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Prediction of peptide retention times in normal-phase liquid chromatography with only a single gradient run

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Abstract

Previous studies of peptide separation by normal-phase liquid chromatography have shown a linear relationship between the logarithm of the capacity factor and the logarithm of the volume fraction of modifier in the mobile phase. This permitted the use of a model to predict isocratic and gradient retention times based on data obtained by two initial gradient runs. In the present study, chromatographic behavior of 25 peptides in normal-phase liquid chromatography with isocratic elution have been studied and a linear relationship between the slope (S) and intercept [log $k(0)$] was obtained. This relationship was combined with the algorithm of prediction reported in the previous paper. The prediction of peptide retention times with only a single experimental gradient retention data was investigated.

Keywords: Retention parameters; Gradient elution; Peptides

1. Introduction

Previous papers [1–3] have described the separation of peptides, using acetonitrile (A)–water+0.1% trifluoroacetic acid (B) gradients with a carbamoyl-silica column. Similar separations of other samples have been reported [4–6], and the technique has been referred to recently as hydrophilic interaction chromatography (HILIC) [7–10]. Separation selectivities in this version of normal-phase liquid chromatography (NPLC) and reversed-phase liquid chromatography (RPLC) differ considerably.

Prediction using two experimental values obtained by either isocratic or gradient elution have been already established by Snyder and co-workers [11,12], Schonmakers and co-workers [13,14] and Jandera and Churacek [15,16] and have been widely used for optimization of high-performance liquid chromatography (HPLC) conditions. In this version of NPLC, a similar approach to predict the isocratic and gradient retention times followed by two initial gradient runs was successfully carried out and has been reported in a previous paper [3]. These methods, however, require two sets of experimental data obtained under different conditions in order to obtain two unknown parameters [slope and intercept of plots of capacity factor (k) and volume fraction (ϕ)]. On the other hand, these two unknown parameters could be reduced to one using a linear relationship [17–22] between the slope and intercept of plots of k–ϕ. Using this relationship, Jandera [20], Cooper and Hurtubise [21] and Hamoir and Massart [22] have predicted isocratic retention times in NPLC using only one experimental value.

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In the present study, chromatographic behavior of 25 peptides in the isocratic elution has been studied. It was found that a linear relationship exists between the slope (S) and intercept [log $k(0)$] in this version of NPLC. Therefore, this relationship was combined with the algorithm of prediction reported in the previous paper [3] in order to predict the retention times using only a single gradient run. To the author's knowledge, this paper is the first to present the prediction of both gradient and isocratic retention times with only a single gradient run in this version of NPLC.

2. Theoretical

2.1. Relationship of k and ϕ

In the present normal-phase system, previous work has established the following relationship

$$^{e}\log k(\phi) = {}^{e}\log k(0) - S \cdot {}^{e}\log \phi \quad (1)$$

where $k(0)$ is the retention factor k for $\phi = 0.01$, and ϕ is the volume-fraction of more polar solvent (B) in a mobile phase A–B. In the present study, B is 0.1% (trifluoroacetic acid) TFA–water and A is acetonitrile. Note that here "^{e}log" refers to the natural logarithm.

2.2. Relationship between slope and intercept

In RPLC, several investigations [17–19] have been conducted on the relationship between S (slope) and $^{e}\log k(0)$ (intercept) derived from the k–ϕ plots. In NPLC, some researchers [20–22] also have reported this relationship. In both modes, it was also reported that this relationship appears to be valid only for structurally similar compounds.

$$S = p \cdot {}^{e}\log k(0) + q \quad (2)$$

where p and q are the linear regression coefficients for the slope and intercept, respectively.

2.3. Gradient elution equation

The gradient elution equation was described based on the algorithm in the previous paper [3]. The gradient retention time (tg) can be calculated using Eq. (3) for any gradient, provided that $k(\phi)$ is known [3,14,23]:

$$\int_{0}^{tg-t_0-t_D} d\left[f^{-1}(\phi)/k(\phi)\right] = t_0 - t_D/k(a) \quad (3)$$

By inserting Eq. (1) into Eq. (3), the gradient retention time t_{gi} ($i=1,2$) is given by

$$tgi = \frac{1}{bi} \cdot \left\{ \left[bi \cdot (S_{NPLC} + 1) \cdot (t_0 \cdot k(0)_{NPLC} - t_D) + a^{(S_{NPLC}+1)} \right]^{\frac{1}{S_{NPLC}+1}} - a \right\} + t_0 + t_D \quad (4)$$

Eq. (4) involves two unknowns, $k(0)_{NPLC}$ and S_{NPLC}, that can be solved by numerical means. If the following gradient condition can be assumed [3,23]:

$$k(0)_{NPLC} \gg \frac{a^{S_{NPLC}+1} + t_D}{bi \cdot (S_{NPLC} + 1) \cdot t_D} \quad (5)$$

Eq. (5) allows explicit solution for $k(0)_{NPLC}$ and S_{NPLC}:

$$S_{NPLC} + 1 = {}^{e}\log(b1/b2) / {}^{e}\log\{[a + b2 \cdot (tg2 - t_0 - t_D)]/[a + b1(tg1 - t_0 - t_D)]\} \quad (6)$$

$$k(0)_{NPLC} = \left\{ \left[a + b1 \cdot (tg1 - t_0 - t_D) \right]^{\frac{1}{S_{NPLC}+1}} / \left[b1 \cdot (S_{NPLC} + 1) \right] + t_D \right\} / t_0 \quad (7)$$

2.4. Prediction retention using only a single gradient run

If a linear relationship between the slope and intercept exists [17–22], Eq. (8) can be derived from Eqs. (2) and (7)

$$k(0)_{NPLC} = \left(\frac{t_0 \cdot [a + b1 \cdot (tg1 - t_0 - t_D)]^{\frac{1}{p + q\,{}^{e}\log k(0)_{NPLC} + 1}}}{[b1 \cdot (p + q \cdot {}^{e}\log k(0)_{NPLC} + t_D]} \right) \quad (8)$$

When only a single gradient experimental reten-

tion time was given, the $k(0)_{NPLC}$ value was solved by numerical calculation and S_{NPLC} can be calculated using Eq. (2). $k(0)_{NPLC}$ and S_{NPLC} allow the prediction of both isocratic and gradient retention times under a variety of experimental conditions.

