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INFLUENCE OF ORGANIC MODIFIERS ON THE RETENTION BEHAVIOUR IN REVERSED-PHASE LIQUID CHROMATOGRAPHY AND ITS CONSEQUENCES FOR GRADIENT ELUTION

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SUMMARY

In a previous paper it was shown that the key to the description and understanding of retention behaviour under gradient conditions in reversed-phase liquid chromatography lies in the relationship between the isocratic capacity factor (k) and the volume fraction of organic modifier (φ). Hence, the presentation of extensive data on this relationship in different organic modifier systems offers possibilities for evaluating and refining existing gradient elution models, such as the one of Snyder and co-workers.

An extensive study of 32 aromatic compounds in aqueous systems containing 10–100% of methanol, acetonitrile and tetrahydrofuran as organic modifiers under isocratic conditions is described. Two major conclusions are drawn that have consequences for gradient elution. Firstly, the relationship between ln k and φ is generally non-linear, in accordance with previous statements. Secondly, the rate of change of ln k with φ can be solute dependent. Systematic changes in the slope of the ln k *versus* φ curves with absolute retention are observed for methanol and tetrahydrofuran as organic modifiers. It is shown that this conclusion leads to optimal gradients, which are convex rather than linear.

THEORETICAL

Gradient elution equation

In a previous paper[1] we have shown that the basic equation for the net retention time (t'_R) in gradient elution is given by

$$\int_0^{t'_R} \frac{d[f^{-1}(\varphi)]}{k(\varphi)} = t_m \qquad (1)$$

where $k(\varphi)$ describes the capacity factor as a function of the volume fraction, φ, of organic modifier in a binary mixture and t_m is the time spent in the mobile phase. It should be noted that the choice of t_m must be consistent with the definition of the capacity factor and that t_m cannot be allowed to vary with composition. These

restrictions are inherent in all gradient elution models. Finally, in eqn. 1 f^{-1} is called the inverse gradient function. If the gradient programme is formulated generally as

$$\varphi = f(t) \tag{2}$$

then

$$t = f^{-1}(\varphi) \tag{3}$$

For the general case of a non-linear gradient and/or a complex $k(\varphi)$ function, eqn. 1 can only be solved by numerical integration.

It should be noted that gradient programmers do not deliver the imposed gradient without transformation and delay[2]. Both should be minimized as much as possible, but delays are easy to account for mathematically[1] or by injecting the sample after the start of the programme. For the special case of a linear gradient, eqns. 2, 3 and 1 change into

$$\varphi = a + bt \tag{4}$$

$$f^{-1}(\varphi) = (\varphi - a)/b \tag{5}$$

and

$$\int_{a}^{a+bt'_R} \frac{d\varphi}{k(\varphi)} = bt_m \tag{6}$$

where eqn. 6 can be solved analytically for linear and quadratic relationships between $\ln k$ and φ (ref. 1).

The case of a linear gradient is of special interest, because of its widespread use and conceptual simplicity. Also, Snyder and co-workers[3,4] have indicated that linear gradients are optimal for reversed-phase high-performance liquid chromatography (HPLC). However, this is only true when a number of conditions are fulfilled, two of which appear to be rather arguable:

(a) For each solute $\ln k$ varies linearly with φ, according to

$$\ln k = \ln k_0 - S\varphi \tag{7}$$

(b) the slope S in eqn. 7 is identical for all solutes, *i.e.*, the straight lines for different solutes in a given modifier system are parallel.

These conditions will now be examined in detail.

Relationship between ln k and φ

It has been predicted from a theoretical model[1] that $\ln k$ varies quadratically with φ. This is confirmed by the experimental data presented below. In agreement with theory, the curvature is more pronounced for less polar organic modifiers, but even in the system methanol–water it is readily apparent if data are collected over a sufficiently wide range of φ. The choice of the time spent in the mobile phase, t_m, does not impair these conclusions.

Consequently $k(\varphi)$ should correctly be expressed as

$$\ln k = A\varphi^2 + B\varphi + C \tag{8}$$

Snyder and co-workers[3,4], conceding this point, nevertheless argue that a linear relationship (eqn. 7) provides an adequate approximation for practical gradient elution. Indeed, for very large capacity factors ($k > 10$) the solute hardly moves through the column, whereas in a well designed programme the solute leaves the column before its capacity factor becomes very small ($k < 1$). Consequently, the $\ln k$ *versus* φ relationship is of practical interest only over the restricted range of $1 < k < 10$ or $0 < \ln k < 2.3$.

To verify this argument, data are given in Table I for quadratic curves of variable curvature drawn through two fixed data points: $\ln k = 2$, $\varphi = 0.3$ and $\ln k = 1$, $\varphi = 0.5$. Obviously a linear relationship between $\ln k$ and φ is represented by the top line in Table I ($A = 0$).

TABLE I

INFLUENCE OF CURVATURE OF THE $\ln k$ *VERSUS* φ CURVE UPON GRADIENT ELUTION BEHAVIOUR

Coefficients in eqn. 8			t_R* *(min)*	φ_e**	$\ln k_e$***	k_e***
A	*B*	*C*				
0	− 5.0	3.5	9.9	0.45	1.28	3.58
1	− 5.8	3.65	10.0	0.45	1.24	3.46
2	− 6.6	3.8	10.0	0.45	1.24	3.44
3	− 7.4	3.95	10.0	0.45	1.23	3.41
4	− 8.2	4.1	10.1	0.46	1.20	3.31
5	− 9.0	4.25	10.1	0.46	1.19	3 29
10	−13.0	5.0	10.2	0.46	1.14	3.11
20	−21.0	6.5	10.2	0.46	1.07	2.92

* Calculated by numerical integration for a 0–100% linear gradient in 20 min; $t_m = 1$ min.

