Sense and nonsense of high-temperature liquid chromatography

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Abstract
High-temperature liquid chromatography (HTLC) is recognized today as a valuable technique in reversed-phase high-performance liquid chromatography (RP-HPLC). Column temperature can play a role in reducing analysis time, modifying retention, controlling selectivity, changing efficiency or improving detection sensitivity. The different effects of high temperatures on reversed-phase separations, the practical limitations due to the instrumentation, the limits and the main advantages of HTLC, especially for the separation of polar and ionized compounds, are reviewed.

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1. Introduction

High-temperature liquid chromatography (HTLC) is a term which refers to any separation carried out at temperatures above room temperature (typically within a range from 40 °C to 200 °C) with a mobile phase in a liquid state. Although the use of the name “HTLC” is quite recent, interest in temperature in LC has been mentioned since the first earlier developments of HPLC [1]. A survey of the literature over the last decade reveals that the interest in high temperatures has considerably grown, as shown by the several reviews that have been recently published [2–7]. However, up to now, temperature is not considered as a key parameter in LC and does not appear very often in routine work. Some aspects probably limit the use of high temperatures, including the lack of commercially available instruments equipped with ovens which can reach very high temperatures (as high as 200 °C) and the lack of reliable and thermally stable stationary phases. Yet, the complexity of the changes involved by the variation of numerous relevant physico-chemical parameters with temperature might also discourage the chromatographers. Only one effect is sometimes emphasized, e.g. the decrease in mobile phase viscosity which leads to a decrease in column pressure drop, whereas some other important effects such as the reduction of eluent strength, the change in selectivity, the increase in diffusivity or the change in dissociation rate for ionizable compounds may be neglected, thereby altering the conclusions about the real benefits and the real limitations of high temperatures. In this review, an attempt is made to encompass all the different effects of high temperatures under reversed-phase conditions while giving an overview of seminal works and most recent studies on the use of HTLC.

2. The driving forces towards the use of high temperatures in liquid chromatography

2.1. Need for faster analysis—reduction of mobile phase viscosity

2.1.1. Theoretical background

In the early days of liquid chromatography, there was already a need for faster separations and some famous studies dealt with this subject [8–11]. Researchers are still very interested in finding more and more efficient ways to increase the analysis speed in order to improve the sample throughput for routine analysis. Twenty years ago, Anitra and Horvath [12] showed that the effect of temperature on both viscosity and diffusivity allowed the use of higher linear velocities thereby leading to faster analysis. They predicted a 15–20-fold decrease in analysis time when a column is operated at 200 °C rather than 25 °C. The relationship between the diffusion coefficient, the solvent viscosity, and the absolute temperature, T, can be expressed by the Wilke-Chang equation [13]:

\[ D_m = 7.4 \times 10^{-8} \left( \frac{\Phi M}{\eta V_a} \right)^{1/2} T \]

where \( D_m \) is the solute diffusion coefficient at a very low concentration in solvent (in cm²/s), M, the solvent molecular weight (in g/mol); T, the absolute temperature (in K), \( \eta \), the solvent viscosity (in cP), \( V_a \), the solute molar volume (in cm³/(g⁻¹ mol⁻¹)) and \( \phi \) is the solvent association factor (dimensionless). Other equations have been reported and compared to experimental values [14–16].

According to Eq. (1), \( D_m \eta / T \) is constant for a given solvent and a given solute. Thus, the reduction of the mobile phase viscosity with temperature leads to an increase in the diffusion coefficient. Numerous relationships giving the variation of viscosity of acetonitrile–water or methanol–water mixtures with both temperature and solvent composition have been published in the literature. Some of them are valid within a small range of temperatures [17,18] while some others were empirically established by measuring the pressure drop across the column, up to temperatures as high as 200 °C for both acetonitrile–water [19] and methanol–water mixtures [20]. According to Guillarme et al. [20], viscosity can be calculated as

\[ \eta = 10^{-2.249-(714/T)} \times (912/T) V_{MeOH} + 1.859 \times (968/T) V_{MeOH} \]

(2)

\[ \eta = 10^{-(12.063+(602/T)} \times (52/T) X_{ACN} + 0.504 \times (346/T) X_{ACN} \]

(3)

for methanol–water and acetonitrile–water mixtures, respectively.

According to Knox and co-workers [10,21] the relationship between the reduced plate height, \( h \), defined as \( H / d_p \) (\( H \) being the plate height and \( d_p \) the particle size) and the reduced velocity, \( V \), defined as \( u dp / D_m \) (\( u \) being the linear velocity of the mobile phase) is given to a good approximation by

\[ h = A v^a + B v + C v \]

(4)

The A term depends on both the quality of column packing and the contribution of slow mass transfer across the moving stream, the B term represents the contribution to band dispersion from longitudinal diffusion, the C term is the mass transfer parameter which reflects the equilibration slowness between moving and stationary or stagnant zones [22] and the exponent \( n \) is generally taken as 1.3. Knox [23] gave typical values for A, B and C-terms: B in the range 2–4, A around 1, and C around 0.1. From data obtained between 20 °C and 60 °C on different silica-based stationary phases, Knox and Vasiari [24] found no dependence of h upon temperature at a specific reduced velocity for any solute and concluded that there was no intrinsic change in properties of the packing material with the temperature. A unique curve for neutral solutes was also experimentally obtained for temperatures up to 180 °C with silica-based and non-silica-based stationary phases [20,25]. For a given stationary phase and solute, it can hence be assumed that the three coefficients only depend on the packing quality and the retention factor [22]: they are independent of the temperature for neutral solutes and provided that the packing quality is kept constant, they do not depend on the particle size. For given coordinates \( (h, v) \), the column dead time, \( t_d \) needed to reach a given plate number, \( N = L / h dp \), is inversely proportional to the diffusion coefficient according to

\[ t_d = N h \frac{d_p}{v D_m} \]

(5)

As a result, the gain in speed when increasing temperature corresponds to the ratio of the diffusion coefficient at high temperature \( (T_2) \) to the diffusion coefficient at low temperature \( (T_1) \) and is therefore given by \( (\eta_1 / \eta_2) \times (T_2 / T_1) \). The viscosity ratio, \( \eta_1 / \eta_2 \), strongly depends on both the type and the volume fraction of organic modifier as shown in Fig. 1. The ratio is higher with methanol–water
than with acetonitrile–water mixtures and of the same order of magnitude from 20 °C to 100 °C as from 100 °C to 200 °C.

The comparison between two techniques is useful provided it is supported by relevant rules. Thus, a global view of the gain in analysis speed when increasing the temperature demands that the overall separation power be kept constant. The concept of peak capacity, first introduced by Giddings [26], is a useful tool for measuring the separation power of a given analysis. The peak capacity can be defined as the number of peaks that can be ideally placed between the first and the last peak with a resolution of 1 between all peaks. If the first and the last peaks are related to non-retained and most retained compounds, respectively, the peak capacity can be easily calculated. In isocratic conditions, it is given by [27]

$$n_c = 1 + \frac{\sqrt{N}}{4} \times \ln(1 + k)$$

(6)

where $k$ is the retention factor of the most retained compound.

The peak capacity (Eq. (6)) is unchanged provided that the volume fraction of organic modifier is decreased thereby keeping constant the solute retention factor (same eluent strength). On the other hand, the viscosity ratio is mostly lower than the one that would be expected if the mobile phase composition was kept constant when increasing the temperature. As underlined by Thompson and Carr [19], the theory of speed must take into account the relationship between retention factor, temperature, and volume fraction of organic modifier. They therefore calculated the real viscosity ratio between 25 °C and 200 °C for acetonitrile–water as mobile phase and butylbenzene as solute. Depending on the required $k$ value, this ratio only varied from 3 to 4 which, when multiplied by $T_2/T_1$, corresponds to a gain in speed not higher than 6.5.

In reversed-phase gradient conditions, the peak capacity can be approximated by the following Eq. (7), assuming that the contribution of the dwell volume to the retention is not significant and the retention factor at the beginning of the gradient is very large [28]:

$$n_c = 1 + \frac{\sqrt{N}}{4} \times \frac{1}{(t_0/T_C)(1 + (B \Delta C))}$$

(7)

where $T_C$ is the gradient time, $t_0$, the column dead time, $\Delta C$, the difference in solvent composition between the initial and final composition of the gradient and $B$, the slope of the relationship between the Neperian logarithm of the retention factor and the organic solvent concentration, which is not expected to vary much with temperature. With a view to assessing the gain in speed in gradient conditions, both the difference in solvent composition, $\Delta C$, and the ratio $t_0/T_C$ should be kept constant in order to maintain the same peak capacity. This is not always done and may lead to an overestimation of the gain in speed.

