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 effect was not identical whatever the temperature and the pressure induced change in retention was surprisingly 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.

KEYWORDS:

UHPLC, ultra-high pressure, retention, molar volume, pressure effect, peptides

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, 22-24]. It was also demonstrated that analyte molecular volume changes under elevated pressure were among the major variables affecting analyte retention [24]. 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 [25].

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 9-24% 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 [25]. 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 [26]. Finally, the effect of pressure was also demonstrated in normal phase mode and chiral separations [2,27].

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 [28]. 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 dependencies constant throughout the column.

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 $k[z]$.

$$\frac{\mathrm{d}z}{\mathrm{d}t} = \frac{u_0}{\left(1 + k\left[z\right]\right)} \quad (6)$$

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).

$$t_R = \int_0^{t_R} \mathrm{d}t = \frac{1}{u_0} \int_0^L \left(1 + k\left[z\right]\right) \mathrm{d}z \tag{7}$$

The integration can be accomplished by substituting Eq. (7) in Eq. (9). Accordingly,

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

and

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

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 × 2.1 mm and 150 x 2.1 mm) were purchased from Waters (Milford, MA, USA).

3.2. Equipment, software

The measurements were performed using a Waters Acquity UPLC™ 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 σ_{ec}^2 ~ 0.5-4 µL^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.

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

Decapapetides (MW = 1277.5 – 1311.5 g/mol) were eluted isocratically with 25:75 v/v ACN : H_2O (containing 0.1 % TFA). 2 µL of test solution was injected on the Acquity BEH300 wide pore C4 (5 cm x 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 80ºC.

The very high pressure was generated by adding restrictor capillaries to the column outlet. At 30ºC, around 193, 295, 590, 750, 870 and 1030 bar column head-pressure were generated, while at 60 and 80ºC 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_2O (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 80ºC. At 30ºC, around 220, 300, 359, 508, 576, 657, 910 and 993 bar column head-pressure were generated. At 60 and 80ºC, 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. Figure 1 shows chromatograms recorded at 190, 540 and 1010 bar, while figure 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 figure 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 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 [29]. 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) [29]. On figure 2 A, 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 figure 2 B. 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 figure 2 B. 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 a factor 3 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 a factor of 6, 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.

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

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 $\left(\frac{\partial \Delta V_m}{\partial T} > 0\right)$. 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. Figure 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. Figure 3 A also suggests this assumption since the absolute retention at 60 and 80 °C, in the conventional 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 [30]. 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 PKa values can also be modified with temperature and can explain thiy type of behavior. 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 [31]. Due to the different conformation-dependent responses of peptides and proteins at elevated temperatures, the change in retention can be unexpected [32,33]. Another study demonstrated the thermally induced inter-conversion of insulin by temperature-dependent changes in the retention parameters [34]. The authors reported irreversible conformational changes for insulin at temperatures between 65 and 85ºC.

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 figure 3 A, 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.

Figure 3 B shows the relative change in retention *vs.* relative head pressure increase. This type of representation highlights that the pressure induced retention increase is strongly temperature dependent. When injecting this 3.5 kDa polypeptide, increasing the pressure by a factor of 3 at 30 °C resulted in 130 % increase while at 60 and 80°C, it caused only 45 and 20 %, respectively.

4.3. Determination of 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. Figure 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 [12,29]. 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

363 kDa polypeptide, the differences between molar volume changes at different temperatures
364 seem to be much more important. At 60 and 80°C, 28 and 52% lower change of molar
365 volume were calculated. These observations can probably be explained by the possible
366 temperature dependent changes of molecular conformation. In addition, Fig. 4 demonstrates
367 clearly, that there is a linear relationship between the change of molar volume and
368 temperature. The derivative of ΔV_m with respect to temperature is positive that means a
369 decrease in the molar volume change.
370 If the ΔV_m and k_0 values are known, (1) the retention time of a compound, (2) the local
371 retention factor at any position on the column, and (3) the position of the band at any time,
372 can be predicted for any inlet pressures and pressure drops. In the case of isocratic elution,
373 a linear pressure gradient occurs along the column, assuming that the phase ratio is constant
374 throughout the column. Therefore, in this case, the pressure for any column length or for any
375 position can be predicted. To validate the reliability of our model, retention times of three
376 decapeptides were predicted for 15, 20 and 30 cm column length, based on initial
377 measurements performed on a 5 cm long column. Columns were coupled in series and
378 operated at the same flow rate. In this case, the various head pressures were generated by
379 applying different column lengths. The retention times were calculated for the observed head
380 pressures and were compared with the experimental retention times. Figure 5 shows the
381 predicted retention times (lines) and the experimentally observed data. Please note that
382 instead of retention times, the retention time per column length ratio was considered for the
383 correct comparison since different pressures correspond to different column lengths. The
384 experimental and predicted values were in good agreement as the deviation between
385 experimental and calculated retention times was lower than 1% for the less retained peptides
386 (866 and 869). The difference was somewhat larger with the most retained peptide (870), but
387 the relative error of prediction was lower than 4.5 % that can be considered as accurate
388 value. Based on our results, the proposed model enables the accurate prediction of peptide
389 retention for various head pressures or column length.
390
391 **4.4. Determination of fundamental thermodynamic parameters**
392 According to Eq. (3), the limiting retention factor at zero pressure, k_0, depends on the
393 temperature, *T*, the phase ratio, ϕ, the change of internal energy, ΔE, and the change of
394 entropy, ΔS, of the system during adsorption. Eq. (3) can be linearized as
395
$$R \ln k_0 = -\frac{\Delta E}{T} + \Delta S + R \ln \phi \qquad (11)$$

