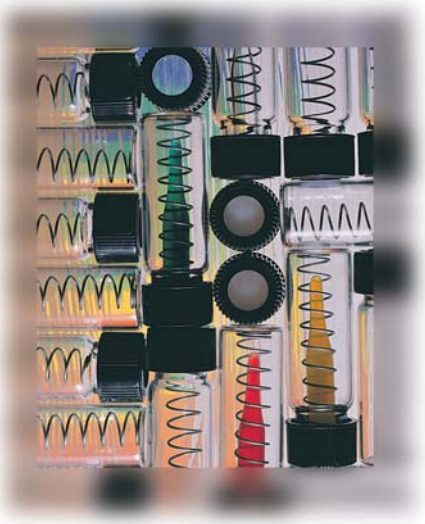
Elevated Temperature HPLC: Principles and Applications to Small Molecules and Biomolecules



The use of elevated and high temperatures in high performance liquid chromatography (HPLC) is reviewed, encompassing the range ambient to 250 °C and with examples covering separations of both small molecules and biomolecules. Generic issues of temperature dependence of retention and plate height are discussed. Use of elevated temperatures allows good chromatographic efficiency to be obtained at flow rates higher than those optimal at ambient temperature, thus increasing the speed of separation. Comparisons are made between temperature gradient and solvent gradient elution, and requirements for successful elevated temperature HPLC are described.

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Introduction to Elevated Temperature HPLC

In development of the technique of high performance liquid chromatography (HPLC), the use of high pressure was the dominant operational and instrumental characteristic in providing high performance. In comparison with earlier systems driven by lower pressures, far better separations were obtained in shorter times (1,2). Temperature has been an overlooked operational parameter in HPLC, and the potential advantages of elevated column temperatures, particularly enhanced kinetic and transport properties, which are based on the decrease of the viscosity of mobile phase and increase of the analyte diffusivity at higher temperature, have begun to be exploited for rapid analysis of biological macromolecules by HPLC in the past 10 years (3). There has been a recent review of high-temperature liquid chromatography (4), and the aim of this article is to extend the discussion of the topic and include a critical analysis of the effects of temperature change on peak shape and efficiency.

In most instances, the objective of using elevated or high temperature is to increase the speed of separation to obtain higher efficiencies and faster results, though there are some situations where selectivity can be manipulated through change of temperature (5,6). By using a backpressure regulator, as in supercritical fluid chromatography, it is possible to use superheated water as the mobile phase at temperatures up to 250 °C (7,8). At temperatures of 140 °C, this eluent, alternatively called subcritical water, has the characteristics of an organic solvent in terms of dielectric constant, and also greatly improved solubilization properties for hydrophobic analytes when compared with water at ambient conditions. This allows reversed-phase HPLC to be performed using a mobile phase containing no organic co-solvents, offering a completely green environment for LC as well as safety and cost benefits. Temperature-programmed HPLC can be used as an alternative to using solvent gradient elution for variation of solvent strength during the run, and this is expected to be of particular utility with small-bore columns which have low thermal mass (4). Considerable effort has been devoted to developing stationary phases, which are sufficiently robust to withstand use at temperatures of 200 °C or higher, and this work recently has been reviewed (9,10).

Only recently has the need for rapid

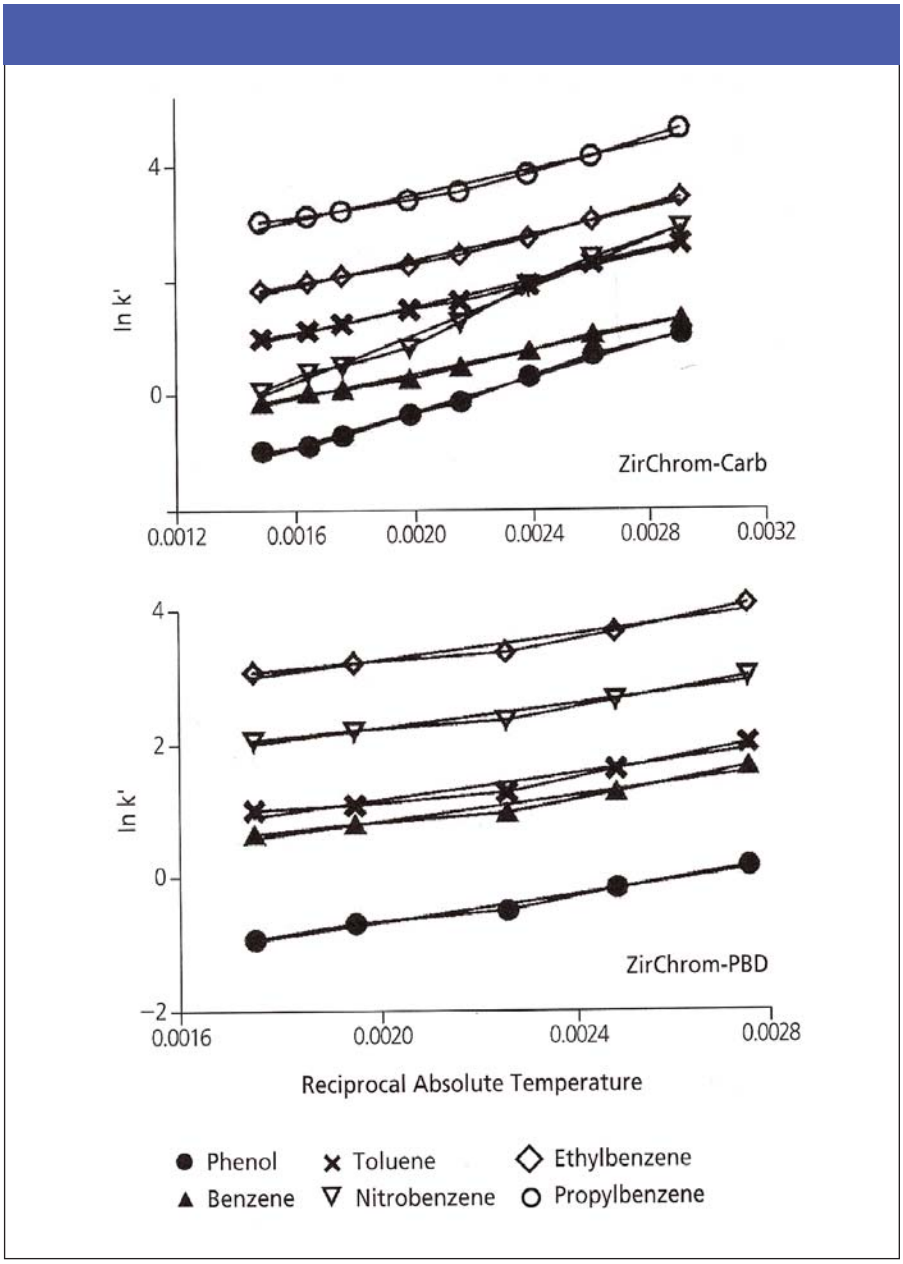

Figure 1: Van't Hoff plot of the retention temperature relationship of benzene derivatives, showing reduced retention with increased temperature and difference in selectivity with the temperature range 90–370 °C (ZirChromCarb column) and 90–300 °C (zirChrom-PBD). Reprinted with permission from reference 6. Copyright (2004) Elsevier.

