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GRADIENT ELUTION IN HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

II. PRACTICAL APPLICATION TO REVERSED-PHASE SYSTEMS

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CONTENTS

1. INTRODUCTION

In Part I[1] we presented a practical theory of gradient elution separation, with emphasis on reversed-phase systems and high-performance liquid chromatography (LC). In this paper we continue this examination of reversed-phase gradient elution (RP-GE) liquid chromatography. Here we shall focus on three separate areas: (1) the nature of the relationship between isocratic capacity factor (k') values and mobile phase composition in reversed-phase LC, (2) an experimental verification of the various conclusions reached in the theoretical study[1] and (3) a practical summary of preferred separation conditions for achieving various goals in RP-GE applications.

2. EXPERIMENTAL

A. Equipment

The LC system consisted of two Waters Model 6000A LC pumps and a Model 660 solvent programmer (Waters Assoc., Milford, Mass., U.S.A.). Samples were injected using an injection valve fitted with a 10-μl sample loop (Model 7120, Rheodyne, Berkeley, Calif., U.S.A.). A 2.0-μm pre-filter (Model 7302, Rheodyne) was placed between the injection valve and the chromatographic column. Unless stated otherwise, separations were performed at ambient temperature with a 23 × 0.46 cm column with 6-μm C_{18} packing (Zorbax ODS, DuPont, Wilmington, Del., U.S.A.). A DuPont Model 901 254-nm fixed-wavelength detector was used with an x–y recorder (Model 2000, Houston Instruments, Austin, Texas, U.S.A.).

Additional columns were used for the study shown in Table 3: Merck C_8 (25 × 0.46 cm, 10-μm particles, EM Labs., Elmsford, N.Y., U.S.A.), Waters C_{18} (30 × 0.39 cm, 10-μm particles, Waters Assoc.), Hypersil C_{18} (16 × 0.5 cm, 5–7-μm particles, Shandon Southern Instruments, Selwickley, Pa., U.S.A.), DuPont C_{18} (23 × 0.46 cm, 6-μm particles; one column prepared with octadecyldimethylchlorosilane and one with octadecyltrichlorosilane, DuPont), DuPont C_8 (23 × 0.46 cm, 6-μm particles).

B. Chemicals

Mobile phases consisted of HPLC-grade methanol (MeOH), acetonitrile (AN) or tetrahydrofuran (THF) (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.) mixed with high-purity water from a Milli-Q unit (Millipore, Bedford, Mass., U.S.A.). For gradient elution, the organic–water mobile phases were mixed 5% organic–95% water for initial solvent A and 95% organic–5% water for final solvent B. This premixing plus helium sparging eliminated solvent de-gassing upon mixing during gradient formation. Thus 0–100% gradients were really 5–95% organic; actual mobile phase compositions are referred to throughout this paper.

C. Procedure

(a) Isocratic

Isocratic data were gathered using either the gradient former to mix the isocratic mobile phase or, for log k' *versus* Φ_b data, precise-composition mobile phases were mixed independently of the gradient device, and one pump was used in the isocratic mode in order to eliminate any bias introduced by the gradient system.

(b) Gradient

All gradients were 0–100% B in 20 min except as noted; b values were changed by changing the mobile phase flow-rates in convenient increments (0.5–1.0 ml/min). The column was regenerated after a gradient run to the initial mobile phase conditions by running a 10-min reverse gradient at 2.0 ml/min followed by at least 10 min of isocratic operation at initial mobile phase conditions before injection of the next sample. All separations were performed in duplicate.

3. SOLVENT EFFECTS IN ISOCRATIC REVERSED-PHASE LC

A brief review and discussion of this topic was presented in Part I[1]. There we concluded, to a first approximation, that solute k' values in reversed-phase systems can be represented by the general equation

$$\log k' = \log k_w - S\Phi_b \qquad (1)$$

Here, for a given sample component or solute X, and a given organic solvent B (*e.g.*, methanol), k' is the isocratic capacity factor for some volume fraction Φ_b of B in the water–organic mobile phase. The quantity k_w is an extrapolated value of k' for $\Phi_b = 0$. Thus, if eqn. 1 holds exactly over the range $0 \leqslant \Phi_b \leqslant 1$, k_w is the k' value of the compound X in pure water as mobile phase. The solvent-strength parameter S is determined by the organic solvent B; *e.g.*, $S \approx 3$ for methanol and $S \approx 4$ for tetrahydrofuran as solvent. S is known to vary somewhat (for a given organic solvent B) for different reversed-phase columns. It was assumed in Part I[1] that S does not vary significantly with solute molecular structure in the case of most samples. However, it was noted that there is a general increase in S with increasing solute molecular weight for samples composed of either a homologous series or certain oligomers.

The validity of eqn. 1 as discussed above forms the basis of:

(1) the general treatment of Part I[1] for RP-GE separation;

(2) the experimental test of that general treatment presented in a later section of this paper;

(3) the practical summary of RP-GE separation found in the final section of this paper.

We feel that eqn. 1 can be accepted as a reliable first approximation for reversed phase systems, without serious reservation. Nevertheless, there is value in further examining this relationship, for two reasons: firstly, to allay any questions concerning the value of eqn. 1 for interpreting RP-GE systems, and secondly, to gain insight into the importance of second-order effects (deviations from eqn. 1, variation of S with solute structure, etc.) in special cases. The present study does not allow final answers to the questions we shall raise, but is intended in part as a stimulus to further experimental investigation.

There are four main points of discussion with respect to the validity of eqn. 1:

(1) deviations from linearity of $\log k'$ *versus* Φ_b plots in reversed-phase systems;

(2) variation of S (other variables fixed) with change in solute structure;

(3) variation of S with different reversed-phase packings;

(4) variation of S for different solvents B.

A. Linearity of log k′ versus Φ_b

As reviewed in Part I[1], most previous experimental studies have shown essentially linear plots of log k' *versus* Φ_b in reversed-phase systems. A few studies suggest curvature of such plots, particularly in the region of $\Phi_b \approx 1$. The most detailed of these studies is that of Schoenmakers *et al.*[2], who summarized data on a large number of solutes and three organic solvents B (methanol, ethanol and propanol). They found that their plots of log k' *versus* Φ_b are better represented by the quadratic expression

$$\log k' = A\,\Phi_b^2 + B\,\Phi_b + C \tag{2}$$

If data are averaged for the various solutes studied by Schoenmakers *et al.*[2], for methanol and propanol as organic solvents, the resulting plots of log k' *versus* Φ_b shown in Fig. 1 are obtained. The curvature of these plots is readily apparent, with the data for propanol showing a distinct minimum in k' in the region of $\Phi_b = 0.9$. If the curves are extrapolated according to eqn. 2 beyond $\Phi_b = 1$ (dashed lines), it is seen that a minimum in k' results for methanol also (for $\Phi_b \approx 1.4$).

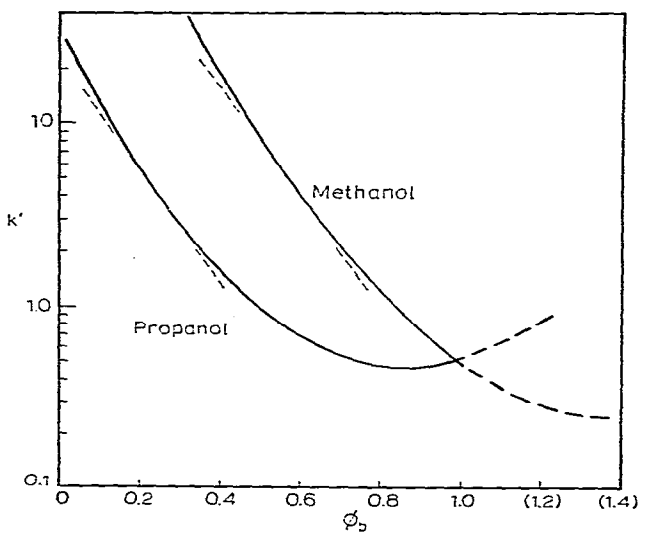

Fig. 1. Averaged data of ref. 2 for variation of log k' *versus* Φ_b for *n*-propanol and methanol as organic solvents B. *n*-Propanol, calculated from eqn. 2 with $A = 2.42$, $B = -4.19$ and $C = 1.50$; methanol, same, with $A = 1.88$, $B = -5.24$ and $C = 3.06$.

In Part I[1] we noted that migration of bands in RP-GE separation occurs mainly during the time (or mobile phase composition) when k' is between 2 and 10. It can be seen in Fig. 1 that plots of log k' over this region (light, dashed lines) hardly differ from the experimental plots (heavy lines). In particular, the minimum in k' found near $\Phi_b = 0.9$ for propanol (and other less polar solvents) is of little practical significance in RP-GE separation.

For $k' > 1$, it is not obvious that significant non-linearity of log k' *versus* Φ_b plots ever occurs. When a limited number of data points are collected (for different values of Φ_b), small errors in one or more points can easily suggest curvature in log-k' *versus* Φ_b plots, even where such curvature is non-existent. That this may be true to some extent in the study of Schoenmakers *et al.*[2] is suggested by examination of values of C from eqn. 2 for the same solute and different solvents B (methanol, ethanol, propanol). If eqn. 2 were a reliable fitting function, values of C for a given solute should be constant, as C is then the value of k' for the solute in question in pure water as the mobile phase, regardless of the solvent B considered. In fact, the data of Schoenmakers *et al.*[2] show differences in C for a given solute, in some cases by as much as 1.74 units (corresponding to differences in k' of 55-fold for that solute with water as mobile phase).

