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Gradient elution in micellar liquid chromatography I. Micelle concentration gradient

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Abstract

Gradient elution in micellar liquid chromatography (MLC) is discussed. On the basis of the gradient elution theory, first developed by Snyder, equations were derived for the prediction of gradient retention times in micelle concentration gradient from isocratic data. Likewise, partition coefficients into micelles and stationary phase, and subsequently isocratic retention at different micelle concentrations can be estimated from two gradient runs. However, more studies need to be done to achieve better agreement between isocratic and gradient data. The equations will be useful for efficient development of practical separations by MLC.

1. Introduction

Gradient elution in reversed-phase high-performance liquid chromatography has been widely studied over the past years. Snyder and his coworkers [1–5] have derived equations describing gradient elution in hydro–organic reversed-phase liquid chromatography (RPLC) and have extensively studied the theoretical and experimental basis of these equations. This technique has been mainly used to solve the general elution problem that exists in the separation of mixtures containing compounds with a wide range of polarities. Likewise, gradient elution has the advantage of increasing the column peak capacity with adequate resolution as well as increasing detection sensitivity and decreasing band tailing and separation time [4,5]. The major disadvantage is solvent demixing, i.e., the preferential uptake of one mobile phase component by the stationary phase. This would result in a change in the composition of the stationary phase during the gradient run and thus leads to variations in the column dead time. In addition, the column will have to be re-equilibrated with the initial mobile phase composition for repetitive analysis.

In micellar liquid chromatography (MLC), gradient elution can be performed by increasing the micelle concentration (and/or an organic modifier concentration) during the course of the separation. Micelles provide hydrophobic and electrostatic sites of interaction with hydrophobic and ionic compounds in the aqueous media. Hence, the retention of hydrophobic and charged solutes is inversely proportional to micelle concentration. In addition to solvent

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strength, selectivity is also greatly influenced by micelle concentration [6].

At moderate concentrations of ionic surfactants, the amount of free surfactant in a micellar solution is approximately constant such that any change in the total surfactant concentration would result only in a change in the micelle concentration [7]. Consequently, the composition of the stationary phase (which is only modified with the monomer surfactant) remains constant during the micelle concentration gradient [8–11]. Therefore, the problem of solvent demixing is solved and column regeneration is not necessary after each gradient run [10,11]. The only re-equilibration process necessary before the next gradient run is to flush the mixer and other pre-column as well as column dead volumes with the initial mobile phase. Hence, this technique can be used for repetitive, routine analysis with considerable savings in time and solvent.

Another alternative to perform gradient elution in MLC is to increase the concentration of an organic solvent (e.g. propanol) within a limited range. In this case, however, the composition of the stationary phase might change since the addition of an organic solvent in the micellar mobile phases results in desorption of ionic surfactants from the stationary phase depending on the concentration of the additive [12]. However, we have observed that limited organic concentration gradient (e.g. 3–15% propanol) can be performed in the presence of micelles without disturbing the column equilibration as will be discussed in the following paper [13]. Cole and Dorsey [14] have recently reported that the presence of a small concentration of propanol can greatly reduce the column re-equilibration time after gradient elution in conventional RPLC. Bear in mind that in the case of adding propanol to the micellar mobile phases, the stationary phase will also be partly modified with the organic modifier as well as with surfactants. In addition, the results from this laboratory have shown that the combination of micelles and organic solvents can provide simultaneous enhancement of separation selectivity and solvent strength [6,15–18].

In this paper, the theory of micelle concentration gradient elution in MLC is discussed and the experimental verification of the theory is presented. In the following paper, the use of organic solvent gradient in MLC will be discussed.

2. Theory

2.1. Gradient retention time

Derivation of the equations to describe micellar gradient elution is analogous to the ones described by Snyder et al. [1,2] for linear solvent strength (LSS) gradients in RPLC. The LSS gradients are linear concentration gradients wherein the concentration of the modifier in the mobile phase is changed with time in a linear manner [19]. This type of gradient was used because it is less complicated to work with and it provides easy calculation of retention as a function of experimental conditions.

The relationship between retention factor and micelle concentration in MLC has been reported [20–23]. The following form of the equation was used as the initial equation in this study:

$$1/k' = 1/P_{sw}\phi + S[\mathrm{M}] \quad (1)$$

where k' is the solute retention factor, P_{sw} is the partition coefficient of a compound between the mobile phase and the stationary phase, ϕ is the phase ratio, S is equal to $K_{mw}/P_{sw}\phi$ (K_{mw} is the binding constant of the solute to the micelle) and [M] is the micelle concentration and is equal to [surfactant] − CMC (CMC is the critical micelle concentration). In order to create a gradient in solvent strength, a linear change in the micelle concentration is created [19] as:

$$[\mathrm{M}] = A + BV \quad (2)$$

This equation shows the change in micelle concentration at the outlet of the gradient forming device as a function of V which is volume of the mobile phase delivered by this device at any time, t, from the start of the gradient, i.e., at the beginning of the gradient, $V = 0$. A and B are the y-intercept and the slope, respectively. Since

$V = 0$ at the beginning of the gradient, A is then equal to the initial micelle concentration and B is the measure of the slope of the total concentration change during the course of the gradient [24].