analysis of biological macromolecules drawn attention to the use of elevated column temperatures, and in this field analytical HPLC is routinely performed at temperatures 20–40 °C above ambient temperature (11). The range of specialist stationary phases is growing steadily and includes various affinity chromatographies based on interactions with chiral selectors, complexing agents, proteins and macrocyclics (5). These separation mechanisms are often influenced by temperature to a much larger extent than in reversed-phase chromatography of small molecule analytes (12). Thus, it is now recognized that temperature is an important tool to optimize chromatographic parameters, such as retention, selectivity and efficiency, particularly for macromolecules (13). Denaturing HPLC, performed on partially renatured double-stranded nucleic acids, recently has been used as a very specific and sensitive method of screening for mutations, and in this technique, temperature is the most critical experimental variable (14).

For the purpose of this review, elevated temperature is taken in the broadest sense to mean a temperature higher than ambient, and in this respect, it also encompasses the alternative descriptive phrase high temperature. The principal reason we prefer to use the term elevated temperature is that many applications of interest to chromatographers reviewed in this article are at temperatures in the range 40–80 °C, which are certainly elevated with respect to ambient but would not normally be categorized as high temperatures. When performing database searching, it is recommended that both keyword descriptors, "elevated temperature HPLC" and "high temperature HPLC" should be used, because both terms are used in the literature.

Effect of Increase of Temperature on Retention Factor and Selectivity

A number of papers have considered the effect of change of temperature on retention (5,15–18). The effect of temperature on retention factor k can be described by the van't Hoff equation

$$ln\, k = -\frac{\Delta H^\circ}{RT} + \frac{\Delta S^\circ}{R} + ln\, \beta \qquad [1]$$

where ΔH° is the standard enthalpy change associated with the transfer of the solute from the mobile to the stationary phases, ΔS° the corresponding standard entropy change, R the molar gas constant, T the absolute temperature, and b the phase ratio of the column (1). From Equation 1, the change of retention factor with temperature results from the ΔH° term. Because the ΔH° term is normally negative, the retention factor decreases with increase of temperature.

Figure 1 illustrates van't Hoff plots obtained for benzene derivatives on ZirChrom-Carb and ZirChrom-PBD columns for a change of temperature in excess of 200 °C; results were obtained using super heated water in a study by Kephart and colleagues (6). For each plot, the standard enthalpy and entropy of the solute transfer from mobile to stationary phase can be calculated from the slope and the intercept, respectively. The results show that ΔH° values change relatively little for the alkylbenzenes in moving from the carbon to the polybutadiene phase, but that there is substantial change for the more polar compounds, phenol and nitrobenzene.

In a study using subcritical water in the temperature range 100–200 °C, retention of

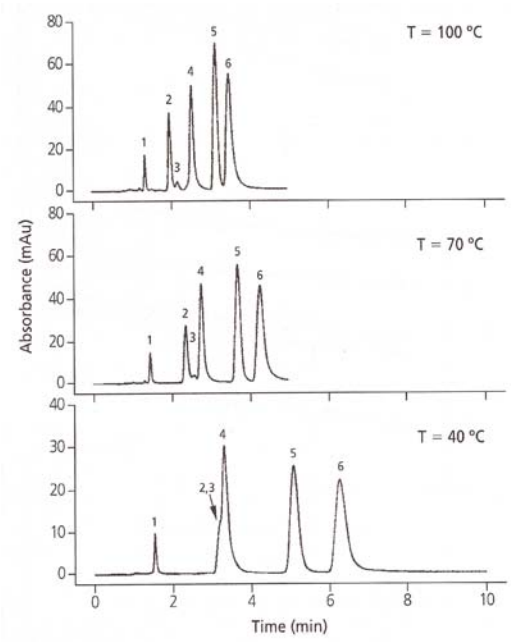

Figure 2: Chromatograms demonstrating the improvement in the separation of theapeutic tricclic antidepressants on PBD coated zirconia with increasing temperature. Solutes: 1= lidocaine; 2 = quinidine; 3 = norephedrine; 4 = tryptamine; 5 = amitriptyline; 6 = nortriptyline. Reprinted with permission from reference 5. Copyright (2004) American Chemical Society.

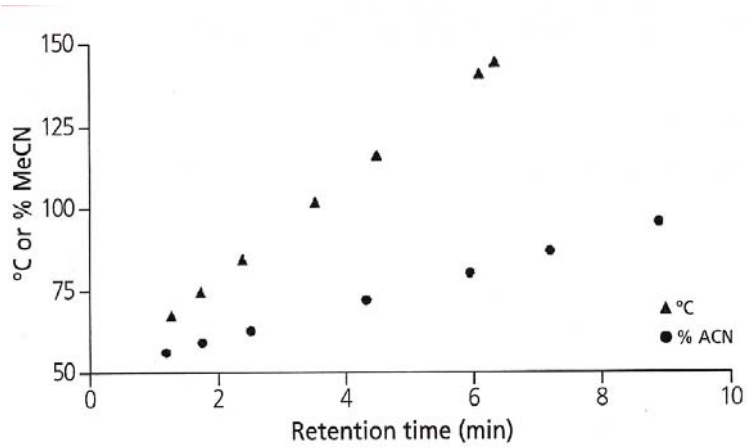

Figure 3: Retention time versus °C and retention time versus % MeCN for the HyperCarb column using acetonitrile–water with 0.1% TFA as the mobile phase. Circles represent retention times for the solvent gradient and triangles represent retention times for the temperature program. Elution order was aniline, styrene glycol, 2-phenyl-2-propanol (one peak), amitriptylene, acetophenone, salicylic acid, ibuprofen. Reprinted with permission from reference 21. Copyright (2004) Elsevier.

anilines and phenols was shown to be more sensitive to temperature than retention of alkylbenzenes (8). Using a Nucleosil C18 AB column, *k* values for 2,3-dichloroaniline and benzene were 20.1 and 9.6, respectively, at 100 °C, but 1.1 and 1.6 at 200 °C. Both studies show that the elution order in a mixture of components of different polarities can be sensitive to temperature.