We feel that a better test of eqns. 1 or 2 for a given set of reversed-phase data is provided by superimposing plots of log k' *versus* Φ_b for different solutes. This can be achieved by shifting such plots horizontally until they roughly coincide, then examining the resulting plot for possible curvature. An example is provided in Fig. 2, for the reversed-phase system water (A)–methanol (B) studied by us (data of Table 1). The solid straight line through these data suggests no curvature of these plots (within experimental error). The average plot from ref. 2, based on eqn. 2, is superimposed on these same data as the dashed curve. While the similarity of the two plots is apparent, the slight curvature noted in ref. 2 appears to be absent in our own data for the same reversed-phase system.

TABLE I

ISOCRATIC k' VALUES FOR DIFFERENT METHANOL–WATER MIXTURES AS MOBILE PHASE USING A DUPONT ZORBAX-ODS COLUMN

*Solute**	*Methanol (%) in methanol–water*			
	70	*60*	*50*	*45*
Phenol (◒) (0.175)		0.74	1.38	1.94
p-Nitrophenol (▼) (0.146)		0.72	1.97	2.86
p-Cresol (●) (0.083)	0.67	1.45	2.87	4.49
2,5-Xylenol (■) (−0.004)	1.18	2.66	6.14	9.65
Methyl benzoate (◉) (−0.037)	1.75	3.54		11.1
Anisole (○) (−0.053)	1.87	3.88		12.1
Benzene (▽) (−0.073)	1.90	5.26		
Phenetole (□) (−0.108)	2.87	6.08		
Toluene (○) (−0.135)	3.36	7.94		

* Symbols refer to experimental points in Fig. 2; numbers in parentheses refer to shift in Φ_b of plots in Fig. 2; *e.g.*, data for phenol (0.175) are plotted at Φ_b values of 0.775, 0.675 and 0.625, respectively.

Finally, even if log k' *versus* Φ_b plots are actually curvilinear for some reversed-phase systems (*e.g.*, as in ref. 2), the effect of such non-linearity on RP-GE separation is minor (see Appendix V in Part I[1]).

Further study of the validity of eqn. 1 in reversed-phase systems is needed, with particular reference to the linearity of log k' *versus* Φ_b plots. Apart from the

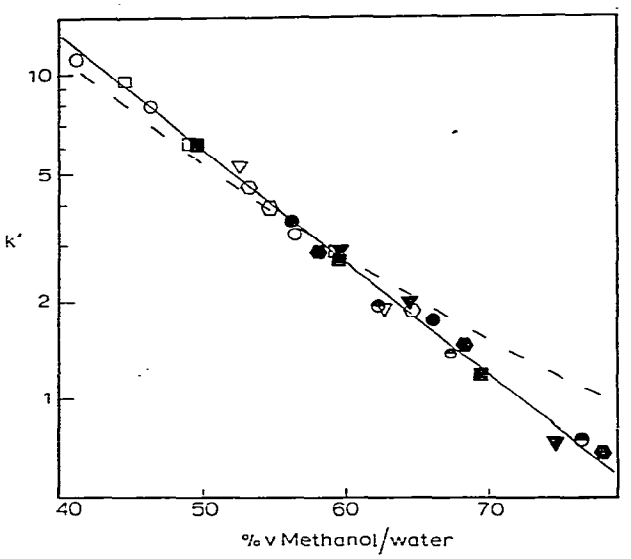

Fig. 2. Dependence of log k' on Φ_b for methanol–water as mobile phase; data of Table 1. Data shifted horizontally to obtain best fit to solid (linear) curve. Methanol curve of Fig. 1 (from ref. 2) similarly shifted and plotted as dashed curve. Experimental points defined in Table 1.

superposition technique described in Fig. 1, emphasis should be given to certain experimental considerations when collecting k' data for such purposes:

(a) complete equilibration of column and mobile phase before collecting data;

(b) verification that k' is not a function of solute concentration, expecially when $k' > 5$;

(c) constancy of the temperature of the column and incoming mobile phase during collection of k' data;

(d) use of column packings that exhibit full coverage of the silica surface by the bonded-phase.

(e) determination of the possible error in t_0 and its effect on reported k' values.

B. Variation of S with solute structure

Few studies have been concerned with the dependence of S on the molecular structure of the solute. A total of 17 solutes were investigated by Schoenmakers *et al.*[2], with the resulting S values (methanol as solvent B) shown in Table 2. Average S values from several columns (see the following section) and a number of different solutes studied by us are also summarized in Table 2. There is no obvious correlation of S with solute structure that appears from these data. Furthermore, for these representative solutes the average variation of S for a given column (and organic solvent B) is only of the order of $\pm$ 10–20%. That is, for typical samples little variation in S among the constituents of the sample is to be expected.

The situation is somewhat different in the case of solutes that form part of a homologous series. S values derived from the study of Tanaka and Thornton[3] are plotted for various homologous series of solutes (methanol–water as mobile phase) in Fig. 3. Here, a strong dependence of S on the alkyl carbon number, n, of the solute is

TABLE 2

VALUES OF S AS A FUNCTION OF SOLUTE STRUCTURE

Methanol–water solutions as mobile phase, ambient temperature.

Solute	S	
	*Ref. 2**	*Data in Table 3***
Phenol	1.7	2.6
Acetophenone	2.0	3.2
Benzene	2.1	2.7
Toluene	2.6	3.4
Ethyl benzene	3.2	
Diethyl phthalate	2.6	
Dibutyl phthalate	4.0	
Benzophenone	2.7	
Aniline	1.8	
N-Methylaniline	2.2	
N,N-Dimethylaniline	2.4	
Quinoline	2.2	
Benzyl alcohol	1.8	
2,4-Xylenol	2.3	
2-Cresol	2.1	
3-Cresol	2.1	
Benzaldehyde		2.9
Nitrobenzene		2.9
Methyl benzoate		3.6
Anisole		3.0
Fluorobenzene		3.0
Average	2.4 ± 0.6	3.0 ± 0.3

* Calculated from ref. 2 for $k' = 1.4$.

** Average values.

clearly evident. The slopes of these various plots for different homologous series are seen to be roughly constant (0.4 unit per methylene group). Extrapolation of the plots in Fig. 3 to $n = 0$ for the n-alkane and alkylbenzene series suggests that the addition of a phenyl group to a solute molecule increases S by about 0.8 unit, or much less per aromatic carbon (0.1 unit) than per aliphatic carbon (0.4 unit).

C. Variation of S among different reversed-phase packings

We have earlier expressed concern over the variability of S values among different reversed-phase packings (and columns). Table 3 summarizes data collected by us for nine different solutes and five different columns. The absolute values of S in Table 3 are found to vary as much for a given solute among the five columns as for a given column among the nine solutes. The effect of the column on S could be corrected for, however, by normalizing S values for each column. This was accomplished by dividing each S value by the average value of S for a given column. The resulting normalized S values for a given solute were then found to remain relatively constant among the five columns (average coefficient of variation in $S = 4\%$).

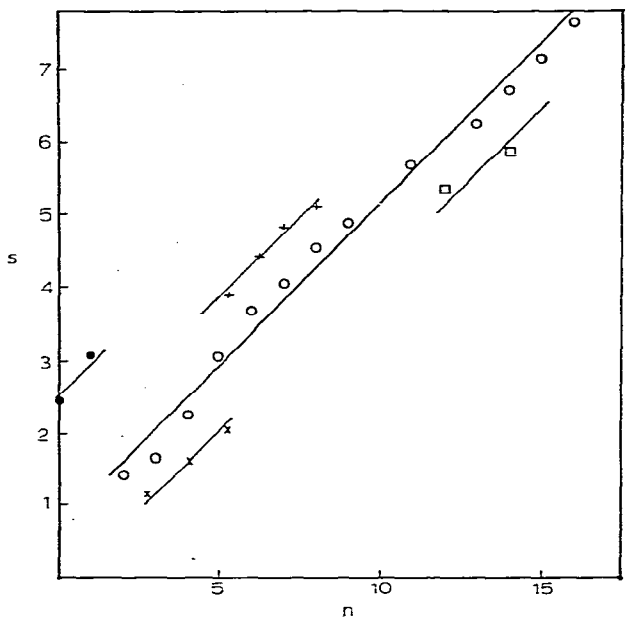

Fig. 3. Dependence of S on alkyl carbon number, n, in homologous series of solutes (from ref. 3). ●, Alkyl benzenes; +, n-alkanes; ○, carboxylic acids; ×, n-alkanols; □, dicarboxylic acids.

TABLE 3

VALUES OF S FOR SELECTED SOLUTES ON FIVE DIFFERENT REVERSED-PHASE COLUMNS

Water–methanol as mobile phase, ambient temperature.

