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GRADIENT ELUTION IN LIQUID CHROMATOGRAPHY

XI. INFLUENCE OF THE ADJUSTABLE GRADIENT PARAMETERS ON THE CHROMATOGRAPHIC BEHAVIOUR OF SAMPLE COMPOUNDS

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SUMMARY

The influence of the initial concentration of the stronger eluting component in the mobile phase and of the slope and shape of the gradient on important retention characteristics, such as retention volume, band width, selectivity, resolution and the position of elution bands in the chromatogram, was investigated both theoretically and experimentally, with particular attention to normal adsorption and reversed-phase chromatography using gradient elution in a binary solvent system. The different effects of the adjustable gradient parameters on the above retention characteristics are discussed.

INTRODUCTION

Gradient elution is generally accepted as the most efficient means for the solution of the so-called "general elution problem" in liquid chromatography[1]. Increasing requirements for the analysis of complex samples of naturally occurring compounds and for pollution analysis have emphasized the practical utility of gradient elution[2–4].

A better understanding of the influence of various adjustable gradient parameters on the separation process is useful when making a rational choice of the gradient profile for a given practical system. Further, the quantitative approach to the problem can be used as an aid to the identification of individual sample compounds in gradient elution chromatograms.

The main aim of gradient elution is to adjust adequately the retention of sample compounds during elution. Most practical separation problems can be solved using two-component (binary) solvent systems in which one component (solvent b) is a much stronger eluent than the other (solvent a), so that the capacity ratios of chromatographed compounds can be varied over a wide range by changing the proportion of the stronger eluent b in the mobile phase from 0 to 1.

The concentration of the stronger eluent in the mobile phase can be changed discontinuously by isocratic steps (so-called "stepwise elution"), continuously according to one monotonous mathematical function (straight lines or convex or concave

curves) or in several subsequent gradient steps with different concentration–time functions. Stepwise elution is discussed in a separate paper[5]. Elution consisting of several gradient steps is rather complex to describe quantitatively. It is meant as a "tailor-made" gradient profile for given separation problems requiring different changes of solvent composition in different parts of a chromatogram. The influence of the gradient profile on the chromatographic behaviour of sample compounds can be studied most adequately for single-curve gradients, which are by far the most frequently used and the simplest for generating gradient profiles.

This study is concerned with simple gradients described by a single mathematical function of the concentration (c) of the stronger eluting component in the mobile phase with time or, better, with the volume (V) of the eluate from the beginning of gradient elution. Various arbitrary mathematical functions can be used for this purpose; the most popular are, however, linear, exponential and logarithmic curves.

It is advantageous to express the mathematical form of the gradient function in such a way that three features of a gradient profile can be clearly distinguished: the initial concentration of the stronger eluting component in the mobile phase (A), the slope (B) and the shape ($\varkappa$) of the gradient[6]:

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

As the three parameters of the gradient function, A, B and $\varkappa$, can be adjusted independently one from the others in order to control separation, it is desirable to know how each of these parameters influences the important characteristics of gradient elution chromatographic behaviour (retention volumes, band width, resolution, etc.).

During the preparation of this manuscript we had the opportunity of studying the latest results of Snyder and co-workers on the above aspects of gradient elution, with particular emphasis on reversed-phase systems[7–9]. These workers used a similar approach and obtained results very similar to ours in many respects. Our treatment, however, is somewhat different and it may be useful to compare the results of the two approaches. In the following discussion the results of our previous experiments with gradient elution in normal adsorption and reversed-phase chromatography are used[6,10–13].

CHROMATOGRAPHIC BEHAVIOUR OF SAMPLE COMPOUNDS IN GRADIENT ELUTION EXPERIMENTS

Retention volume

A quantitative treatment of retention volumes and other characteristics in gradient elution chromatography requires that the relationship between the capacity ratios, k', of sample compounds and the concentration of the more efficient eluting component in the mobile phase, c, be known, at least in a simplified manner. As we have shown previously[14], two simple equations can be used to describe many practical chromatographic systems:

$$k' = k'_0 c^{-n} \tag{2}$$

for chromatography based on adsorption or ion-exchange mechanisms and

$$k' = k'_0 \cdot 10^{-cn} \tag{3}$$

for partition chromatography, reversed-phase chromatography and related systems. Here, k'_0 and n represent experimental parameters characteristic of a given column material, solute and binary mobile phase system and do not depend on the composition of the mobile phase.

Much can be discussed about the validity of eqns. 2 and 3 and deviations from these equations can be found in numerous practical systems. It should be stressed, however, that these equations provide a good fit to a great variety of practical chromatographic systems in the range of capacity ratios k' = 1–10.

The sample compounds in gradient elution chromatography migrate down the column with capacity ratios changing within the above range[9,13] and deviations from eqns. 2 and 3 for $k' > 10$ or $k' < 1$ are relatively small in practice. Some deviations at $k' < 1$ can be attributed to systematic errors in the measurement of V_m (the precision of the determination of k' is poor here). We found a good validity of eqn. 2 in different adsorption systems[10,12,15] and eqn. 3 was found to fit well in a variety of reversed-phase systems (see, for example, works cited in refs. 9, 13 and 14). Thus, the deviations from eqn. 3, such as those found by Schoenmakers *et al.*[16], are not very important in most instances.

We derived two equations making possible calculations of net retention volume, $V'_{R(g)}$, for gradient elution chromatography[6,13] using the gradient function defined by eqn. 1 for systems where eqn. 2 or 3 can be applied, provided the parameters k'_0 and n in eqn. 2 or 3 are known for the compounds to be separated.

We have verified the validity of the relationships derived in adsorption chromatography of azo dyes on silica with gradients in various binary solvent systems[10,12] and in the reversed-phase chromatography of xanthine alkaloids, barbiturates and substituted uracils with a concentration gradient of methanol in a methanol–water mobile phase[13]. These relationships were further verified by Hartwick *et al.*[17] for the reversed-phase chromatography of nucleosides and bases.

The approach of Snyder and co-workers[7–9] is based on the so-called linear solvent strength gradients[18], in which the capacity ratios for individual sample compounds decrease during gradient elution according to the equation

$$\log k' = \log k_a - b\left(\frac{t}{t_0}\right) \tag{1a}$$

where k_a refers to the k' value at the beginning of gradient elution (*i.e.*, for the starting eluent). In reversed-phase chromatography, Snyder and co-workers make use of eqn. 3, as we did[7–9,13]. A linear solvent strength gradient in reversed-phase chromatography means a linear change of c with time (or V)[7,9] *i.e.*, eqn. 1 can be used for $\varkappa = 1$. Combining this equation with eqn. 3 and comparing it with eqn. 1a, we obtain

$$\log k' = \log k'_0 - nA - nBV \tag{1b}$$

$$\log k_a = \log k'_0 - nA \tag{1c}$$

and

$$b = nBt_0F = nBV_m \tag{1d}$$

where F is the flow-rate of the mobile phase and V_m is the column void volume.

In this instance, the two approaches yield essentially identical equations for retention volumes (compare the derivations in refs. 6, 7, 9 and 13). The parameter b, used by Snyder and co-workers, involves both the adjustable slope of the concentration gradient and the parameter n, which is a property of the sample compound and the two solvents, a and b, forming the gradient.

The parameter n can be adjusted by changing the nature of solvent b or a. In our approach, the parameter B is the property of the gradient profile only and does not depend on the chromatographic system.