As noted earlier, Eq. (2) is generally valid only for compounds of similar structure. Thus, Eq. (8) should not be extended to sample other than peptides, until values of p and q for such samples have been determined. It is also possible that peptides of very different structure than those studied here may also exhibit different values of p and q that those assumed here.

3. Experimental

3.1. Materials

HPLC-grade acetonitrile (ACN) was obtained from Nacalai Tesque (Kyoto, Japan), and trifluoroacetic acid (TFA) and formic acid from Wako (Osaka, Japan). A Milli-Q system (Japan Millipore, Tokyo, Japan) was used for water purification. Most of the peptides were purchased from Sigma (St. Louis, MO, USA) and Peptide Institute (Osaka, Japan) and the others obtained by cyanogen bromide degradation of myoglobin or synthesized by a peptide synthesizer (Perkin-Elmer Applied Biosystems Division). TSKgel Amide-80 (25 cm×0.46 cm I.D.) and TSKgel ODS-80Ts (15 cm×0.46 cm I.D.) columns were from Tosoh (Tokyo, Japan).

3.2. Apparatus

The HPLC system was a Tosoh liquid chromatograph equipped with a SC-8020 micro-computer, CCPM-II pump, UV-8020 detector, AS-8020 auto-sample injector and CO-8020 column oven.

3.3. Methods

In this version of NPLC, the peptides were dissolved in 5 μl formic acid, followed by the addition of 40 μl ACN. Eluent A (initial eluent) was 0.1% TFA in ACN–water (97:3) and eluent B, 0.1% TFA in ACN–water (55:45). The peptides were separated by a linear gradient from eluents A to B over 70 min (0.6% water/min). The flow-rate was 1.0 ml/min. For NPLC, t_0 of TSK gel Amide-80 was determined by the retention of water (2.55 ml).

The mobile phase for isocratic elution in both modes was 0.1% TFA in ACN–water. Elution was monitored by UV absorption at 215 nm. The temperature in the column oven was 40°C.

4. Results

Chromatograms for separating peptides by RPLC (A) and this version of NPLC (B), are shown in Fig. 1.

4.1. Relationship of k and ϕ

The retention times of 25 peptides are listed in Table 1, where ϕ values are also included. Among them, the 10 peptides with differing molecular masses, shown in Fig. 1, were used for the investigation of the relationship between k and ϕ. Fig. 2 shows the relationship between logarithm of k and logarithm of ϕ(water) in this version of NPLC.

As shown in Fig. 2, the chromatographic behavior in this version of NPLC using aqueous mobile phase was also in agreement with that in usual NPLC, on which there are many reports, using non-aqueous mobile phase. The logarithm of k decreased as the logarithm of volume fraction of water in the mobile phase increased. The data points fell on straight lines. Almost all the correlation coefficients were above 0.99.

4.2. Correlation between S and ^{e}log k(0)

Fig. 3 shows the relationship between S_{NPLC} and $^e\log k(0)_{NPLC}$ for the 25 peptides in this version of NPLC, which was obtained from Table 1. Using Eq. (2), the linear regression coefficients (p and q) for S_{NPLC} and $^e\log k(0)_{NPLC}$ were calculated by the least-squares method.

