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Gradient elution in micellar liquid chromatography II. Organic modifier gradients

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Abstract

The application of organic modifier gradients in micellar liquid chromatography (MLC) is discussed. The equation derived by Snyder and co-workers describing gradient elution in hydro–organic reversed-phase LC was verified for organic solvent gradients in the presence of micelles. It is also demonstrated that the use of these gradients require little re-equilibration time due to the limited range of organic modifier concentration used in the gradient. This would result in shorter analysis time. Lastly, a practical application of the use of propanol and acetonitrile gradients in MLC is described.

1. Introduction

In gradient elution the mobile phase composition is varied during the course of the separation process in order to provide a continuous increase in the strength of the mobile phase entering the column. Hydro–organic gradients involve an increase in the percentage of the organic modifier in the mobile phase during the run. The result will be faster separation of later-eluting peaks while separating the early-eluting solutes as well as enhanced detectability. Hence, gradient elution provides a solution to the general elution problem.

The theoretical and experimental aspects of gradient elution in reversed-phase liquid chromatography (RPLC) have been studied extensively over the past years [1–7]. Snyder et al. [1] have derived an equation to describe gradient elution in hydro–organic RPLC:

$$t_g = [(t_0/b)\ \log\ 2.3k_0'b(1-f)+1] + t_D + t_0 \qquad (1)$$

where t_g is the gradient retention time, t_0 is the column dead time, b is the gradient steepness parameter, k_0' is the isocratic retention factor at initial mobile phase condition, f is the fraction of the column the solute has already traveled before the gradient reaches it, and t_D is the system delay time. The validity of this equation has been verified experimentally [2]. This equation can be used to predict gradient retention time from gradient data but more importantly, it can also be used to predict isocratic retention data from gradient runs (scouting technique) [8].

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The major disadvantage commonly associated with gradient elution is the additional time required to re-equilibrate the column with the initial gradient conditions after each run [2,5]. Studies show that significant quantities of the organic modifier in the mobile phase are extracted by the stationary phase [9,10]. As the concentration of the organic solvent changes during the gradient run, the alkyl bonded phase is solvated to a varying extent [11]. The changing solvation of the stationary phase makes it necessary to re-equilibrate the column to its starting conditions. Complete column re-equilibration usually requires flushing the column with about 15 column volumes of the initial mobile phase [7] before the next sample injection. This re-equilibration time results in longer analysis times.

Different methods to reduce the column re-equilibration time have been studied extensively [2,7,11,12]. The use of a reverse gradient following the completion of the initial gradient has been suggested [2]. However, this does not show any significant advantage as compared to returning directly to the starting conditions [7]. Another study done by Frenz and Horváth [12] suggested the use of a series of different solvents in the order of decreasing affinity for the stationary phase as column regenerants. A recent study on column regeneration was done by Cole and Dorsey [11]. The addition of a constant volume of 3% 1-propanol in the mobile phase throughout the solvent gradient provides consistent solvation of the stationary phase. This provides a dramatic reduction in the column re-equilibration time of up to 78%.

In micellar liquid chromatography (MLC), gradient elution can be performed by increasing the micelle concentration and/or by increasing the concentration of an organic modifier. No column re-equilibration is needed after a micellar gradient due to the constant composition of the stationary phase during the course of the gradient. Solvent strength in MLC can also be controlled by changing the concentration of an organic modifier.

Reduced chromatographic efficiency has been the major drawback of MLC due to poor resistance to mass transfer. It has been shown that the addition of 3% 1-propanol to the micellar mobile phase provides better "wetting" of the stationary phase which improves the mass transfer of the solutes between the mobile phase and the stationary phase and consequently the chromatographic efficiency [13].

In addition, the results from this laboratory have proven that not only does the presence of an organic modifier compensate for the otherwise weak solvent strengths of micellar eluents, but also the combination of micelles and organic solvent provides pronounced selectivity for the separation [14–19]. As a result, a study of both micellar gradients (i.e. increasing the micelle gradient) and organic modifier gradients (i.e. increasing the concentration of the organic modifier) is worth pursuing. In the first part of this study, we reported the theory and experimental verification for a micelle concentration gradient [20].

In this paper, the use of organic solvent gradient in MLC is discussed. It is demonstrated that Eq. 1 can be used to describe organic solvent gradients in the presence of micelles. The use of gradient elution to predict the retention under isocratic conditions is also presented. In addition, it will be shown that the column re-equilibration time after each organic solvent gradient run is very short. Lastly, the gradient capability of this technique is illustrated.

