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CHEMISTRY

Behaviour of proteins on reversed-phase supports during high performance liquid chromatography on C18 stationary phase

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Abstract. The separation of high-molecular compounds is very difficult, if possible at all, under isocratic conditions. For this gradient elution is needed. The theory of gradient elution for small molecules is well established; however, its applications to reversed-phase gradient separations of biopolymers are not straightforward because of specific problems, such as slow diffusion, limited accessibility of the stationary phase for larger molecules, or possible sample conformation changes during the elution.

We used high performance liquid chromatography to investigate the reversed-phase chromatographic behaviour of 14 proteins. The first step was the determination of the experimental data, and then these data were used to predict gradient retention times. A water–organic solvent–trifluoroacetic acid system was used to examine the influence of experimental parameters. The chromatographic results from four C18-chain-length supports were comparable.

Key words: high-performance liquid chromatography, mobile phase composition, protein, reversed phase.

INTRODUCTION

Reversed-phase liquid chromatography (RPLC) separations of proteins can easily be tuned by changing the gradient slopes, operating temperature, additives, pH, or organic modifier [1–3]. Optimization of protein separations in RPLC has generally been achieved via the manipulation of the mobile phase with a given column; however, the use of different stationary phases, preferably with complementary selectivity, has also been successful [4]. The best approach to improve selectivity and thus resolution for peptides and proteins is to change the chemical nature or concentration of the organic modifier (e.g., acetonitrile, methanol, or isopropanol) and to select a suitable ion-pairing reagent [5].

The retention of peptides and proteins can be influenced by adding ion-pairing reagents to the mobile phase [6–8]. The ion-pairing reagents interact with the ionized groups of the proteins. Anionic counterions (e.g., hexanesulphonic acid, orthophosphoric acid, and trifluoroacetic acid) interact with the basic residues (i.e., arginine, lysine, and histidine) of a protein and with the protonated N-terminus. Cationic counterions (e.g., triethylammonium and tetrabutylammonium) interact with ionized acidic residues (i.e., glutamic and aspartic or cysteic acid) and ionized free C-terminal carboxylic groups.

The mobile phase temperature plays a key role for improving the peak shapes of proteins. Indeed, an elevated temperature improves the diffusion coefficients and reduces secondary ionic interactions [4].

The major difference in method development for small analytes and large molecules such as proteins is related to the number of interactions (e.g., hydrophobic and ionic) that occur between the mobile and the stationary phase, the latter being strongly influenced by the conformations of proteins [9].

Gradient elution covers a large retention range of peptides and proteins and enables separation of complex samples widely differing in polarities and (or) molecular size, shape and structure, surface charge, or isoelectric point [10]. Theoretical description of the effects of the

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mobile phase on the retention, selectivity, and resolution of sample components enables prediction and optimization of gradient operation conditions and significantly reduces the number of experiments and time necessary for high-performance liquid chromatography (HPLC) separation method development. The theory of gradient elution of small molecules is now well understood and can be used for predicting retention and optimizing gradient separations in various LC separation modes [10].

There are few studies on peptide and protein retention prediction. For predicting peptide and protein retention, we need to explore the behaviour of proteins on different columns with acetonitrile and methanol as organic modifiers on reversed-phase HPLC.

The aim of this work was to explore the behaviour of proteins on different C18 stationary phases with acetonitrile and methanol as organic solvents on reversed-phase HPLC.

EXPERIMENTAL

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). Methanol of HPLC gradient grade, acetonitrile of HPLC gradient grade, and trifluoroacetic acid (TFA) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Insulin (MW ~ 5800, No. 1), aprotinin (MW ~ 6512, No. 2), cytochrome c (MW ~ 12 384, No. 3, ≥95%), ribonuclease (MW ~ 13 700, No. 4), α-lactalbumin (MW ~ 14 175, No. 5, ≥85%), lysozyme (MW ~ 14 388, No. 6, ≥90%), myoglobin (MW ~ 17 670, No. 7, ≥90%), β-lactoglobulin (MW ~ 18 400, No. 8, ≥90%), elastase (MW ~ 25 900, No. 9, ≥98%), carbonic anhydrase (MW ~ 29 114, No. 10, ≥95%), peroxidase (MW ~ 44 000, No. 11), albumin (MW ~ 66 463, No. 12, ≥96%), enolase (MW ~ 93 068, No. 13, ≥50%), and L-glutamic dehydrogenase (MW ~ 290 000, No. 14) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Aniline, phenol, toluene, and benzene were also purchased from Sigma-Aldrich (St. Louis, MO, USA).

