution required in isocratic elution chromatography[13]. In part XI[2], the influence of A, B and $\varkappa$ on the resolution is discussed in detail. It has been shown that there are certain values of the slope of the gradient function, B and/or of the initial concentration of the efficient eluting agent in the mobile phase, A, at which maximal or zero resolution of compounds 1 and 2 can eventually occur in gradient elution chromatography, if $n_1 \neq n_2$ in eqn. 2a or 2b.

The occurrence of an extreme in the $R_{S(g)} = f(A)$ or $R_{S(g)} = f(B)$ function within the practically useful range of these functions, however, is likely only with major differences in n_1 and n_2, which rarely happens in practical systems. Maxima of $R_{S(g)} = f(\varkappa)$ functions are more likely to occur, but they are rather flat. By analogy with isocratic elution chromatography, the resolution in gradient elution chromatography using a given pair of solvents is limited by minimal and maximal values, which cannot be exceeded at any practical combination of A, B and $\varkappa$.

A can be varied within the possible concentration limits of the more efficient eluting agent in the mobile phase (from 0 to c_{max}). The slope of the concentration gradient is limited by the requirement that the retention volume of the last compound eluted, $V'_{R(g)z}$, must not exceed the volume of the mobile phase delivered on to the column from the beginning of the gradient until the maximal possible concentration of

the more efficient eluting agent in the mobile phase is achieved, otherwise the elution is finished under isocratic conditions with the pure, more efficient eluting agent, and that the two components of the mobile phase must remain miscible. If two of the parameters A, B and $\varkappa$ are known, the maximal admissible (m.a.) value of the remaining parameter can be calculated, *e.g.*, the parameter $B_{\text{m.a.}}$ or $A_{\text{m.a.}}$ assuming the gradient function according to eqn. 3:

$$B_{\text{m.a.}} = \frac{c_{\max}^{\frac{1}{\varkappa}} - A^{\frac{1}{\varkappa}}}{V'_{R(g)z}} \tag{4a}$$

$$A_{\text{m.a.}} = (c_{\max}^{\frac{1}{\varkappa}} - V'_{R(g)z}B)^{\varkappa} \tag{4b}$$

The values of $B_{\text{m.a.}}$ or $A_{\text{m.a.}}$ can be calculated after the introduction of the appropriate relationship for $V'_{R(g)z}$ in accordance with the validity of eqn. 2a or 2b for the experimental system studied[16].

METHODS OF CALCULATION

If the required resolution of a two-component mixture is in principle possible in a given chromatographic system, it can be achieved using optimized isocratic elution and the application of gradient elution chromatography is unnecessary. On the other hand, it is extremely difficult to programme the composition of the mobile phase so as to obtain just the resolution necessary for baseline separation of all of the components of a complex, multicomponent mixture. This aim, if necessary at all often cannot be achieved by using a simple monotonous gradient function and the elution conditions have to be programmed separately, step-by-step, for the resolution of subsequent neighbouring compounds. Stepwise elution can be used to give an approximate solution to this problem.

Gradient elution using a simple gradient function with three parameters (like eqn. 3) can be suitably optimized so that two, or maximally three, independent requirements could be satisfied. Thus, the parameters of a gradient function could be calculated for two or three required values of the resolution at different points on a chromatogram or for a required resolution of two compounds and a required retention volume of another compound. Such an approach to the optimization of gradient elution would not only be very complex, but also unlikely to be meaningful, as the values of A, B and $\varkappa$ for one of the requirements could often fall into the range precluded by another requirement and the calculation would fail. In our opinion, it is more reasonable to base the optimization approach on only one required value for the resolution of the two components of a sample mixture that are most difficult to resolve. Consequently, one of the parameters A, B and $\varkappa$ is determined and the other two can be varied, as is illustrated in Figs. 1 and 2. Fig. 1. shows the relationship between B and $\varkappa$ at a fixed value of A and the required value of the resolution of two neighbouring substances in adsorption gradient elution chromatography, and Fig. 2 shows a plot of B *versus* A at a fixed value of $\varkappa$ and with the same required resolution. In both figures, a plot of the retention volume of another sample compound against

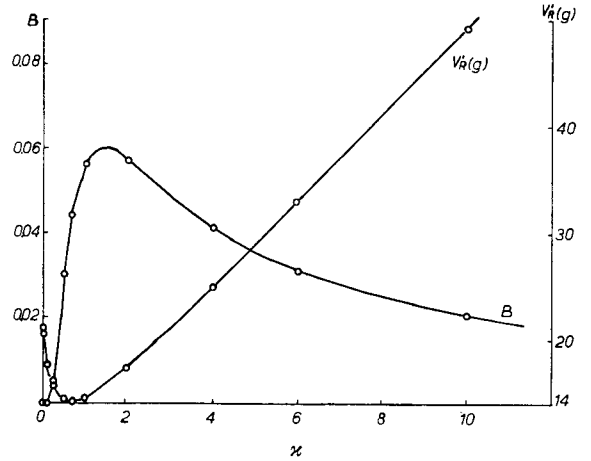

Fig. 1. Influence of $\varkappa$ in eqn. 3 on B and the retention volume of the last-eluted compound ($V'_{R(g)4}$) in the adsorption chromatography of four azo compounds. Column, Porasil A; binary mobile phase composed of cyclohexane and ethyl acetate. Compounds: 1 = di-(*n*-butyl)amide, 2 = di-(*n*-propyl)-amide, 3 = diethylamide and 4 = dimethylamide of *p*-N,N-dimethylamino-*p'*-azobenzoic acid. A in eqn. 3 = 0. $R_{s(g)1,2} = 0.5$ required. Eqns. 5, 6 and 7 were used in calculations. $V_m = 2.0$ ml; $N = 137$; $k'_{01} = 0.242$; $k_{02} = 0.330$; $k'_{03} = 0.65$; $k'_{04} = 1.94$; $n_1 \approx n_2 \approx n_3 \approx n_4 \approx n = 1.68$.