$$S_{NPLC} = 0.296 \cdot {}^e\log k(0)_{NPLC} + 0.272 \quad (9)$$

4.3. Prediction of retention time using a single gradient run

Using the excellent correlation between S_{NPLC} and

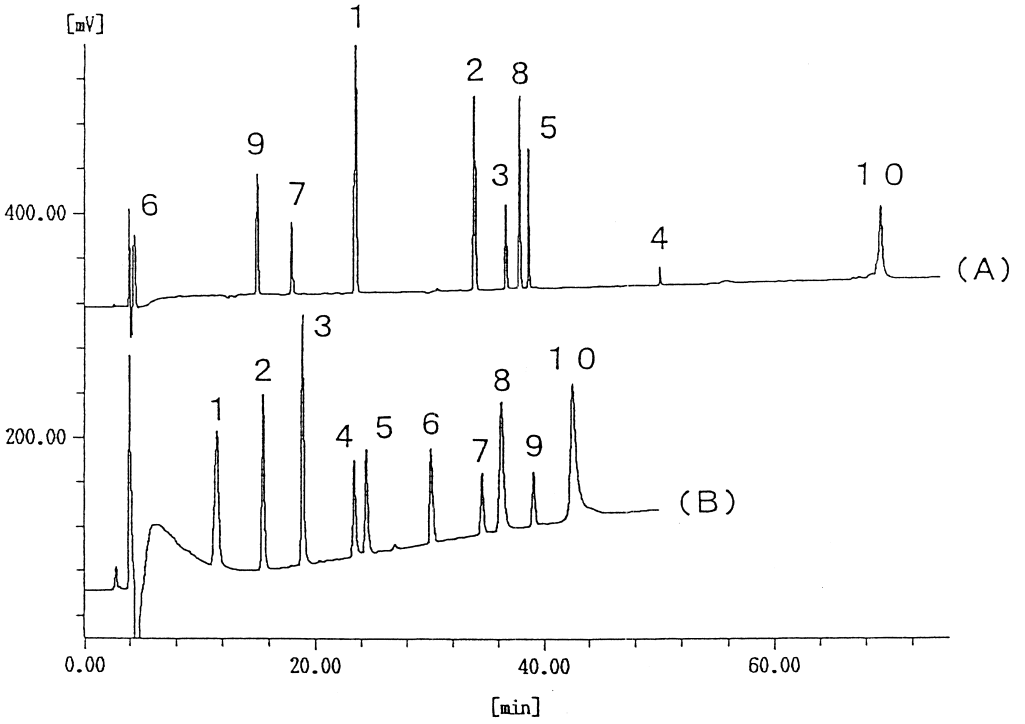

Fig. 1. Chromatograms of peptides separated on (A) TSKgel ODS-80Ts (RPLC) and (B) TSKgel Amide-80 (NPLC). The peptide mixture was separated with (A) 83.3-min linear gradients of ACN from 5 to 55% in 0.1% TFA (0.6% ACN per min) and (B) 70-min linear gradients of water from 3 to 45% in 0.1% TFA (0.6% water per min). Peaks: 1=FY; 2=FGGF; 3=FLEEI; 4=DYMGWMDP-NH2; 5=NFTYGGF; 6=AGSE; 7=WAGGDASGE; 8=YGGFMTSQKSQTPLVT; 9=ASTTTNYT; 10=VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAE. This figure has been generated from Ref. [1].

$^{e}\log k(0)_{NPLC}$ shown in Eq. (9), the approach for predicting the retention times using only a single gradient run was carried out. The 10 peptides shown in Fig. 1 were used as probes. Values of $^{e}\log k(0)_{NPLC}$ were calculated numerically by inputting a single experimental data obtained by a 90 min gradient elution into Eq. (8). Next, values of S_{NPLC} were calculated using $^{e}\log k(0)_{NPLC}$ and Eq. (9). Then, retention times in a 120 min gradient elution were predicted using S_{NPLC} and $^{e}\log k(0)_{NPLC}$ calculated by the single 90 min gradient run. The mean deviation between observed and predicted gradient times was only 1.43%. Table 2 summarizes comparisons of observed and predicted retention time. Prediction results obtained by two gradient runs, derived from the previous paper [3], are also listed in Table 2.

From the observed peak widths obtained by the 120 min linear gradient elution, the resolution (R_s) for the separation of adjacent bands with the gradient elution were calculated by three ways: (a) using observed retention time; (b) using predicted retention time by two gradient runs; and (c) using predicted retention time by the single gradient run. The results are listed in Table 3.

Isocratic retention times predicted by the single gradient run are listed in Table 4. The average error between the observed and predicted isocratic retention times was 14.44%. In a similar manner to the gradient elution, values of R_s for gradient separation of adjacent bands with the isocratic elution were calculated. The results are listed in Table 5.

5. Discussion

The results for separating the typical peptides by this version of NPLC, together with those of the

Table 1
Isocratic retention times of peptides in this version of NPLC

Peptide[b]	ϕ (retention time, min)						S_{NPLC}[a]	[e]Log $k(0)_{NPLC}$[a]
1	0.0770	0.0535	0.0418					
	(6.47)	(8.91)	(10.96)				1.25	3.00
2	0.218	0.171	0.124	0.0770	0.0535			
	(5.28)	(6.93)	(10.26)	(19.90)	(32.47)		1.70	5.36
3	0.124	0.0770	0.0535	0.0300				
	(6.43)	(9.12)	(11.48)	(15.04)			0.82	2.55
4	0.124	0.101	0.0770	0.0535	0.0300			
	(5.17)	(6.73)	(10.34)	(18.20)	(35.93)		1.80	4.68
5	0.101	0.0770	0.0535	0.0300				
	(5.52)	(7.70)	(11.73)	(19.42)			1.41	3.52
6	0.230	0.195	0.171	0.136	0.124	0.101		
	(6.67)	(8.67)	(10.90)	(17.12)	(20.69)	(35.58)	2.49	8.27
7	0.242	0.218	0.195	0.171	0.124	0.101		
	(5.61)	(6.92)	(8.68)	(11.48)	(22.05)	(39.30	2.76	9.03
8	0.042	0.218	0.195	0.171	0.148	0.136		
	(7.54)	(9.53)	(12.64)	(17.73)	(26.37)	(33.78)	3.17	10.77
9	0.101	0.0770	0.0535	0.0418				
	(6.47)	(9.23)	(15.56)	(21.73)			1.81	4.64
10	0.124	0.101	0.0770	0.0653				
	(5.88)	(8.58)	(16.30)	(24.00)			2.93	7.64
11	0.159	0.124	0.101	0.0770				
	(6.92)	(12.13)	(21.41)	(49.82)			3.27	9.58
12	0.277	0.230	0.195	0.159				
	(6.86)	(10.65)	(17.31)	(33.39)			3.57	12.35
13	0.159	0.124	0.101	0.0770				
	(5.84)	(10.43)	(19.48)	(48.91)			3.64	10.31
14	0.277	0.230	0.195	0.0171	0.136			
	(5.46)	(7.76)	(11.64)	(16.85)	(36.23)		3.44	11.51
15	0.171	0.148	0.124	0.101	0.888			
	(6.36)	(9.08)	(15.06)	(29.02)	(44.72)		3.66	10.79
16	0.195	0.171	0.148	0.124				
	(5.38)	(7.61)	(12.29)	(22.33)			4.32	12.95
17	0.218	0.195	0.171	0.148				
	(6.97)	(10.32)	(18.20)	(37.48)			5.31	16.90
18	0.218	0.195	0.171	0.148				
	(6.21)	(8.58)	(15.45)	(35.44)			5.68	17.78
19	0.265	0.218	0.195	0.171				
	(5.80)	(11.08)	(17.83)	(35.73)			5.27	17.47
20	0.230	0.195	0.171	0.159	0.148			
	(7.24)	(13.13)	(23.03)	(32.17)	(48.39)		5.12	16.64
21	0.230	0.218	0.195	0.171	0.148			
	(5.37)	(6.28)	(9.46)	(16.52)	(35.02)		5.51	17.35