396 ΔE and ΔS can be determined by fitting Eq. (11) on a $R\ln k_0$ vs. 1/*T* dataset. The slope of
397 the curve is equal to the negative change of internal energy, while the intercept includes the
398 change of entropy and the phase ratio. Fig. 6 shows the $R\ln k_0$ values of the four
399 decapeptides as a function of reciprocal temperatures. Fig. 6 clearly demonstrates the linear
400 relationship between $R\ln k_0$ vs. 1/*T*. The lines in the figure represent the fitted curves. It can
401 be seen that Eq. (11) fitted well on the measured data, and determination coefficients, r^2
402 varied between 0.9957 and 0.9977. The fundamental thermodynamic parameters (ΔE and
403 ΔS) of four decapeptides calculated from the fitted parameters have been reported in Table
404 2. As expected, the results clearly show that the retention of decapeptides is controlled by
405 molecular forces rather than the degree of randomness. It can also be seen that values of
406 ΔE correlates well with the retention order of decapeptides.
407 Finally, the determined ΔE and ΔS values and the linear relationship between ΔV_m and *T*
408 can be used to predict the retention behavior of the investigated decapeptides at any
409 temperature, inlet pressure and pressure drop.
410
411 **5. CONCLUSION**
412 UHPLC is a well established strategy and allows improving significantly kinetic performance,
413 compared to old-fashion HPLC. However, the effect of pressure on thermodynamic
414 parameters may be of importance when dealing with pressure range between 400 and 1200
415 bar. In this study, we focused on the effect of pressure on the retention of therapeutic
416 peptides possessing molecular weights between ~1 and ~4 kDa. For this purpose, restrictor
417 tubing was located between the column outlet and the UV detector, to evaluate the pure
418 effect of pressure, while neglecting as much as possible the influence of frictional heating.
419 Under such conditions, the retention increases with pressure. As example, the relative
420 retention increase of glucagon reached ~ 200 % at around 1000 bar compared to ~ 100 bar.
421 This is in agreement with the theoretical expectations, because of the change in molar
422 volume with pressure. In parallel, a model was developed to estimate the change in molar
423 volume and it was experimentally observed that this model fits perfectly the experimental
424 data points (determination coefficients, r^2, were higher than 0.9992 in each case).
425 Additionally, these experiments were conducted at various temperatures comprised between
426 30 and 80°C. Surprisingly, the pressure induced change in retention was found to be
427 temperature dependent and was more pronounced at 30 °C vs. 60 or 80 °C. Again, the
428 models were tested at different temperatures and still remain valid.
429 Using the proposed models, it was possible to easily calculate the expected increase in
430 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 Vm 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.

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485
486
487

FIGURE CAPTIONS

Figure 1: Effect of pressure on the retention of related peptides (1.1 – 1.3 kDa). Column: Acquity BEH300 C4 (50 x 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).

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

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

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

Figure 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.

Figure 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.