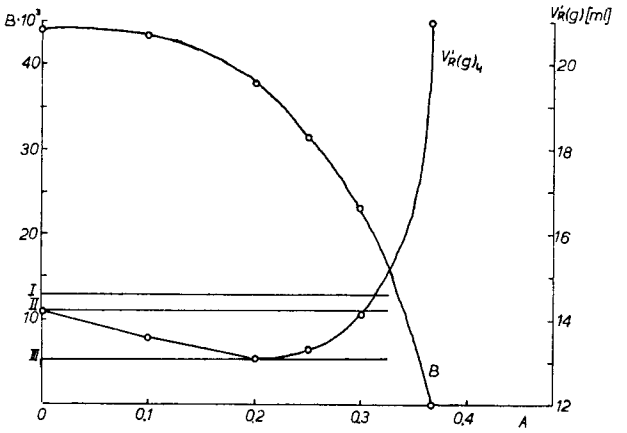

Fig. 2. Influence of A in eqn. 3 on B and the retention volume of the last-eluted compound ($V'_{R(g)4}$) in the adsorption chromatography of four azo compounds. Chromatographic system, compounds and operating conditions as in Fig. 1. $\varkappa$ in eqn. 3 = 0.68. $R_{s(g)1,2} = 0.5$ required. Eqns. 5, 6 and 7 were used in calculations. For comparison, the values of $V'_{R(g)4}$ are given for gradients with different A and $\varkappa$, where B is optimized with respect to the achievement of $R_{s(g)1,2}$ required: I, $c = 0.0564V$ (linear gradient, $\varkappa = 1$); II, $c = (0.0441V)^{0.68}$ (gradient with zero initial concentration of ethyl acetate, A = 0); III, $c = (0.1302 + 0.0378V)^{0.68}$ (gradient optimized with respect to the minimal time of separation) (c = %, v/v·10^{-2} of methanol in the mobile phase at the inlet of the column at V ml of the eluate from the beginning of the gradient elution).

$\varkappa$ and A is shown. The plots of retention volume show a minimum at a certain value of $\varkappa$ and A. Thus, it seems reasonable to accept the achievement of the minimal retention volume of an arbitrary compound as the second condition of the optimization approach. These two requirements are sufficient to determine A and B.

The parameter $\varkappa$ is less significant than the other two with respect to the requirements of the optimization approach, but it determines the compression of a chromatogram expressed by means of a compression criterion, Q (ref. 2), (if eqn. 2a applies) and, if necessary, it can be determined from the required value of Q before the optimization of A and B.

The optimization of A and B consists in an appropriate choice of the compound i, the retention volume of which should be minimal, the determination of the minimum of function $V'_{R(g)i} = f(A,B)$ at given value of $\varkappa$, while A and B are further interrelated so that the required value of resolution $R_{S(g)1,2}$ of two appropriately chosen compounds with adjacent bands is kept constant. The usual Lagrange mathematical solution to the problem would suffer from severe difficulties and a modifled method of solution is therefore preferred. An interval of possible values of A is defined, $A > 0$; $A < A_{max}$, where A_{max} represents the concentration of the more efficient eluting agent in the mobile phase, at which the resolution required is just achieved in isocratic elution chromatography. From this interval, the values of A that are used for calculation of the corresponding values of B necessary to achieve the required $R_{s(g)1,2}$ are subsequently chosen. Then, the corresponding values of the retention volume, $V'_{R(g)i}$, are calculated and compared. The comparison of the values of $V'_{R(g)i}$ corresponding to the chosen values of A is used for a subsequent reduction of the interval of values of A (the interval is halved in each step) until the minimal value of $V'_{R(g)i}$ is found with a pre-set precision (*e.g.*, 1 %). Then, the arithmetic mean of this interval represents the required solution for A with the corresponding value of B. This approach is based on the assumption that there is only one minimum of $V'_{R(g)i}$ in the interval of the values of A, as in Fig. 2; it is universal and can be applied with different $k' = f(c)$ and gradient functions. However, a computer is required for the optimization calculations. In the present work, a TI 58 programmable pocket calculator with a program capacity of 480 steps was used. Fig. 3 shows the block diagram of the calculation.

The equations used for the calculations in the above optimization approach will differ according to the gradient function and the function $k' = f(c)$ that apply for a given system.

I. Gradient function according to eqn. 3: eqn. 2a applies for a given chromatographic system. Then,

$$B = \frac{\sqrt{N}(X_2 - X_1)}{2V_m R_{s(g)1,2}\left(\frac{k'_{01}}{X_1^{\varkappa n_1}} + \frac{k'_{02}}{X_2^{\varkappa n_2}} + 2\right)} \tag{5}$$

$$V'_{R(g)i} = \frac{X_i - A^{\frac{1}{\varkappa}}}{B} \tag{6}$$

where N is the number of plates of the column, which is assumed to be approximately equal for all the sample components, V_m is the volume of the mobile phase in the column, $\varkappa$ is selected before the calculation and

$$X_j = \left[(\varkappa n_j + 1)Bk'_{0j}V_m + A^{\frac{\varkappa n_j + 1}{\varkappa}}\right]^{\frac{1}{\varkappa n_j + 1}} \tag{7}$$