Good validity of the equation derived by Snyder and co-workers for reversed-phase gradient elution has been obtained[8,19]. The approach of Snyder and co-workers yields identical equations for retention volume in reversed-phase and in normal adsorption chromatography, provided a linear solvent strength gradient is employed. In normal adsorption chromatographic systems, where eqn. 2 can be applied, this means an exponential gradient, as can easily be demonstrated. Here, Snyder and co-workers' approach and the resulting equation differ essentially from our treatment using gradients according to eqn. 1. However, similar conclusions concerning the influence of the gradient profile on retention behaviour in both reversed-phase and normal adsorption systems result from our approach and that of Snyder and co-workers.

From the equations for retention volumes in gradient elution chromatography according to gradient function given by eqn. 1, *i.e.* in different possible linear and non-linear solvent strength gradients[6,13], it can clearly be seen that for a given gradient function the ratio $V'_{R(g)}/V_m$ remains constant when A, $\varkappa$ and BV_m are kept constant (in a given separation system of stationary phase and solvents a, b, the parameters k'_0 and n in eqns. 2 and 3 are always constant). This means that sample compounds are eluted with the same retention volume (in column void volume units) and with the mobile phase of the same composition, even if the flow-rate of the mobile phase is changed, but B (in % of solvent b per millilitre of the eluent) is kept constant. Retention behaviour in agreement with this theoretical conclusions was found experimentally by Engelhardt and Elgass[20]. On the other hand, if we require a constant $V'_{R(g)}/V_m$ ratio when the length or diameter of the column is changed, B should be adjusted so as to keep BV_m constant (constant parameter b of Snyder and co-workers, eqn. 1a), which is in agreement with suggestions in refs. 7 and 9.

For convenience, in the following discussion we shall refer to the slope of the gradient as represented by B, the shape (curvature) by $\varkappa$ and the initial concentration of solvent b at the beginning of the gradient by A.

According to the relationships for retention volumes derived using the two approaches discussed, the net retention volumes in gradient elution chromatography, $V'_{R(g)}$, decrease with increasing parameters A and B in eqn. 1. As predicted by this theory, a linear decrease in log $V'_{R(g)}$ with increasing log B was found to apply well in the adsorption chromatography of azo dyes in different solvent systems[10,12], but could also be found in the reversed-phase chromatography of alkaloids, barbiturates and substituted uracils, where eqn. 3 applies[13] (Figs. 1–3).

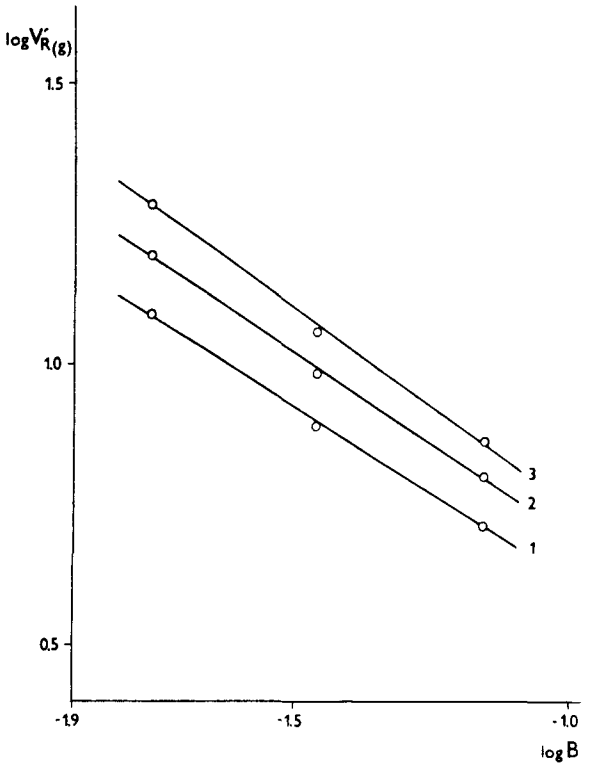

Fig. 1. Logarithmic plots of retention volumes $V'_{R(g)}$, of alkaloids *versus* the slope of the gradient, B, in reversed-phase gradient elution chromatography. Column: reversed-phase C_{18} on LiChrosorb Si-100 (10 μm), 300 × 4.2 mm; V_m = 3.2 ml. Mobile phase: gradient of methanol in water; $A = 0$; $\varkappa = 1$ (eqn. 1); flow-rate, 0.98 ml/min. $V'_{R(g)}$ in ml, B in % CH_3OH per millilitre of the mobile phase·10^{-2}. Compounds: 1 = theobromine; 2 = theophylline; 3 = caffeine.

The parameter A influences the retention volumes of later eluted peaks far less than those of earlier eluted peaks[7].

The parameter $\varkappa$ characterizes the shape of the gradient in such a way that as $\varkappa$ increases from 0 to 1, the concavity of the gradient diminishes; the gradient profile is linear at $\varkappa = 1$ and becomes more and more convex as $\varkappa$ increases further. Thus, with increasing $\varkappa$ and A and B constant, the actual concentration of more efficient eluting agent at a given time (or volume of the eluate) decreases and the net retention volumes, $V'_{R(g)}$, of sample compounds increase, as can be seen from the example of adsorption chromatography of azo dyes in Fig. 4.

Band width

The width of a peak in gradient elution chromatography results from three effects: the spreading of the solute band with time as it moves along the column, the instantaneous value of the capacity ratio at the moment of elution of the peak maximum and the compression of the band resulting from the fact that the front of the band moves in the mobile phase with a lower eluting strength than the end of the band[7–9,18].

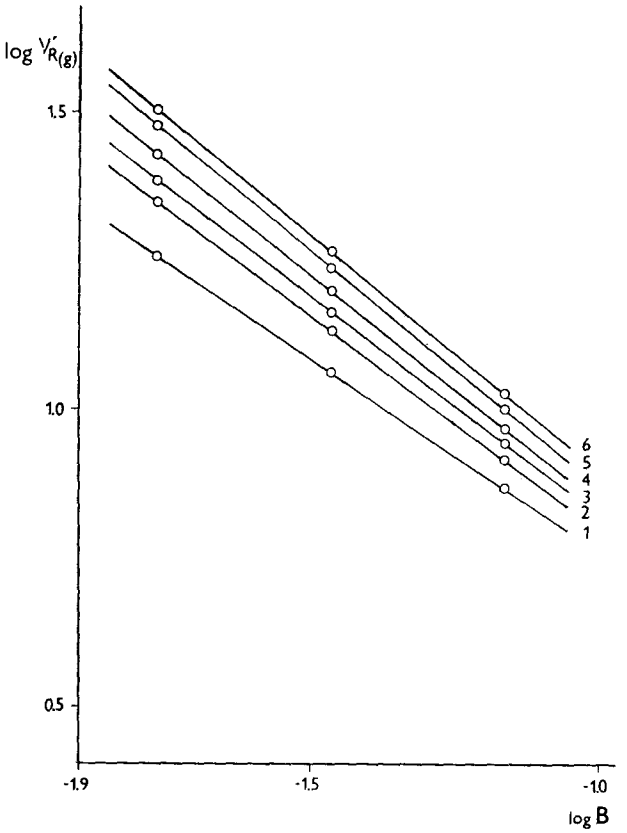

Fig. 2. Logarithmic plots of retention volumes, $V'_{R(g)}$, of barbiturates *versus* the slope of the gradient, B, in reversed-phase gradient elution chromatography. Conditions as in Fig. 1. Compounds: 1 = barbital; 2 = heptobarbital; 3 = allobarbital; 4 = aprobarbital; 5 = butobarbital; 6 = hexobarbital.