Figure

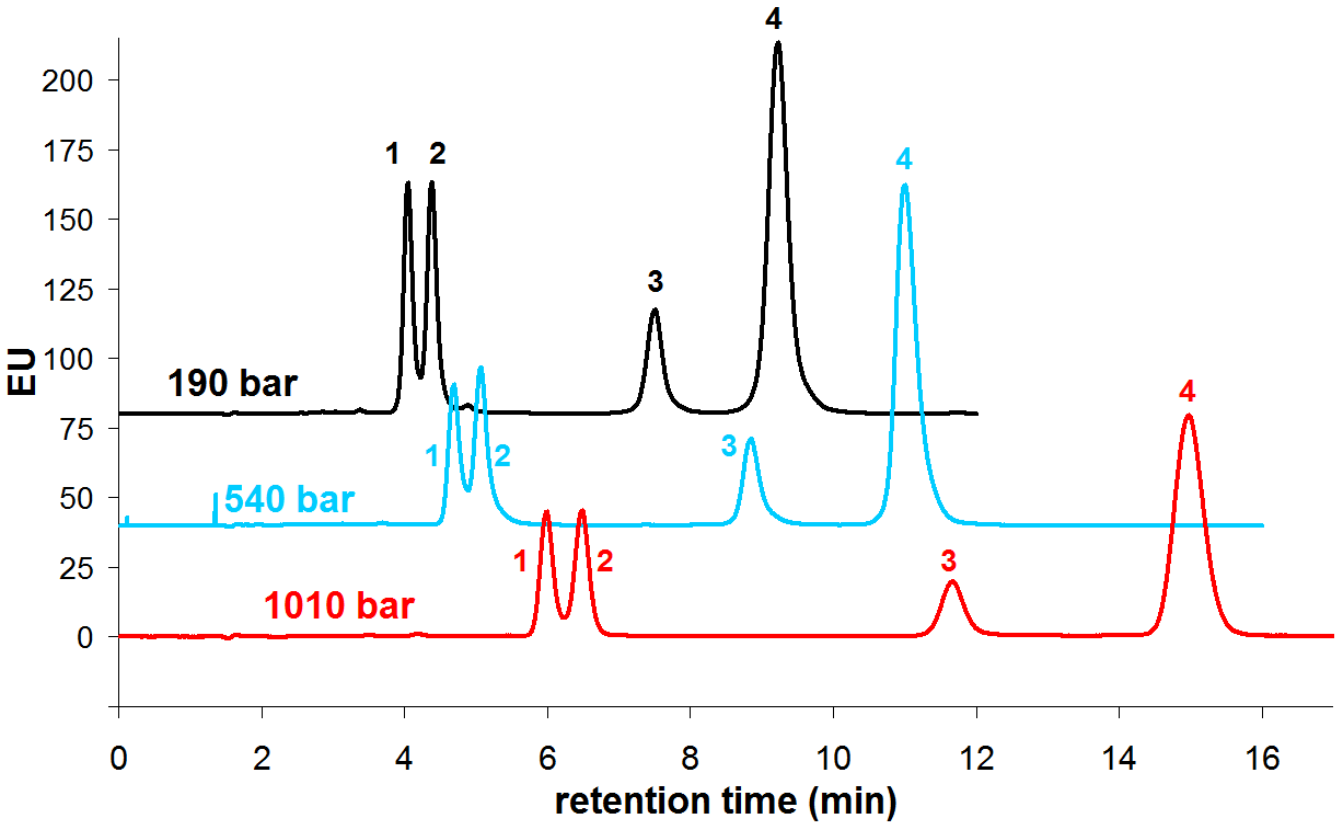
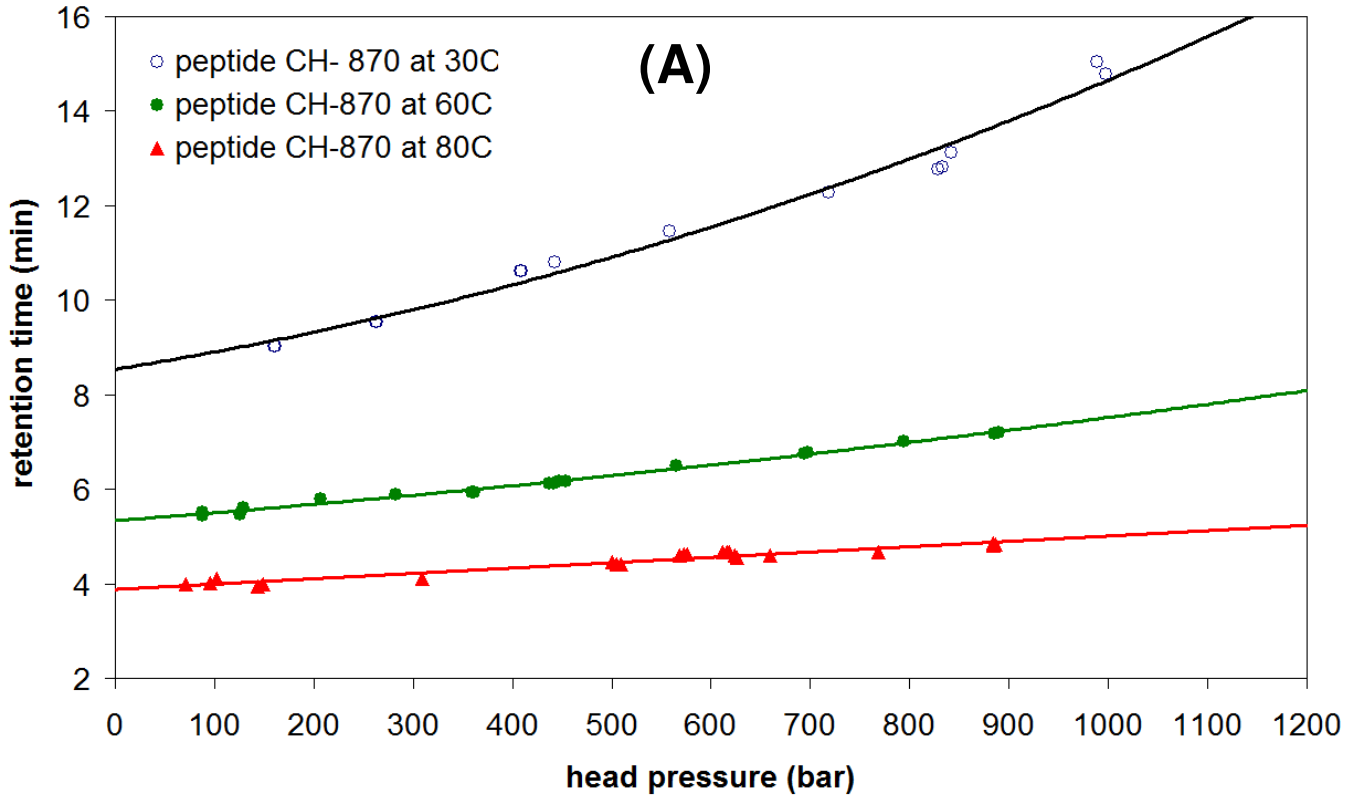