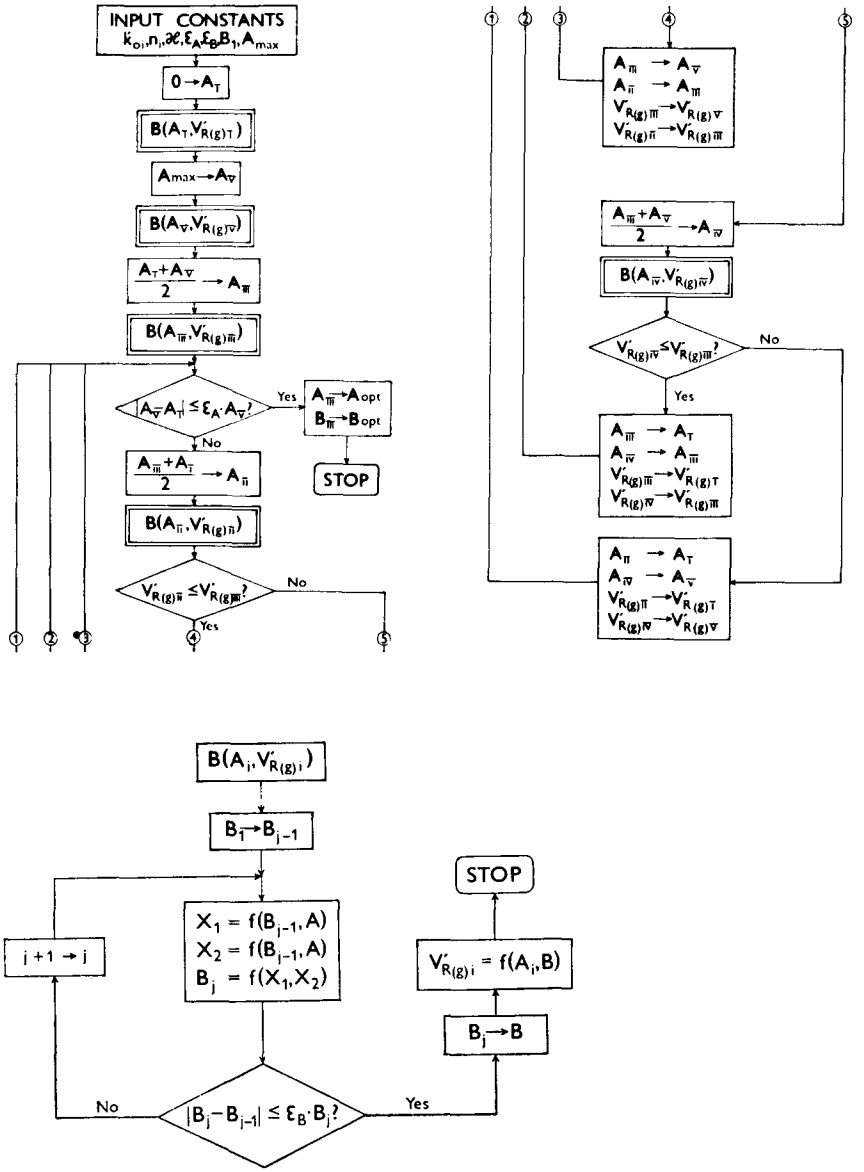

Fig. 3. Block diagram of the program used in the optimization approach.

The subscript $j = 1$, 2 or i relates to compound 1, 2 or i, respectively. The parameter B must be calculated from eqn. 5 using an iteration method (see the block diagram in Fig. 3).

The upper limit of the interval of A, A_{max}, is calculated as the concentration of the more efficient eluting agent in the mobile phase for a required resolution $R_{s1,2}$ in isocratic elution chromatography[13]:

(1) by an iteration method if $n_1 \neq n_2$:

$$A_{max} = \left[\frac{k'_{02}\left(1 - \frac{2R_{s1,2}}{\sqrt{N}}\right)}{\frac{k'_{01}}{A_{max}^{n_1}} \cdot \left(1 + \frac{2R_{s1,2}}{\sqrt{N}}\right) + \frac{4R_{s1,2}}{\sqrt{N}}} \cdot \right]^{\frac{1}{n_2}} \tag{8}$$

(2) if $n_1 \approx n_2 = n$:

$$A_{max} = \left\{ \left[\frac{\sqrt{N}}{2R_{s1,2}} \cdot (k'_{02} - k'_{01}) - k'_{01} - k'_{02} \right] \cdot \frac{1}{2} \right\}^{\frac{1}{n}} \tag{9}$$

II. Gradient function according to eqn. 3 with $\varkappa = 1$; eqn. 2b applies for a given chromatographic system. Then,

$$B = \frac{\sqrt{N}(\log X_2 - \log X_1)}{2V_m R_{s(g)1,2}\left(\frac{k'_{01}}{X_1^{n_1}} + \frac{k'_{02}}{X_2^{n_2}} + 2\right)} \tag{10}$$

$$V'_{R(g)i} = \frac{\log X_i - A}{B} \tag{11}$$

Here,

$$X_j = [2.31BV_m n_j k'_{0j} + 10^{n_j A}]^{\frac{1}{n_j}} \tag{12}$$

and the meaning of the subscripts j, i, 1, 2, and of V_m and N is as above. Eqn. 10 is solved for B by an iteration method.

The upper limit of the interval of A, A_{max}, is calculated as the concentration of the more efficient eluting agent in the mobile phase necessary to achieve the resolution $R_{s1,2}$ under isocratic conditions. Using an approach analogous to that in ref. 13 for eqn. 2a. we can derive the following relationships based on eqn. 2b:

Eqn. 13 for $n_1 \neq n_2$, which is solved using an iteration method:

$$A_{max} = \frac{1}{n_1} \cdot \log \left[\frac{k'_{01}\left(1 + \frac{2R_{s1,2}}{\sqrt{N}}\right)}{\frac{k'_{02}}{10^{n_2 A_{max}}} \cdot \left(1 - \frac{2R_{s1,2}}{\sqrt{N}}\right) - \frac{4R_{s1,2}}{\sqrt{N}}} \right] \tag{13}$$

(2) eqn. 14 for $n_1 \approx n_2 = n$:

$$A_{max} = \frac{1}{n} \cdot \log \left\{ \left[\frac{\sqrt{N}}{2R_{s1,2}} (k'_{02} - k'_{01}) - k'_{01} - k'_{02} \right] \cdot \frac{1}{2} \right\} \tag{14}$$

The same approach can be used for other gradient functions. For example, if eqn. 2a applies and we use a logarithmic gradient function:

$$c = \log(A^{\frac{1}{\varkappa}} + BV)^{\varkappa} \tag{15}$$

we can calculate B, $V'_{R(g)i}$ and X_j from eqns. 5–7, as in case I above. The value of

A_{max}, however, should be calculated from the equation

$$A_{max} = 10^{A'_{max}} \tag{16}$$

where A'_{max} represents the value of A_{max} calculated from eqn. 13 or 14.