Li and Carr examined the effects of temperature on the selectivity of a PBD-coated zirconia column (5). With isocratic elution using a mobile phase consisting of a 40:60 (v/v) mixture of acetonitrile and water containing 20 mM ammonium fluoride and 50 mM Tris adjusted to pH 10 with sodium hydroxide, there was a significant improvement in selectivity for a mixture of tricyclic antidepressants on increasing the temperature from 40 to 100 °C (Figure 2). Peaks for quinidine, norephidrine, and tryptamine, which were coeluted at 40 °C, were well resolved at 100 °C. The temperature dependence of separation of alkylbenzenes was also investigated on this column (5), using 40:60 acetonitrile: water as mobile phase. While all components were baseline resolved under all conditions and the elution times of the last-eluted species *n*-pentylbenzene decreased from 35 to 12 min on going from 40 °C to 100 °C, the selectivity decreased for all species. This is consistent with a parallel increase in both k and the magnitude of $\Delta H°$ in the sequence benzene to *n*-pentylbenzene. It was suggested that the selectivity is only likely to change if the retention mechanisms for the solutes in a mixture include more than one process, and this is consistent with the results shown in Figure 1 and discussed in the previous paragraph.

In HPLC with ion exchange stationary phases, cation exchange resins show exceptional thermal stability and can be used over a wide temperature range. Linear van't Hoff plots are the norm, and divalent and monovalent cations were shown to fall into two distinct groups regarding their retention behavior and temperature dependence of *k* (19). Within each group, increase in *k* is matched by an increase in $\Delta H°$; values range from 22 to 212 kJ/mol from Li^+ to Cs^+, and this correlates with decreasing size of the hydrated cation. However, *k* values are higher for divalents than for monovalents and in a separation of nine cations at 27 °C there is partial overlap of the peaks for Cs^+ and Mg^{2+}. Resolution and speed of separation were both improved on changing the

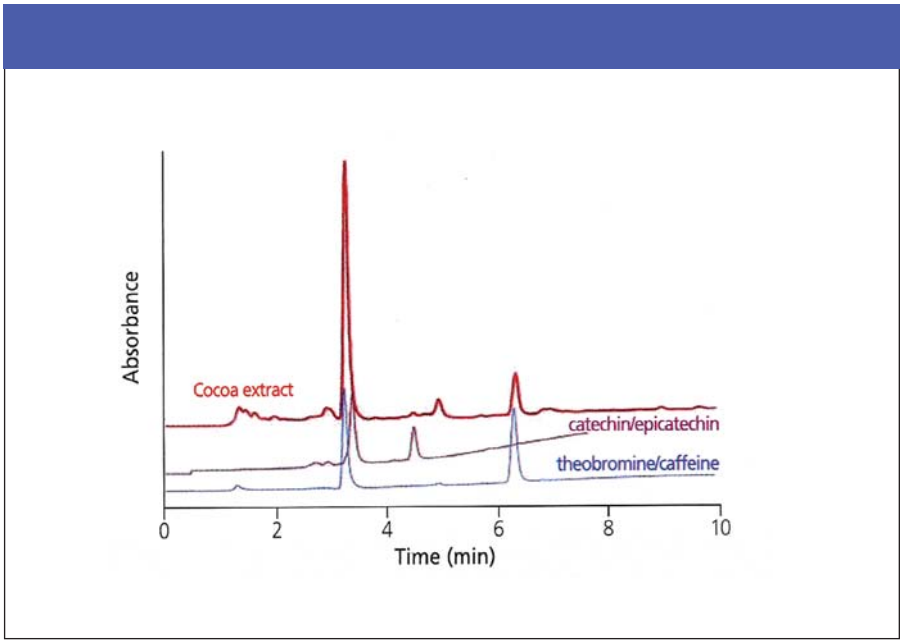

Figure 4: Separation of cocoa extract components using thermal gradient from 40 °C to 90 °C at 10 °C/min^{-1}. Column: Selerity Blaze C8, 100 mm × 4.6 mm. Reproduced from reference 31 courtesy of Selerity Technologies, Inc.

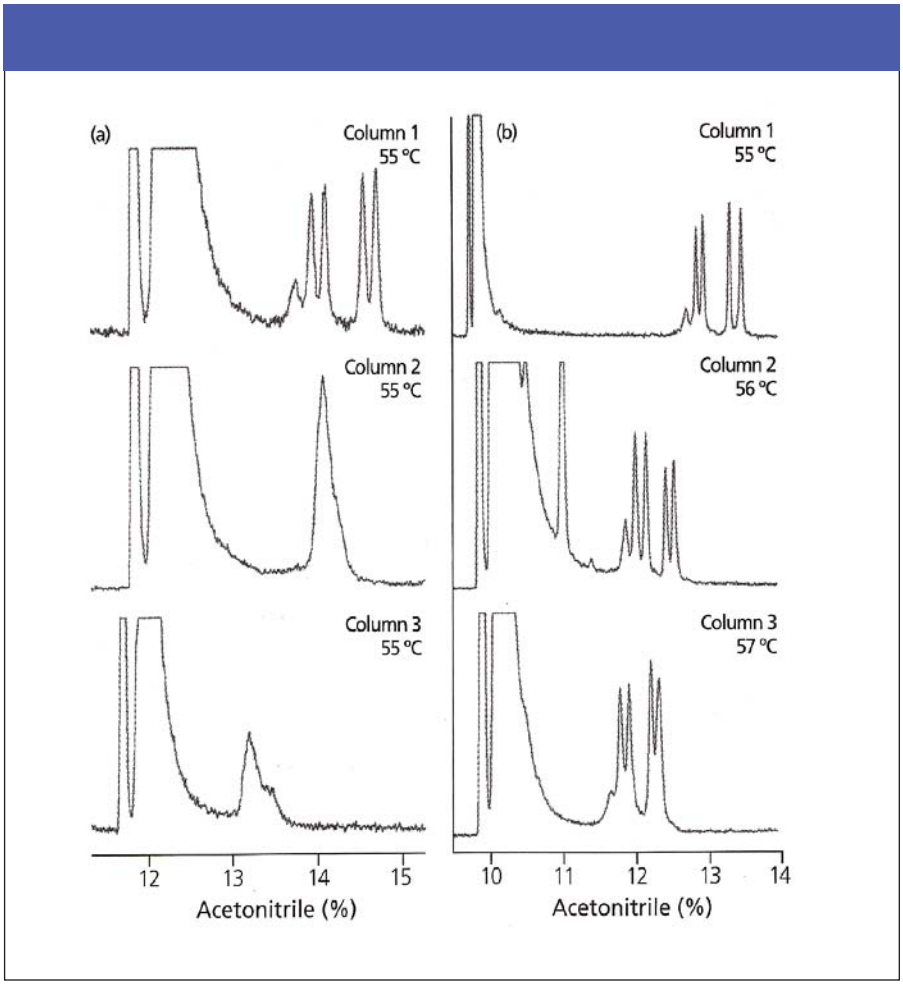

Figure 5: Harmonization of chromatographic elution profiles of different PS-DVB monolithic columns by means of modulation of individual column temperatures. The same DNA mutation standard injected onto three different columns (a) kept at the same temperature and (b) using individual column thermostats to vary temperatures. Reprinted with permission from reference 14. Copyright (2004) Advanstar Communications.

temperature from 27 °C to 60 °C, with a particularly marked improvement in resolution for the peak pair Cs^+ and Mg^{2+} (19).