The column pressure drop, $\Delta P$, is related to the characteristics of both column and mobile phase through the well-known Darcy equation which is usually written as

$$\Delta P = \frac{\eta L}{K_0}$$

(8)

where $K_0$ is the specific column permeability.

For given coordinates ($h, \nu$), the column pressure drop needed to reach $N$ is then given by

$$\Delta P = \frac{N h v}{K_0} \eta D_m$$

(9)

As a result, when keeping in mind that $D_m \eta/T$ is constant, the required column pressure drop increases when temperature is increased. This is true for any point of the $h$–$\nu$ curve and in particular for the minimum corresponding to the optimum column performance. This pressure increase has been taken into account in some papers [20,29]. If not, conclusions about temperature effect on the speed of HTLC separations compared to classical HPLC ones could be overly optimistic. Higher temperatures do not allow lower pressures when separations are carried out at the same $\nu$ value, which is not the case if they are incorrectly carried out at the same $u$ value.

Yet, although the pressure drop increase is limited from 20 °C to 100 °C (about 30%), it is more significant at 200 °C (80%). Moreover, the additional tubing needed at high temperature as heat exchanger leads to an additional pressure drop in the system thereby reducing the pressure available for the column. This issue will be discussed in the fourth section.

2.1.2. Understanding speed through kinetic plots

The preceding equations do not take into account the maximum pressure drop allowed by the different chromatographic systems and therefore do not reflect the potential of a given temperature. If pressure would not be limited, the ideal temperature in terms of analysis speed would be the highest possible one and the gain in speed would be the diffusivity ratio for all points of the $h$–$\nu$ curve. A good way for comparing the speed performance of different techniques consists in making use of kinetic plots [30] which were originally proposed by Giddings [8] by plotting the separation time versus $N$ and later by Poppe by plotting $t_0/N$ versus $N$ in logarithmic coordinates [31]. The kinetic plots were used to evaluate the performance of HTLC [25,32,33], the combined use of HTLC and ultra-high-pressure liquid chromatography (UHPLC) [34] and to compare HTLC to other fast chromatographic techniques [35]. For a given maximum pressure, $\Delta P_{max}$, the kinetic plots translates any points ($h, \nu$) into a plot of the minimum $t_0$ needed to reach a given plate number, $N$, according to the two following equations:

$$t_0 = \Delta P_{max} \frac{K_0 d_p^2}{v^2 D_m \eta}$$

(10)

$$N = \Delta P_{max} \frac{K_0}{h v D_m \eta}$$

(11)

Hence, for each temperature, the plot of $t_0$ versus $N$ gives a curve, as shown in Fig. 2 for two different temperatures, 30 and 90 °C [36]. As indicated by the arrows, the gain in speed decreases when the desired plate number increases. In these conditions, the two curves intersect at a critical plate number close to 50,000 plates, meaning that for efficiency lower than this value, 90 °C is beneficial while it is detrimental for higher values. Lestremau et al. [32] did not observe any experimental crossing of the curves at 30 and 80 °C as it should be theoretically observed. Such a result was ascribed by the authors to a lower experimental $B$-term at 80 °C than at 30 °C, resulting from a lower $k$ value at high temperature. In addition, it could be due to
the significant difference in the flow resistance values observed at 30 and 80 °C (520 and 464, respectively) while such values should not have to vary very much with temperature.

2.1.3. Concluding remarks
To summarize, increasing temperature is a practical way to reduce the analysis time while keeping the same efficiency. If a unique $h$–$u$ curve exists which is mostly the case with neutral compounds, some relevant conclusions can be drawn.

1. At fixed column length (variable column pressure drop), the theoretical gain in speed for a given efficiency is equal to the $D_m$ ratio and the column pressure drop increases.
2. At fixed column pressure drop (variable column length), the theoretical gain in speed is dependent on the desired plate number. It is maximum (close to the $D_m$ ratio) for small efficiencies (short columns). It is smaller (or even reversed) for high efficiencies (long columns).
3. When a plate number cannot be reached at a given temperature, it can neither be reached at any higher temperature. In particular, with a high column length, a small particle size or a very viscous mobile phase, if a given plate number cannot be reached at room temperature, it is neither attainable at higher temperature. As sometimes reported, an increase in plate number can be induced by an increase in temperature. This is usually obtained by working in non-optimum conditions, usually by comparing separations at a fixed analysis time (fixed flow-rate) [37].

2.1.4. Applications
A review of recent papers suggests that applications for fast analysis fall into two categories: (1) those that use conventional pressures (<400 bar) and temperatures up to 200 °C [37,38–41] and (2) those that combine the use of sub-2 μm particles, ultra-high pressures (>400 bar) and temperatures up to 90 °C [29,42,43]. A very fast gradient separation on a sub-2 μm stationary phase by combining high temperature (90 °C) and UHPLC (650 bar) is shown in Fig. 3 [36]. It is important to note that such a separation, performed in less than 15 s, must require a very high data acquisition rate of the detection system (>50 Hz).

Many of the very fast separations found in the literature concern synthetic mixtures of standard test compounds (parabens, alkylbenzenes, etc.) showing that HTLC research is still in progress and that this technique is used only scarcely in routine analysis. Unfortunately, as discussed above, the comparison of analysis speed between low and high temperature is often reported without adjusting either the mobile phase composition in isocratic elution to keep the same eluent strength or the gradient time in gradient elution to keep the same gradient volume thereby leading to conclusions which are too much promising.

The three techniques currently investigated to speed up analysis times (i.e. HTLC, monoliths, sub-2-μm particles associated to very high pressures) were recently compared as part of the quantitative analysis of a pharmaceutical sample [35]. With respect to the analysis speed they concluded that sub-2 μm particles at 30 °C and 1000 bar are more attractive than 5 μm particles at 90 °C. However, in these latter conditions the flow-rate was not increased to its maximum value, namely to the one leading to the maximum pressure allowed by the instrument and therefore to the minimum analysis time.

Overall, the implementation of a LC analysis includes several steps, including sample treatment, additional time required by the chromatographic instrument to inject and data analysis which all, are time consuming and consequently can limit the interest for ultra-fast separations.

2.2. Need for “green chromatography”—reduction of the percentage of organic solvent

2.2.1. Effect of temperature on the retention
The effect of temperature on solute retention factor, $k$, can be expressed by the van’t Hoff equation:

$$\log(k) = -\frac{\Delta H_0}{2.303R} + \frac{\Delta S_0}{2.303R} + \log \Phi$$  \hspace{1cm} (12)

where $\Delta H_0$ and $\Delta S_0$ are respectively the enthalpy and the entropy of solute transfer from the mobile phase to the stationary phase, $T$ is the absolute temperature, $R$ is the universal gas constant and $\Phi$ is the phase ratio of the column (the volume of the stationary phase divided by the volume of the mobile phase). $\log \Phi$ may be considered as independent of the temperature although Coyne and Chester [44] examined the possibility of such a dependence. When $\Delta H_0$ and $\Delta S_0$ are also invariant with temperature which is usually
the case for neutral compounds, the plot of \( \log k \) versus \( 1/T \) is linear with a slope of \( \Delta H_0 / 2.3R \) and an intercept of \( \Delta S_0 / 2.3R + \log \Phi \). However a quadratic dependence of \( \log(k) \) versus \( 1/T \) over a wide range of temperatures and using silica-based as well as non-silica-based stationary phases was observed by different authors [20,29,45–48]. This phenomenon is not yet fully explained. In case of silica-based columns, curved van’t Hoff plots were attributed to dissimilar enthalpies involved in the solvophobic and silanophilic interaction [49]. Increasing the water content of the mobile phase gives rise to a concave curvature in the van’t Hoff plot as predicted by the dual retention mechanism. The examination of retention behaviour as a function of temperature was therefore used as a reliable method for evaluating the retention mechanism in RPLC [50,45]. Alternatively, deviations from linearity were observed on silica-based columns and attributed to the so-called “phase transition” phenomenon. The transition is a consequence of a conformational change of the stationary phase going from a solid-like (low temperature) to a liquid-like (high temperature) state. As a result, the curve can be sometimes divided into two linear plots which intersect at the transition temperature. However, most time the transition is more diffuse and may occur over a large temperature range [51]. The transition usually appears in the 20–50 °C range for C18 silica-based stationary phases [50,52] although Liu et al. [53] observed a transition temperature of about 100 °C for a hybrid C18 stationary phase. With non-silica-based columns, curved plots are probably explained by a variation of both enthalpy and entropy with temperature. However, Guillarme et al. [20] observed a curious dependence of the solute behaviour on the type of solvents when an organic polymer was used as stationary phase. This phenomenon was not explained. As illustrated in Fig. 4, van’t Hoff plots were indeed linear with water–methanol mixtures (Fig. 4a) while curved with water–acetonitrile (Fig. 4b).