Neglecting the last effect, we derived the relationship for the band width, $w_{(g)}$, in gradient elution chromatography, where eqn. 2 or 3 applies[6].

The widths of peaks calculated when the band compression effect was neglected were approximately 10–20% higher than the experimental values measured in the gradient elution chromatography of azo dyes on silica[10,12] and in the gradient elution reversed-phase chromatography of barbiturates, substituted xanthines and uracils[13].

Snyder and Saunders[21] presented a method of calculation of the band compression factor, which expresses quantitatively the band compression effect, for linear solvent strength gradients, where this is only a function of b from eqn. 1a and can be determined for the corresponding value of b using a plot constructed by Snyder and co-workers[7,9,21] or by direct calculation[9]. The results of the experimental verification of the band width calculations according to Snyder presented in ref. 9 indicate that the calculated band widths are underestimated for $b > 0.2$, where the band compression factor acquires values lower than 0.8, while the agreement is satisfactory for $b < 0.2$. Thus, taking into account that complete neglect of band compression yields

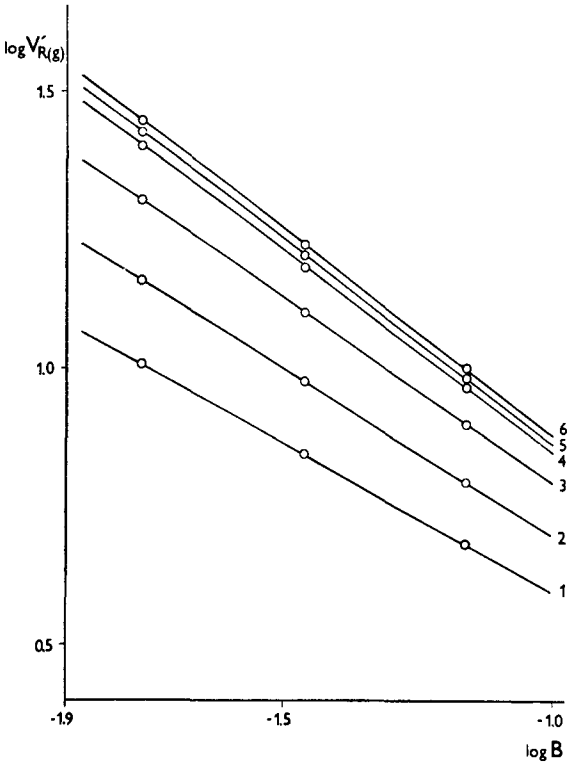

Fig. 3. Logarithmic plots of retention volumes, $V'_{R(g)}$, of substituted uracils *versus* the slope of the gradient, B, in reversed-phase gradient elution chromatography. Conditions as in Fig. 1. Compounds: 1 = 3,6-dimethyluracil; 2 = 3-ethyl-6-methyluracil; 3 = 3-*n*-propyl-6-methyluracil; 4 = 3-*sec.*-butyl-6-methyluracil; 5 = 3-*n*-butyl-6-methyluracil; 6 = 3-*tert.*-butyl-6-methyluracil.

overestimated calculated band widths, it might be expected that the use of a semi-empirical band compression factor of 0.8–0.9 for practically used gradient slopes would not introduce gross errors in comparison with more accurate determinations from the above-mentioned plot or calculation. However, this simplified approach would require further investigation.

Like retention volumes, peak widths in gradient elution chromatography decrease to a certain extent with increase in A and B in eqn. 1, as expected from both Snyder and co-workers' and our approaches, but this effect is less distinct here than with retention volumes. The rate of decrease in $w_{(g)}$ with increase in B in the adsorption chromatography of azo dyes on silica was dependent on the shape of the gradient ($\varkappa$) and the peak widths showed a tendency to reach constant values at high B[11]. In the reversed-phase chromatography of barbiturates, substituted xanthines and uracils, the peak widths, $w_{(g)}$ were far less influenced by the gradient slope, B[13]. Increasing the initial concentration of methanol in the mobile phase had a negligible effect on the peak width, and the values of $w_{(g)}$ were very similar for all the compounds studied with little regard to the differences in retention[13]. This is also in fair agreement with the behaviour predicted from Snyder's theory of linear solvent strength gradients[9].

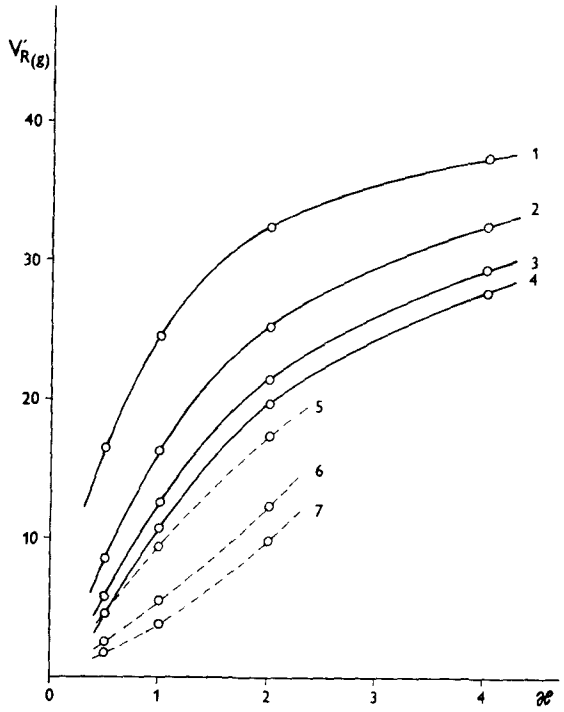

Fig. 4. Plots of the experimental retention volumes, $V'_{R(g)}$ (ml), of four azo compounds in gradient elution adsorption chromatography on Porasil A *versus* $\varkappa$ in eqn. 1. Column: Porasil A (60) (37–75 μm), 400 × 3 mm; V_m = 2.00 ml. Mobile phase: gradient of ethyl acetate in cyclohexane (curves 1–4) and of *n*-propanol in *n*-heptane (curves 5–7). Flow-rate, 0.64 ml/min; $A = 0$; $B = 0.026$ (% of alcohol per millilitre of mobile phase·10^{-2}). Compounds: dimethylamide (curve 1); diethylamide (curves 2 and 5); di-(*n*-propyl)amide (curves 3 and 6) and di-(*n*-butyl)amide (curves 4 and 7) of *p*-N,N-dimethyl-*p'*-aminobenzeneazobenzoic acid.

The influence of the shape (curvature) of the gradient on peak widths is complex and depends on the character of the compound and the mobile phase (parameters k'_0 and n in eqn. 2 or 3) and on the other parameters of the gradient (A, B). All of these parameters play a certain role in the determination of the actual composition of the eluent at the moment at which the peak maximum is eluted. Experimental plots of $w_{(g)}$ *versus* $\varkappa$ for four azo compounds in gradient elution chmatography on silica are shown in Fig. 5. In reversed-phase chromatographic experiments with gradient elution of barbiturates and other compounds, a change in the shape of the gradient ($\varkappa$) had only a minor effect on peak widths[13].