A detailed theoretical analysis has been performed on the effect of temperature variation on selectivity for situations where retention processes are complex (20). These include ionizable compounds, solutes that experience two independent retention modes, such as amine compounds and ion-pair chromatography of carboxylic acids. The importance of the effect of temperature on the buffer was also stressed.

All studies of small molecules and ions show that temperature should be considered as a useful variable to control resolution only when components in a mixture are of different types: in reversed-phase LC, this might be polarity, while in ion exchange LC, it might be charge number.

In developing and validating an HPLC method, the most common approach is to optimize the mobile phase composition after an appropriate column has been selected (1). The effect of the solvent strength is usually stronger than the effect of temperature on solute retention. For example, Chen and Horváth (11) found that a 1% (v/v) increase in percentage of the organic modifier had almost the same effect as a temperature increase of 5 °C on elution times and retention factors for alkylbenzenes using an acetonitrile–water mobile phase on a Zorbax SB ODS-silica column.

Marin and colleagues have compared solvent and temperature gradient effects for a set of seven analytes, consisting of three neutral, two acidic and two basic compounds, separated on three stationary phases: a C8 phase supported on polydentate silica, a poly(styrene-divinylbenzene) (PS-DVB) polymer, and a graphitic carbon phase (21). With the C8 phase, working either in 50:50 (v/v) acetonitrile–water using a linear temperature gradient from 35 °C to 100 °C, or at 35 °C and using a linear solvent gradient from 50 to 100% (v/v), slopes of graphs comparing the effects of increasing the temperature or increasing acetonitrile content show that a 1% (v/v) increase in percentage acetonitrile has the same effect as a 3 °C increase in temperature. The same relative effects were found when investigating the same analyte set and the same column but under acidic rather than neutral conditions: here the mobile phase was acetonitrile: water with 0.1% trifluoroacetic acid.

On the PS-DVB column, starting at 50% organic content and 50 °C, and ramping lin-

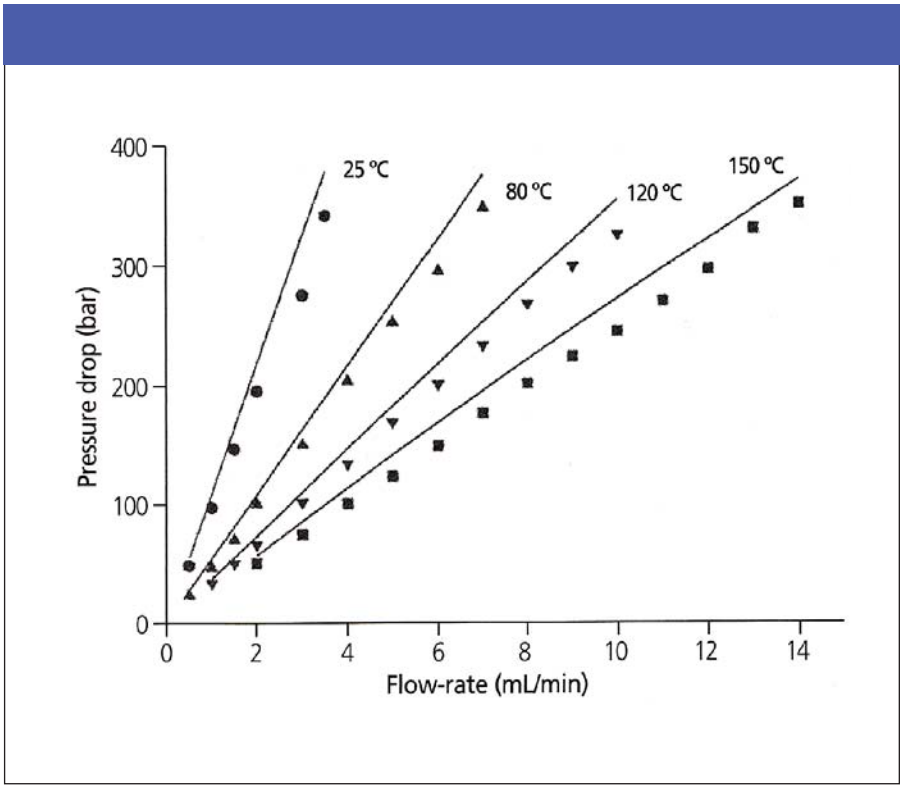

Figure 6: Theoretical and experimental pressure drops at different temperatures for a polystyrene-coated zirconia column, mobile phase 25:75 (v/v) acetonitrile–water. Lines are based upon the theoretically calculated values of the back pressure resulting from reference 37. Copyright (2004) American Chemical Society.

early to 100% organic or 150 °C, the relative effects of temperature and solvent increase were more similar: a 1% (v/v) increase of % acetonitrile was equivalent to a 1.7 °C temperature increase. For the graphitic carbon column, starting at 50% organic content and 50 °C, and ramping linearly to 100% organic or 200 °C (Figure 3), a 1% (v/v) increase of % acetonitrile was equivalent to a 1.7 °C temperature increase (21).