The HPLC analyses were performed using Agilent 1290 Infinity Quaternary LC System (Agilent Technologies, Santa Clara, CA, USA). This instrument includes a UV detector, a binary pump, a TCC column oven, and an autosampler. Data acquisition, data handling, and instrument control were performed by ChromSword Auto 4.0 Professional software.

The columns used Zorbax 300SB-C18 (column 1; 50 mm × 2.1 mm ID, 1.8 µm), Zorbax 300Extend-C18 (column 2; 50 mm × 2.1 mm ID, 3.5 µm), Poroshell 300SB-C18 (column 3; 75 mm × 2.1 mm ID, 5 µm), and Poroshell 300Extend-C18 (column 4; 75 mm × 2.1 mm ID, 5 µm).

Table 1. Gradient profile and time in the elution with organic solvents

Organic solvent	Gradient profile, B%*	Gradient time, min
Acetonitrile	15–50	5
	15–50	10
Methanol	35–85	5
	35–85	10

* B% – organic solvent concentration in the mobile phase.

Table 2. Mobile phase flow rates on Zorbax and Poroshell columns

Column	Flow rate, mL/min	Organic solvent
Zorbax 300SB-C18	0.50	Acetonitrile
Zorbax 300SB-C18	0.35	Methanol
Zorbax 300Extend-C18	0.50	Acetonitrile
	0.50	Methanol
Poroshell 300SB-C18	1.0	Acetonitrile
Poroshell 300Extend-C18	1.0	Methanol

Gradient elution was carried out with a mixture of two solvents. Solvent A consisted of 0.1% TFA in water, and solvent B was 0.1% TFA in acetonitrile or methanol (see Table 1). The flow rates were from 0.35 to 1.0 mL/min (Table 2). The stationary phase temperature was kept at 60 °C, and detection was carried out at 210 nm. The injection volume was 5 µL. Proteins were dissolved in water at a concentration of 0.05 mg/mL.

Isocratic elution was carried out following Golushko test conditions [11]. A mixture of two solvents was used. Solvent A was water and solvent B was methanol. The flow rate was 0.2 mL/min for Zorbax 300SB-C18 and Zorbax 300Extend-C18 and 0.5 mL/min for Poroshell 300SB-C18 and Poroshell 300Extend-C18. The stationary phase temperature was kept at 30 °C and the detection was carried out at 210 nm. The injection volume was 5 µL. Aniline and phenol were dissolved in water at a concentration of 0.2 mg/mL and toluene and benzene were dissolved in methanol at a concentration of 0.2 mg/mL.

RESULTS AND DISCUSSION

In most cases, the available technical information is not sufficient to objectively select the most suitable column for a particular separation. Furthermore, since manufacturers use different tests and evaluation parameters for their columns, their product claims were difficult to compare. We used the Golushko test for different C18 stationary phase tests and comparisons.

As the first step, we tested the C18 stationary phase with the Golushko test. The testing protocols were strictly followed. In the Golushko test, hydrophobicity is defined and calculated from the average retention factors (k) of toluene and benzene:

$$\text{Hydrophobicity} = \frac{k_{\text{toluene}} + k_{\text{benzene}}}{2}. \quad (1)$$

The silanol activity was defined and calculated from the average retention factors (k) of aniline and phenol:

$$\text{Silanol activity} = 1 + 3\left(\frac{k_{\text{aniline}}}{k_{\text{phenol}}} - 1\right). \quad (2)$$

Stationary phase Zorbax was more hydrophobic than the Poroshell columns (Fig. 1). The silanol activity for Zorbax and Poroshell sorbents was similar, except for Zorbax 300Extend-C18. Columns with the Poroshell sorbents showed similar chemical properties, whereas the Zorbax sorbents showed minor differences in chemical properties. The Golushko test revealed differences in the chemical properties of the C18 stationary phase, and these test results were used for the stationary phase collation.

As the second step, the reversed-phase retention times of insulin, aprotinin, cytochrome c, ribonuclease, α-lactalbumin, lysozyme, myoglobin, β-lactoglobulin, elastase, carbonic anhydrase, peroxidase, albumin, enolase, and L-glutamic dehydrogenase were determined using the two linear gradients method (with different gradient times). Eluent A was 0.1% aqueous TFA and eluent B was 0.1% TFA in organic solvent (acetonitrile or methanol). Table 3 and Table 4 show the protein retention times with methanol and acetonitrile as organic solvents on Zorbax and Poroshell stationary phases, respectively.

