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Behaviour of proteins on reversed-phase supports during high-performance liquid chromatography and prediction of protein retention times on different Zorbax stationary phases

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Abstract. The separation of high-molecular compounds under isocratic conditions is very difficult, if possible at all, and thus 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.

The first step of our study was the determination of the experimental data, and then these data were used to predict gradient retention times. High performance liquid chromatography was used to investigate the reversed-phase chromatographic behaviour of four proteins. The influence of experimental parameters was examined using a water/organic solvent/trifluoroacetic acid system. Chromatographic results from four Zorbax stationary phases supports were comparable.

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

1. INTRODUCTION

The reversed-phase liquid chromatography (RPLC) separations of proteins can easily be tuned by changing the gradient slopes, operating temperature, additives, pH, or organic modifier [1,2]. The 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 selectivities, has also been successful [3].

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 [4]. Once the initial conditions of the mobile and stationary phases are fixed, further optimization should concentrate on less relevant parameters, such as the mobile phase temperature and gradient profile, that could help improve the resolution [3].

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 [3].

To optimize the gradient profile, the best approach is to choose two linear gradient conditions that differ by a factor of 3 in their gradient run times, all other chromatographic parameters being held unchanged. This helps to evaluate the influence of the gradient run times on the overall resolution, and these two experiments can also be employed to predict the RPLC retention times of each protein as a function of the gradient program using optimization software [5–7].

Therefore, development of methods that utilize automated computer-assisted techniques for predicting the retention properties on the basis of protein structure is not quite accurate. However, computer simulations of

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the retention behaviour of peptides and proteins that are based on experimental chromatographic runs can still be a useful tool.

A few papers can be found in the literature that discuss applications of DryLab software for the computer-assisted method development of RPLC, for example, in cases of dialkylphthalate and nitroalkane separations [8,9].

The work by Gritti and Guiochon [10] marked significant progress in the investigation of protein band broadening by developing a theoretically rigorous model for packed columns. Unfortunately, the model was rather complicated for routine use with numerical calculations.

The aim of this work was to explore the behaviour of proteins on different Zorbax stationary phases with acetonitrile and methanol as organic modifiers during reversed-phase high-performance liquid chromatography. Protein retention time predictions in the gradient elution mode were based on experimental chromatographic runs.

2. 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). Cytohrome c (MW ~ 12 384; ≥95%), enolase (MW ~ 93 068; ≥50%), lactate dehydrogenase (MW ~ 142 000; ~50%), and L-glutamic dehydrogenase (MW ~ 290 000) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

The HPLC analyses were performed using the 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 using ChromSword Auto 4.0 Professional software.

The columns used Zorbax 300SB-C8 (50 mm × 2.1 mm ID, 1.8 µm), Zorbax 300SB-C3 (50 mm × 2.1 mm ID, 1.8 µm), Zorbax 300SSB-Diphenyl (50 mm × 2.1 mm ID, 1.8 µm), and Zorbax 300SB-CN (50 mm × 4.6 mm ID, 3.5 µm).

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. Detection was carried out at 210 nm (acetonitrile UV cut-off ~190 nm; proteins are best absorbed at 210 nm). The injection volume was of 5 µL. Protein was dissolved in water at a concentration of 0.05 mg/mL.

Table 1. Gradient profiles for different organic solvents (time 10 min)

Solvent	Gradient profile, B%*
Acetonitrile	29–50
	30–80
Methanol	50–80
	70–80

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

Table 2. Mobile phase flow rates on different columns

Column	Flow rate, mL/min	Organic solvent
Zorbax 300SB-C8, Zorbax 300SB-C3, Zorbax 300 Diphenyl	0.50	Acetonitrile
Zorbax 300SB-C8, Zorbax 300SB-C3, Zorbax 300SB-Diphenyl	0.35	Methanol
Zorbax 300SB-CN	1.0	Acetonitrile, Methanol

3. RESULTS AND DISCUSSION

The reversed-phase retention times of cytochrome c, enolase, lactate dehydrogenase, and L-glutamic dehydrogenase were determined using linear gradients.