Combining Eqs. 1 and 2 yields:

$$1/k' = 1/P_{sw}\phi + SA + SBV \quad (3)$$

Let

$$1/k'_0 = 1/P_{sw}\phi + SA \quad (3a)$$

and

$$b = SBV_m \quad (3b)$$

where k'_0 is the retention factor at the initial mobile phase, b is the gradient steepness parameter and V_m is the column dead volume. Then,

$$1/k' = 1/k'_0 + b(V/V_m) \quad (4)$$

If k'_a is the instantaneous or "actual" value of k' for the band of interest, then

$$1/k'_a = 1/k'_0 + b(V/V_m) \quad (5)$$

Therefore, solving for k'_a, the following equation is obtained

$$k'_a = (V_m k'_0)/(V_m + (bVk'_0)) \quad (6)$$

At any given time, the instantaneous or "actual" value of the corrected retention volume, V'_a, can be expressed as

$$V'_a = V_m k'_a = V_a - V_m \quad (7)$$

where V_a is the total retention volume for the solute. Substituting Eq. 7 for k'_a

$$(V'_a/V_m) = (V_m k'_0)/(V_m + (bVk'_0)) \quad (8)$$

For gradient elution, Snyder and co-workers [1–4] expressed retention as

$$\int_0^{V_g} (dV/V'_a) = 1 \quad (9)$$

where V_g is the corrected gradient retention volume ($V_g = V_R - V_m$) of the band of interest, dV is the differential volume of the mobile phase that has passed through the band center during the migration of the band along the column. Using Eq. 8, the expression given below is obtained:

$$\int_0^{V_g} [(V_m + k'_0 bV)/(k'_0 V_m^2)]\, dV = 1 \quad (10)$$

Performing the integration and rearranging the terms the final equation is obtained:

$$V_g = [V_m/b]\{(-1/k'_0) + [(1/k'_0)^2 + 2b]^{1/2}\} \quad (11)$$

To change retention volume to retention time, the following relationship can be written, using the volume flow rate, F:

$$t_g = (V_g/F) + t_0 \quad (12)$$

Substituting Eq. 11 for V_g, the equation for the gradient retention time (t_g) in MLC is derived:

$$t_g = ((t_0/b) \cdot \{(-1/k'_0) + [(1/k'_0)^2 + 2b]^{1/2}\}) + t_0 \quad (13)$$

However, this equation does not take into account the delay time, i.e. the time before the gradient actually reaches the top of the column. This delay time, t_D, includes the delay time from the instrument (tubings and other connections) as well as the intentional delay time added by the chromatographer. Thus the observed gradient retention time is actually the calculated t_g plus t_D, i.e.,

$$t_{R,g} = t_g + t_D \quad (14)$$

During this time, t_D, elution of compounds is essentially isocratic. Thus, when the gradient reaches the top of the column, the solute band has already traveled a fraction of the column. This is known as solute pre-elution. Consequently, correction for this should also be made.

The gradient reaches the solute band when

$$X_s = X_g \quad (15)$$

where X_s and X_g are the distances the solute and the gradient have traveled through the column. This equation can also be written as

$$u_s(t_D + t) = u_0 t \quad (16)$$

where u_s is the velocity of the solute, u_0 is the velocity of the gradient and t is the time elapsed

since the start of the gradient. Rearranging terms and solving for t,

$$t = t_D/[(u_0/u_s) - 1] \quad (17)$$

The term $[(u_0/u_s) - 1]$ is equal to the retention factor in the initial mobile phase, k'_0, as shown below:

$$k'_0 = (t_R - t_0)/t_0 = (t_R/t_0) - 1$$
$$= [(L/u_s)/(L/u_0)] - 1 = (u_0/u_s) - 1 \quad (18)$$

Therefore, Eq. 17 becomes

$$t = (1/k'_0)t_D \quad (19)$$

Consequently, the corrected delay time will be

$$t'_D = t_D + t \quad (20)$$

Using Eq. 20 and rearranging terms,

$$t'_D = t_D(1 + 1/k'_0) \quad (21)$$

Another parameter which should be corrected for solute pre-elution is the column dead time, t_0 [3].

The length of the column used in the calculation of t_g is actually shorter than the original length due to the solute pre-elution. The fraction of the column that the solute band has traveled, f, can be expressed as

$$f = t'_D/t_R \quad (22)$$

where t_R is the retention time of the solute.

Using Eq. 21 and the relationship

$$t_R = t_0(1 + k'_0) \quad (23)$$

then simplifying, f can be calculated using the equation

$$f = t_D/(t_0 k'_0) \quad (24)$$

The corrected dead time is then

$$t'_0 = (1 - f)t_0 \quad (25)$$

and the corrected gradient steepness parameter is

$$b' = (1 - f)b \quad (26)$$

Using the corrected values of t_D and t_0 in Eq. 13 yields:

$$t_{R,g} = ((t'_0/b')\{(-1/k'_0) + [(1/k'_0)^2 + 2b(1-f)]^{1/2}\}) + t'_0 + t'_D \quad (27)$$

Simplifying, the final equation for the gradient retention time for MLC is

$$t_{R,g} = ((t_0/b)\{(-1/k'_0) + [(1/k'_0)^2 + 2b(1-f)]^{1/2}\}) + t_0 + t_D \quad (28)$$

2.2. Estimates of values of $P_{sw}\phi$ and K_{mw}

The $P_{sw}\phi$ and K_{mw} values for individual solutes can be determined using isocratic data from the slopes and intercepts of the linear plots of $1/k'$ vs. [M] as illustrated by Eq. 1. This approach, however, is time consuming and is experimentally inconvenient because most of the samples requiring separation by gradient elution cannot be easily studied using isocratic techniques. An alternative approach would be to obtain the values of these physicochemical parameters based on two gradient runs as described below.