Kondo and Yang (18) performed isocratic elution on sets of (i) polyhydroxybenzenes and (ii) three basic compounds uracil, aniline and pyridine, using two C18 and one PS-DVB stationary phase. Comparisons were made of subcritical water as eluent at elevated temperature with methanol:water and acetonitrile:water mixtures at ambient temperature. For the C18 columns, a 1% acetonitrile increase was equivalent to a 3 °C rise in temperature for subcritical water and a 1% methanol increase was equivalent to about 2 °C rise in water temperature. For the PS-DVB column, the comparisons are a 1% acetonitrile increase was equivalent to a 5–8 °C rise in temperature for subcritical water, and a 1% methanol increase was equivalent to about 3.5 °C rise in water tem-

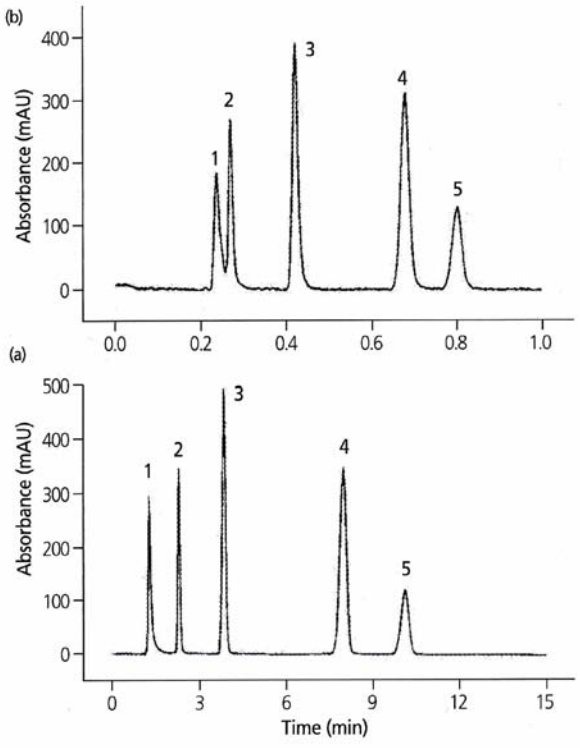

Figure 7: Chromatograms of a reversed-phase test mixture. The mobile phase composition was 20% MeCN, the column was PBD-coated zirconia material. Plot A is the chromatogram at 30 °C and 1 mL/min, and plot B is the chromatogram at 100 °C and 5 mL/min. Solutes: 1 = uracil; 2 = *p*-nitroaniline, 3 = methyl benzoate; 4 = phenetole; 5 = toluene. Reprinted with permission from reference 17. Copyright (2004) American Chemical Society.

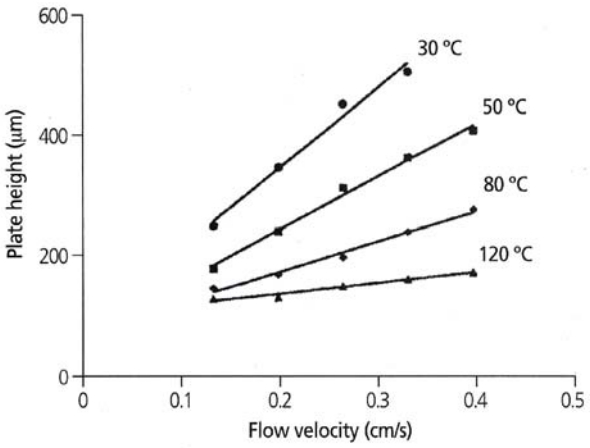

Figure 8: Plots of plate height versus linear flow velocity measured with ribonuclease A at elevated temperatures in the domain of high reduced flow velocities. Column: 30 × 4.6 mm, packed with 5-μm macroreticular cross-linked polystyrene. Concentration (v/v) of acetonitrile in water containing 0.1% (v/v) TFA: ● = 25%; ■ = 25%; ♦ = 22%; ▲ = 18%. Reprinted with permission from reference 3. Copyright (2004) John Wiley & Sons Limited.

perature (18).

A number of studies have shown the benefits in terms of environmental impact and cost of using pure water as a mobile phase (6,8,18,22–25). For aniline and phenol, *k* values in subcritical water separation at 150 °C are similar to those achieved by using 43% methanol in water or 40% acetonitrile in water (8).

In their series papers, Zhu and colleagues (26–29) have explored the combined effects of variation of temperature and solvent strength for predicting separation of neutral and ionizable analytes by a combination of experiments and computer simulation. The highest temperature used was 75 °C. Their research suggested that HPLC method development should commence with studying the effects of changing the gradient steepness and that if further changes in band spacing and resolution are required, changes in temperature should then be explored. The changes in selectivity as a result of varying both parameters are generally independent of each other. Ionizable compounds were found to show greater temperature effects than nonionizable compounds. An example is in resolution of a drug sample containing 47 acids, bases and neutrals (28). The column used was 5-μm Zorbax RX-C18, 25 cm × 0.46 cm, the mobile phase a solvent gradient with component A 0.15 M H_3PO_4–0.05 M triethylamine and component B acetonitrile, the gradient from 0 to 100% B, and flow rate 2 mL/min (26). With temperature 30 °C and a 20-min gradient, eight band pairs were resolved incompletely. When the temperature was increased to 66 °C, an additional five of the eight pairs were resolved, including two (salicylic acid/butabarbital and codeine/acetaminophen) that changed their elution order. Changing the steepness of the gradient, to 60 min instead of 20 min at 30 °C, baseline resolution was obtained for six of the previously unresolved peak pairs, with two pairs changing elution order. All peaks could be resolved using one or other of the strategies (28).

In a recently published review paper, Dolan (30) pointed out that temperature can be programmed quite simply in HPLC operating systems, and that during method development changes in temperature can be more convenient than solvent composition or pH changes. Temperature programming for HPLC is now being promoted commercially and comparisons are available on the effects of temperature gradients and solvent

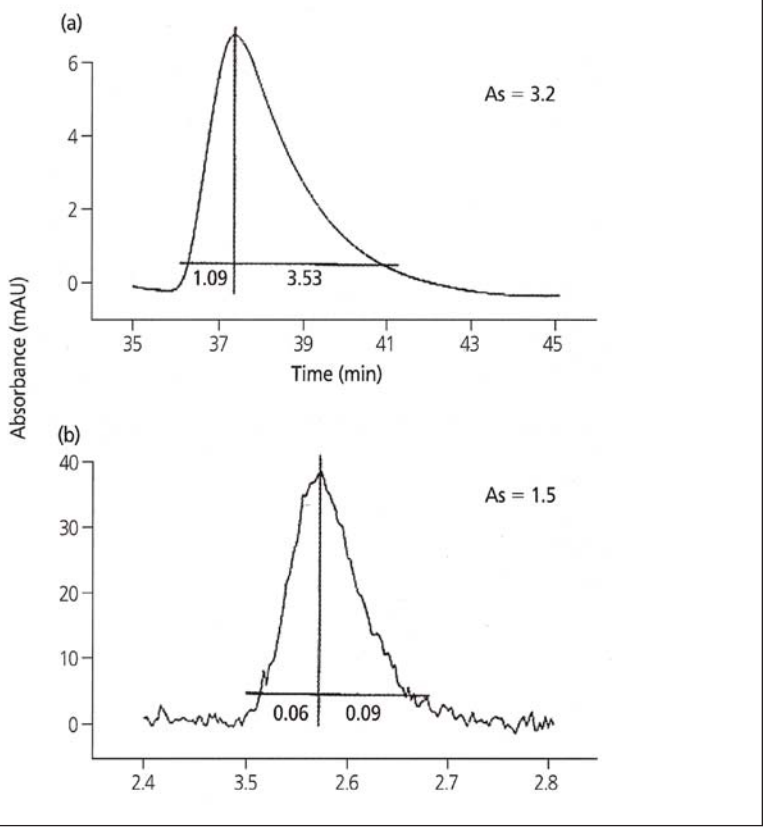

Figure 9: Comparison of peak symmetry of benzo[*a*]pyrene at different temperatures. Plot A is the peak at 30 °C and 1 mL/min^{-1}, and plot B is the peak at 100 °C and 5 mL/min^{-1}. The peak symmetry is measured at 10% peak height. Reprinted with permission from reference 17. Copyright (2004) American Chemical Society.