Figure 1


(A)
peptide CH- 870 at 30C
peptide CH-870 at 60C
peptide CH-870 at 80C
retention time (min)
head pressure (bar)
16
14
12
10
8
6
4
2
0
100
200
300
400
500
600
700
800
900
1000
1100
1200

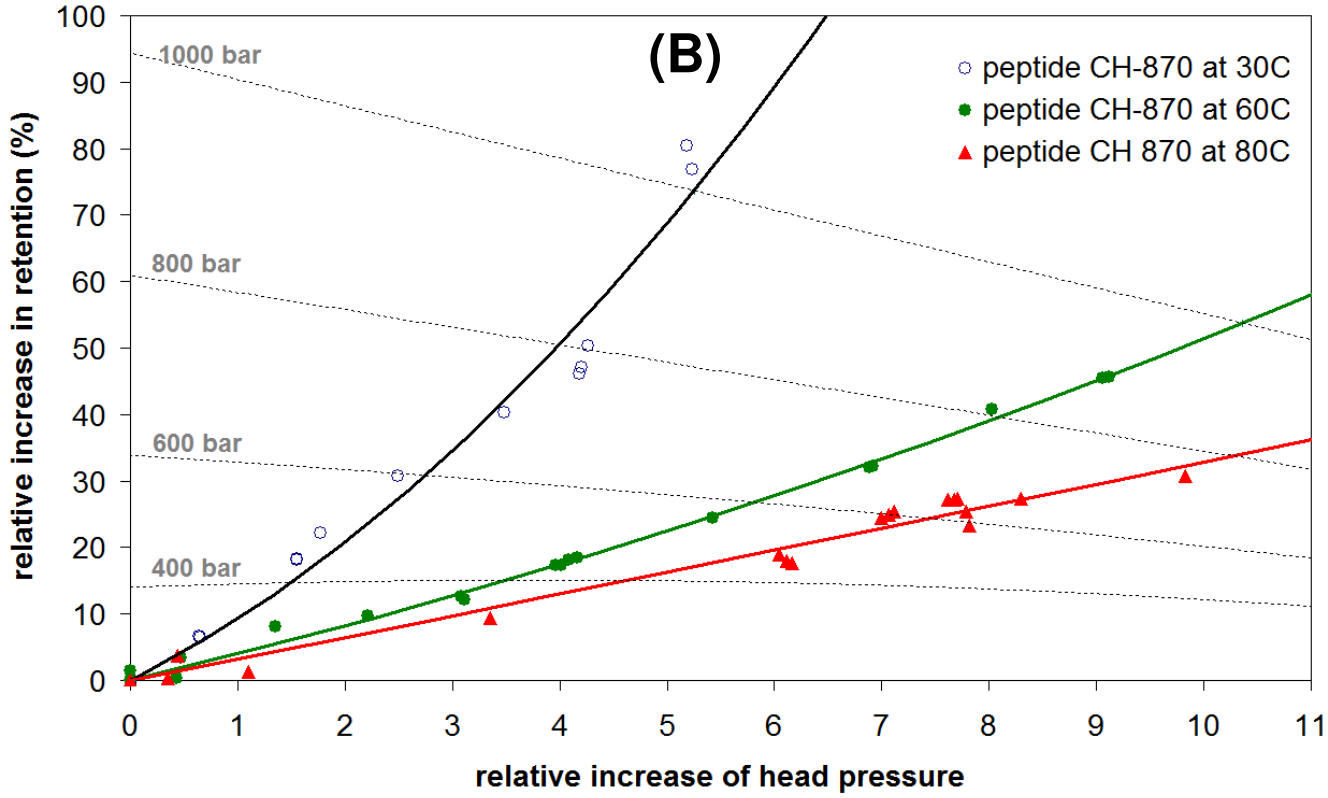
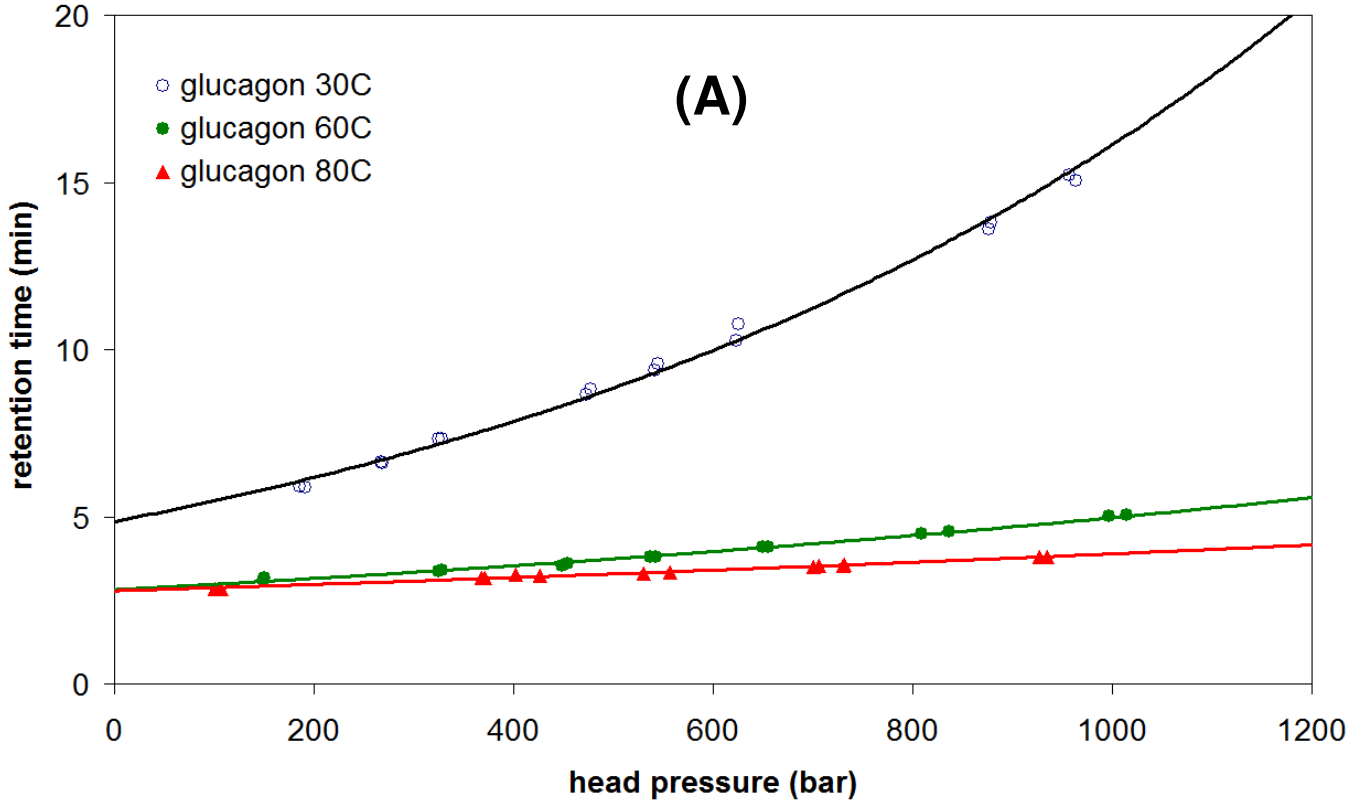