Rearranging Eq. 28 yields:

$$(t_{R,g} - t_D - t_0)(b/t_0) = (-1/k'_0) + [(1/k'_0)^2 + 2b(1-f)]^{1/2} \quad (29)$$

Let $T = (t_{R,g} - t_D - t_0)/t_0$, thus

$$Tb = (-1/k'_0) + [(1/k'_0)^2 + 2b(1-f)]^{1/2} \quad (30)$$

Rearranging terms, squaring both sides of the equation and simplifying:

$$T^2b^2 + (2Tb/k'_0) = 2b(1-f) \quad (31)$$

Recalling, $b = SBV_m$, $1/k'_0 = 1/P_{sw}\phi + SA$ and $S = K_{mw}/P_{sw}\phi$. Substituting these in Eq. 31, Eq. 32 is obtained:

$$K_{mw}(T^2BV_m + 2TA) = [2P_{sw}\phi(1-f)] - 2T \quad (32)$$

Values of $P_{sw}\phi$ and K_{mw} can therefore be obtained by the numerical solution of two simultaneous equations obtained from two gradient runs.

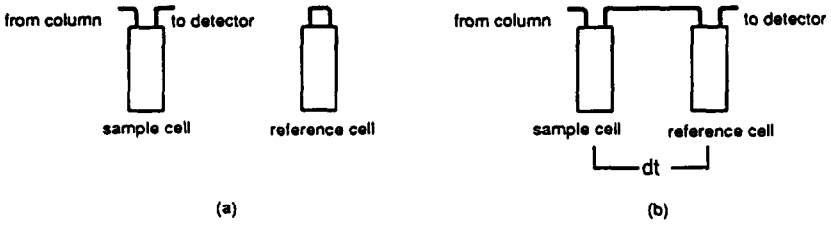

Fig. 1. Configuration of the detector cells: (a) normal detection; (b) differential detection.

$$K_{mw} = 2(T_2 - T_1)/[(T_1^2 B_1 V_m) + (2T_1 A_1) - (T_2^2 B_2 V_m) - (2T_2 A_2)] \quad (33)$$

where 1 and 2 refers to two different gradient runs with different steepness as defined by B_1 and B_2.

In this case, T_2 should be greater than T_1 meaning gradient retention time in gradient 2 should be greater than that in gradient 1 so that a positive value of the K_{mw} is obtained. By substituting the value of K_{mw} in Eq. 32, the value of $P_{sw}\phi$ can be calculated. Once the values of K_{mw} and $P_{sw}\phi$ are known, Eq. 1 can be used to predict the isocratic retention time at different micelle concentrations.

3. Experimental

3.1. Equipment

All experiments were performed using an ISCO gradient liquid chromatograph incorporating two ISCO Model 2350 pumps and an IDS PC-88 computer as the controller. Two kinds of detectors were used, i.e., a Spectra-Physics UV detector and a Kratos fluorescence detector used to confirm the data obtained from the UV detector. Chromatographic data were collected using the ISCO Chemresearch chromatographic data management/system controller version 2.4 and an IDS PC-88 computer.

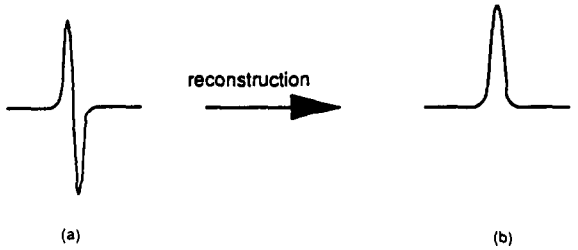

Fig. 2. Appearance of the peaks obtained: (a) from differential detection; (b) from normal detection or after reconstruction of the differential peak.

The flow-rate was 1 ml/min for all measurements. The critical micelle concentration of 8 m*M* was used in all calculations of gradient retention times.

The analytical and the guard columns were water jacketed and thermostated at 40°C with a Lauda refrigerating circulator Model RMS-6 (Brinkmann Instruments).

3.2. Reagents

Sodium dodecyl sulfate (SDS) was obtained from Sigma (St. Louis, MO, USA) and was used as received. Surfactant solutions were prepared using deionized, distilled water (Milli-Q reagent water system) and were filtered using a 0.45-μm nylon-66 membrane filter (Schleicher & Schuell, Keene, NH, USA). All the mobile phases contained 3% (v/v) 2-propanol (HPLC grade) and 0.02 *M* phosphate buffer obtained from Fischer Scientific (Raleigh, NC, USA). The pH was adjusted to 2.5. Solutes were obtained from various manufacturers and were used as received. Solutions were prepared either by dissolving solutes in 2-propanol or in aqueous micellar solution. All other solvents were HPLC grade and obtained from Fisher Scientific.

3.3. Column

The column was laboratory-packed, 15 × 0.46 cm I.D., packed with 5 μm particle size and 300 Å pore size C_{18} Nucleosil packing from Phenomenex (Torrance, CA, USA) using a column

packer was obtained from Alltech (Deerfield, IL, USA). The slurry and the packing solvents were acetone and methanol respectively and the packing pressure was 6000 p.s.i. (1 p.s.i. = 6894.76 Pa).

The void volume of the system was determined by injecting pure water. A value of 1.78 ± 0.01 ml was obtained for the laboratory-packed column which was used for all k' and $t_{R,g}$ calculations.

