Influence of pressure and temperature on molar volume and retention properties of peptides in ultra-high pressure liquid chromatography

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ABSTRACT

In this study, pressure induced changes in retention were measured for model peptides possessing molecular weights between ∼1 and ∼4 kDa. The goal of the present work was to evaluate if such changes were only attributed to the variation of molar volume and if they could be estimated prior to the experiments, using theoretical models. Restrictor tubing was employed to generate pressures up to 1000 bar and experiments were conducted for mobile phase temperatures comprised between 30 and 80 °C. As expected, the retention increases significantly with pressure, up to 200% for glucagon at around 1000 bar compared to ∼100 bar. The obtained data were fitted with a theoretical model and the determination coefficients were excellent ($r^2 > 0.9992$) for the peptides at various temperatures. On the other hand, the pressure induced change in retention was found to be temperature dependent and was more pronounced at 30 °C vs. 60 or 80 °C. Finally, using the proposed model, it was possible to easily estimate the pressure induced increase in retention for any peptide and mobile phase temperature. This allows to easily estimating the expected change in retention, when increasing the column length under UHPLC conditions.

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1. Introduction

Liquid solutions are normally considered to be incompressible, however several studies showed that in conventional HPLC pressure ranges (<400 bar), solute retention can be significantly altered [1–6]. Obviously, the pressure effects are much more important in ultra-high pressure LC (UHPLC) conditions [7–11]. In order to study the pure effect of pressure, data are generally gathered using a restriction capillary at the column outlet to increase pressure, while avoiding frictional heating effects related to high mobile phase flow rates. Generally, pressure influences the mobile phase density, viscosity and temperature, the analytes' diffusion rate and the strength of interactions between solute, stationary phase and mobile phase [12]. Therefore, the pressure in LC can significantly influence the retention (k) of any type of analytes.

First, Giddings showed that pressure could induce important changes in molecular volume and also alter the ability of molecules to crowd together, to reduce molecular volume [13,14]. Numerous theoretical aspects of the influence of pressure on the chromatographic process have been explained and described by Martin and Guiochon [15]. Several publications have shown that k increases with a pressure enhancement [16,17]. While the increase for low-molecular-weight analytes is relatively modest, the increase for large analytes (e.g. peptides, proteins) has been found to be much larger [18–20]. This effect is primarily related to the changes in molecular molar volume (V_m). However, pressure also has a strong influence on the solvation layer of the alkyl-bonded phase; on the solvation shell of the hydrophobic regions of the protein; and on the hydration shell of the hydrophilic parts [18]. A reduced solvation layer increases molecule hydrophobicity, and therefore increases k under reversed-phase conditions. Pressure can also affect other intrinsic parameters of chromatographic separation, such as column void volume and intrinsic column porosity [15,21].

The effects of pressure on thermodynamic equilibrium were studied and it was reported that changes in column pressure could produce equilibrium changes in the distribution of the analyte between stationary and mobile phases. The magnitude of these changes depends on the analyte–solvent interconnection and on the changes in solvent structure within the mobile and stationary phase [7,22,23,13]. It was also demonstrated that analyte molecular volume changes under elevated pressure were among the major variables affecting analyte retention [13]. Other studies have shown that the variation of retention with pressure can be related to changes in mobile phase pH and to the extent of analyte ion-dissociation [24].

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Numerous works have demonstrated the importance of pressure on the analyte retention for small molecular weight compounds. Under conventional HPLC conditions, an increase in retention factors between 9 and 24% was observed for fatty acids when increasing the pressure from 100 to 350 bar [5,6]. An important change in retention and selectivity of weak acids and bases, and even a change in elution order were also demonstrated [24]. Another study showed retention changes between −7 and +12% under conventional HPLC conditions as well [3]. With short, narrow bore columns packed with conventional 5 μm particles, 2 to 12% increase in retention was reported for neutral solutes, while the most important changes (35–50%) were observed for acidic and basic analytes [7,8]. The changes in retention were significantly larger (by a factor of 3) when ion-pairing reagent was added to the mobile phase in RPLC mode [25]. Finally, the effect of pressure was also demonstrated in normal phase mode and chiral separations [2,26].

For large molecules such as peptides and proteins, it has been observed that pressure can have a rather strong influence on retention [18–20]. Even in gradient elution mode, important changes in retention and slight changes in selectivity and resolution were reported [20]. A recent study demonstrated that pressure induced conformational changes of proteins are highly probable under RP conditions [20]. As mentioned, the change in V_m caused by the pressure is a suitable parameter to model the effect of pressure on solute retention. However, ΔV_m is complex as it can be correlated with several associated phenomena taking place during the adsorption process [18]. It should be expected, especially in the case of macromolecules that changes in V_m may originate from different sources, such as the variations in the energy of molecular interactions, solvation, aggregation or changes in the energy density of these interactions [27]. These effects probably play a key role in the retention of peptides or proteins. Conformational changes induced by pressure, besides affecting V_m directly, can also modify the surface hydrophobicity of the molecule [18]. The conformational change (folding or unfolding) of a protein molecule upon adsorption is a well-known phenomenon that leads to the exposure of its hydrophobic core. At higher pressures, the adsorption of proteins onto the stationary phase could therefore be more pronounced. Therefore, modelling the pressure effects on retention could become very complex for proteins.