(B)
1000 bar
800 bar
600 bar
400 bar
peptide CH-870 at 30C
peptide CH-870 at 60C
peptide CH 870 at 80C
relative increase in retention (%)
relative increase of head pressure
100
90
80
70
60
50
40
30
20
10
0
0
1
2
3
4
5
6
7
8
9
10
11

Figure 2


(A)
glucagon 30C
glucagon 60C
glucagon 80C
retention time (min)
head pressure (bar)
0
5
10
15
20
0
200
400
600
800
1000
1200

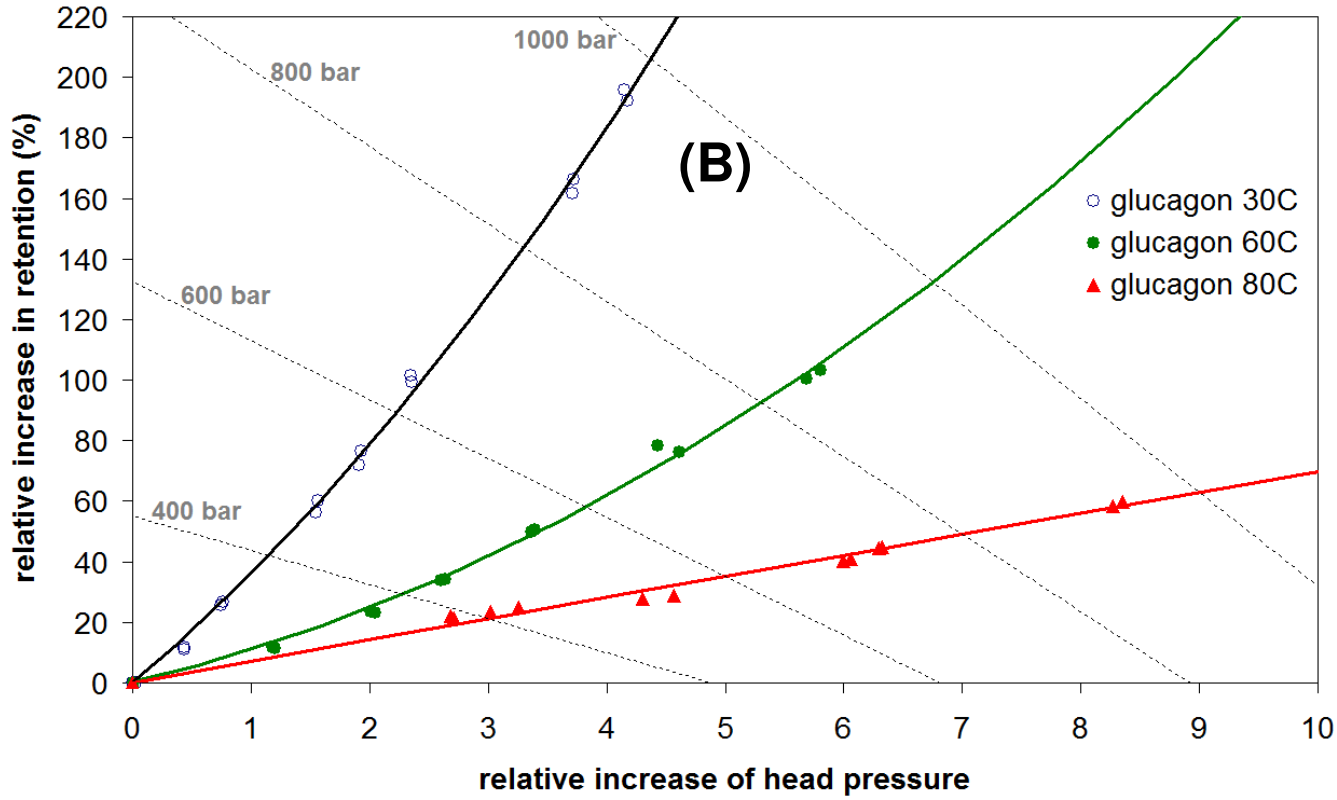

(B)
1000 bar
800 bar
600 bar
400 bar
glucagon 30C
glucagon 60C
glucagon 80C
relative increase in retention (%)
relative increase of head pressure
0
20
40
60
80
100
120
140
160
180
200
220
0
1
2
3
4
5
6
7
8
9
10