2. Experimental

2.1. Equipment

All experiments were done using an ISCO gradient liquid chromatograph incorporating two ISCO Model 2350 pumps and an IDS PC-88 computer as the controller. The detector used was an Applied Biosystems Model 783A programmable absorbance detector (wavelength set at 254 nm). Chromatographic data were collected using a Yokogawa Model 3021 pen recorder and the ISCO Chemresearch chromatographic data management/system controller version 2.4 with an IDS PC-88 computer.

The flow-rate used for all measurements was 1 ml/min. The analytical column and the guard column were water jacketed and thermostated at 40°C with a Lauda refrigerating circulator Model RMS-6 (Brinkmann Instruments).

2.2. Reagents and solutions

The surfactant used was sodium dodecyl sulfate (SDS) obtained from Sigma (St. Louis, MO, USA) and 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 0.02 *M* phosphate buffer and varying concentrations of organic modifier (2-propanol or acetonitrile which are HPLC grade) and SDS depending on the gradient used. The pH was adjusted to 2.5. In addition, all the mobile phases were degassed by sonicating for 15 min prior to use.

Solutes were obtained from various manufacturers and were used as received. Solutions were prepared by either dissolving the solutes in 2-propanol, in acetonitrile or in aqueous micellar solution. Concentrations of the solute solutions were adjusted such that reasonable peak heights are obtained.

All solvents were HPLC grade and obtained from Fisher Scientific.

2.3. Column

The column used was a laboratory-packed column, 15×0.46 cm I.D. Blank column (Supelco, Bellefonte, PA, USA) packed with 5 μm particle size and 300 Å pore size Nucleosil C_{18} packing from Phenomenex (Torrance, CA, USA) using a column packer from Alltech (Deerfield, IL, USA). The slurry and the packing solvents were acetone and methanol, respectively and the packing pressure used was 6000 p.s.i. (1 p.s.i. = 6894.76 Pa).

The void volume of the system was evaluated by injecting pure water. The first disturbance of the baseline was assumed to be V_m. A value of 2.11 min was obtained for the laboratory-packed column which was used for all k' and t_g calculations.

3. Results and discussion

3.1. Verification of the gradient equation

The equation for gradient elution developed by Snyder et al. has already been found to be applicable to gradient elution in conventional RPLC with hydro–organic mobile phases. However, the validity of this equation for organic solvent gradients in the presence of micelles should be examined. This was done using 9 dansylated amino acids.

In order to calculate gradient retention times using Eq. 1, the values for b and k_0' were determined under isocratic conditions from the linear relationship between log k' and volume fraction of organic modifier in the mobile phase, φ as:

$$\log k' = \log k'_m - S\phi \quad (2)$$

where k' is the isocratic retention factor, k'_m is the retention factor in a purely aqueous micellar mobile phase at a fixed micelle concentration and S is the slope of the log k' vs. φ in the micellar eluent. The values of S and k'_m were calculated from the slope and intercept, respectively.

The validity of this equation in MLC has been demonstrated for different types of organic modifiers and for a broad range of ionic and non-ionic compounds [14–19].

The gradient steepness parameter, b, was calculated using the expression

$$b = \frac{S\,\Delta\varphi\,t_0}{t_G} \quad (3)$$

where $\Delta\varphi$ is the change in the volume fraction of the organic modifier during the gradient. By substituting the values of k_0' and b into Eq. 1, calculated values of the gradient retention time were obtained.

The experimental gradient retention times were then obtained for two gradients with differ-

ent gradient times, t_G (15 and 60 min). A total delay time of 5 min was incorporated in all the gradients used. The mobile phase contained 2-propanol, 0.06 *M* SDS and 0.02 *M* phosphate buffer with the pH adjusted to 2.5. The propanol concentration was varied from 3 to 15% (v/v) for the two gradients used. Note that in addition to the organic solvent content, the elution strength of micellar mobile phases is also influenced by concentration of micelles.