UV Detector baseline shift

The experiments were done using a UV detector. However, under normal detection (Fig. 1a),

Table 1
Calculated and experimental gradient retention times using a reconstructed chromatogram from the UV detector and the fluorescence detector

Compound	Gradient retention time (min)			
	Gradient 1		Gradient 2	
	Calculated	Experimental	Calculated	Experimental
UV detector				
Nap	15.39	16.67	19.67	18.80
Ant	19.18	20.61	26.74	26.17
Ph	17.34	20.06	23.28	25.28
P	20.46	21.79	29.17	28.66
D-G	6.64	7.01	6.64	6.99
D-F	11.38	11.74	12.35	12.02
D-K	15.37	15.85	18.68	17.75
DD-K	14.23	14.99	16.78	16.19
D-W	9.06	9.15	9.33	9.27
D-Y	26.47	26.89	38.76	34.41
DD-Y	23.58	24.56	33.30	32.36
D-M	11.70	11.97	12.79	12.08
D-L	16.53	16.92	20.31	18.91
D-R	16.21	16.73	20.24	18.93
D-Nor-L	15.74	16.08	19.00	17.64
Fluorescence detector				
Nap	15.39	16.68	19.67	18.77
Ant	19.18	20.77	26.74	26.13
Ph	17.34	20.22	23.28	25.16
P	20.46	21.68	29.17	28.57
D-G	6.64	7.03	6.64	6.97
D-F	11.38	11.72	12.35	11.99
D-K	15.37	15.93	18.68	17.67
DD-K	14.23	14.86	16.78	16.09
D-W	9.06	9.17	9.33	9.25
D-Y	26.47	26.55	38.76	35.53
DD-Y	23.58	23.88	33.30	31.12
D-M	11.70	11.99	12.79	12.02
D-L	16.53	16.92	20.31	18.85
D-R	16.21	16.71	20.24	18.92
D-Nor-L	15.74	16.00	19.00	17.60

Mobile phase: 0.10–0.50 *M* SDS, 0.02 *M* phosphate buffer, 3% PrOH, pH 2.5.

steeply sloping baselines were obtained when performing micelle concentration gradient due to large changes in the refractive index of the mobile phase. In order to alleviate this problem, the detector set-up was modified to differential detection (Fig. 1b).

Fig. 2a shows the appearance of the peaks obtained from differential detection. Therefore, some sort of an integration or reconstruction step is needed in order to achieve a normal peak (Fig. 2b).

The reconstruction step was done using the equation given below.

$$A_{\text{sample}}[t] = A_{\text{observed}}[t] + A_{\text{sample}}[t - \mathrm{d}t] \qquad (34)$$

Retention data were then collected after reconstruction.

This reconstruction step, however, might introduce additional error in the determination of retention. To verify that the results of the integration is acceptable, a fluorescence detector was connected in series with the UV detector. It was determined that the difference in retention times measured from the two detectors due to additional tubing is negligible.

The results of the verification are shown in Table 1 and Fig. 3. As shown from the figure, no significant difference is evident when comparing the plots for the reconstructed chromatograms (UV detector) and that for the unreconstructed ones (fluorescence detector). Therefore, the retention data from the UV detector are acceptable.

4. Results and discussion

4.1. Verification of equation

In MLC, gradient retention times, $t_{R,g}$, can be predicted using Eq. 28 provided that K_{mw} and $P_{sw}\phi$ are known. The values of K_{mw} and $P_{sw}\phi$ for the test solutes were determined from the slopes and intercepts of the linear $1/k'$ vs. [M] plots measured under isocratic elution. Results are given in Table 2 and as shown, excellent linearity ($r^2 > 0.997$) was obtained for all compounds under study with the exception of DF. For very hydrophobic solutes, the numerical analysis sometimes produces negative values to

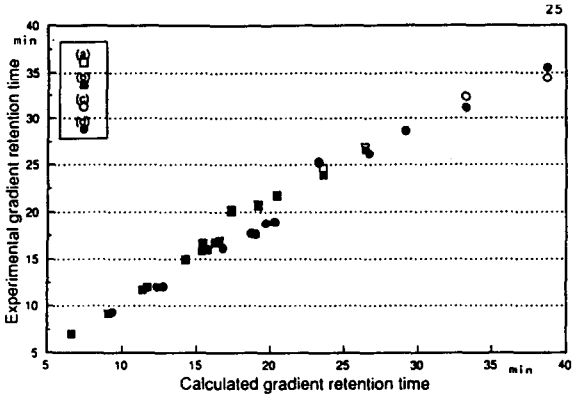

Fig. 3. Comparison of calculated and experimental gradient retention data using a reconstructed chromatogram from the UV detector and an unreconstructed chromatogram from the fluorescence detector; $t_0 = 1.78$ min, $t_D = 5$ min. Mobile phase: 0.10–0.50 M SDS, 0.02 M phosphate buffer, 3% PrOH, pH 2.5: $t_G = 15$ min, $A = 0.092$, $B = 0.267$; $t_G = 60$ min, $A = 0.092$, $B = 0.00667$. (a) UV detector, $t_G = 15$ min; (b) fluorescence detector, $t_G = 15$ min; (c) UV detector, $t_G = 60$ min; (d) fluorescence detector, $t_G = 60$ min.

