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PREDICTION OF PEPTIDE RETENTION TIMES

YASUHIRO SAKAMOTO*, NAMI KAWAKAMI and TATSURU SASAGAWA
Scientific Instrument Div. Tokyo Research Center, TOSOH Co., 2743-1, Ayase-shi, Kanagawa 252 (Japan)
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SUMMARY

A new approach for predicting the retention times of peptides, either with isocratic or gradient elution is described. The isocratic capacity factors of peptides are correlated with their molecular weights and with their hydrophobicities. Given the experimental conditions, and the amino acid composition, it is possible to calculate the retention time of a peptide eluted by a gradient, for any slope of gradient, flow-rate and column length.

INTRODUCTION

High-performance liquid chromatographic (HPLC) separation of peptides is one of the most important techniques in protein chemistry. Although this method is extremely powerful it is also very laborious and time-consuming. The conditions for gradient elution are usually selected by a trial-and-error method which is strongly dependent on the experience of the chromatographer involved. In many cases it would be useful, however, to be able to calculate the optimum gradient elution conditions for a given separation problem from the properties of the chromatographic system and peptides to be separated. This would require a good understanding of the retention behaviour of peptides.

The calculation of peptide retention times was first reported by Martin[1] in 1948. Subsequent important contributions were made[2,3] and in 1980 Meek[4] reported the calculation of peptide retention times using HPLC. Since then, several empirical prediction methods have been presented by us[5,6] and others[7–11]. We assumed that the contribution of each amino acid residue to retention would be additive and that the retention time would be related to the sum of the contributions of each residue:

$$T = A \cdot \ln (1 + \Sigma D_j \cdot n_j) + B \quad (1)$$

Here D_j (Table I) is an empirical retention parameter that takes account of the hydrophobicity, A and B are constants and n_j is the number of residues j in the peptide. With these assumptions, a good correlation between observed and calculated retention times was observed. One of the disadvantages, however, in this approach is that it is valid only under fixed chromatographic conditions, *i.e.*, a linear gradient with

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TABLE I

RETENTION CONSTANTS, D_j, OF AMINO ACIDS[5]

Amino acid	D_j
Tryptophan	2.34
Phenylalanine	1.71
Isoleucine	1.38
Leucine	1.34
Tyrosine	1.23
Methionine	0.85
Proline	0.48
Valine	0.38
Threonine	0.12
Histidine	0.34
Alanine	0.13
Glutamine	0.36
Glutamic acid	0.27
Glycine	0.22
Serine	0.18
Arginine	0.26
Aspartic acid	0.10
Asparagine	−0.45
Lysine	0.05
Carboxymethylcysteine	1.57
Homoserine	0.23

the same slope, a fixed flow-rate, a fixed column length, etc. Accordingly, the application of this method is very limited. An alternative, more versatile approach is the use of a computer as an interactive tool, without the restriction of a single optimization algorithm. Schoenmakers *et al.*[12] and Quarry *et al.*[13] have demonstrated that exact mathematical solutions can be derived for certain gradient conditions and that these solutions can predict the retention times of compounds with good accuracy. Such mathematical solutions can then form the basis for subsequent optimization procedures. In this paper, we have modified our previous treatment in order to obtain a much more flexible method which is based on gradient elution theory.

THEORETICAL

The reversed-phase retention time is generally well approximated over a practical range in k' by:

$$\ln k'(\varphi) = \ln k'(0) - S\varphi \tag{2}$$

Here φ is the volume fraction of the less polar component in the water–organic mobile phase, S and $k'(0)$ are constants for a given solute and system.

The gradient retention time can be calculated using eqn. 3 for any gradient, provided that $k'(\varphi)$ is known[12]:

$$\int_{a}^{T-DT-T0} \mathrm{d}[f^{-1}(\varphi)]/k'(\varphi) = T0 - DT/k'(a) \tag{3}$$

Here DT is the gradient delay time, $k'(a)$ is the capacity factor at the initial concentration of organic solvent ($\varphi = a$) and φ is a function of time, $\varphi = f(t)$.

In case of a linear gradient of the form

$$\varphi = b \cdot t + a \tag{4}$$

Eqn. 3 results in

$$T = 1/(b \cdot S) \cdot \ln\{1 + b \cdot S[T0 \cdot k'(a) - DT]\} + \\ + T0 + DT \quad \text{if} \quad T \geqslant DT + T0 \tag{5}$$

$$T = T0 \cdot [1 + k'(a)] \quad \text{if} \quad T < DT + T0 \tag{6}$$

where T0 is the retention time of an unretained sample. In the above two equations, we have assumed that the isocratic capacity factor is expressed by eqn. 2, and the slope of the gradient is b.