The retention times of the four proteins for each stationary phase of Zorbax are shown in Figs 1–4. The sorption of cytochrome c (Fig. 1) with acetonitrile as the organic solvent on Zorbax 300SB-C3 was similar to that on Zorbax 300SB-CN, and its sorption on Zorbax 300SB-Diphenyl was similar to that on Zorbax 300SB-C8. Furthermore, its sorption on Zorbax 300SB-C3 or

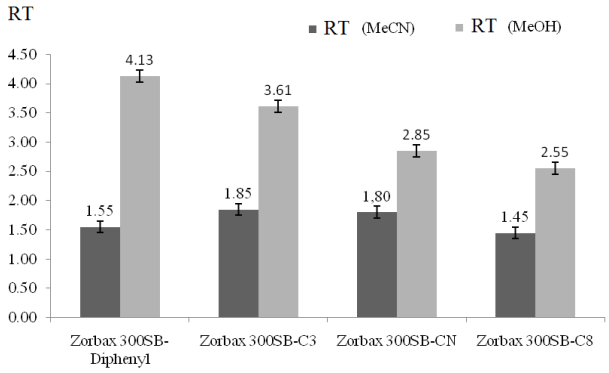

Fig. 1. Cytochrome c retention times in min (gradient profile 29–50% with acetonitrile and 50–80% with methanol) on different stationary phases.

Zorbax 300SB-CN was stronger than on Zorbax 300SB-Diphenyl or Zorbax 300SB-C8. When methanol was the organic solvent, the sorption strength decreased as the stationary phase was changed from diphenyl to C3 to CN to C8.

The sorption of enolase (Fig. 2) with acetonitrile as the organic solvent on Zorbax 300SB-C3 was similar to that on Zorbax 300SB-CN and Zorbax 300SB-C8. On Zorbax 300SB-Diphenyl its sorption was stronger than on any other tested stationary phase. When however methanol was used as the organic solvent, its sorption strength decreased as the stationary phase was changed from diphenyl to CN to C8 to C3.

The sorption of lactate dehydrogenase (Fig. 3) was similar on all used stationary phases when acetonitrile was the organic solvent. However, when methanol was used as the organic solvent, its sorption strength decreased as the stationary phase changed from diphenyl to C3 to CN to C8 and its chromatographic behaviour was similar to that of cytochrome c on all used stationary phases.

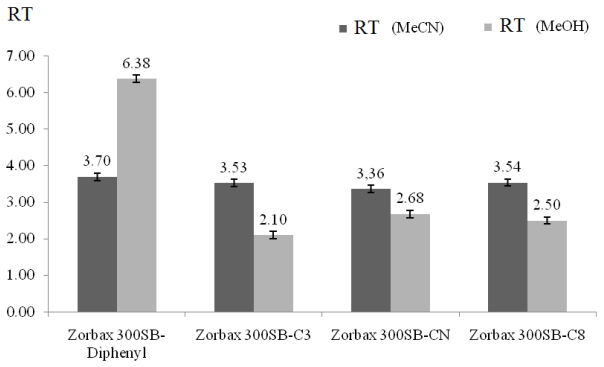

Fig. 2. Enolase retention times in min (gradient profile 30–80% with acetonitrile and 70–80% with metanol) on different stationary phases.

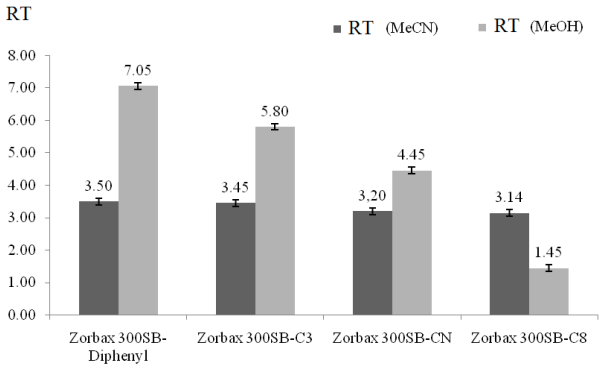

Fig. 3. Lactate dehydrogenase retention times in min (gradient profile 30–80% with acetonitrile and 70–80% with metanol) on different stationary phases.