The goal of this study was to experimentally measure the pressure induced changes in retention for model peptides possessing molecular weights between ∼1 and ∼4 kDa. These experiments were performed using restrictor tubing, to evaluate the pure effect of pressure, while neglecting as much as possible the influence of frictional heating. Finally, the experimental work was conducted for pressures up to ∼1000 bar and for various mobile phase temperatures comprised between 30 and 80 °C. The experimental data were then fitted with theoretical models, to evaluate if theory was able to predict pressure induced changes in retention, only taking into account the variation of molar volume.

2. Theory

The dependence of the retention factor, k, of a compound on the pressure, p, can be derived from Gibbs free energy as follows:

$$\ln k = -\frac{\Delta G}{RT} + \ln \phi = -\frac{\Delta E}{RT} - p\frac{\Delta V_m}{RT} + \frac{\Delta S}{R} + \ln \phi \quad (1)$$

where ΔG is the change of Gibbs free energy, ΔE the change of internal energy of the system, ΔV_m the change of molar volume of solute during adsorption, ΔS the change of system entropy, ϕ the phase ratio, R the universal gas constant, and T the absolute temperature.

By rearranging Eq. (1), k can be calculated at any pressure as:

$$k = k_0 \exp \left(-\frac{\Delta V_m}{RT} p \right) \quad (2)$$

where k_0 is the limiting value of the retention factor at zero pressure:

$$k_0 = \phi \exp \left(-\frac{\Delta E}{RT} + \frac{\Delta S}{R} \right) \quad (3)$$

Eqs. (2) and (3) cannot be used directly for the determination of the compound retention time since the retention factor changes gradually during the compound migration, due to the pressure gradient inside the column. In the case of isocratic elution, a linear pressure gradient can be assumed. In this case, the local pressure at any position, z, in the column is given as:

$$p[z] = p_{in} - \frac{\Delta p}{L} z \quad (4)$$

where p_{in} is the inlet pressure (pressure at the head of column), and Δp the pressure drop across the column. Note, that Eq. (4) is valid only in case of isocratic elution assuming that the phase ratio of the column is constant throughout the column. In our case, the change of phase ratio was negligible (see Section 3 for more details).

By combining Eqs. (2)–(4), the local value of the compound retention factor can be written as:

$$k[z] = k_0 \exp \left[-\frac{\Delta V_m}{RT} \left(p_{in} - \frac{\Delta p}{L} z \right) \right] = k_{in} \exp \left(\frac{\Delta V_m}{RT} \frac{\Delta p}{L} z \right) \quad (5)$$

where k_{in} is the retention factor of solute at the head of column (at the inlet pressure, p_{in}).

The local migration velocity of the zone of a compound at any position, z, in the column can be calculated with the knowledge of $k[z]$.

$$\frac{dz}{dt} = \frac{u_0}{(1 + k[z])} \quad (6)$$

where u_0 is the linear velocity of the eluent ($u_0 = L/t_0$, where t_0 is the hold-up time of the column). Accordingly, the retention time, t_R, can be calculated by integrating Eq. (8).

$$t_R = \int_0^{t_R} dt = \frac{1}{u_0} \int_0^L (1 + k[z]) dz \quad (7)$$

The integration can be accomplished by substituting Eq. (5) in Eq. (7). Accordingly,

$$t_R = \frac{1}{u_0} \int_0^L \left(1 + k_0 \exp \left[-\frac{\Delta V_m}{RT} \left(p_{in} - \frac{\Delta p}{L} z \right) \right] \right) dz \quad (8)$$

and

$$t_R = t_0 \left[1 + \frac{k_0 RT}{\Delta p \Delta V_m} \exp \left[-\frac{p_{in} \Delta V_m}{RT} \right] \left(\exp \left[\frac{\Delta p \Delta V_m}{RT} \right] - 1 \right) \right] \quad (9)$$

Eq. (9) can be used for the migration time calculation of any compounds through the separator column with the knowledge of operating parameters of the chromatographic system and fundamental molecular and thermodynamic properties.