Solute	*S for indicated columns*				*Relative S value*[§§]
	Waters C_{18}*	*Shandon* C_{18}**	*DuPont* C_{18}***	*DuPont* C_8[§]	
Phenol	2.21	2.52	2.35, 2.97	3.13	0.87 ± 0.06
Benzaldehyde	2.52	2.72	2.92, 3.07	3.08	0.95 ± 0.03
Acetophenone	2.82	3.04	3.08, 3.63	3.39	1.06 ± 0.03
Nitrobenzene	2.61	2.78	2.79, 3.18	3.16	0.96 ± 0.01
Methyl benzoate	3.17	3.46	3.44, 3.82	3.78	1.18 ± 0.02
Anisole	2.61	2.93	2.90, 3.29	3.28	1.00 ± 0.01
Fluorobenzene	2.70	3.07	2.90, 3.27	3.28	1.01 ± 0.02
Benzene	2.32	2.66	2.58, 2.94	3.02	0.90 ± 0.02
Toluene	2.90	3.24	3.13, 3.52	3.56	1.13 ± 0.06
Average	2.65	2.94	2.90, 3.29	3.29	(1.00)

* Monochlorosilane plus additional silanization ("capping").

** Trichlorosilane plus additional silanization.

*** Trichlorosilane (first column), monochlorosilane (second column), no additional silanization.

[§] Monochlorosilane, no additional silanization.

[§§] Average S value for given solute, relative to S for all columns.

D. Variation of S for different organic solvents B

Tables 4 and 5 summarize isocratic k' values as a function of mobile phase composition for two additional binary mixtures: acetonitrile–water and tetrahydrofuran–water. Values of S for these various solutes are also tabulated. Apart from a general increase in S for tetrahydrofuran, and a decrease in S for acetonitrile, these data follow the same pattern as for the methanol data in Table 1. They add little to our general understanding of the dependence of S on solute structure.

TABLE 4

ISOCRATIC k' VALUES FOR DIFFERENT TETRAHYDROFURAN–WATER MIXTURES AS MOBILE PHASE USING A DUPONT ZORBAX-ODS COLUMN AT AMBIENT TEMPERATURE

Solute	k'				S
	55% THF	*50% THF*	*45% THF*	*40% THF*	
Phenol			0.79	1.27	4.1
p-Nitrophenol			0.99	1.80	5.2
p-Cresol			1.00	1.76	4.9
2,5-Xylenol	0.69	1.05	1.55	2.79	4.0
Methyl benzoate	0.76	0.93	1.31	2.11	3.0
Anisole	0.92	1.35	1.87	3.09	3.4
Benzene	1.17	1.76	2.41	4.00	3.5
Phenetole	1.17	1.80	2.61	4.69	3.9
Toluene	1.49	2.33	3.39	6.08	4.0
Butyl benzoate	1.45	2.53	3.84		4.2
Anthracene	1.65	2.87	4.76		4.6
Benzanthracene	1.87	3.49	6.19		5.2
Average					4.2 ± 0.6

TABLE 5

ISOCRATIC k' VALUES FOR DIFFERENT ACETONITRILE–WATER MIXTURES AS MOBILE PHASE USING A DUPONT ZORBAX-ODS COLUMN AT AMBIENT TEMPERATURE

Solute	k'						S
	80% AN	*70% AN*	*60% AN*	*50% AN*	*40% AN*	*30% AN*	
p-Nitrophenol				0.61	1.39	4.12	3.6
Phenol				0.64	1.18	3.24	2.7
p-Cresol				0.98	1.99	10.0	3.1
2,5-Xylenol			0.95	1.68	3.71	14.5	3.0
Methyl benzoate			1.57	2.66	5.68		2.8
Anisole			1.64	2.80	6.00		2.8
Benzene			1.78	3.10	6.42		2.2
Phenetole			2.37	4.46	10.4		3.1
Toluene	0.95	1.57	2.78	5.13			2.5
Butyl benzoate	1.57	2.85	5.58				3.0
Anthracene	2.71	5.03	10.4				2.9
Average							2.9 ± 0.4

4. RETENTION, BAND WIDTH AND RESOLUTION IN LSS-GE

A. Retention time

As was discussed in Part I[1], eqn. 3*, and thus eqn. 3a*, has been experimentally verified in another study[4]. The present study provides further confirmation of eqn. 3* for RP-GE. For convenience in calculation, eqn. 3a* is modified to read

$$t_g = (t_0/b) \log (2.3\, k_0 b + 1) + t_0 + t_d \qquad (3)$$

Here, t_d is the delay time of the system corresponding to the time from initiation of the gradient until a change in mobile phase composition is observed at the head of the column. In our case, $t_d = 2.0$ ml/F (F = flow-rate), and is accounted for by the volumes of the pulse dampener, pre-column connecting tubing, injection valve and filter. Eqn. 3 assumes that solutes do not move along the column during t_d. There is, in fact, little or no migration during t_d except for compounds which elute close to t_0 (*e.g.*, uracil).

The compounds listed in Table 6 elute over the range of the AN–water gradient. The b values were calculated for each compound (eqn. 14*) and the k_0 values are extrapolated from individual log k' *versus* Φ_b curves. The experimental and calculated retention times agree well (coefficient of variation = 0.6%), confirming the validity of eqn. 3. Here, the importance of using individual b or S values is shown from the last column in Table 6, where use of average b values for the retention calculation results in a coefficient of variation significantly greater than for the individual compounds and, in this case, a prediction of retention order which is incorrect.

TABLE 6

PREDICTION OF RETENTION IN RP-GE SEPARATION

5–95% AN–water; $t_0 = 2.15$ min, $t_d = 2.0$ min, $t_s = 20$ min, $F = 1.0$ ml/min.

Solute	b*	k_0**	t_g *(min)*		
			Expt!.	*Calc.***	*Calc.*§
p-Cresol	0.30	24	13.2	13.1	13.5
Benzene	0.27	59	16.5	16.7	16.4
Phenetole	0.31	134	17.7	17.9	19.1
Toluene	0.24	63	18.1	18.1	16.6
Butyl benzoate	0.27	180	20.5	20.5	20.00

* b value calculated for each solute.

** k_0 extrapolated to 5% water from log k' *versus* Φ_b curve.

*** Calculated from eqn. 3 using individual b values; coefficient of variation for deviation from experimental values = 0.6%.

§ Calculated from eqn. 3 using average $b = 0.28$ for AN; coefficient of variation = 4.3%.

B. Initial mobile phase concentration

The effect of varying the initial mobile phase composition, Φ_0 (value of Φ for mobile phase entering the column at time $t = 0$), is illustrated in Fig. 4A–E and in

* All Figure, Table and equation numbers followed by an asterisk are taken from Part I[1].

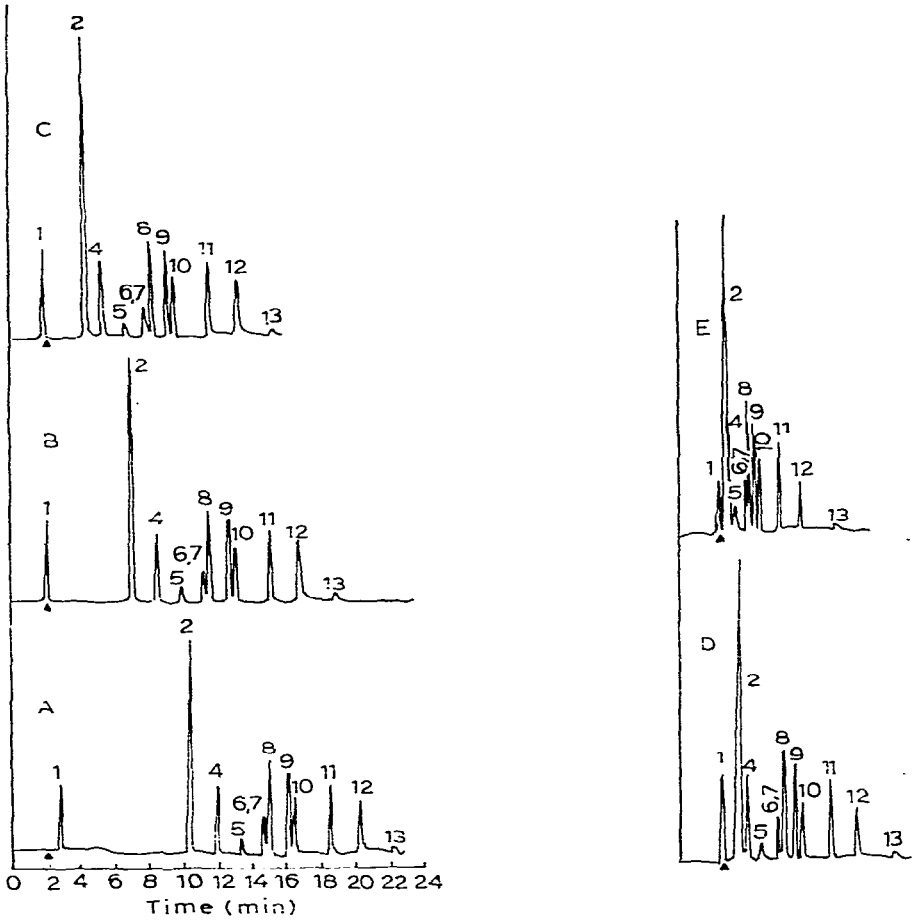

Fig. 4. Influence of initial mobile phase composition on gradient chromatogram. Solutes: 1, uracil; 2, phenol; 3, *p*-nitrophenol; 4, *p*-cresol; 5, 2,5-xylenol; 6, anisole; 7, methyl benzoate; 8, benzene; 9, phenetole; 10, toluene; 11, anthracene; 12, butyl benzoate; 13, benzanthracene. All gradients: $\Phi_f = 95\%$ AN–water, $b = 0.28$, $F = 1$ ml/min. ▲ = start of gradient at head of column (t_d). (A) $\Phi_0 = 5\%$ AN, $t_s = 20$ min; (B) $\Phi_0 = 26\%$ AN, $t_s = 16$ min; (C) $\Phi_0 = 46\%$ AN, $t_s = 12$ min; (D) $\Phi_0 = 64\%$ AN, $t_s = 8$ min; (E) $\Phi_0 = 79\%$ AN, $t_s = 4$ min.