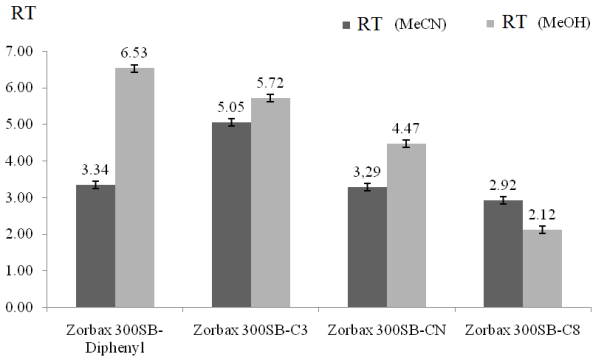

Fig. 4. L-Glutamic dehydrogenase retention times in min (gradient profile 30–80% with acetonitrile and 70–80% with metanol) on different stationary phases.

The sorption of L-glutamic dehydrogenase (Fig. 4) with acetonitrile as the organic solvent on Zorbax 300SB-Diphenyl was similar to that on Zorbax 300SB-CN, and the sorption strength decreased as the stationary phase changed from C3 to diphenyl to CN to C8. When methanol was the organic solvent, its chromatographic behaviour was similar to that of cytochrome c and lactate dehydrogenase on all used stationary phases. If this was the case, sorption (methanol was used as the organic solvent) decreased as the stationary phase was changed from diphenyl to C3 to CN to C8 (Figs 1, 3, and 4).

The peptide or protein interacts with the immobilized hydrophobic ligands through the hydrophobic chromatographic contact region (adsorption) [11]. The most common ligand is *n*-octadecyl (C18), but *n*-butyl (C4) and *n*-octyl (C8) are commonly used for the analysis of more hydrophobic proteins. Additionally, phenyl and cyanopropyl ligands can provide alternative selectivity. The type of *n*-alkyl ligand significantly influences the retention of peptides and proteins and can therefore be used to manipulate the retention, recovery, and, to a lesser extent, selectivity for peptides and proteins [3].

Once the chromatographic behaviours were characterized, we used data from a small number of 'well-chosen' experiments to predict retention in other conditions. The initial data sets were used as inputs to build retention models, and the relationships between retention times and the concentration of organic solvents in the mobile phase were predicted. Linear solvent strength theory requires data from at least two runs to predict retention. For retention time prediction the LC simulator ChromSword was used. Experimental data were obtained using protein standards. We were able to determine the protein sorption of the chosen chromatographic conditions with the data obtained. Experimental data will be used for the LC simulator ChromSword to

develop methods for various divisions of protein mixtures.

In reversed-phase liquid chromatography, retention behaviour can normally be described by the linear retention model:

$$\ln k' = \ln k'_0 + a \cdot C,$$

where k' is the retention factor and C is the concentration of organic solvent in the mobile phase [9].

The predicted retention times of the studied compounds were nearly identical to those found experimentally, yielding a correlation coefficient of 0.9990 (Fig. 5 and Table 3).

The linear retention model provided a good correlation between the experimental and predicted retention times of these proteins when acetonitrile or methanol was used in the mobile phase and the stationary phases were Zorbax 300SB-Diphenyl, Zorbax 300SB-C3, Zorbax 300SB-CN, or Zorbax 300SB-C8. The correlation coefficients are shown in Table 3. The organic solvents used here (acetonitrile and methanol) are suitable for such protein retention time predictions.

Figure 6 shows the retention time of enolase on the Zorbax 300SB-Diphenyl stationary phase with acetonitrile as the organic solvent. Figure 7 shows the retention time of lactate dehydrogenase on the Zorbax 300SB-C8 stationary phase with acetonitrile as the organic solvent.

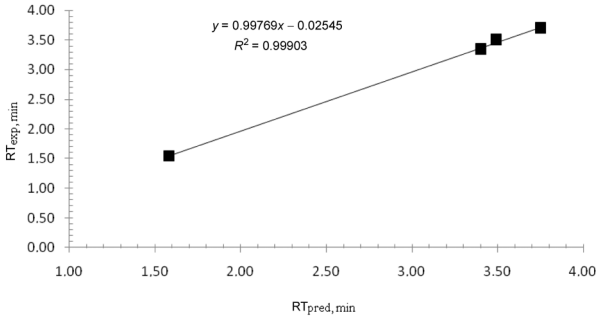

Fig. 5. Correlation of predicted and experimental retention times of the used proteins from the data of two experiments on the stationary phase Zorbax 300SB-Diphenyl with acetonitrile as the organic solvent.

