Journal of Chromatography A, 1228 (2012) 135–147

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

# Effect of pH additive and column temperature on kinetic performance of two different sub-2 μm stationary phases for ultrafast separation of charged analytes

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ARTICLE INFO

*Article history:*
Available online 19 August 2011

*Keywords:*
HETP
Column efficiency
UHPLC
High temperature
HT-UHPLC
Peptides
Ionisable compounds
Sub-2 μm particles
Volatile buffers

ABSTRACT

The performance characteristics of separation were studied for small pharmaceuticals and larger charged molecules (peptides) in various mobile phase conditions on two 5-cm long narrow bore columns packed with 1.7 μm core-shell and totally porous particles respectively. The effect of temperature and pH additives (formic acid, trifluoroacetic acid, ammonium formate, ammonium acetate and ammonia) on column efficiency was investigated through a kinetic study based upon data obtained under gradient elution conditions. Sample peak capacities were calculated and compared in all studied conditions for a sample of ten representative peptides having masses ranging from 500 to 2000 Da. The elevation of temperature was found to be significantly beneficial. The effect of flow-rate on peak shape was also investigated. Ammonium acetate at neutral pH led to the best results in terms of both efficiency and peak capacity. It was found that column performance was strongly dependent on the type of stationary phase, especially in acidic medium.



## 1. Introduction

Nowadays, the analysis of small ionisable compounds such as pharmaceuticals or larger charged molecules such as peptides is mostly carried out in reversed phase liquid chromatography (RPLC) with mass spectrometry (MS) detection using an electrospray (ESI) interface. Separations are usually achieved at acidic pH either with TFA or with formic acid as additive. The advantage of low pH is the suppression of ionisation of silanol groups thereby limiting secondary interaction with cationic compounds and thus preventing peak tailing. TFA is known to perform well with cationic analytes probably due to its action as an ion-pairing agent thereby improving the peak shapes [1]. As a result, it is widely used for the analysis of pharmaceutical and biological compounds. However the use of TFA can also have a negative effect on the detection sensitivity in MS detection. Furthermore, the low mobile phase pH when using TFA as additive (pH < 2.5) may be detrimental to the column life. On the other hand, the problem of unusual overloading of ionised bases at acidic pH, especially with low ionic strength additive such as formic acid 0.1%, was often cited [2–7] although the reasons for this behaviour are still debated [8]. The use of neutral or even basic buffers instead of acid buffers has been reported [9] but the comparison of their performance in terms of mass overload was only related to non-volatile buffers. The use of alternative volatile additives and their comparison with TFA and formic acid in terms of column performance for charged compounds has been little studied up to now.

Very fast separations are of great interest to increase the analysis throughput. Very short analysis times are also demanded in the second dimension of a comprehensive on-line two-dimensional separation [10]. The combined use of high temperature and sub-2 μm particles at ultra-high pressure was often proved to be very efficient to speed up the analysis [11,12]. The use of the new core-shell particles was also recommended for fast separations due to their excellent mass transfer properties [13–16] which make the use of very high linear velocities possible without significant loss in efficiency.

The objective of this study was to make a critical comparison of pH additives that are recommended for mass spectrometry detection. This study was carried out on two common 5-cm long narrow bore columns, packed with 1.7 μm core-shell and 1.7 μm totally porous particles respectively, both columns being famous for their ability to achieve ultrafast separation. Experimental measurements were conducted under gradient elution conditions using a novel method described in Section 2. The kinetic characteristics of both columns were assessed via reduced HETP plots for basic compounds and peptides at different temperatures under different mobile phase conditions. Finally sample peak capacities were calculated on both columns for a sample of ten representative peptides and compared in different mobile phase conditions at different temperatures.

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doi:10.1016/j.chroma.2011.08.041

**Table 1**
Physical properties of peptides.

| No. | Peptide name | Mw (g) | Isoelectric point |
|---|---|---|---|
| 1 | Influenza Hemagglutinin (HA) peptide | 1102.15 | 3.5 |
| 2 | FLAG peptide | 1012.97 | 3.9 |
| 3 | WDDHH | 708.68 | 5.2 |
| 4 | Leucine enkephalin | 555.62 | 6 |
| 5 | Bombesin | 1619.85 | 7.6 |
| 6 | [arg8]-Vasopressin | 1084.23 | 8.2 |
| 7 | [ile]-Angiotensin | 897.08 | 9.4 |
| 8 | Bradykinin fragment 1–5 | 572.66 | 10.6 |
| 9 | Substance P | 1347.63 | 11.7 |
| 10 | Bradykinin | 1060.21 | 12.5 |

## 2. Experimental

### 2.1. UHPLC system

All data were acquired with the Acquity UPLC system (Waters, Milford, USA). This instrument includes a high pressure binary pump with a maximum delivery flow-rate of 2 mL/min, an autosampler with a 5 μL sample loop, a column oven with a maximum temperature of 90 °C, a photo diode array detector with a 500 nL flow-cell. The instrument was controlled by Empower software. The maximum backpressure is 1000 bar for flow-rates up to 1 mL/min, 800 bar for flow-rates up to 1.5 mL/min and 630 bar for flow-rates up to 2 mL/min.

The signal sampling rate was set at 80 Hz for the measurements of extra-column variances and at 40 Hz for the measurements of band profiles eluted from the columns. The wavelength was set at 220 nm and the time constant at 25 ms.

The flow-rate accuracy was checked at 30 °C, 60 °C and 90 °C by collecting water at different flow-rates ranging from 100 μL/min to 2 mL/min. The relative errors were less than 0.5%.

The mobile phase was preheated by a heat exchanger made up of a stainless steel tube (50 cm × 0.127 mm) located between the Rheodyne injection valve and the column inlet. A polyether ether ketone (PEEK) tube (15 cm × 0.1 mm) was located between the column outlet and the detector. A total extra-column volume of 13 μL was determined in the absence of column. The measured dwell volume was 120 μL. A time offset of 0.8 s was observed after the zero injection time was recorded. It was taken into account for the retention time measurements. The needle wash cycle included a strong wash using water–acetonitrile (20/80, v/v) and a weak wash using water–acetonitrile (80/20, v/v).

### 2.2. Chemicals and reagents

The sample mixture for experiments under gradient elution mode contained diphenhydramine (0.05 mg/mL, p*K*a = 9.0 [17]), caffeine (0.05 mg/mL) and ten peptides (0.03 mg/mL) which were prepared by diluting their stock solutions with water. Ten representative peptides were chosen in order to have a large variety of molecular weights and isoelectric points. They were obtained from Sigma–Aldrich (Steinheim, Germany). One peptide, WDDHH, was custom synthesized (Genecust, Luxembourg). Their isoelectric point and molecular weight are reported in Table 1. Diphenhydramine, protryptilline, ethylparaben and caffeine were also obtained from Sigma–Aldrich (Steinheim, Germany). The tryptic digest of BSA, lysozyme and myoglobin from Sigma Aldrich (Steinheim, Germany) was injected after preparation without further dilution.

Acetonitrile (HPLC grade) was obtained from SDS (Peypin, France). Water was obtained from an Elga water purification system (Veolia water STI, Le Plessis Robinson, France). The mobile phase pH was controlled thanks to various additives selected in order to be compatible with mass spectrometry detection: formic acid 0.1% (FA, pH 2.8), trifluoroacetic acid 0.05% (TFA, pH 2.4), ammonium formate 10 mM (AF-pH 2.8 adjusted with formic acid and AF-pH 10.4 adjusted with ammonia), ammonium acetate 10 mM (AA, pH 6.8) and ammonium hydroxide 0.1% (AMO), all from Sigma–Aldrich (Steinheim, Germany). Buffered eluents prepared from salts were filtered through a 0.2-μm nylon filter before use. In order to keep the ionic strength constant all along the gradient, the pH adjuster was added in both aqueous and organic phases except for ammonium salts, not enough soluble in organic solvents at such concentrations.

### 2.3. Columns

Two different columns with the same particle size were studied: a core-shell Kinetex-C18 column (5 cm × 2.1 mm I.D., particle diameter 1.7 μm) from Phenomenex (Torrance, CA, USA) and a totally porous Acquity BEH-C18 column (5 cm × 2.1 mm I.D., particle diameter 1.7 μm) from Waters (Milford, MA, USA). The core- particles are made of a 1.25 μm solid silica core and a 0.23 μm thick layer of porous silica. Total column porosity was determined as 0.5 and 0.6 for Kinetex-C18 and Acquity BEH-C18 respectively by injecting a non-retained solute (uracile). The mobile phase linear velocity, $u_0$ was determined by

$$u_0 = \frac{4F}{\pi d_i^2 \varepsilon_t} \quad (1)$$

where $F$ is the volumetric flow-rate, $d_i$, the column internal diameter and $\varepsilon_t$, the total column porosity. HETP data were collected on new columns before performing any other experiments.

### 2.4. Determination of viscosities and diffusion coefficients

The mobile phase viscosities, $\eta$, were calculated for every mobile phase conditions according to a method previously described [18]. The diffusion coefficients of caffeine, protriptylline and diphenhydramine were estimated from the Wilke and Chang equation [19]. The diffusion coefficients of peptides were estimated by

$$D_m = \frac{0.9}{298}\frac{T}{\eta} D_{m,w,25\ ^\circ C} \quad (2)$$

$\eta$ is the solvent viscosity (cP) at the temperature $T$ (K) and $D_{m,w,25\ ^\circ C}$, the diffusion coefficient ($m^2/s$) in water at 25 °C which was calculated using the empirical correlation of Stadalius et al. [20]:

$$D_{m,w,25\ ^\circ C} = 10^{-9}\left(2.2M^{-1/3} + \frac{62}{M}\right) \quad (3)$$

where $M$ is the molecular weight

### 2.5. Measurement of column pressure drop, retention times and peak variances

The contributions of extra-column volumes including extra-column residence time ($t_{ext}$), extra-column pressure drop ($\Delta P_{ext}$) and extra-column peak variance ($\sigma^2_{ext}$), were determined by replacing the column by a zero dead volume connector and injecting 1 μL ethylparaben in an aqueous eluent in all conditions of temperature and flow-rate. $\sigma^2_{ext}$ was calculated from the second order moment using a home-made program written in Microsoft Visual Basic 6.0 which made use of data exported by Empower. The obtained values were 5.4, 5.8 and 6.2 $\mu L^2$ at 0.5, 0.7 and 1 mL/min respectively. Under gradient conditions, the contribution of extra-column band

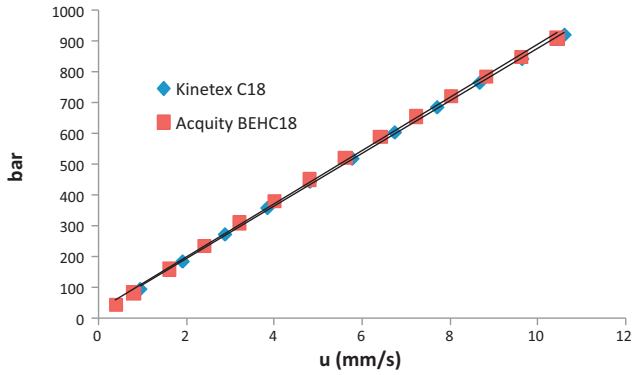


**Fig. 1.** Column pressure drop versus linear velocity at 30 °C with 1.7 μm fully porous column (Acquity BEH-C18, 5 cm × 2.1 mm) and 1.7 μm core-shell column (Kinetex-C18, 5 cm × 2.1 mm). Mobile phase: water–acetonitrile (70–30, v/v).