It was shown, for neutral compounds, on silica-based columns, that a temperature increase of 4–5 °C had roughly the same effect on retention as a 1% increase in organic solvent concentration [54,55]. This correspondence was shown to be the same on zirconia-based columns [36]. Thus, by elevating the temperature under reversed-phase conditions, it is possible to significantly reduce the content of organic solvent while keeping the same eluent strength. A low content of organic solvent is attractive as it allows handling more “green” mobile phases. However with polar or ionized compounds which exhibit very low retention, high temperatures may result in an excessive decrease in retention even with pure water as mobile phase. When a gradient elution is needed, if the early peaks elute close to 100% water, the gradient has to start with a fully aqueous mobile phase, even at room temperature. Recently Neuhe [56] has examined the effect of temperature on peak capacity in gradient elution when working close to the maximum performance of the column. Elevated temperatures always reduce peak capacity if the initial composition of the gradient is held constant (i.e. 100% water). In this case, there is a decrease in the final composition of the gradient (decrease of \( \Delta C \) in Eq. (7)) due to a reduction in retention thereby leading to a decrease in peak capacity with increasing temperature.

2.2.2. Use of pure water as mobile phase

At high temperatures, water has many of the characteristics of aqueous–organic eluents in terms of eluotropic strength. Its polarity can be controlled by changing temperature [57]. The use of pure water as mobile phase has been investigated, mostly at temperatures higher than 100 °C so that the eluent strength is high enough. Two recent reviews [6,58] have covered this topic in detail including the equipment required and the current applications, so we will only briefly mention the possibilities and the limits of this technique which is called either “superheated water chromatography”, “subcritical water chromatography” or “chromatography in very hot water”, depending on the authors.

In addition to its attractive advantage of non-toxicity, superheated water offers the possibility of hyphenation of interest with special detectors [4] including the flame ionization [59–62], the low wavelength UV [63], the inductively coupled plasma mass spectrometry [64] and the nuclear magnetic resonance spectroscopy detectors using hot deuterium oxide as mobile phase [65–67]. However, some problems can arise when using superheated water. (1) The eluent strength cannot be increased during the analysis except if the solvent gradient is replaced by a temperature gradient. (2) Non-polar solutes can be still too retained even at high temperatures. Indeed, the eluent strength of pure water at 150 °C is only roughly the same as a mixture of water–methanol 50–50 (v/v) at room temperature [68]. (3) Very hot water is a very aggressive solvent. As reported by Teutenberg et al. [69], the number of available high temperature stationary phases suitable for superheated water is very limited. (4) The electrospray ionization mass spectrometry detection is expected to be less sensitive when the percentage of organic solvent decreases [70,71]. (5) Some problems may arise when the solute has a low solubility in cold water. In this case, a stronger injection solvent can be necessary which may lead to further peak distortion after peak injection as discussed in a recent paper [72].

2.3. Need for alternative methods

2.3.1. Temperature programming

Temperature programming instead of gradient elution could be useful in a few situations. (1) For separations with micro- or nanocolumns when a gradient elution is not easy to operate at very low flow-rates on a given gradient system although a few instruments are currently especially designed for gradient elution in nano-LC and work very well. (2) When working with superheated water

![Fig. 4](Image). Plot of log k versus 1/T for the PLRP-S column: (a) with water–methanol mobile phases; (a) 50:50 (v/v), solute m-toluidine; (b) 50:50 (v/v), solute ethylparaben; (c) 60:40 (v/v), solute benzene. From Ref. [20] with permission.
since a temperature gradient represents the only way of increasing the eluent strength during the analysis. (3) With instruments possessing large dwell volumes thereby leading to large isocratic holds in gradient elution. A review entirely dedicated to temperature programming and its related problems of instrumentation has been published in 2004 [73]. More recently, Vanhoenacker and Sandra [5] reviewed some applications. The use of capillary columns is usually recommended for temperature programming due to a better heat transfer with small inner diameters [54,74,75]. However, some specific problems contribute to limit the applications. (1) The range of compositions needed for a composition gradient is usually wider than 50% which is consistent with a range of temperatures wider than 200 °C. However, current commercially available equipments are still unable to cover such a large range of temperatures. (2) The required ramp of temperature is often too steep (> 20 °C/min) to be applied to LC-separations, heat transfer in liquids being very slow compared with those in gas. In addition, heating of the massive amount of steel associated with HPLC columns can be too slow as well. As a result, temperature programming is mostly used to improve isocratic isothermal analysis rather than to replace gradient elution conditions [3,38,76]. As soon as 1970, Snyder [77] compared gradient elution and temperature programming and concluded that this latter was limited in application compared with gradient elution. On the other hand, flow programming should accompany temperature programming to maintain an optimum velocity during the whole analysis. This is rarely done although the combined use of both gradient modes was proved to be attractive [78]. In some particular cases, a temperature gradient can also be combined with a solvent gradient to enhance the selectivity [5] as illustrated in Fig. 5: it was impossible to reach the complete resolution of all pairs of peaks under isothermal conditions whereas the use of a moderate temperature gradient made it possible to achieve higher resolution between solute pairs. It can also be noted that an inverse temperature programming in packed capillary LC was applied by Andersen et al. [79] to improve the separation of polyethylene glycol (PEG) oligomers.

2.3.2. Temperature as useful parameter for method development

According to the van’t Hoff equation (Eq. (12)), column temperature has a strong effect on retention. The influence of temperature on selectivity is also widely acknowledged and a recent review has been dedicated to this topic [2]. Zhu et al. [80] have listed the different situations favoring change in selectivity with temperature. Those include (1) when the relative retention of two solutes is sensitive to changes in the conformation of the stationary phase as temperature is varied, (2) when the relative size or shape of two molecules is different, leading to differences in their entropies, (3) when two molecules have different functional groups with a different temperature dependence for the retention and (4) when a ionizable solute is partially ionized, so that the molecule exists in both ionized and neutral forms. This latter situation will be extensively highlighted in the next section.

Change in selectivity or even reversed selectivity with temperature was observed for polymers such as PEG oligomers [79,81] or Triton X100 [82,83] on various stationary phases. At low temperature, oligomers with a high molecular weight (long ethylene oxide chain) elute before those with a smaller degree of polymerization (more hydrophobic). A non-linear van’t Hoff behaviour leading to an increase in the retention with temperature can be observed. At moderate temperature (e.g. 50 °C) with the same mobile phase composition, there is no selectivity between the oligomers. At higher temperatures a satisfactory oligomeric separation can be obtained whereas the elution order is reversed and identical to the one observed in normal phase. This irregular temperature-dependent retention behaviour was attributed to the existence of different conformational states of the molecule depending on the temperature [81].