3. Experimental

3.1. Chemicals, columns

Water was obtained from a Milli-Q Purification System from Millipore (Bedford, MA, USA). Acetonitrile and Methanol (gradient

grade) were purchased from Sigma–Aldrich (Buchs, Switzerland). Model peptides such as CH-866 (MW = 1311.5 g/mol), CH-868 (MW = 1311.5 g/mol), CH-869 (MW = 1277.5 g/mol) and CH-870 (1295.5 g/mol) decapeptides were purchased from ChinaPeptides Co., Ltd. (Shanghai, China). These model peptides are analogues of the commercial therapeutic peptide, triptorelin in which only one amino acid was altered. Glucagon as the largest test peptide (MW = 3485 g/mol) was purchased from Sigma–Aldrich. Trifluoroacetic acid (TFA) and uracil were also purchased from Sigma–Aldrich.

Waters Acquity BEH C4 columns packed with 1.7 μm particles (50 mm × 2.1 mm and 150 mm × 2.1 mm) were purchased from Waters (Milford, MA, USA).

3.2. Equipment, software

The measurements were performed using a Waters Acquity UPLC^{TM} I-Class system equipped with a binary solvent delivery pump, an autosampler and UV detector and/or fluorescence detector (FL). The system includes a flow through needle (FTN) injection system with 15 μL needle, a 0.5 μL UV flow-cell and a 2 μL FL flow-cell. The connection tube between the injector and column inlet was 0.003″ I.D. and 200 mm long (active preheating included), and the capillary located between the column and detector was 0.004″ I.D. and 200 mm long. The overall extra-column volume (V_{ext}) was about 8.5 μL and 11 μL as measured from the injection seat of the auto-sampler to the detector cell (UV and FL, respectively). The average extra-column peak variance of our system was found to be around $\sigma_{ec}^2 \sim 0.5 - 3 \, \mu L^2$ (depending on the flow rate, injected volume, mobile phase composition and solute). Data acquisition and instrument control was performed by Empower Pro 2 Software (Waters).

Column backpressure was increased by connecting capillary tubes of 25 μm I.D. and lengths of 5, 15, and 20 cm (it was possible to couple up to 45–50 cm tubing length to generate the required pressure). The capillary tubes were purchased from SGE Analytical Science (Kiln Farm Milton Keynes, UK). The tubes were connected between the end of the columns and the detector cell using zero dead volume connectors. The volume of the longest tube was 0.24 μL which is negligible compared to the total extra-column volume of the instrument.

Retention data were corrected for system transit time (the time that solutes spend in the extra-column volume) and offset time (the difference between the moment when the zero time is recorded and the moment when the sample leaves the injection needle). Pressure data were also corrected for system pressure. The corrected hold-up time of the column differed by 0.02 min between the extremes of head pressures indicating that the change of the phase ratio was negligible in this experiment.

Calculations were achieved by using Mathematica 8.0 (Wolfram Research) ran under Debian GNU Linux operating system (v. 6.0.6).

3.3. Apparatus and methodology

In this study the effect of very high pressure was dissociated from frictional heating effects. Indeed, all the measurements were performed at constant flow rate and the column head pressure was varied by adding restrictors to the column outlet. This experimental setup allowed studying the effect of pressure only, independently from frictional heating. Moreover, narrow-bore columns (2.1 mm I.D.) were used to minimize as much as possible the possible frictional heating effects.

3.3.1. Effect of pressure on decapeptides

Decapeptides (MW = 1277.5–1311.5 g/mol) were eluted isocratically with 25:75 (v/v) ACN: H$_2$O (containing 0.1% TFA). 2 μL of test solution was injected on the Acquity BEH300 wide pore C4 (5 cm × 2.1 mm, 1.7 μm) column and the chromatograms were acquired in both UV (λ = 210 nm, 40 Hz) and fluorescence emission mode (excitation at 280 nm, emission at 360 nm, 20 Hz). The flow rate was set to 100 μL/min. This flow rate generated relatively small pressure and provided reasonable retention times. The effect of pressure on retention properties and molar volume was studied at different temperatures, namely 30, 60 and 80 °C. The very high pressure was generated by adding restrictor capillaries to the column outlet. At 30 °C, around 193, 295, 590, 750, 870 and 1030 bar column head-pressure were generated, while at 60 and 80 °C the head-pressure was set at about 115, 154, 234, 310, 387, 474, 592, 723, 821, 915 bar and 163, 194, 238, 401, 597, 710, 861, 976 bar, respectively. The stock solution of peptides was prepared in the mobile phase (∼1 mg/ml) and uracil (1 mg/ml) was also added as column dead time marker. The solutions for the chromatographic runs were diluted from the stock solutions with mobile phase. The final concentration of the test solution was 100 μg/ml.