The experimental retention time ($t_{R,expt}$) obtained were compared with the calculated retention time ($t_{R,calc}$). The results of this verification is given in Table 1 and Fig. 1. As shown, the calculated gradient retention times agree closely with the experimental gradient retention time. Errors obtained were less than 5% which were mainly negative errors, i.e., the experimental values lie below ideal curve. This can be attributed to systematic errors contributed by the instrument used [20]. The close agreement between the experimental and calculated data indicates that the integrity of the micelles is maintained during the gradient runs. Since there is no discernible difference in the prediction errors of the early- and late-eluting compounds, a breakdown of the micelles at higher organic modifier concentration is not apparent.

Table 1
Verification of Eq. 1 used to predict retention times from characteristic values derived from isocratic runs for organic modifier gradients in the presence of micelles

Compound	t_G = 15 min		t_G = 60 min	
	$t_{R,calc}$ (min)	$t_{R,expt}$ (min)	$t_{R,calc}$ (min)	$t_{R,expt}$ (min)
D-G	7.15	7.22	7.15	7.24
D-F	11.94	11.98	12.31	12.26
DD-K	16.13	15.71	17.10	16.76
D-W	9.46	9.32	9.53	9.41
D-K	18.85	18.34	20.45	19.76
D-M	12.16	12.38	12.68	12.74
D-L	18.37	18.73	20.65	20.61
D-R	20.14	19.65	22.48	21.72
D-Nor-L	17.42	17.49	19.16	19.07

Shown are the calculated and experimental gradient times, $t_{R,calc}$ and $t_{R,expt}$, respectively, as determined for a number of dansylated amino acids in two gradients, both 3 to 15% propanol but with different gradient times t_G. Other mobile phase conditions: 0.06 *M* SDS, 0.02 *M* phosphate buffer, 3–15% PrOH, pH 2.5. Standard abbreviations for amino acids were used.
D = dansylated amino acids; DD = didansylated amino acids.

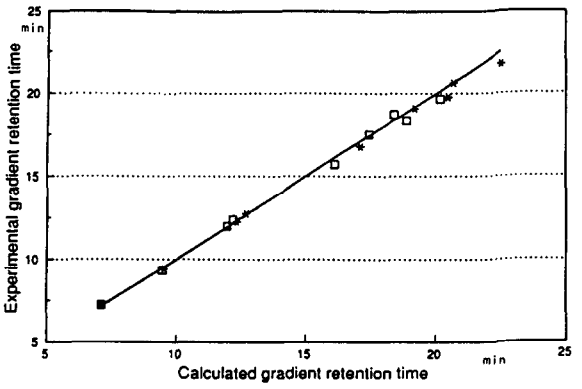

Fig. 1. Verification of Eq. 1 for organic solvent gradient in MLC: comparison of the calculated and experimental gradient retention times (□: t_G = 15 min; *: t_G = 60 min; line: ideal). Mobile phase: 0.06 *M* SDS, 0.02 *M* phosphate buffer, pH 2.5; gradients: 3 to 15% 2-propanol.

Eq. 1 can also be used to predict isocratic retention from gradient retention data. This capability has been effectively used in HPLC and is known as the scouting method. Further verification of Eq. 1 and the scouting method in MLC was performed by predicting the isocratic parameters using the retention data from two gradient runs with different t_G values [8]. This gives two equations for t_g, i.e.,

$$t_{g,1} = [(t_0/b_1) \log 2.3k_0'b_1(1-f) + 1] + t_D + t_0 \quad (4)$$

and

$$t_{g,2} = [(t_0/b_2) \log 2.3k_0'b_2(1-f) + 1] + t_D + t_o \quad (5)$$

From Eq. 3:

$$\beta = b_1/b_2 = t_{G,2}/t_{G,1} \quad (6)$$

The values for k_0', b_1 and b_2 can be obtained by solving the three simultaneous Eqs. 4–6. Consequently, the values for the solvent strength parameter, S, can be estimated from Eq. 3. The results of this procedure are given in Table 2 and

Table 2
Comparison of gradient steepness b for the 15- (b_1) and 60-min (b_2) gradients, retention factor in 3% propanol (k'_0) and slope (S) of log k' vs. organic modifier concentration as determined from isocratic runs and the two gradient runs

