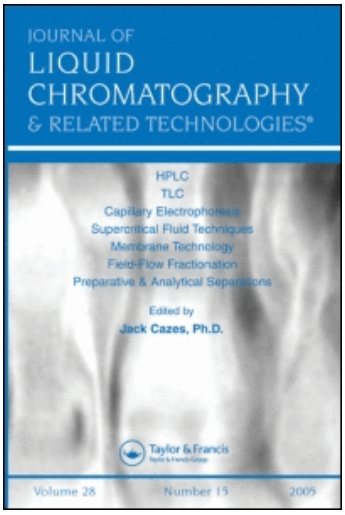

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Influence of Acetyl and Amide Groups on Peptides RP-LC Retention Behavior

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Influence of Acetyl and Amide Groups on Peptides RP-LC Retention Behavior

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Abstract: Reversed phase liquid chromatography (RP-LC) is still one of the most efficient analytical techniques for separation of complex biologically active analytes, including proteins and peptides. However, the separation of those analytes is not easy to perform in practice. Therefore, the appropriate optimization of the separation conditions is necessary.

The aim of the study was to explore the influence of acetyl and amide groups in peptides on their retention in gradient elution reversed phase LC. Moreover, the investigation regards the influence of temperature and gradient time for such peptides. Those results were suggested in view of their usefulness in predictions of peptides RP-LC retention on the basis of quantitative structure retention relationships for proteomic research.

Keywords: Peptides, Proteomics, Retention behavior, Reversed phase liquid chromatography (RP-LC)

INTRODUCTION

Nowadays, products of the genes – proteins and a comprehensive analysis and characterization of all expressed proteins called proteomics, are from day to day the highest point of interest. However, the

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complexity of proteome analysis has been noted. It results from the continual change in concentration of the protein in a cell and their multiple forms due to post translational modifications. Considering 30–40 thousands of genes in human cells, there may be 10–20 times more proteins to be examined.[1,2] The issue of proteome identification is more difficult because of the wide dynamic concentration range and the diversity in protein properties, the lack of an amplification procedure for proteins and their varied biological functions. Today, the most widely used procedure for analyzing complex protein mixtures is two dimensional gel electrophoresis.[3–8] But it is well known that even more efficient separation is required prior to mass spectrometry analysis and bioinformatics database searching enabling the correct identification of proteins.[9–11] The high resolution separation techniques like multidimensional chromatography, liquid chromatography coupled to tandem mass spectrometry (LC/MS/MS) is widely used to identify the components of protein complexes and subcellular compartments.[12–15]

One of the challenging aspects of proteomic analysis[16–20] is a practically useful processing of information, which becomes available after the separation of peptides with the use of HPLC. In fact, retention time is a characteristic and strictly structurally dependent parameter for a given analyte chromatographed at fully described experiment conditions (mobile phase composition, stationary phase, temperature, pH). Prediction of the retention time for a given peptide, combined with MS/MS data analysis, could therefore be useful in proteomics. It could improve the confidence of peptide identifications and, hence, increase the number of correctly identified peptides.

The aim of the study was to explore the influence of acetyl and amide groups in peptides on their retention in gradient elution reversed-phase HPLC. The investigation searched, also, the influence of temperature and gradient time for such peptides. The results are considered in the view of additional information during predictions of peptides HPLC retention with the use of quantitative structure retention relationships,[21] which in combination with MS/MS data analysis could serve to improve the confidence of peptide identifications and to increase the number of correctly identified peptides in proteomic research.

EXPERIMENTAL

Equipment

Chromatographic measurements were performed with HPLC apparatus (Waters Corporation, Milford, MA, USA) comprised of a pump,

variable wavelength UV/VIS detector, autosampler, and thermostat. Data were collected with the use of the Waters Millenium 2.15 software. The following column was employed in the studies: XTerra MS C18, 15.0 × 0.46 cm I.D., particle size 5 μm (Waters, Millford, MA, USA).

Materials and Methods

HPLC measurements were performed with the use of a mobile phase comprising water and 0.12% of trifluoroacetic acid and acetonitrile with the addition of 0.10% of trifluoroacetic acid. Gradient elution from 0 to 60% of acetonitrile in gradient time of 20 and 60 min was executed. Temperature was 40°C and 60°C. Flow rate of the mobile phase was 1 mL/min. Detection at wavelength equal to 223 nm was used. Dead time determined with the signal of the stronger solvent was 2.30 min. All peptide samples were diluted in water with the addition of 0.12% trifluoroacetic acid. Injection volume of 20 μL was used.

