Knowledge note #0033

The Importance of Column Temperature in Liquid Chromatography

INTRODUCTION

Column temperature is an important variable in liquid chromatography (LC). Modern LC systems are typically equipped with column ovens, which allow the column temperature to be precisely controlled. Maintaining a stable temperature is a major benefit for LC method reproducibility and robustness.

Additionally, the column temperature can typically be varied over a wide range, often from around 10 to >100 °C. The chosen temperature set-point can have a profound impact on the separation obtained by affecting the peak width, back pressure and significantly, the separation selectivity. It is therefore an important parameter to consider during method development and can be a powerful tool for fine tuning a separation. This Knowledge Note discusses the impact that column temperature can have on LC separations, why it is important to consider during method development and limitations to avoid column and sample stability issues.

WHY CONSIDER COLUMN TEMPERATURE?

Column ovens are common components of modern LC systems and allow the chromatographer to accurately control the temperature of the LC column. In general, changes in column temperature will typically affect analyte retention. Not all sample analytes will necessarily be affected by a change in temperature to the same degree, so the selectivity obtained may also be affected. One of the key benefits to controlling the column temperature is that it provides stable analyte retention and selectivity, thereby improving the reproducibility and robustness of the analysis.

The fact that column temperature can affect analyte retention and selectivity means that it is also often a valuable method development tool. Whilst the changes in selectivity that can be achieved are typically not as great as can be achieved by varying column stationary phase or mobile phase composition,[1] column temperature never-the-less warrants investigation as

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part of method development strategies to help optimise analyte separation.

In reversed-phase, as column temperature increases, analyte retention typically decreases, along with the mobile phase viscosity, leading to shorter analysis times with reduced back pressure. Additionally, analyte mass transfer is improved, leading to narrower chromatographic peaks. This combination leads to the potential to develop faster LC methods through the use of elevated column temperatures.

The combined effects on column performance and separation selectivity means that the impact of temperature set-point on LC separations can be somewhat complex and it is therefore important to assess and optimise the temperature during method development. The remaining sections of this Knowledge Note examine each of these points in more details and discusses how they can be applied to improve LC methods. Finally, some cautionary considerations of working with elevated temperature are discussed.

METHOD REPRODUCIBILITY

Often, in particular for older legacy and pharmacopeia methods, "ambient temperature" may be specified in the LC method conditions, or no temperature may be specified at all. In these cases, the column may be operated without use of a column oven or temperature control. Unfortunately for such methods, variations in temperature can affect both analyte retention time and the selectivity (i.e. peak spacing) of the separation. Ambient air temperature in laboratories may also vary by several degrees throughout the day and indeed, may show significant long term variability (e.g. seasonally). Additionally, ambient temperature can vary significantly between different labs in different geographic locations. If the column is used at ambient temperature without a column oven, variability in retention and separation may be observed for what are nominally the same LC conditions. This could be problematic if the method involves marginally resolved peaks. Some methods will naturally be more prone to such effects than others. This situation can be improved by utilising a column oven for the analysis, with an appropriate temperature set-point (e.g. 20 °C), to at least ensure intra-lab reproducibility.

With increased availability of column ovens, it is highly recommended to develop new methods using temperature control and specify an exact temperature for the analysis to avoid possible reproducibility issues and improve the overall method robustness.

EFFECT ON RETENTION TIME AND SELECTIVITY

In general, for small molecule reversed-phase separations, as the column temperature increases, analyte retention decreases. This is demonstrated in Figure 1, which compares an isocratic separation of aspirin and related substances at two different temperatures. In this example, a 15 °C increase in temperature results in a 3.1 minute (18.3%) decrease in retention for the final peak. As the temperature increases, taller, narrower peaks are also obtained, which increases analyte response (this is discussed later in this article).