Table 3. Correlation of predicted and experimental retention times of proteins from the data of two experiments

Organic solvent	Correlation, R^2			
	Zorbax 300SB-Diphenyl	Zorbax 300SB-C3	Zorbax 300SB-CN	Zorbax 300SB-C8
Acetonitrile	See Fig. 1	0.9998	0.9993	0.9995
Methanol	0.9991	0.9995	0.9996	0.9992

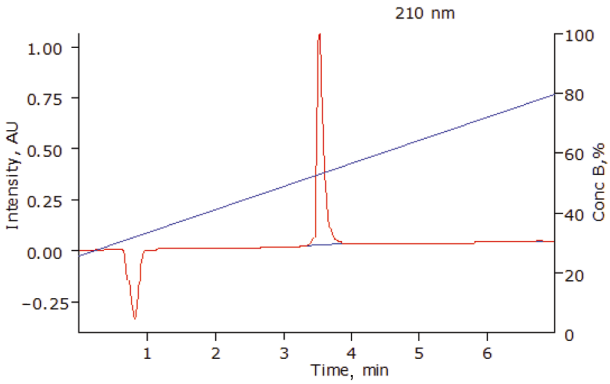

Fig. 6. Enolase chromatogram on Zorbax 300SB-Diphenyl with acetonitrile as the organic solvent (Grad 30–80, 10 min).

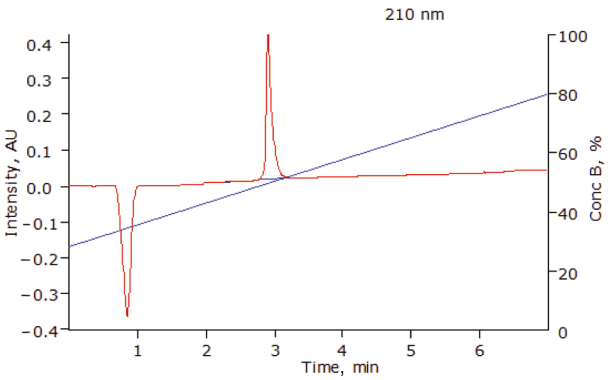

Fig. 7. Lactate dehydrogenase chromatogram on Zorbax 300SB-C8 with acetonitrile as the organic solvent (Grad 30–80, 10 min).

4. CONCLUSIONS

The protein sorption used is dependent both on the organic solvent of the mobile phase (acetonitrile and methanol) and on the stationary phase.

A simulated run can be carried out in less than a minute, saving more than 90% of the time required for an actual gradient elution separation. At the same time, the computer can present results of a wider range of possible experiments.

A linear retention model was applied to the prediction of protein retention in gradient reversed-phase high-performance liquid chromatography. This model enabled prediction of initial conditions from two experimental data points for different types of reversed-phase stationary phases with water–acetonitrile–TFA and water–methanol–TFA mobile phases. The described method for the prediction of retention can substantially reduce the time needed to find optimal conditions in gradient elution chromatography.

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Valkude käitumine kõrgefektiivses vedelikkromatograafias pöördfaaskandjatel ja retentsiooniaegade ennustamine erinevate Zorbaxi tüüpi statsionaarsete faaside korral

Oksana Rotkaja, Jelena Golushko ja Peteris Mekss

Kõrgmolekulaarsete ainete vedelikkromatograafiline eraldamine isokraatilistes tingimustes on väga keeruline, kui üldse võimalik, mistõttu vajatakse gradientelueerimist. Väikeste molekulide gradientelueerimise teooria on hästi välja töötatud; selle rakendamine biopolümeeride eraldamisel pöördfaasgradientmeetodil ei ole aga spetsiifiliste probleemide, nagu aeglane difusioon, statsionaarse faasi piiratud kättesaadavus suurtele molekulidele või eraldatavate molekulide konformatsioonides elueerimise ajal toimuvad muutused, tõttu niisama lihtne.

Eksperimendi esimeses etapis saadi tulemused, mida seejärel kasutati retentsiooniaegade ennustamiseks gradientsüsteemis. Kõrgefektiivset vedelikkromatograafiat kasutati nelja erineva valgu käitumise uurimiseks pöördfaaskolonnil. Erinevate parameetrite mõju uurimiseks süsteemis vesi – orgaaniline solvent – trifluoräädikhape neljal erineval Zorbaxi tüüpi statsionaarsel faasil läbiviidud kromatografeerimiste tulemused olid omavahel võrreldavad.