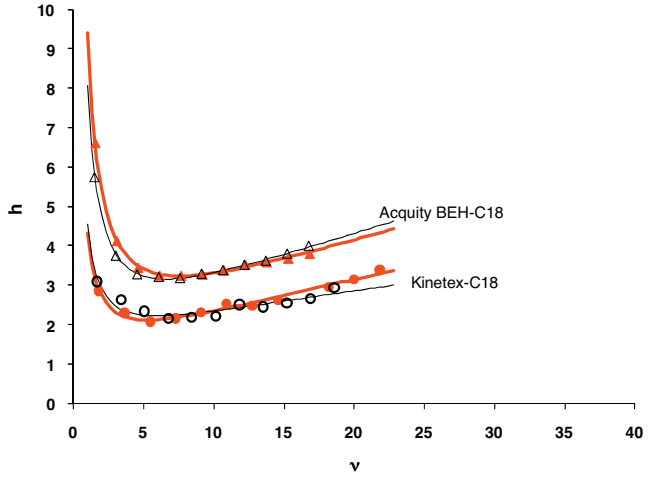


**Fig. 2.** Plot of reduced plate height versus reduced linear velocity. Comparison of common standard isocratic method (open symbols and black fitted curve, $k$ around 3.5) and proposed gradient method (full symbols and red fitted curve, $k_e$ around 3.5) on Kinetex-C18 with caffeine as solute (○ and ●) and on Acquity BEH-C18 with ethylparaben as solute (△ and ▲). Mobile phase: water–acetonitrile. Temperature: 30 °C. See Table 1 for other conditions.

broadening before the column inlet was assumed to be negligible due to the sample concentration at the column inlet (focusing effect). The extra-column variance due to the contribution of both detector and connecting tube between the column outlet and the detector cell was estimated as about 2 μL$^2$ at the same flow-rates.

The total peak variance was established from the Dorsey–Foley equation [21], which was shown to be well correlated to the true peak variance for small peak asymmetry (<2.5) [22]:

$$\sigma^2_{\text{tot}} = \frac{(b/a + 1.25) \times w_{0.1}}{41.7} \quad (4)$$

where $w_{0.1}$ is the peak width and $b/a$, the ratio of the right half width to the left half width, measured at 10% of the peak height. It should be underlined that the Dorsey–Foley equation might become strongly inaccurate in case of very high asymmetry values (i.e. >2.5) and thus might underestimate plate height values. Accordingly, corresponding values should rather be considered as the best possible performance that would be achieved in such conditions.

The column characteristics were subsequently calculated according to $\sigma^2_{\text{col}} = \sigma^2_{\text{tot}} - \sigma^2_{\text{ext}}$, $\Delta P_{\text{col}} = \Delta P_{\text{tot}} - \Delta P_{\text{ext}}$ and $t_{r,\text{col}} = t_{r,\text{tot}} - t_{r,\text{ext}}$. The variation of the column pressure drop as a function of the linear velocity, $u_0$, at 30 °C for the two columns is given in Fig. 1. As shown by the very small difference between the slopes of the two linear fitting curves, it can be concluded that the $u_0$-based column permeability is nearly the same for the two columns.

### 2.6. Getting kinetic curves

The column plate height can be expressed as

$$H_{\text{col}} = \frac{\sigma^2_{L,\text{col}}}{L} \quad (5)$$

where $L$ is the column length and $\sigma^2_{L,\ \text{col}}$, the peak variance due to the dispersion inside the column at the time the solute band leaves the column, both in length units. The variance in length units is related to the variance in time units by

$$\sigma^2_{L,\text{col}} = u_e^2 \times \sigma^2_{t,\text{col}} \quad (6)$$

where $u_e$ is the band velocity at the time the solute is eluted and thus, the following equation can be obtained from Eqs. (5) and (6):

$$H_{\text{col}} = \frac{u_e^2 \times \sigma^2_{t,\text{col}}}{L} \quad (7)$$

In isocratic elution the band velocity is constant all along the column and given by the ratio of the column length to the retention time and thus, $H_{\text{col}}$ can be readily calculated from the solute retention time and the peak variance according to

$$H_{\text{col}} = \frac{L \times \sigma^2_{t,\text{col}}}{t_r^2} \quad (8)$$

The universal method to construct Van Deemter curves consists in collecting data in isocratic elution and then using Eq. (8). With the aim of enabling a fair comparison of Van Deemter curves, it is essential that all curves be obtained with the same retention factors as both the B- and the C-terms depend on solute retention factor. However, this cannot be achieved under isocratic conditions within a single run. Furthermore, for large molecules such as peptides, the variation of the retention factor with eluent composition is much more significant than for small molecules and hence more difficult to control. Finally, the contribution of extra-column band broadening can be much more a concern in isocratic than in gradient conditions wherein a focusing effect can significantly reduce the extra-column band broadening which takes place before the column inlet.

For these various reasons, gradient data were used to construct Van Deemter curves. In gradient elution, Eq. (8) is not valid since the band velocity increases as the solute band moves inside the column. The band velocity at the time the solute is eluted depends on its retention factor at this time, $k_e$ and is given by

$$u_e = \frac{u_0}{1 + k_e} \quad (9)$$

For linear solvent strength gradients [23] and very large retention factor at initial composition, $k_e$ can be expressed as:

$$k_e = \frac{1}{2.3b} = \frac{t_G}{2.3S\Delta C t_0} \quad (10)$$

where $b$ is the gradient steepness, $S$ is the slope of the relationship between the logarithm of the retention factor and the eluent composition, $\Delta C$ is the difference between final and initial compositions, $t_0$ is the column dead time and $t_G$ is the gradient duration. The product $\Delta C \times (t_0/t_G)$ is the normalized gradient slope.

Recently, Neue et al. [24] proposed a method to calculate protein plate counts at a given flow-rate from gradient data. Their method consisted in plotting the measured peak width as a function of the inverse of the normalized gradient slope in order to determine $S$ values which in turn allow the calculation of plate counts via Eqs. (7),

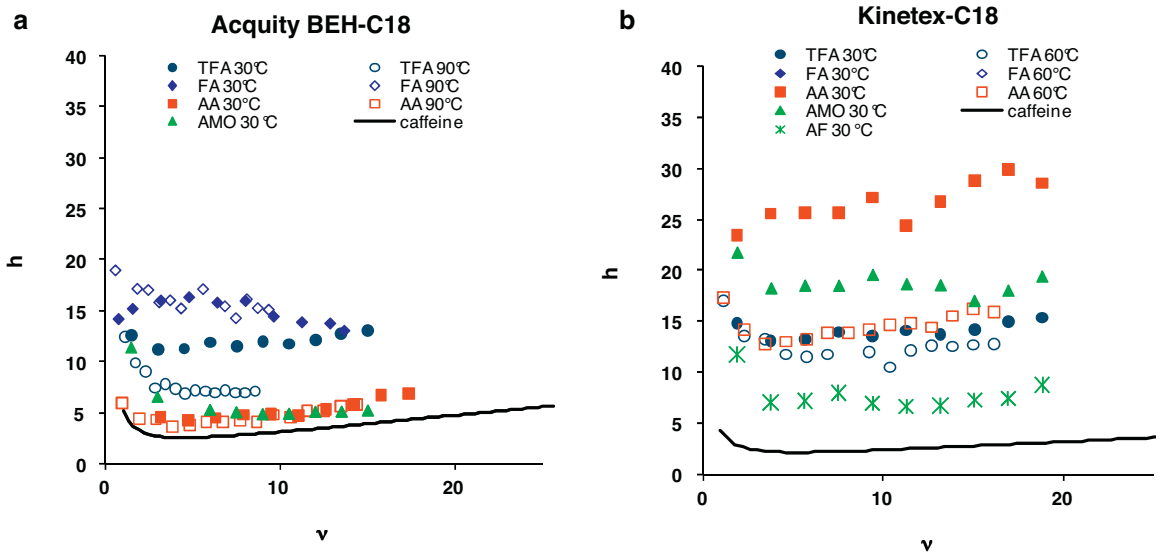


**Fig. 3.** Performance comparison of various analytical conditions for the analysis of diphenhydramine ($k$ about 5). Experimental $h$–$\nu$ plots of (a) Acquity BEH-C18, 5 cm × 2.1 mm, 1.7 μm and (b) Kinetex-C18, 5 cm × 2.1 mm, 1.7 μm. Mobile phase: water/acetonitrile with pH additive, TFA 0.05% (TFA); formic acid 0.1% (FA); ammonium acetate 10 mM (AA); ammonia 0.1% (AMO); ammonium formate 10 mM-pH10.4 (AF), 1 μL injected. The plots of formic acid for Kinetex-C18 are out of scale.