This application demonstrates that faster, higher throughput methods can often be developed using elevated column temperatures. Additionally, as temperature increases, mobile phase viscosity decreases, hence reducing column back pressure. This can potentially enable the separation to be run at a higher flow rate to maximise sample throughput. However, it is important to note that temperature can also affect the separation selectivity, so this approach is not necessarily applicable to all applications.
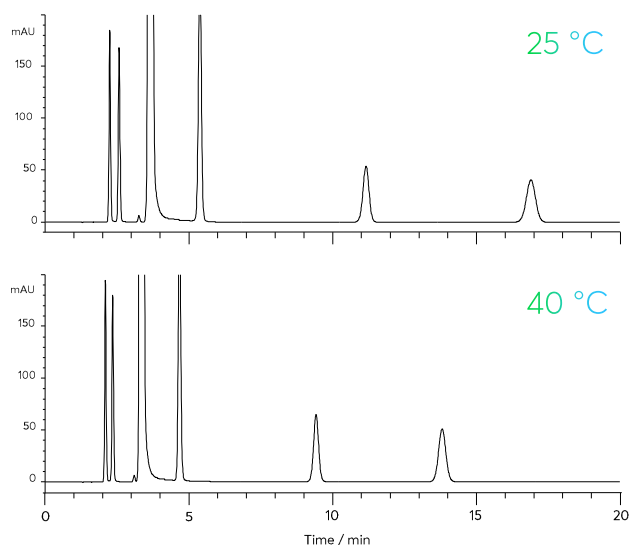

Figure 1: Separation of aspirin and related substances at two different temperatures.

Column: Avantor® ACE® Excel 5 SuperC18, 150 x 4.6 mm, Mobile phase: MeCN/H_2O (40:60 v/v) + 0.2% H_3PO_4; Flow rate: 1 mL/min; Injection volume: 5 µL; Detection: UV, 237 nm. Sample: 1. 4-Hydroxybenzoic acid, 2. 4-Hydroxyisophthalic acid, 3. Acetylsalicylic acid (aspirin), 4. Salicylic acid, 5. Acetylsalicylsalicylic acid, 6. Salsalate. Impurities were spiked at 0.5% w/w.

The influence of temperature on the retention of analyte molecules can be further demonstrated by considering the Van 't Hoff equation, which is derived from the following two thermodynamic equations.

$$\Delta G^0 = \Delta H^0 - T\Delta S^0 \qquad (1)$$

$$\Delta G^0 = -RTlnK \qquad (2)$$

Where:
ΔG^0 = Gibbs free energy of transfer between stationary and mobile phase
ΔH^0 = Enthalpy of transfer
T = Absolute temperature (Kelvin)
ΔS^0 = Entropy of transfer
R = Universal gas constant
K = Equilibrium constant

These can be rearranged, with some assumptions, to obtain the Van 't Hoff equation, which relates the retention factor (k) to the entropy and enthalpy values[2].

$$lnk = \left(\frac{-\Delta H^0}{RT}\right) + \left(\frac{-\Delta S^0}{R}\right) + ln\varphi \qquad (3)$$

Where:
k = Retention factor
φ = Phase ratio

From this equation, it is possible to model the retention behaviour of analytes as a function of temperature. In reversed-phase analysis, the relationship is often linear and the relationship can be simplified to:

$$lnk = a + \frac{b}{T} \qquad (4)$$

A plot of lnk vs 1/T (with temperature in Kelvins) for each analyte will therefore typically provide a linear plot. Figure 2 shows such a plot for a series of neutral analytes run at different temperatures. As the column temperature is increased, the retention time of all analytes decreases in a linear fashion. Note that in this case, the plots
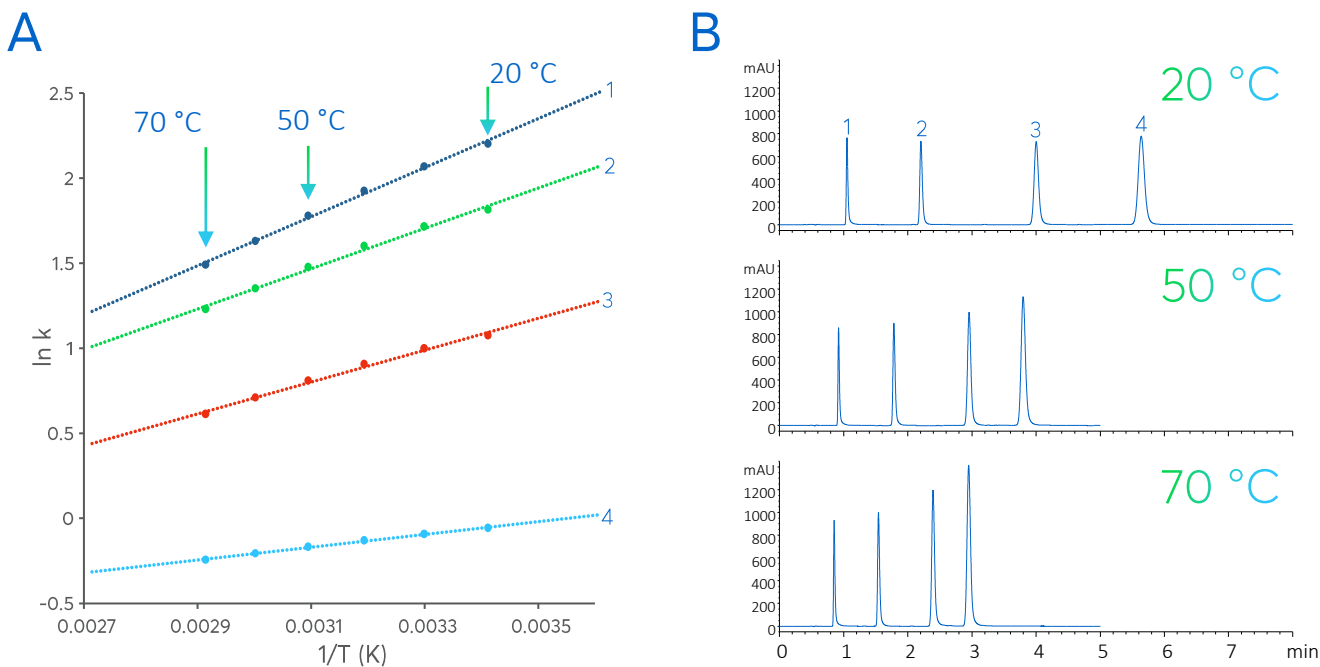
Figure 2: A. Plot of lnk vs 1/T for four neutral compounds chromatographed isocratically at different temperatures using the same mobile phase conditions. B. Chromatograms corresponding to the temperature points in A, showing the effect of column temperature on analyte retention and confirming the absence of any change in selectivity.

Column: Avantor® ACE® ACE Excel 2 C18-PFP, 100 x 2.1 mm; Mobile phase: MeCN/H_2O (60:40 v/v) + 0.1% formic acid; Flow Rate: 0.4 mL/min. Sample: 1. Dimethyl phthalate, 2. Toluene, 3. Biphenyl, 4. Phenanthrene.

possess similar slopes and do not intersect one another. This demonstrates that for this application, the selectivity of the separation remains constant, irrespective of the temperature set point (Figure 2B).

However, the slope of lnk vs 1/T can differ between analytes. Different analytes may possess differing enthalpies of transfer (equation 3), which is the driving force for temperature driven changes in selectivity.[3] Figure 3A demonstrates an isocratic separation where the selectivity changes dramatically with the column temperature. In this case, there are several temperatures at which the plots for individual analytes intersect one another, which equates to analyte co-elution. Quinine (peak 1) shows very little change in retention as the temperature is varied and co-elutes at 40 and 50 °C, with acetanilide and salicylic acid respectively. In contrast, the retention times for the remaining 4 analytes decreases as the column temperature increases. In addition, examining the plot for salicylic acid, at high temperature the plot intersects with acetanilide, whilst at low temperature it intersects with the plot for

acetylsalicylic acid. The selectivity for this application is therefore highly sensitive to the column temperature (Figure 3B). In this case, thorough assessment of the column temperature was essential during method development. The chromatograms show that full separation is achieved at 30 °C, whilst at 40 and 50 °C co-elution occurs.