Table 1. Continued

Peptide[b]	ϕ (retention time, min)					S_{NPLC}[a]	[e]Log $k(0)_{NPLC}$[a]
22	0.265	0.242	0.218	0.195	0.171		
	(5.45)	(8.11)	(12.78)	(24.42)	(56.29)	6.60	21.77
23	0.265	0.242	0.218	0.195			
	(7.02)	(10.89)	(21.29)	(49.16)		7.63	25.52
24	0.265	0.242	0.218	0.195			
	(5.27)	(10.27)	(19.98)	(42.64)		8.59	28.32
25	0.253	0.230	0.218	0.206	0.195		
	(6.27)	(12.75)	(20.48)	(36.59)	(70.68)	11.01	35.91

[a] The data were obtained by fitting to Eq. (1).

[b] Sequences: 1=FY; 2=GE; 3=GP; 4=EVF; 5=VYV; 6=AGSE; 7=GGYR; 8=TKPR; 9=FGGF; 10=FLEEI; 11=NFTYGGF; 12=ASTTTNYT; 13=DYMGWMDP-NH2; 14=WAGGDASGE; 15=GNLWATGHFM; 16=PHPFHFFVYK; 17=YGGFMRRVGRPE; 18=DAVYIHPFHLVIH; 19=RRLIEDAEYAARG; 20=YGGFMTSQKSQTPLVT; 21=YGGFMTSEKSQTPLVTL; 22=NLAKGKEESLDSDLYAELR; 23=DAEFRHDSYQNHHQKLVFFAEDV; 24=HSDAVFTDNYTRLRKQMAVKKYLNSILN; 25=VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAE.

RPLC, are shown in Fig. 1 [1]. Under these conditions, peptides are retained through a normal-phase mechanism. Although the hydrophilic peptide such as AGSE (peak No. 6) was not often retained on an octadecyl silica column in the RPLC mode, it was retained on the TSK gel Amide-80 column in this version of NPLC. Separation selectivities for the NPLC and RPLC differed significantly. Furthermore,

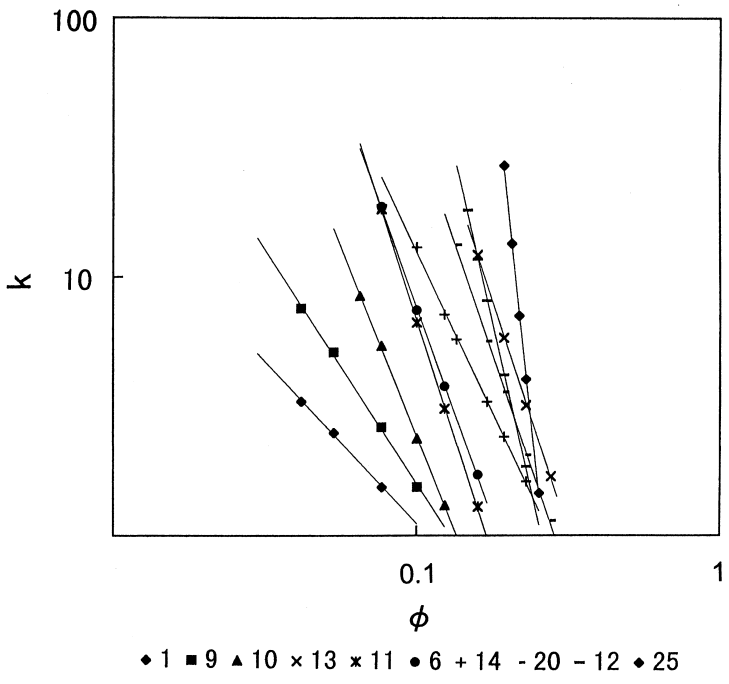

Fig. 2. Dependence of capacity factor, $k(\phi)$, of peptides on volume fraction of water, ϕ, in the mobile phase. Isocratic data (Table 1) in this version of NPLC are plotted. Peptide identification is shown in Table 1. Column, TSKgel Amide-80; mobile phase, ACN–water–0.1% TFA.

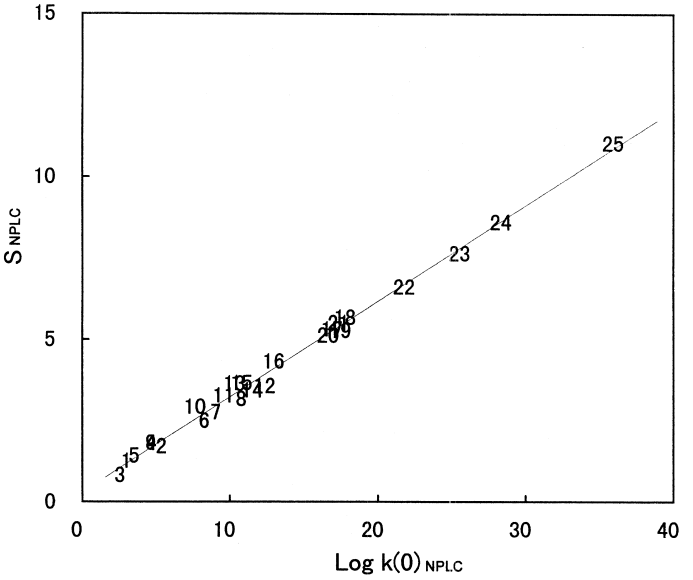

Fig. 3. Relationship between $^{e}\log k(0)_{NPLC}$ and S_{NPLC} in this version of NPLC [Eq. (9)]. The slope and intercept of the straight line were 0.296 and 0.272, respectively. The correlation coefficient is 0.99. Peptide identification as in Table 1. Column, TSKgel Amide-80; mobile phase, ACN–water–0.1% TFA.