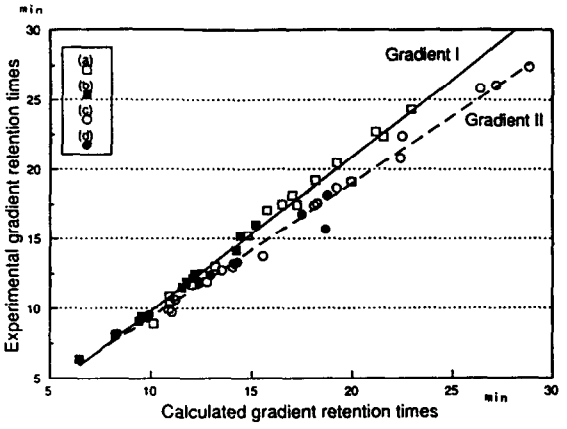

Fig. 4. Comparison of calculated and experimental gradient retention data; $t_0 = 1.78$ min, $t_D = 5$ min or 8.5 min. Mobile phase: 0.02 M phosphate buffer, 3% PrOH, pH 2.5: (a) 0.10–0.50 M SDS gradient, $t_G = 15$ min, $A = 0.092$, $B = 0.02667$; (b) 0.04–0.20 M SDS gradient, $t_G = 15$ min, $A = 0.032$, $B = 0.01067$; (c) 0.10–0.50 M SDS gradient, $t_G = 60$ min, $A = 0.092$, $B = 0.00667$; (d) 0.04–0.20 M SDS gradient, $t_G = 60$ min, $A = 0.032$, $B = 0.00267$.

Table 2
Regression data from $1/k'$ vs. [M] plots for 40 different kinds of compounds

Compound	r^2	$P_{sw}\phi$	K_{mw}
F	0.998	26.5 ± 16.1	37.4 ± 23.1
FF	0.998	145.0 ± 263.1	113.9 ± 207.1
FFF	0.998	173.5 ± 251.2	83.1 ± 120.8
FFFF	0.999	920.0 ± 2169.9	178.7 ± 421.7
DF	0.915	−51.9 ± 729.1	−131.3 ± 1845.8
RF	1.000	−77.3 ± 2.6	−63.0 ± 2.1
KF	1.000	−57.1 ± 13.4	−64.3 ± 15.2
Y	1.000	16.2 ± 0.7	64.7 ± 2.8
AY	1.000	14.6 ± 0.8	108.7 ± 6.0
LY	1.000	45.0 ± 3.1	137.5 ± 9.3
GLY	1.000	58.7 ± 8.7	189.4 ± 26.2
W	1.000	96.0 ± 4.5	153.5 ± 7.1
LW	1.000	161.9 ± 17.7	193.0 ± 21.1
D-G	0.997	11.2 ± 2.0	38.5 ± 6.9
D-F	0.998	24.2 ± 3.6	45.7 ± 7.0
D-W	0.998	19.8 ± 3.4	52.7 ± 9.3
D-K	0.999	76.9 ± 0.2	102.7 ± 0.4
DD-K	0.999	55.4 ± 11.5	82.3 ± 17.1
D-M	0.998	24.8 ± 4.2	44.8 ± 7.8
D-L	0.998	51.7 ± 11.3	56.1 ± 12.4
D-R	0.999	136.2 ± 68.0	172.7 ± 86.3
D-Nor-L	0.999	46.6 ± 10.4	54.8 ± 12.4
D-Y	0.999	377.1 ± 217.3	187.7 ± 108.3
DD-Y	0.999	252.7 ± 106.1	156.6 ± 65.8
BZA	0.999	5.8 ± 0.2	11.1 ± 0.5
B	1.000	15.7 ± 0.4	17.0 ± 0.4
BZO	0.999	5.9 ± 0.2	11.8 ± 0.6
NB	0.999	10.0 ± 0.5	15.4 ± 0.8
T	1.000	45.1 ± 1.1	40.7 ± 1.0
Nap	1.000	138.5 ± 5.3	103.8 ± 4.0
Ant	1.000	581.6 ± 140.3	278.9 ± 67.3
Ph	1.000	306.4 ± 29.8	187.6 ± 18.3
Py	1.000	864.5 ± 335.7	361.0 ± 140.2
2-CP	0.998	8.1 ± 0.6	17.9 ± 1.4
3-CP	0.999	13.5 ± 1.0	27.3 ± 2.0
2,3-DCP	0.999	28.9 ± 5.2	51.4 ± 9.3
2,5-DCP	0.999	30.9 ± 3.6	48.9 ± 5.7
2,4,5-TCP	1.000	78.0 ± 14.3	95.4 ± 17.6
2,4,6-TCP	0.999	62.1 ± 13.0	80.2 ± 16.9
PCP	1.000	161.5 ± 40.3	133.8 ± 33.4

$P_{sw}\phi$ due to small values of the intercepts relative to the experimental errors and because of the high correlation between K_{mw} and $P_{sw}\phi$. However, highly accurate retention data can still be predicted using these two parameters combined, despite the fact that $P_{sw}\phi$ no longer has a physical meaning. A compilation of characteristic constants for micelle interaction is available in the literature [25]. In so far as the data set presented here overlaps with the ones discussed in Ref. [25], there is good agreement with respect to the order of magnitude of the solute–micelles interaction after correction for the aggregation number. However, a more thorough comparison of the values is not appropriate due to differences in the applied experimental conditions, most notable, the temperature.