** $\varphi_e = \varphi(t_R - t_m)$ is the local composition of the mobile phase when the solute leaves the column.

*** k_e is the k value of the solute on leaving the column.

It can be seen from Table I that the only parameter that changes significantly with increasing curvature is the capacity factor, k_e, of a solute as it leaves the column. This parameter is of interest for the calculation of peak broadening and detector sensitivity under gradient conditions. On the other hand, the net retention time of the solute (t'_R) and the composition of the solvent (φ_e) at which it leaves the column are extremely insensitive to the imposed curvature. In other words, over the restricted range $0 < \ln k < 2.3$ the linear approximation expressed by eqn. 7 provides a good description of gradient elution behaviour. It should be emphasized that in using this approximation the intercept $\ln k_0$ has no distinct physical significance. Specifically, k_0 is not equal to, but rather is much smaller than, the capacity factor of a solute in pure water (compare Fig. 2a–c below). In fact, the slope S and the intercept

$\ln k_0$ in eqn. 7 can be readily expressed in the coefficients of the quadratic expression (eqn. 8):

$$S = \frac{-3[A(\varphi_1^2 + \varphi_2^2) - B(\varphi_1 + \varphi_2)](\varphi_1 + \varphi_2) + 2[A(\varphi_1 + \varphi_2) - 2B](\varphi_1^2 + \varphi_1\varphi_2 + \varphi_2^2)}{(\varphi_1 - \varphi_2)^2} \quad (9)$$

$$\ln k_0 = \frac{3A(\varphi_1^2 + \varphi_2^2)(\varphi_1 + \varphi_2) + 4(B + S)(\varphi_1^2 + \varphi_1\varphi_2 + \varphi_2^2) + 6C(\varphi_1 + \varphi_2)}{6(\varphi_1 + \varphi_2)} \quad (10)$$

where φ_1 and φ_2 are the solvent compositions for which $\ln k = 2.3$ and 0, respectively.

Variation of slope S for different solutes

As the first condition for optimal gradients to be linear does not appear to be critical, we now turn to the second condition. Some workers have indicated for different solutes that the slope S in eqn. 7 occasionally varies systematically throughout the chromatogram[3–6]. Indeed, the extensive data presented below show that in methanol–water and in tetrahydrofuran–water (but not in acetonitrile–water) the slope, S, calculated from eqn. 9 varies systematically with the intercept, $\ln k_0$, calculated from eqn. 10. It appears that the correlation is approximately linear:

$$S = p + q \ln k_0 \quad (11)$$

The importance of this correlation becomes clear if we wish to define an optimal gradient. The ultimate aim is a gradient programme such that all solutes elute with the same peak width and sensitivity. According to Snyder and co-workers[3,4] this is realized when during the chromatographic run

$$\beta = \frac{d\varphi}{dt} \cdot St_m \quad (12)$$

is kept constant. In this expression $d\varphi/dt$ follows from the gradient function (eqn. 2) and is obviously constant for a linear gradient (eqn. 4). However, a linear gradient can be reconciled with the condition of constant β only as long as S is constant. As we have seen above, this is not true. Firstly, for a particular solute the slope S (*i.e.*, $-\mathrm{d}\ln k/\mathrm{d}\varphi$) decreases slightly with increasing φ (eqn. 8). Fortunately, if we restrict ourselves to a limited range ($1 < k < 10$), this variation is very small and hardly affects the elution behaviour of the solute (Table I). Consequently, the non-linear relationship between $\ln k$ and φ presents an insufficient reason to use a non-linear gradient programme.

Secondly, however, for different solutes the slope S in eqn. 12 may increase significantly with retention (eqn. 11), *i.e.*, with increasing φ_e. In turn, this means that the parameter β, defined by eqn. 12, can be kept constant only if the increase in S is compensated for by a gradual decrease in $d\varphi/dt$ during the chromatographic run. This results in a non-linear, convex gradient programme designed to yield a constant β.

Mathematical expression for the constant-β gradient

A mathematical expression for the optimal gradient can be obtained by selecting some k value for which we shall make the elution conditions similar for all solutes. Such a reference value should be in the range over which we expect solute migration to take place ($1 < k < 10$). It seems reasonable and convenient to choose $\ln k = 1$ as the reference k value for the migration of solute zones (see also refs. 3 and 4).

The approach of making conditions equal at one particular k value is a plausible one, as a constant β value means that the elution pattern (*i.e.*, the variation in k with time and hence migration velocity and migration distance with time) is similar for all solutes. It can readily be shown that the choice of the reference value does not affect the resulting expression for an optimal gradient shape. The gradient time t_G (see below) does vary with the choice of k_{ref}, but only to a limited extent. Choosing $\ln k = 1$ to make conditions similar has the advantage that an optimal β value of about 0.5 can be taken from Snyder and co-workers' work[3,4].

According to eqn. 2, the constant-β gradient is now defined by

$$\left(\frac{d\varphi}{dt}\right)_{\ln k=1} = \frac{\beta}{St_m} = \frac{\beta}{(p + q \ln k_0)t_m} \tag{13}$$

where eqn. 11 has been used. Substituting $\ln k = 1$ and eqn. 11 in eqn. 7 we find

$$\ln k_0 = \frac{1 + p\varphi}{1 - q\varphi} \tag{14}$$

and substitution of eqn. 14 in eqn. 13 then yields

$$\frac{d\varphi}{dt} = \frac{\beta(1 - q\varphi)}{(p + q)t_m} \tag{15}$$

which can be integrated to yield

$$\int_0^{\varphi} \frac{d\varphi}{1 - q\varphi} = \frac{\beta}{(p + q)t_m} \int_0^{t} dt \tag{16}$$

or

$$-\ln (1 - q\varphi) = \frac{\beta q t}{(p + q)t_m} \tag{17}$$

If we define a time t_G needed to run a complete gradient, *i.e.*, φ from 0 to 1, we have

$$t_G = t_{(\varphi=1)} = -\frac{(p + q)t_m \ln(1 - q)}{\beta q} \tag{18}$$

If the reference value for k is not taken as $\ln k_{ref} = 1$, but retained in the derivation, this expression becomes

$$t_G = -\frac{(p + q \ln k_{ref})t_m \ln(1 - q)}{\beta q} \tag{18a}$$

Now, eqn. 17 can be expressed as

$$\frac{\ln(1 - q\varphi)}{\ln(1 - q)} = \frac{t}{t_G} = \tau \tag{19}$$

or

$$\varphi = 1/q[1 - (1 - q)^{\tau}] \tag{20}$$

Clearly, τ is a dimensionless parameter, expressing the running time t as a fraction of the total time t_G needed to go from pure water to pure organic modifier. Provided the parameters p and q are known, this total time can be calculated from eqn. 18 for any value of the gradient steepness parameter β. From eqn. 20, we conclude that the shape of the gradient programme (φ as a function of τ) is determined only by the coefficient q, which describes the correlation between S and $\ln k_0$ (eqn. 11).