(9) and (10). However, they observed an improvement in the calculated plate count values for steeper gradients (higher normalized gradient slopes) and attributed this phenomenon to band compression. The band is indeed subjected to a compression inside the column due to the fact that the band tail moves faster with a mobile phase that is stronger than the band front. As a result the band width is reduced by a factor $G$, called the band compression factor. Band compression in gradient elution has been discussed by Snyder et al. since the very beginning of liquid chromatography [23,25]. It was shown that $G$, decreases with gradient steepness with values usually ranging from 0.6 to 1 [23,26]. It is likely that $G$ also depends on both solute and mobile phase composition for charged compounds. As a result, the measure of column plate heights from gradient data should take into account the band compression effect by replacing Eq. (7) by Eq. (11):

$$H_{\mathrm{col}} = \frac{u_e^2 \sigma^2_{t,\ \mathrm{col}}}{G^2 L} \tag{11}$$

It should be noted that Eq. (11) is valid if we assume that $H_{\mathrm{col}}$ remains independent of the mobile phase composition [27]. Combining Eqs. (9) and (11) leads to the following relationship

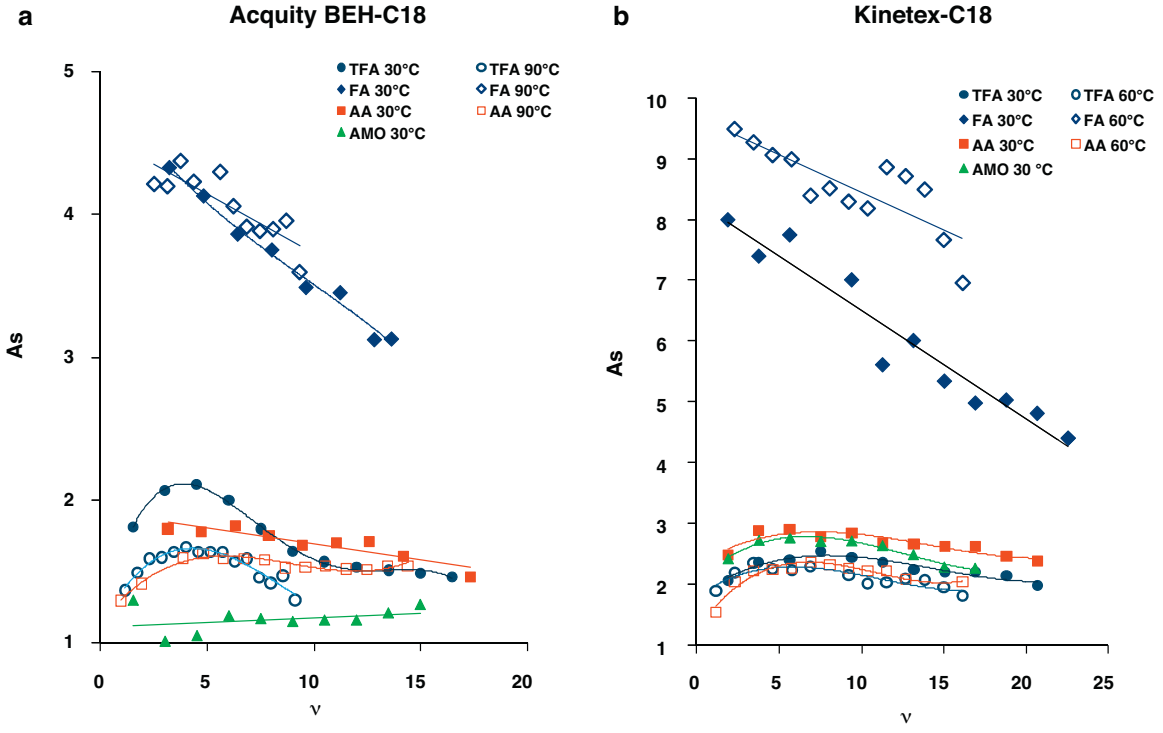


**Fig. 4.** Variation of peak asymmetry as a function of the reduced linear velocity in gradient elution. Solute: diphenhydramine. Columns: (a) Acquity BEH-C18 and (b) Kinetex-C18. Gradient conditions are given in Section 2. Same other conditions as in Fig. 3.

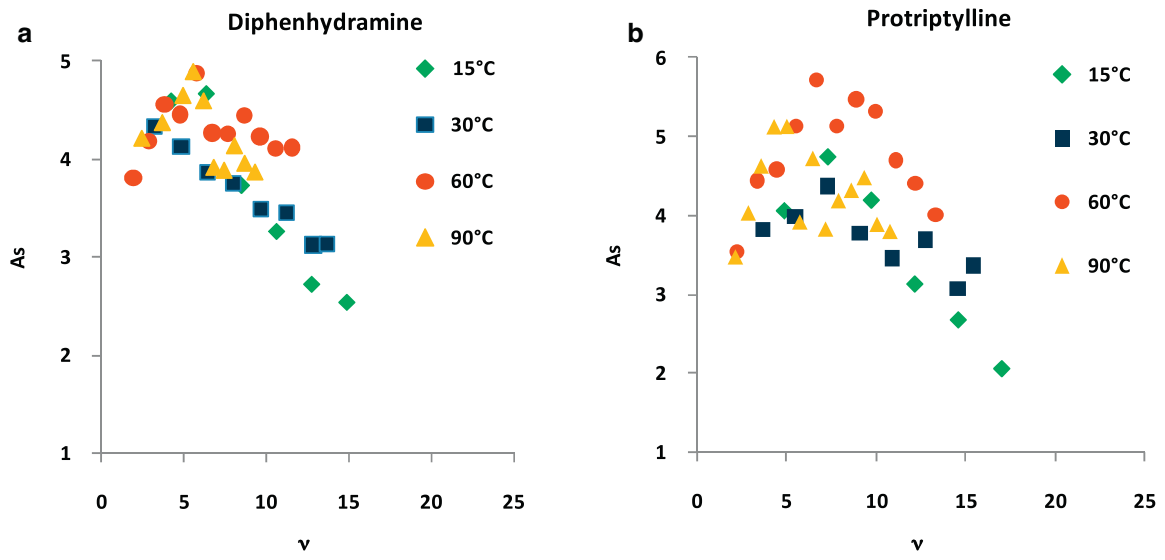


**Fig. 5.** Variation of peak asymmetry as a function of the reduced linear velocity in isocratic elution at different temperatures with formic acid as additive. Column: Acquity BEH-C18; Mobile phase: 29% ACN at 15 °C; 27% ACN at 30 °C; 25% at 60 °C; 21% at 90 °C. Solutes: (a) diphenhydramine ($k$ about 5) and (b) protryptilline ($k$ about 10).

which gives the estimation of column plate heights from gradient data:

$$H_{\mathrm{col}} = \frac{u_0^2}{(1+k_e)^2} \times \frac{\sigma^2_{t,\ \mathrm{col}}}{G^2 L} \tag{12}$$

In Neue's method [24], the band compression is assumed to be negligible. In addition Eq. (8) can be strongly affected by a deviation from LSS theory. Thus, in order to overcome these limitations, a prior determination of both $k_e$ and $G$ was done for each component using the following steps:

1. The whole sample was first separated in gradient elution with a normalized gradient slope of 1% (with solvent concentration expressed as the volume percentage) and a flow-rate of 0.5 mL/min. The initial and final acetonitrile compositions were 1% and 65% respectively ($\Delta C = 64\%$).
2. The mobile phase compositions at the point of elution were calculated according to

$$C_e = C_i + \Delta C \frac{t_0}{t_G}\left(\frac{t_r}{t_0} - \frac{t_D}{t_0} - 1\right) \tag{13}$$

where $t_r$ is the solute retention time and $t_D$, the instrument dwell time. It should be underlined that this method assumes perfectly linear and non-distorted gradients.
3. An isocratic experiment using the corresponding solvent composition as mobile phase was performed for each component at the same flow-rate. Both compression factor and retention

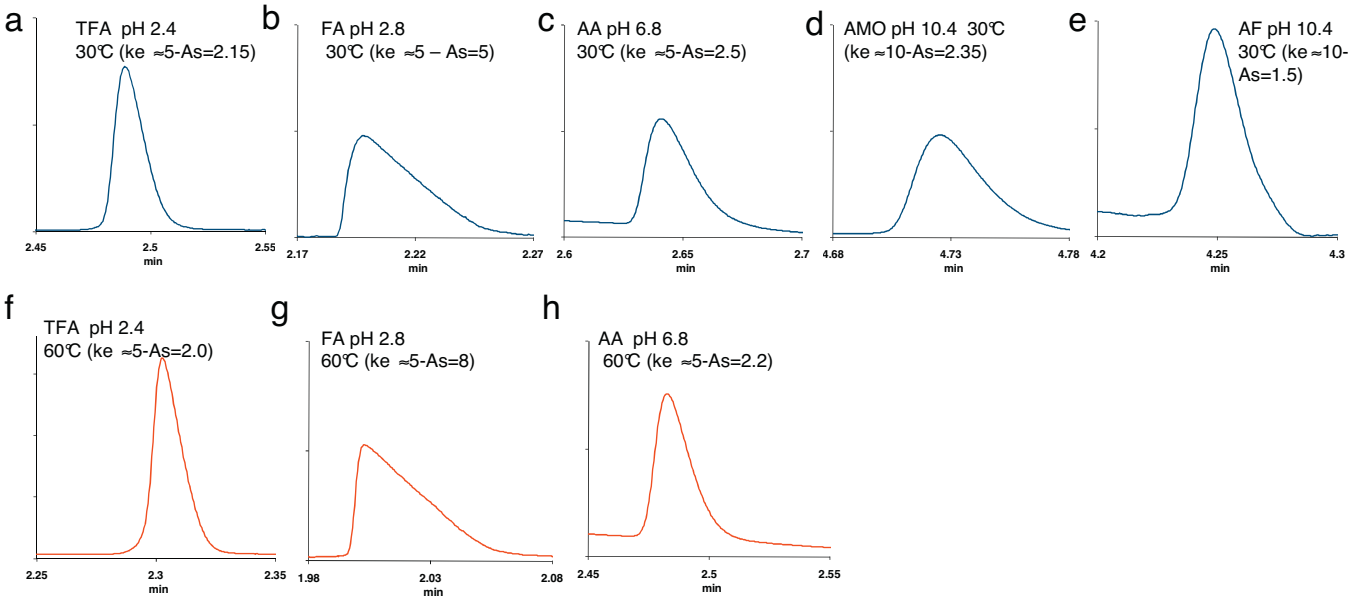


**Fig. 6.** Chromatograms on Kinetex-C18 (5 cm × 2.1 mm, 1.7 μm particles) for diphenhydramine with various volatile mobile phase additive at 30 °C and 60 °C. Flow-rate: 1 mL/min, 1 μL injected, mobile phase additive in water-acetonitrile, normalized gradient slope of 1% (see Section 2 for the gradient conditions). Retention factor at elution and peak asymmetry are given into brackets. Other conditions given in Fig. 3.