Table 2
Comparison of observed and predicted retention times of peptides in the gradient elution

Peptide	Retention time (min)			S_{NPLC}[d]	$^{e}\text{Log } k(0)_{NPLC}$[e]
	Observed[a]	Two gradient runs[b]	One gradient run[c]		
1	11.68	12.24 (−0.56)[f]	11.98 (−0.29)[f]	1.03	2.56
9	17.39	17.55 (−0.16)	17.27 (0.12)	1.45	3.99
10	23.87	23.89 (−0.02)	23.21 (0.65)	1.90	5.52
13	32.20	32.26 (−0.06)	31.52 (0.68)	2.59	7.84
11	32.93	32.98 (−0.05)	32.54 (0.39)	2.68	8.16
6	37.96	37.99 (−0.03)	38.56 (−0.60)	3.28	10.21
14	48.45	48.48 (−0.03)	48.91 (−0.46)	4.69	14.95
20	53.12	53.12 (0.00)	53.10 (0.01)	5.47	17.59
12	54.35	54.39 (−0.04)	55.36 (−1.01)	5.98	19.29
25	64.95	64.97 (−0.02)	64.52 (0.43)	9.03	29.63
Mean deviation (%)		0.64	1.43		

[a] Observed retention times for 120 min gradient elution were generated from Ref. [3].

[b] Predicted retention times for 120 min gradient elution were calculated by two gradient runs. Values of $^{e}\log k(0)_{NPLC}$ and S_{NPLC} values were calculated based on observed retention times for 70 min and 90 min gradients in Ref. [3]. Predicted retention times were calculated using Eq. (5). These data were generated from Ref. [3].

[c] Predicted retention times for 120 min gradient elution were calculated by one gradient run. Values of $^{e}\log k(0)_{NPLC}$ were calculated numerically using Eq. (9). The observed retention times for 90 min gradient time in Ref. [3] were used as input to Eq. (9). Next, values of S_{NPLC} were calculated using Eq. (2). Thus, retention times were calculated using Eq. (4).

[d] Values of S_{NPLC} calculated by one gradient run.

[e] Values of $^{e}\log k(0)_{NPLC}$ calculated by one gradient run.

[f] The numbers in parentheses are the error values (=observed−predicted). Peptide identification as in Table 1.

Table 3
Comparison of predicted and observed R_s value of adjacent peptides in gradient elution

Peptide	R_s		
	Observed[a]	Two gradient runs[b]	One gradient run[c]
1–9	7.16	6.66 (0.50)[d]	6.63 (0.52)[d]
9–10	9.36	9.16 (0.20)	8.58 (0.77)
10–13	14.72	14.78 (−0.07)	14.67 (0.04)
13–11	1.22	1.19 (0.02)	1.69 (−0.48)
11–6	7.76	7.73 (0.02)	9.28 (−1.52)
6–14	16.21	16.21 (0.00)	16.00 (0.21)
14–20	6.65	6.61 (0.04)	5.97 (0.68)
20–12	1.73	1.78 (−0.05)	3.17 (−1.44)
12–25	13.10	13.08 (0.02)	11.32 (1.78)

[a] Values of R_s were calculated using observed gradient retention times shown in Table 2.
[b] Values of R_s were calculated using the gradient retention times obtained by two gradient runs.
[c] Values of R_s were calculated using the gradient retention times obtained by one gradient run.
[d] The numbers in parentheses are the error values (=observed−predicted).

it is noted that the elution order of peptides in this version of NPLC was not a simple reversal of that in RPLC.

5.1. Correlation between S and ^{e}log k(0)

Several studies [24,25] have demonstrated that S_{RPLC} often increased with increasing solute molecular mass in RPLC. In this study, the relationship between $^e\log\ k(0)_{\mathrm{NPLC}}$ and molecular mass was investigated as well as the relationship between S_{NPLC} and the molecular mass in this version of NPLC. The coefficient for the molecular mass was calculated by the least-squares method. The results are shown in Figs. 4 and 5.

$$S_{\mathrm{NPLC}} = 0.0305 \cdot (\mathrm{MW})^{0.68} - 0.417 \qquad (10)$$

$$^e\log k(0)_{\mathrm{NPLC}} = 0.0655 \cdot (\mathrm{MW})^{0.73} - 1.555 \qquad (11)$$

These figures indicated that both S_{NPLC} and $^e\log\ k(0)_{\mathrm{NPLC}}$ correlate with molecular mass.

Similarly, the relationship S_{RPLC} and $^e\log\ k(0)_{\mathrm{RPLC}}$ in RPLC was investigated using the same peptide shown in Table 1. The plot of S_{RPLC} versus $^e\log\ k(0)_{\mathrm{RPLC}}$ is shown in Fig. 6.

$$S_{\mathrm{RPLC}} = 1.932 \cdot {}^e\log k(0)_{\mathrm{RPLC}} + 16.286 \qquad (12)$$

In spite of the same eluting system using ACN–water containing 0.1% TFA, differences in S–$^e\log\ k(0)$ relationships exist between RPLC and NPLC. Snyder et al. [17] and Schoenmakers et al. [13] reported less correlation for the ACN system. These results were consistent with their report. Therefore, the prediction approach in RPLC cannot be carried out.