gradients using a range of columns and a test set of analytes spanning neutral, acidic and basic molecules (21). Representative results from this study have been presented in Figure 3. An example of use of thermal gradient elution is shown in Figure 4, where an isocratic mix of 80:20 acidified water: methanol is used with a linear temperature gradient from 40 °C to 90 °C and a C8 column to separate and characterize components in a commercial cocoa preparation (31).

For larger molecules, such as peptides and proteins, the selectivity effects of solvent strength and temperature are often complementary (3,31–32). The simultaneous optimization of mobile phase composition and temperature has been regarded as a powerful and convenient way to control retention (33). The enthalpies of transfer between mobile and stationary phase are generally greater for large molecules than for small ones (15). Chen and Horváth compared the effects of temperature change on reversed-phase HPLC behavior of lysozyme and nitrobenzene. $\Delta H°$ values were found to be in the range 250 to 2160 kJ/mol for the protein, in comparison with 215 to 221 kJ/mol for the small molecule. In both instances, values decreased on increasing the percentage of acetonitrile in water–acetonitrile mixtures containing 0.1% trifluoroacetic acid. For large molecules, therefore, their retention is generally quite sensitive to a change in temperature, and this shows that temperature can be an effective retention modulator. As in the instance of small molecules discussed in the previous paragraph, it has been suggested that temperature programming could be a powerful adjunct or even an alternative to mobile phase gradients in the analytical separation of macromolecules by reversed-phase chromatography with columns having low heat capacity (15,16).

In a separation of a standard protein mixture of ribonuclease A, cytochrome c, lysozyme and β-lactoglobulin B, gradient elution was performed from 0 to 70% (v/v) acetonitrile in water containing 0.1% trifluoroacetic acid. Superimposing a gradient of 10 °C/min, with a start temperature of 30 °C, provided better resolution than was obtained for gradient elution at a constant temperature of either 30 °C or 80 °C (16).

Denaturing HPLC is used as a sensitive screening method for mutations in chromosomes (14). In the presence of a mutation, two heteroduplex DNA fragments are formed in addition to the two original homoduplex fragments. These are separated using elevated temperature HPLC, using a PS-DVB stationary phase and an acetonitrile–aqueous pH 7, 100 mM TEAA, 0.1 mM Na_4EDTA solvent gradient (14,34). The column temperature must be carefully chosen to maintain partial denaturation, and the optimum value is normally in the range 50–70 °C. The heteroduplices, which are thermally less stable than the homoduplices, are retained less on the stationary phase and consequently have shorter elution times. Figure 4 shows results obtained during research on column arrays for multiplexed denaturing HPLC (34). Four monolithic capillary columns with independently controlled thermostatting column sleeves were run in parallel. The same sample, a DNA fragment with 209 base pairs differing only in a mutation from A to G at position 168, was injected onto each column.

In Figure 5a, all are run under the same conditions, with temperature of 55 °C. While column 1 shows resolution into the four heteroroduplex peaks, the peaks coelute on columns 2 and 3. Differences in chromatographic behavior were ascribed to differences in porosity of the individual monolithic PS-DVB columns. Tuning the temperature (Figure 5b) allows the four peaks to be seen on all three columns, with optimum temperatures for columns 2 and 3 of 56 °C and 57 °C, respectively. Increase in percentage of acetonitrile and in temperature both serve to denature the double stranded DNA, and a 0.8% increase in percentage of acetonitrile corresponds to an increase in column temperature of 1 °C. This helps to account for why column 3, on which the DNA fragments eluted at 13% acetonitrile, needed to be run at a temperature 1 °C higher than column 2, where the fragments eluted at 14% acetonitrile. This result shows that for any separations that are very sensitive to temperature, it is necessary to use proper standardization to optimize

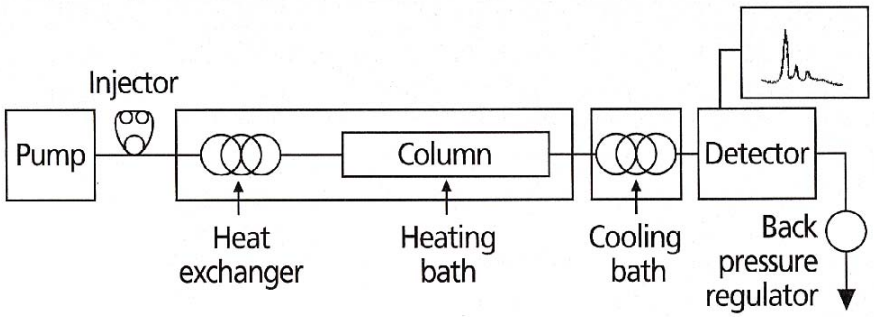

Figure 10: Block diagram of a simple high-temperature HPLC system. Reprinted with permission from reference 55. Copyright (2004) American Chemical Society.

conditions to allow for any column-to-column variability.

Effect of Increase of Temperature on Separation Time, Plate Height, Efficiency, and Peak Shape

Increasing the efficiency and speed of separations in LC always has been a key objective for separations scientists. Developments in column technology have been mainly responsible for the advances in these directions (15). Use of small particles and high flow rates result in fast separation but require high pressure of the operating system (35–37). The maximum operating pressure normally sets the upper limit for speed of analysis. Operation at elevated temperatures decreases mobile phase viscosity: for acetonitrile–water mixtures, the viscosity is decreased by up to a factor of 4 as the temperature is increased from 30 °C to 120 °C (3). The column backpressure is decreased as well, as shown in Figure 6, allowing use of higher flow rates (38). It also permits the use of longer columns or smaller particles for separating complex mixtures requiring large plate numbers (35,38).