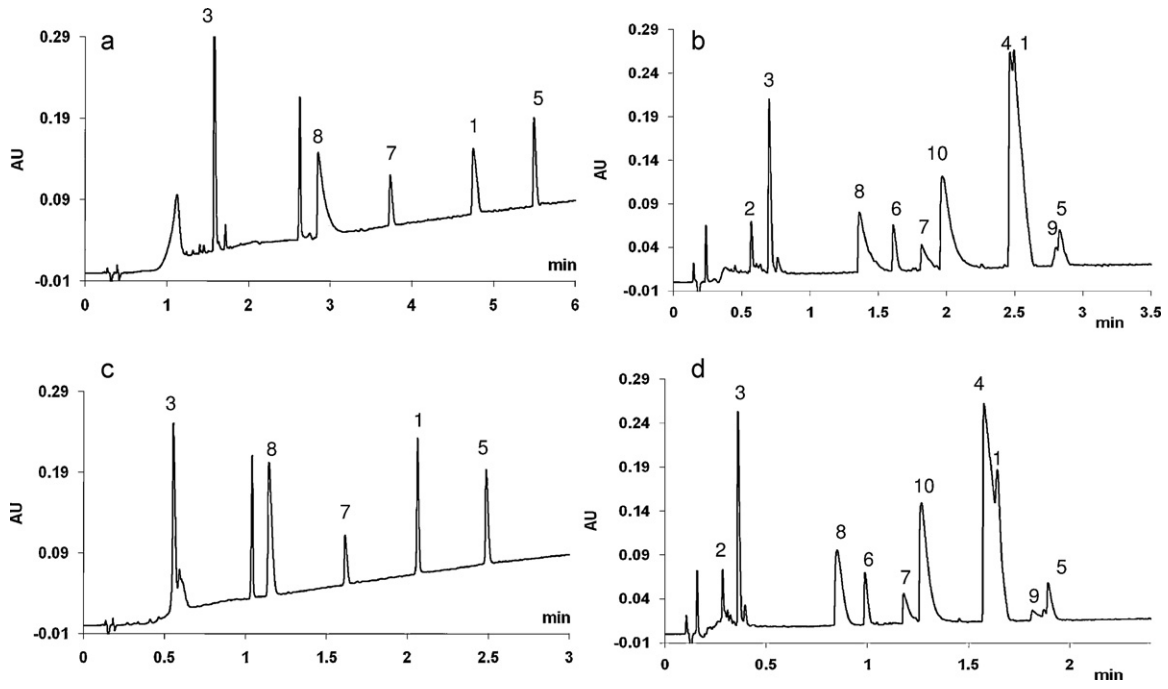


**Fig. 7.** Sample chromatograms of peptide mixture on Aquity BEH C18 (a, c) and on Kinetex-C18 column (b, d); presence of formic acid 0.1% in the mobile phase. Gradient runs from 1% ACN to 36% ACN. 1.0 μL injected. Normalized gradient slope: 1%. (a) Gradient time: 8 min; 0.5 mL/min; 30 °C; (b) gradient time: 4.35 min; 0.7 mL/min; 30 °C; (c) gradient time: 4 min; 1 mL/min; 90 °C; (d) gradient time: 3.03 min; 1 mL/min; 60 °C. Solutes are given in Table 1.

factor at the point of elution were calculated according to Eqs. (14) and (15) respectively:

$$G^2 = \frac{\sigma^2_{\text{col, Grad}}}{\sigma^2_{\text{col, Iso}}} \tag{14}$$

$$k_e = \frac{t_{r,\ \text{iso}}}{t_0} - 1 \tag{15}$$

where $t_{r,\text{iso}}$ is the solute retention time under isocratic mode.

Gradient data were then collected at various flow-rates. The gradient time was changed along with the flow-rate in order to keep the normalized gradient slope constant and hence maintaining the same $k_e$ and $G$ values. Column plate heights were then calculated according to Eq. (12).

## 3. Results and discussion

In the first part of this work, the validity of the present method involving gradient data is illustrated by comparing the resulting kinetic curves to those obtained from isocratic data. In a second part, the effect of both mobile phase additive and temperature on the kinetic performance is discussed for charged compounds via reduced HETP plots. Usually the construction of kinetic plots [28,29] can offer a fair comparison of kinetic performances between different chromatographic systems. However, as previously highlighted,

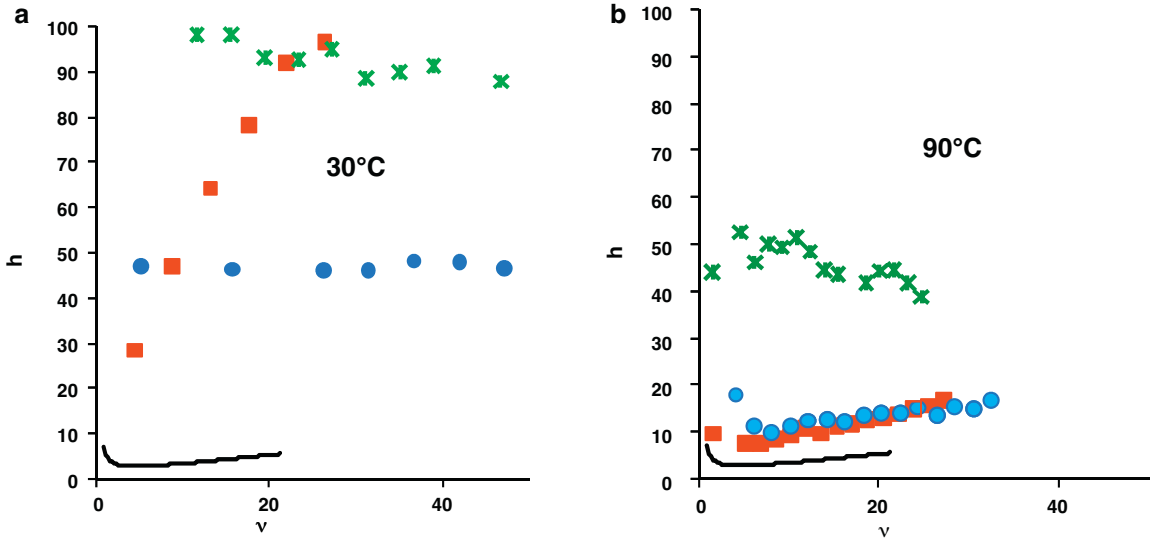


**Fig. 8.** Effect of temperature on $h$–$\nu$ plots in the presence of formic acid 0.1% in the mobile phase. Conditions: 30 °C (a) and 90 °C (b) Acquity BEH-C18 column (5 cm × 2.1 mm, 1.7 μm). Solutes: 1-Influenza Hemagglutinin (■), 5-bombesin (●) and 7-[ile]-angiotensine (×). $k$ about 3 for all solutes.

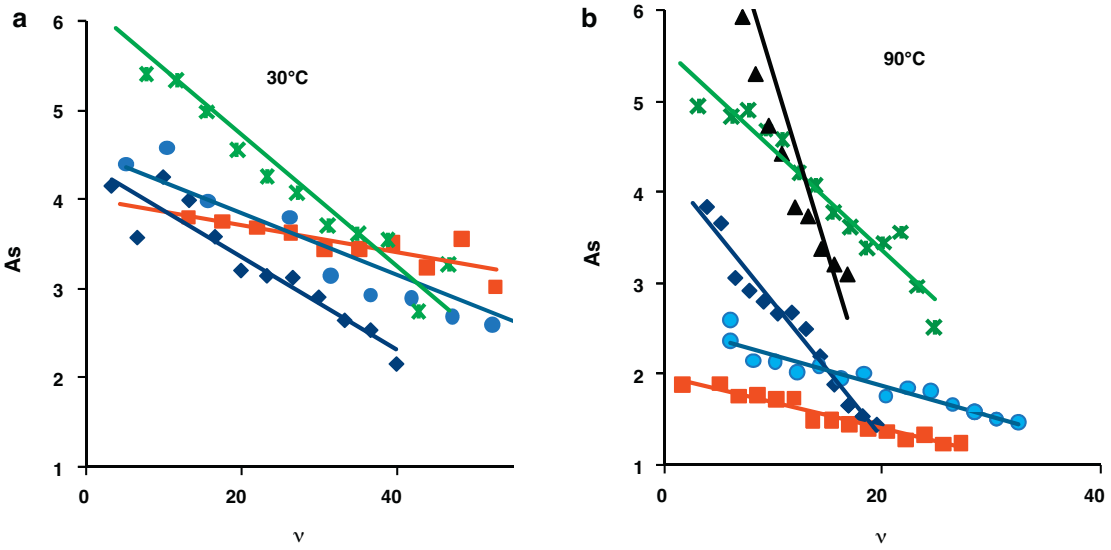


**Fig. 9.** Peak asymmetry as a function of the reduced linear velocity at 30 °C (a) and 90 °C (b). Conditions: Acquity BEH-C18 column (5 cm × 2.1 mm, 1.7 μm). Solutes: 1-Influenza Hemagglutinin (■),3-WDDHH (♦), 5-bombesin (●) and 7-[ile]-angiotensine (✕), 8-bradykinin (▲). Same conditions as in Fig. 7.

both columns have the same permeability and their upper limiting pressure is the same (1000 bar). Accordingly kinetic plots become useless in this case and hence reduced HETP plots are quite suitable to compare kinetic performance between the two studied columns in different mobile phase conditions. All studied pH additives were volatile so that they can be compatible with mass spectrometry detection. In the last part, sample peak capacities are calculated for a sample of ten representative peptides on both columns and compared in different mobile phase conditions at three temperatures.