Alternatively, the capacity factor, $k'(a)$, can be solved from eqn. 5:

$$k'(a) = \{\exp[(T - DT - T0) \cdot b \cdot S] - 1\}/(b \cdot S \cdot T0) + \\ + DT/T0 \quad \text{if} \quad T \geqslant DT + T0 \tag{7}$$

$$k'(a) = (T - T0)/T0 \quad \text{if} \quad T < DT + T0 \tag{8}$$

This allows the prediction of the isocratic capacity factor, $k'(a)$, on the basis of gradient data, if we know the slope, S.

EXPERIMENTAL

Materials

Almost all peptides were obtained by either tryptic or cyanogen bromide degradation of sperm whale myoglobin or hen lysozyme. The sequences were confirmed by amino acid analysis. Other oligopeptides were obtained from the indicated sources: methionine-enkephalin, angiotensin I(human), γ-endorphin (Peptide Institute, Osaka, Japan); Gly–Gly, Gly–Ala, Phe–Ala, Leu–Val, Phe–Gly–Gly–Phe, Gly–Ser, Ala–Gly–Ala, Gly–Gly–Gly, Gly–Thr, Gly–Glu(Sigma).

Methods

Retention times of peptides were measured on a 5-μm, 8-nm pore ODS column capped with trimethylchlorosilane (ODS 80TM, 15 cm × 0.46 cm; TOSOH, Japan) using a TOSOH liquid chromatograph at 25°C. The mobile phase was 0.1% trifluoroacetic acid and the mobile phase modifier was acetonitrile. For gradient elution, the concentration of the modifier was increased linearly from 0 to 50% over 30 min (1.67%/min) at a flow-rate of 1 ml/min unless stated otherwise. Other retention data both in isocratic or gradient elution modes were obtained from refs. 5 and 14. The column dead-time, T0, was taken as the retention time of sodium nitrate (1.3 min) at a flow-rate of 1 ml/min. The time required for the front of the gradient to reach the top of the column (gradient delay time, DT) was determined by observing the extrapolated onset of a gradient whose mobile phase absorbance increased with the

TABLE II

DEPENDENCE OF PEPTIDE RETENTION TIMES (min) ON MOBILE PHASE COMPOSITION

1 = GG; 2 = AG; 3 = LV; 4 = FA; 5 = FGGF; 6 = YGGFM; 7 = DRVYIHPFHL; 8 = CKGTDVQAW; 9 = WWCNDGR; 10 = IVSDGDGMNAW; 11 = NAWVAWRNRCKGTDVQAWIRGCRL; 12 = YGGFMTSEKSQTPLVTL; 13 = HGLDNYR; 14 = NTDGSTDYGILQINSR; 15 = KVFGRCELAA; C = carboxymethylcysteine; X = homoserine.

No.	*% Acetonitrile*									
	0	*5*	*10*	*15*	*20*	*25*	*28*	*30*	*35*	*40*
1	2.14	2.02	1.92	1.80	1.71					
2	2.70	2.19	1.98	1.83	1.74					
3			9.70	4.72	3.15	2.55				
4			7.75	4.01	2.84	2.39				
5						5.33	3.90	3.20	2.53	2.11
6						5.35	3.88	3.28	2.53	2.11
7						10.89	4.83	3.33	2.23	
8					7.47	3.57	2.77	2.30		
9					10.21	4.04	2.94	2.53		
10					12.34	4.48	3.15	2.67		
11							10.10	4.55	2.20	
12						15.60	6.60	4.32	2.48	2.11
13				5.07	2.49	2.34		1.58		
14					15.20	8.69	2.51	2.32	2.24	
15					8.86	2.98	2.34	2.31		

volume fraction of the B reservoir at a flow-rate of 1 ml/min (DT = 2). Note that the column was replaced with an equivalent length of 0.25 mm I.D. stainless-steel tubing.

Regression analyses were performed using an Operate 7000 personal computer, programmed in BASIC.

RESULTS AND DISCUSSION

The first step in our approach to predicting retention times was to extract values of S and $k'(0)$ in eqn. 2 for many peptides. The next step was to correlate these values with currently known parameters.