Figure 3

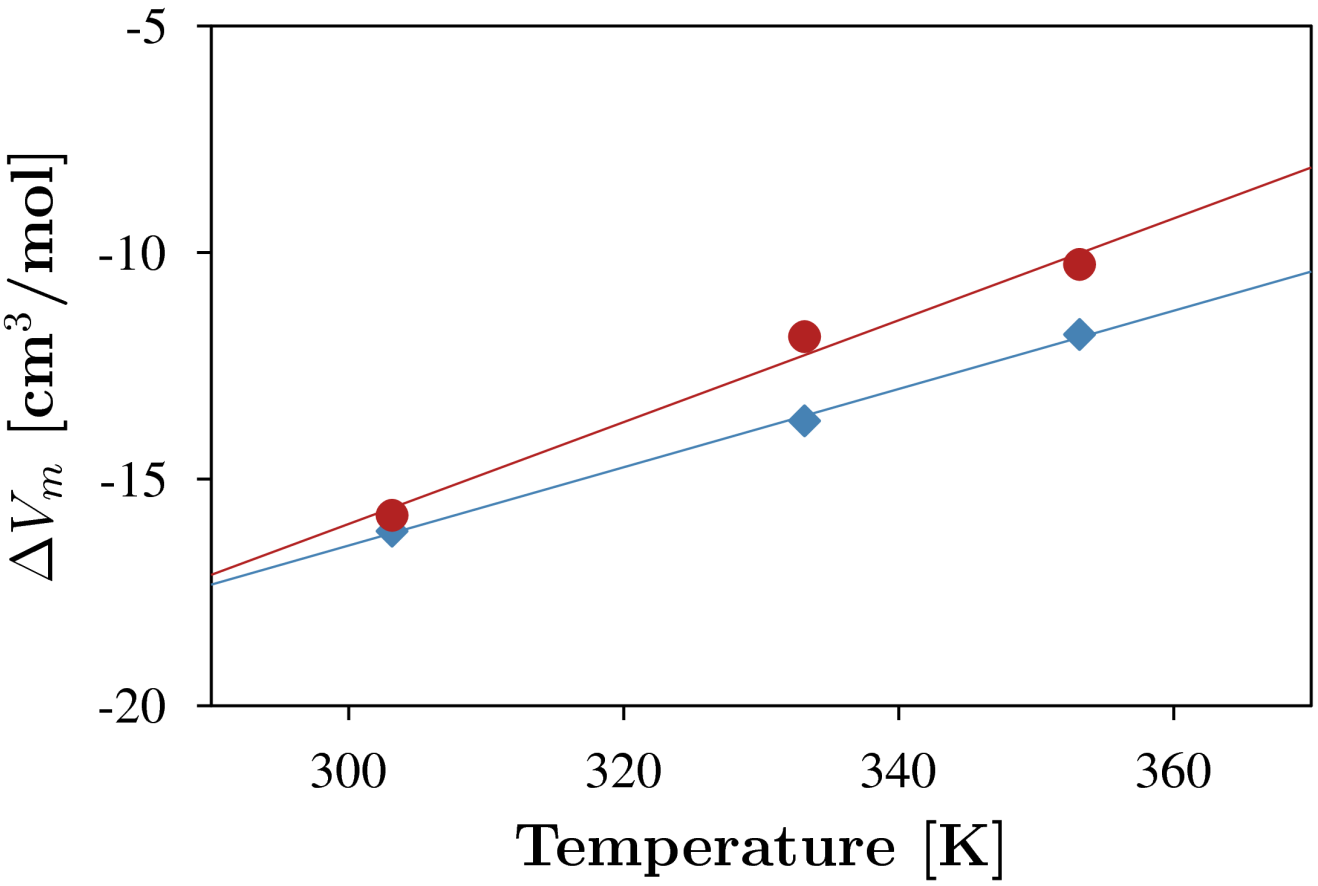


Figure 4.

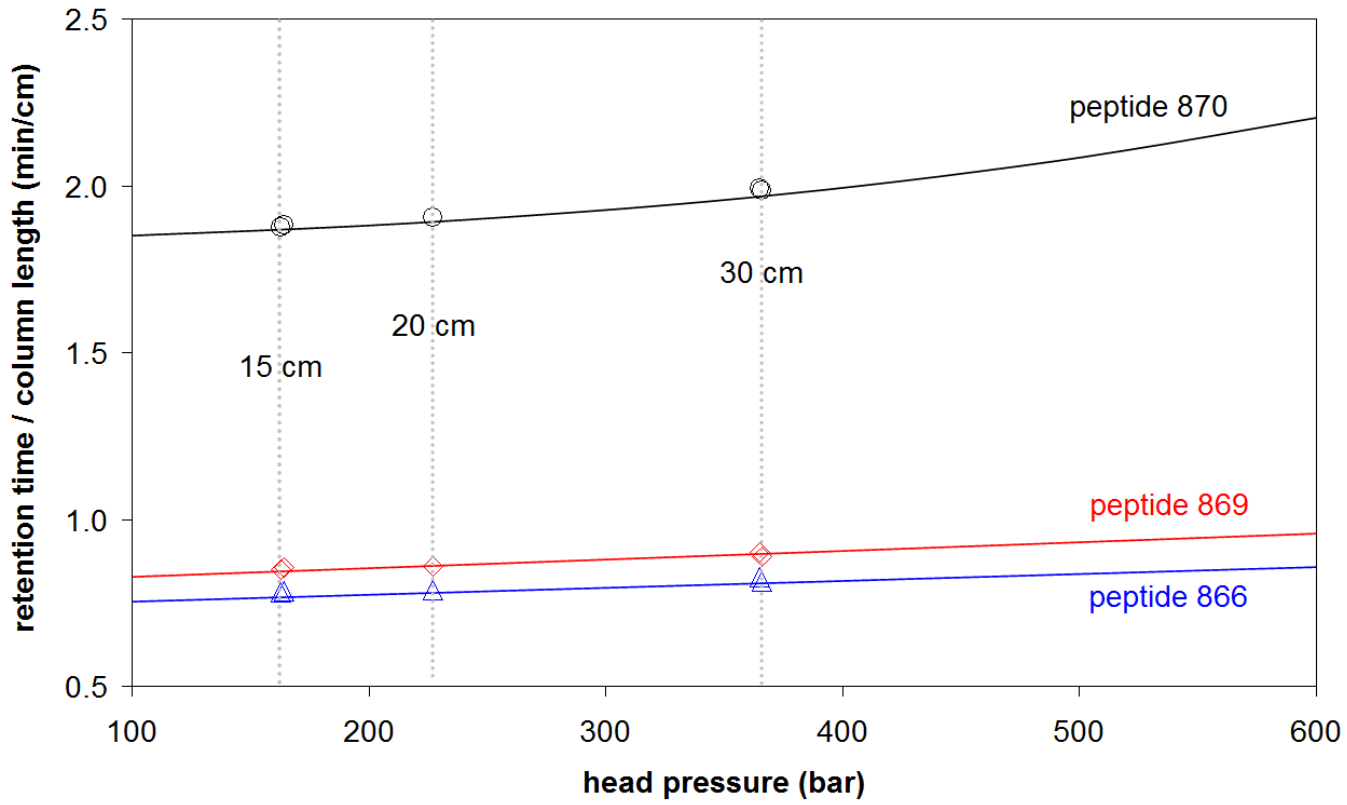

2.5
2.0
1.5
1.0
0.5
retention time / column length (min/cm)
peptide 870
30 cm
20 cm
15 cm
peptide 869
peptide 866
100
200
300
400
500
600
head pressure (bar)