### 3.1. Comparison of isocratic and gradient methods for the construction of the kinetic curves

As previously pointed out, *h* measures under gradient conditions are useful when a wide variety of compounds are studied since these measures are obtained in a single run for a given flow-rate. Under isocratic conditions, it is necessary to inject the compounds separately with an appropriate mobile phase for each compound (providing similar retention factors). Besides being time consuming, this method runs the risk of some column degradation before completing the whole study due to the huge amount of experiments that are required when different conditions of pH and/or temperature are investigated. Fig. 2 shows reduced plate height ($h = H/d_p$) versus reduced linear velocity ($\nu = u_0.d_p/D_m$) plots for neutral solutes with k value around 3 using fully porous and core-shell sub-2 μm particles. The reduced plate heights values which were obtained from gradient runs are represented with full symbols in Fig. 2. The gradient data for caffeine were obtained together with those for peptides (same runs) and that is the reason of the presence of ammonium acetate in gradient elution. Experimental data were fitted to the Knox equation [30] using the least square method. The good agreement of the data obtained from gradient runs (full symbols) with those obtained from isocratic runs (open symbols) emphasizes the excellent reliability of the proposed gradient method.

The minimum reduced HETP value is 2 for the Kinetex-C18 column whereas it is close to 3 for the Acquity BEH-C18 column. These results confirm those obtained in recent studies on neutral compounds and sub 2-μm narrow-bore columns [16,31,32]. The minimum reduced plate height was found even lower with sub-3 μm particles but it is probably more difficult to pack efficiently 1.7 μm particles than 2.6 μm particles. The slope of the curve beyond the optimum velocity (close to the *c*-term value) which expresses the effect of resistance to mass transfer in both stagnant mobile and stationary phases is flatter with shell particles (about 0.05 for shell particles while 0.09 for totally porous particles). Better performance of shell particles was explained by thickness of the shell, narrower particle size distribution and/or better quality of packing [15]. It was also suggested that the difference in kinetic performance between the two particles might be related to the different degree of external roughness of these two particles [33].

### 3.2. Effect of mobile phase additive and temperature on the reduced HETP plots for basic compounds

The sample with ten representative peptides, caffeine and diphenhydramine was injected on both columns in various mobile phase pH, first at 30 °C, and then at the maximum allowed temperature (60 °C for Kinetex-C18 and 90 °C for Acquity BEH-C18). Separations were performed in gradient elution with different flow-rates ranging from 0.1 mL/min to the maximum flow-rate allowed by the authorized pressure limit (1000 bar for both columns). The plate height calculation was performed according to the procedure described above.

Fig. 3 shows the *h*–ν plots of diphenhydramine on Acquity BEH-C18 (Fig. 3a) and Kinetex-C18 (Fig. 3b). In all studied conditions the results with diphenhydramine (basic compound) are quite different from those with caffeine (neutral compound) with much higher reduced plate height in case of diphenhydramine even at high pH (ammonia or ammonium formate adjusted at pH 10.4) although the dissociation rate of diphenhydramine is expected to be very small in these high pH conditions. *h*–ν plots scatter in the range of very high h values. It is due to the great difficulty of measuring peak variances in this range due to very bad peak shapes. As was pointed out in Section 2, the measure of variances by the Dorsey–Foley equation becomes inaccurate for As >2.5 and thus usually underestimate the actual value. However, an accurate measure of such high *h* values is much less important than in the low range values and therefore the presented *h* values are just expected to give a general trend.

Best results, with *h*–ν plots very close to those of caffeine, were obtained on Acquity BEH-C18 (Fig. 3a) with ammonium acetate

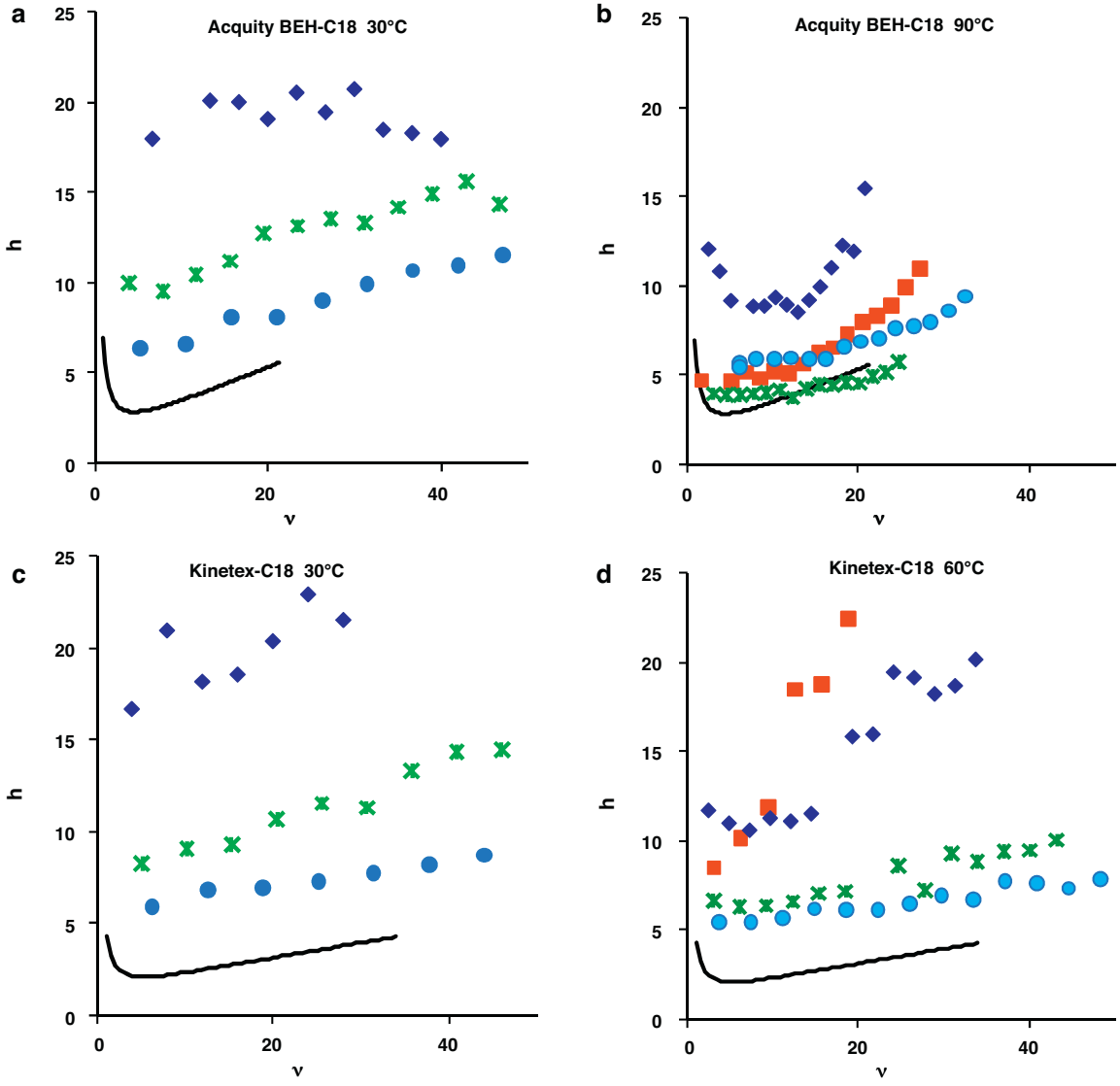


**Fig. 10.** Plots of $h$ versus $\nu$ for peptides with TFA 0.05% as pH mobile phase additive. Columns: Acquity BEH-C18 (5 cm × 2.1 mm, 1.7 μm) at 30 °C (a) and 90 °C (b) and Kinetex-C18 (5 cm × 2.1 mm, 1.7 μm) at 30 °C (c) and 60 °C (d). Solutes: 1-Influenza Hemagglutinin (■), 3-WDDHH (♦), 5-bombesin (●) and 7-[ile]-angiotensine (✕), caffeine (—-). $k$ about 3 for all solutes. Note the significant loose in column performance in all studied conditions compared to the neutral caffeine. Plots of 1-Influenza Hemagglutinin at 30 °C are out of scale.

10 mM as pH additive. Surprisingly reduced plate height values do not vary very much when increasing the reduced linear velocity, especially with Kinetex-C18 (Fig. 3b). The flat shape of these curves might be explained by an unexpected decrease in peak asymmetry with the reduced linear velocity as shown in Fig. 4 for both columns. The decrease in peak asymmetry is particularly significant at low pH with formic acid as pH additive. The same trend was also observed in isocratic elution with different strong bases and various RP-silica columns. Results obtained with formic acid at different temperatures with diphenhydramine and protryptilline are shown in Fig. 5. Similar decrease in peak asymmetry can be observed at the four studied temperatures suggesting that increasing temperature has no effect on peak shape when using formic acid. Similarly, McCalley has recently pointed out a continuous decrease in the asymmetry factor with flow-rate for bases on monolithic RP-silica columns in buffered mobile phases at pH 3 and suggested that this effect might be due to a radial inhomogeneity of the monolith [8]. Yet, this explanation cannot be applied to shell particles which are expected, unlike monoliths, to have a good radial packing homogeneity [34]. We therefore propose an alternative explanation. The continuous decrease in peak asymmetry might result of a positive effect of pressure which can counteract the inhability of the protonated bases to penetrate the pores because of a mutual repulsive effect of charged analytes adsorbed on the packing surface [8]. As a result, high flow-rates might reduce the overloading effect which takes place at low pH with low ionic strength. This explanation is also supported by the fact that an elevation of temperature has no effect on peak asymmetry with formic acid (Figs. 4 and 5) unlike with other additives (Fig. 4) where a higher temperature significantly reduces the peak asymmetry and hence the plate height value. With TFA (pH 2.4), ammonium acetate (pH 6.8) and ammonia (pH 10.4), reduction of peak asymmetry with reduced linear velocity is less significant and only occurs in the c-term dominated region. It appears that for both columns, formic acid is a very bad additive in terms of column efficiency. It is likely that bad peak shapes are due to a low ionic strength thereby leading to mass overload which can occur even with very small amounts of ionised bases [4].

It appears that, with diphenhydramine, Acquity BEH-C18 performs better than Kinetex-C18 in all studied conditions. This was

not expected from the results with caffeine (see Fig. 2). Also this is not in good agreement with recent studies on pharmaceuticals that found comparable or better performance with sub sub-2 μm [35] and even with sub-3 μm [32] superficially porous particles compared to sub-2 μm fully porous particles. However these referenced studies were carried out with TFA as additive and actually the present results show little differences between the two columns. The problem involved by formic acid is much more important with Kinetex-C18 (Fig. 3b) possibly due to different properties of the stationary phases (surface coverage, carbon load, pore volumes) which might lead to a larger amount of C18-chains accessible to ionized compounds on Acquity BEH-C18. Indeed as discussed above, very bad peak shapes with formic acid may be caused by mutual repulsion of charged analytes adsorbed on the packing surface resulting in overloading even at very low analyte concentration. This overloading effect might increase with a decrease of weak sites (hydrophobic interaction). The lower sample capacity of shell particles compared to totally porous particles was recently found to be in line with the proportion of the particle that was porous (i.e. 60%) [7].