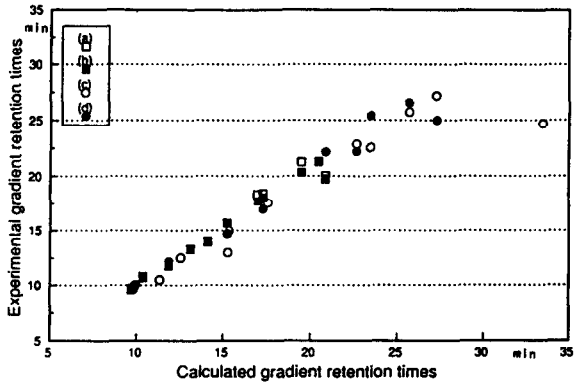

Fig. 5. Comparison of calculated and experimental gradient retention data with pump going from 0 to 100% solvent B vs. 10–90% solvent B; $t_0 = 1.78$ min, $t_D = 5$ min. Mobile phase: 0.02–0.10 M SDS, 0.02 M phosphate buffer, 3% PrOH, pH 2.5: $t_G = 15$ min, $A = 0.012$, $B = 0.00533$; $t_G = 60$ min, $A = 0.012$, $B = 0.00133$. (a) 0–100% solvent B, $t_G = 15$ min; (b) 10–90% solvent B, $t_G = 15$ min; (c) 0–100% solvent B, $t_G = 60$ min; (d) 10–90% solvent B, $t_G = 60$ min.

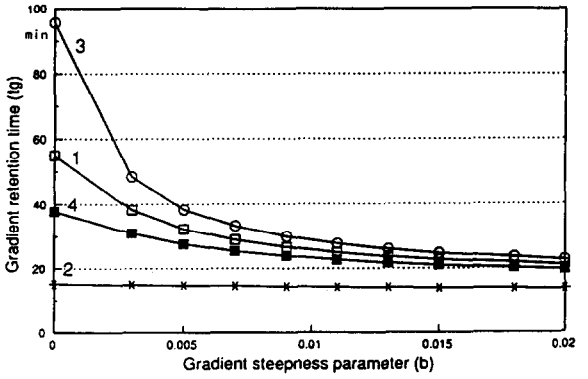

Fig. 6. Effect of gradient steepness parameter (b) on the gradient retention time: (1) $P_{sw}\phi = 291$ and $K_{mw} = 70$; (2) $P_{sw}\phi = 291$ and $K_{mw} = 560$; (3) $P_{sw}\phi = 1164$ and $K_{mw} = 70$; (4) $P_{sw}\phi = 1164$ and $K_{mw} = 560$.

Table 3
Calculated and experimental gradient retention times

Compound	Gradient retention time (min)			
	Gradient 1		Gradient 2	
	Calculated	Experimental	Calculated	Experimental
0.10–0.50 M SDS gradient				
F	10.92	10.87	10.95	10.37
FF	17.01	18.12	19.93	19.09
FFF	21.15	22.70	27.16	26.05
KF	15.76	17.03	18.24	17.54
RF	18.14	19.19	22.46	22.37
2,4,5-TCP	13.16	13.00	13.49	12.72
2,4,6-TCP	12.51	12.47	12.76	11.89
PCP	16.51	17.45	18.10	17.38
T	14.78	15.18	15.56	13.73
Ph	19.22	20.45	22.41	20.79
P	22.92	24.30	28.84	27.39
B	10.88	9.94	11.21	10.59
Nap	17.24	17.43	19.19	18.63
Ant	21.53	22.33	26.37	25.82
W	10.12	8.90	11.03	9.72
LW	12.04	11.71	14.03	12.95
0.04–0.20 M SDS gradient				
DF	14.22	14.13	18.64	15.65
2-CP	9.53	9.40	9.84	9.31
3-CP	11.73	11.91	12.91	12.37
2,3-DCP	14.40	15.16	17.47	16.74
2,5-DCP	15.18	15.97	18.73	18.12
BZA	8.26	8.12	8.33	8.16
NB	11.54	11.48	12.36	11.76
BZO	8.25	8.07	8.33	8.12
Y	9.41	9.07	9.91	9.51
AY	6.47	6.33	6.48	6.37
LY	12.07	12.15	14.07	13.20
GLY	12.16	12.44	14.28	13.29

Mobile phase: x *M* SDS, 0.02 *M* phosphate buffer, 3% PrOH, pH 2.5.

Subsequently, the values for the gradient steepness parameter, b, and k'_0 were calculated from Eqs. 3a and 3b which allowed the calculation of $t_{R,g}$ from Eq. 28.

Calculated gradient retention times were obtained by first calculating the values of A and B using Eq. 3 for gradient 1 (gradient time, $t_G = 15$ min) and for gradient 2 ($t_G = 60$ min). These values and the $P_{sw}\phi$ and K_{mw} values obtained from isocratic elution were substituted into Eq. 28 resulting in the calculated gradient retention times.

Eq. 28 was then verified by comparing calculated and experimental gradient retention time values for 39 different solutes (polar, non-polar and ionic compounds consisting of amino acids, peptides, polyaromatic hydrocarbons, substituted benzenes and chlorophenols).

The gradient retention times for these solutes were experimentally determined using two SDS gradients (0.10–0.50 *M* and 0.04–0.20 *M* SDS) depending on the hydrophobicity of the compounds. Likewise, two gradient times (gradient 1 and gradient 2) were used.

Good agreement was observed between the experimental and calculated retention times with a slope of 1.00 and $r = 0.99$ (Table 3 and Fig. 4). The largest error observed was less than ± 15%.

It has been reported that for reciprocating pumps, better results can be obtained by performing a 10 to 90% solvent B gradient (the stronger solvent in the gradient) rather than going from 0 to 100%. This was examined for the instrument used. As shown in Table 4 and Fig. 5, there is no significant difference between the plots for the gradients performed at 10 to 90% solvent B and at 0 to 100%. Thus, for the instrument used, retention in gradient elution is not greatly influenced by this factor. The slopes of predicted vs. observed gradient retention times for the two gradients (0–100% and 10–90%) were 1.00 and 0.97, respectively.