Compound	Isocratic				Gradient			
	b_1	b_2	k'_0	S	b_1	b_2	k'_0	S
D-G	0.049	0.012	2.39	2.92	0.045	0.011	2.43	2.67
D-F	0.037	0.009	4.90	2.21	0.032	0.008	4.88	1.90
D-K	0.027	0.007	9.02	1.59	0.025	0.006	8.60	1.48
DD-K	0.028	0.007	7.29	1.65	0.029	0.007	7.05	1.72
D-W	0.032	0.008	3.53	1.91	0.029	0.007	3.46	1.72
D-M	0.048	0.012	5.11	2.85	0.044	0.011	5.21	2.61
D-L	0.041	0.010	9.30	2.41	0.037	0.009	9.40	2.19
D-R	0.031	0.008	10.17	1.86	0.029	0.007	9.67	1.72
D-Nor-L	0.037	0.009	8.45	2.22	0.033	0.008	8.34	1.96

Fig. 2. As shown, there is good agreement between values obtained from gradient and those from isocratic runs. Knowing the values for k'_0 *and* S from the gradient data, one can predict the isocratic retention from:

$$\log k' = \log k'_0 - S\varphi_0$$

where φ_0 represents the initial organic modifier concentration. As shown in Fig. 3 and Table 3, there is an excellent agreement between the retention factors determined from isocratic experiments and those predicted from two gradient runs.

3.2. Re-equilibration studies

One problem observed for gradient elution in RPLC with hydro–organic eluents is the need for column re-equilibration between gradient runs [2,5]. It has been observed that many column volumes of initial mobile phase composition are needed to re-equilibrate the column before the next gradient analysis. This is due to the fact that the composition of the stationary phase is altered by solvation of the bonded alkyl chains as the concentration of the organic modifier is increased during a gradient run. Generally, column re-equilibration can be achieved by flushing the column with 15 column volumes of the starting mobile phase [7].

When using micelles in the mobile phase, the surfactant monomers are adsorbed onto the stationary phase. It has been reported that the presence of 3% propanol in the mobile phase would provide over 90% monolayer coverage of the alcohol on the stationary phase [21]. An increase in the concentration of the organic modifier in the micellar mobile phase during the course of an organic solvent gradient might alter the composition of the stationary phase through the displacement of the adsorbed surfactant monomers from the stationary phase by the organic modifier. In addition, the critical micelle concentration (CMC) of a surfactant depends on the concentration of the organic co-solvent and a change in the CMC would lead to a change in the surface concentration of the adsorbed monomer surfactant. This would disturb the equilibration of the column that might require long re-equilibration times. Fortunately, however, this was not the case here as shown below.

Re-equilibration studies were performed by determining the effect of a 15-min re-equilibration time (or 15-ml re-equilibration volume) after each gradient run on the reproducibility of the retention time of 10 dansylated amino acids. If the stationary phase equilibrium is disturbed, one would observe poor reproducibility of the retention behavior especially for early-eluting compounds. Each solute was injected 15 times to determine the reproducibility of their retention

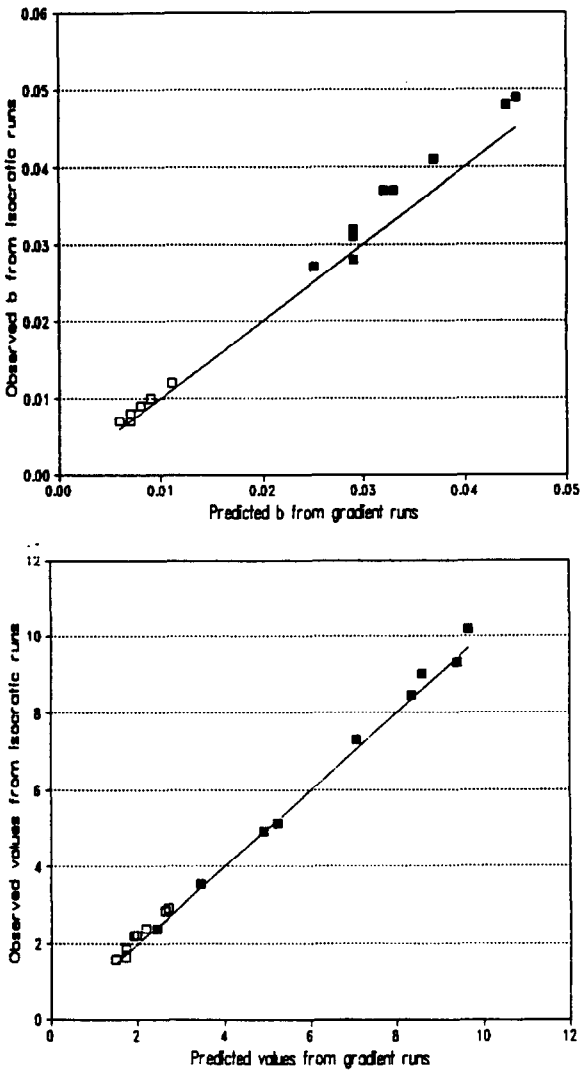

Fig. 2. Comparison of values of b_1 (top, ■), b_2 (top, □), k'_0 (*bottom*, ■) *and* S (bottom, □) obtained from isocratic and gradient runs. Lines = ideal.