Correlation between S and molecular weight

It has been shown that there exists a linear relationship between log $k'(\varphi)$ of peptides and the mobile phase composition, φ. Several studies[15,16] have also shown that the constant S in eqn. 2 often increases with solute molecular weight, although factors other than molecular weight are believed to affect the values of S. Stadalius *et al.*[17] showed that the value of the slope of the straight line is proportional to the 0.44 power of the solute molecular weight. Schoenmakers *et al.*[18] showed that the slope of the straight line is related to the $k'(0)$ of the solute. The $k'(\varphi)$ values of fifteen peptides, varying in molecular weight from 132 to 2976 daltons, were measured at different mobile phase compositions (Table II). The slopes of plots of log $k'(\varphi)$ *vs.* φ were calculated by least squares, and then plotted against the logarithms of the

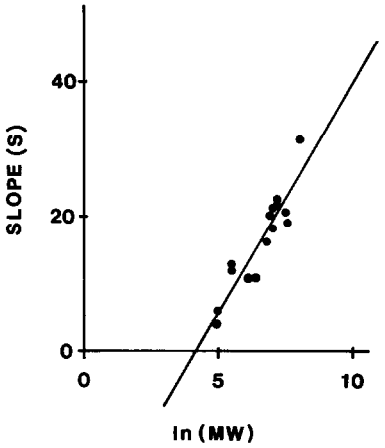

Fig. 1. Dependence of the slope, S, on the molecular weights of fifteen peptides.

molecular weights. The resulting correlation (Fig. 1) shows some scatter (correlation coefficient = 0.92) but generally confirms the trend in S *vs.* molecular weight. S, therefore, was expressed as

$$S = P \cdot \ln \mathrm{MW} - Q \tag{9}$$

where the calculated coefficients were $P = 6.79$ and $Q = 28.24$.

With the application of eqn. 9, only a single unknown, $k'(a)$, remains in eqn. 5. By combining eqns. 7 and 9, the $k'(0)$ values of 33 peptides were calculated (Fig. 2) using the observed gradient retention times (Table III).

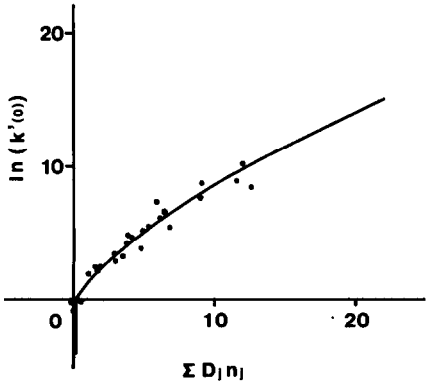

Fig. 2. Dependence of $k'(0)$ on the sum of the retention parameters. The calculated ln $k'(0)$ values for 33 peptides and the observed ln $k'(0)$ values for 7 small peptides were plotted against the sum of the retention parameters of the constituent amino acid residues. The regression curve corresponds to eqn. 11.

TABLE III

COMPARISON OF PREDICTED AND OBSERVED RETENTION TIMES

Number	*Retention time (min)*		*Sequence*
	Observed	*Predicted*	
1	1.9	1.9	GS
2	2.0	2.2	AGA
3	2.0	2.8	GGG
4	2.1	1.8	AG
5	2.2	1.8	GT
6	2.2	2.0	GG
7	2.4	2.2	GE
8	6.2	5.5	YK
9	7.6	7.9	FK
10	7.8	7.6	KDIAAK
11	8.0	7.6	DIAAK
12	8.7	7.0	TEAEMK
13	8.7	8.5	ASEDLKK
14	8.9	8.6	FA
15	9.2	8.9	FDR
16	9.2	8.5	ASEDLK
17	9.4	7.9	LV
18	10.4	11.3	SHPETLEK
19	11.8	13.6	LFK
20	11.8	13.9	HKIPIK
21	12.3	11.4	HGLDNYR
22	12.5	14.1	ELGYQG
23	13.7	16.6	YKELGYQG
24	13.9	12.8	HPGNFGADAGGAMNK
25	16.6	15.9	KVFGRCELAAX
26	16.6	13.8	CKGTDVQAW
27	16.9	19.0	VEADVAGHGEDILIR
28	17.2	16.9	WWCNDGR
29	17.3	16.6	FGGF
30	17.9	17.2	IVSDGDGMNAW
31	18.2	17.5	NTDGSTDYGILQINSR
32	18.6	16.6	YGGFM
33	19.2	17.9	ALELFR
34	21.1	19.3	DRVYVHPFHL
35	21.3	16.7	GHHEAELKPLAESHATK
36	21.8	25.4	NAWVAWRNRCKGTDVQAWIRGCRL
37	21.9	22.5	YGGFMTSEKSQTPLVTL
38	25.1	26.4	VLSEGEWQLVLHVWAK
39	28.4	27.0	YLEFISEAIIHVLHSR
40	25.6	23.2	HGVTVLTALGALGAILK