Thus, the parameters A, B and $\varkappa$ of the gradient function affected the peak widths in gradient elution in a relatively low-efficiency adsorption chromatographic system (non-linear solvent strength gradients) far more than those in a high-efficiency reversed-phase chromatographic system (linear solvent strength gradients), where the experimental peak widths were approximately constant (to within 20–40%) for all of the compounds and gradient functions studied[13].

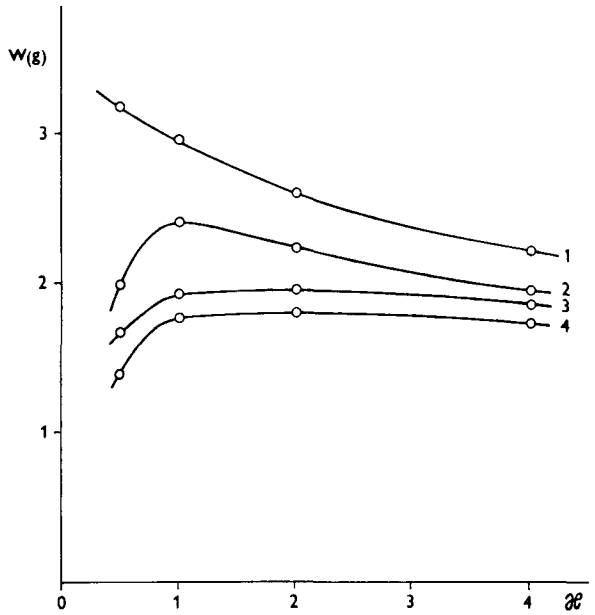

Fig. 5. Plots of the experimental peak widths, $w_{(g)}$, of four azo compounds *versus* $\varkappa$ in gradient elution adsorption chromatography on Porasil A. Conditions and numbers of compounds as in Fig. 4. Gradient of ethyl acetate in cyclohexane.

Retention ratio

By analogy with isocratic elution chromatography, we can calculate an apparent retention ratio in gradient elution chromatography, $\alpha_{(g)}$, as the ratio of net retention volumes for a pair of sample compounds 1 and 2, $V'_{R(g)1}$, $V'_{R(g)2}$, after substitution from the appropriate relationships[6,7,13] into

$$\alpha_{(g)} = \frac{V'_{R(g)2}}{V'_{R(g)1}} \tag{4}$$

In contrast to the isocratic retention ratio, $\alpha_{(g)}$ defined in this way does not represent the function of the parameters k'_{01}, k'_{02}, n_1 and n_2 only, but depends also on the parameters of the gradient (A, B and $\varkappa$). As a rule, relatively minor differences in n can be expected for most structurally similar compounds and their isocratic retention ratio is virtually independent of the composition of the mobile phase prepared from a given pair of solvents[9,10,14,15]. In systems where eqn. 3 applies, a more or less significant increase in $\alpha_{(g)}$ would be expected with decreasing B and increasing A in eqn. 1, and this could be observed in practical reversed-phase systems[13] (Table I). [Increasing A diminishes the retention volumes, whereas it does not cause great differences in $V'_{R(g)}$.]

In systems described by eqn. 2, $\alpha_{(g)}$ is not expected to depend on the slope B and decreases with increase in the parameter $\varkappa$ in eqn. 1, provided $A = 0$ (ref. 6).

TABLE I

INFLUENCE OF THE INITIAL CONCENTRATION OF THE MORE EFFICIENT ELUTING AGENT IN THE MOBILE PHASE AND OF THE SLOPE OF THE GRADIENT FUNCTION (PARAMETERS A AND B IN EQN. 1) ON THE RETENTION RATIOS OF BARBITURATES IN GRADIENT ELUTION REVERSED-PHASE CHROMATOGRAPHY

Column, mobile phase gradient and experimental conditions as in Table III. The retention ratios, $\alpha_{(g)i/1}$ of all of the compounds are relative to barbital (compound 1). The experimental values are compared with those calculated using eqn. 4 in ref. 13. Compounds: 1 = barbital, $k'_0 = 21.81$, $n = 3.2$; 2 = heptobarbital, $k'_0 = 58.44$, $n = 3.71$; 3 = allobarbital, $k'_0 = 69.44$, $n = 3.55$; 4 = aprobarbital, $k'_0 = 106.96$, $n = 3.66$; 5 = butobarbital, $k'_0 = 187.41$, $n = 3.78$; 6 = hexobarbital, $k'_0 = 252.29$, $n = 3.77$; 7 = pentobarbital, $k'_0 = 470.65$, $n = 4.07$ [k'_0 and n are the experimental parameters in eqn. 3 evaluated by linear regression from experimental $\log k' = f(c)$ plots; c = concentration of methanol in the mobile phase in isocratic elution experiments].

A	*Retention ratio*	*B*					
		0.06872		*0.03436*		*0.01718*	
		$\alpha_{(g)}$ *calc.*	$\alpha_{(g)}$ *exptl.*	$\alpha_{(g)}$ *calc.*	$\alpha_{(g)}$ *exptl.*	$\alpha_{(g)}$ *calc.*	$\alpha_{(g)}$ *exptl.*
0	$\alpha_{(g)2/1}$	1.13	1.12	1.19	1.17	1.26	1.24
	$\alpha_{(g)3/1}$	1.21	1.20	1.28	1.27	1.37	1.36
	$\alpha_{(g)4/1}$	1.29	1.28	1.38	1.37	1.50	1.49
	$\alpha_{(g)5/1}$	1.39	1.38	1.51	1.50	1.67	1.67
	$\alpha_{(g)6/1}$	1.46	1.46	1.60	1.59	1.78	1.78
	$\alpha_{(g)7/1}$	1.50	–	1.66	1.66	1.90	1.90
0.1	$\alpha_{(g)2/1}$	1.16	1.14	1.23	1.22	1.32	1.31
	$\alpha_{(g)3/1}$	1.26	1.25	1.35	1.34	1.47	1.49
	$\alpha_{(g)4/1}$	1.35	1.35	1.47	1.46	1.64	1.68
	$\alpha_{(g)5/1}$	1.48	1.48	1.64	1.64	1.87	1.94
	$\alpha_{(g)6/1}$	1.57	1.56	1.75	1.75	2.02	2.09
	$\alpha_{(g)7/1}$	1.61	1.61	1.84	1.84	2.18	2.26

Table II shows experimental results from a normal adsorption system, which are in agreement with these considerations.

For certain pairs of compounds, however, n_1 differs significantly from n_2 and, depending on the values of k'_{01} and k'_{02}, $\alpha = 1$ under isocratic conditions for a certain composition of the binary mobile phase, as we have discussed in detail and demonstrated on practical examples elsewhere [15]. In gradient elution chromatography the situation is analogous and $\alpha_{(g)} = 1$ for such a pair of compounds for certain combinations of A, B and $\varkappa$, where these compounds cannot be separated. This situation is illustrated by two practical examples of isocratic and gradient reversed-phase separations[13] in Table III and has been discussed qualitatively by Snyder[9].