Table 7. As was discussed in Part I[1], only the initial part of a gradient elution chromatogram is affected by a change in Φ_0. An increase in Φ_0 generally leads to poorer resolution and higher bands for initially eluted compounds that elute near t_0. This effect is obvious in Fig. 4 and is quantified in Table 7. In the latter, we have tabulated values of Φ_g for each solute in each separation in Fig. 4, where Φ_g is the value of Φ at the column inlet at the time t_g of elution of the given band, *i.e.*,

$$\Phi_g = \Phi_0 + \Phi' t_g \tag{4}$$

It can be seen that, in most cases, a given band elutes at a characteristic value of Φ_g, until Φ_0 is increased to the point where it is similar in value to Φ_g. As Φ' is constant

TABLE 7

EFFECT OF INITIAL MOBILE PHASE COMPOSITION ON RETENTION AND DETECTABILITY

All gradients: $\Phi_f = 95\%$ AN–water, $b = 0.28$.

Solute	Φ_0									
	0.05		*0.26*		*0.46*		*0.64*		*0.79*	
	Φ_g*	*Detectability***	Φ_g*	*Detectability***	Φ_g*	*Detectability***	Φ_g*	*Detectability***	Φ_g*	*Detectability***
Phenol	0.54	127	0.56	148	0.60	—	0.71	—	0.83	—
p-Nitrophenol	0.63	39	0.64	40	0.63	43	0.73	51	0.84	—
Phenetole	0.86	48	0.87	48	0.88	49	0.89	55	0.92	66
Toluene	0.89	30	0.89	30	0.90	30	0.91	34	0.94	42
Anthracene	0.95	44	0.95	42	0.95	43	0.95	46	0.95	52

* From eqn. 4.

** Peak height, arbitrary units.

for the various separations in Fig. 4, this effectively means that a given solute band is eluted by the same composition of mobile phase, provided that $\Phi_0 \ll \Phi_g$.

As Φ_0 has no effect on the separation or resolution of later eluting bands, provided that $\Phi_0 \leqslant \Phi_g$, in practice the largest possible value of Φ_0 should be selected. This in turn minimizes the separation time. For example, t_s for the separation in Fig. 4A can be reduced significantly by changing Φ_0 to the conditions shown in Fig. 4C, while maintaining adequate resolution.

C. Band width

The band width in RP-GE is predictable by eqn. 7a* of ref. 1. The validity of this equation for RP-GE is shown in Table 8 for several compounds in an AN–water gradient. One can see that it makes little difference whether individual or average b values are used to calculate σ_t, with either method giving predictions in agreement with experimental values. The data in Table 8 show that the band width is relatively constant throughout the RP chromatogram (coefficient of variation = 10%), whereas under isocratic conditions the band width increases in proportion to $k' + 1$.

D. Resolution

For maximal resolution R_s, the discussion in Part I[1] predicts that $b = 0.2$ is roughly optimal. More precisely, for $t_s = 20$ min and the 5-μm particles as used in this study, Appendix II in Part I[1] predicts that $b = 0.1$ is optimal. The chromatograms in Figs. 5–7 show the effect of varying b while holding the separation time (and column length, L) constant. These examples provide a rough confirmation for an intermediate value of $b \approx 0.1$–0.2 being preferred, so far as resolution is concerned.

Another (more precise) measure of R_s as a function of b (or $\bar{k}$) is provided by the peak capacity, PC, equal here to the difference in retention times for the first- and

TABLE 8

COMPARISON OF EXPERIMENTAL AND THEORETICAL BAND WIDTHS IN REVERSED-PHASE GRADIENT ELUTION

5–95% AN, t_0 = 129 sec, F = 1 ml/min.

Solute	σ_t *(sec)*				b[§]	G[§§]	N ($\times 10^{-4}$)[§§§]
	Gradient elution			*Isocratic elution*[***]			
	Exptl.	*Calc.*[*]	*Calc.*[**]				
p-Cresol	2.5	3.5	3.6	2.4	0.30	0.81	0.79
Benzene	2.4	2.5	2.4	3.0	0.27	0.82	1.77
Phenetole	2.4	2.3	2.4	3.8	0.31	0.81	1.67
Toluene	2.4	2.6	2.4	4.1	0.24	0.83	1.79
Butyl benzoate	3.0	2.7	2.6	8.6	0.27	0.82	1.46

[*] From eqn. 7a[*] in Part. I[1], using individual b values; coefficient of variation = 12%, calc. *vs.* exptl.

[**] From eqn. 7a[*] in Part I[1], using average value of b = 0.28.

[***] Isocratic value, 64% AN, F = 1 ml/min.

[§] Calculated for each compound from eqn. 7a[*] in Part I[1].

[§§] From Fig. 5[*] in Part I[1].

[§§§] Isocratic N value.

last-eluted compounds in a given sample, divided by average band widths. As N and b are changed (*e.g.*, Figs. 5–7), PC should vary as NQ^2. In Fig. 8 experimental values of PC are plotted against b valves from Figs. 5–7, and the theoretical plot of NQ^2 *versus* b is superimposed on these data (calculated as described in Appendix II in Part I[1]). The data follow the theoretical plot reasonably closely, and confirm a maximal resolution in the range of $0.05 < b < 0.2$. Within this range of b values, there is little change in PC or NQ^2 with b.

Visual examination of the chromatograms in Fig. 5 suggests a maximal resolution of this sample for b = 0.28 (Fig. 5B), rather than for lower values of b. This is the result of selectivity changes which accompany variation in b, and is not an atypical result (*i.e.*, better separation for a slightly non-optimal value of b). Similar observations concerning the separation of Figs. 6 and 7 can also be drawn.

The separations in Figs. 5–7 and the data plot in Fig. 8 provide general confirmation of an optimal value of b in these cases of about 0.1. However, even more important is the finding that (as predicted) NQ^2 is not very sensitive to changes in b (with parallel changes in F, as in Figs. 5–7), when the separation time is held constant. Similarly, small differences in NQ^2 can be overshadowed by changes in α with variation in b. Finally, it should not be overlooked that larger values of b give greater detection sensitivity (see next section).

E. Detection sensitivity

Eqn. 11a[*] predicts an increased detection sensitivity as b increases. The data in Table 9 illustrate this effect. To increase the detection sensitivity in the case of a fixed t_s (as in this study), b is increased by lowering the flow-rate. Table 9 indicates that s_g and peak height increase by about 3-fold from b = 0.07 to b = 0.56. This is

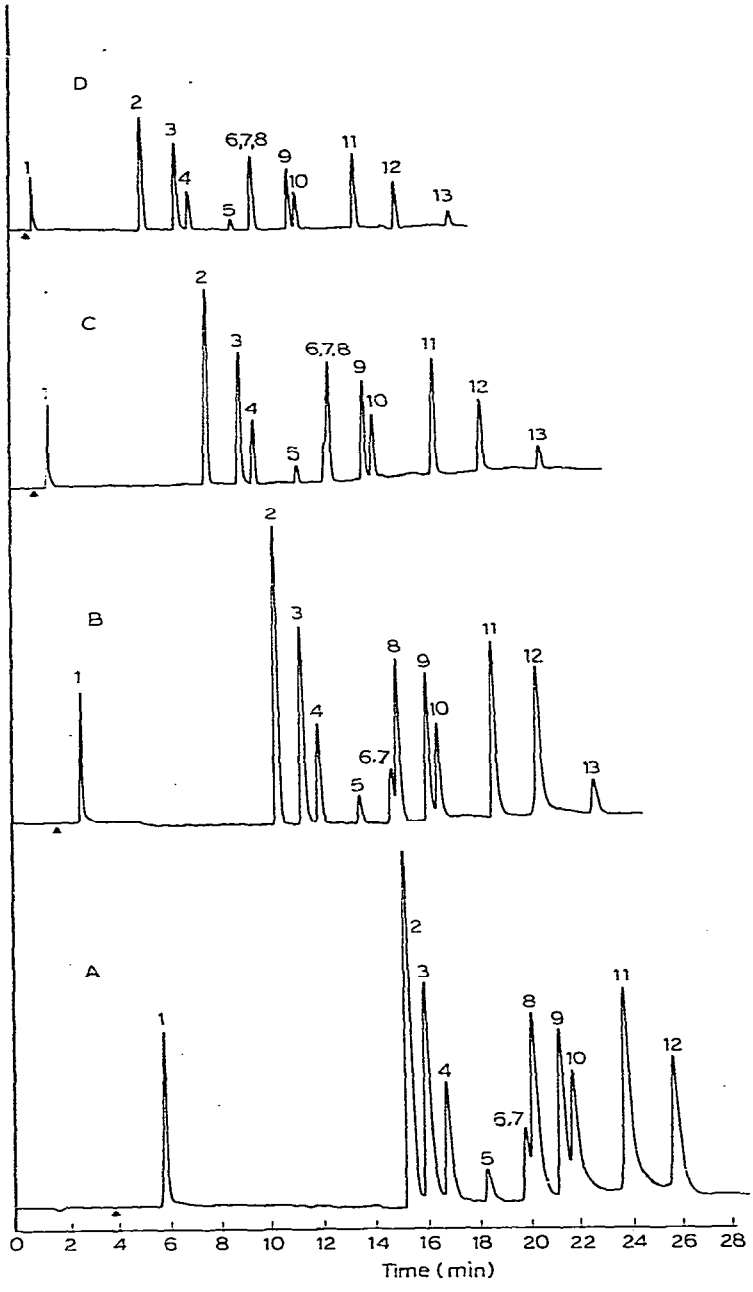

Fig. 5. Influence of b on chromatographic parameters with AN mobile phase. Solutes as in Fig. 4. All gradients: 5–95% AN–water; $t_s = 20$ min; ▲ $= t_d$. (A) $b = 0.56$, $F = 0.5$ ml/min; (B) $b = 0.28$, $F = 1.0$ ml/min; (C) $b = 0.14$, $F = 2.0$ ml/min; (D) $b = 0.07$, $F = 4.0$ ml/min.