Acetonitrile (HPLC grade) was from P.C. Odczynniki (Gliwice, Poland) and trifluoroacetic acid was from Fluka (Buchs, Switzerland). Water was prepared with the use of Milli-Q Water Purification System (Millipore Corporation, Bedford, MA, USA). All peptides tested were synthesized in the University of Gdañsk, Poland.[18,19]

Chromatographic Measurements

In the first part of the experiments, 32 peptides were chromatographed with the use of gradient elution 0–60% of acetonitrile, at gradient time of 20 min and temperature equal to 40°C. Equilibration of the column was performed by flushing it with the initial mobile phase during a period of 10 min. When retention data for all peptides were collected, peptides were divided into several groups allowing the influence of the peptides' modifications on their retention to be studied.

In the second part of experiments, eight peptides were chromatographed in gradient elution 0–60% of acetonitrile with variable values of gradient time and temperature. Measurements were conducted at gradient time, $t_G = 20$ and 60 min as well as at temperature, $T = 40$ and 60°C. In that way, the additional influence of gradient time and temperature was tested with regards to the studied modifications of the peptides.

RESULTS

Influence of Acetyl and Amide Groups on Peptides Retention in RP-HPLC System

Peptides were divided into eight sets. Each set consisted of four peptides with the same amino acid sequence. In each set there was a peptide without any additional group at amino or carboxyl groups. There was a peptide with an amido group ($-CONH_2$) instead of carboxyl group, a peptide with acetyl group connected to an amino group of peptide, and a peptide with acetyl connected to an amino group of peptide and an amide group instead of a carboxyl group. The general scheme for such a set of peptides is presented in Table 1.

A good example of one of the eight peptides sets studied is depicted in Figure 1.

Four sets of hexapeptides and four sets of decapeptides were chosen for experiments. Retention data for 32 peptides chromatographed in gradient elution 0–60% B at gradient time, $t_G = 20$ min and temperature, $T = 40°C$ were collected in Table 2. The influence of the amino acid sequence length was observed in this set of peptides. Also hexapeptides had shorter retention times in comparison to decapeptides. Peptides with shorter sequences comprising 6 amino acids can be followed according to the increasing retention times (Table 3).

On the basis of the performed experiments it can be noticed that the introduction of the appropriate functional group influences the retention times of peptides. Considering retention times of peptides with the additional $-CONH_2$ groups and without such ones, it can be noted that peptides with $-CONH_2$ groups have shorter retention times. On the other hand, introducing a –COOH group into peptide's amino group caused increasing retention times. The differences between retention times in all sets of hexapeptides are very similar. It can be concluded that the introduction of a $-CONH_2$ group into a peptide decreased retention

Table 1. The general scheme of a set of peptides used in the studies

Abbreviation	Explanation
X	peptide without any additional group
X-NH_2	peptide with amide group instead of carboxyl group
Ac-X	peptide with acetyl group connected to amino group
Ac-X-NH_2	peptide with amide group instead of carboxyl group and acetyl group connected to amino group

Where: X is amino acid sequence.