times which is an indication of the column re-equilibration. Two gradients with different gradient times (as given above) were used. The mobile phase contained 0.15 *M* SDS, 0.02 *M* phosphate buffer (pH 2.5) and 2-propanol concentration varying from 3 to 15% (v/v). The average values and relative standard deviations of the first three and last three injections were calculated. Results of this series of experiments are given in Table 4. As shown, standard deviation values are less than 0.10 min after a 15-min re-equilibration time. Even smaller S.D. values are observed for the shorter retained compounds. These are the compounds which are most affected by the existence of column non-equilibration. Since the retention times of these early peaks do not vary significantly, additional washing of the column with the initial mobile phase is no longer necessary. This means that the column is already completely re-equilibrated after 15 min or 15 ml of initial mobile phase. Since the process of column re-equilibration actually starts when the initial mobile phase reaches the top of the column, the delay time of the chromatographic system (the time it takes for the starting mobile phase to travel from the pump to the top of the column) was measured and found to be 3.5 min. Therefore the column re-equilibration time is actually only 11.5 min or 11.5 ml of the starting mobile phase (since the flow-rate used in the experiment was 1 ml/min).

A more precise value of the re-equilibration time was then determined using the procedure reported by Cole and Dorsey [11] using phenylalanine as the test solute. Under the mobile phases conditions, phenylalanine is a short retained compound ($k' < 2$) and therefore it will be greatly affected by lack of column equilibrium. The gradient was held at the final mobile phase composition for at least 20 min (equivalent to 20 ml of final mobile phase) to ensure complete equilibration of the stationary phase with the final mobile phase. Following this 20-min equilibration period, the mobile phase was immediately returned to the initial composition. Phenylalanine was injected at a rate of 1 injection per minute for 28–30 min. The retention time was then plotted against the time elapsed after the gradient run. The column was considered to be completely equilibrated when the retention time of phenylalanine reached a constant value. Experiments were repeated at two different SDS concentrations, i.e., 0.30 and 0.15 *M*, in order to determine the effect of surfactant concentration on the column re-equilibration. The propanol concentration was varied from 3 to 15% (v/v). In addition, experiments were performed at 0.30 *M* SDS where the acetonitrile

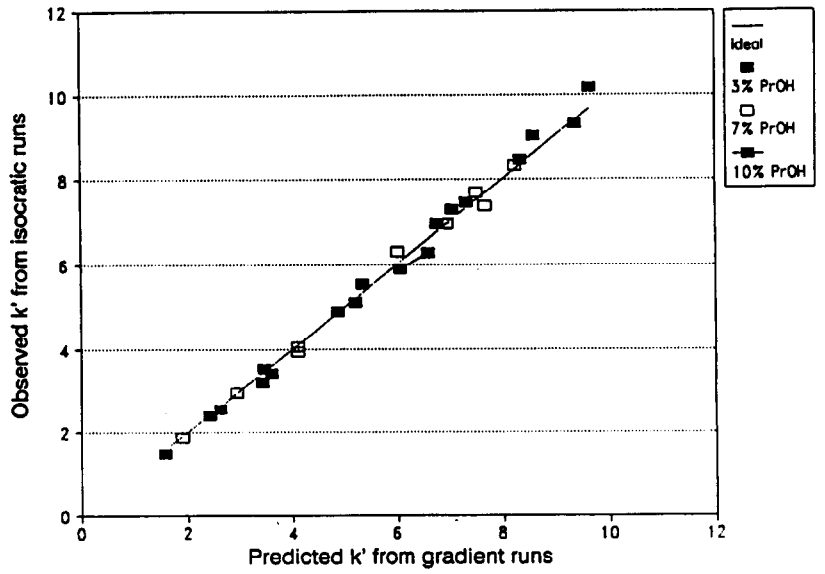

Fig. 3. Comparison of k' values obtained from isocratic and gradient runs.

concentration was varied from 3 to 20%. Conductivity experiments showed that the micelles remained intact within this range of acetonitrile concentration.