Chromatograms from a typical reversed-phase test mixture separated under temperatures 30 °C and 100 °C are shown in Figure 7 (17). The flow rate at 100 °C was 5 mL/min, five times greater than that at 30 °C. The analysis time was decreased from

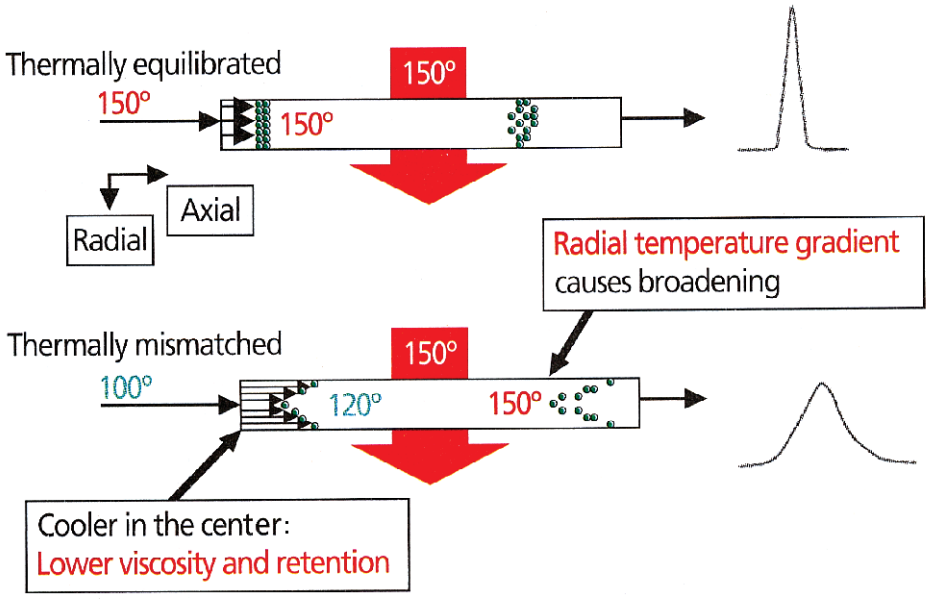

Figure 11: Band broadening caused by thermal mismatch. Reproduced from reference 56, courtesy of Metalox Technologies, Inc.

11 min to 50 s with acceptable resolution. This demonstrates that rapid separation is practicable on conventional LC instrumentation using high temperatures and high flow rates.

When considering the dependence of column efficiency on flow rate, the relationship of most practical utility is the Knox equation, which describes the relationship between the reduced plate height, h, and the reduced velocity v:

$$h = Av^{1/3} + \frac{B}{v} + Cv \quad [2a]$$

$$v = \frac{ud_p}{D_m} \quad [2b]$$

where d_p is the diameter of the stationary phase particle, D_m is the diffusion coefficient of the solute in the mobile phase, and the coefficients A, B, and C are related to the packing quality, longitudinal diffusion of the solute, and the mass transfer contributions, respectively (1,2). The effect of increase of temperature on the A term, which is a measure of how well the column is packed, is uncertain (5). If anything, it should be beneficial because of an improvement of the laminar flow and lateral mixing of molecules among different flow channels; however, this improvement might not be significant (5). The B term should be essentially independent of temperature, in the situation where diffusion is the only source of variance contributing to this term. The C term is expected to decrease with increasing temperature because the mass transfer resistance should decrease: diffusion coefficients in both the stationary phase and the stagnant regions of the mobile phase, together with the stationary phase desorption rate, increase with increasing temperature. It is generally predicted that an increase in column temperature should decrease the reduced plate height, particularly if the mass transfer resistance in the stationary phase is dominant in determining h. At high reduced velocity, where the C term dominates, there should be a beneficial effect with increasing temperature on speed without compromising resolution (3,5,13,15,22,39).

Li and Carr have shown that the use of elevated temperatures could improve column efficiency by as much as 30%: the optimum reduced plate height for 1-phenylnonane was 5.5 at 25 °C, and this reduced to 4 at 65 °C (5). Improvements were particularly significant in the high linear velocity region (a decrease in the C term of the Knox equation).

Figure 8 gives results obtained with ribonuclease A as analyte using a stationary phase with 5-μm particles of macroreticular cross-lined polystyrene (3). The mobile phase composition was adjusted at each temperature to provide a constant k across the temperature range studied, 30–120 °C. Plate height is given as a function of flow velocity and it is evident that the best results are obtained at the highest temperature. There is also least variation of plate height with flow rate at 120 °C.

Thus, in general, it will be advantageous to operate columns at elevated temperatures and flow rates; this will not only improve the speed of separations but also increase the column efficiency because the contribution of longitudinal diffusion should be small relative to the contribution from mass transfer under these conditions. When working at elevated temperatures, use of low flow rates should be avoided. This follows because of the increase of longitudinal diffusion as temperature increases (5,40,41).

McCalley investigated the effect of temperature on peak shape of basic compounds, using an ODS stationary phase (Inertsil ODS-3V) and acetonitrile: aqueous phosphate buffers (pH 3.0 and 7.0) as mobile phases (42). With temperature increase, the three compounds used in the experiment (pyridine, quinine, nortriptyline) showed significant increases in column efficiency in both the acidic and neutral buffers. At pH 7, this was accompanied by considerable reduction in peak asymmetry for quinine and nortripyline: there was no effect for the neutral compound benzene. These reductions in peak asymmetry and increases in efficiency were ascribed at least in part to kinetic effects associated with desorption from silanol binding sites (42). Guiochon and colleagues have discussed how raising the column temperature is probably the only practical tool to reduce peak tailing when tailing is associated with slow mass transfer kinetics (43).

Smith showed that both the efficiency and the asymmetry factor improved noticeably with increase of temperature on a PS-DVB column (25). The peak asymmetry factors for catechol, aniline and pyridine on C18 and poly(styrene-divinylbenzene) columns have been examined by Kondo and Yang (18): peak symmetry was significantly improved either by increasing temperature for separations using subcritical water or by increasing the percentage of organic solvent in a water-organic mobile phase mixture at ambient temperature.

Li and colleagues have compared PAH separations on a PBD-coated zirconia column at 30 and 100 °C (17). The peak asymmetry of benzo[a]pyrene at the two temperatures are shown in Figure 9 and the temperature dependence is characteristic of all PAH species. While the improvement in peak symmetry was judged to be caused by both the decrease in the retention and the faster diffusion of solute in the stationary phase zone, it was the latter that it was felt had given the more significant contribution (17).

Requirements for Successful Elevated Temperature HPLC

As discussed in the previous section, elevated temperature HPLC allows fast and efficient separations. There are several practical constraints to its use. The first and most important requirement is that the analytes must be stable at the temperatures used during the chromatographic run (44). Denaturation of DNA has been used to advantage in elevated temperature HPLC, but this is a very specific application to nucleic acids (14).