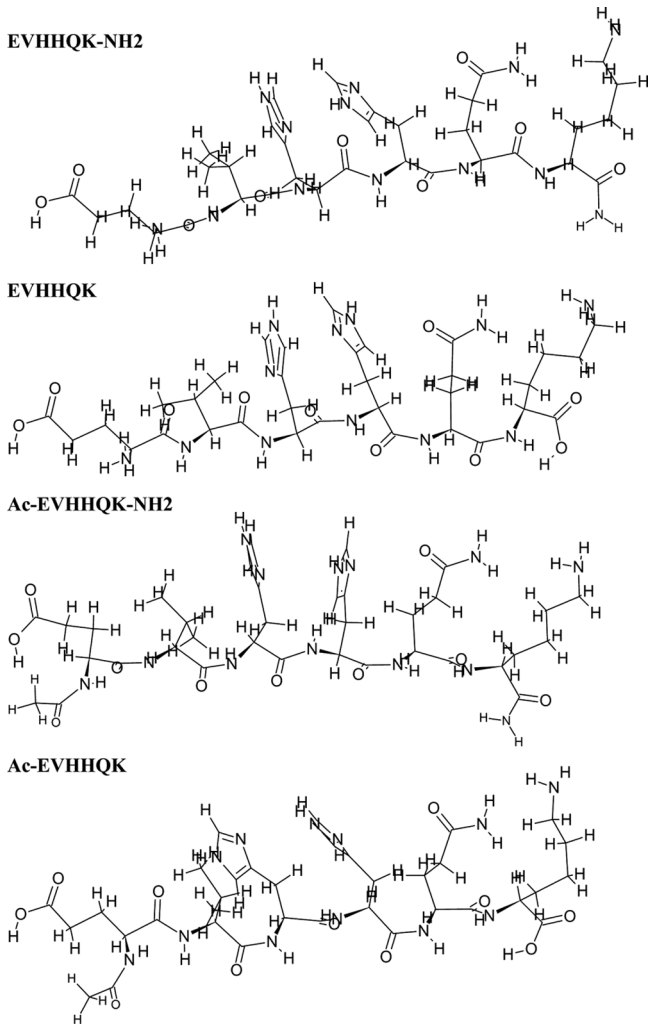

Figure 1. Exemplary molecular structures of four analyzed hexapeptides.

times approximately 0.26 min. Adding the –COOH group to a free amino group increased retention times approximately 1.01 min. Comparative data for all analyzed peptides are collected in Table 4.

Similar results can be observed in the next four sets of peptides comprising ten amino acid sequences (Figure 2). Those sets can be presented according to the increasing retention times (Table 5). Retention data for those peptides are collected in Table 2.

Table 2. Retention data obtained for 32 peptides chromatographed in gradient elution 0–60% B at gradient time, $t_G = 20$ min and temperature, $T = 40°C$

No.	Set no.	Peptide	t_R (min)
1	1	EVHHQK-NH_2	8.22
2		EVHHQK	8.52
3		Ac-EVHHQK-NH_2	9.33
4		Ac-EVHHQK	9.50
5	2	EVRHQK-NH_2	8.53
6		EVRHQK	8.82
7		Ac-EVRHQK-NH_2	9.43
8		Ac-EVRHQK	9.73
9	3	DAEFRH-NH_2	10.62
10		DAEFRH	10.90
11		Ac-DAEFRH-NH_2	11.68
12		Ac-DAEFRH	11.90
13	4	DAEFGH-NH_2	10.93
14		DAEFGH	11.18
15		Ac-DAEFGH-NH_2	11.95
16		Ac-DAEFGH	12.25
17	5	DAEFRHDSGY-NH_2	11.60
18		DAEFRHDSGY	11.62
19		Ac-DAEFRHDSGY-NH_2	12.50
20		Ac-DAEFRHDSGY	12.63
21	6	DAEFGHDSGF-NH_2	13.13
22		DAEFGHDSGF	13.35
23		Ac-DAEFGHDSGF –NH_2	14.02
24		Ac-DAEFGHDSGF	14.23
25	7	EVRHQKLVFF-NH_2	15.53
26		EVRHQKLVFF	16.00
27		Ac-EVRHQKLVFF-NH_2	16.00
28		Ac-EVRHQKLVFF	16.47
29	8	EVHHQKLVFF-NH_2	15.52
30		EVHHQKLVFF	16.02
31		Ac-EVHHQKLVFF-NH_2	15.92
32		Ac-EVHHQKLVFF	16.42

Table 3. Peptides with shorter sequences comprising 6 amino acids followed according to the increasing retention times

Set no.	Peptides followed according to the increasing retention times
1	EVHHQK-NH_2 < EVHHQK < Ac-EVHHQK-NH_2 < Ac-EVHHQK
2	EVRHQK-NH_2 < EVRHQK < Ac-EVRHQK-NH_2 < Ac-EVRHQK
3	DAEFRH-NH_2 < DAEFRH < Ac-DAEFRH-NH_2 < Ac-DAEFRH
4	DAEFGH-NH_2 < DAEFGH < Ac-DAEFGH-NH_2 < Ac-DAEFGH

Table 4. The differences between retention times, t_R of the testes peptides after the addition of amide and acetyl group

Set no.	X	The difference after the addition of amide group		The difference after the addition of acetyl group	
		X−(X-NH_2)	(Ac-X) −(X-NH_2)	(Ac-X)−X	(Ac-X-NH_2) −(X-NH_2)
1	EVHHQK	0.30	0.17	0.98	1.11
2	EVRHQK	0.29	0.30	0.91	0.90
3	DAEFRH	0.28	0.22	1.00	1.06
4	DAEFGH	0.25	0.30	1.07	1.02
5	DAEFRHDSGY	0.02	0.13	1.01	0.90
6	DAEFGHDSGF	0.22	0.21	0.88	0.89
7	EVRHQKLVFF	0.47	0.47	0.47	0.47
8	EVHHQKLVFF	0.50	0.50	0.40	0.40