Fig. 4 shows the re-equilibration time for the three different mobile phases. A constant value of the retention was initially observed due to the delay time of the system. For the three different mobile phases, a re-equilibration time of about 11 min or 11 ml of initial mobile phase is required after each gradient run. Considering the delay time of the chromatographic system, it actually requires 7.5 min or 7.5 ml of the initial mobile phase to re-equilibrate the column. Note that some of the test solutes are charged and therefore their retention is very sensitive towards changes in the stationary phase composition, especially the concentration of the adsorbed surfactant.

The main reason behind the short re-equilibra-

Table 3
Comparison of isocratic k' values obtained from isocratic runs and calculated from two gradient runs for various propanol concentrations, φ

Compound	k'					
	Isocratic			Gradient		
	$\varphi = 0.03$	$\varphi = 0.07$	$\varphi = 0.10$	$\varphi = 0.03$	$\varphi = 0.07$	$\varphi = 0.10$
D-G	2.39	1.87	1.48	2.43	1.90	1.58
D-F	4.90	4.05	3.40	4.88	4.10	3.59
D-K	9.02	7.69	6.93	8.60	7.50	6.77
DD-K	7.29	6.30	5.54	7.05	6.02	5.34
D-W	3.53	2.95	2.56	3.46	2.95	2.62
D-M	5.11	3.93	3.21	5.21	4.10	3.42
D-L	9.30	7.37	6.28	9.40	7.68	6.60
D-R	10.17	8.32	7.45	9.67	8.25	7.33
D-Nor-L	8.45	6.93	5.89	8.34	6.96	6.08

Table 4
Reproducibility studies of retention after a 15-min column re-equilibration

Compound	t_G = 15 min		t_G = 60 min	
	t_R (min)	R.S.D. (%)	t_R (min)	R.S.D. (%)
D-G	3.74	0.02	3.79	0.02
D-F	5.75	0.03	5.86	0.03
DD-K	7.15	0.05	7.32	0.05
D-R	8.81	0.02	9.01	0.04
D-W	4.51	0.01	4.60	0.02
D-K	8.28	0.03	8.47	0.03
DD-Y	14.79	0.07	15.88	0.05
D-M	5.86	0.01	5.99	0.04
D-L	9.08	0.02	9.36	0.04
D-Y	17.63	0.04	19.07	0.09

Mobile phase: 0.15 *M* SDS, 0.02 *M* phosphate buffer, 3–15% PrOH, pH 2.5. Retention time values reported are the averages of the first three and last three injections. R.S.D. = Relative standard deviation.

tion time is the limited range of gradient. The amount of change in the concentration of organic modifier (i.e. 3–15%) is too small to cause any significant (or noticeable) effect on the composition of the stationary phase. One can then anticipate similar results to be observed in ion-pair LC and conventional RPLC with hydro–organic mobile phases. In order to verify this theory, additional experiments were performed using the same range of 2-propanol and acetonitrile concentrations (i.e., 3–15% for 2-propanol and 3–20% for acetonitrile), in hydro–organic RPLC (no surfactant present) and in ion-pair LC. Acetone was used as the test solute because with these mobile phases, phenylalanine is no longer shortly retained. The type of solute should not have an effect of column re-equilibration.

Results of this experiment are shown in Fig. 5. A re-equilibration time of about 11 min was also obtained for both pure hydro–organic (no surfactant) and hydro–organic with surfactant concen-

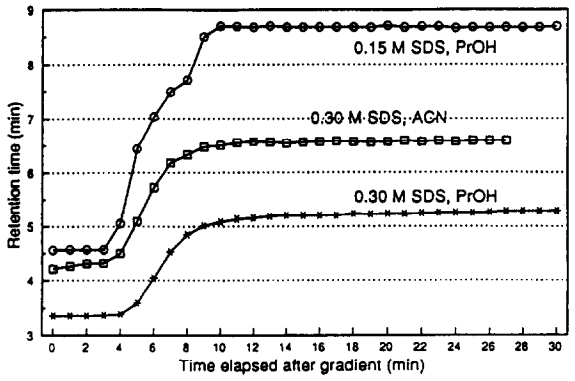

Fig. 4. Determination of column re-equilibration time with micelles in the mobile phase using phenylalanine as the test solute. Mobile phase: 0.30 or 0.15 *M* SDS, 0.02 *M* phosphate buffer, pH 2.5; gradients: 3–15% 2-propanol or 3–20% acetonitrile (ACN).