Experimental data were obtained using protein standards. We can determine the protein sorption under the chosen chromatographic conditions with the obtained data. Experimental data will be used for the program to develop methods for the various divisions of protein mixtures. The aim of this part of the study was to obtain experimental data on different C18 columns, and to find the stationary phase and mobile phase effects on protein sorption. From the obtained retention times, we see that the sorption of proteins affected both the organic solvent of the mobile phase and stationary phase.

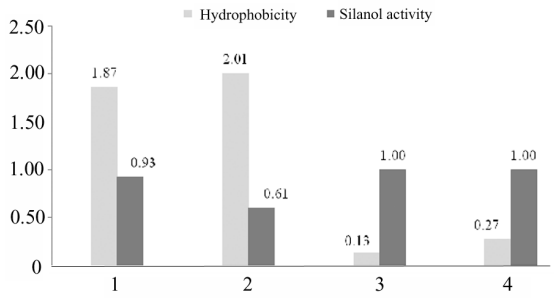

Fig. 1. Hydrophobicity and silanol activity on the Zorbax 300SB-C18 (No. 1), Zorbax 300Extend-C18 (No. 2), Poroshell 300SB-C18 (No. 3), and Poroshell 300Extend-C18 (No. 4) columns.

Table 3. Protein retention times (min) on Zorbax stationary phases at gradient times of 5 or 10 min. Gradient profiles 15–50 B% for acetonitrile and 35–85 B% for methanol

Protein No.*	Zorbax 300SB-C18				Zorbax 300Extend-C18			
	Acetonitrile		Methanol		Acetonitrile		Methanol	
	5 min	10 min	5 min	10 min	5 min	10 min	5 min	10 min
1	3.30	5.73	3.29	5.72	3.11	5.37	2.66	4.70
2	1.66	2.50	1.24	1.60	1.65	2.55	0.85	1.02
3	2.78	4.18	3.08	5.52	2.84	4.95	2.68	4.69
4	2.46	4.25	3.11	5.55	2.26	3.81	2.47	4.18
5	3.65	6.54	3.80	6.37	3.31	5.86	2.98	5.23
6	4.70	8.65	4.66	8.53	4.31	7.93	3.90	7.23
7	4.26	7.76	4.25	7.63	3.93	7.14	3.51	6.35
8	4.77	8.82	4.69	8.60	4.43	8.17	4.00	7.40
9	3.60	5.47	3.21	4.79	3.29	4.87	2.43	3.40
10	4.98	9.41	4.76	8.71	4.74	8.75	4.03	7.44
11	4.18	7.77	4.23	7.76	4.19	7.77	4.02	7.38
12	4.14	7.71	4.25	7.81	3.78	6.97	3.50	6.43
13	5.14	9.48	4.94	9.24	4.68	8.73	4.42	8.26
14	4.79	8.71	4.62	8.62	4.36	8.10	4.09	7.60

* See Experimental for the numbers of proteins.

Table 4. Protein retention times (min) on Poroshell stationary phases at gradient times of 5 or 10 min. Gradient profiles 15–50 B% for acetonitrile and 35–85 B% for methanol

Protein No.*	Poroshell 300SB-C18				Poroshell 300Extend-C18			
	Acetonitrile		Methanol		Acetonitrile		Methanol	
	5 min	10 min	5 min	10 min	5 min	10 min	5 min	10 min
1	2.52	4.48	2.33	4.04	2.40	4.23	1.98	3.32
2	0.93	1.33	1.01	1.19	1.13	1.79	0.75	0.83
3	2.86	5.16	3.62	6.41	2.43	4.34	2.66	4.86
4	2.21	3.83	2.05	3.52	1.86	3.19	1.17	1.86
5	3.09	5.68	2.96	5.42	2.74	4.99	2.31	4.04
6	2.55	4.13	4.06	7.69	3.79	7.11	3.35	6.26
7	4.01	7.54	3.44	6.39	3.39	6.30	3.29	6.15
8	4.30	8.02	4.09	7.74	4.04	7.39	3.62	6.80
9	1.60	2.69	1.00	1.18	1.59	2.12	0.84	0.94
10	4.47	8.39	4.13	7.77	4.22	7.95	3.64	6.82
11	4.08	7.72	4.05	7.65	3.89	7.30	3.15	5.58
12	3.93	7.31	3.73	7.01	3.53	6.46	3.34	6.14
13	4.8	9.11	4.51	8.29	4.41	8.36	3.83	7.22
14	4.65	8.72	4.92	7.90	3.89	7.33	3.78	6.57

* See Experimental for the numbers of proteins.