For the limiting case of $q = 0$, eqn. 20 correctly becomes a straight line:

$$\varphi = \tau \tag{21}$$

and the gradient time becomes, from eqn. 18

$$t_{G(q=0)} = \frac{pt_m}{\beta} = \frac{St_m}{\beta} \tag{22}$$

because for $q = 0$, $S = p =$ constant. Eqns. 21 and 22 and 12 can be combined to yield

$$\varphi = \frac{\beta t}{St_m} = \frac{d\varphi}{dt} \cdot t = bt \tag{23}$$

which resembles the simplest form of a linear gradient starting at pure water (eqn. 4 for $a = 0$).

When $q \neq 0$, eqn. 20 describes curved gradients, convex for $q > 0$ and concave for $q < 0$. Some examples for $q > 0$ are given in Fig. 1. Our data suggest that in reversed-phase liquid chromatography (RPLC) q is not negative. From Fig. 1 it can be seen that optimal gradient curves become increasingly convex if q increases until $q = 1$. Both eqns. 18 and 20 become invalid for $q > 1$. Such q values would indicate that in going from pure water to pure organic modifier the order of elution of the solutes would be reversed. Apart from the physical reality of this phenomenon, such a system would not be of any practical value in gradient elution chromatography.

Finally, it should be noted that t_G represents the real gradient time only if the gradient is actually run from pure water to pure organic modifier. In practice, a gradient programme can be started and stopped at any arbitrary φ value. In all instances, however, the gradient programme should follow the curve prescribed by eqn. 20. For limited intervals of φ, eqn. 19 gives the initial and final values of τ, from which the actual gradient time can be calculated with eqn. 18. An example is

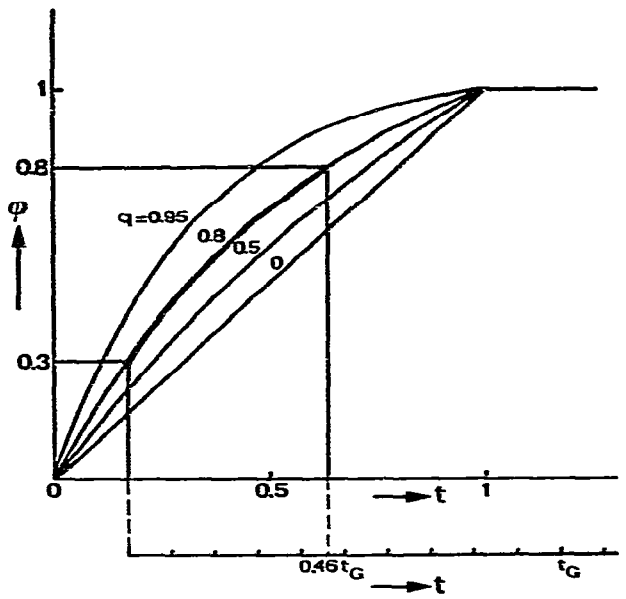


Fig. 1. Shape of constant-β gradients for various q values.

shown in Fig. 1 for a gradient running from $\varphi = 0.3$ to 0.8 (modifier content varying from 30 to 80%) in a system with $q = 0.8$. The running time starts at $\tau = 0.17$ and lasts until $\tau = 0.83$, so that the gradient is run in over a period equal to $t = 0.66$ t_G. This can be calculated from eqn. 18 with known values of p, t_m and β.

EXPERIMENTAL

All data were collected with the same equipment, consisting of one (isocratic) or two (gradients) Model 6000A pumps working at a total flow-rate of 1.5 ml/min, a U6K injector, a Model 440 UV detector operating at 254 nm and a Model 660 solvent programmer, all from Waters Assoc. (Milford, Mass., U.S.A.). The injector and column were thermostated at 25° using a circulating water-bath. All experiments were performed with a single column (30 cm × 4.6. mm I.D.), home packed with Merck RP-18 material (Merck, Darmstadt, G.F.R.). The solvents used were methanol and tetrahydrofuran (THF) from Baker (Philipsburg, N.J., U.S.A.) and acetonitrile from Merck. Water was specially treated with ion-exchange resins and carbon filters after distillation. All data were collected by means of an on-line coupled PDP 11 computer. Retention times were obtained from the first central moments of the eluted peaks.

Special attention was paid to the choice of the time spent in the mobile phase (t_m), as will be reported in a later publication. For the present column $t_m = 107$ sec, representing the elution time of water and D_2O from about 60 to 80% modifier and that of potassium bromide over the whole range of φ, excluding very high concentrations (>90%) of acetonitrile and THF. The conclusions drawn below do not change significantly if t_m is varied within reasonable limits. Each gradient run was followed

by a reversed linear gradient of 15 min and a re-equilibration period of about 5 min. Injection of the sample was held up until 1 min after the start of the gradient programme as a rough compensation for the delay time in the present system[2]. Solvents used to compose gradients were continuously deaerated with helium and all runs were performed with the injector in the load mode.

The gradient delivery system used does not deliver ideal gradients of any arbitrary shape. We are at present constructing a solvent delivery system that qualifies for a more reliable test of the conclusions reached in this study[2]. In the present study we are restricted to a linear and some convex gradients.