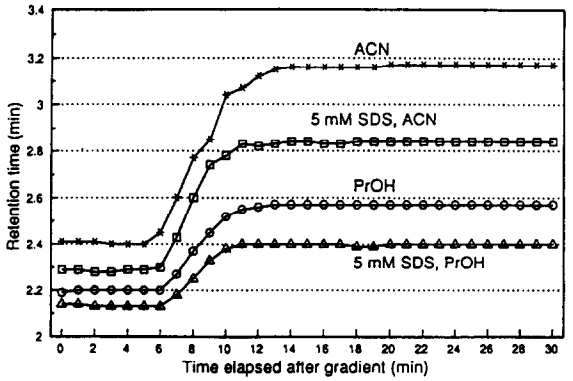

Fig. 5. Determination of column re-equilibration time without the presence of micelles in the mobile phase using acetone as the test solute. Mobile phases: either 0 or 5 m*M* SDS, 0.02 *M* phosphate buffer, pH 2.5; gradients: 3–15% 2-propanol or 3–20% acetonitrile.

tration at 5 m*M* (which is about or less than the CMC, i.e. no micelles) which is the same as that observed in MLC. Therefore, the short re-equilibration time is mainly due to the limited range of organic modifier concentration used in the gradient. The change in the concentration of the organic modifier is apparently not large enough to change the concentration of the adsorbed surfactant monomer on stationary phase and/or the amount of the extracted organic solvent. It is important to note that a limited range of organic modifier is of limited use for solving general elution problem in conventional RPLC and in ion-pair chromatography. In MLC, however, organic modifier gradients are useful since one can use a limited range of organic modifier concentration and compensate the solvent strength with a concurrent micelle concentration gradient. (Note that no column re-equilibration is needed after a micelle concentration gradient.) As can be seen in the following examples, the presence of micelles makes it possible to elute very hydrophobic compounds with a relatively low concentration of organic modifier. In addition, this will provide an opportunity to incorporate unique selectivities in MLC with the enhancement of solvent strength [15–19].

3.3. Test of gradient capability

The capability of an organic modifier gradient in MLC was studied using a seven-component mixture composed of phenylalanine (F), aspartic acid–phenylalanine (DF), lysine–phenylalanine (KF), phenylalanine–phenylalanine (FF), triphenylalanine (FFF), tetraphenylalanine (FFFF) and pentaphenylalanine (FFFFF), under isocratic and gradient conditions. Four isocratic runs and two gradient runs were performed using mobile phases containing 2-propanol and acetonitrile as the organic modifiers. The mobile phases also contained 0.30 *M* SDS, 0.02 *M* phosphate buffer and the pH was adjusted to 2.5.

Fig. 6 shows the separation of the mixture using propanol as the organic modifier. When using 3% (v/v) 2-propanol in the mobile phase

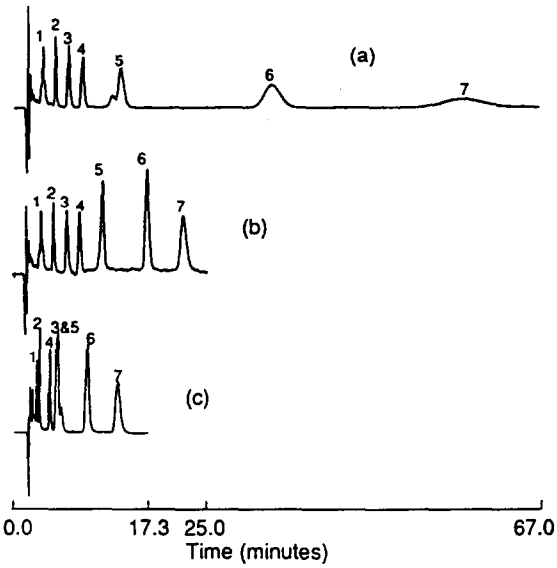

Fig. 6. Separation of a seven-component test mixture. Mobile phase: 0.30 *M* SDS, 0.02 *M* phosphate buffer, pH 2.5 with propanol added. (a) Isocratic separation with 3% 2-propanol, (b) gradient separation with 3 to 15% 2-propanol and (c) isocratic separation with 15% 2-propanol. Peaks: 1 = DF; 2 = F; 3 = KF; 4 = FF; 5 = FFF; 6 = FFFF; 7 = FFFFF.