3.3.2. Effect of pressure on glucagon

In this current study, glucagon was selected as the largest model peptide (MW = 3485 g/mol). It was eluted with 32:68 (v/v) ACN: H$_2$O (containing 0.1% TFA). 3 μL of test solution was injected. The chromatograms were recorded in both UV (λ = 210 nm, 40 Hz) and fluorescence emission mode (excitation at 280 nm, emission at 360 nm, 20 Hz). The flow rate was set to 100 μL/min. The mobile phase temperature was set to 30, 60 and 80 °C. At 30 °C, around 220, 300, 359, 508, 576, 657, 910 and 993 bar column head-pressure were generated. At 60 and 80 °C, the head-pressure was increased to about 183, 360, 485, 574, 687, 855, 1040 bar and 134, 400, 440, 573, 733, 761, 960 bar, respectively. The stock solution of glucagon and uracil was prepared in water (∼1 mg/ml). The solutions for the chromatographic runs were diluted from the stock solutions with mobile phase. The final concentration of the test solution was approximately 100 μg/ml.

3.3.3. Validity of the model, coupling columns in series

To verify our theoretical model, the retention times estimated for different column head pressures were experimentally verified. For this purpose, columns were coupled in series and operated at the same flow rate that was applied in the previous measurements when creating the model (100 μL/min). In this study, 50, 150, 200 and 300 mm column lengths were employed to generate different head pressures. The observed retention factors were compared to the predicted values. These experiments were achieved with three different decapeptides at 30 °C.

4. Results and discussion

4.1. The effect of pressure and temperature on the retention of peptides (1.2–1.3 kDa)

A mixture of four decapeptides was analyzed to evaluate the impact of pressure on solute retention. Fig. 1 shows chromatograms recorded at 190, 540 and 1010 bar, while Fig. 2 illustrates the changes in retention afforded by using various narrow restrictor capillaries generating a variety of pressure at three different temperatures. Since all peptides showed similar behaviour, only one representative (CH-870) peptide is discussed in Fig. 2. A significant increase in retention was observed for all peptides between a pressure drop of 100 up to 1100 bar. In this range, ∼80% relative increase in retention was observed at a temperature of 30 °C. Surprisingly at higher temperature, the change in retention was somewhat lower. At 60 °C only 55% relative increase was noticed, while at 80 °C around 35% increase in retention was observed. Because the change in retention was significantly higher
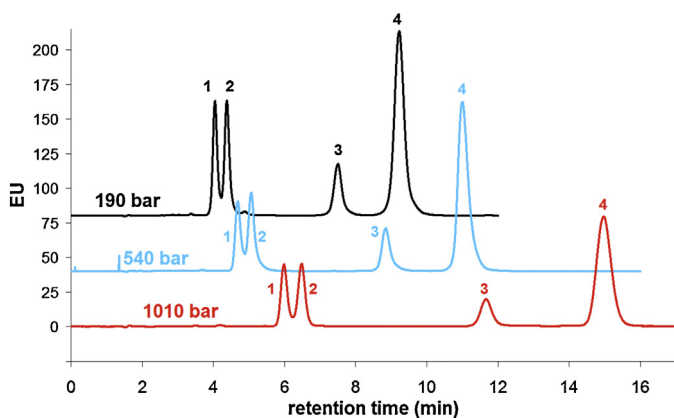
Fig. 1. Effect of pressure on the retention of related peptides (1.1–1.3 kDa). Column: Acquity BEH300 C4 (50 mm × 2.1 mm, 1.7 µm), mobile phase: water (0.1% TFA)/acetonitrile (0.1% TFA): 75/25 (v/v), flow-rate: 100 µL/min, temperature: 30 °C, injected volume: 2 µL, detection: fluorescence ex.: 280 nm, em.: 360 nm. Peaks: P-868 (1), P-866 (2), P-870 (3), P-869 (4).