RESULTS AND DISCUSSION

The results of the isocratic measurements on the ln k *versus* φ relationship are summarized in Tables II and III, where the coefficients for the quadratic expression

TABLE II

ln k *VERSUS* φ RELATIONSHIP IN METHANOL–WATER

No.	*Compound*	*A* (eqn. 8)	*B* (eqn. 8)	*C* (eqn. 8)	*S.D.*	$\ln k_0$ (eqn. 7)	*S* (eqn. 7)
1	Acetophenone	3.73	−10.01	5.51	0.08	4.34	5.74
2	Anethole	1.74	−11.24	9.35	0.07	8.13	8.31
3	Aniline	1.84	− 6.09	3.20	0.07	2.94	4.60
4	Anisole	8.54	−18.70	9.75	0.14	5.77	6.92
5	Anthracene	—	—	—	—	(12.84)	(12.60)
6	Benzaldehyde	1.88	− 6.98	4.21	0.13	3.72	5.00
7	Benzene	0.30	− 5.95	5.09	0.08	4.95	5.54
8	Benzonitrile	3.01	− 8.75	4.79	0.06	4.00	5.59
9	Benzophenone	7.39	−18.98	11.09	0.06	6.96	7.86
10	Benzyl alcohol	2.58	− 7.30	3.71	0.11	3.26	5.05
11	Biphenyl	1.18	−11.08	9.77	0.08	8.91	9.06
12	Chlorobenzene	3.56	−12.45	8.49	0.06	6.44	7.01
13	*o*-Cresol	2.40	− 8.32	4.95	0.09	4.23	5.64
14	Diethyl *o*-phthalate	7.27	−17.83	9.74	0.07	6.46	7.98
15	N,N-Dimethylaniline	3.67	−12.39	8.37	0.05	6.26	6.78
16	2,4-Dimethylphenol	2.75	− 9.94	6.35	0.08	5.22	6.37
17	Dimethyl *o*-phthalate	6.46	−14.47	7.07	0.06	5.09	7.23
18	*m*-Dinitrobenzene	0.08	− 5.10	4.01	0.13	3.99	5.01
19	Diphenyl ether	2.35	−12.84	10.21	0.07	8.58	8.91
20	Ethylbenzene	1.86	−10.70	8.60	0.06	7.38	7.67
21	N-Methylaniline	3.99	−11.30	6.54	0.07	5.01	6.29
22	Naphthalene	−0.59	−11.11	11.36	0.18	11.81	12.15
23	*p*-Nitroacetophenone	2.32	− 8.12	4.87	0.09	4.18	5.52
24	*o*-Nitroaniline	2.07	− 7.39	4.34	0.11	3.81	5.23
25	Nitrobenzene	1.45	− 7.21	4.94	0.09	4.42	5.44
26	*m*-Nitrophenol	2.14	− 7.64	4.43	0.08	3.89	5.43
27	Phenol	2.14	− 6.70	3.47	0.07	3.13	4.90
28	1-Phenylethanol	3.24	− 9.07	4.89	0.05	4.04	5.66
29	2-Phenylethanol	2.94	− 8.65	4.74	0.08	3.98	5.58
30	3-Phenylpropanol	3.18	−10.21	6.09	0.10	4.94	6.33
31	Quinolone	8.12	−17.77	9.23	0.03	5.58	6.76
32	Toluene	2.75	−10.95	7.87	0.05	6.27	6.72

TABLE III

ln k *VERSUS* φ RELATIONSHIP IN ACETONITRILE-WATER AND TETRAHYDROFURAN-WATER

*No.**	*Acetonitrile-water*						*Tetrahydrofuran-water*					
	A (eqn. 8)	*B* (eqn. 8)	*C* (eqn. 8)	*S.D.*	$\ln k_0$ (eqn. 7)	*S* (eqn. 7)	*A* (eqn. 8)	*B* (eqn. 8)	*C* (eqn. 8)	*S.D.*	$\ln k_0$ (eqn. 7)	*S* (eqn. 7)
1	4.24	− 9.18	4.17	0.05	3.42	5.47	4.46	− 9.54	3.62	0.02	3.21	6.68
2	3.76	−11.21	7.10	0.02	5.22	5.83	6.43	−14.92	7.07	0.03	5.35	8.19
3	4.02	− 8.01	3.16	0.11	2.79	5.38	1.28	− 6.05	2.95	0.13	2.83	5.20
4	3.63	− 9.40	5.08	0.03	4.02	5.38	5.58	−12.36	5.43	0.03	4.36	7.36
5	2.21	− 9.53	7.42	0.03	5.95	5.89	7.93	−17.60	8.24	0.02	5.95	8.99
6	3.48	− 8.13	3.79	0.03	3.23	5.19	3.64	− 8.51	3.37	0.05	3.04	6.19
7	3.20	− 8.81	5.01	0.02	4.00	5.11	5.00	−11.78	5.51	0.05	4.41	6.99
8	3.73	− 8.85	4.21	0.03	3.51	5.50	4.10	− 9.53	3.87	0.09	3.41	6.65
9	5.31	−12.92	7.08	0.03	4.94	6.08	7.61	−15.92	6.81	0.06	5.18	8.79
10	5.38	− 9.42	3.16	0.12	2.81	6.47	4.18	− 8.36	2.66	0.06	2.50	6.52
11	4.14	−12.25	7.84	0.04	5.58	6.07	6.96	−16.09	7.70	0.02	5.70	8.55
12	4.27	−11.11	6.40	0.04	4.64	5.53	6.96	−15.17	6.93	0.09	5.17	8.08
13	4.76	− 9.93	4.26	0.04	3.51	6.01	6.90	−14.07	5.67	0.04	4.53	8.34
14	5.23	−12.36	6.36	0.03	4.69	6.34	7.27	−14.55	5.75	0.06	4.58	8.61
15	5.21	−11.74	6.44	0.06	4.07	4.54	4.94	−12.42	6.13	0.05	4.85	7.31
16	5.44	−11.47	5.27	0.03	4.03	6.15	7.63	−15.63	6.49	0.06	5.00	8.80
17	5.36	−11.23	4.94	0.06	3.92	6.42	6.05	−11.55	4.00	0.06	3.47	7.83
18	3.82	− 9.71	4.79	0.04	3.96	6.05	6.22	−13.84	6.04	0.03	4.79	8.18
19	6.20	−15.31	8.77	0.07	5.61	6.36	7.17	−16.43	7.82	0.01	5.76	8.66
20	3.45	−10.47	6.72	0.03	5.01	5.54	5.39	−13.50	6.83	0.03	5.23	7.56
21	3.43	− 8.46	4.39	0.05	3.56	4.96	2.51	− 8.85	4.60	0.09	4.10	6.55
22	1.86	− 8.02	5.82	0.05	4.91	5.38	7.98	−16.95	7.67	0.09	5.56	8.64
23	4.26	− 9.86	4.60	0.04	3.77	5.98	5.16	−11.55	4.79	0.04	4.02	7.47
24	4.18	− 9.40	4.21	0.04	3.52	5.88	7.08	−14.05	5.47	0.03	4.41	8.45
25	3.50	− 9.09	4.69	0.02	3.85	5.57	5.95	−12.69	5.32	0.04	4.30	7.66
26	5.62	−10.89	4.24	0.06	3.53	6.74	9.12	−17.27	6.65	0.06	5.10	9.64
27	4.82	− 9.20	3.39	0.09	2.97	6.19	4.22	− 9.70	3.87	0.11	3.41	6.79
28	6.25	−11.08	4.05	0.13	3.37	6.77	4.65	− 9.62	3.49	0.09	3.11	6.83
29	6.77	−11.68	4.16	0.13	3.44	7.08	5.64	−10.64	3.52	0.06	3.14	7.54
30	6.72	−12.23	4.83	0.06	3.77	6.74	7.22	−13.46	4.79	0.07	3.98	8.50
31	—	—	—	—	—	—	6.44	−11.65	4.05	0.11	3.45	7.56
32	3.82	−10.45	6.20	0.03	4.60	5.42	6.01	−13.81	6.60	0.06	4.99	7.51