(Fig. 6a), all peaks as well as the early-eluting peaks (peaks 1–4) are very well resolved. The later peaks, however, have very large retention time values which means long separation time such as peak 7 which elutes at about 58 min. In addition, peaks 6 and 7 are very broad which severely limits the detection sensitivity.

An increase in the percentage of the organic modifier does not give a better separation as shown in Fig. 6c. The use of 15% 2-propanol shortened the retention time dramatically resulting in a decrease in the resolution of peaks 1 and 2 which elute very near t_0. Peak reversal between peaks 3 and 4 is observed as well as coelution of peaks 3 and 5.

Gradient elution was then used for the separation. The 2-propanol concentration was varied from 3 to 15% for 5 min and was held at 15% 2-propanol for 20 min. The chromatogram is shown in Fig. 6b. The separation is complete after 25 min. Including the re-equilibration time, this would result in an overall run time of about 32.5 min. Peaks 1–4 are very well resolved and

Table 5
Comparison of theoretical plates obtained from isocratic and gradient runs using propanol and acetonitrile

Compound	Theoretical plates					
	Propanol[a]			Acetonitrile[a]		
	a	b	c	a	b	c
FF	2007	3233	1995	1839	3184	2315
FFFF	1047	4793	2015	930	3987	2099
FFFFF	467	3597	1880	Not eluted	4278	2102

[a] a = isocratic separation with 3% propanol or 3% acetonitrile; b = gradient separation with 3–15% 2-propanol or 3–20% acetonitrile; c = isocratic separation with 15% propanol or 20% acetonitrile.

peaks 5–7 elute within a reasonable amount of time. Detection sensitivity of the last peaks was also increased.

Acetonitrile was also used as shown in Fig. 7. Isocratic separation was done with 3% acetonitrile in the mobile phase (Fig. 7a). Peak 7 does not elute after 2 h or could be too broad to be detected. Good resolution, however, is observed for peaks 1–4.

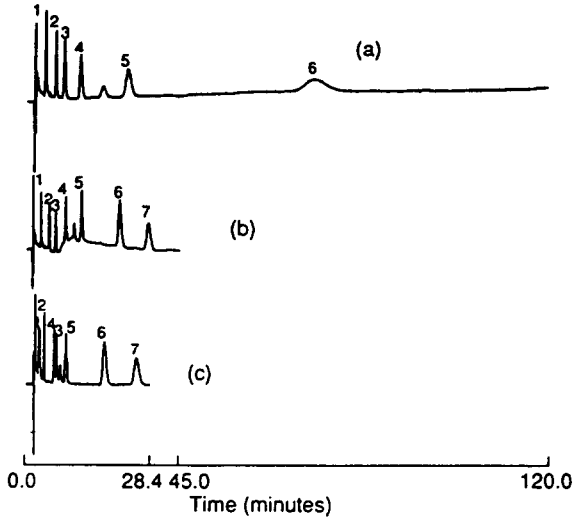

Fig. 7. Separation of a seven-component test mixture. Mobile phase: 0.30 *M* SDS, 0.02 *M* phosphate buffer, pH 2.5 with acetonitrile added. (a) Isocratic separation with 3% acetonitrile, (b) gradient separation with 3 to 20% acetonitrile and (c) isocratic separation with 20% acetonitrile. Peaks: 1 = DF; 2 = F; 3 = KF; 4 = FF; 5 = FFF; 6 = FFFF; 7 = FFFFF.

Another isocratic run was done using 20% acetonitrile (Fig. 7c). A reduction of the separation time to about 25 min and a dramatic increase in the detection sensitivity are observed. However, poor resolution is observed for the early-eluting peaks especially for peaks 3 and 4; peak reversal between these two peaks was observed as well. Likewise, peaks 1 and 2 elute very near t_0.

A better separation is again obtained when using gradient elution as shown in Fig. 7b. The acetonitrile concentration was varied from 3 to 20% for 5 min and was held at 20% acetonitrile for 40 min. The advantages of gradient elution are again observed.

Finally, it is worth noting that the peak efficiencies under gradient elution conditions were better than those under isocratic condition for both propanol and acetonitrile systems (Table 5.)

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