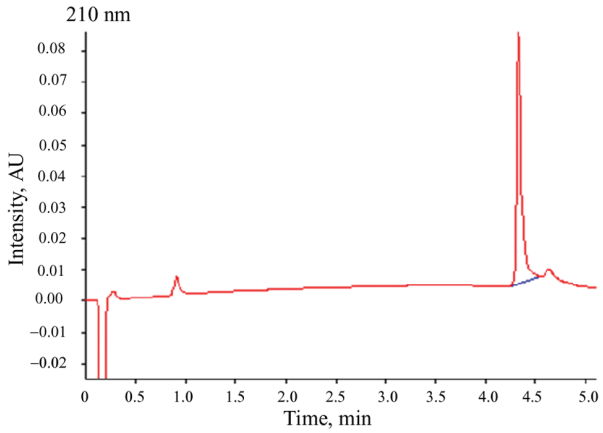

Fig. 2. Enolase retention time on Poroshell 300Extend-C18 with acetonitrile as the organic solvent (gradient profile 15–50 B%, time 5 min).

Figure 2 shows enolase retention time on Poroshell 300Extend-C18 stationary phase with acetonitrile as the organic solvent.

The value of the gradient retention factor (k^*) depends on the solute (its value of *S*-slope in Eq. (4)) and experimental conditions: gradient time t_G, flow rate F, column dimensions, and the gradient range $\Delta\varphi$ [12]:

$$k^* = \frac{0.87 t_G F}{V_m \Delta\varphi S}, \tag{3}$$

where V_m is the column dead volume (mL);

$$S = \frac{b t_G}{t_0 \Delta\varphi}, \tag{4}$$

where b is gradient steepness and t_0 is dead time. Thus, let the gradient times for the two experiments be t_{G1} and t_{G2} ($t_{G1} < t_{G2}$), with a ratio $\beta = t_{G2}/t_{G1}$. Given values of t_R for a given solute in run 1 (t_{R1}) and run 2 (t_{R2}), the value of b can be calculated as:

$$b = \frac{t_0 \log \beta}{t_{R1} - (t_{R2}/\beta) - (t_0 + t_D)(\beta - 1)/\beta}, \tag{5}$$

where t_D is dwell time.

Figures 3 and 4 show the gradient retention factor (k^*) of the 14 proteins studied with acetonitrile and methanol as organic solvents on four C18 stationary phases.

The retention mechanism of proteins is complex. It is a combination of the hydrophobic mechanism (i.e. between the hydrophobic amino acid residues and the bonded C4 or C18 alkyl chains), possible hydrogen bonding, or even ion-exchange mechanisms (between the charged amino acid residues and residual silanols) [13]. Proteins with Zorbax-C18 sorbents will exhibit both hydrophobic and ion-exchange interactions, but the Poroshell-C18 sorbent proteins can only be bound by ion-exchange interactions.

The sorption of proteins such as insulin, aprotinin, α-lactalbumin, ribonuclease, myoglobin, carbonic anhydrase, peroxidase, and enolase was largely unaffected by the sorbent when the mobile phase organic solvent used was acetonitrile. However, the sorption of proteins