A knowledge of the parameters of the gradient for which $\alpha_{(g)} = 1$ may be useful for appropriate selection of gradients for certain separation problems. These values can be calculated after substitution of the appropriate relationships into eqn. 4 while setting $\alpha_{(g)} = 1$. The resulting equations usually must be solved by using approximate methods and the relationships for the required parameters can be expressed in the explicit form in special instances only. Thus, if eqn. 2 applies in a given system and the gradient is started with pure solvent a, we obtain the value of B for which $\alpha_{(g)} = 1$:

TABLE II

INFLUENCE OF THE SHAPE OF THE GRADIENT FUNCTION (PARAMETER $\varkappa$ IN EQN. 1) ON THE RETENTION RATIOS OF AZO COMPOUNDS IN GRADIENT ELUTION ADSORPTION CHROMATOGRAPHY ON SILICA

Column: Porasil A (37–75 μm), 400 × 3.0 mm; V_m = 2.0 ml. Gradient of ethyl acetate in cyclohexane according to the gradient function given by eqn. 1 with different parameters B and $\varkappa$ (A = 0). $\alpha_{(g)2/1}$, $\alpha_{(g)3/1}$ and $\alpha_{(g)4/1}$ are the retention ratios for compounds 2–4 relative to compound 1. The experimental values are compared with those calculated using eqn. 10 in ref. 6. Flow-rate of mobile phase, 0.64 ml/min; detection, photometric (440 nm). Compounds, amides of *p*-N,N-dimethyl-*p'*-aminobenzeneazobenzoic acid: 1 = di(*n*-butyl) amide, k'_0 = 0.242, n = 1.68; 2 = di(*n*-propyl)-amide, k'_0 = 0.330, n = 1.68; 3 = diethylamide, k'_0 = 0.65, n = 1.76; 4 = dimethylamide, k'_0 = 1.94, n = 1.93 [k'_0 and n are the experimental parameters in eqn. 2 evaluated by linear regression from experimental log k' = f (log c) plots. The average value of n = 1.86 was taken for calculations of $\alpha_{(g)}$].

$\varkappa$	*Retention ratio*	*B*							
		0.00162		*0.00649*		*0.02597*		*0.1039*	
		$\alpha_{(g)}$ *calc.*	$\alpha_{(g)}$ *exptl.*	$\alpha_{(g)}$ *calc.*	$\alpha_{(g)}$ *exptl.*	$\alpha_{(g)}$ *calc.*	$\alpha_{(g)}$ *exptl.*	$\alpha_{(g)}$ *calc.*	$\alpha_{(g)}$ *exptl.*
0.5	$\alpha_{(g)2/1}$	1.24	1.25	1.24	1.24	1.24	1.25	1.24	1.19
	$\alpha_{(g)3/1}$	1.86	1.84	1.86	1.86	1.86	1.87	1.86	1.76
	$\alpha_{(g)4/1}$	3.68	–	3.68	3.57	3.68	3.62	3.68	3.50
1.0	$\alpha_{(g)2/1}$	1.16	1.15	1.16	1.16	1.16	1.17	1.16	1.16
	$\alpha_{(g)3/1}$	1.52	1.49	1.52	1.50	1.52	1.52	1.52	1.52
	$\alpha_{(g)4/1}$	2.41	2.18	2.41	2.22	2.41	2.29	2.41	2.39
2.0	$\alpha_{(g)2/1}$	1.09	1.08	1.09	1.09	1.09	1.09	1.09	1.09
	$\alpha_{(g)3/1}$	1.29	1.25	1.29	1.27	1.29	1.28	1.29	1.28
	$\alpha_{(g)4/1}$	1.70	1.59	1.70	1.62	1.70	1.64	1.70	1.66
4.0	$\alpha_{(g)2/1}$	1.05	1.05	1.05	1.05	1.05	1.06	1.05	1.05
	$\alpha_{(g)3/1}$	1.15	1.15	1.15	1.15	1.15	1.16	1.15	1.15
	$\alpha_{(g)4/1}$	1.35	1.34	1.35	1.34	1.35	1.35	1.35	1.35

$$B_{(\alpha=1)} = \frac{1}{V_m}\left\{\frac{[k'_{01}(\varkappa n_1 + 1)]^{(\varkappa n_2+1)}}{[k'_{02}(\varkappa n_2 + 1)]^{(\varkappa n_1+1)}}\right\}^{\frac{1}{\varkappa(n_1-n_2)}} \tag{5}$$

For the systems described by eqn. 3, we can write under analogous conditions an approximate equation, assuming large k'_0 values and $\varkappa = 1$:

$$B_{(\alpha=1)} \approx \frac{1}{2.31\ V_m} \cdot \frac{(k'_{02}n_2)^{\frac{n_1}{n_2-n_1}}}{(k'_{01}n_1)^{\frac{n_2}{n_2-n_1}}} \tag{6}$$

Resolution

As the main aim of chromatography is to separate substances, resolution is the most important characteristic of the chromatographic process. The resolution of two compounds 1 and 2 in gradient elution chromatography, $R_{s(g)}$, can be defined by analogy with isocratic elution as

$$R_{s(g)} \approx \frac{V'_{R(g)2} - V'_{R(g)1}}{w_{(g)2}} \tag{7}$$

TABLE III

INFLUENCE OF PARAMETERS A AND B OF THE GRADIENT FUNCTION (EQN. 1) ON THE ELUTION SEQUENCE IN GRADIENT ELUTION REVERSED-PHASE CHROMATOGRAPHY

Column: octadecylsilica bonded on LiChrosorb Si-100 (10 μm), 300 × 4.2 mm; V_m = 3.2 ml. Isocratic elution: mobile phase composed of methanol (different concentrations, c) in water; k'_1–k'_4 are experimental capacity ratios for compounds 1–4; $\alpha_{2/1}$, $\alpha_{4/3}$ are experimental retention ratios for compounds 2, 1 and 4, 3, respectively. Gradient elution: gradient of methanol in water according to the gradient function given by eqn. 1 with different parameters A and B ($\varkappa = 1$). $V'_{R(g)1}$–$V'_{R(g)4}$ are experimental net retention volumes for compounds 1–4; $\alpha_{(g)2/1}$, $\alpha_{(g)4/3}$ are retention ratios for compounds 2, 1 and 4, 3, respectively, found experimentally and calculated from eqn. 4 in ref. 13. Flow-rate of the mobile phase, 0.97 ml/min; detection, UV (254 nm), 0.16 a.u.f.s. Compounds: 1 = caffeine, k'_0 = 32.28, n = 3.78; 2 = barbital, k'_0 = 21.81, n = 3.20; 3 = 3-n-butyl-6-methyluracil, k'_0 = 67.20, n = 3.25; 4 = aprobarbital, k'_0 = 106.96, n = 3.66 [k'_0 and n are the experimental parameters of eqn. 3 evaluated by linear regression from experimental $\log k' = f(c)$ plots].