5.2. Prediction of retention time using only a single gradient run

In terms of the gradient retention times shown in Table 2, the retention times of all peptides predicted by two gradient runs were slightly smaller than those of observed. The two earlier eluted peptides (Nos. 1 and 9) had relatively large errors compared to the others. The precision (mean deviation 0.64%) in two gradient runs was in close agreement with the experimentally determined data. In contrast, the predicted retention times obtained by one gradient run scattered in either the positive or negative. The average error in predicting the retention times by one gradient run was also 0.44 min. Those errors were also not correlated with elution time, in contrast to the results in the case of two gradient runs. The average error in predicting the retention times obtained by one gradient run was 1.43%. It was about twice as much as that obtained by two gradient runs (mean deviation 0.64%). The error (1.43%), can be considered as follows: although the excellent correlation between S_{NPLC} and $\log\ k(0)_{\mathrm{NPLC}}$ was displayed in Fig. 3, there is small error in regression fitting by

Table 4
Comparison of predicted and observed retention times of peptides in isocratic elution

Peptide							Mean deviation (%)
1							
ϕ	0.0770	0.0535	0.0418				4.78
Observed (min)	6.47	8.91	10.96				
Predicted (min)[a]	6.61	8.46	10.18				
Error (min)[b]	−0.14	0.45	0.78				
9							
ϕ	0.101	0.0770	0.0535	0.0418			8.36
Observed (min)	6.47	9.23	15.56	21.73			
Predicted (min)	7.41	9.69	14.68	19.94			
Error (min)	−0.93	−0.47	0.88	1.79			
10							
ϕ	0.124	0.101	0.0770	0.0635			18.34
Observed (min)	5.88	8.58	16.30	24.00			
Predicted (min)	7.83	10.43	15.63	20.48			
Error (min)	−1.95	−1.84	0.67	3.52			
13							
ϕ	0.159	0.124	0.101	0.770			18.85
Observed (min)	5.84	10.43	19.48	48.91 (18.18)[c]			
Predicted (min)	7.55	12.11	19.02	35.38			
Error (min)	−1.71	−1.68	0.47	13.53			
11							
ϕ	0.159	0.124	0.101	0.0770			10.78
Observed (min)	6.92	12.13	21.41	49.82 (18.54)			
Predicted (min)	7.84	12.91	20.75	39.75			
Error (min)	−0.93	−0.78	0.66	10.07			
6							
ϕ	0.230	0.195	0.171	0.136	0.124	0.101	15.08
Observed (min)	6.67	8.67	10.90	17.12	20.69	35.58 (12.95)	
Predicted (min)	4.85	6.53	8.63	15.53	20.04	37.45	
Error (min)	1.82	2.14	2.27	1.58	0.66	−1.88	
14							
ϕ	0.277	0.230	0.195	0.171	0.136		18.25
Observed (min)	5.46	7.76	11.64	16.85	36.23 (13.21)		
Predicted (min)	3.91	5.81	9.67	15.57	40.98		
Error (min)	1.55	1.95	1.98	1.29	−4.76		
20							
ϕ	0.230	0.195	0.171	0.136	0.148		4.27
Observed (min)	7.24	13.13	23.03	32.17 (11.62)	48.39 (17.98)		
Predicted (min)	6.51	12.39	22.50	31.94	47.26		
Error (min)	0.74	0.74	0.57	0.22	1.13		

Table 4. Continued

Peptide						Mean deviation (%)
12						
ϕ	0.277	0.230	0.195	0.155		29.52
Observed (min)	6.86	10.65	17.31	33.39 (12.10)		
Predicted (min)	4.02	7.03	14.67	42.58		
Error (min)	2.84	3.62	2.64	−9.18		
25						
ϕ	0.253	0.230	0.218	0.206	0.1945	16.17
Observed (min)	6.27	12.75	20.48	36.59 (13.35)	70.68 (26.72)	
Predicted (min)	6.52	12.11	17.91	27.89	45.58	
Error (min)	−0.25	0.64	2.57	8.71	25.09	
					Average	14.44

[a] Predicted isocratic retention times by one gradient run were calculated using Eq. (1). Values of $^{e}\log k(0)_{NPLC}$ and S_{NPLC} were calculated by one gradient run. Both values are shown in Table 2.

[b] Error = predicted − observed.

[c] The numbers in parentheses are the capacity factors in the case of $k > 10$. Peptide identification as in Table 1.

the least-squares method. Furthermore, error of log $k(0)_{NPLC}$ becomes large when it is calculated numerically using the regression results. Finally, error becomes more large when the retention time is predicted using Eq. (3). Therefore, it is assumed that this error (1.43%) may be merely due to both imprecision (a large random error) and inaccuracy (a systematic error).

In terms of the gradient R_s values shown in Table 3, all the predicted R_s values of adjacent peptides calculated by two gradient runs had also good accuracy within ± 0.5. The predicted R_s values for only half of the peptides calculated by one gradient run had an accuracy within ± 0.5. Although the error for peptides (11–6, 14–20 and 12–25) are not within ± 0.5, there may not be large error. Because those R_s values have large value. Concerning R_s values around 1, the predicted R_s value (1.69) for peptide 13–11 had good accuracy within ± 0.5 as compared to observed values (1.22). The predicted value (3.17) for peptide 20–12 had major error as compared to the observed value (1.73). The prediction method by one gradient run could not give the precise value for this peptide.