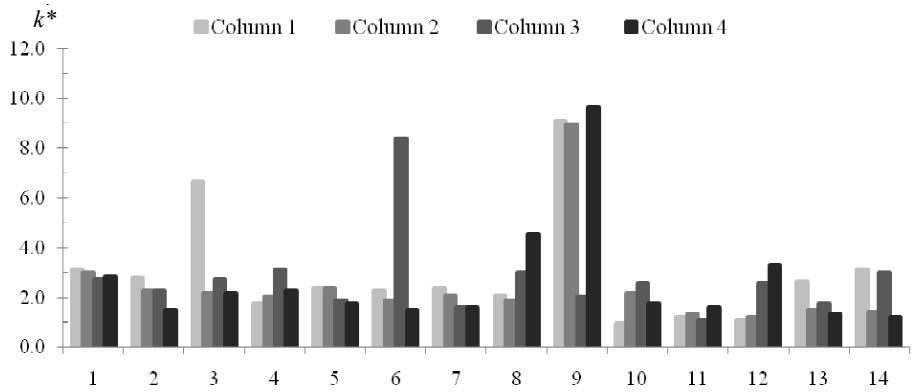

Fig. 3. Gradient retention factor (k^*) with acetonitrile on Zorbax 300SB-C18 (No. 1), Zorbax 300Extend-C18 (No. 2), Poroshell 300SB-C18 (No. 3), and Poroshell 300Extend-C18 (No. 4) columns. Proteins: 1 – insulin, 2 – aprotinin, 3 – cytochrome c, 4 – α-lactalbumin, 5 – ribonuclease, 6 – lysozyme, 7 – myoglobin, 8 – β-lactoglobulin, 9 – elastase, 10 – carbonic anhydrase, 11 – peroxidase, 12 – albumin, 13 – enolase, and 14 – L-glutamic dehydrogenase.

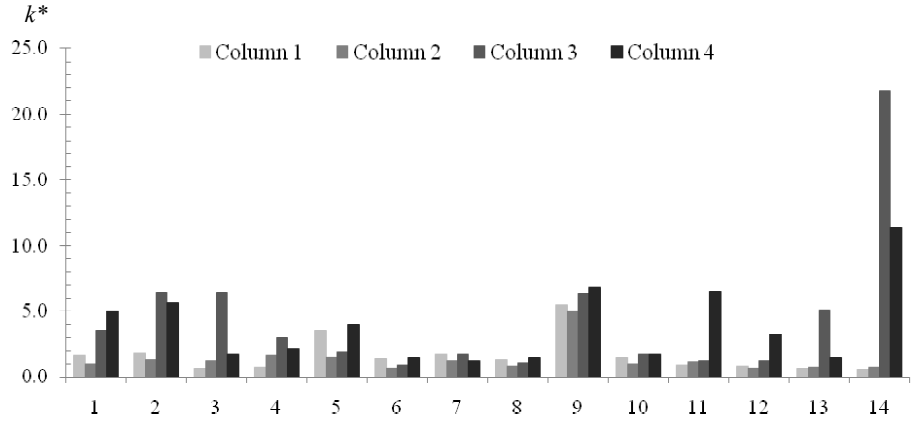

Fig. 4. Gradient retention factor (k^*) with methanol on Zorbax 300SB-C18 (No. 1), Zorbax 300Extend-C18 (No. 2), Poroshell 300SB-C18 (No. 3), and Poroshell 300Extend-C18 (No. 4) columns. Designation of proteins as in Fig. 3.

such as cytochrome c, lysozyme, β-lactoglobulin, elastase, albumin, and L-glutamic dehydrogenase was affected by the sorbent when acetonitrile was used as the mobile phase organic solvent.

When methanol was used in the mobile organic phase, the sorption of proteins such as insulin, aprotinin, cytochrome c, α-lactalbumin, ribonuclease, peroxidase, albumin, enolase, and L-glutamic dehydrogenase was affected by the sorbent, but the sorption for proteins lysozyme, myoglobin, β-lactoglobulin, elastase, and carbonic anhydrase was unaffected. The sorption of myoglobin and carbonic anhydrase was practically unaffected by either the nature of the organic solvent or sorbent ($k^* \sim 2$).

From the experimental data it can be concluded that not for all tested proteins sorption was affected by the organic solvent and sorbent. The protein gradient slope was greater than 10 (Figs 5 and 6).

We can also see that the protein slope was affected by both the organic solvent and sorbent. It is interesting to note that the effect of the sorbent on the protein gradient slope was different for the acetonitrile and methanol cases. From these data we can also conclude that not all proteins were sensitive to small changes in the mobile phase of the organic solvent.