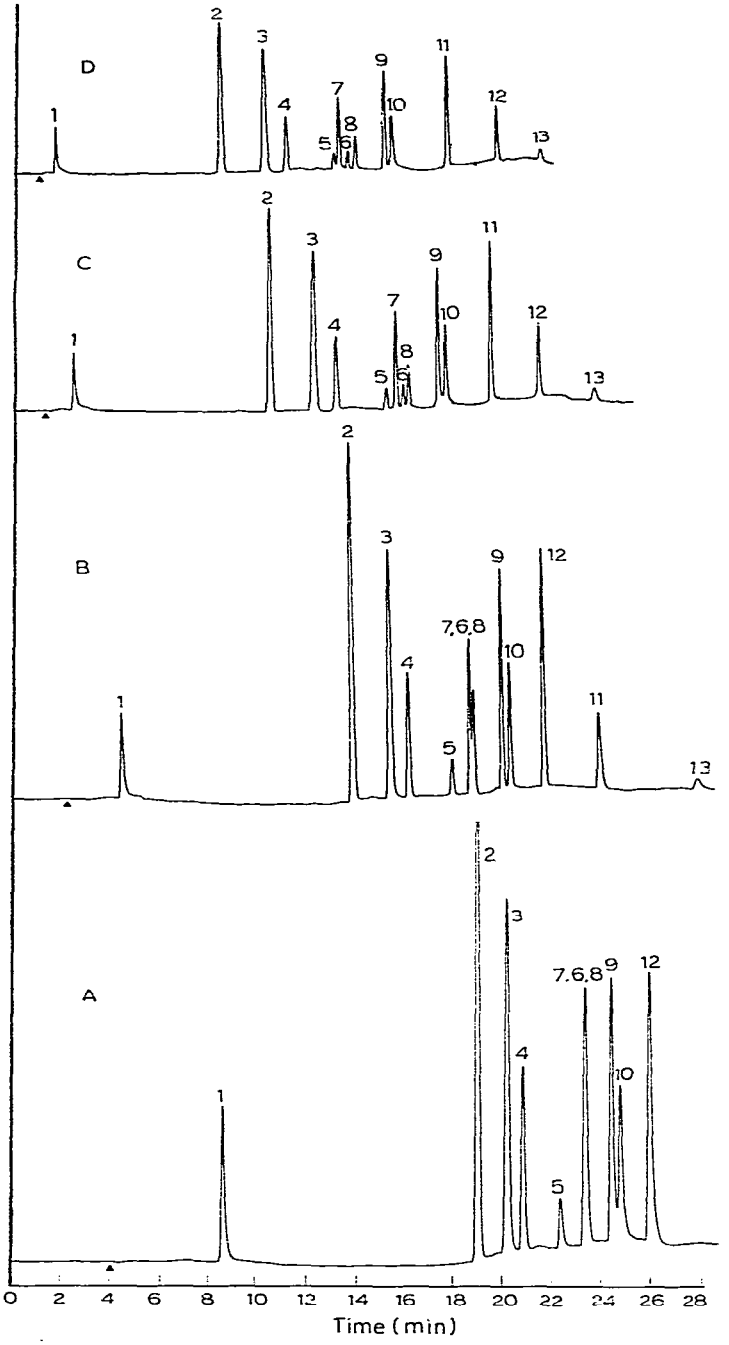

Fig. 6. Influence of b on chromatographic parameters with MeOH mobile phase. Solutes as in Fig. 4. All gradients: 5–95% MeOH–water; t_s = 20 min; ▲, t_d. (A) b = 0.68, F = 0.5 ml/min; (B) b = 0.34, F = 1.0 ml/min; (C) b = 0.17, F = 2.0 ml/min; (D) b = 0.11, F = 3.0 ml/min.

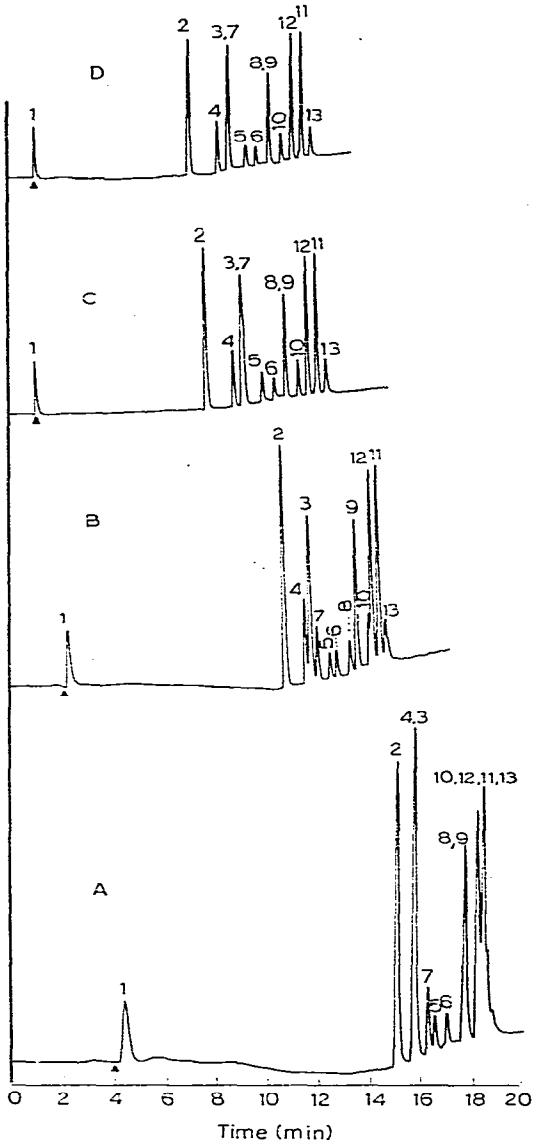

Fig. 7. Influence of b on chromatographic parameters with THF mobile phase. Solutes as in Fig. 4. All gradients: 5–95% THF–water; t_s = 20 min; ▲ = t_d. (A) b = 0.81, F = 0.5 ml/min; (B) b = 0.41, F = 1.0 ml/min; (C) b = 0.20, F = 2.0 ml/min; (D) b = 0.17, F = 2.5 ml/min.

visually apparent for AN as solvent by comparing Fig. 5A (b = 0.56) with Fig. 5D (b = 0.07). Similarly, we can compare Table 9 with Fig. 6A and D for MeOH or Fig. 7A and D for THF. Thus, as predicted in eqn. 11a*, we achieve a predictable increase in detection sensitivity in GE by increasing b. We must, of course, keep in mind that this simultaneously decreases R_s.

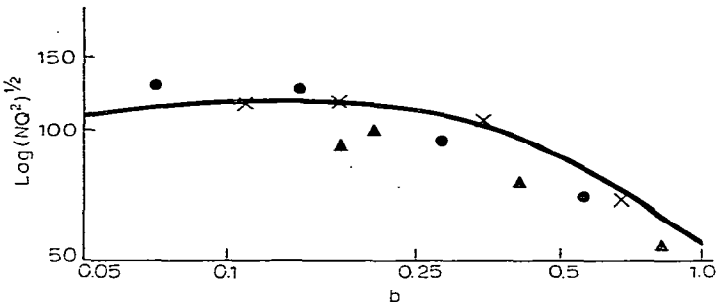

Fig. 8. Resolution as a function of b. Solid line, theoretical curve; individual points, experimental data for AN (●), MeOH (×) and THF (▲) shifted vertically for superposition onto theoretical curve.

F. Separation selectivity

As was discussed in Part I[1], we can change the selectivity (or resolution) of a given solute pair by changing α. The two options for GE are to change the type of mobile phase or the slope (b value) for the gradient. Let us take as an illustration the solute pair benzene–phenetole. Assume we chose for our initial separation the optimal THF–water gradient shown in Fig. 7C ($b = 0.2$). Under these conditions (THF as mobile phase) we see that the two solutes are completely merged into one peak. In a case such as this where we have essentially no separation under "ideal" conditions, it is preferable to try a mobile phase with different solvent properties. If we chose either MeOH or AN and ran an optimal ($b = 0.2$) LSS gradient we observe (Figs. 5C and 6C) baseline resolution for benzene and phenetole.