* For identification see Table II.

(eqn. 8) are given together with an overall standard deviation (for ln k). The quadratic curve describes k values up to about 50, with an accuracy that can be estimated from the standard deviation. For example, a standard deviation of 0.05 implies an average error in k of about 5%.

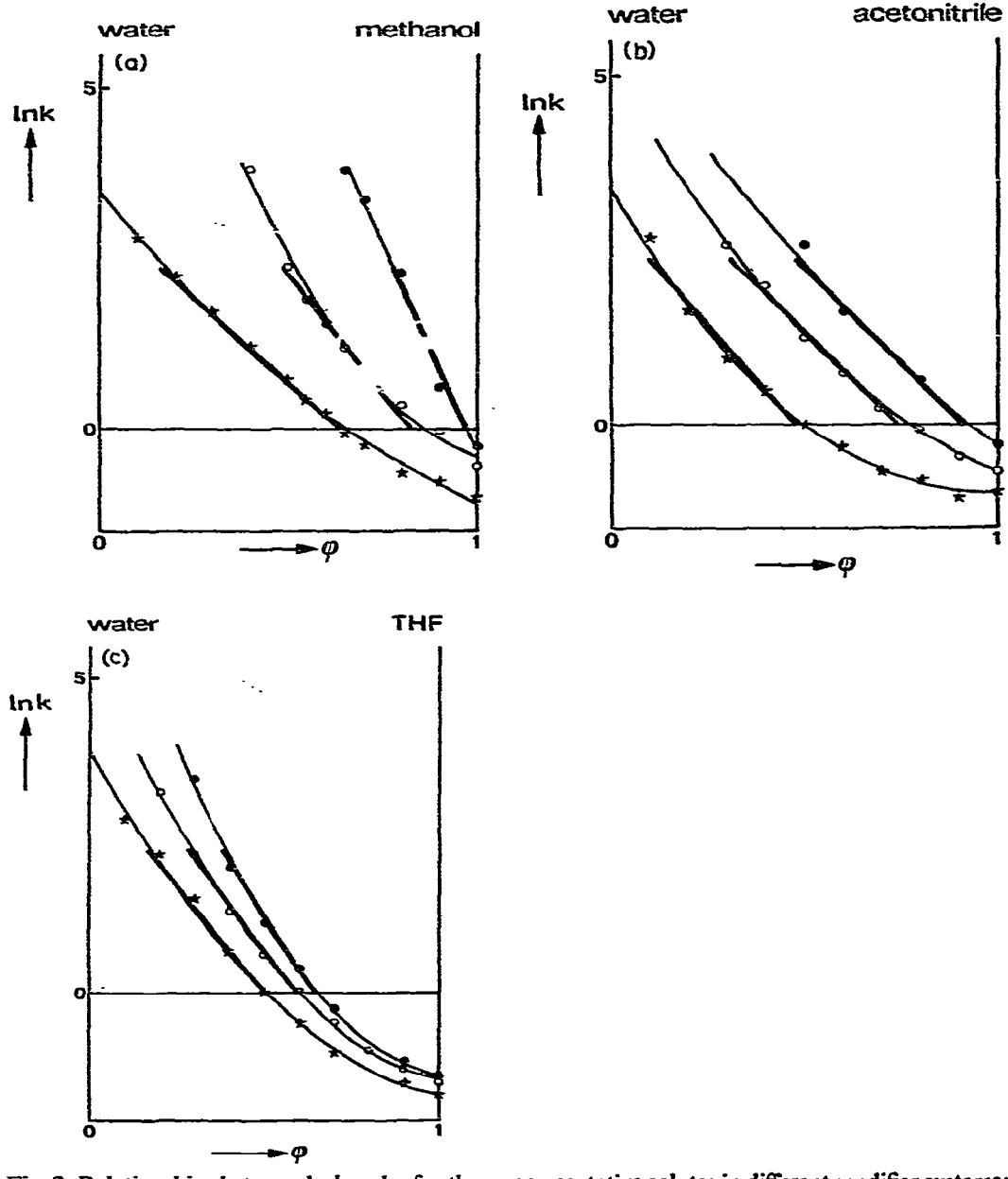


Fig. 2. Relationships between ln k and φ for three representative solutes in different modifier systems: (a) methanol–water; (b) acetonitrile–water; (c) THF–water. ●, Naphthalene; ○, anisole; ★, phenol. Thin lines, eqn. 8 ($k < 50$); thick lines, eqn. 7 ($1 < k < 10$).

Fig. 2a–c show some typical examples of ln k *versus* φ plots in the three solvent systems studied. The three solutes shown in each diagram are representative of the whole set of 32 solutes. Some of the differences between the three systems are readily apparent, and can be summarized as follows.