at 30 °C vs. 60 or 80 °C, it appears that the pressure induced increase in retention is temperature dependent. This observation suggests that temperature has an influence on the mechanism of the pressure induced perturbations in the molecular structure of these decapeptides. In contrast, Szabelski et al. observed that pressure and temperature affect the retention behaviour of insulin (5.8 kDa) in a separate and different way [28]. They concluded that temperature has no (or very little) influence on the pressure induced retention change. Probably, insulin shows different behaviour due to a possible conformational change at elevated pressure that cannot be observed with the investigated decapeptides (no
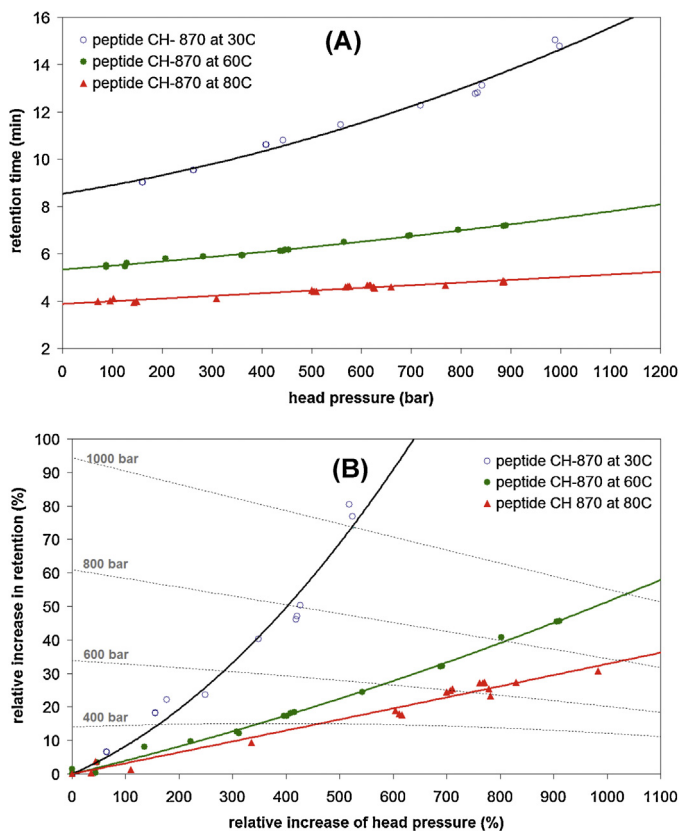
secondary or tertiary structures for such small peptides). Indeed, the temperature induced conformational changes of insulin is well known but it remains hard to believe that different conformations (molar volumes) are equally sensitive to the pressure induced changes. Moreover, in that study, the authors investigated only a narrow pressure range, between 47 and 147 bar and narrow temperature range (25–50 °C) [28]. On Fig. 2A, the slopes of the fitted curves are indeed very similar in the range of 50–150 bar, suggesting that for relatively low pressures (e.g.<200 bar), temperature does not affect significantly the retention changes attributed to pressure alteration. However, in the ultra-high pressure range (>400 bar), temperature has a clear impact on the mechanism of the pressure induced perturbations in molecular structure.

At different temperatures, the retention observed without restrictors at the same flow rate are obviously different, because of the mobile phase polarity reduction with temperature. For correctness–and for better visualizing the influence of temperature on pressure induced changes–the relative increase in retention was plotted against the relative increase in pressure, on Fig. 2B. The reference values of retention and pressure were selected as the lowest observed values (without restrictor). Since the same relative pressure change corresponds to different absolute pressures at different temperatures, the absolute pressure was also indicated on Fig. 2B. This plot clearly shows that the pressure induced retention increase is more important at lower than at higher temperature. As example, increasing the pressure by 300% at 30 °C manifests in 35% retention increase, while at 60 and 80 °C, it corresponds to 13 and 10%, respectively. When increasing the pressure by 600%, the retention increases by 90% at 30 °C, and only 27 and 20% at 60 and 80 °C, respectively.

The same conclusion can be drawn by calculating the derivative of k (Eq. (2)), with respect to pressure and temperature.

$$\frac{\partial^2 k}{\partial p \, \partial T} = \left(\Delta V_m - T \frac{\partial \Delta V_m}{\partial T} \right) \frac{(RT - p\Delta V_m)}{R^2 T^3} k \tag{10}$$

Since k is positive, and ΔV_m is negative, the last two terms of the above expression are positive and the sign of Eq. (10) depends on the sign of the first term. It will be shown later on (see Section 4.3) that ΔV_m increases as the temperature increases ($\partial \Delta V_m / \partial T > 0$). Accordingly, the first term of Eq. (10) is negative, as well as the whole expression. It means that the change in retention as a function of pressure decreases at higher temperature, just as it was shown above for the four model decapeptides.

4.2. The effect of pressure and temperature on the retention of glucagon (3.5 kDa)

Glucagon was injected to evaluate the impact of pressure and temperature on solute retention. This molecule is a 29 amino acids polypeptide that can form slightly different conformational states depending on the conditions. This is the reason why it was interesting to study its behaviour under different pressures and temperatures conditions. Fig. 3 represents the changes in retention caused by the pressure at different temperatures. A more significant retention increase was observed at elevated pressure compared to the smaller decapeptides. The relative retention increase reached ~200% at around 1000 bar compared to ~100 bar at 30 °C. At higher temperature, the change in retention was again lower, similarly to what was observed with decapeptides. At 60 and 80 °C, about 130 and 60% relative increase in retention were measured. In agreement with our previous findings, the pressure induced retention shift depends on the temperature. Probably, this observation can be attributed to a variation of the glucagon conformation (molar volume) with temperature. Fig. 3A also suggests this assumption since the absolute retention at 60 and 80 °C, in the conventional
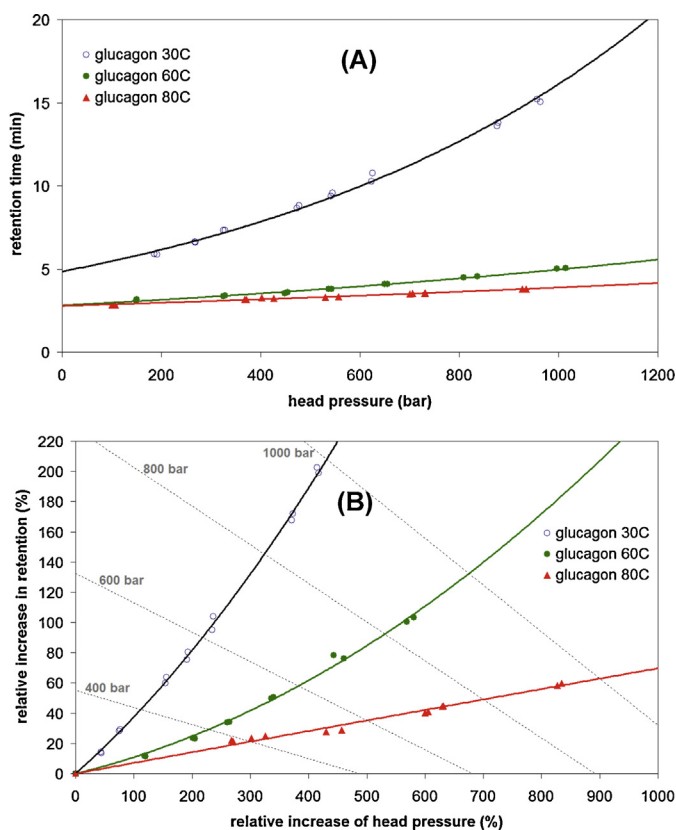