EXPERIMENTAL

The same gradient elution instrumentation was used as in Part X[17]. A reversed-phase column, packed with an octadecylsilica reversed phase and a mobile phase composed of water and methanol was treated as described in Part X[17], and the same samples, substituted alkyluracils and barbiturates, were also used.

RESULTS AND DISCUSSION OF THE VERIFICATION OF THE OPTIMIZATION APPROACH

Reversed-phase chromatography of substituted uracils and barbiturates on an octadecylsilica column using methanol and water as the components of the binary mobile phase was used for verification experiments. To illustrate the optimization approach suggested above, three model mixtures were chosen. The first mixture contained homologous lower alkyluracils, 3,6-dimethyluracil, 3-ethyl-6-methyluracil, 3-*n*-propyl-6-methyluracil and 3-*n*-butyl-6-methyluracil. The second mixture contained 3-*sec.*-butyl-6-methyluracil and 3-*tert.*-butyl-6-methyluracil in addition to the components of the first mixture. The third mixture was composed of barbital, heptobarbital, allobarbital, aprobarbital, butobarbital, hexobarbital and amobarbital.

For each of these mixtures, the concentrations of methanol in the mobile phase necessary to achieve the required resolution for each pair of the compounds with adjacent chromatographic bands were calculated first. An iteration method of solution of eqn. 13 was used. The separation of the most difficult to separate pair required the lowest concentration of methanol in the mobile phase, which represented the optimal concentration for isocratic elution, c_{opt}.

Then, the optimized conditions for gradient elution with a linear gradient function were calculated using eqns. 10–12. The required resolution of the most difficult to resolve pair of compounds should be obtained and the minimal elution volume of the last-eluted compound was required simultaneously. The calculated optimized gradient functions are shown in Fig. 4. The chromatographic experiments were performed under optimized isocratic and gradient elution conditions and the important retention data from these experiments are summarized and compared with calculated values in Tables I –III. The method of calculation of retention volumes, peak widths and resolution in reversed-phase chromatography under isocratic and gradient elution conditions was described in Parts IX[14] and X[17] and elsewhere[3,11,18].

The parameters k'_0 and n in eqn. 2 for the individual compounds in the chromatographic system used were determined from the values of the capacity ratios, k', in isocratic experiments using mobile phases with different concentrations of methanol (linear regression analysis of the experimental log k' *versus* c function; see ref. 17). These values of k'_0 and n are given in Tables I–III and were used in both optimization calculations and calculations of retention characteristics.

TABLE I

COMPARISON OF EXPERIMENTAL AND CALCULATED RETENTION VOLUMES, PEAK WIDTHS AND RESOLUTION FOR THE REVERSED-PHASE SEPARATION OF A MIXTURE OF HOMOLOGOUS SUBSTITUTED URACILS UNDER OPTIMIZED ISOCRATIC AND GRADIENT ELUTION CONDITIONS

Column: octadecylsilica chemically bonded on LiChrosorb Si-100 (10 μm), 300 × 4.2 mm; V_m = 3.20 ml, N = 3350. Mobile phase, methanol–water; flow-rate, 0.96 ml/min. Sample compounds: 1 = 3,6-dimethyluracil; 2 = 3-ethyl-6-methyluracil; 3 = 3-*n*-propyl-6-methyluracil; 4 = 3-*n*-butyl-6-methyluracil. The optimal concentration of methanol in the mobile phase (c_{opt}) was calculated from eqn. 13 for isocratic resolution, R_s = 1.95, of compounds 1 and 2 and the optimal values A_{opt} and B_{opt} for gradient elution according to the function given by eqn. 3 ($\varkappa$ = 1) were calculated for the required optimal conditions, $R_{s1,2}$ = 2.15, $V'_{R(g)4}$ = minimum, using eqns. 10–12. The parameters k'_0 and n in eqn. 2 were determined according to ref. 17 and the values of V'_R, w, R_s, $V'_{R(g)}$, $w_{(g)}$ and $R_{s(g)}$ (retention volumes, peak widths and resolution under isocratic and gradient elution conditions) were calculated according to refs. 14 and 17.

Compound	k'_0	n	A_{opt}	B_{opt}	$V'_{R(g)}$ *(ml)*		$w_{(g)}$ *(ml)*		$R_{s(g)}$	
					Calc.	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*
1	9.51	4.40	0.358	0.165	0.51	0.79	0.24	0.23		
2	15.63	3.69			1.05	1.37	0.26	0.24	2.15	2.47
3	29.98	3.31			1.74	2.05	0.27	0.26	2.60	2.72
4	67.20	3.25			2.40	2.72	0.27	0.30	2.44	2.39
			c_{opt} *(%, v/v* × 10^{-2}*)*		V'_R *(ml)*		*w (ml)*		R_s	
					Calc.	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*
1	9.51	4.40	0.509		0.17	0.38	0.23	0.25		
2	15.63	3.69			0.66	0.93	0.27	0.30	1.95	2.00
3	29.98	3.31			1.98	2.06	0.36	0.37	4.19	3.37
4	67.20	3.25			4.78	4.54	0.55	0.54	6.15	5.45

TABLE II

EXPERIMENTAL AND CALCULATED RETENTION VOLUMES, PEAK WIDTHS AND RESOLUTION FOR THE REVERSED-PHASE SEPARATION OF A MIXTURE OF LOWER ALKYL-SUBSTITUTED URACILS UNDER OPTIMIZED ISOCRATIC ELUTION CONDITIONS

Column, operating conditions, methods of calculation and meaning of symbols as in Table I. Sample compounds: 1 = 3,6-dimethyluracil; 2 = 3-ethyl-6-methyluracil; 3 = 3-*n*-propyl-6-methyluracil; 4 = 3-*sec.*-butyl-6-methyluracil; 5 = 3-*n*-butyl-6-methyluracil; 6 = 3-*tert.*-butyl-6-methyluracil. Conditions of optimization: isocratic elution, $R_{s4,5}$ = 1.95.