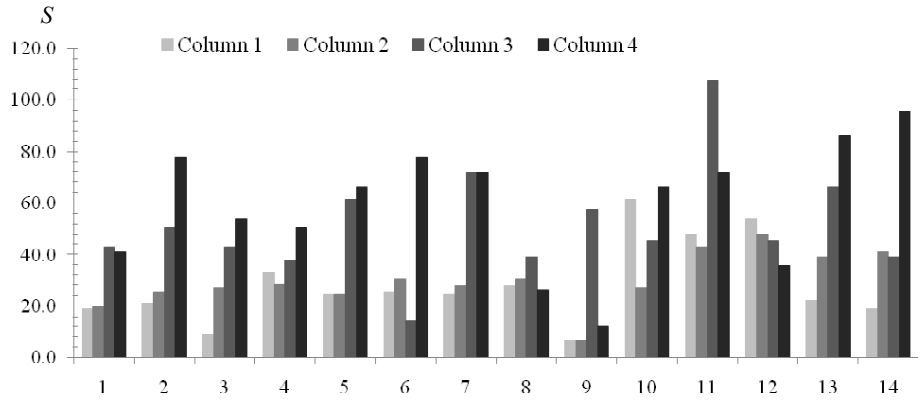

Fig. 5. Gradient slope (*S*) with acetonitrile on Zorbax 300SB-C18 (No. 1), Zorbax 300Extend-C18 (No. 2), Poroshell 300SB-C18 (No. 3), and Poroshell 300Extend-C18 (No. 4) columns. Designation of proteins as in Fig. 3.

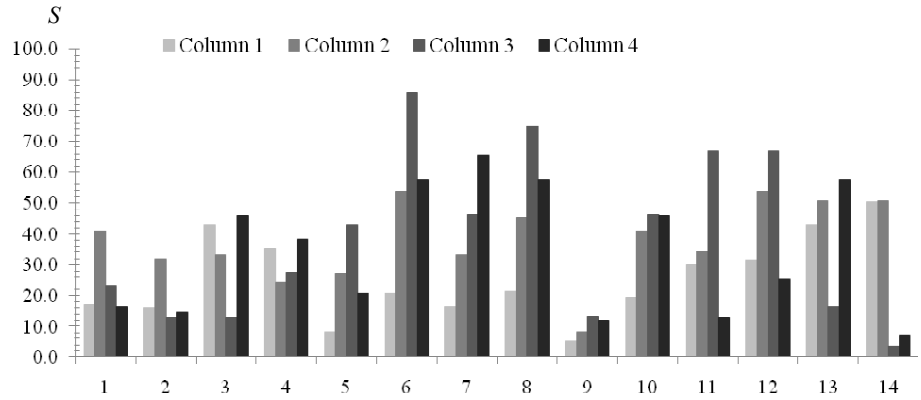

Fig. 6. Gradient slope (*S*) with methanol on Zorbax 300SB-C18 (No. 1), Zorbax 300Extend-C18 (No. 2), Poroshell 300SB-C18 (No. 3), and Poroshell 300Extend-C18 (No. 4) columns. Designation of proteins as in Fig. 3.

CONCLUSIONS

We concluded that the two Poroshell-C18 sorbents had similar chemical properties whereas the Zorbax-C18 stationary phase showed minor differences in chemical properties. Protein sorption was affected by both the nature of the organic solvent and the chemical properties of the sorbent. Increasing protein molecular weight was not observed to increase the retention time of the chromatographic system.

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Proteiinide käitumine pööratud faasi kolonnis kõrgrõhu vedelikkromatograafia ajal C18 statsionaarses faasis

Oksana Rotkaja, Jelena Golushko ja Peteris Mekss

Isokraatilises režiimis on kõrgmolekulaarsete ühendite lahutamine väga keeruline (kui see on üldse võimalik) ja tuleb kasutada gradientelueerimist. Samal ajal kui väikeste molekulide jaoks on gradientelueerimisteooria olemas, ei ole kõrgmolekulaarsete ühendite gradientlahutamisprotsess selge, sest on spetsiifilised probleemid, nagu aeglane molekulide difusioon, kõrgmolekulaarsete ühendite piiratud võime absorbeeruda statsionaarsel faasil või molekulide konformatsiooni võimalik muutus elueerumise jooksul.

Artiklis on esmase sammuna mõõdetud eksperimentaalseid andmeid, mida on kasutatud gradientelueerimise retensiooniaegade ennustamiseks. Kõrgrõhu vedelikkromatograafiat on kasutatud neljateistkümne proteiini lahutamiseks pööratud faasi kolonnis, kasutades vee / orgaanilise lahusti / trifluoratsetaathappe eluenti. On uuritud eksperimentaalsete parameetrite mõju lahutamisele. On leitud, et tulemused, mis on saadud nelja C18 kolonni korral, on võrreldavad.