(1) Separation factors (relative retention) can be identified as the vertical differences between the curves for a given φ value:

$$\ln \alpha_{21} = \ln k_2 - \ln k_1 = \Delta \ln k \tag{24}$$

It is clear from Fig. 2a–c that separation factors increase in the order THF < acetonitrile < methanol. Generally, therefore, methanol can be expected to yield the highest selectivity for an arbitrary sample. Note, however, that this is a rule of thumb, as in some particular instances acetonitrile and especially THF show considerable specificity[7].

(2) The rather higher separation factors in methanol–water have the disadvantage that samples containing widely varying components cannot easily be analysed under isocratic conditions. *E.g.*, for the three solutes in Fig. 2a it is impossible to find a single isocratic composition for which all peaks will elute in the range $1 < k < 10$. If we take φ to be 0.65, phenol will elute rapidly with $k = 1$, but then naphthalene will take a very long time to leave the column, as it has a capacity factor of $k \approx 50$. On the other hand, in water–THF (1:1) both components elute reasonably fast, as $k \approx 1$ for phenol and $k \approx 3$ for naphthalene. Hence, we can say that in the case of a more than adequate separation in methanol–water, THF is a useful alternative modifier to decrease the analysis time under isocratic conditions or to avoid the use of gradient elution. In general, however, methanol is a more useful modifier for gradient elution analysis of complex samples.

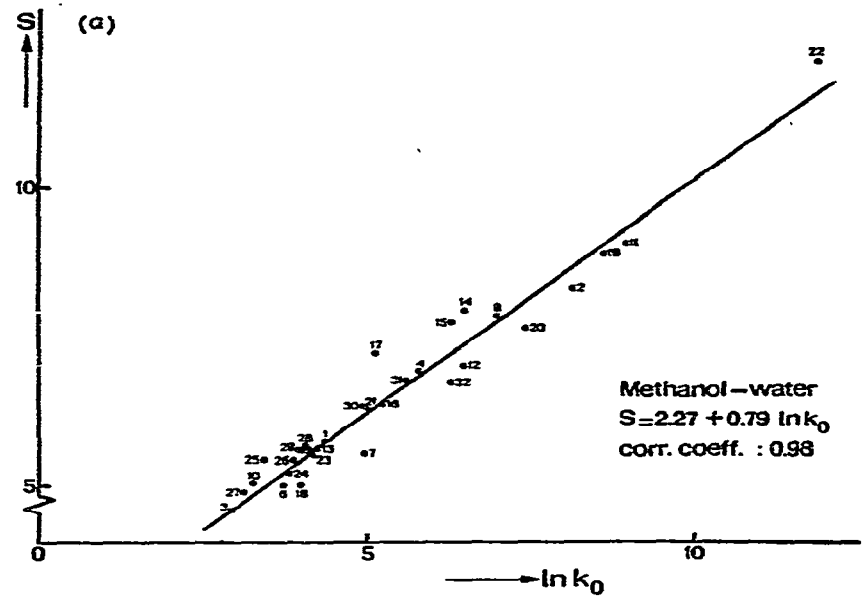
(3) For methanol and THF the curves for different solutes appear to be convergent, whereas for acetonitrile they are more or less parallel. This conclusion can be generalized to at least the 32 solutes studied (see Fig. 3). Therefore, the degree of separation is expected to increase when the methanol or THF concentrations are lowered. The increase in separation time can be compensated for by using shorter columns. In this way, separations can purposely be varied in methanol and THF. In acetonitrile–water systems, however, separation is roughly independent of binary composition and of separation time, at least over a limited range of k values.

The last conclusion is substantiated in Fig. 3, where for the linear approximation to the ln k *versus* φ curves (eqn. 7), the slopes are plotted against the intercepts for all 32 solutes in three different systems. Obviously, a positive correlation exists between φ and ln k_0 in both methanol–water and THF–water, but not acetonitrile–water. The resulting parameters for these correlations according to eqn. 11 are given in Table IV. For acetonitrile the absence of any correlation implies a q value of zero and a p value representing the average of all S values. Table IV also includes total gradient times (t_G) for a 0–100% modifier gradient for $\beta = 0.5$ and $t_m = 1.8$ min. Here eqn. 18 is used for THF and methanol and eqn. 22 for acetonitrile. The time for a 0–100% THF–water gradient is relatively long, but this is compensated for by the fact that k values between 1 and 10 usually occur over a limited range of φ and hence the actual gradient time can be limited to a much shorter period.

As derived under Theoretical, a non-zero value of q implies that the optimal

gradient curve is non-linear. Because methanol and THF fortuitously exhibit almost identical values of q, the same, convex gradient curve is predicted to be optimal in either system. This curve, calculated from eqn. 20, is shown in Fig. 4. For acetonitrile, however, $q = 0$ and hence a linear gradient should be optimal for this modifier (Fig. 4).

The validity of the theoretical predictions has been verified in two different ways. Firstly, Fig. 5 shows rigorously calculated chromatograms for a mixture of solutes subjected to two different water–methanol and water–THF gradients. Here



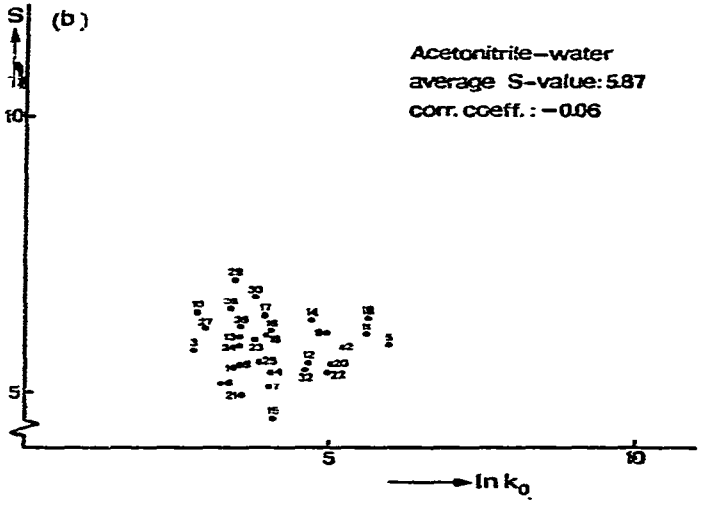


Fig. 3.