Other studies have shown variation in selectivity through the variation of stationary phase properties with temperature. The retention can be modulated by a temperature-responsive polymer with reversible hydrophilic–hydrophobic conformation [84,85]. Snyder et al have published a series of articles on the combined use of temperature and solvent strength in gradient elution [80,86–88]. Column temperature together with mobile phase

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**Fig. 5.** Analysis of a mixture of ten triazine and ten phenylurea pesticides on a Zorbax StableBond-C18 column (150 mm × 4.6 mm I.D., 1.8 μm d.) Flow-rate: 1 mL/min, gradient: water/ACN 80:20 to 45/55 in 30 min. Temperature program (lower chromatogram): initial 40 °C hold 0–1 min, ramp 40–60 °C at 1.3 °C/min, and ramp 60–90 °C at 10 °C/min. From Ref. [5] with permission.
composition can be considered as an important parameter for controlling selectivity [89] and for developing RPLC methods [90–96]. Melander et al. [97] examined various relationships between logarithmic retention factor, mobile phase composition and column temperature in order to find a convenient equation to describe the retention behaviour of numerous polar and non-polar compounds. They concluded that the retention data could be very well fitted by the linear equation given below:

$$\log(k) = A_1 \phi \left(1 - \frac{T_c}{T}\right) + \frac{A_2}{T} + A_3$$  \hspace{1cm} (13)

where $\phi$ is the volume fraction organic co-solvent of the hydro-organic mobile phase, $T_c$, the so-called compensation temperature [98,99] which was found to be usually constant in RP chromatography and $A_1, A_2, A_3$ are parameters varying with solutes and stationary phases. Gant et al. [100] proposed a similar equation. This equation was used to develop optimization software in order to rapidly optimize both temperature and mobile phase composition. For a given sample, four initial experiments are carried out at two different temperatures, using either isocratic or gradient elution. On the basis of Eq. (13), the corresponding data allow to calculate the resolution map as a function of both temperature and either mobile phase composition in isocratic mode or gradient slope in gradient elution mode [90,95,96].

Finally, Mao and Carr [101–103] have proposed the concept of thermally tuned tandem column for the optimization of selectivity. It consists in serially coupling two columns exhibiting quite different selectivity. Each column temperature can be independently controlled and consequently, each temperature can be individually optimized. The mobile phase composition is kept constant so that the selectivity could be changed without a large change in retention. This technique is attractive provided that the two columns can separate different critical pairs. It is quite similar to an iso-elutropic ternary solvent optimization [104], the two organic solvents being replaced by the two stationary phases and the iso-elutropic solvent composition by the temperature.

3. The use of high temperatures for the separation of pharmaceutical compounds

Most of the pharmaceutical and biological substances possess ionizable functions such as carboxylic or amino groups. The separation of these compounds and more especially the basic ones is still a difficult challenge. For basic compounds, the separation is often performed at low pH where the ionization of the residual silanols at the silica surface is suppressed thereby reducing the problems of peak tailing but giving rises to low solute retention. The use of high temperature can overcome many of the problems encountered at room temperature.

3.1. Effect of temperature on dissociation rate

The acid base equilibrium being given by $HB_\text{z} \rightleftharpoons B_\text{z}^{-1} + H^+$ ($z$ being the charge ($z \leq 0$ for acids; $z = +1$ for bases), the acid dissociation constant, $K_a$, is given by

$$K_a = \frac{a_{H^+} a_{B}^{-1}}{a_{HB}}$$  \hspace{1cm} (14a)

which can be usually approximated by

$$K_a \approx \frac{a_{H^+} c_{B}^{-1}}{c_{HB}}$$  \hspace{1cm} (14b)

where $a_i$ and $c_i$ are the activity and the concentration of the indicated species, respectively. From Eq. (14b), the dissociation rate of a given solute at a temperature, $T$, in a given mobile phase can be expressed by

$$\tau = \frac{1}{1 + 10^{pH - \frac{pK_{a,\text{solute},T}}{2.3R}}}$$  \hspace{1cm} (15)

where $pH$ and $pK_{a,\text{solute},T}$ refer to pH of the mobile phase and $pK_a$ of the solute, respectively, both measured at $T$ in the same medium using a pH-meter calibrated with standards in the same medium.

For sake of convenience, the pH is most often measured at room temperature in aqueous medium and the resulting measure cannot be relied upon. For a given mobile phase, the variation of the acid dissociation constant with the absolute temperature is given by

$$\frac{d(pK_a)}{d(1/T)} = \frac{\Delta H^0_a}{2.3R}$$  \hspace{1cm} (16)

where $\Delta H^0_a$ is the standard enthalpy of ionization and $R$, the gas constant. As a result

$$\frac{d(pH - \frac{pK_{a,\text{solute},T}}{2.3R})}{d(1/T)} = \frac{(\Delta H^0_{a,\text{buffer}} - \Delta H^0_{a,\text{solute}})}{2.3R}$$  \hspace{1cm} (17)

According to Eqs. (15) and (17), the variation of the dissociation rate with temperature is then directly dependent on the difference in enthalpies of ionization of buffer and solute and is therefore significant when this difference is large. Since the enthalpies of ionization are usually large for basic compounds (cationic acids, $z = 1$) and small for acidic ones (neutral or anionic acids, $z \leq 0$) [105–107], large variations can be expected with basic solutes in acidic buffers.

Fig. 6. Variation of the dissociation rate versus $1/T$ for amitriptyline with different buffer–acetonitrile compositions: 70:30 (v/v) (○); 60:40 (v/v) (▲); 50:50 (v/v) (●); 40:60 (v/v) (■); 30:70 (v/v) (×). Phosphate buffer (a) and Tris buffer (b). From Ref. [111] with permission.
Using a method based upon chromatographic data and described elsewhere [110], Heinisch et al. [111,112] have studied the variation of the dissociation rate with temperature for various ionizable compounds (acid and basic), in different buffers (phosphate, citrate and Tris) by determining the so-called chromatographic $pK_a$, \( pK_{a,\text{chrom}} = pK_{a,\text{solute, T}} - \Delta pK_{a,\text{buffer}} \). Form 30 °C to 90 °C, pH values can be shifted of 2 pH units and of up to 3 pH units from 30 °C to 120 °C for a basic solute using a phosphate buffer whereas no shift was observed with the same solute using a Tris buffer. The resulting variations of dissociation rate are given in Fig. 6 for different buffer-acetonitrile compositions. Consequently, as mobile phase pH, temperature may be a powerful parameter to vary the dissociation rate of basic compounds. According to the preceding results, the dissociation of a basic compound in acidic buffer is quite identical when the pH is varied by 2 pH units at a given temperature as when the column temperature is varied by 60 °C at a given pH.

### 3.2. Effect of temperature on retention and selectivity of ionizable compounds

The effect of temperature on the retention of partially ionized compounds which exist in two forms is also well described in Eq. (12). However, both enthalpy and entropy are expected to be different for the two forms (molecular and ionized forms) and as a result, both \( \Delta H_0 \) and \( \Delta S_0 \) can vary with temperature when both forms are present to a significant extent. Melander et al. [106] and later Castells et al. [107] have developed a complex relationship to describe the retention of a partially ionized solute with a unique acid-base equilibrium, according to the classical assumption that the retention factor is the weighted mean of the retention factors of the individual forms. An increase in retention with temperature has been noted by several authors but the form of the curve was often assumed to be a straight line [107,109,113,114]. The theory predicts [106,115] and experimental data show [111,116,117], that in a chromatographic system operated at a mobile phase pH close to the $pK_a$ values of both the buffer and the solute, van’t Hoff plots may not yield straight lines depending on the relative magnitude of the enthalpy changes. As shown in Fig. 7 for a silica-based column and in Fig. 8 for an organic polymer column, the shape of the curves depends on the type of buffer although the same pH was adjusted in the aqueous medium. With a phosphate buffer, van’t Hoff plots for basic compounds are not linear and furthermore, the retention increases with temperature. This atypical phenomenon is less important with an ammonium acetate buffer and can be related to a difference in the variation of the dissociation rate with temperature depending on the buffer (see Eqs. (15) and (17)). As a result, contrary to neutral compounds where only four experiments are sufficient for optimizing both temperature and mobile phase composition (see above), it was recommended to perform nine initial experiments in case of basic compounds in order to get reliable optimized conditions [111].

From a theoretical study about selectivity variation with temperature, Li [115] concluded that great changes in selectivity with temperature can be expected for ionizable compounds and that buffer composition, $pK_a$ and enthalpy of ionization have a significant effect on this variation. From a rapid examination of the curves in Fig. 7a and b, it is clear that temperature can be a powerful tool to vary selectivity and could replace the mobile phase pH in method development. There are probably different reasons to prefer temperature rather than pH for selectivity tuning. (1) The relationship between retention and temperature is smooth and therefore the retention modelling as a function of temperature is more reliable than as a function of pH. (2) Temperature is a more flexible and easily adjustable parameter than pH. (3) Optimizing temperature instead of mobile phase pH can give rise to more rugged analysis. As a matter of fact, a 2 °C change in temperature can lead to the same change in solute dissociation rate as a 0.05 pH unit change. Such a variation can therefore be controlled more easily. (4) Finally, as recently underlined by Dolan [118], when small errors in buffer preparation have been made leading to a reduction in the expected peak resolution, a small change in temperature might be sufficient to adjust the method conditions in order to improve the separation of peaks poorly resolved.