Second, a thermally stable stationary phase is required. Many commonly used silica-based C18 phases are easily degraded using aqueous organic mixtures with high water contents at temperatures above 60 °C (44). Hydrolysis of siloxane bonds (Si-O-Si) causes loss of bonded phase from the silica support and eventually leads to column failure. This hydrolysis is more acute at low pH (< 3) and elevated temperatures. Silica-based phases stable at elevated temperatures include Zorbax SB products, available from Agilent, which use special diisobutyl silanes for the bonded phase (45). The bulky isobutyl groups on these silanes sterically protect the siloxane bonds from hydrolysis and reduce the loss of bonded phase, allowing operation up to 90 °C. Other stable silica-based columns are those in the Blaze series from Selerity Technologies, which use polydentate bonding chemistry to provide a protective shield against degradation of siloxane bonds and can be used up to 100 °C (21).

A number of speciality stationary phases have been developed that are extremely robust over a wide temperature range, and

Table I: Stationary phases used in elevated temperature reversed-phase HPLC

Company	Base Material (Functional Group)	Max. Temperature (°C)	Application Examples
Agilent Technologies www.agilent.com	Silica with sterically protected siloxane (C3, C8, C18, CN, phenyl)	80 (C18, 90)	Wheat protein
Hamilton Company www.hamiltoncomp.com	Poly(styrene-divinylbenzene)	150	Corn syrup, sugars, organic acids
Jordi www.jordiassoc.com	Highly crosslinked divinylbenzene	150	Phenolic antioxidants
MicroSolv Technology www.microsolvtech.com	Type-C silica bidentate (C18 & C8, cholesterol) DVB-RP	100 150	Steroids, vitamins Polar organic acids
Selerity Technologies www.selerity.com	Silica polydentate (C4, C8, phenyl)	100	Analgesics
Shimadzu www.eu.shimadzu.de	Polymer encapsulated silica (C18 and others)	200	Polyaromic hydrocarbons
Supelco, Inc. www.sigma-aldrich.com	Zirconia (C18)	200	Peptide tryptic digests
Thermo Electron www.thermo.com	Porous graphitic carbon	200	Narcotics, analgesics
ZirChrom Separations www.zirchrom.com	Zirconia (C18, PS, PBD, Carb) Zirconia-carbon (C18)	150 (Carb, 200) 200	Anticonvulsants, barbiturates, PAHs, steroids

can withstand temperatures up to 200 °C. Helpful review articles of column technologies are produced annually to accompany the Pittcon exhibition (46–49). A comparison of stationary phases used for high temperature liquid chromatography has recently been published in this journal (9). The temperature ranges over which the columns are applicable are documented in this review and stability issues are discussed. Table I lists manufacturers of phases for which performance has been documented at elevated temperatures and also gives the maximum operating temperatures as well as typical applications.

Zirconia-based phases, which were introduced by Carr and colleagues (50) have very good thermal stability, and negligible aqueous solubility even at elevated temperatures. Any chemical modification that is made to the surface functional group must also be compatible with both the temperature and the mobile phase conditions used. Both elemental carbon and polybutadiene (PBD)-modified zirconia based stationary phases are compatible with aqueous eluents and are commercially available (6,46). Carr's group has conducted many studies on PBD-modified zirconia materials, and shown them to give high efficiencies in LC separations at elevated temperatures (5,17,51). Their studies show that this PBD-coated zirconia column was stable for at least 1300 column volumes at 200 °C (17). However, there has been a report of potential problems with degradation of zirconia columns with aqueous-acetonitrile eluents under temperature programming conditions (21). Another class of speciality phases are those based on polymers, especially poly(styrene-divinylbenzene) (PS-DVB). There are no solubility problems associated with use of PS-DVB, and robust performance with neutral, acidic and basic solutions has recently been documented to temperatures up to 150 °C (21). In a study of the long-term stability of polymer columns in separations using superheated water, a PS-DVB column was used at 100 °C for over 11,000 column volumes, then at 150 °C for a further 9000 column volumes (52). Both retention and efficiency under these two sets of conditions showed no decrease with usage, proving the excellent column stability under these conditions. The PS-DVB columns should not be used at extreme temperatures, because depolymerization occurs above 250 °C (6). Graphitic carbon columns are very robust, showing no evidence of degradation over a wide pH range and with temperature programming up to 200 °C (21,53).

The third consideration with elevated temperature HPLC concerns the need to ensure that all equipment is compatible with conditions used. A schematic diagram for high-temperature operation is shown in Figure 10 (54). The backpressure regulator is required to ensure that the mobile phase is present in the liquid state: this is essential when working at temperatures above the normal boiling point. It should be emphasized that metal rather than PEEK hardware and fittings must be used for all components in the apparatus subject to high temperatures. A heat exchanger is necessary to avoid any temperature difference between the incoming eluent and the column. Any temperature mismatch can cause peak broadening (44,54–56). The origin and effect of band broadening caused by thermal mismatch is illustrated in Figure 11 (56), with an input stream at a lower temperature than the column heater. There is a radial temperature gradient, resulting in higher temperature at the wall than at the center. This means that mobile phase will have lower viscosity and stationary phase will have lower retention at the wall; both effects cause analyte at the center to lag behind that at the wall. Methods to ensure appropriate preheating have been recently reviewed (21), and ovens and column heaters specifically designed for elevated temperature HPLC and temperature-programmed HPLC are now available (56,57). Proper thermal preconditioning is also vital in denaturing HPLC and a heat exchanger must be incorporated prior to the column (14). In instances such as this, where the quality of the chromatography is extremely sensitive to temperature, measurements with certified reference probes should be performed to ensure that the temperature displayed on the column oven corresponds to the actual temperature in the column (14).

Conclusions

Use of elevated temperatures can bring benefits to HPLC, particularly in instances where columns are stable over an extended temperature range. When working with mixtures of analytes in different compound classes, selectivity can be dependent on temperature. Decrease in viscosity of the mobile

phase on increasing the temperature allows run times to be reduced, and the improvement in mass transfer rates between mobile and stationary phases allows good chromatographic efficiency to be obtained at flow rates higher than those optimal at ambient temperature. While solvent gradient elution normally is the preferred method for use with mixtures of analytes with widely varying retention factors, temperature gradient elution offers an alternative open to more separation scientists now that speciality suppliers are promoting the technology. In the field of gas chromatography, high-speed temperature gradients obtained using resistive heating have been used to advantage in developing fast separations with capillary columns. We see potential for analogous developments in the field of capillary HPLC separations, where rapid thermal equilibration is possible with thermostated fused-silica columns. There could be a particular niche in the field of capillary HPLC and biomolecular separations, because retention of such molecules is more sensitive to temperature change than is the situation with small molecules.

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