If, on the other hand, because of some other restriction, we need to use THF–water as the mobile phase, we might try to separate the two solutes by changing the b value of the gradient. This possibility is based on the (normally) small

TABLE 9

COMPARISON OF EXPERIMENTAL AND THEORETICAL DETECTION SENSITIVITY IN REVERSED-PHASE GRADIENT ELUTION

Mobile phase	b	S_g*	
		*Exptl.***	*Calc.****
AN–water	0.07	0.15 ± 0.03	0.15
	0.14	0.23 ± 0.05	0.28
	0.28	0.35 ± 0.07	0.48
	0.56	0.41 ± 0.10	0.76
MeOH–water	0.11	—	0.23
	0.68	—	0.85
THF–water	0.18	—	0.34
	0.81	—	0.92

* Average value for *p*-cresol, phenetole, toluene and butyl benzoate.
** s_g (exptl.) = h_2/h_1, where h_1 = peak height at t_0 and h_2 = GE peak height, ± 1σ.
*** s_g (calc.) = $2.3b/(1 + 2.3b)G$.

differences in S values for various solutes in a given mobile phase. The net result of these small variations in S is that b can be changed slightly in order to improve the separation of the two solutes. In the case of benzene–phenetole, we can increase b (or reduce $\bar{k}$) by reducing the flow-rate to 1.0 ml/min (with t_s constant). Observe that benzene and phenetole now have baseline resolution (Fig. 7B) (in this case reducing F also increases N). However, it is usually much easier to obtain the necessary resolution by changing the type of mobile phase (*e.g.*, from THF to MeOH) than by adjusting the b value with the same mobile phase. Tanaka *et al.*[5] have recently investigated the influence of organic modifiers on solvent selectivity in isocratic RP-LC. Their findings emphasize the possibility of improving separation in RP by change in organic solvent.

5. MISCELLANEOUS CONSIDERATIONS

A. Design of isocratic separations

In the discussion in Part I[1], it was predicted that preferred isocratic elution conditions ($k' = 4$) can be obtained by using the mobile phase composition corresponding to the gradient mobile phase at the head of the column at $t_g - 2.5\,t_0$. Taking t_d into account, we then require the mobile phase at $t_g - 2.5t_0 - t_d$. The data in Table 10 show that the experimental values obtained from the log k' *versus* Φ_b curves are in agreement with predicted values for $k' = 4$ (coefficient of variation = 2%).

TABLE 10

PREDICTION OF ISOCRATIC CONDITIONS FROM REVERSED-PHASE GRADIENT ELUTION

Mobile phase, acetonitrile–water.

Solute	t_g* *(min)*	*Isocratic mobile phase***	
		*Exptl.****	*Calc.*§
p-Cresol	13.2	0.30	0.31
Benzene	16.5	0.47	0.46
Phenetole	17.7	0.52	0.52
Toluene	18.1	0.54	0.53
Butyl benzoate	20.5	0.65	0.64

* 5–95% AN, $b = 0.28$, $t_0 = 2.15$ min, $t_d = 2.0$ min.

** Φ_b for $k' = 4$.

*** From log k' *vs.* Φ_b plots.

§ Mobile phase at head of column at $t_g - 2.5\,t_0 - t_d$, coefficient of variation = 2%, calc. *vs.* exptl.

Thus GE greatly simplifies the optimization of isocratic conditions. Instead of an "educated guess" of isocratic conditions followed by trial-and-error optimization, we can run a single LSS-GE separation at optimal b and predict the desired isocratic elution conditions within a few percent.

B. Calculation of column plate number in GE

Eqn. 15a* was derived in Part I[1] to allow for correct estimates of N with GE. Table 11 compares N values under isocratic conditions with N values for GE for five

TABLE 11

CALCULATION OF COLUMN EFFICIENCY IN REVERSED-PHASE GRADIENT ELUTION

Isocratic: 64% AN, $F = 1$ ml/min. Gradient: 5–95% AN, $F = 1$ ml/min, $b = 0.28$.

Solute	$N (\times 10^{-3})$			
	*Isocratic**	*Gradient*		
		*Correct***	*Correct****	*Incorrect*§*
p-Cresol	8	13	14	100
Benzene	18	16	15	170
Phenetole	17	13	15	190
Toluene	18	19	15	200
Butyl benzoate	15	10	10	260
Average	15	14	14	180

* Proper application of eqn. 15* in Part I[1].
** Proper application of eqn. 15a* using individual b values, coefficient of variation = 25%.
*** Proper application of eqn. 15a* using average $b = 0.28$, coefficient of variation = 28%.
§ Improper application of eqn. 15* to compute N for gradient elution.

solutes. The two sets of data correlate well when one considers that manual measurements of N are only precise to about 10%. We also see that an average b value predicts approximately the same plate count as the use of b values for individual compounds. The last column indicates the large discrepancy in N values when they are calculated improperly, using eqn. 15* of ref. 1.

C. Summary

In this section we have experimentally verified the theoretical predictions of Part I[1]. This greatly increases the practical utility of GE-LC, by allowing systematic and predictable optimization of gradients, as well as the use of gradient data to predict reliably isocratic separation conditions.

In the next section we shall discuss the actual measurement of the gradient parameters discussed above, plus some practical "rules-of-thumb" for successful use of GE-LC.

6. APPLICATION OF THEORY TO PRACTICE IN REVERSED-PHASE GRADIENT ELUTION: INITIAL SEPARATION

In this section, a simple procedure for designing "general, optimal" RP gradients will be presented. Evaluation of the gradient chromatograms by means of the theory verified above enables rapid, logical "tuning" of the gradient, *i.e.*, optimization of resolution, detection sensitivity and gradient time. The so-called "general, optimal" gradient results as a compromise among the latter three goals. Table 12 summarizes the important instrumental and mobile phase parameters in designing the gradient, thus serving as an outline of the following discussion. A typical example will be developed during this discussion, as summarized in Table 13.

TABLE 12

SUMMARY OF PARAMETERS IMPORTANT IN DESIGNING RP-GE SEPARATIONS

Type of parameter	*Important parameters*
Instrument parameters	t_0
	t_d
	Gradient profile
Mobile phase characteristics	Solvent selectivity
	Φ'
	Range
	Gradient blank

TABLE 13

TYPICAL INITIAL GRADIENT CONDITIONS FOR RP-GE

Parameter	*Value*
Gradient profile	Linear
Solvent A	95% water, 5% AN
Solvent B	5% water, 95% AN
Gradient steepness, Φ'	6.5%/min
t_0	1.07 min
Flow-rate	2.0 ml/min
Gradient range	0–100% B
Gradient time, t_s	14 min
Gradient delay, t_d	1.0 min

A. Instrument parameters

It is necessary to determine several parameters characteristic of a given instrument (*i.e.*, the pump, injector, column and connecting tubes) in order to efficiently develop a GE separation.

In order to estimate Φ' (see below), t_0 must be known. A good estimate of t_0 can be obtained easily by isocratic elution of uracil, using a mobile phase with greater than 60% organic modifier. In our present example, t_0 was found to be about 1.07 min ($V_m = 2.15$ ml; $F = 2.0$ ml/min).

Another important characteristic of a given instrument is the gradient delay. This is easily determined by using a UV-absorbing solute dissolved in the strong solvent, B, (*e.g.*, uracil in methanol) and the same mobile phase without any solute as A (methanol). The detector is connected where the column is usually attached, and the gradient of interest run. Typical results are illustrated in Fig. 9. The gradient delay time, t_d, is determined from this trace by simply measuring the time between the start of the gradient program and the initial increase in the baseline due to the arrival of solvent B at the detector. A knowledge of delay time is useful in fine tuning the gradient for a particular sample, as will be illustrated shortly.

The gradient profile produced by the instrument is also illustrated in Fig. 9. This nominally linear gradient is in fact seen to be slightly convex, with deviations from the theoretical value as great as 6%. However, the discussions in Part I[1] and above indicate that the results obtained in RP-GE are relatively insensitive to devia-

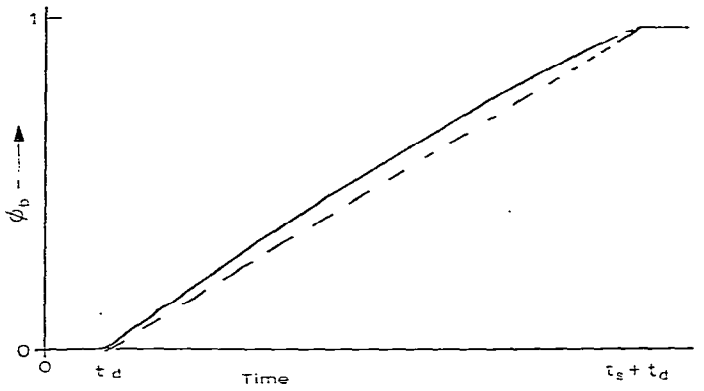

Fig. 9. Gradient profile for pre-set system. Solid line, actual gradient; broken line, theoretical gradient.

tions of this magnitude. It is convenient to determine the gradient delay (and verify gradient profile) after the initial solvent program has been designed (see below). The gradient delay volume (t_d/F) and the relative gradient profile need be checked only once for a given instrumental configuration, as they should remain constant for a properly functioning instrument.

B. Mobile phase characteristics

The first step entails selection of the gradient profile. LSS gradients are preferred, and linear solvent programs generate this type of gradient for most RP-GE separations[1]. Therefore, we assume a linear solvent program.