Figure 5

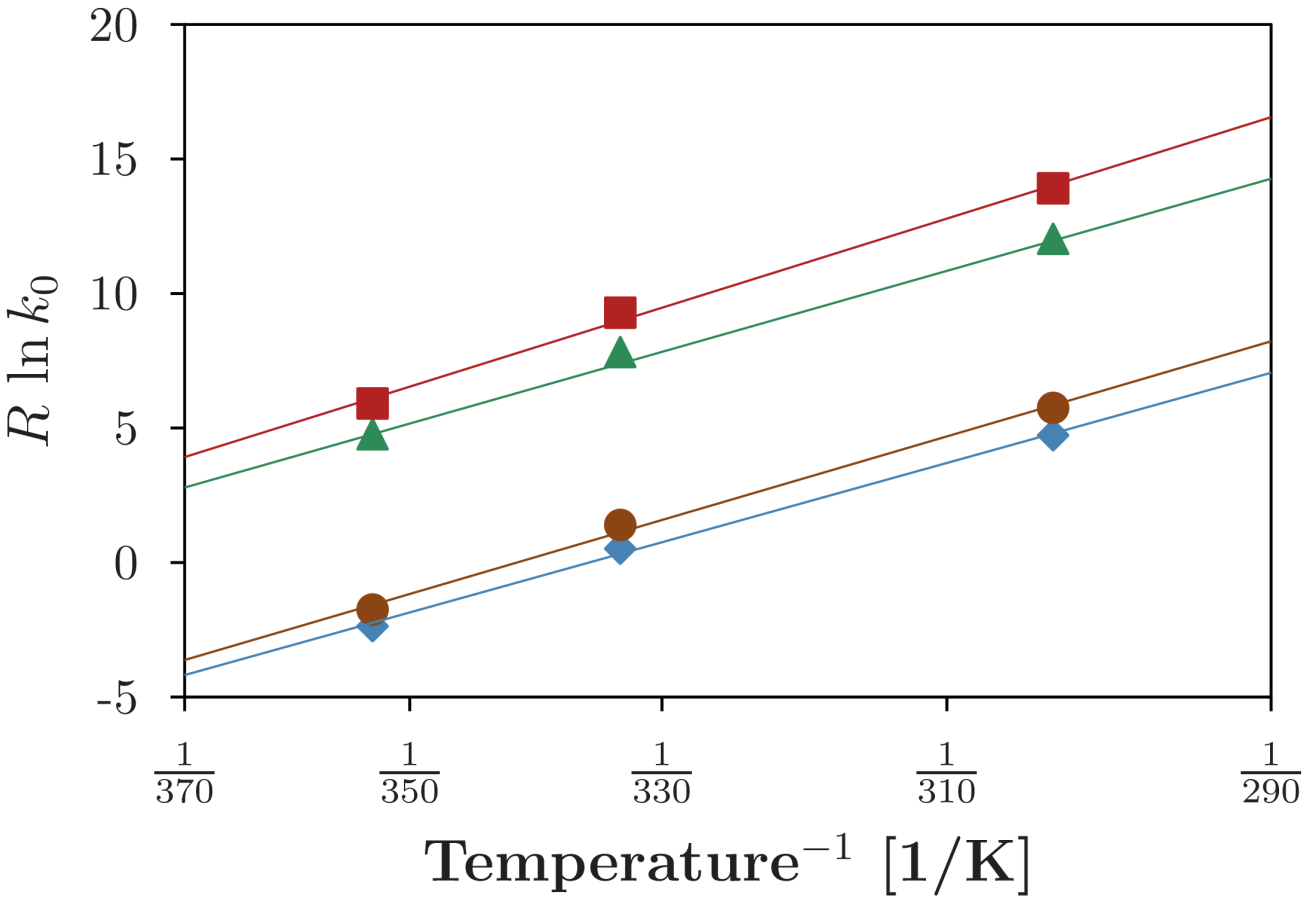

20
15
10
5
0
-5
$R \ln k_0$
$\frac{1}{370}$
$\frac{1}{350}$
$\frac{1}{330}$
$\frac{1}{310}$
$\frac{1}{290}$
Temperature^{-1} [1/K]

Figure 6

Tables

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

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