As can be seen in Fig. 3, the effect of temperature is dramatic. When temperature is raised from 30 °C to 90 °C (Fig. 3a for Acquity BEH-C18) or from 30 °C to 60 °C (Fig. 3b for Kinetex-C18), $h$–$\nu$ plots are significantly shifted towards the bottom. A major improvement in both efficiency (Fig. 3) and peak asymmetry (Fig. 4) is observed with all studied pH additives except formic acid. The change in peak shape with temperature is shown in Fig. 6 for diphenhydramine. The separations were obtained in gradient elution in different pH conditions at a flow-rate of 1 mL/min on the Kinetex-C18 column. McCalley also investigated the effect of temperature with phosphate as buffer and also observed a better efficiency for strong bases at pH 7 while no change occurred at pH 3 [36]. Better results at higher temperature were usually attributed to an increase in sorption–desorption kinetics and/or a decrease in the dissociation rate with temperature [8,37]. Unlike with other additives, higher temperatures do not improve peak shapes and hence peak efficiencies with formic acid (Fig. 6b and g).

Finally, ammonia at pH 10.4 does not seem to be a good additive either, particularly with the Kinetex-C18 column. At this pH, diphenhydramine is still partly protonated and ionized silanols are likely to be present in a larger extent on the surface of the silica-based Kinetex-C18 column than on the surface of the hybrid organic silica-based BEH column. A significant improvement can be observed when ammonium formate 10 mM (AF-10.4) is adjusted at the same pH (Figs.3b, 6d and e) due to a higher ionic strength and therefore a larger amount of competing cations in the mobile phase.

### 3.3. Effect of mobile phase additive and temperature on the reduced plots for peptides

A similar study was carried out with peptides. Reduced plate height values were measured from gradient data with $k_e$ values close to 3 for all solutes in all mobile phase conditions. With formic acid at 30 °C, the obtained results were similar to those obtained with diphenydramine. That is, for most of the peptides, the peak shapes were very bad, especially on the Kinetex-C18 column as shown in Fig. 7 by the separations obtained with the same normalized gradient slope of 1% on both columns at two different temperatures. As for small basic compounds, overloading effect might be explained by mutual repulsion of charged peptides adsorbed on the material surface and hence by difference in peptide behaviour due to different properties between the two columns. Surprisingly, the effect of temperature on peak efficiency in these mobile phase conditions seems to be more favourable for peptides than for basic compounds as also highlighted by the obtained reduced plate height values given in Fig. 8 for the Acquity BEH-C18 column. Increasing temperature results indeed in an overall downward shift in reduced HETP data suggesting that ionic interactions are more complex for peptides than for monocharged analytes. It should be noted for peptide 7 that a twofold improvement of $h$-values with h values as high as 100 (i.e. about 300 plates) is less than satisfactory. On the other hand, improvement in peak efficiency is particularly attractive for both Peptides 1 and 5 since their $h$–$\nu$ plots get almost mixed up with those of caffeine. It should be noted that the $h$–$\nu$ plots for all peptides on Kinetex-C18 were out of scale at 30 °C and even at 60 °C and hence not shown in Fig. 8. Enhancement of peak efficiency with temperature is associated with an important reduction of peak asymmetry as shown in Fig. 9. On the other hand it is interesting to notice that increasing flow-rate has also a positive effect on peak shape when formic acid is used as additive. As previously discussed for basic compounds, we speculate that the reduction of peak asymmetry could be due to the increase in pressure with flow-rate thereby extending the accessible surface of the stationary phase by forcing charged analytes which are subjected to a repulsive effect, to penetrate into the pores.

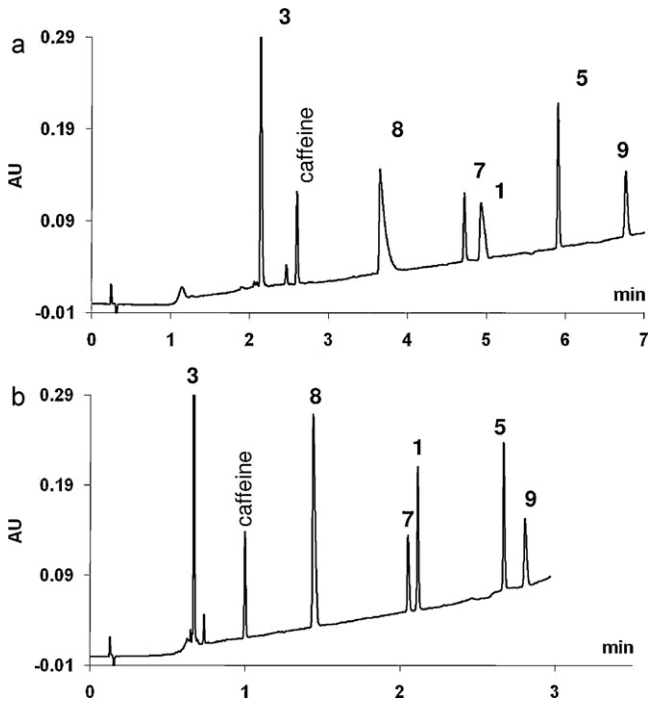


**Fig. 11.** Separation of a peptide mixture on Acquity BEH-C18. Conditions: gradient runs from 1% ACN to 36% ACN in 8 min at 30 °C and 0.5 mL/min (a); in 4 min at 90 °C and 1 mL/min (b); presence of TFA 0.05% in the mobile phase. 1.0 μL injected. Normalized gradient slope: 1%. Peptide names are given in Table 1.

With TFA and its ion-pairing role (Fig. 10), the results at 30 °C are markedly better than those obtained with formic acid. However, it is clear that kinetic performance of both columns at 30 °C (Fig. 10a and c) are totally dependent on the solute and always worse for peptides than for neutral solutes (solid black line for caffeine in Fig. 10). Both columns provided similar reduced plate height values at 30 °C whatever the peptides with very bad results obtained for Peptide 1 ($h > 25$), probably due to its low isoelectric point (3.5) and hence to a difference in the total number of positive charges compared to other peptides. Increasing temperature was found to be very attractive (Fig. 10b and d), especially with the Acquity BEH-C18 column (Fig. 10b). In this latter case, the minimum reduced plate height values of peptides turn out to be close to those of caffeine.

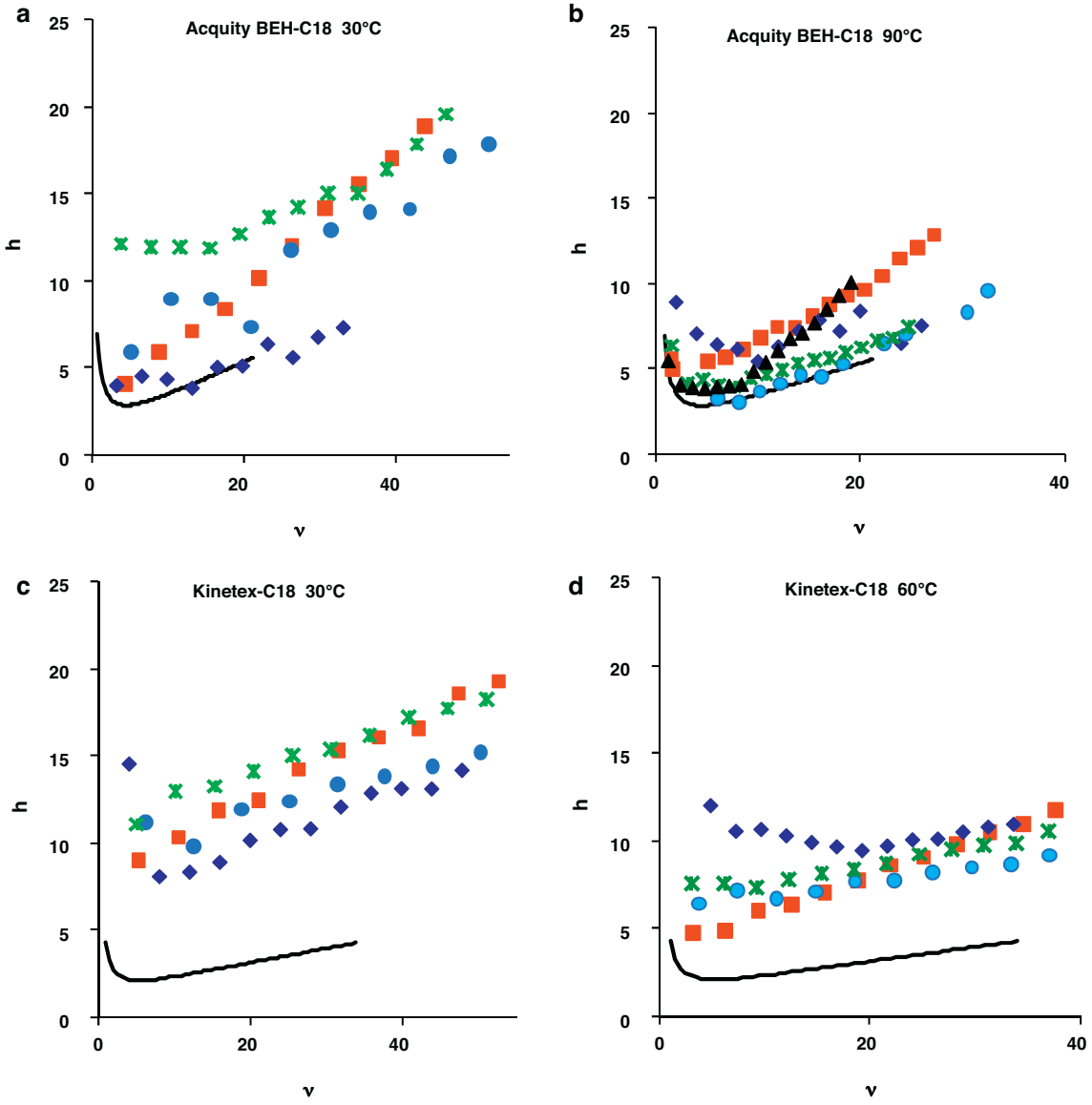


**Fig. 12.** Plots of h versus $\nu$ for peptides with ammonium acetate 10 mM as pH mobile phase additive. Columns: Acquity BEH-C18 (5 cm × 2.1 mm, 1.7 μm) at 30 °C (a) and 90 °C (b) and Kinetex-C18 (5 cm × 2.1 mm, 1.7 μm) at 30 °C (c) and 60 °C (d). Solutes: 1-Influenza Hemagglutinin (■), 3-WDDHH (♦), 5-bombesin (●) 7-[ile]-angiotensine (✕),8-bradykinin (▲), caffeine (—-). $k$ about 3 for all solutes.