Isocratic elution

% CH_3OH (c)	k'_1	k'_2	$\alpha_{2/1}$	k'_3	k'_4	$\alpha_{4/3}$
20	5.82	5.12	0.88	21.89	21.06	0.96
30	1.78	2.29	1.29	7.49	8.42	1.12
40	0.90	1.16	1.29	3.24	3.47	1.07

Gradient elution

A	B	$V'_{R(g)1}$ (ml)	$V'_{R(g)2}$ (ml)	$\alpha_{(g)2/1}$		$V'_{R(g)3}$ (ml)	$V'_{R(g)4}$ (ml)	$\alpha_{(g)4/3}$	
				Exptl.	Calc.			Exptl.	Calc.
0	0.06872	7.26	7.25	1.00	1.02	9.58	9.28	0.97	1.00
	0.03436	11.39	11.56	1.01	1.00	15.97	15.88	0.99	1.01
	0.01718	19.21	17.92	0.93	0.96	26.63	26.75	1.00	1.04
0.1	0.06872	5.64	5.84	1.04	1.03	8.02	7.90	0.98	0.99
	0.03436	8.72	8.90	1.02	1.01	13.23	13.03	0.98	1.01
	0.01718	13.11	12.36	0.94	0.97	20.65	20.79	1.01	1.04

If the relationship between the capacity ratios of sample compounds and composition of the mobile phase is given by eqn. 2, the introduction of the corresponding relationships for $V'_{R(g)}$ and $w_{(g)}$ yields the following relationship for resolution:

$$R_{s(g)} = \underbrace{\frac{\sqrt{N_2}}{4}}_{\text{I}} \cdot \underbrace{\frac{\alpha_{(g)} - 1}{\alpha_{(g)}}}_{\text{II}} \cdot$$

$$\cdot \underbrace{\frac{(\varkappa n_2 + 1)Bk'_{02}V_m + A^{\frac{\varkappa n_2+1}{\varkappa}} - A^{\frac{1}{\varkappa}}\left[(\varkappa n_2 + 1)Bk'_{02}V_m + A^{\frac{\varkappa n_2+1}{\varkappa}}\right]^{\frac{\varkappa n_2}{\varkappa n+1}}}{V_m B\left\{\left[(\varkappa n_2 + 1)Bk'_{02}V_m + A^{\frac{\varkappa n_2+1}{\varkappa}}\right]^{\frac{\varkappa n_2}{\varkappa n_2+1}} + k'_{02}\right\}}}_{\text{III}}$$

N_2 is the number of plates for compound 2, which is assumed not to depend significantly on the composition of the mobile phase.

By analogy with isocratic elution chromatography, three terms for different contributions to the resolution can be distinguished: I, the efficiency, is essentially the same as in isocratic elution operation; II, the selectivity, employs $\alpha_{(g)}$ instead of α; and III, the capacity, is a function only of the retention of compound 2 in gradient elution work.

Introducing the relationship for $\alpha_{(g)}$ into eqn. 8, we can express resolution as the function of the parameters k'_{01}, n_1, k'_{02} and n_2 of the two chromatographed compounds (eqns. 2) and of A, B and $\varkappa$:

$$R_{s(g)} = \frac{\sqrt{N_2}}{4V_m B} \times$$

$$\times \frac{\left[(\varkappa n_2 + 1)Bk'_{02}V_m + A^{\frac{\varkappa n_2+1}{\varkappa}}\right]\left[\frac{1}{\varkappa n_2+1} - \right](\varkappa n_1 + 1)Bk'_{01}V_m + A^{\frac{\varkappa n_2+1}{\varkappa}}\Big]^{\frac{1}{\varkappa n_1+1}}}{1 + k'_{02}\left[(\varkappa n_2 + 1)Bk'_{02}V_m + A^{\frac{\varkappa n_2+1}{\varkappa}}\right]^{-\frac{\varkappa n_1}{\varkappa n_2+1}}} \quad (9)$$

In systems described by eqn. 3, relationships analogous to eqns. 8 and 9 can be derived in the same manner ($\varkappa = 1$):

$$R_{s(g)} = \underbrace{\frac{\sqrt{N_2}}{4}}_{\text{I}} \cdot \underbrace{\frac{\alpha_{(g)} - 1}{\alpha_{(g)}}}_{\text{II}} \cdot \underbrace{\frac{\log(2.31n_2BV_mk'_{02} + 10^{n_2A}) - n_2A}{n_2BV_m[1 + k'_{02}(2.31n_2BV_mk'_{02} + 10^{n_2A})^{-1}]}}_{\text{III}} \quad (10)$$

and

$$R_{s(g)} = \frac{\sqrt{N_2}}{4V_m B} \times$$

$$\times \frac{\frac{1}{n_2} \cdot \log(2.31n_2BV_mk'_{02} + 10^{n_2A}) - \frac{1}{n_1} \cdot \log(2.31n_1BV_mk'_{01} + 10^{n_1A})}{1 + k'_{02}(2.31n_2BV_mk'_{02} + 10^{n_2A})^{-1}} \quad (11)$$

The influence of A, B and $\varkappa$ on the resolution in gradient elution chromatography depends on combination of the parameters k'_{01}, k'_{02}, n_1 and n_2 of the two compounds 1 and 2, like the influence of the concentration of the more efficient eluting agent in the mobile phase on the resolution in isocratic elution chromatography.

As has been discussed above, there are certain combinations of A, B and $\varkappa$ which may yield $\alpha_{(g)} = 1$ and no resolution in gradient elution chromatography. In such an instance, as one of A and B increases while the other two parameters are held constant, the capacity term III decreases and the selectivity term II in eqn. 8 or 10 decreases first to zero [$\alpha_{(g)} = 1$] and then increases again, as when the concentration of the more efficient eluting agent in the mobile phase is increased in isocratic elution chromatography[15].

Consequently, a maximum occurs on the $R_{s(g)} = f(B)$ or $R_{s(g)} = f(A)$ curve, which can be calculated by solving the equation $dR_{s(g)} = 0$ for one of the above parameters. For example, the value of $B_{(max)}$ for maximum resolution can be solved by an approximate method using the relationship obtained from eqn. 9 for $A = 0$ and a constant value of $\varkappa$, in the following form:

$$B_{(max)} = \left\{ \frac{k'_{02}\left(\frac{\varkappa n_1}{\varkappa n_1 + 1} - \frac{\varkappa n_2}{\varkappa n_2 + 1}\right) \cdot \frac{[(\varkappa n_1 + 1)k'_{01}V_m]^{\frac{1}{\varkappa n_1+1}}}{[(\varkappa n_2 + 1)k'_{02}V_m]^{\frac{\varkappa n_2}{\varkappa n_2+1}}} + B_{(max)}^{\frac{\varkappa n_2}{\varkappa n_2+1}} \cdot \frac{\varkappa n_1}{\varkappa n_1 + 1}[(\varkappa n_1 + 1)k'_{01}V_m]^{\frac{1}{\varkappa n_1+1}}}{\frac{\varkappa n_2}{\varkappa n_2 + 1} \cdot [(\varkappa n_2 + 1)k'_{02}V_m]^{\frac{1}{\varkappa n_2+1}}} \right\}^{\frac{\varkappa n_1+1}{\varkappa n_1}} \quad (12)$$

In most practical systems, no reversal of the order of elution with changing composition of the mobile phase occurs. Here, A has no significant influence on the differences in retention volumes or peak widths, which leads to a relatively small influence of A on resolution. Thus, the increase in the selectivity term II and the decrease in the capacity term III with increasing A tend to counterbalance each other, up to a certain value of A, where the retention of the two compounds is decreased to such an extent that the resolution decreases with a further increase in A.

In these systems, the capacity term III is influenced by B more significantly than the selectivity term II and the resolution increases with decreasing B[11].