Correlation between $k'(0)$ and the sum of the retention parameters of the constituent amino acids

Solvophobic theory[19] predicts a linear dependence of ln $k'(\varphi)$ on the hydrophobic surface area of a solute. In previous reports[5,6], we assumed that the hydrophobic surface area of an amino acid is related to the retention parameter for each amino acid residue. Furthermore, we assumed the sum of these retention parameters

to be equal to the retention parameter of the peptides. The reason for this assumption is that the total hydrophobic surface area of a polypeptide chain in its extended form is found to be related to the sum of D_j, as it should be since each additional residue contributes a constant area to the peptide. In Fig. 2, the calculated ln $k'(0)$ values for 33 peptides and the observed ln $k'(0)$ values of 7 small peptides were plotted against the sum of the retention parameters of the constituent amino acid residues ($\Sigma D_j \cdot n_j$). The plot deviates from the expected linearity, probably due to the folding of peptide chains. The major question concerns the form of the fitting function. It has been shown[20] that the accessible surface area of a monomeric protein varies as the 2/3 power of the molecular weight. To take account of the observed curvature, the form of the function was tested simply by replacing $\Sigma D_j \cdot n_j$ with $\Sigma(D_j \cdot n_j)^{2/3}$:

$$\ln k'(0) = E(\Sigma D_j \cdot n_j)^{2/3} + F \tag{10}$$

The calculated best-fit values of E and F were 2.02 and -0.76 respectively. Eqn. 10 is inadequate for describing $k'(0)$ at small values of $\Sigma D_j \cdot n_j$. As the value of $\Sigma D_j \cdot n_j$ approaches zero, ln $k'(0)$ should tend to $-\infty$. To take account of the above discrepancy, it was found necessary to add a second term which tends to $-\infty$ as $\Sigma D_j \cdot n_j$ approaches zero. We modified eqn. 10 to:

$$\ln k'(0) = E(\Sigma D_j \cdot n_j)^{2/3} + G/(\Sigma D_j \cdot n_j) + F \tag{11}$$

Using the same set of data, best-fit values of G were computed to be -0.40 without changing the value of E or F. By using eqns. 5, 9 and 11 or eqns. 5 and 10 when $T < DT + T0$, the retention times of the peptides were predicted, as shown in Fig. 3. The correlation coefficient between the observed and the predicted retention time was 0.98. The mean deviation was 8.9%, which is better than that (11.0%) obtained by eqn. 9. The calculated correlation coefficient and mean percent deviation were very close to those observed previously (0.98 and 9.9%)[5].

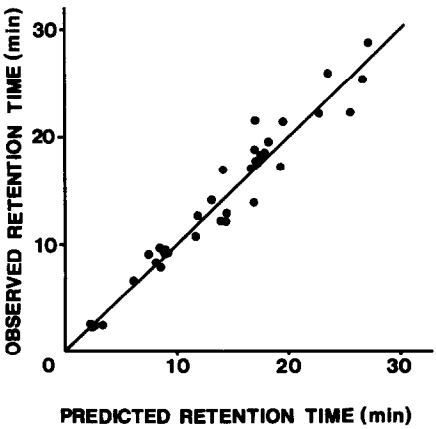

Fig. 3. Relationship between the observed and calculated retention times. The correlation coefficient was 0.98 and the mean deviation was 8.9%.

Evaluation of present model under different conditions

Table IV summarizes both predicted and observed retention times of 20 tryptic peptides of myoglobin under various experimental conditions involving different starting concentrations, a, slope of gradient, b, flow-rates and column lengths.

Effect of the shape of the gradient. Retention times were measured with a shallower gradient (b = 1.5%/min) with the initial concentration, a, at 5% instead of 0%, while keeping the flow-rate at 1 ml/min. The retention times of these peptides were calculated simply by using new values (Table IV, columns III and IV). The mean deviation was 8.2%.