Compound	k'_0	n	c_{opt} *(%, v/v·*10^{-2}*)*	V'_R *(ml)*		*w (ml)*		R_s	
				Calc.	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*
1	9.51	4.40	0.392	0.57	0.93	0.26	0.28		
2	15.63	3.69		1.79	2.02	0.34	0.38	4.07	3.32
3	29.98	3.31		4.85	4.79	0.56	0.55	6.80	5.96
4	54.56	3.21		9.66	9.78	0.89	0.91	6.63	6.83
5	67.20	3.25		11.51	11.99	1.02	1.04	1.95	2.27
6	96.25	3.40		14.39	14.44	1.22	1.18	2.57	2.21

TABLE III

COMPARISON OF EXPERIMENTAL AND CALCULATED RETENTION VOLUMES, PEAK WIDTHS AND RESOLUTION FOR THE REVERSED-PHASE SEPARATION OF A MIXTURE OF BARBITURATES UNDER OPTIMIZED ISOCRATIC AND GRADIENT ELUTION CONDITIONS

Column, operating conditions, methods of calculation and meaning of symbols as in Table I. Sample compounds: 1 = barbital; 2 = heptobarbital; 3 = allobarbital; 4 = aprobarbital; 5 = butobarbital; 6 = hexobarbital; 7 = amobarbital. $N \approx 2330$. Conditions of optimization: isocratic elution, $R_{s1,2} = 1.60$; gradient elution, (a) $R_{s(g)1,2} = 1.70$; $V'_{R(g)7}$ = minimum; (b) $R_{s(g)6,7} = 1.75$; $V'_{R(g)1}$ = minimum.

Compound	k'_0	n	c_{opt}		V'_R *(ml)*		w *(ml)*		R_s	
			(%, v/v·10^{-2})		*Calc.*	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*
1	21.81	3.20	0.523		1.48	1.53	0.39	0.42		
2	58.44	3.71			2.15	2.08	0.44	0.47	1.60	1.24
3	69.44	3.55			3.11	3.03	0.52	0.52	2.00	1.92
4	106.96	3.66			4.18	4.14	0.61	0.61	1.89	1.96
5	187.41	3.78			6.38	6.16	0.79	0.75	3.14	2.97
6	252.29	3.77			8.69	8.10	0.98	0.92	2.61	2.32
7	617.73	4.29			11.36	10.80	1.21	1.06	2.44	2.73
			A_{opt}	B_{opt}	$V'_{R(g)}$ *(ml)*		$w_{(g)}$ *(ml)*		$R_{s(g)}$	
					Calc.	*Exptl.*	*Calc.*	*Exptl.*	*Calc.*	*Exptl.*
1	21.81	3.20	0.368*	0.061*	2.51	2.58	0.39	0.29		
2	58.44	3.71			3.17	3.27	0.39	0.29	1.70	2.38
3	69.44	3.55			3.76	3.96	0.41	0.29	1.48	2.38
4	106.96	3.66			4.26	4.56	0.41	0.29	1.23	2.07
5	187.41	3.78			4.98	5.32	0.41	0.30	1.76	2.58
6	252.29	3.77			5.53	5.95	0.41	0.30	1.34	2.10
7	617.73	4.29			5.79	6.29	0.40	0.31	0.64	1.11
1	21.81	3.20	0.523**	0.0082**	1.41	1.40	0.38	0.32		
2	58.44	3.71			2.00	1.86	0.42	0.35	1.48	1.37
3	69.44	3.55			2.82	2.67	0.48	0.37	1.82	2.28
4	106.96	3.66			3.66	3.55	0.53	0.43	1.66	2.23
5	187.41	3.78			5.23	4.98	0.62	0.48	2.73	3.14
6	252.29	3.77			6.72	6.32	0.70	0.55	2.26	2.60
7	617.73	4.29			7.99	7.70	0.75	0.57	1.75	2.46

* Conditions of optimization: (a).
** Conditions of optimization: (b).

The optimal (maximal) isocratic concentration of methanol necessary to achieve $R_s = 1.95$ for 3,6-dimethyluracil and 3-ethyl-6-methyluracil is 50% (v/v) (Table I). Fig. 4 (curve 1) shows the gradient function optimized for the required resolution, $R_{s(g)} = 2.15$, for these two compounds and the minimal retention volume of 3-*n*-butyl-6-methyluracil. The retention volume of the last-eluted compound in gradient elution chromatography is half that in optimized isocratic elution.

In the chromatography of the six-component mixture of lower alkyluracils, the separation of 3-*sec.*-butyl-6-methyluracil from 3-*n*-butyl-6-methyluracil is more difficult than the separation of the other pairs of compounds and requires a lower concentration of methanol in the mobile phase for isocratic elution than the separation

of the homologous mixture, *ca.* 40% (v/v) (Table II). If gradient elution is optimized for this resolution of the two butyl isomers and the minimal retention time of 3-*tert.*-butyl-6-methyluracil is required, the calculation gives the values $A = 0.392$ and $B = 0$, which represents isocratic elution with the same composition of mobile phase as above. Thus, gradient elution chromatography cannot diminish the time of separation for this mixture in comparison with isocratic elution.