In terms of the isocratic retention times, Cooper and Hurtubise [21] successfully predicted the isocratic retention times of hydroxyl aromatics using a single experimental isocratic data. They used Eqs. (1) and (2), and reported that the average error in k between predicted and observed values was 16.4%. In the present study, the average relative error in predicting the isocratic retention times by one gradient run was 14.4%, as shown in Table 4. This error is slightly less than that in their method. Especially, the errors of peptide Nos. 1 and 20 were small as compared to others for observed range ϕ. In the case of $k > 10$, the errors tend to be large, however. As for the gradient prediction method, it is assumed that the error may be merely due to imprecision and inaccuracy.

In terms of the isocratic R_s values shown in Table 5, the predicted R_s values of most adjacent peptides calculated by two gradient runs had also good accuracy within ± 0.5. In contrast, the predicted R_s values of most adjacent peptides calculated by one gradient run had not accuracy within ± 0.5. The error in predicting the value of R_s would become higher because it is affected by errors in two retention times. Especially, the prediction method by one gradient run is not suitable for the prediction the values of R_s in the isocratic elution because the errors in predicted isocratic retention times are higher than those in the gradient elution.

6. Conclusions

Using a linear relationship between S and $^{e}\log$

Table 5
Comparison of predicted and observed R_s value of adjacent peptides in isocratic elution

Peptide	R_s		
	Observed[a]	Two gradient runs[b]	One gradient run[c]
$\phi = 0.418$			
1–9	8.83	10.10 (−1.28)[d]	8.00 (0.83)[d]
$\phi = 0.535$			
1–9	10.20	11.40 (−1.20)	9.54 (0.66)
$\phi = 0.770$			
1–9	6.88	8.53 (−1.65)	7.69 (−0.82)
9–10	9.55	9.55 (0.00)	8.01 (1.54)
10–13	16.94	18.47 (−1.53)	10.26 (6.68)
13–11	0.33	0.96 (−0.63)	1.59 (−1.26)
$\phi = 0.101$			
9–10	4.94	5.04 (−0.10)	7.06 (−2.12)
10–13	12.33	12.97 (−0.64)	9.72 (2.61)
13–11	1.55	1.21 (0.34)	1.39 (0.15)
11–6	10.17	9.94 (0.22)	11.98 (−1.82)
$\phi = 0.124$			
10–13	8.60	8.62 (−0.02)	8.10 (0.50)
13–11	2.37	2.28 (0.09)	1.12 (1.25)
11–6	10.98	9.58 (1.40)	9.14 (1.84)
$\phi = 0.136$			
6–14	15.51	15.93 (−0.43)	20.66 (−5.15)
$\phi = 0.159$			
13–11	2.52	2.62 (−0.10)	0.69 (1.83)
$\phi = 0.171$			
6–14	9.22	9.23 (−0.01)	10.75 (−1.53)
14–20	5.05	5.08 (−0.04)	5.63 (−0.59)
$\phi = 0.195$			
6–14	6.06	5.56 (0.50)	6.39 (−0.33)
14–20	1.70	1.68 (0.02)	3.10 (−1.40)
20–12	4.12	4.03 (0.09)	2.25 (1.87)
12–25	16.58	16.33 (0.25)	9.60 (6.96)?
$\phi = 0.230$			
6–14	3.00	1.93 (1.06)	2.63 (0.37)
14–20	0.95	0.83 (0.12)	1.29 (0.12)
20–12	5.75	5.51 (0.24)	0.89 (4.87)
12–25	2.07	1.79 (0.28)	5.01 (−2.94)
$\phi = 0.277$			
14–12	3.90	3.66 (0.24)	0.31 (3.59)

[a] Values of R_s were calculated using observed isocratic retention times shown in Table 1.
[b] Values of R_s were calculated using the isocratic retention times obtained by two gradient runs.
[c] Values of R_s were calculated using the isocratic retention times obtained by one gradient run.
[d] The numbers in parentheses are the error values (observed − predicted).

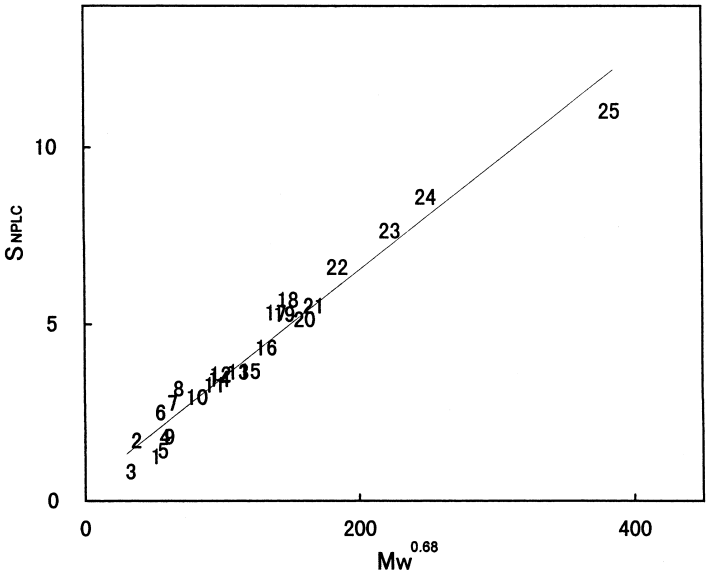

Fig. 4. Relationship between 0.68 power of the peptide's molecular mass and S_{NPLC} in this version of NPLC [Eq. (10)]. The slope and intercept of the straight line were 0.0305 and −0.417, respectively. The correlation coefficient is 0.96. Peptide identification as in Table 1. Column, TSKgel Amide-80; mobile phase, ACN–water–0.1% TFA.