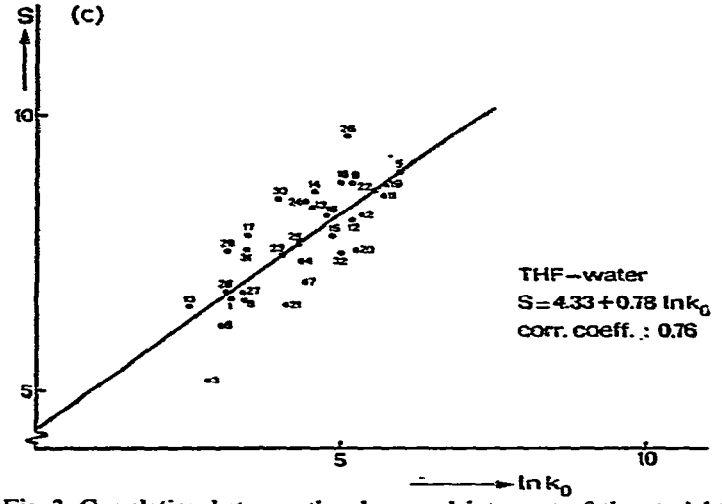


Fig. 3. Correlation between the slope and intercept of the straight-line approximation of the ln k *versus* φ relationship in different modifier systems. Solute identification numbers as in Table II. (a) Methanol–water, $S = 2.27 + 0.79 \ln k_0$, correlation coefficient = 0.98; (b) acetonitrile–water, average S value = 5.87, correlation coefficient = −0.06; (c) THF–water, $S = 4.33 + 0.78 \ln k_0$, correlation coefficient = 0.76.

TABLE IV

GRADIENT SHAPE PARAMETERS

System	p	q	n^*	r^{**}	t_G^{***}
Methanol–water	2.27	0.79	31	0.98	21.8
Acetonitrile–water	5.87[‡]	0[‡‡]	31	−0.06[‡‡]	21.1
THF–water	4.33	0.78	32	0.76	35.7

* Number of data points.
** Correlation coefficient.
*** For $\beta = 0.5$ and $t_m = 1.8$ min.
‡ Average S value.
‡‡ No correlation, hence $q = 0$.

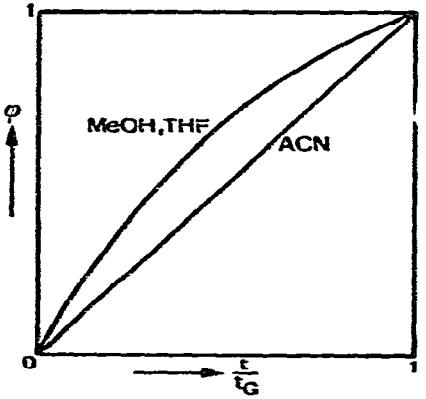


Fig. 4. Optimal gradient shapes according to eqn. 20; q values taken from Table IV.

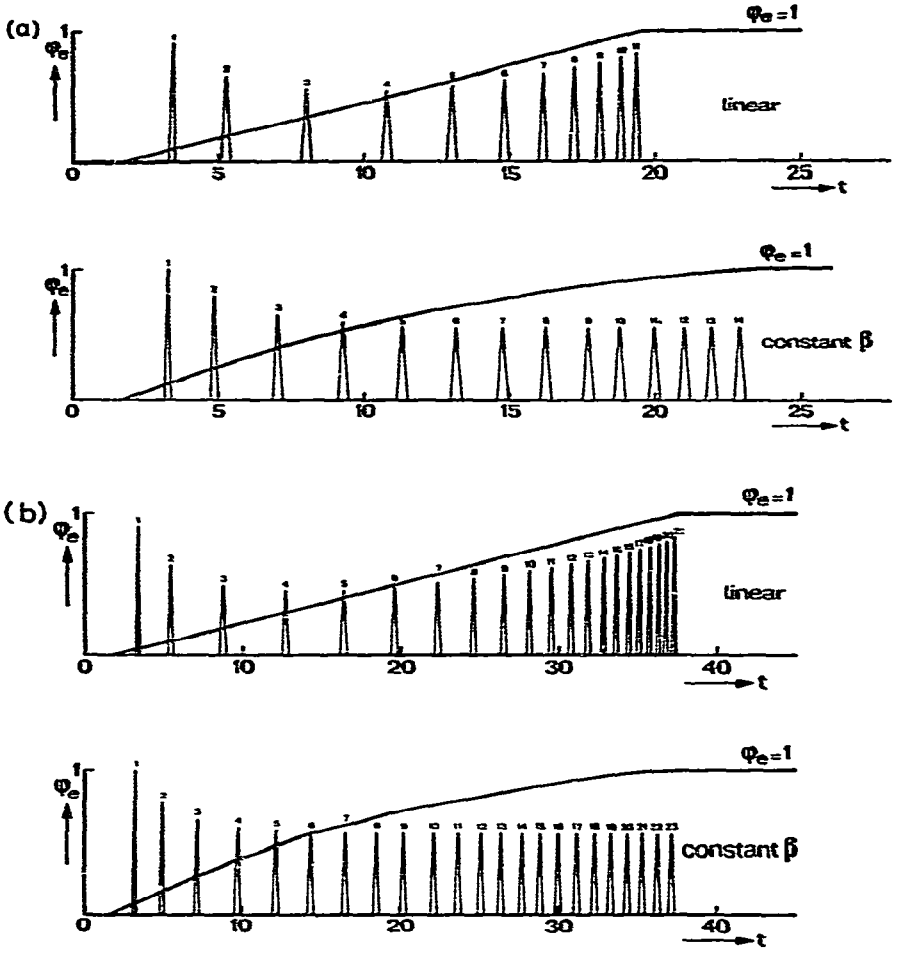


Fig. 5. (a) Calculated chromatograms showing the difference between linear and constant-β gradients (0–100% methanol–water) for a series of solutes for which $\ln k_0 = n$. $t_m = 1.78$ min; $G = 0.85$; $N = 2500$; $p = 2.27$; $q = 0.79$; t_G for constant-β gradient (eqn. 18, $\beta = 0.5$), 21.6 min; t_G for linear gradient (eqn. 22, $\beta = 0.5$ for $S = 5$), 17.8 min. All peak areas identical. (b) As in (a), but for 0–100% THF–water gradients. Conditions as in (a), except t_G (linear gradient) = t_G (constant-β gradient) = 35.7 min; $p = 4.33$; $q = 0.78$.