The beneficial effect of temperature is also clearly highlighted in Fig. 11 with the comparison of two separations of peptides at 30 °C and 90 °C with TFA as additive on Acquity BEH-C18. Both separations were performed with the same normalized gradient slope. The flow-rate was twice larger at 90 °C thereby leading to nearly the same column pressure drop. In such conditions, diffusion coefficients are nearly twice larger and hence the reduced linear velocity should remain nearly constant from 30 °C to 90 °C. Accordingly, for a neutral compound such as caffeine, the conditions at 90 °C should provide the same efficiency than those at 30 °C but with a significant gain in analysis time (close to a factor 2) whereas for all peptides, in addition to the same gain in analysis time, the conditions at 90 °C offer a great improvement in peak efficiency. With the Kinetex-C18 column, the benefit of increasing temperature is less noticeable with reduced HETP plots staying well above those of caffeine (Fig. 10d). This can be explained by a lower temperature (60 °C for the Kinetex-C18 instead of 90 °C for the Acquity BEH-C18) and perhaps by a difference in column properties resulting in more overloading effect on Kinetex-C18 compared to Acquity BEH-C18.

Finally the results obtained with ammonium acetate are given in Fig. 12 for both columns at two different temperatures. The trends are similar to those observed with TFA, namely better efficiencies on the Acquity BEH-C18 column and significant improvement on both columns by elevating the temperature. The beneficial effect of temperature is also highlighted in Fig. 13 with the separation of peptides on the Kinetex-C18 column. At neutral pH, ionized silanols are expected to play a role in the interactions between the peptides and the stationary phase and the amount of ionized silanols is likely to be larger on the surface of silica phases (Kinetex-C18) than on the surface of silica-organic hybrid phases (Acquity BEH-C18) thereby explaining the difference in reduced HETP data between the two columns shown in Fig. 12. However, on both columns, the higher ionic strength yielded by ammonium acetate 10 mM permits to reach lower plate height values compared to those obtained with formic acid (Fig. 8) or even with TFA (Fig. 10). Higher pH

with ammonium acetate is also probably responsible of such an improvement as pointed out by the difference in reduced HETP data between ammonium acetate 10 mM (pH 6.8) and ammonium formate 10 mM (pH 2.8) (Fig. 14). Reduced plate height values are markedly smaller with ammonium acetate, especially for Peptides 7 and 8, suggesting that a neutral pH is more suited to the separation of peptides in terms of column efficiency.

### 3.4. Comparison of sample peak capacities of peptides in gradient elution

Peak capacities were measured and compared for the same mixture of ten peptides on the two columns with four different mobile phase additives at three temperatures. Conditions and results are given in Table 2. The experimental peak capacities were determined according to the concept of "sample peak capacity" as derived by Dolan et al. [38]:

$$n_c = \frac{t_n - t_1}{w_{\text{average}}} \quad (16)$$

where $t_n$ and $t_1$ are the gradient retention times of the most and the least retained peptides (peptide 5 and peptide 3 respectively) and $w_{\text{average}}$, the average $4\sigma$ peak width. For a given sample in gradient elution, the sample peak capacity provides a reliable comparison of the quality of separation in different analytical conditions. Peak capacities were measured with the same normalized gradient slope of 1% in order to have a similar retention window between the more and the less retained peptides. Peak capacities were determined at maximum pressure allowed by the solvent delivery system. Maximum pressure depends on the flow-rate, namely 900 bar with 1.1 mL/min at 30 °C, 700 bar with 1.4 mL/min at 60 °C and 600 bar with 1.6 mL/min at 90 °C. As a result, these conditions provided the minimum column dead time (maximum velocity) that could be achieved under the studied conditions, namely 5.8 s, 4.3 s for both columns at 30 and 60 °C respectively and 3.9 s for Acquity BEH-C18 at 90 °C. Such very fast separations are of prime interest for the second dimension of on-line comprehensive two-dimensional liquid chromatography which combines two LC modes with different retention mechanisms. Fig. 15 displays the resulting peak capacities for both columns. With all additives, peak capacities were 15–30% higher on the Acquity BEH-C18 column.

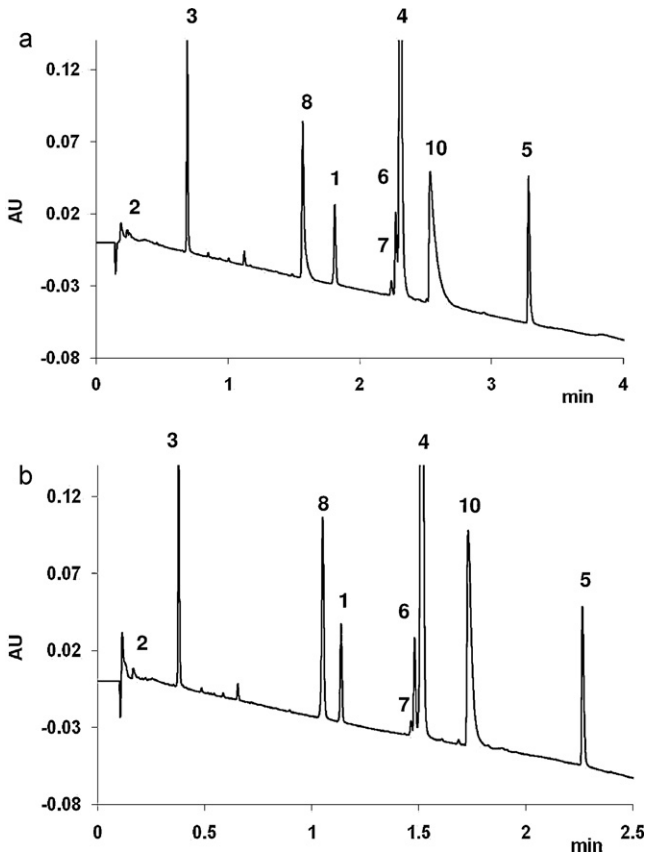


**Fig. 13.** Separation of a peptide mixture on Kinetex-C18. Conditions: gradient from 1% ACN to 35% ACN; 30 °C; 0.7 mL/min; gradient time 4.23 min (a), 60 °C; 1 mL/min; gradient time 2.94 min (b); presence of ammonium acetate 10 mM in the mobile phase. 1.0 μL injected. Normalized gradient slope: 1%. Peptide names are given in Table 1.

On the other hand, for both columns, much higher peak capacities were obtained with ammonium acetate. As an example, it can be seen in Table 2 that a gradient run at 30 °C with ammonium acetate 10 mM should generate a peak capacity twice larger as a gradient run at 30 °C with formic acid 0.1%. Also, higher peak capacities

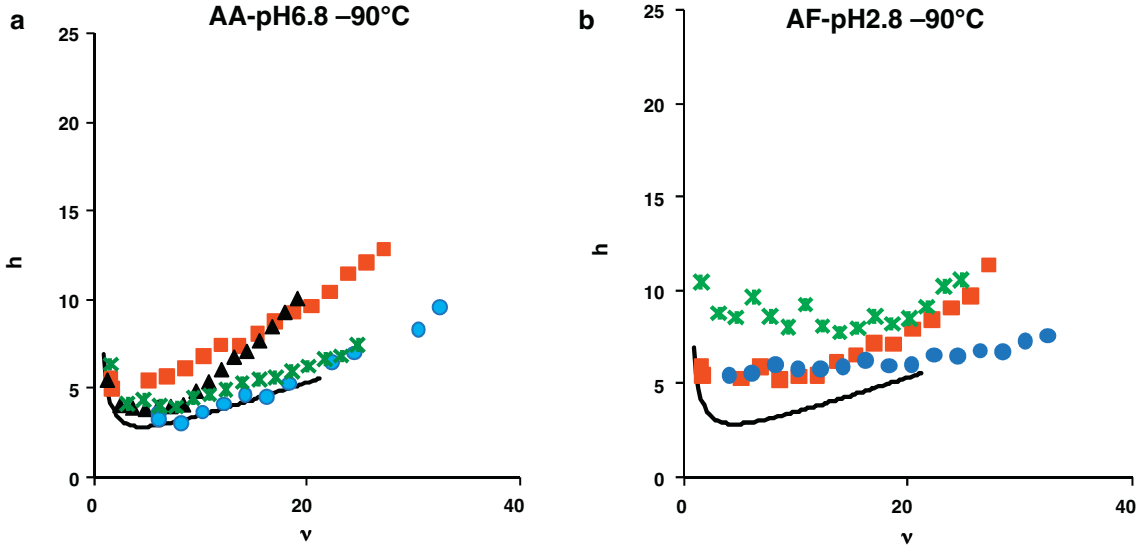


**Fig. 14.** Comparison of $h$–$\nu$ plots on Acquity BEH-C18 between ammonium acetate 10 mM pH6.8 (AA) (a) and ammonium formate 10 mM pH 2.8 (AF) (b). Column temperature: 90 °C; solutes: 1-Influenza Hemagglutinin (■), 5-bombesin (●), 7-[ile]-angiotensine (✕), 8-bradykinin (▲), caffeine (—·—). The plots of 8-bradykinin in ammonium formate are out of scale.