The resolution of barbital and heptobarbital requires a lower concentration of methanol in the mobile phase under isocratic conditions than the separation of the other pairs from the mixture of barbiturates, 52% (v/v), for a resolution $R_s = 1.6$ (Table III). If a resolution of barbital and heptobarbital of $R_s = 1.7$ and a minimal retention volume of the last-eluted amobarbital are required, the optimization calculation yields $A = 0.368$ and $B = 0.061$ for the linear gradient function (curve 2 in Fig. 4) and the time of separation is almost half that in the optimized isocratic experiment.

The resolution of hexobarbital from amobarbital is too low, however (Fig. 5).

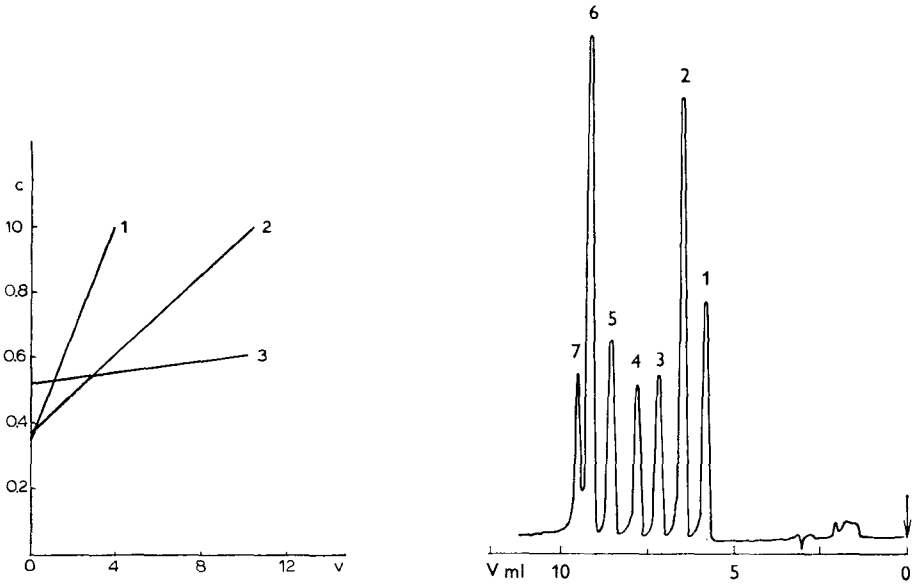

Fig. 4. Plots of optimized linear gradient functions for separation of homologous alkyluracils and barbiturates. Curve 1: $c = 0.358 + 0.165V$, optimized for separation of alkyluracils (optimization and operating conditions in Table I). Curve 2: $c = 0.368 + 0.061V$, optimized for separation of barbiturates; resolution of barbital and heptobarbital, $R_{s(g)} = 1.7$ required [optimization and operating conditions in Table III, conditions (a)]. Curve 3: $c = 0.523 + 0.0082V$, optimized for separation of barbiturates; resolution of hexobarbital and amobarbital, $R_{s(g)} = 1.75$ required [optimization and operating conditions in Table III, conditions (b)]. c = Concentration of methanol in the mobile phase at the inlet of the column, %, v/v·10^{-2}; V ml = volume of the mobile phase delivered on to the column from the beginning of gradient elution.

Fig. 5. Optimized gradient elution reversed-phase separation of a mixture of seven barbiturates. Numbers of compounds and operating conditions as in Table III [conditions (a)]; resolution of barbital and heptobarbital (compounds 1 and 2), $R_{s(g)} = 1.7$ required; elution according to linear gradient function (curve 2 in Fig. 4). V ml = volume of the eluate from the beginning of gradient elution. Detection: UV (254 nm), range 0.32 a.u.f.s.

Therefore, the optimization approach was repeated with other requirements: the resolution of hexobarbital and amobarbital should be $R_s = 1.75$ and the retention volume of the first-eluted compound, barbital, should be minimal. The calculated gradient function ($A = 0.523$ and $B = 0.0082$, curve 3 in Fig. 4) is much less steep and begins at a higher concentration of methanol in the mobile phase than in the previous optimization. The separation of all of the components in the mixture is satisfactory (Fig. 6) and the separation time is slightly increased (*ca.* 75% of the time for isocratic elution) in comparison with the above optimized experiment.

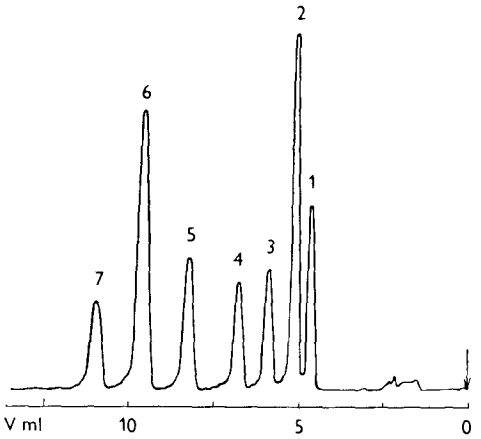

Fig. 6. Optimized gradient elution reversed-phase separation of a mixture of seven barbiturates. Numbers of compounds and operating conditions as in Table III [conditions (b)]; resolution of hexobarbital and amobarbital (compounds 6 and 7), $R_{s(g)} = 1.75$ required; elution according to linear gradient function (curve 3 in Fig. 4). V ml = volume of the eluate from the beginning of gradient elution. Detection: UV (254 nm); range 0.32 a.u.f.s.

The calculated and experimental retention characteristics under isocratic and gradient elution conditions, compared in Tables I–III, are in satisfactory agreement. The maximal difference in retention volumes is *ca.* 0.3–0.4 ml, which is an error comparable to that in the previous experiments with gradient elution reversed-phase chromatography[17]. The differences between the experimental and calculated peak widths were less than 0.1–0.15 ml.