Fig. 2. Pressure induced absolute (A) and relative (B) change in retention of 1.3 kDa peptide (CH-870) at 30, 60 and 80 °C.

Fig. 3. Pressure induced absolute (A) and relative (B) change in retention of 3.5 kDa glucagon at 30, 60 and 80 °C.

Table 1
k_0 and ΔV_m (cm^3/mol) values of peptides determined by fitting Eq. (9) to the retention database.

Compound	T (K)	k_0	ΔV_m
CH-868	303	1.77	−16.15
	333	1.06	−13.71
	353	0.75	−11.81
CH-866	303	2.00	−15.60
	333	1.18	−12.74
	353	0.81	−11.79
CH-869	303	4.17	−14.97
	333	2.51	−11.87
	353	1.74	−11.57
CH-870	303	5.33	−15.80
	333	3.06	−11.86
	353	2.04	−10.26
Glucagon	303	3.06	−33.15
	333	1.16	−23.95
	353	1.13	−15.91

the pressure induced retention increase is strongly temperature dependent. When injecting this 3.5 kDa polypeptide, increasing the pressure by 300% at 30 °C resulted in 130% increase while at 60 and 80 °C, it caused only 45 and 20%, respectively.

4.3. Determination of molar volume change and limiting retention factors

Eq. (9) allows the determination of molar volume change values, ΔV_m, and limiting retention factors, k_0, by least squares fitting. Figs. 2A and 3A show the fitted curves (based on our model) applied to our experimental dataset of retention times versus column head pressure for a decapeptide (870) and glucagon, respectively. The model fits well with the experimental data as the corresponding determination coefficients, r^2, were higher than 0.9992 in each case.

The calculated values of molar volume change and limiting retention factors for the four decapeptides and glucagon at three temperatures have been reported in Table 1. The typical values for the molar volume change of decapeptides ranged between −10 and −16 cm^3/mol, while it was clearly larger (between −16 and −33 cm^3/mol) for glucagon. This is in agreement with our expectations as typical molar volume changes around 5–10 cm^3/mol were reported in the literature for small molecular weight test analytes and around 100 cm^3/mol for insulin [12,28]. Based on our experimental results, the molar volume change seems to be also temperature dependent. Compared to 30 °C (303 K), the absolute value of molar volume change for decapeptides was 15–25% lower at 60 °C and 20–35% lower at 80 °C. For the 3.5 kDa polypeptide, the differences between molar volume changes at different temperatures seem to be much more important. At 60 and 80 °C, 28 and 52% lower change of molar volume were calculated. These observations can probably be explained by the possible temperature dependent changes of molecular conformation. In addition, Fig. 4 demonstrates clearly, that there is a linear relationship between the change of molar volume and temperature. The derivative of ΔV_m with respect to temperature is positive that means a decrease in the molar volume change.