### 3.3. Effect of temperature on peak shape

Poor peak shape of basic compounds can be due to different factors including kinetic phenomena, dual retention mechanism or overloading effects. Alternatively, a mutual repulsion of charged species in the stationary phase was also mentioned to explain poor peak shape at acidic pH [119,120], this effect being reduced when
the ionic strength is increased [119,121]. The influence of these different factors on peak shape can be significantly reduced by increasing temperature. As a matter of fact, the effect of temperature on peak shape is widely acknowledged [102,111,113,122,115] but not yet fully explained. McCalley [113] reported a significant increase in efficiency with temperature, higher at pH 7 than at pH 3, often accompanied by a considerable reduction in peak asymmetry. He suggested that this increase in efficiency might be due to kinetic effects and improvement in mass transfer of solute with silanols. It was indeed proposed that peak tailing could be involved by slow kinetics of “strong” sites such as silanols together with fast kinetics of hydrophobic sites [124,125]. So, increasing the temperature may increase the kinetics of the slow sites in such a way that they tend towards those of the fast sites thereby improving the peak shape for basic compounds. Moreover, the dissociation rate of both analytes and silanols changes with temperature, thereby probably contributing also to the improvement in peak shape. The effect of temperature on van Deemter curves for basic compounds was examined in two papers [122,126] with a phosphate buffer at both acidic and neutral pH. This study revealed a significant decrease of the minimum plate height by raising the temperature from 20 °C to 60 °C, particularly at neutral pH. According to the authors, these results are consistent with the above explanation in view of the fact that there is a larger number of ionised silanols at neutral than at acidic pH.

Increases in efficiency for basic and polar compounds were also reported with alternative materials: zirconia-based [20,101], Hypercarb [117], organic polymer [60] and bare silica [116]. Such a significant increase in peak efficiency on a zirconia-based column is illustrated in Fig. 9 from 25 °C to 150 °C for the separation of caffeine derivatives. These experiments were carried out with the same eluent strength, obtained by adjusting the mobile phase composition [20]. Peak shape improvement observed on a carbon-coated zirconia [102] was explained by a reduction of the strong electronic interactions between the polar groups of the solutes and the carbon surface, those probably responsible of the dramatic peak tailing at low temperature. On the other hand, a continual increase in efficiency up to the highest tested temperature (80 °C) was noted on bare silica with reversed-phase conditions for different basic compounds and was explained by the effect of temperature on the adsorptive and electrostatic forces which contribute to retention [116].

3.4. Effect of temperature on gradient re-equilibration time

The gradient re-equilibration time is a limiting parameter when a high throughput is needed. It represents the time necessary to
re-equilibrate the column at the initial mobile phase composition prior to the next injection. Recent studies have examined the influence of temperature on the re-equilibration volume needed for neutral as well as ionizable compounds. The conclusions about the effect of higher temperature were different depending on the authors. Schellinger et al. [127–129] have distinguished two distinct states of re-equilibration: the repeatable equilibrium which provides excellent run-to-run reproducibility and the full equilibrium which is expected to be reached when all retentions times no longer change when the re-equilibration time varies. They did not observe a significant effect on the time required for full equilibrium when the temperature was increased from 40 °C to 80 °C. It should be noted yet, that the analysis was performed by adding a small amount of n-butanol to the mobile phase [129] throughout the solvent gradient to provide constant solvation of the stationary phase as recommended by Cole and Dorsey [130]. Moreover, the initial mobile phase contained 10% of acetonitrile which is a much less critical initial composition than pure water with respect to the column re-equilibration. Such conditions are quite favourable for a low re-equilibration time and as a result it was probably difficult to highlight any effect of temperature. In contrast, the beneficial role of temperature was clearly highlighted with a neutral compound as probe solute and with pure water as initial solvent [131]. The authors studied three different stationary phases: a polar embedded, a polar endcapped and a classical C18 phase and concluded that there was a significant decrease of the re-equilibration volume when the temperature was increased from 10 °C to 50 °C. They also noted a reduction of the difference in re-equilibration volume between the three phases at higher temperature. Finally, the re-equilibration volume needed for full equilibrium was established at acidic [36] and neutral [132] pH starting with a 100% aqueous mobile phase and for a set of ionizable compounds. At both pH, a drastic reduction of the required re-equilibration volume was observed which results, considering the necessary increase in flow-rate with temperature, in a re-equilibration time divided by a factor 20–30 when going from 30 °C to 90 °C.

4. Practical aspects and limitations of high-temperature liquid chromatography

4.1. Thermal stability of solutes

The instability of some solutes at high temperature can limit the use of high temperatures for the analysis of complex mixtures. The occurrence of the on-column reaction depends on both the solute residence time in the column and the reaction rate in the column conditions. The rate of reaction can be higher into the column than in free mobile phase if catalytically active sites exist on the stationary phase [7,133]. From a theoretical approach, Antia and Horvath [12] predicted that the on-column reaction could be insignificant if the increase in the reaction rate with an increase in temperature was well compensated by the decrease in the residence time due to an increase in flow-rate. According to these authors, the interaction of the retention process and the on-column reaction can be quantitatively measured by the Damkohler number (Da) which is defined as the ratio of the residence time in the column to the relaxation time for the on-column reaction [134]. The Da number may keep low values under fast analysis conditions and in this case, no on-column reaction can be expected to occur. This was recently illustrated by the separation of three proteins at 120 °C [39]. The separation was performed in less than 25 s using a 3.3 cm x 0.21 cm silica-based column in acidic mobile phase. The unexpected stability of the peptide bonds of the proteins under the operating temperature and pH can be fully explained by the short residence time of the proteins in the chromatographic column. The combined use of HTLC and UHPLC using columns packed with small particles could be very useful for further reducing the analysis time and thereby improving the on-column stability of analytes. It should be pointed out that the reaction rate is also dependent on the mobile phase pH and usually expected to be lower in neutral than acidic or basic medium. Neutral pH conditions should be therefore preferred at high temperature. On the other hand, in some particular cases where the increase in reaction rate is small compared to the decrease in residence time, high temperatures are expected to give more interesting analysis conditions than room temperature would do. Anyway, in case of unstable compounds, high temperatures should not be used without making sure that the analysis is reliable. Thompson and Carr examined criteria to check whether a given analyte can be analyzed at high temperature [135]. From the calibration curves of thermally unstable pharmaceuticals at very high temperatures, they demonstrated that reliable separations could be obtained even though the compound was decomposed on the column, provided that the decomposition peak was not separated from the parent peak.

4.2. Thermal stability of stationary phases

The thermal stability of stationary phases is a real problem which considerably limits the use of HTLC. The maximum allowed temperature is low for most silica-based columns used in reversed-phase conditions, particularly in acidic or basic buffered eluents and usually it should not exceed 60 °C. High temperatures can result in a significant reduction in column life due to increased rate of silica hydrolysis, which is not the case for silica-based stationary phases especially designed for their improved stability at high pH. There are currently different approaches to improve the pH stability of silica-based columns at high pH and probably at high temperature: (1) grafting an organic protective layer on the silica surface, (2) modifying silica with bonding and endcapping technology so that the packing material is more stable at high pH, (3) synthesizing a hybrid particle resulting in organic groups incorporated into the silica matrix [5,136]. On the other hand, a novel polydentate C18 silica column was recently evaluated at high temperatures under acidic, basic and neutral mobile phase conditions [137]. The good performances (see Table 1) were explained by the bridged structure which can protect the underlying silica from hydrolysis and dissolution. Other factors can lead to further reduction in the thermal stability of stationary phases: a recent study has shown that the stability of silica-based columns at high temperature in acidic medium may be affected by the presence of metal impurities released from the stainless steel inlet frit which could catalyze the hydrolysis of the siloxane bonds [138]. Alternative packing materials have been developed to withstand much higher temperatures. Those include metal oxide-based columns [139,140], porous graphitic carbon and organic polymer. Unfortunately, these stationary phases are often less efficient than silica-based ones, especially with polar and basic compounds.