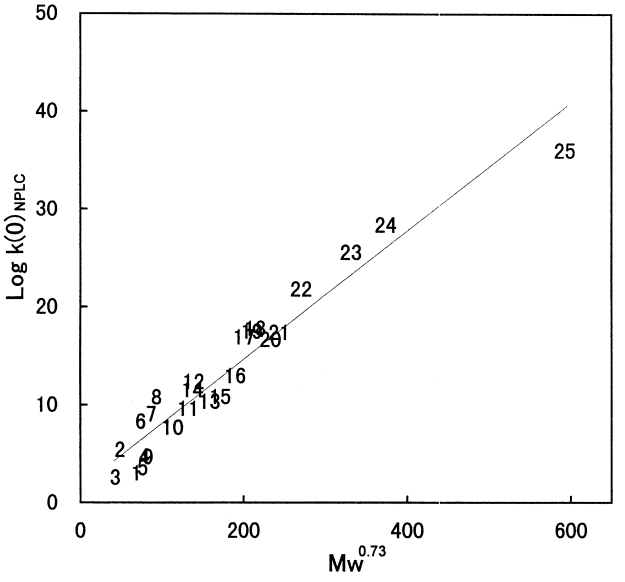

Fig. 5. Relationship between the 0.73 power of the peptide's molecular mass and $^{e}\log k(0)_{NPLC}$ in this version of NPLC [Eq. (11)]. The slope and intercept of the straight line were 0.0655 and −1.555, respectively. The correlation coefficient is 0.93. Peptide identification as in Table 1. Column, TSKgel Amide-80; mobile phase, ACN–water–0.1% TFA.

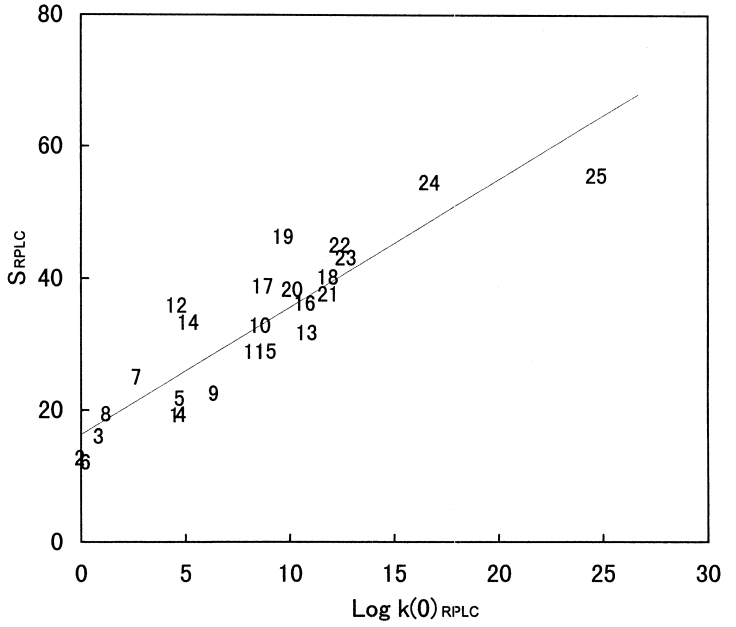

Fig. 6. Relationship between $^{e}\log k(0)_{RPLC}$ and S_{RPLC} in RPLC [Eq. (12)]. The slope and intercept of the straight line were 1.932 and 16.286, respectively. The correlation coefficient is 0.80. Peptide identification as in Table 1. Column, TSKgel ODS-80Ts; mobile phase, ACN–water–0.1% TFA.

$k(0)$, prediction of both retention times and the values of R_s of peptides with only a single gradient run was attempted. The main advantage of this method is the simplicity which requires starting data for only a single gradient run in order to predict both isocratic and gradient retention times under a variety of experimental conditions. Furthermore, this method could be applicable to the peptides with relatively wide range k in one experiment. The gradient retention times could be predicted with an average error of 1.43%, about twice that compared with two gradient runs. The isocratic retention times could be predicted with an average error of 14.44%, about three-times higher than that obtained two gradient runs. However, this method is not suitable for exact prediction of R_s values.

7. Symbols

ϕ	Volume fraction of modifier in the mobile phase $\phi = a + bi \cdot t$ $(i = 1, 2)$
a	Value of ϕ at the beginning of the gradient
bi	Gradient steepness parameter
$b1$, $b2$	Value of b for two gradient runs differing only in gradient times
$f(t)$	Shape of gradient program as a function of time
k, $k(\phi)$	Solute capacity factor
$k(0)_{NPLC}$	Capacity factor at $\phi = 0.01$ for this version of NPLC
$k(0)_{RPLC}$	Capacity factor at the water for RPLC
p, q	Linear regression coefficients for S and $^{e}\log k(0)$ in this version of NPLC and RPLC
t	Time (min)
tgi	Retention time in gradient elution (min)
t_0	Time required for a non-retained solute to elute from the column (min)
t_D	Dwell time for gradient elution (min); equal to the time it takes a change in the mobile phase composition to pass from the gradient mixer to the column inlet (min)
S_{NPLC}	Equal to $-d[^{e}\log k]/d[^{e}\log \phi]$
S_{RPLC}	Equal to $-d[^{e}\log k]/d\phi$
R_s	Resolution. The resolution R_s of two

adjacent bands 1 and 2 is defined equal to the distance between the two band centers (tr$i$, $i=1,2$), divided by average band width (tw$i$, $i=1,2$)

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