If the ΔV_m and k_0 values are known, (1) the retention time of a compound, (2) the local retention factor at any position on the column, and (3) the position of the band at any time, can be predicted for any inlet pressures and pressure drops. In the case of isocratic elution, a linear pressure gradient occurs along the column, assuming that the phase ratio is constant throughout the column. Therefore, in this case, the pressure for any column length
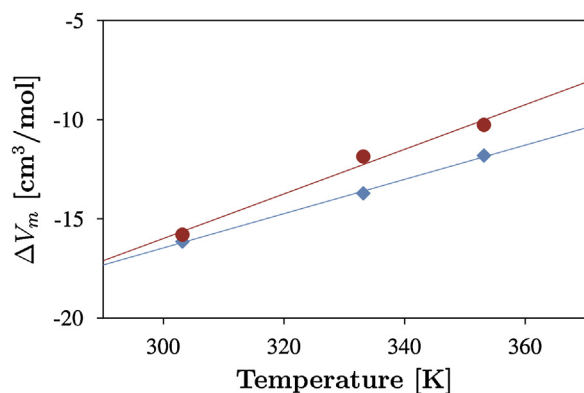
pressure range (100–400 bar) is practically the same (unchanged). However, if there is no change in molecular structure, generally a decrease in retention is expected when increasing the temperature (due to the reduction of mobile phase polarity and strength of interactions). In the case of large biomolecules, the effect of temperature on retention is not as simple, and it was previously observed that little or even no change in retention occurred with some therapeutic peptides and the 5.7 kDa insulin [29]. In some cases, an increase or no change in retention as a function of temperature can be observed, depending on the investigated compounds and the temperature range. In the case of peptides or proteins, which contain many charged amino acids, it is much more difficult to predict the evolution of retention with temperature. On the other hand PK_a values can also be modified with temperature and can explain thiy type of behaviour. Depending on the stability of the secondary structure, the molecules unfold to various extents at different temperatures and hence interact with the stationary phase with various strength [30]. Due to the different conformation-dependent responses of peptides and proteins at elevated temperatures, the change in retention can be unexpected [31,32]. Another study demonstrated the thermally induced interconversion of insulin by temperature-dependent changes in the retention parameters [33]. The authors reported irreversible conformational changes for insulin at temperatures between 65 and 85 °C.

This example with glucagon also suggests that temperature has an impact on the mechanism of the pressure induced changes in the molecular structure of peptides. On Fig. 3A, the slopes of the fitted curves are again quite similar in the range of 100–300 bar, but becomes significantly different in the ultra-high pressure range.

Fig. 3B shows the relative change in retention vs. relative head pressure increase. This type of representation highlights that

Fig. 4. Change of molar volume for two decapeptides (diamonds: CH-868, circle: CH-870) as a function of temperature.

Fig. 6. $R \ln k_0$ values of the four decapeptides as a function of reciprocal temperatures. The lines represents Eq. (10) fitted on the measured datapoints.

or for any position can be predicted. On the basis of the determined ΔV_m and k_0 values, it can be shown that the retention factor of the decapeptides studied decreased by ~10% during their migration through the column (e.g. k of CH-868 decreased from 5.3 (column inlet) to 4.8 (column outlet)). Even if the pressure drop was rather small in our experiments, the change of retention factor was not negligible. Thus, the use of Eq. (9) instead of the much simpler Eq. (2) for the determination of ΔV_m and k_0 was reasonable.

To validate the reliability of our model, retention times of three decapeptides were predicted for 15, 20 and 30 cm column length, based on initial measurements performed on a 5 cm long column. Columns were coupled in series and operated at the same flow rate. In this case, the various head pressures were generated by applying different column lengths. The retention times were calculated for the observed head pressures and were compared with the experimental retention times. Fig. 5 shows the predicted retention times (lines) and the experimentally observed data. Please note that instead of retention times, the retention time per column length ratio was considered for the correct comparison since different pressures correspond to different column lengths. The experimental and predicted values were in good agreement as the deviation between experimental and calculated retention times was lower than 1% for the less retained peptides (866 and 869). The difference was somewhat larger with the most retained peptide (870), but the relative error of prediction was lower than 4.5% that can be considered as accurate value. Based on our results, the proposed model
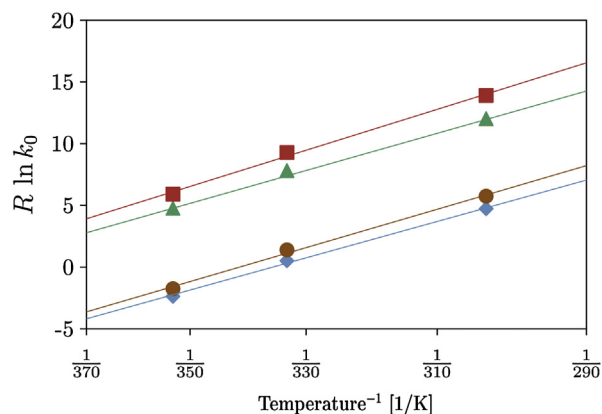
enables the accurate prediction of peptide retention for various head pressures or column length.