Elongation of the amino acid sequence caused increasing the retention times for all decapeptides in these sets. Similar influence of the additional functional groups can be noted. Addition of a $–CONH_2$ group decreases retention but adding –COOH group to amino group increased retention. Also, the sequence of the peptide had the significant influence on retention. In the fifth and sixth sets, where differences between sequences regarding two amino acids were observed, the differences between retention times were very similar. Addition of the $–CONH_2$ group caused a decrease of retention approximately 0.15 min and adding the –COOH group caused increase of retention approximately 0.92 min. On the other hand, considering the seventh and eight group, which differed only with one amino acid, the introduction of the $–CONH_2$ group caused increasing the retention approximately 0.48 min and adding the –COOH group shortened retention approximately 0.43 min (Table 4).

Influence of Temperature and Gradient Time on Peptides' Retention Studied

Investigation was performed with the use of eight peptides. Increasing gradient time from $t_G = 20$ min to $t_G = 60$ min at temperature, $T = 40°C$, generated increasing of peptides retention. Similar tendencies were noted at temperature, $T = 60°C$. However, the differences between retention times measured at 60°C is not so distinct, as in the case of 40°C (Tables 6 and 7). Higher temperature influenced larger changes in peptides

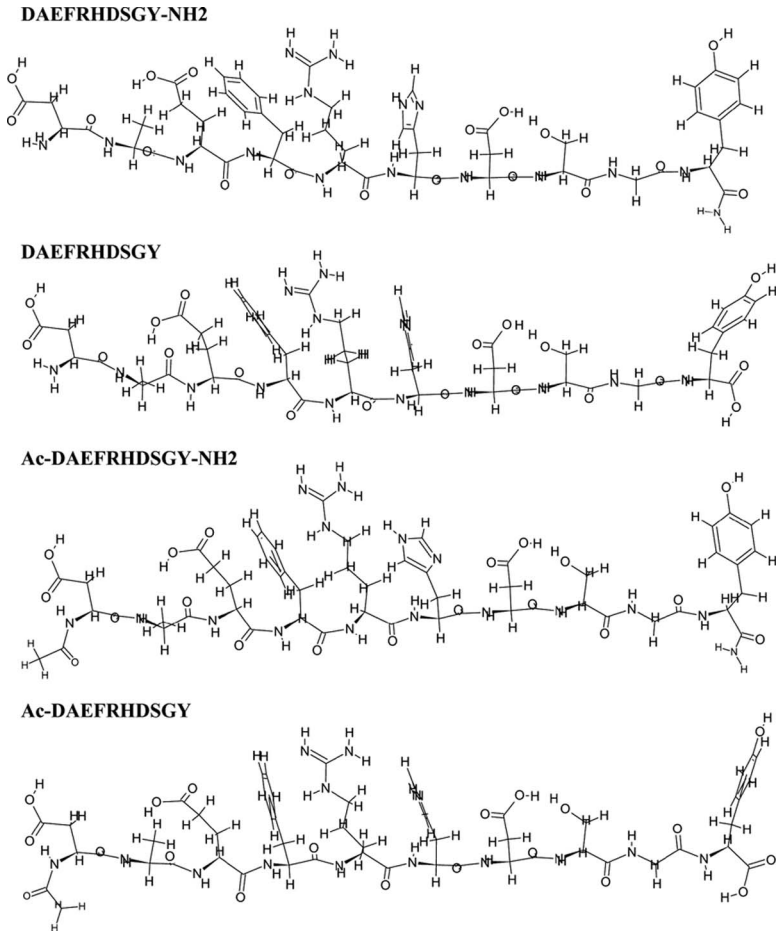

Figure 2. Exemplary molecular structures of four analyzed decapeptides.

retention in comparison to the changes observed among gradient time. At the same time, it was noticed that increasing the gradient time at 60°C did not significantly influence the peptides retention which did not possess the –COOH group at the amino group. More distinct alterations were observed for peptides with the –COOH group at the amino group.