The shape of the concentration gradient characterized by the parameter $\varkappa$ in eqn. 1 has a more complex influence on resolution. It can be shown theoretically that maximal resolution can be found for certain values of $\varkappa$ even in the most simple instances, where $n_1 \approx n_2$ and $A = 0$ in eqn. 8. Here, the resolution approaches zero for very large $\varkappa$ and becomes identical with the resolution in isocratic elution using the pure, more efficient eluent for $\varkappa = 0$. The resolution obtained in gradient elution chromatography is always larger than in the above two extreme situations and a maximum on the $R_s = f(\varkappa)$ curve must occur. In practice, however, these maxima are likely to be rather flat and even a large change in $\varkappa$ would not influence the resolution very significantly, as shown by the example in Fig. 6.

Snyder *et al.*[7,9] published a relatively simple equation for linear solvent strength gradients, using certain simplifying assumptions, such as $n_1 = n_2$, low values of A and large values for k'_{01} and k'_{02}, but small differences between them. As a result of these simplifications, this equation is much simpler than eqn. 10, which can be applied to this system. Another difference is in the band compression factor, which is incorporated in the denominator of their equation as a function of the parameter b from eqn. 1a. Like eqn. 10, their relationship may be written in the form of terms I, II and III, where $\alpha_{(g)} = k'_{02}/k'_{01}$ and the term III is also much simpler (approximately equal to $1/(1 + 1.15\,b)$ (ref. 9). This equation allows for rapid estimations of resolution and its application requires only a rough guess of the parameter n (which is contained implicitly in b and is usually known in reversed-phase systems, see eqn. 1d). Eqns. 8–10 are much more complex but allow direct calculations of resolution without further simplifying assumptions, in linear and non-linear solvent strength gradients, for compounds with different values of n and for any values of A. The band compression factor can be incorporated into denominators of the above equations, either as a function of the gradient slope or as a semi-empirical factor of 0.8–0.9; see preceding discussion of band width.

To use Snyder's equation for the calculation of resolution in linear solvent systems where $n_1 \neq n_2$ and/or $A \gg 0$, a series of subsequent corrections have to be introduced, as suggested in ref. 9.

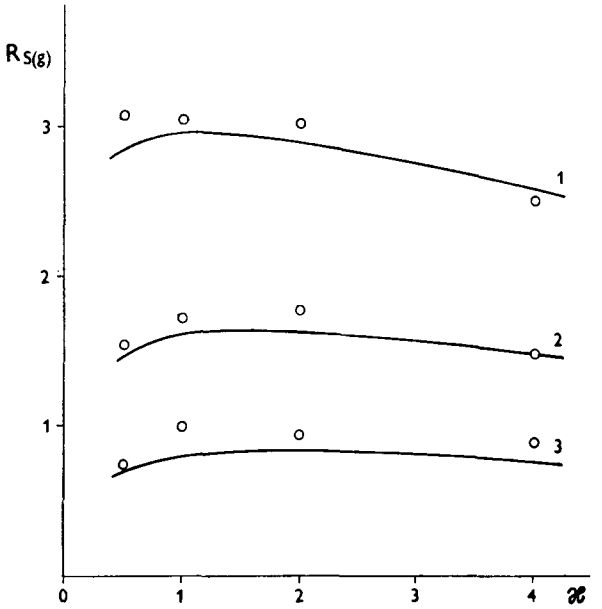

Fig. 6. Plots of the experimental (points) and calculated (eqn. 13, $A = 0$; full curves) resolution, $R_{s(g)}$, *versus* $\varkappa$ in gradient elution adsorption chromatography on Porasil A. Conditions and numbers of compounds as in Fig. 4. Gradient of ethyl acetate in cyclohexane. Curve 1, resolution of compounds 1 and 2, $R_{s(g)1,2}$; curve 2, resolution of compounds 2 and 3, $R_{s(g)2,3}$; curve 3, resolution of compounds 3 and 4, $R_{s(g)3,4}$.

Using his approach, Snyder[9] arrived at very similar conclusions about the influence of the initial concentration and gradient slope on resolution in systems where $n_1 = n_2$ and where $n_1 \neq n_2$.

Compression criterion

It is difficult to find a convenient criterion that is useful for the characterization of the chromatographic behaviour of more than two compounds during gradient elution. An important feature of gradient elution is its capability to compress the chromatogram, *i.e.*, to shorten the retention time of the last eluted compounds with respect to the early eluted solutes. We attempted to introduce a new appropriate criterion to characterize quantitatively this effect, namely the compression criterion, Q, which is defined as

$$Q = \frac{V'_{R(g)z} - V'_{R(g)1}}{V'_{R(g)2} - V'_{R(g)1}} = \frac{\alpha_{(g)z/1} - 1}{\alpha_{(g)2/1} - 1} \tag{13}$$

where $V'_{R(g)1}$, $V'_{R(g)2}$ and $V'_{R(g)z}$ denote the net retention volumes of the first, the second and the last eluted compound, respectively, and $\alpha_{(g)2/1}$ and $\alpha_{(g)z/1}$ are the retention ratios of the second and the last compounds with respect to the first compound. Thus, Q expresses the length of the chromatogram in multiples of its relative portions necessary for the elution of the first two sample compounds. If the first two

compounds are eluted with $R_{s(g)} = 1$ and the peak widths are equal for all of the compounds eluted, then Q becomes identical with peak capacity in gradient elution chromatography defined according to Horváth and Lipsky[22], but generally no limiting assumptions are involved in the definition of Q.

The compression criterion can be useful evaluating the influence of A, B and $\varkappa$ on the compression of a chromatogram, if $V'_{R(g)1}$, $V'_{R(g)2}$ and $V'_{R(g)z}$ are expressed from the appropriate equation[6,13]. An adequate compression of the chromatogram may be desirable with respect to the number of compounds to be resolved and the time of analysis.

Assuming that eqn. 2 applies and that the respective parameters n for all of the compounds are close to each other, and using gradient functions with zero initial concentration of the efficient eluting component, $A = 0$, we obtain the following relationship for Q:

$$Q = \frac{\left(\frac{k'_{0z}}{k'_{01}}\right)^{\frac{1}{\varkappa n + 1}} - 1}{\left(\frac{k'_{02}}{k'_{01}}\right)^{\frac{1}{\varkappa n + 1}} - 1} \tag{14}$$

According to eqn. 14, the compression of a chromatogram in this instance should depend on the curvature only, and not on the slope of the gradient function. This conclusion was confirmed experimentally in the adsorption chromatography of azo dyes on silica, as is shown in Fig. 7, where the calculated values of Q are plotted against $\varkappa$ together with the experimental points obtained at four different values of the slope of the gradient function (B).

As confirmed experimentally, the differences in retention volumes are not influenced much by the initial concentration of the efficient eluting agent at the beginning of gradient elution, A (see preceding discussion). It can also be assumed that Q is not influenced much by A.

This is not the case, however, in the relatively rare instances where the differences in the values of n are large for individual sample compounds. In reversed-phase chromatography, where eqn. 3 fits approximately to describe the dependence of capacity ratios on the composition of mobile phase, eqn. 14 cannot be used and the compression criterion Q depends on the slope of the gradient function, as shown in Table IV.