Next, we select the organic modifier: methanol, acetonitrile and tetrahydrofuran are most commonly used in RP-GE. The choice of a given organic solvent is dictated mainly by the selectivity required for a given sample. This is generally not known in advance of the separation, so the choice of initial solvent is somewhat arbitrary. For this discussion, we shall assume the selection of water–acetonitrile as mobile phase. Gradient steepness is estimated from Table 4*. Using the t_0 value determined above (1.07 min), Φ' is found to be approximately 6.5%/min for acetonitrile.

At this point, the gradient range must be considered. The latter refers to the range in k_i values during the separation; the gradient range is greater for larger S values of the organic solvent and for larger changes in Φ_b (*e.g.*, 0–1.0) during separation. As the gradient range increases, it is more likely that a given compound will be successfully separated, *i.e.*, eluting neither near t_0 nor long after the completion of the gradient. For unknown samples, we recommend an initial gradient of 5% to 95% acetonitrile–water, using pre-mixed solutions of 5% and 95% acetonitrile–water as solvents A and B, and running the gradient from 0 to 100% B. This provides a reasonable gradient range, yet avoids certain practical problems. For example, de-gassing frequently occurs if pure water and organic solvents are mixed on-line. Also, some reversed-phase columns show poor efficiency with mobile phases that contain 90–100% of water, because wetting of the packing is poor. In subsequent

sections we shall discuss possible alteration of the gradient range for various reasons.

The gradient time, t_s, can be calculated by dividing gradient range by Φ'. In our example, this is approximately 14 min (90/6.5). At this point, all of the parameters necessary to run our initial RP gradient have been determined and are summarized in Table 13 for our example. However, before running gradients of actual samples, it is advisable to run a blank gradient at the most sensitive detector attenuation anticipated in the ensuing gradients. This provides valuable information about baseline fluctuations and ghost peaks, which can be a problem with solvents of insufficient purity.

The importance of solvent purity in GE-LC cannot be overemphasized. Solvents which are acceptable for isocratic LC may be useless in GE, as is illustrated in Fig. 10A and B. Fig. 10A shows a MeOH–water gradient using ACS-quality anhydrous methanol, which is often used without any problems in routine isocratic LC. One can see that the impurities are concentrated on the column when the mobile phase is weak and then elute later in the gradient. On the other hand, highly purified HPLC-grade MeOH under the same conditions provides an acceptable blank, as shown in Fig. 10B. Similar results can be shown with water of varying quality; here one must be careful to remove all UV-absorbing impurities before use. A further problem of not using highly purified solvents is that blank runs are not reproducible, as only rarely are the recycle and equilibration times exactly the same from run to run, and varying amounts of impurities can build up on the column prior to each gradient run.

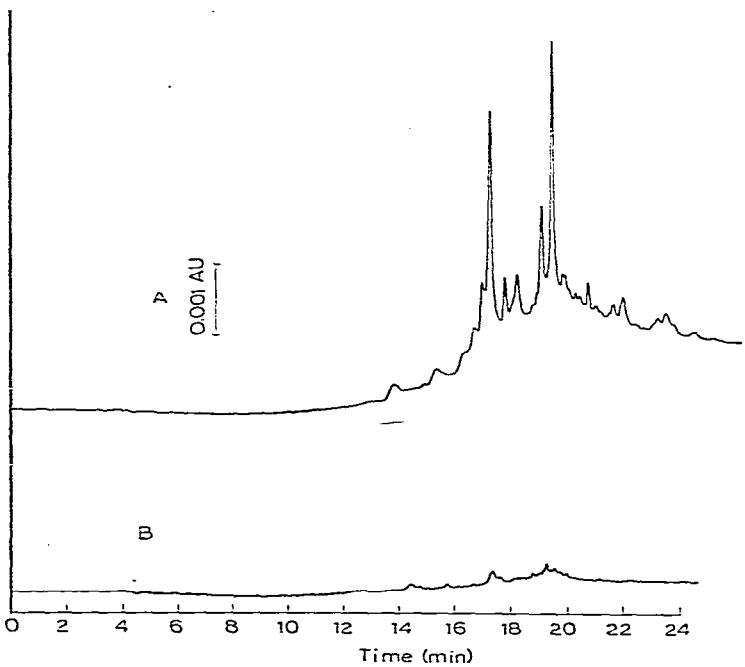

Fig. 10. Influence of solvent purity on gradient. 5–95% MeOH–water, t_s = 20 min. (A) ACS-grade anhydrous MeOH; (B) HPLC-grade high-purity MeOH.

Given an acceptable blank, we are now prepared to run our initial RP-GE separation.

C. Analysis of samples

As in isocratic LC, it is useful first to obtain an acceptable chromatogram of standard compounds of interest if these are available. Thus, the first gradient should be of standards. Next, a gradient of the actual sample is obtained. By using the standard chromatogram as a guide, attention can be focused on the most important parts of the separation. At this point, we have designed and run our initial RP-GE separation. The steps summarizing this development are presented in Table 14, and can be used as a check list in developing a RP-GE separation.

In the following section, we shall discuss our initial solvent program, decide what improvements are necessary, and present straightforward procedures for implementing these improvements.

TABLE 14

STEPS IN DESIGNING A "GENERAL OPTIMAL" RP-GE SEPARATION

No.	*Step*
1	Select a linear gradient profile
2	Choose organic modifier (acetonitrile)
3	Determine Φ' (given t_0); an approximate value is adequate
4	Choose gradient range (0–100% B for unknown sample)
5	Calculate gradient time, t_s
6	Determine gradient delay, t_d
7	Gradient blank
8	Gradient of standards
9	Gradient of sample(s)
10	Modify gradient range
11	Fine tune the system (see Table 15 and accompanying discussion)

D. Column regeneration

The importance of re-equilibration to initial gradient conditions cannot be overemphasized. Too often, irreproducible results are due to inadequate re-equilibration. A generally accepted procedure is to run a 10–15-min reverse gradient followed by 10 min under the initial conditions (with a typical flow-rate of 2.0 ml/min). It should also be noted that column regeneration is dependent on the total volume of liquid passing through the column, not the time of column reconditioning. Therefore, regeneration will be faster using a steep reverse gradient at a high flow-rate.

7. "FINE TUNING"

As our "general optimal" RP gradient represents a compromise among resolution, detection sensitivity and analysis time, it stands to reason that we can optimize each of these parameters individually (at the expense of one or both of the

remaining two). In this section, we shall illustrate procedures for adjusting resolution and improving detection sensitivity, so that satisfactory separations can be obtained. Finally, procedures for minimizing the analysis time will be discussed (see Table 15 for a summary).

TABLE 15

SUMMARY OF FINE-TUNING PROCEDURES

Parameter	*Procedures*
Resolution	Increase gradient range if necessary Increase N Decrease b Change organic modifier
Detection sensitivity	Increase sample size Increase b Increase N
Optimizing analysis time	Increase initial %B (beginning) Decrease final %B (end) Increase b via decreasing t_s (R_s initially "too good")

A. Improving resolution in RP-GE

The principles of resolution in LSS gradient elution have been summarized in Part I[1]. In general, these principles are similar to those associated with isocratic RP-LC, as long as the analogy between k' and b is understood. In isocratic RP-LC, R_s can be improved by making appropriate changes in N, α and/or k'. Likewise, R_s in RP-GE can be improved by appropriate adjustments in N, α and/or b. We shall now discuss how each of these parameters can be adjusted to improve R_s in RP-GE.

(a) Gradient range

Before considering adjustments to N, α or b, we must check for elution of all sample compounds of interest prior to the end of the gradient, with no compounds eluting near t_0. If peaks continue to elute after the end of the gradient (*i.e.*, with gradient "hold", and pure B as mobile phase), a stronger solvent B is required. In this case, it is possible to substitute a solvent of higher S value (*e.g.*, tetrahydrofuran) for the original solvent acetonitrile. Alternatively, for very strongly retained compounds, it may be necessary to consider ternary gradients such as water–tetrahydrofuran–*n*-hexane (which requires a more complex pumping system). When one or more compounds of interest elute at t_0, it is necessary to consider some means for increasing their retention, *e.g.* by changing the pH or by use of ion pairing.

(b) Varying k' or b

Having approximately optimized b as discussed in Part I[1] and above, there is usually little reason to consider further adjustment of b for the purpose of increasing R_s (however, it may be worthwhile increasing b for increased detection sensitivity; see below). However, two minor points should be mentioned in passing as they relate to the question of optimal b in RP-GE.

First, as discussed in Appendix I, the optimal values of k' and of b in isocratic or gradient elution, respectively, do vary somewhat with the particle size of the column packing, and with separation time. For a relatively fast separation (14 min) as assumed in Table 13, and the use of 10-μm particles, a value of b close to 0.2 is indeed correct. However, for longer separation times and/or smaller particles, a value of $b = 0.1$ would have a slight advantage. Similarly, for larger particles (*e.g.*, 50-μm) and/or still shorter separation times (< 10 min), a value of b as large as 0.3 might be preferable. In any of these cases, however, we are talking of an increase in R_s (other factors being equal) of generally no more than 5–10%. At the same time, a change in b can result in small changes in α, which could largely cancel the increase in R_s as a result of change in b.