Presently, there is a lack of information about the long-term thermal stability of the different chromatographic supports and moreover there is no universal test which could provide an objective comparison. Many stability studies on various stationary phases have been reported [69,137,141–145]. However the studied parameters are often too much different to have a good idea of the relative stability of the studied stationary phases. It should be noted that a good thermal stability in given conditions is not a guarantee for obtaining long-term stability in alternative conditions. The decisive question for the chromatographer remains the number of runs that can be performed on a given column, in given mobile phase conditions without damaging the separation. An overview of studies on the long-term thermal stability of different reversed-
<table>
<thead>
<tr>
<th>Ref</th>
<th>Column</th>
<th>Column dimensions</th>
<th>Column classification</th>
<th>Temperature (°C)</th>
<th>Mobile phase conditions</th>
<th>Probe solutes</th>
<th>Performance parameters</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>[53]</td>
<td>Xbridge-C18</td>
<td>100 mm × 4.6 mm, 5 μm</td>
<td>Hybrid organic silica-based zirconia-based</td>
<td>200</td>
<td>Water (5 mL/min)</td>
<td>Butylbenzene</td>
<td>k</td>
<td>10% change in k over 1-month</td>
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<tr>
<td>[143]</td>
<td>PBD-ZrO2</td>
<td>100 mm × 0.5 mm, 3 μm</td>
<td>Zirconia-based</td>
<td>100 and temperature programming (50–100 °C in 5 min)</td>
<td>ACN–20 mM phosphate buffer, pH 7.0 (50:50) (10 μL/min)</td>
<td>Toluene, fluorene, Amtripitline</td>
<td>k, N</td>
<td>Rapid decrease in both N and k with the cycles of temperature programming</td>
</tr>
<tr>
<td>[142]</td>
<td>Zorbx RX-C8</td>
<td>150 mm × 4.6 mm, 5 μm</td>
<td>Silica-based</td>
<td>100</td>
<td>Water (1 mL/min)</td>
<td>Caffeine, methyl benzoate, benzene Benzene, toluene, methyl benzoate</td>
<td>k, N</td>
<td>No change in both k and N over 6000 column volumes</td>
</tr>
<tr>
<td>[144]</td>
<td>Nucleosil C18 AB</td>
<td>150 mm × 4 mm, 5 μm</td>
<td>Silica-based</td>
<td>100</td>
<td>Water (0.4 mL/min)</td>
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<td>Change in both k and N over 8000 column volumes</td>
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<tr>
<td>[142]</td>
<td>Hypersil BDS C18</td>
<td>150 mm × 4.6 mm, 5 μm</td>
<td>Silica-based</td>
<td>100</td>
<td>Water (1 mL/min)</td>
<td>Mixture of chlorophenols Naphthalene, m-xylene, toluene</td>
<td></td>
<td>Change in both k and N over 700 column volumes</td>
</tr>
<tr>
<td>[144]</td>
<td>ZirChrom-PS</td>
<td>100 mm × 2.1 mm, 3 μm</td>
<td>Zirconia-based</td>
<td>100</td>
<td>Water (0.4 mL/min)</td>
<td>Caffeine, m-cresol, p-acetophenotride</td>
<td></td>
<td>No significant change in both k and N over 7600 column volumes</td>
</tr>
<tr>
<td>[142]</td>
<td>Hamilton PRP-1</td>
<td>100 mm × 2.1 mm, 10 μm</td>
<td>PS-DVB polymer</td>
<td>100, 150</td>
<td>Water (0.4 mL/min)</td>
<td></td>
<td></td>
<td>No significant change in both k and N over 12000 column volumes at 100 °C, 8000 column volumes at 150 °C</td>
</tr>
<tr>
<td>[40]</td>
<td>PBD-ZrO2</td>
<td>100 mm × 4.6 mm, 2.5 μm</td>
<td>Zirconia-based</td>
<td>100</td>
<td>Water (5 mL/min)</td>
<td>Alkylbenzenes</td>
<td>k</td>
<td>No significant change in k over 7000 column volumes</td>
</tr>
<tr>
<td>[144]</td>
<td>Hypercarb</td>
<td>100 × 4.6 mm, 7 μm</td>
<td>Graphitic carbon</td>
<td>Temperature programming (40–200 °C at 15 °C/min)</td>
<td>ACN–water (50:50) (2 mL/min)</td>
<td>Alkylbenzenes</td>
<td>k</td>
<td>No column bleed under temperature programming, no loss of efficiency or retention after 3 runs</td>
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<td>ACN–water (50:50)</td>
<td>7 Acidic, basic and neutral compounds</td>
<td>Baseline drift, average k and N</td>
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<td>No column bleed under temperature programming, no loss of efficiency or retention after 3 runs</td>
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<td>Significant column bleed under temperature programming</td>
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<tr>
<td>[145]</td>
<td>Zorbx RX-C18</td>
<td>150 mm × 4.6 mm, 5 μm</td>
<td>Silica-based</td>
<td>40, 60</td>
<td>ACN–250 mM phosphate buffer pH 7.0 (20:80) (1 mL/min)</td>
<td>Amount of silica dissolved</td>
<td></td>
<td>Minor amount of silica dissolved at 40 °C after 141 L of eluent while continuous amount at 60 °C after 21 L eluent</td>
</tr>
<tr>
<td>[69]</td>
<td>ZirChrom-Carb</td>
<td>150 mm × 4.6 mm, 3 μm</td>
<td>Zirconia-based</td>
<td>185</td>
<td>Water (1 mL/min)</td>
<td>p-Cresol, ethylbenzen, nitrobenzene</td>
<td>k, Rs, As, system pressure</td>
<td>Continuous decrease in k over 50 h heating time; no significant change in Rs, As and system pressure</td>
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<td>Continuous decrease in peak widths over 50 h heating time; no significant change in Rs and system pressure</td>
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<td>Continuous decrease in peak widths over 50 h heating time; no significant change in Rs and system pressure</td>
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<tr>
<td>[137]</td>
<td>Blaze C18</td>
<td>100 mm × 2.1 mm, 3 μm</td>
<td>Polysiloxane</td>
<td>100–200 °C in 20 °C increments</td>
<td>ACN–water, MeOH–water, ACN–10 mM aqueous buffer pH 10, 9, 3, 2</td>
<td>Acetophenone</td>
<td>N, tr, As</td>
<td>About 20% change in performance parameters at 200 °C with 80% organic solvent at 100 °C with pure water, at 60 °C with ACN-aqueous buffer pH 10</td>
</tr>
</tbody>
</table>
phase stationary phases is given in Table 1. The differences in the reported studies involve mobile phase conditions (acidic, neutral, basic, rich in organic solvent or not), column dimensions, column performance parameters (retention factor, peak asymmetry, column efficiency, column pressure drop and amount silica dissolved), probe solutes, test procedures and column temperatures.

Teutenberg et al. [69] suggested testing various performance parameters for evaluating the columns. Their resulting test separations prior to and after 50 h heating time in water at 185 °C are shown in Fig. 10 for zirconia-based and carbon-based columns. A significant decrease in retention and changes in selectivity can be observed on the carbon-clad zirconia column (Fig. 10a and b) while other parameters seem to be unaffected. In contrast, the treatment at high temperature gives rise to a severe loss in peak efficiency with the Hypercarb column (Fig. 10c and d). Other parameters are unaffected. This example clearly shows that a unique performance parameter is not sufficient to evaluate the long-term thermal stability of columns. On the other hand, the results of Table 1 show that temperature programming has a much more detrimental effect on column stability than isothermal analysis. Moreover, Marin et al. [144] evaluated the column bleed under temperature programming using different mobile phase pH conditions (acidic, neutral and basic) and found, for the three tested zirconia-based columns, an excessive rise in the baseline which can be very detrimental to detection techniques such as mass spectrometry, evaporative light scattering detection and flame ionization detection, which are very sensitive to column bleed. The bleeding effect was also studied in isothermal analysis on two separate identical C18 sub-2 μm columns [29]. The authors compared with positive ion electrospray MS detection mode, the baseline data obtained at 30 and 90 °C and concluded that there was a bleeding at 90 °C but that its intensity varied depending on the column. However, the extent of the problem of column bleed and its effect on the background signal has not yet been investigated.