Using the same optimization requirements as for the second gradient curve, we calculated the optimized logarithmic gradient function for the separation of the mixture of barbiturates (eqn. 15, $\varkappa = 1$). The parameters of this function are given in Table IV, where the profiles of the logarithmic and linear gradient functions are compared. It is obvious that the two gradient functions are essentially identical in the part of gradient useful for the separation of the mixture of barbiturates. This result seems to give further support to the opinion that the curvature of the gradient function is much less important than the slope and the initial conditions for the optimization of gradient elution.

TABLE IV

COMPARISON OF THE OPTIMIZED LINEAR AND LOGARITHMIC GRADIENT FUNCTIONS FOR THE REVERSED-PHASE SEPARATION OF A MIXTURE OF BARBITURATES

Operating conditions as in Table I, sample compounds in the mixture as in Table III. Conditions of optimization as in Table III, (b), *i.e.*, $R_{s(g)6,7} = 1.75$; $V'_{R(g)1}$ = minimum; $N \approx 2330$. Gradient function: (I), $c = A + BV$ (eqn. 3), $A = 0.523$, $B = 0.0082$, calculated with use of eqns. 10–13; (II) $c = \log(A + BV)^{\varkappa}$ (eqn. 15), $\varkappa = 1$, $A = 3.331$, $B = 0.067$, calculated with use of eqns. 5–7, 13 and 16. V = ml of the eluate from the beginning of the gradient elution; c = concentration of methanol (%, v/v·10^{-2}) in the mobile phase at the inlet of the column, corresponding to gradient functions I(c_I) and II(c_{II}).

Concentration	*V (ml)*										
	0	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*
c_I	0.523	0.531	0.539	0.547	0.555	0.564	0.572	0.580	0.588	0.596	0.605
c_{II}	0.523	0.531	0.540	0.548	0.556	0.564	0.572	0.580	0.587	0.595	0.602

SIMPLIFIED GENERAL APPROACH FOR OPTIMIZATION OF REVERSED-PHASE GRADIENT ELUTION CHROMATOGRAPHY

Let us now examine the possibilities for the optimization of gradient elution in the reversed-phase chromatography of an unknown sample mixture in a different way to that of Snyder *et al.*[11]. In this instance, significant simplifications of the theory must be accepted. Firstly, identical values of n in eqn. 2a will be assumed. Then, the equation for the difference in retention volumes, $\Delta V'_{R(g)}$, of two compounds, 1 and 2, can be written as follows:

$$\Delta V'_{R(g)} = V'_{R(g)2} - V'_{R(g)1} = \frac{1}{nB} \cdot \log\left(\frac{2.31nBV_m k'_{02} + 10^{nA}}{2.31nBV_m k'_{01} + 10^{nA}}\right) \tag{17}$$

Assuming a low value of A, we neglect the term 10^{nA} as a first approximation:

$$\Delta V'_{R(g)} \approx \frac{1}{nB} \cdot \log\left(\frac{k'_{02}}{k'_{01}}\right) \approx \frac{\log \alpha_0}{nB} \approx \frac{\log \alpha}{nB} \tag{18}$$

where α_0 denotes the retention ratio of compounds 1 and 2 in the pure, less efficient eluting agent in the binary mobile phase under isocratic conditions, which should be equal to the retention ratio α at an arbitrary composition of the binary mobile phase (α should not depend on the composition of the mobile phase in isocratic elution if $n_1 = n_2 = n$). In practice, however, n is not strictly constant for the members of a homologous series and, consequently, α depends to certain extent on the composition of the binary mobile phase. Therefore, it is reasonable to consider α in eqn. 18 as the retention ratio in the mobile phase with a composition corresponding to the arithmetic mean in the composition interval effective during the gradient elution.

In a homologous series, the plots of log k' *versus* the number of carbon atoms in aliphatic substituents are close to straight lines. Thus, the logarithms of the retention ratios remain approximately constant for two neighbouring homologues differ-

ing by one CH_2 group ($\log \alpha_c \approx$ constant). Further, it can be reasonably expected that the values of α_c and n do not depend much on the type of organic compound in a given system of reversed phase and binary mobile phase. Thus, in agreement with other workers, we found $n \approx 3.0$–4.0 and $\log \alpha_c \approx 0.3$ for octadecylsilica reversed phase in a water–methanol mobile phase containing 50% of methanol[17].

From eqn. 18, it follows that, with known $\log \alpha_c$ and n, we can estimate the slope of the concentration gradient in reversed-phase chromatography necessary for achievement of the required difference in retention volumes between neighbouring members of a homologous series. The difference in retention volumes, $\Delta V'_{R(g)}$ can be correlated with the resolution, $R_{s(g)}$, if we assume that the peak widths in gradient elution chromatography, $w_{(g)}$, are approximately equal to peak widths under isocratic conditions with $k' = 1$:

$$\Delta V'_{R(g)} = R_{s(g)} w_{(g)} \approx \frac{8 V_m R_{s(g)}}{\sqrt{N}} \tag{19}$$

where V_m is the volume of the mobile phase in the column and N is the number of plates (isocratic conditions).

The use of eqn. 18 is illustrated by Table V. The values of n and $\log \alpha_c$ were taken from the experiments in Part X[17]. The estimated values of the differences between the retention volumes of the two compounds differing by one CH_2 group in the aliphatic substituent are compared with the experimental values for three different slopes, B, of the linear gradient function (start of the gradient in pure water, $A = 0$, system I).

A similar correlation was shown for experiments performed by Elgass[20] and Engelhardt and Elgass[21] on the gradient elution chromatography of phenacyl esters of saturated fatty acids on a C_8 reversed-phase column using a binary mobile phase composed of water and acetonitrile. The values of n and $\log \alpha_c$ for this system were estimated from two isocratic experiments with C_8–C_{12} acids using 100% and 70% acetonitrile – water. The parameter n was calculated as the average of the ratio of the difference in $\log k'$ and the corresponding difference in the concentration of acetonitrile in the mobile phase; $\log \alpha_c$ was estimated as the arithmetic mean of the differences in $\log k'$ between two neighbouring homologous acids in the two mobile phases. These calculations are shown in the bottom section of Table V.