CONCLUSIONS

To summarize the influence of the gradient profile on the chromatographic behaviour of sample compounds in gradient elution chromatography, from the preceding discussion and from the comparison of the approaches of Snyder and co-workers and ourselves, we can draw the following conclusions:

(a) As the initial concentration of solvent b (parameter A in eqn. 1) is increased, retention volumes decrease, but band widths, differences in retention volumes of

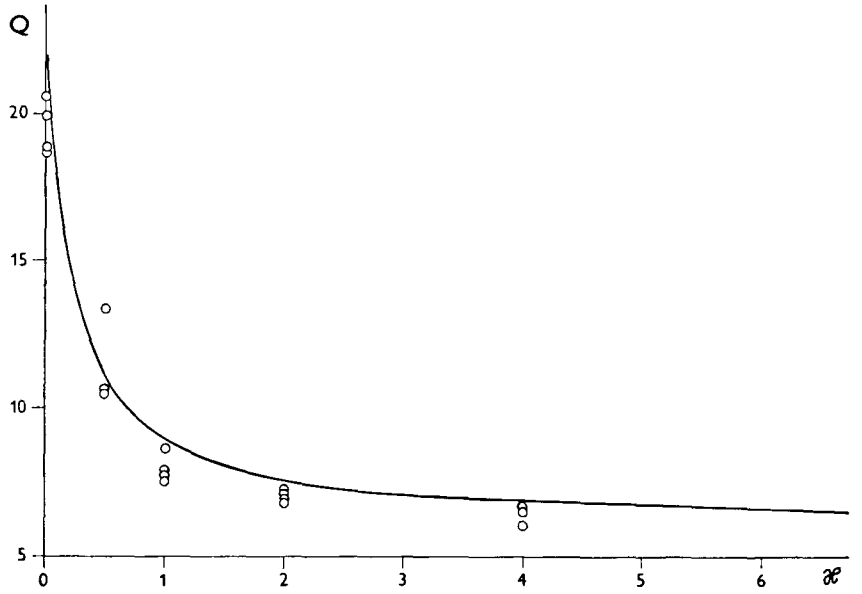

Fig. 7. Plots of the experimental (points) and calculated (full curve) values of the compression criterion, Q, *versus* $\varkappa$ in gradient elution adsorption chromatography on Porasil A. Experimental points were measured at different values of B (0.0016–0.1039); $A = 0$. Eqn. 14 was used for calculations; k_{01} is k_0' of compound 4; k_{0z}' is k_0' of compound 1; $n = 1.86$ is an average value for the four azo compounds studied. Conditions and numbers of compounds as in Fig. 4. Gradient of ethyl acetate in cyclohexane.

TABLE IV

INFLUENCE OF PARAMETERS A AND B OF THE GRADIENT FUNCTION (EQN. 1) ON THE COMPRESSION CRITERION, Q, IN GRADIENT ELUTION REVERSED-PHASE CHROMATOGRAPHY

Column, mobile phase gradient and experimental conditions as in Table III. The experimental values of Q are compared with those calculated using eqns. 13 and 4 in ref. 13. Compounds: barbiturates: 1 = barbital; 2 = heptobarbital; z = hexobarbital; the values of k_0' and n (eqn. 3) are given in Table I; substituted uracils: 1 = 3,6-dimethyluracil, $k_0' = 9.51$, $n = 4.40$; 2 = 3-ethyl-6-methyluracil, $k_0' = 15.63$, $n = 3.69$; z = 3-*n*-butyl-6-methyluracil, $k_0' = 67.20$, $n = 3.25$ [k_0' and n were evaluated by linear regression from experimental $\log k' = f(c)$ plots].

Compounds	*A*	*B*					
		0.06872		*0.03436*		*0.01718*	
		Q calc.	*Q exptl.*	*Q calc.*	*Q exptl.*	*Q calc.*	*Q exptl.*
Barbiturates	0	3.53	3.74	3.18	3.41	2.98	3.21
	0.1	3.63	3.92	3.31	3.48	3.16	3.49
Substituted	0	3.40	3.37	3.65	3.67	3.96	3.92
Uracils	0.1	3.43	3.39	3.70	3.76	4.08	4.26

sample compounds and resolution are usually not much influenced, if A does not exceed practically useful limits. In this range, A can be used to control the time of analysis, while the resolution is kept approximately constant.

(b) An increase in the gradient slope (parameter B in eqn. 1) usually results in a decrease in retention volumes, resolution and band width (the influence of B increases in this order). The chromatogram becomes more compressed. B can be utilized for control of resolution within certain limits, while other system parameters are kept constant. If the parameters n_1 and n_2 in eqns. 2 or 3 are significantly different for compounds to be separated, maxima and minima of resolution may be found occasionally when B (or A or $\varkappa$, to a lesser extent) is changed in a systematic manner, but this effect is likely to be of minor importance only.

(c) Increasing the parameter $\varkappa$ in eqn. 1 [changing the shape (curvature) of the gradient] leads to changes in the relative positions of sample bands in a chromatogram, usually corresponding to some increase in retention volumes. The compression of the whole chromatogram can be controlled, but the influence of $\varkappa$ on other retention characteristics is usually not as significant as that of A and B.

REFERENCES

1 L. R. Snyder, in J. J. Kirkland (Editor), *Modern Practice of Liquid Chromatography*, Wiley-Interscience, New York, 1971, p. 149.
2 K. Aitzetmüller, *J. Chromatogr. Sci.*, 13 (1975) 454.
3 W. E. May , S. N. Chesler, S. P. Cram, B. H. Gump, M. S. Hertz and S. M. Dyszel *J. Chromatogr. Sci.*, 13 (1975) 535.
4 C. G. Creed, *Res./Develop.*, 27 (1976) 40.
5 P. Jandera and J. Churáček, *J. Chromatogr.*, 170 (1979) 1.
6 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 223.
7 L. R. Snyder, J. W. Dolan and J. R. Gant, *J. Chromatogr.*, 165 (1979) 3.
8 J. W. Dolan, J. R. Gant and L. R. Snyder, *J. Chromatogr.*, 165 (1979) 31.
9 L. R. Snyder, in Cs. Horváth (Editor), *Liquid Chromatography*, Academic Press, New York, London, in press.
10 P. Jandera and J. Churáček, *J. Chromatogr.*, 93 (1974) 17.
11 P. Jandera and J. Churáček, *J. Chromatogr.*, 104 (1975) 9.
12 P. Jandera, M. Janderová and J. Churáček, *J. Chromatogr.*, 115 (1975) 9.
13 P. Jandera, J. Churáček and L. Svoboda, *J. Chromatogr.*, 174 (1979) 35.
14 P. Jandera and J. Churáček, *J. Chromatogr.*, 91 (1974) 207.
15 P. Jandera, M. Janderová and J. Churáček, *J. Chromatogr.*, 148 (1978) 79.
16 P. J. Schoenmakers, H. A. H. Billiet, R. Tijssen and L. de Galan, *J. Chromatogr.*, 149 (1978) 519.
17 R. A. Hartwick, C. M. Grill and P. R. Brown, *Anal. Chem.*, 51 (1979) 34.
18 L. R. Snyder, *Chromatogr. Rev.*, 7 (1965) 1.
19 H. Elgass, *Untersuchung zur Gradient Elution*, Thesis, Universität des Saarlandes, Saarbrücken, 1978.
20 H. Engelhardt and H. Elgass, *J. Chromatogr.*, 158 (1978) 249.
21 L. R. Snyder and D. L. Saunders, *J. Chromatogr. Sci.*, 7 (1969) 195.
22 C. G. Horváth and S. R. Lipsky, *Anal. Chem.*, 39 (1967) 1893.