Secondly, if our initial separation involves a reduced velocity, $\nu \approx 3$ (*i.e.*, at minimal h), and if we do not want to change the solvent B or increase column length L, there is only one option available for increasing R_s: a decrease in b by 2–5-fold can in this case provide an increase in R_s of 10–20%. However, this would be accompanied by a corresponding increase in separation time of 2–5-fold, with a loss in detection sensitivity by the same factor.

(c) Varying N

The same options are available for increasing N in gradient elution as for isocratic elution. The two major approaches are a decrease in F (holding the column length L constant), or an increase in L with a proportionate increase in F (*i.e.*, holding pressure P constant). A predictable change in resolution in either of these two ways can be effected exactly as in the case of isocratic elution[7]. The only requirements during this change in L and/or F is that the gradient steepness be held constant, in terms of the change in $\%B/t_0$ (*i.e.*, b must be held constant). A summary of the necessary changes in the gradient steepness accompanying these two options for increasing N and R_s is given in Table 16, together with the necessary change in other separation variables.

TABLE 16

INCREASING RESOLUTION AND N IN GRADIENT ELUTION

Variable	*Column length constant*	*Column length varied*
Flow-rate, F	Decrease by factor x	Decrease by x
Column length, L	No change	Increase by x
Gradient steepness, %B/min	Decrease by x	Decrease by x^2
Separation time, t	Increase by x	Increase by x^2
Column pressure, P	Decrease by x	No change

It is important to note that if the gradient steepness (measured simply as Φ') is left unchanged when the flow-rate is decreased or the column length is increased, the true steepness in terms of b actually increases, because t_0 is increased in each case. This in turn means a decrease in the effective value of k' during separation, and a loss in resolution in some cases. Another reason for keeping b constant during a change in N is that then (and only then) will the relative elution order of different sample bands remain absolutely the same.

(d) Varying α

As in isocratic separation, α values in gradient elution do not vary as N is varied. Sometimes changes in the α values of adjacent bands result when b is changed. These changes in α are analogous to those occurring in isocratic separation when k' is varied by adjusting Φ_b. This has recently been discussed by Karger *et al.*[5,8]. To change α values in RP-GE deliberately, one must usually change either the mobile phase or the stationary phase, while holding b constant. Normally, the mobile phase composition will be varied in one of two ways. Firstly, another organic modifier can be selected, and the gradient re-optimized for this new solvent. For example, if our initial gradient were 7%/min methanol–water, 4.5%/min tetrahydrofuran–water can be substituted (see Table 4*). Hopefully this change in mobile phase will provide some change in α values, but leave average k' and N values at their original optimal levels. This is dramatically illustrated by comparing Figs. 5C and 6C, where the only difference is a change from AN to MeOH as organic modifier. Looking at components 5, 6, 7 and 8, remarkable changes in separation are observed.

In the second approach for changing α values, a third solvent C can be added to both solvents A and B (*e.g.*, ref. 9).

B. Detection sensitivity

If the detection sensitivity must be improved, there are two possible approaches in RP-GE. If R_s is not a problem, b can be increased to improve detection. This is analogous to decreasing k' in isocratic LC. This has been discussed in detail above, and Table 17 summarizes the relationship between b and detection sensitivity (increasing b increases detection sensitivity).

TABLE 17

RELATIONSHIP OF RESOLUTION AND PEAK SENSITIVITY IN GRADIENT ELUTION TO THE GRADIENT STEEPNESS, b

b	*Relative* R_s	*Relative sensitivity****
0.05	0.94	0.1
0.1	0.79	0.2
0.2*	0.63	0.4
0.3**	0.54	0.5
0.5	0.39	0.7
1.0	0.20	1.0
2.5	0.06	1.4

* Optimal value when column length L is held constant.
** Optimal value when column pressure P is held constant.
*** Relative to an isocratic band at t_0.

When resolution is more critical and cannot be attained at higher b values, the detection sensitivity can be improved by charging a larger sample to the column. Again, the analogy with isocratic LC is valid. Large samples can be charged to the column, provided that the solvent in which the sample is dissolved is sufficiently weak (so as not to cause significant migration of the sample bands of interest), and Φ_0 is as small as possible.

In general, the first step in increasing detection sensitivity should be to increase sample size when possible.

C. Minimizing analysis time

Having obtained the desired resolution and detection sensitivity, the final step in fine tuning our RP gradient is to minimize the analysis time. The two most general cases in which analysis time is wasted in RP-GE will now be discussed.

In the first case, the polarity range of the solvent program may be larger than required to elute the sample(s) of interest. This is easily recognized when there is empty space (*i.e.*, no peaks) at the beginning and/or end of the chromatogram. In this case, optimal use of the analysis time results by adjusting the initial and/or final Φ_b with concurrent adjustment of t_s in order to maintain b constant. This procedure effectively eliminates wasted time, while keeping R_s constant. A similar situation exists when early eluting peaks are present, but are of no interest for the particular sample(s). In this case the initial Φ_b is increased to the point where the sample component(s) of interest are resolved, but the early eluting peaks elute close to t_0. Fig. 4 provides an illustration of time wasted at the beginning of the gradient. Fig. 4B–E illustrates the decrease in t_s resulting from increasing the initial Φ_b at constant b.

In the second case, R_s is larger than required at optimal values of b, but the full gradient range is required (*i.e.*, 0–100% B). The most direct solution in this case is to increase Φ' (and hence b) by decreasing t_s, while keeping F constant. As t_s determines the analysis time, the improvement here is obvious. Alternatively, N can be offset against t_s by decreasing L and/or increasing F. Finally, simultaneously increasing b and F can result in significant time savings, when we have a higher N than is required.

8. SYMBOLS*

Φ_0	value of Φ for mobile phase entering column at $t = 0$.
Φ_g	value of Φ for mobile phase entering column at $t = t_g$.
t_d	delay time between initiation of gradient and actual change in Φ at head of column.
V_0	void volume of chromatographic column.

9. APPENDIX I

Optimal values of k′ and b for isocratic and gradient elution

In isocratic separations on large-particle (> 20-μm) columns, it has been shown[6] that the optimal value of k' is related to the slope, n, of the log k' *versus* log u plot for that column. If the column length L is held constant,

$$k' \text{ (optimal)} = 2/n \tag{i-1}$$

If the column pressure is held constant (L allowed to vary), then the optimal value of k' is

$$k' \text{ (optimal)} = 4/(1 + n) \tag{i-2}$$

* See also the symbols in Part I[1] (Section 7).

The values of n for large-particle columns generally range from 0.4 to 0.6, so that optimal values of k' vary between 2.5 and 5. As resolution is relatively insensitive to k' in this range, while separation time and detection sensitivity are adversely affected by an increase in k' beyond 5, there is little reason to consider adjustment in k' for most cases.

The situation is somewhat more complex in the case of separations on small-particle columns. In a preceding paper[7] values of n were derived for various values of the reduced velocity ν, and values of ν are in turn roughly related to particle size, d_p (for typical separation conditions). We can summarize these preceding treatments for porous particles as follows:

Reduced velocity ν	n	*Typical* d_p *(μm)*	*Optimal k' (eqns. i-1 and i-2)*	
			Fixed L	*Fixed P*
3	0.02	—	100	3.9
10	0.35	5	5.7	3.0
30	0.53	15	3.8	2.6
100	0.68	45	2.9	2.4

Again, the above optimal values of k' refer to the maximization of R_s, without regard to possible loss in detection sensitivity. The main conclusion to draw is that the optimal k' tends to increase somewhat as the particle size becomes smaller, and this effect is more pronounced when the column length is fixed.

The situation is precisely analogous in the case of gradient elution. While a value of $b = 0.2$ is a good general compromise for most separations, resolution can be increased somewhat by using lower values of b in the case of separations on small-particle columns. This trend is apparent in Fig. 7 in Part I[1], where a 10-μm column shows optimal values of b that are generally closer to 0.1. Similarly, in Fig. 5 in the present paper, it is apparent that maximal resolution occurs at $b \approx 0.1$ for the 5-μm column used. Also apparent in Fig. 7 in Part I[1] is the fact that longer separation times generally favor smaller values of b, and this is true also of small-particle separations by isocratic elution.

10. SUMMARY

The theory developed in Part I is verified experimentally in gradient separations with C_{18} columns and solvent systems consisting of water–methanol, water–acetonitrile and water–tetrahydrofuran. Linear solvent strength separations correspond to gradients that vary linearly with time. Some practical rules for optimizing such separations are presented.

REFERENCES

1 L. R. Snyder, J. W. Dolan and J. R. Gant, *J. Chromatogr.*, 165 (1979) 3.
2 P. J. Schoenmakers, H. A. H. Billiet, R. Tijssen and L. de Galan, *J. Chromatogr.*, 149 (1978) 519.
3 N. Tanaka and E. R. Thornton, *J. Amer. Chem. Soc.*, 99 (1977) 7300.
4 L. R. Snyder and H. D. Warren, *J. Chromatogr.*, 15 (1964) 344.
5 N. Tanaka, H. Goodell and B. L. Karger, *J. Chromatogr.*, 158 (1978) 233.
6 L. R. Snyder, *J. Chromatogr. Sci.*, 7 (1969) 352.
7 L. R. Snyder, *J. Chromatogr. Sci.*, 15 (1977) 441.
8 B. L. Karger, J. R. Gant, A. Hartkopf and P. H. Weiner, *J. Chromatogr.*, 128 (1976) 65.
9 L. B. Sybrandt and E. F. Montoya, *Amer. Lab.*, 9, No. 8 (1977) 79.