4.3. Specific instrumentation

One of the major problems of HTLC is the design of a chromatographic system that minimizes both thermal mismatch and extra-column band broadenings. Thermal mismatch band broadenings can result from (1) insufficient mobile phase preheating and (2) viscous heat dissipation across the column length. Both phenomenon produce longitudinal and radial thermal gradients. Radial thermal gradients decrease efficiency whereas longitudinal thermal gradients affect solute retention factors.

4.3.1. Mobile phase preheating

When the mobile phase entering the column is not at the column set temperature, a longitudinal thermal gradient takes place with a difference in temperature between the column outlet and the column inlet which results in a decrease in retention factor from the cold column inlet to the warmer column outlet. In the meantime, the solvent molecules entering the wall region of the column reach the column set temperature before those entering the centre of the column. An efficient mobile phase preheating is therefore needed to avoid temperature mismatch. Fig. 11 illustrates the effect of inadequate preheating on the separation of alkylbenzenes at 150 °C with a flow-rate of 4 mL/min using a forced circulating air oven [20]. The use of one meter tubing length led to dramatic peak distortion (Fig. 11b), especially for more retained compounds while two meters tubing length provided acceptable peak shapes (Fig. 11a). A significant increase in the retention of last eluted solutes can also be observed with an inadequate preheating.

The difference in temperature between the incoming mobile phase and the column itself is recommended to be smaller than ±5 °C in order to keep good column performance [147–149]. Because specifications for valve rotor give usually an upper limit of 75 °C, mobile phase preheating must be achieved after the injector valve. Jones [73] reviewed the different designs for mobile phase preheating, including long lengths of connection tubing between
the injector and the column, shorter length in contact of heater blocks and counter current heat exchanger transferring the energy exiting the column to the incoming mobile phase entering the column. A heat transfer correlation was used to estimate the tubing length needed to heat the mobile phase using both air and liquid heat-transfer media [149]. A major conclusion was that adequate control of mobile phase and column temperature can be more easily achieved by using narrow bore columns (2.1 mm I.D.) rather than conventional ones (4.6 mm I.D.). In addition to mobile phase preheating, mobile phase cooling is required when using a UV detector and cooling blocks (metal-to-metal contact). Thermal output is controlled by measuring the temperature immediately downstream from the heater. Such active preheating requires shorter tubing. The efficiency of heat transfer with a stainless steel tube in a block heater is expected to be two to five times greater than in a forced air oven [148].

4.3.2. Extra-column dispersion

A major problem associated with HTLC analysis is the need for some additional tubing length resulting in both additional extra-column dispersion and additional system pressure [20,150]. According to Thomson and Carr [19], the full speed of LC will not be available in the absence of major improvements in commercially available equipment. The required length of additional tubing can be critical for shorter columns. The tubing must be long enough to avoid thermal mismatch broadening and short enough to avoid excessive extra-column broadening. A good compromise is obtained by reducing both column and tube internal diameters [20]. This is yet difficult with instruments only equipped with stainless steel tubing, such as UHPLC instruments, because reliable tubes having very small internal diameters (<127 μm) are presently not commercially available. As a result, extra-column band broadening can be significant with narrow bore columns (2.1 mm I.D.) and unacceptable for micro-bore columns (1 mm I.D.). This is illustrated in Fig. 12 by four separations, obtained in UHPLC conditions, at a low and a high temperature (30 and 90 °C) with the same 5 cm column length but different column internal diameters (2.1 and 1 mm) [34]. The increase in plate number with the retention of the three parabens clearly indicates that peak broadening is principally due to extra-column dispersion, at least for the less retained compounds. The solute dispersion in the tube is the result of the resistance to mass transfer across the tube due to the parabolic velocity profile of the mobile phase under conditions of laminar flow. According to the theoretical Golay equation, the variance in volume units can be approximated by [148]

\[
\sigma_{tubing}^2 = \frac{\pi \tau t L F}{24D_m}
\]

where \( \tau \), and \( L \) are the tubing radius and tubing length, respectively and \( F \) the mobile phase flow-rate. While band broadening in the injector valve and in the detector cell is to a first approximation independent of the flow-rate [151], Eq. (18) shows that it increases in tubing with flow-rate. While the linearity of variance with flow-rate was experimentally verified with rather long tubing [152], it does not apply with coiled and/or short tubing [153,154]. As a result, although the variance increases with flow-rate, it is usually smaller at high velocities than in the corresponding ideal long straight tube (Eq. (18)) [34]. On the other hand, it was shown that for a given reduced linear velocity (a given ratio \( F/D_m \) inside the column), the overall extra-column variance was larger at high temperature although the ratio \( F/D_m \) was expected to remain constant in the tubes as well [36]. This is most probably due to lower \( D_m \) values in the tubes than in the column because a significant part of the tubing is subjected to lower temperatures (the cooling tube for example). It should be yet noted that in gradient elution, the band broadening in both the injection unit and the preheating tube, is subjected to a focusing effect and hence less detrimental than the band broadening in the cooling tube. The detrimental effect of tubing on extra-column dispersion and extra-column pressure drop has been investigated by means of the so-called “total instrument” kinetic plot equations [33,34] which are very useful to investigate the performance limits of a given type of support if used in a given type of instrument with given instrumental limitations (extra-column volume, maximum flow-rate, maximum column length, and maximum detector frequency). With a conventional HPLC system, an oven equipped with a heater block to preheat the mobile phase and conventional columns (4.6 mm I.D.) with 3 μm particle size, the combined effect of both extra-column band broadening and pressure drop has involved about 30% loss in the gain factor which was potentially available when going from 30 °C to 120 °C [33]. However, it was also shown that the speed loss caused by the extra-column band broadening and the additional pressure drop was smaller than the separation speed limitation coming from the flow-rate limitation and the detector frequency limitation which both depend on the specifications of conventional HPLC instruments. With a UHPLC system and narrow-bore columns (2.1 mm I.D.) with 1.7 μm particle size [34], the effect of pressure on performance was found to be negligible. In contrast, the presence of the extra-column band broadening significantly increased the min-

Fig. 11. Chromatograms showing the effect of efficient (a) and inefficient (b) preheating on the peak shape of alkybenzenes. Column: ZirChrom-DRC18 50 mm × 4.6 mm, flow-rate: 4 mL/min, temperature 150 °C, preheating tube length: 2 m (a) and 1 m (b). From Ref. [20] with permission.
Fig. 12. Isocratic separation of seven aromatic compounds on two Acquity BEH C18 columns, 50 mm × 2.1 mm, 1.7 μm and 50 mm × 1 mm, 1.7 μm at 30 and 90°C. Solutes: uracil; caffeine; methylparabene; o-cresol; ethylparabene; β-naphthol; propylparabene. From Ref. [34] with permission.

imal time needed to achieve a given plate number and this effect was significantly larger at 90°C than at 30°C. As a result, the gain in analysis time when elevating the temperature was less than the expected one and as a result, the use of high temperatures may be sometimes not justified. Because of the significance of extra-column dispersion, the authors suggested that 2.1 and 1 mm I.D. columns packed with 2.5–3.5 μm particles could be more attractive, in terms of speed, for the current HT-UHPLC instruments than columns with the same bore diameter but packed with sub-2 μm particles.

4.3.3. Column ovens

Column temperature can be controlled either by block heaters, forced circulating air ovens (converted GC oven), jackets with circulating liquids in them (water or oil) or direct heating of the column through electrical resistance in contact with the column. Presently, most available column ovens use either still air (all UHPLC instruments) or forced circulating air.