4.4. Determination of fundamental thermodynamic parameters

According to Eq. (3), the limiting retention factor at zero pressure, k_0, depends on the temperature, T, the phase ratio, ϕ, the change of internal energy, ΔE, and the change of entropy, ΔS, of the system during adsorption. Eq. (3) can be linearized as

$$R \ln k_0 = -\frac{\Delta E}{T} + \Delta S + R \ln \phi \qquad (11)$$

ΔE and ΔS can be determined by fitting Eq. (11) on a $R \ln k_0$ vs. $1/T$ dataset. The slope of the curve is equal to the negative change of internal energy, while the intercept includes the change of entropy and the phase ratio. Fig. 6 shows the $R \ln k_0$ values of the four decapeptides as a function of reciprocal temperatures. Fig. 6 clearly demonstrates the linear relationship between $R \ln k_0$ vs. $1/T$. The lines in the figure represent the fitted curves. It can be seen that Eq. (11) fitted well on the measured data, and determination coefficients, r^2 varied between 0.9957 and 0.9977. The fundamental thermodynamic parameters (ΔE and ΔS) of four decapeptides calculated from the fitted parameters have been reported in Table 2. As expected, the results clearly show that the retention of decapeptides is controlled by molecular forces rather than the degree of
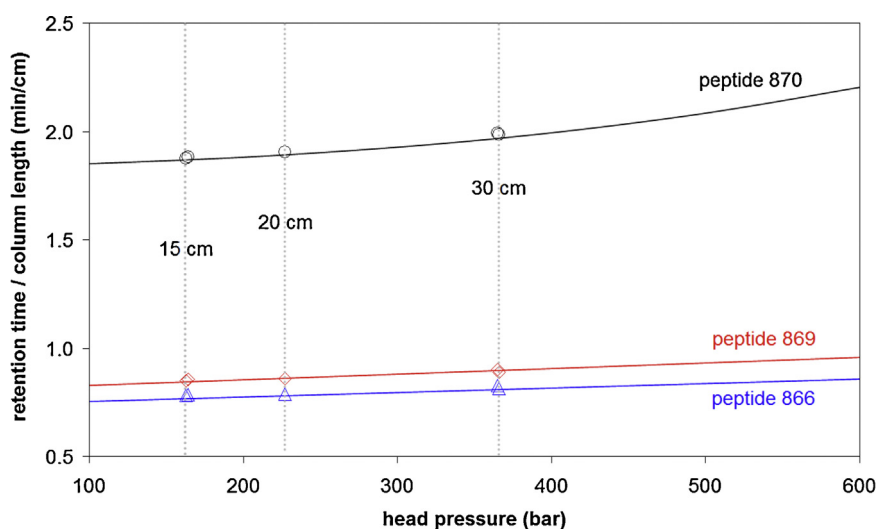

Fig. 5. Predicted and measured retention of decapeptides for 15, 20 and 30 cm column length. Please note that retention time/column length was considered on the y-axis to make the data comparable.

Table 2
ΔE (kJ mol^{-1}) and ΔS (J mol^{-1} K^{-1}) values of decapeptides determined by fitting Eq. (11) to the retention database.

Compound	ΔE	ΔS
CH-868	15.07	−34.95
CH-866	15.89	−36.60
CH-869	15.40	−28.86
CH-870	16.95	−31.91

randomness. It can also be seen that values of ΔE correlates well with the retention order of decapeptides.

Finally, the determined ΔE and ΔS values and the linear relationship between ΔV_m and T can be used to predict the retention behaviour of the investigated decapeptides at any temperature, inlet pressure and pressure drop.

5. Conclusion

UHPLC is a well established strategy and allows improving significantly kinetic performance, compared to old-fashion HPLC. However, the effect of pressure on thermodynamic parameters may be of importance when dealing with pressure range between 400 and 1200 bar. In this study, we focused on the effect of pressure on the retention of therapeutic peptides possessing molecular weights between ∼1 and ∼4 kDa. For this purpose, restrictor tubing was located between the column outlet and the UV detector, to evaluate the pure effect of pressure, while neglecting as much as possible the influence of frictional heating. Under such conditions, the retention increases with pressure. As example, the relative retention increase of glucagon reached ∼200% at around 1000 bar compared to ∼100 bar. This is in agreement with the theoretical expectations, because of the change in molar volume with pressure. In parallel, a model was developed to estimate the change in molar volume and it was experimentally observed that this model fits perfectly the experimental data points (determination coefficients, r^2, were higher than 0.9992 in each case). Additionally, these experiments were conducted at various temperatures comprised between 30 and 80 °C. Surprisingly, the pressure induced change in retention was found to be temperature dependent and was more pronounced at 30 °C vs. 60 or 80 °C. Again, the models were tested at different temperatures and still remain valid.

Using the proposed models, it was possible to easily calculate the expected increase in retention with pressure for any mobile phase temperature. This is particularly useful to estimate the alteration of retention due to pressure, when increasing column length under UHPLC conditions.

Finally, it was demonstrated that the retention increase with pressure was much more pronounced for glucagon (MW of ∼4 kDa) than triptorelin derivatives (MW of ∼1 kDa). To move one step forward, we are currently working on small proteins under isocratic mode, to evaluate if V_m is the only parameter explaining the change in retention with pressure or if conformational changes could also play a role. For this purpose, our models may be helpful.

Acknowledgements

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