The effect of b on the gradient retention times for different $P_{sw}\phi$ and K_{mw} values was examined using Eq. 28 and the results are shown in Fig. 6. As expected, for a steeper gradient, smaller gradient retention times were obtained. This change is more pronounced for compounds having larger $P_{sw}\phi$ and smaller K_{mw} values i.e. compounds having more interaction with the stationary phase.

The majority of observations related to gradient 2 showed experimental retention times that were less than the predicted values (Tables 3 and 4). This might indicate the presence of systematic errors. In order to figure out the source of the systematic errors, the gradient shape delivered by the instrument and the flow-rate error of the instrument were measured.

The actual gradient shapes at two gradient times were obtained by using pure methanol as the initial mobile phase and methanol with a few drops of acetone as the final mobile phase. The increase in the absorbance as the concentration of acetone is increased was determined. This was compared with the ideal gradient shape. The difference between the %B of the actual and the ideal gradient, ΔB, at 12 min was measured and the results are given in Table 5. The ΔB obtained for the pump operating from 0–100% B and from 10–100% B are almost equal for both gradients. The same is true for the two gradients which have different gradient times.

Likewise, the %B for the actual gradient is slightly higher than that for the ideal gradient. This would correspond to a slight decrease in the gradient retention time as compared to the theoretical value.

Flow-rate studies also illustrate this effect as shown in Fig. 7. The pumps were set such that the total flow-rate is equal to 1 ml/min. The figure shows the deviation of the actual flow-rate from this value. The errors observed were all positive which means that the flow-rates observed were greater than 1 ml/min. At 0% B, only pump A is pumping the solvent and a 0% error was observed. When pump B was used together with pump A, the observed error was increased. When using pump B only, an error of about 2.5% was observed. Therefore, the error in the flow-rate is due mostly to pump B. An excellent discussion on the various factors contributing to errors in flow-rate has been published by Foley et al. [26] where potential sources include the differences in pressure coefficients of viscosity of the solvents used for a gradient. Although the relevant coefficients were not available for the system under discussion, a significant difference in viscosity exists between the A and B solvents.

Therefore, the systematic error observed was due primarily to the errors in the operation of the instrument for the gradient runs. Thus, the use of a better instrument should produce better correlation, i.e., less error, between the experimental and the calculated values.

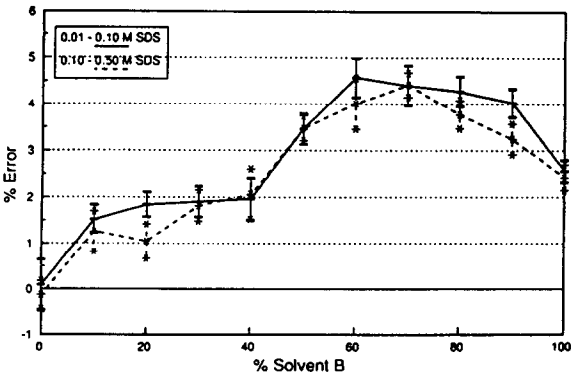

Fig. 7. Flow-rate errors generated by the instrument using different concentrations of A and B solvents.

Table 4
Calculated and experimental gradient retention times with pump going from 0 to 100% solvent B vs. 10–90% solvent B

Compound	Gradient retention time (min)			
	Gradient 1		Gradient 2	
	Calculated	Experimental, $A = 0.012$, $B = 0.00533$	Calculated	Experimental, $A = 0.012$, $B = 0.00133$
0–100% Solvent B gradient				
DF	20.88	20.01	33.47	24.67
2-CP	11.87	11.87	12.56	12.54
3-CP	15.23	15.62	17.56	17.55
2,3-DCP	19.50	20.29	25.66	25.71
2,5-DCP	20.48	21.24	27.27	27.18
BZA	9.70	9.69	9.86	9.93
NB	14.12	13.99	15.35	14.92
BZO	9.80	9.71	9.98	10.03
Y	13.12	13.34	15.27	13.02
AY	10.38	10.89	11.36	10.46
LY	16.99	18.33	22.66	22.83
GLY	17.29	18.41	23.46	22.53
10–90% Solvent B gradient				
DF	20.88	19.73	33.47	24.99
2-CP	11.87	11.79	12.56	12.21
3-CP	15.23	15.67	17.56	16.92
2,3-DCP	19.50	20.29	25.66	25.40
2,5-DCP	20.48	21.30	27.27	26.55
BZA	9.70	9.54	9.86	9.72
NB	14.12	13.95	15.35	14.68
BZO	9.80	9.68	9.98	9.60
Y	13.12	13.30	15.27	14.66
AY	10.38	10.65	11.36	10.85
LY	16.99	17.68	22.66	22.10
GLY	17.29	18.03	23.46	22.16

Mobile phase: 0.02–0.10 *M* SDS, 0.02 *M* phosphate buffer, 3% PrOH, pH 2.5.