the sample is assumed to consist of components with regularly increasing values of $\ln k_0$ (*e.g.*, a homologous series). Retention times are calculated from eqn. 6 in the case of a linear gradient. For the optimal constant-β gradient, the parameters from Table IV are used to derive the gradient curve (eqn. 20) and the retention times are found by numerical integration of eqn. 1. In each chromatogram, the peaks are presented as triangles with $4\sigma_t$ base width calculated from an expression derived from Snyder and co-workers' model[3,4]:

$$4\sigma_t = \frac{4(\beta k_0 + k_0 + 1)Gt_m}{(\beta k_0 + 1)\sqrt{N}} \tag{25}$$

where G is a peak compression factor, which can be taken to be constant ($G = 0.85$), and N is the column plate number. Obviously, the convex, constant β-gradient is superior to the linear gradient, because it provides a constant peak width and a constant sensitivity throughout the gradient elution chromatogram.

The main difference between the methanol–water and the THF–water chromatograms is caused by the eluotropic strength of the organic modifier. Because THF is stronger than methanol, more homologues can be eluted under similar gradient conditions. Notice that the gradient time with THF is also longer, but that the peak widths in the two solvent systems are essentially identical, as long as optimal gradient shapes are applied.

As a second test, Fig. 6 illustrates the use of optimal gradients in practice. The top chromatogram shows a linear acetonitrile–water gradient applied to some of the solutes from Table II, resulting in a good chromatogram with roughly constant peak widths and a good distribution of peaks over the chromatogram. The centre chromatogram is the result of a linear methanol–water gradient applied to the same solutes. Clearly, the result is inferior to the top chromatogram, because the peaks

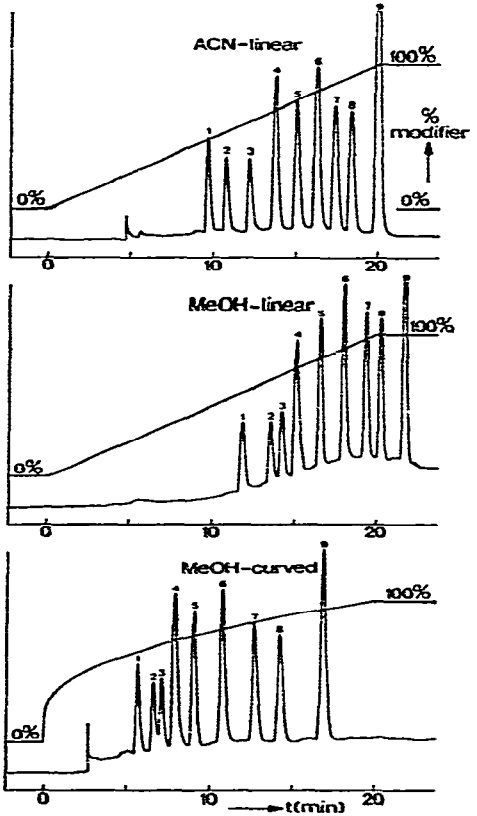


Fig. 6. Gradient elution chromatograms of a test mixture; 0–100% modifier gradients as indicated. See text for chromatographic conditions. Peaks: 1 = benzyl alcohol; 2 = 2-phenylethanol; 3 = *o*-cresol; 4 = nitrobenzene; 5 = diethyl *o*-phthalate; 6 = benzophenone; 7 = naphthalene; 8 = biphenyl; 9 = anthracene.

become significantly sharper and more closely spaced towards the end of the gradient run. The bottom trace presents the results of a convex methanol–water gradient. Although not exactly representing the optimal gradient shape of Fig. 4, the improvements in comparison with the centre chromatogram are evident.

It can be argued that to improve the middle chromatogram in Fig. 6 it is not necessary to use a curved gradient. From the centre chromatogram it can be seen that no bands occur for $\varphi < 0.5$. Hence, keeping analysis time roughly constant, a linear gradient from 50 to 100% methanol with a two-fold decrease in steepness would lead to increased separation. It can also be seen from Fig. 4 that over limited ranges of φ the theoretically optimal gradient can be approximated reasonably well by a straight line. Indeed, the main difference between the centre and bottom chromatograms in Fig. 6 can essentially be reduced to an effective decrease in the gradient steepness parameter β.

Therefore, if we know that the sample components will elute over a limited range of φ, then a straight-line approximation of the optimal gradient (Fig. 4) will be adequate. In that event a linear gradient should be preferred because of its conceptual simplicity. However, if the gradient is applied for the investigation of samples in which bands can be expected to occur throughout the chromatogram ($0 < \varphi < 1$), then the optimal gradient in Fig. 4 will usually give the best results.

Our conclusions with respect to optimal gradient shapes can be supported by evidence in the literature. Engelhardt and Elgass[8] include linear gradients for both acetonitrile–water and methanol–water systems applied to a series of fatty acid phenacyl esters. The decreased spacing and peak width towards the end of the methanol–water gradient programme is in sharp contrast with the constant peak width observed for acetonitrile–water, in agreement with our conclusions drawn from Fig. 5. Jordi[9] gives chromatograms for convex acetonitrile–water gradients applied to a series of *p*-bromophenacyl esters of fatty acids, which shows that the gradients are too convex, with a collection of closely spaced sharp peaks at the beginning of the chromatogram. An indication of the validity of the conclusion for THF gradients can be found in the work of Van der Maeden *et al.*[10], where also for the dioxane–water system optimal gradients are suggested to be convex.

CONCLUSIONS

The ln k *versus* φ relationship in RPLC is generally non-linear, but this has no consequences for the shape of optimal gradients.

In methanol–water and THF–water systems the slope of the ln k *versus* φ curves varies systematically with the absolute retention and this leads to optimal gradient shapes that are convex rather than linear.

In acetonitrile–water the slopes of the ln k *versus* φ curves show no systematic changes and therefore optimal gradients for this case are linear.

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