Effect of the flow-rate. Retention times at a lower flow-rate (0.5 ml/min) were measured while keeping other parameters constant (a = 0, b = 1.67) using the same set of peptides. The retention times were calculated simply by changing the values of T0 (=2.6) and DT (=4.0) (Table IV, columns V and VI). The mean deviation was 5.8%.

Effect of column length. Our 15-cm column was cut in half. The retention times of the same set of peptides were measured on this column at a flow-rate of 1 ml/min, while keeping the same initial concentration (a = 0) and gradient (b = 1.67). Using a new T0 (=0.65), retention times on this short column were calculated (Table IV, columns VII and VIII). The mean deviation was 8.4%.

TABLE IV

COMPARISON OF OBSERVED AND PREDICTED RETENTION TIMES (min)

The numbers in parentheses are predicted retention times. Columns I and II show retention times under standard conditions (T0 = 1.3, DT = 2.0, b = 1.67, a = 0), III and IV show retention times with a different gradient programme (T0 = 1.3, DT = 2.0, b = 1.5, a = 5), V and VI show retention times at a flow-rate of 0.5 ml/min (T0 = 2.6, DT = 4.0, b = 1.67, a = 0) and VII and VIII show retention times on a 7.5-cm ODS column (T0 = 0.65, DT = 2.0, b = 1.67, a = 0). All the peptides were from a tryptic digest of myoglobin.

Sequence	*I*	*II*	*III*	*IV*	*V*	*VI*	*VII*	*VIII*
YK	6.2	(6.0)	5.2	(4.3	12.7	(11.1)	5.0	(3.4)
FK	7.6	(8.5)	7.4	(6.5)	15.0	(14.5)	6.4	(5.3)
KDIAAK	7.8	(8.1)	7.0	(5.6)	13.3	(13.4)	7.2	(5.3)
DIAAK	8.0	(8.1)	7.2	(5.7)	14.7	(13.5)	6.9	(5.2)
TEAEMK	8.7	(7.4)	7.6	(5.0)	14.0	(12.5)	8.0	(4.8)
ASEDLKK	8.7	(8.9)	7.6	(6.3)	14.7	(14.2)	7.7	(6.2)
FDR	9.2	(9.5)	8.2	(7.1)	14.7	(15.3)	8.1	(6.4)
ASEDLK	9.2	(8.9)	8.2	(6.4)	14.7	(14.3)	8.1	(6.1)
SHPETLEK	10.4	(11.7)	9.4	(9.1)	16.5	(17.0)	9.3	(8.9)
LFK	11.8	(14.1)	11.8	(11.8)	19.0	(20.3)	10.4	(10.6)
HKIPIK	11.8	(14.2)	10.9	(11.8)	17.0	(19.8)	11.0	(11.3)
ELGYQG	12.5	(14.4)	11.6	(12.0)	18.8	(20.1)	11.0	(11.4)
YKELGYQG	13.7	(16.7)	12.8	(14.6)	19.0	(22.1)	12.7	(14.0)
HPGNFGADAGGAMNK	13.9	(13.0)	12.8	(10.5)	19.2	(18.1)	12.7	(10.5)
VEADVAGHGEDILIR	16.9	(19.0)	15.8	(17.1)	22.1	(24.0)	15.4	(16.5)
ALELFR	19.2	(18.1)	18.8	(16.1)	25.2	(23.7)	17.7	(15.2)
GHHEAELKPLAESHATK	21.3	(16.7)	12.1	(14.6)	17.9	(21.7)	12.2	(14.3)
VLSEGEWQLVLHVWAK	25.1	(26.0)	24.0	(24.9)	29.4	(31.0)	23.4	(23.7)
YLEFISEAIIHVLHSR	28.4	(26.6)	27.0	(25.5)	32.4	(31.5)	26.4	(24.2)
HGVTVLTALGALGAILK	25.6	(22.9)	24.6	(21.5)	30.2	(28.0)	23.9	(20.5)