Table 5
Measured difference of %B between the actual and the ideal gradient at 12 min

Gradient time (min)	Pump setting (%B)	ΔB (%)
15	0–100	2
	10–90	3
60	0–100	2
	10–90	2

4.2. Estimation of K_{mw} and $P_{sw}\phi$ values

The validity of Eq. 32 was verified by calculating the values of K_{mw} and $P_{sw}\phi$ using the calculated gradient retention times. The observed errors from this verification were within the expected experimental errors. The next step would then be to use the experimental gradient retention times. However, this gave K_{mw} and $P_{sw}\phi$ values which do not agree with the data in

Table 6
Comparison of isocratic k' values calculated from two gradient runs and measured experimentally from isocratic runs

Compound	k' at two micelle concentrations			
	Gradient		Isocratic	
	0.092 *M*	0.192 *M*	0.092 *M*	0.192 *M*
FF	9.19	7.83	11.01	5.17
FFF	13.91	10.93	17.59	8.41
RF	12.77	8.20	13.69	5.85
KF	8.09	7.23	9.92	4.16
Y	3.06	1.74	4.41	0.90
AY	3.26	2.26	1.01	0.90
LY	4.29	2.30	2.70	1.27
GLY	4.65	2.57	2.62	1.21
W	4.84	1.33	4.93	2.57
LW	6.65	4.08	6.83	3.52
D-F	5.57	4.43	3.27	1.63
D-W	4.33	3.30	2.33	1.11
D-K	7.74	5.21	5.43	2.56
DD-K	7.12	4.95	4.66	2.25
D-M	5.80	5.64	3.42	1.71
D-L	8.36	5.78	6.17	3.08
D-R	8.21	5.37	6.00	2.77
D-Nor-L	7.72	5.35	5.68	2.82
D-Y	17.87	10.70	15.81	7.44
DD-Y	15.79	8.64	12.42	5.90
BZA	2.44	1.51	2.26	1.45
BZO	2.04	1.15	2.22	1.41
NB	4.67	3.17	3.35	2.04
Nap	9.45	7.55	11.10	5.55
Ant	15.64	11.22	18.65	9.01
Ph	14.77	10.66	14.30	6.97
Py	18.76	12.50	21.74	10.40
2-CP	3.09	1.85	2.42	1.44
3-CP	4.48	2.67	3.10	1.72
2,3-DCP	5.95	3.31	4.08	2.17
2,5-DCP	6.52	3.65	4.61	2.43

Table 2. Better correlation is observed for neutral compounds and for gradient runs using low surfactant concentrations.

An inherent error in gradient elution is the fact that the actual [M] at a certain time, t, is not exactly equal to the predicted [M]. This difference would affect the retention behavior of compounds. This effect is larger for ionic compounds because these types of compounds are more sensitive to variations in the [M].

Another possible source of error arises from the use of high surfactant concentrations so as to elute hydrophobic compounds from the reversed-phase column in a reasonable amount of time. One assumption for Eq. 1 is that CMC, aggregation number and the structure of the micelle do not change as a result of a change in the surfactant concentration. The use of high surfactant concentration in the gradient (from 0.10 to 0.50 *M* SDS) would lead to variation in the CMC and consequently, altering the amount of adsorbed surfactant on the stationary phase. This would drastically increase errors made in the calculation of K_{mw} and $P_{sw}\phi$.

Another important source of error would be the uncertainties in the measurement of the column dead volume. During the gradient the mobile phase composition is constantly changing which subsequently influences the void volume.

One way of reducing this error would be to use a less hydrophobic stationary phase as well as the use of shorter columns. This would allow the use of lower surfactant concentration yet eluting the hydrophobic compounds in a reasonable amount of time.

Despite large errors obtained for the K_{mw} and $P_{sw}\phi$ values, the isocratic k' values were still calculated for different [M] using Eq. 1. This is then compared with the experimental isocratic k'. As shown in Table 6, two gradient runs can be used to estimate isocratic k' value. However, the average % difference between the isocratic k' values calculated from the two gradient runs and from isocratic elution is quite large. Likewise, this method may not give accurate values for K_{mw} and $P_{sw}\phi$ values but can still be used as a scouting technique for the estimation of isocratic k' as well as predict band positions in isocratic elution for a mixture of compounds. However, the results from gradient elution could still be improved by using more accurate gradient formers, shorter columns and less hydrophobic stationary phases.

Acknowledgement

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Appendix A

List of compounds used

Compound	Abbreviation
Amino acids and peptides	
Phenylalanine	F
Aspartic acid–phenylalanine	DF
Arginine–phenylalanine	RF
Lysine–phenylalanine	KF
Tyrosine	Y
Alanine–tyrosine	AL
Leucine–tyrosine	LY
Glycine–leucine–tyrosine	GLY
Trytophan	W
Leucine–tryptophan	LW
Dansylated amino acids	
Dansyl-glycine	D-G
Dansyl-phenylalanine	D-F
Dansyl-trytophan	D-W
Dansyl-lysine	D-K
Didansyl-lysine	DD-K
Dansyl-methionine	D-M
Dansyl-leucine	D-L
Dansyl-norleucine	D-Nor-L
Dansyl-arginine	D-R
Dansyl-tyrosine	D-Y
Didansyl-tyrosine	DD-Y
Aromatic compounds	
Benzaldehyde	BZA
Benzene	B
Benzonitrile	BZO
Nitrobenzene	NB
Toluene	T
Napthalene	Nap
Anthracene	Ant
Phenanthrene	Ph
Pyrene	P
Chlorophenols	
2-Chlorophenol	2-CP
3-Chlorophenol	3-CP
2,3-Dichlorophenol	2,3-DCP
2,5-Dichlorophenol	2,5-DCP
2,4,5-Trichlorophenol	2,4,5-TCP
2,4,6-Trichlorophenol	2,4,6-TCP
Pentachlorophenol	PCP

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