Elevating temperature from 40°C to 60°C at gradient time, $t_G =$ 20 min, the decreasing of retention was noted. Especially at $t_G = 60$ min, elevation of temperature influenced, more significantly, retention of peptides without a –COOH group at an amino group. That in turn, caused decreasing of peptides' retention (Table 7).

Table 5. Peptides with shorter sequences comprising 10 amino acids followed according to the increasing retention times

Set no.	Peptides followed according to the increasing retention times
5	DAEFRHDSGY-NH_2 < DAEFRHDSGY < Ac-DAEFRHDSGY-NH_2 < Ac-DAEFRHDSGY
6	DAEFGHDSGF-NH_2 < DAEFGHDSGF < Ac-DAEFGHDSGF-NH_2 < Ac-DAEFGHDSGF
7	EVHHQKLVFF-NH_2 < EVHHQKLVFF < Ac-EVHHQKLVFF-NH_2 < Ac-EVHHQKLVFF
8	EVRHQKLVFF-NH_2 < Ac-EVRHQKLVFF-NH_2 < EVRHQKLVFF < Ac-EVRHQKLVFF

DISCUSSION

Concluding the results of the performed studies, it can be first noticed that the significant influence of the amino acid sequence length on peptides retention was observed. Longer sequence generated higher retention. Also important was the influence of functional groups on peptides retention. Adding of an amide group decreased retention of peptides and adding of an acetyl group increased retention.

Analyzing the influence of temperature and gradient time on peptides retention, it can be concluded that elevation of temperature generated shorter retention times of peptides. On the other hand, longer gradient time produced increasing of retention. Moreover, the influence of temperature on peptides with $–CONH_2$ group in comparison to peptides with –COOH group at amine group is stronger. However, the

Table 6. The differences between retention times, Δt_R, obtained in four gradient experiments 0–60% B at gradient time, $t_G = 20$ and 60 min and temperatures, $T = 40$ or 60°C for the eight tested peptides

		$T = 40$°C			$T = 60$°C		
No.	Peptide	$t_G = 20$ min	$t_G = 60$ min	Δt_R	$t_G = 20$ min	$t_G = 60$ min	Δt_R
1	EVHHQK-NH2	8.22	9.13	0.91	4.97	5.17	0.20
2	EVHHQK	8.52	10.15	1.63	6.86	7.76	0.90
3	Ac-EVHHQK-NH_2	9.33	12.25	2.92	8.73	10.63	1.90
4	Ac-EVHHQK	9.50	12.90	3.40	8.95	11.22	2.27
5	EVRHQK-NH2	8.53	10.03	1.50	7.72	7.62	0.10
6	EVRHQK	8.82	10.88	2.06	8.05	9.20	1.15
7	Ac-EVRHQK-NH2	9.43	13.03	3.60	9.10	11.50	2.40
8	Ac-EVRHQK	9.73	13.47	3.74	9.18	11.85	2.67
Mean				2.47	Mean		1.45

Table 7. The differences between retention times, Δt_R, obtained in four gradient experiments 0–60% B at gradient time, $t_G = 20$ or 60 min and temperatures, $T =$ 40 and 60°C for the eight tested peptides

		$t_G = 20$ min			$t_G = 60$ min		
No.	Peptide	$T = 40$°C	$T = 60$°C	Δt_R	$T = 40$°C	$T = 60$°C	Δt_R
1	EVHHQK-NH2	8.22	4.97	3.25	9.13	5.17	3.96
2	EVHHQK	8.52	6.86	1.66	10.15	7.76	2.39
3	Ac-EVHHQK-NH_2	9.33	8.73	0.60	12.25	10.63	1.62
4	Ac-EVHHQK	9.50	8.95	0.55	12.90	11.22	1.68
5	EVRHQK-NH2	8.53	7.72	0.81	10.03	7.62	2.41
6	EVRHQK	8.82	8.05	0.77	10.88	9.20	1.68
7	Ac-EVRHQK-NH2	9.43	9.10	0.33	13.03	11.50	1.53
8	Ac-EVRHQK	9.73	9.18	0.55	13.47	11.85	1.62
Mean				1.07	Mean		2.11

increasing of gradient time generated larger changes of retention of peptides with a –COOH group at an amine group in comparison to peptides with a –$CONH_2$ group.

The results presented in the paper can be further considered as useful additional information during predictions of peptides HPLC retention with the use of quantitative structure retention relationships,[18–20] which in combination with MS/MS data analysis could serve to improve the confidence of peptide identifications and to increase the number of correctly identified peptides in proteomic research.

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