Frictional heating between the mobile phase and the column bed together with poor heat dissipation in packed columns also induce both longitudinal and radial thermal gradients across the column [147]. According to Halász et al. [11], the magnitude of this effect increases linearly with operating pressure. This problem is of topical interest with the current use of smaller particles and the combined use of HTLC and UHPLC. A theoretical [155] and two experimental [156,157] studies have recently shown the evidence and the effects of temperature gradients due to frictional heating. With a still-air column oven leading to approximate adiabatic conditions, a longitudinal temperature gradient dominates, resulting in a gradient of retention factors across the column. However, no significant loss of plates is observed. In contrast, with a water bath leading to almost ideal heat transfer, the temperature gradient is mainly radial resulting in additional band broadening and therefore in a significant loss of efficiency [156]. Both situations can be encountered simultaneously with available ovens. It was suggested that the mobile phase enters the column at a lower temperature than the column walls so that an opposite radial gradient temperature can be formed in the first part of the column which may compensate for the positive gradient in the second part, coming from frictional heating [148,158]. Alternatively, like preheating, frictional heating can be less difficult to handle with capillary or micro-bore columns which can produce faster heat dissipation. An additional problem may arise from the use of temperature programming conditions. Ovens should rapidly come back to the initial temperature of the temperature program which eliminates numerous column heaters. In fact, only a forced circulating air oven is a possible candidate. Yet, in this case, heat transfer into the column is very slow which often results in thermal lag between the oven set-point and the interior of the column [73]. Once again, only the use of capillary columns can readily overcome this problem.

5. The future possibilities and development of high-temperature liquid chromatography

It is not easy to imagine the future of HTLC because although not really new this technique is not widely used yet. The beneficial effects of medium temperatures (up to 100°C) are noticeable and besides, efficient equipments, including chromatographic instruments and fairly stable stationary phases, are nowadays commercially available. In spite of additional advantages of higher temperatures it is often not worth working at very high temperatures when considering the risk of either column life reduction or efficiency, retention and/or selectivity decrease. As previously discussed the development in more stable silica-based columns or in alternative efficient packing materials, as well as advances in instrumentation are necessary before the technique can move from the domain of academic research into the domain of routine analysis. Very often, the temperature is neglected as a relevant tool to vary selectivity and moreover to enhance peak efficiency. In contrast the benefits of high temperatures in the domain of very fast separations are most often overestimated. The decrease in eluent strength when increasing temperature is sometimes integrated into
the real benefit of temperature on the analysis speed. This leads to overestimation of the temperature benefit and therefore, to optimistic conclusions. It is obvious that HTLC should be combined with UHPLC because these techniques are fully complementary, HTLC leading to faster separations for a given efficiency and UHPLC allowing higher efficiencies. Looking at the different fields into which HTLC should appear as clearly of interest, it seems that two major domains could be very promising: (1) the use of high temperatures to speed up the separation of very complex mixtures and (2) the use of high temperatures for the separation of biomolecules.

5.1. Increasing the separation power per time unit

There is a growing interest for columns with high separation power (i.e. leading to high peak capacity, e.g. >300), because of increasingly complex samples, particularly those derived from metabolomic and proteomic research. A high peak capacity is necessary for the successful resolution of a large number of compounds [159]. For a given column, highest peak capacities are obtained in gradient elution mode since all peaks are roughly of the same peak width [160]. According to Eq. (7), if both $\Delta C$ and $t_o/T_C$ cannot be further improved, the only variable for increasing peak capacity is the column plate number. High peak capacities can therefore be achieved by coupling several LC columns in series. In such cases, the price that has to be paid is both high pressures and long analysis times. This was highlighted in the second section and has been extensively discussed on the basis of theoretical equations in a recent review [161]. The problem of pressure can be partially overcome by carrying out separations either with a large particle size on a conventional LC instrument with very long columns, at the cost of huge analysis time or with a lower particle size on an UHPLC instrument [162], thereby allowing faster analysis with shorter columns. An alternative method for achieving high peak capacities with suitable pressures consists in using monolithic columns with low permeability [163]. From a theoretical point of view, all these techniques can be combined with high temperatures in order to further reduce the analysis time. It is important to point out that HTLC is not a need for achieving separations with a high plate number but it is an efficient way of speeding up separations to acceptable analysis times. Moreover, as reported in the third section, there are additional attractive advantages of high temperature for polar and basic compounds. As regards monolithic columns, commercially available silica-based monolithic columns are presently not designed for withstanding high temperatures whereas organic monolithic columns, more stable at high temperature, usually provide poor peak efficiency due to slower mass transfer. Recently, a 1-m long alkyl methacrylate-based monolithic stationary phase has been operated at 80 °C and yet, only 20 000 plates/m could be reached [164]. Conventional silica-based columns carried out at a medium temperature seem to be more attractive. Sandra and Vanhoenacker [165] obtained a peak capacity of 900 by coupling 8 × 25 cm × 2.1 mm conventional packed LC columns for the gradient analysis of human serum tryptic peptides at 60 °C. The combined use of UHPLC and HTLC is a very promising technique for complex biological samples so as to achieve very high-resolution separations within an acceptable analysis time. Plumb et al. [166] have obtained a very high peak capacity (>1000) in 1 h for a rat urine sample analysis by combining UHPLC (11 000 psi and sub-2 μm particles) with a reversed-phase gradient at 90 °C as shown in Fig. 13. At 90 °C, they also measured 100 000 plates on three linked 150 mm × 2.1 mm columns packed with 1.7 μm particles [29]. This separation of neutral solutes under isocratic conditions was performed with a low flow-rate, lower than the optimum one because of the system pressure limitation at 1000 bar. The gain in analysis time which can be achieved by going from 30 °C to 90 °C has not been reported.

A recent theoretical study based on graphical method has highlighted the advantage of high temperatures for enhancing the kinetics of peak capacity production under gradient conditions for the separation of peptides [167]. This method is quite similar to the one used for the construction of Poppe plots (or kinetic plots) which are intended to evaluate the potential of isocratic separations. Here, the plate number is replaced by the peak capacity and the column dead time by the ratio of the gradient time to the peak capacity. It should be noted that the curves calculated at 40 and 100 °C and shown in Ref. [167], are analogous to those shown in Fig. 2 for isocratic conditions. Temperature affects principally the range of low peak capacities (low plate range in Fig. 2) which correspond to short gradient time (short dead time in Fig. 2). Thus, in terms of separation speed, the use of high temperatures seems to be useless in the range of ultra-high peak capacities (i.e. >800 according to the results given in Ref. [167]) while it remains relevant for lower ones. On the other hand, according to the authors this figure is evidence of the great benefit of a high-temperature ultra-fast gradient elution as the second dimension in comprehensive two-dimensional liquid chromatography (2D-LC). A faster separation in the second dimension allows performing a faster separation in the first dimension while keeping an acceptable first dimension-sampling rate. Accordingly, a significant gain in speed can be obtained. An additional advantage of a gradient elution in the second dimension is the possibility of re-focusing the solute bands and thereby limiting the solute band broadening. In the ideal case of fully orthogonal separation mechanisms, the overall peak capacity can theoretically attain the product of the peak capacities of the two dimensions [168]. An interesting review on comprehensive 2D-LC with a particular focus on the use of high temperatures has been recently published [169]. The authors have pointed out that the great advantage of fast 2D-LC, in addition to the quantification of compounds or groups of compounds in a sample, is the rapid characterization of complex biological mixtures via multi-dimensional detectors. The option of a gradient elution at high temperature as the second dimension was investigated by Stoll et al. [170]. They concluded that a peak capacity production rate of 2100/h could be possible by using such an approach.

5.2. Separation of biomolecules

With the current developments in proteomic and metabolomic research, the interest for the separation of peptides and proteins has
considerably grown. Horvath and co-workers [18,171,172] first used high temperatures to separate peptides and proteins. They recommended the use of pellicular stationary phases for their stability at high temperature and highlighted the great interest of high temperatures for fast and efficient separations of macromolecules having low diffusivity and slow sorption kinetics. Their results suggested for such compounds that high temperatures could give rise to a significant improvement in efficiency, speed and sensitivity. Hancock et al. [173,174] made use of the significant effect of temperature on selectivity to optimize the separation of peptide and protein samples by varying both mobile phase composition and temperature on a silica-based stationary phase.

It is now widely recognized that temperature is an important tool to vary selectivity and to improve efficiency [39] of macromolecules such as peptides and proteins. The effect of temperature on both selectivity and efficiency is expected to be more pronounced for these compounds because their various ionized sites can lead to mixed retention mechanisms. In addition, changes in selectivity can arise from conformational differences depending on temperature [175]. In 2001, the theoretical study of Neue and Mazzeo [28] has shown the great advantage of combining the use of high temperatures and ultra-high pressures, especially for the chromatography of macromolecules. Finally as previously discussed in the third section, the limitation which arises from the usual thermal instability of these compounds can be mostly circumvented by the very short column residence times obtained with the combination of HTLC and UHPLC.

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References