TABLE V

COMPARISON OF PREDICTED AND OBSERVED RETENTION TIMES

Number	*Retention times (min)*		*Sequence*
	Observed	*Predicted*	
1	2.5	2.98	PG
2	2.8	2.85	ARKX
3	5.0	4.75	TEEQ
4	6.5	5.75	MTAK
5	7.5	8.81	MARKX
6	7.8	6.54	MAR
7	8.0	6.89	YK
8	8.1	6.71	TPGSR
9	8.2	9.08	KYE
10	8.5	8.40	GY
11	9.2	9.90	TEAEMK
12	9.6	8.77	EY
13	9.8	10.51	HLK
14	9.9	10.96	FK
15	10.3	11.80	IRE
16	10.3	11.68	PL
17	10.9	11.02	IAE
18	11.5	12.68	GF
19	11.5	12.76	KMKDTDSEEE
20	12.0	13.23	AFR
21	12.0	10.77	DIAAK
22	12.0	13.29	QIAE
23	13.0	12.39	ASEDLK
24	13.5	14.56	EAFR
25	13.8	12.88	FDR
26	14.8	19.61	VFDKDGRNGY
27	15.0	12.64	FKE
28	15.6	15.74	KVFGR
29	15.8	16.11	SLGQNPTEAE
30	16.3	17.51	GW
31	16.5	16.76	MIRE
32	16.7	16.94	SHPETLEK
33	17.0	17.06	HGLDNYR
34	18.2	19.66	LFK
35	19.5	20.57	IAEFK
36	19.8	20.81	ADIDGDGQVNYEE
37	20.2	23.92	VFDKDGNGYI
38	20.3	20.47	ISAAELR
39	20.3	17.87	FESNFNTQATNR
40	21.2	19.14	ELGTVMR
41	21.3	16.57	GHHEAELK
42	22.0	20.72	LQDMINE
43	22.5	23.82	FVQMMTQ
44	23.8	21.53	QIAEFK
45	24.0	23.56	RSLGQNPTEAELQDX
46	24.8	27.98	MIREADIDGDGQVNYEE

(Continued on p. 78)

TABLE V (*continued*)

Number	*Retention times (min)*		*Sequence*
	Observed	*Predicted*	
47	25.1	26.34	FLTMMAR
48	25.3	22.57	VDADGNGTIDFPE
49	25.8	25.85	LGTVMRSLGQNPTEAE
50	26.9	26.31	NTDGSTDYGILQINSR
51	26.9	28.80	VEADVAGHGQDILIR
52	27.0	28.91	FLTMMARKMKDTDSEEE
53	27.5	29.02	VFDKDGNGYISAAELR
54	28.6	30.06	AFRVFDKDGNGYISAAE
55	29.0	28.78	VFDKDGNGYISAAEL
56	29.1	27.08	GYSLGNWVC
57	29.2	32.09	IREADIDGDGQVNYEEFVQX
58	30.0	30.55	EAFSLFDKDGDGTITTK
59	30.2	26.33	ALELFR
60	30.4	30.55	AFSLFDKDGDGTITTKE
61	34.2	36.81	NKALELFRKDIAAKYKELGYQG
62	34.8	37.25	PGYPGVYTEVSYHVDWIK
63	35.9	34.26	DDYGADEIFDSMICAGVPEGGK
64	40.5	31.00	HGVTVLTALGAILK
65	45.0	40.43	YLEFISEAIIHVLHSR
66	37.2	31.64	EADIDGDGQVNYEEFVQMMTAK
67	37.5	33.57	INEVDADGNGTIDFPEFLTX
68	38.5	42.92	IILHENFDYDLLDNDISLLK

Application to other systems. In order to demonstrate the applicability of the present method, retention times of a different set of peptides on a different ODS column which were listed in refs. 5 and 14 were predicted. Taking the same approach as above, the coefficients in eqns. 9 and 11 were calculated:

Eqn. 9: $P = 8.70$, $Q = 32.29$, correlation coefficient = 0.997 (from ref. 14)
Eqn. 11: $E = 2.60$, $G = -0.45$, $F = -1.42$ (from ref. 5)

Using these values and the system constants, T0 = 1.5, DT = 4, the retention times of 68 peptides were calculated as shown in Table V. The correlation coefficient between the observed and the predicted retention times was 0.97. The mean deviation was 8.84%, very close to that observed previously (9.9%).

It seems that the present method gives good estimates of the retention times of peptides. Our prediction model is based on gradient elution theory with *a priori* knowledge of the amino acid composition of peptides. The slope S and $k'(0)$ are predicted on the basis of amino acid composition. Our method, therefore, would be useful for the optimization of the initial chromatographic conditions for known peptides. The method reported by Stadalius *et al.*[17] which is also based on the theory of gradient elution requires no *a priori* knowledge of the amino acid composition. The slope, S, and $k'(0)$, alternatively, have to be determined after two initial experiments. The latter method therefore, would be useful for predicting how a separation will change with the conditions after initial experiments.

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