**Table 2**
Comparison of sample peak capacity and sample peak capacity production on a 5 cm Kinetex-C18 column and on a 5 cm Acquity BEH-C18 column at maximum pressure with different volatile additives at 30 °C, 60 °C and 90 °C. Normalized gradient slope: 1%.

| Additive | | Formic acid 0.1% | | | Ammonium formate 10 mM – pH 2.8 | | | TFA 0.05% | | | Ammonium acetate 10 mM | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $T$ (°C) | | 30 | 60 | 90 | 30 | 60 | 90 | 30 | 60 | 90 | 30 | 60 | 90 |
| $\Delta P$[a] (bar) | | 900 | 700 | 600 | 900 | 700 | 600 | 900 | 700 | 600 | 900 | 700 | 600 |
| Kinetex-C18 | $F$ (mL/min) | 0.9 | 1.2 | | | | | 0.9 | 1.2 | | 0.9 | 1.2 | |
| | $t_0$ (s) | 5.8 | 4.3 | | | | | 5.8 | 4.3 | | 5.8 | 4.3 | |
| | $t_n - t_1$ (s) | 98.5 | 75.3 | | | | | 93.8 | 75.0 | | 124.0 | 94.0 | |
| | $n_c$ [b] | 30 | 38 | | | | | 48 | 63 | | 62 | 80 | |
| | $n_c/t_0$ | 5.2 | 8.8 | | | | | 8.3 | 14.7 | | 10.7 | 18.6 | |
| Acquity BEH-C18 | $F$ (mL/min) | 1.1 | 1.4 | 1.6 | 1.1 | 1.4 | 1.6 | 1.1 | 1.4 | 1.6 | 1.1 | 1.4 | 1.6 |
| | $t_0$ (s) | 5.7 | 4.5 | 3.9 | 5.7 | 4.5 | 3.9 | 5.7 | 4.5 | 3.9 | 5.7 | 4.5 | 3.9 |
| | $t_n - t_1$ (s) | 102.4 | 80.6 | 70.0 | 108.0 | 84.6 | 72.6 | 103.0 | 83.7 | 75.7 | 126.0 | 94.0 | 79.6 |
| | $n_c$ [b] | 35 | 51 | 59 | 54 | 64 | 72 | 54 | 74 | 87 | 74 | 89 | 93 |
| | $n_c/t_0$ | 6.1 | 11.3 | 15.1 | 9.5 | 14.2 | 18.5 | 9.5 | 16.4 | 22.3 | 13.0 | 19.8 | 23.8 |

[a] Colum pressure drop at starting gradient conditions.
[b] Sample peak capacity calculated using Eq. (16).

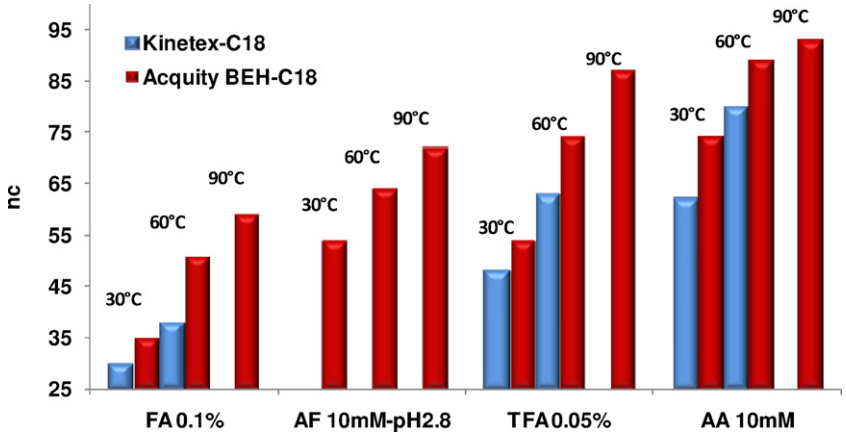


**Fig. 15.** Sample peak capacity for 5 cm Kinetex-C18 and 5 cm Acquity BEH-C18 for the sample of ten peptides. Experimental data points at a pressure of 900 bar at 30 °C, 700 bar at 60 °C and 600 bar at 90 °C. Comparison between different additives: formic acid 0.1% (FA); Ammonium formate 10 mM (FA); TFA 0.05% and ammonium acetate 10 mM (AA) as additives. Normalized gradient slope: 1%. Conditions and results are given in Table 2.

were obtained with ammonium acetate compared with TFA. Peak capacities with ammonium formate at pH 2.8 were nearly the same as with TFA, and hence significantly lower than those with ammonium acetate. In addition to higher efficiencies, the use of ammonium acetate provides larger retention windows ($t_{10} - t_1$ in Table 2) thereby amplifying the difference in peak capacities. Finally, it is worth noting that the elevation of temperature is very attractive in all mobile phase conditions as previously discussed. A major issue with the use of high temperatures is the reduction of retention. In gradient elution this usually results in a reduction in the retention window which may affect peak capacities. This situation was indeed encountered as shown by the difference between the retention times of the most and the least retained peptides given in Table 2. However the loss in retention was largely compensated by a dramatic enhancement of peak efficiency with all studied additives including formic acid, thereby leading to much higher peak capacities at high temperature. Furthermore, in addition to a great improvement in peak capacity, higher temperatures made the use of higher mobile phase velocities possible and hence higher peak capacities were attained in a shorter analysis time as highlighted by the peak capacity production given in Table 2. As an example, with a normalized gradient slope of 1% and ammonium acetate, a peak capacity of about 100 was obtained at 90 °C on Acquity BEH-C18 with a column dead time of less than $s$ (Fig. 16a) while half of this peak capacity value was obtained at 30 °C with TFA (same other conditions). Meanwhile the analysis time (i.e. the dead time and hence the gradient time) was 50% higher. It should

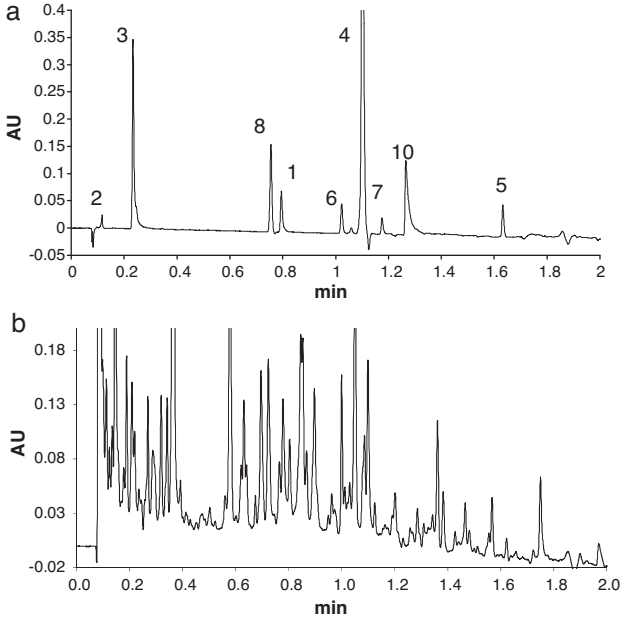


**Fig. 16.** Separation of peptide on Acquity BEH-C18. Gradient from 1% ACN to 32% ACN in 2 min; presence of ammonium acetate 10 mM in the mobile phase; 90 °C; 1.6 mL/min; normalized gradient slope: 1%; 1.0 μL injected; (a) sample of 10 representative peptides (see Table 1) (b) sample of 3 proteins digested by trypsin (BSA, lysozyme, myoglobin). The blank has been subtracted.

be stressed that these conditions do not maximize the peak capacity production on each column as the limiting pressure on this instrument was only 700 bar at 1.2 mL/min (60 °C for Kinetex-C18) and only 600 bar at 1.6 mL/min (90 °C for Acquity BEH-C18) suggesting that the gain in speed should be greater at higher flow-rate provided that the limiting pressure was higher. Finally the fact that the limiting temperature is higher on Acquity BEH-C18 (90 °C), also contributes to achieve better kinetic performances compared to Kinetex-C18 as highlighted by the very high peak capacity values obtained at 90 °C (see Fig. 15 and Table 2). The more attractive found gradient conditions were applied to get an ultrafast analysis (less than 1.8 min) of a complex tryptic digest of three proteins (Fig. 16b). Peak widths and hence peak capacity were in very good agreement with those predicted by the test mixture shown in Fig. 16a.

## 4. Conclusions

In this work, we demonstrated that using gradient elution for collecting HETP data was straightforward and very convenient for the RP-separation of peptides.

It was shown that the kinetic behaviour of charged compounds was totally different from that of neutral compounds and furthermore fully dependent on mobile phase conditions. Thus, results derived from the comparison of columns by means of either reduced HETP plots or kinetic plots have to be carefully evaluated before concluding and should be always related to the studied component. For example, in the present study unlike many other reported studies, the kinetic performance of Acquity BEH-C18 was found to be better than that of Kinetex-C18 in all studied mobile phase conditions. This was demonstrated by considering basic compounds and peptides as analytes and columns packed with 1.7 μm particles. The difference is probably caused by difference in the stationary phase (silanol activity, hybrid silica versus silica) but the difference in particle structure which leads to difference in carbon coverage may also explain the very bad peak shapes obtained with formic acid on Kinetex-C18.

The comparison of kinetic performance between different volatile additives allowed to identify ammonium acetate at neutral pH as the best mobile phase additive for basic compounds as well as for peptides. Despite its poor buffer capacity, the interest in using this pH additive was also pointed out in a recent study on gradient equilibration time [39].

Formic acid should be avoided unless temperature is increased. Elevating temperature was indeed proved to be very useful for drastically reducing plate height values of charged compounds. Furthermore the reduction of solvent viscosity with temperature permitted to increase the flow-rate and hence to decrease the analysis time. As a result, the peak capacity production was increased two or even threefold from 30 °C to 90 °C on Acquity BEH-C18 while the peak capacity was 20–50% higher depending on the mobile phase conditions (Table 2). Such conditions might be very attractive to speed up the second dimension of a comprehensive on-line 2D-LC separation while maintaining a reasonable peak capacity. An illustrative separation of a tryptic digest of three proteins was in good accordance with the prediction.

It is obvious that reduced HETP plots of charged compounds are more difficult to interpret via the A, B and C-terms of the Knox equation compared with neutral compounds. The role of the component dissociation (silanols and solutes), of the pH and of the ionic strength on the performance of columns is likely to be essential. However the role of the pressure on the reduction of peak asymmetry was also pointed out. Enhancement of the peak shape was attributed to a decrease in overloading effect thanks to the increase in pressure with flow-rate. However, more work is still needed to confirm our hypothesis.

## Acknowledgement

The authors would like to thank Waters and Phenomenex for the generous gift of the columns used in this work

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