The experimental differences in the retention volumes for two neighbouring homologues in Table V are in satisfactory agreement with the estimated values, if we take into account the simplifications involved in the estimation approach.

CONCLUSIONS

The experimental results of reversed-phase gradient elution chromatography suggest a good validity of the optimization approaches described. The exact calculation procedure requires that the parameters of the function $k' = f(c)$ be known for sample compounds in a given chromatographic system. Under this condition, the calculations with the use of a computer or a programmable calculator make it possible to plan the conditions for gradient elution with a required resolution of a chosen pair of sample compounds with minimal retention volume of another chosen compound

TABLE V

OPTIMIZATION OF GRADIENT ELUTION OF HOMOLOGOUS MIXTURES IN REVERSED-PHASE CHROMATOGRAPHY

System I. Column: C_{18} on LiChrosorb Si-100 (10 μm). Mobile phase: methanol–water. Sample compounds: barbiturates, substituted uracils. The values of log α_c and n were taken as average values from experiments under isocratic conditions; log α_c is the average difference in log k' between two compounds differing by one CH_2 group in 50% methanol–water from the two series of compounds[17].

System II. Column: C_8 on LiChrosorb Si-100 (10 μm). Mobile phase: acetonitrile–water. Sample compounds: phenacyl esters of saturated fatty acids, n-C_6–C_{18}. The values of log α_c and n were taken from two isocratic experiments in 70% ($c = 0.7$) and 100% ($c = 1.0$) acetonitrile–water, as shown in the bottom section. These two experiments and two experiments with linear gradient elution were performed by Elgass[20] and Engelhardt and Elgass[21].

B represents the slope of linear gradient function [eqn. 3; $\varkappa = 1$, $A = 0$ (0–100% CH_3OH) in system I; $A = 0.7$ (70–100% CH_3CN) in system II].

System	*Compounds*	*Reversed phase*	*Mobile phase*	*Log* α'_c	n	B	$\Delta V'_{R(g)}$ *(ml)*	
							Calc.	*Exptl.*
I	Barbiturates; substituted uracils	C_{18}	CH_3OH–H_2O	0.3	3.5	0.069	1.2	0.8
						0.035	2.4	1.8
						0.017	5.0	4.0
II	Saturated fatty acid derivatives	C_8	CH_3CN–H_2O	0.1	3.9	0.0075	3.5	3.0
						0.015	1.7	1.6

Fatty acid	*Concentration of acetonitrile in the mobile phase (%, v/v·10⁻²)*				$P = \log k'_{0.7} - \log k'_{1.0}$	$n = \frac{P}{\Delta c}$
	$c = 0.7$		$c = 1.0$			
	log k'	$\Delta \log k'_{CH_2}$	*log* k'	$\Delta \log k'_{CH_2}$		
C_8	0.32		−0.77		1.09	3.63
		0.13		0.09		
C_{10}	0.58		−0.59		1.17	3.90
		0.125		0.07		
C_{12}	0.83		−0.45		1.28	4.27
Arithmetic mean	–	0.127*	–	0.08*	–	3.93

* Log $\alpha_c = 0.103$ (arithmetic mean of $\Delta \log k'_{CH_2}$ at $c = 0.7$ and $c = 1.0$).

(if this resolution can be achieved under isocratic conditions in the given system). The slope of the gradient in the reversed-phase chromatography of homologous mixtures for the achievement of a required difference in retention volumes between two neighbouring homologues can be estimated.

REFERENCES

1 C. Liteanu and S. Gocan, *Gradient Liquid Chromatography*, Wiley, Chichester, New York, 1974.
2 P. Jandera and J. Churáček, *J. Chromatogr.*, 192 (1980) 1.
3 L. R. Snyder, in C. Horváth (Editor), *Liquid Chromatography*, Academic Press, New York, London, in press.
4 H. Schwab, W. Rieman and P. A. Vaughan, *Anal. Chem.*, 29 (1957) 1357.
5 E. C. Freiling, *J. Phys. Chem.*, 61 (1957) 543.
6 S. Ohashi, N. Tsuji, Y. Ueno, M. Takeshita and M. Muto, *J. Chromatogr.*, 50 (1970) 349.

7 F. Molnár, A. Horváth and V. A. Khalkin, *J. Chromatogr.*, 26 (1967) 215.
8 D. L. Massart and W. Bossaert, *J. Chromatogr.*, 32 (1968) 195.
9 L. R. Snyder, *Chromatogr. Rev.*, 7 (1965) 1.
10 L. R. Snyder and D. L. Saunders, *J. Chromatogr. Sci.*, 7 (1969) 195.
11 L. R. Snyder, J. W. Dolan and J. R. Gant, *J. Chromatogr.*, 165 (1979) 3.
12 J. W. Dolan, J. R. Gant and L. R. Snyder, *J. Chromatogr.*, 165 (1979) 31.
13 P. Jandera, M. Janderová and J. Churáček, *J. Chromatogr.*, 148 (1978) 79.
14 P. Jandera and J. Churáček, *J. Chromatogr.*, 170 (1979) 1.
15 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 207.
16 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 223.
17 P. Jandera, J. Churáček and L. Svoboda, *J. Chromatogr.*, 174 (1979) 35.
18 P. J. Schoenmakers, H. A. H. Billiet, R. Tijssen and L. de Galan, *J. Chromatogr.*, 149 (1978) 519.
19 P. Jandera and J. Churáček, *J. Chromatogr.*, 93 (1974) 17.
20 H. Elgass, unpublished results.
21 H. Engelhardt and H. Elgass, *J. Chromatogr.*, 158 (1978) 249.