Figure 4 demonstrates that the temperature set-point can also be important for gradient separations. For this mixture of basic pharmaceutical compounds, a general decrease in retention time is again observed as the temperature increases. Note also that at higher temperature the peak shape narrows and analyte response increases (see next section). At lower temperature, peaks 5 and 6 show poor separation; as temperature increases the elution order is reversed and full separation of these two analytes is achieved. Conversely, peaks 8 and 9 are fully resolved at lower temperatures but are found to co-elute at 60 °C. A temperature set-point of 40 °C was found to provide optimal separation for this application.
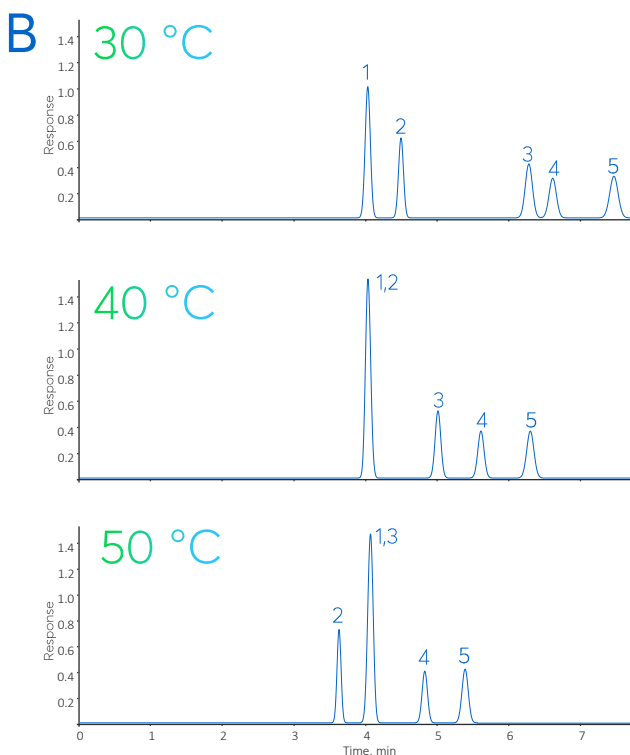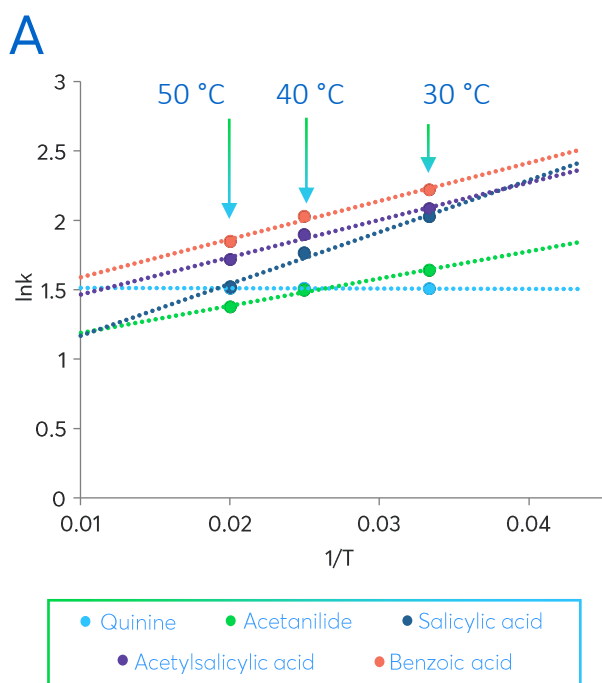

Figure 3: A. Plot of lnk vs 1/T for five compounds separated isocratically at different temperatures using the same mobile phase conditions. B. Chromatograms used to construct A, showing the clear differences in selectivity obtained at different temperatures.

Column: Avantor® ACE® ACE Excel 2 C18-AR, 100 x 3.0 mm; Mobile phase 20 mM KH_2PO_4 pH 2.7 in $MeCN/H_2O$ (15:85 v/v); Flow Rate: 0.6 mL/min. Sample 1. Quinine, 2. Acetanilide, 3. Salicylic acid, 4. Acetylsalicylic acid, 5. Benzoic acid.

Column:	Avantor® ACE® Excel 3 C18-PFP
Dimensions:	50 x 2.1 mm
Mobile phases:	A: 20 mM KH_2PO_4 (pH 2.5) in H_2O
	B: 20 mM KH_2PO_4 (pH 2.5) in $MeOH/H_2O$ (65:35)
Gradient:	

Time (mins)	%B
0	3
5	100
6	100
6.5	3

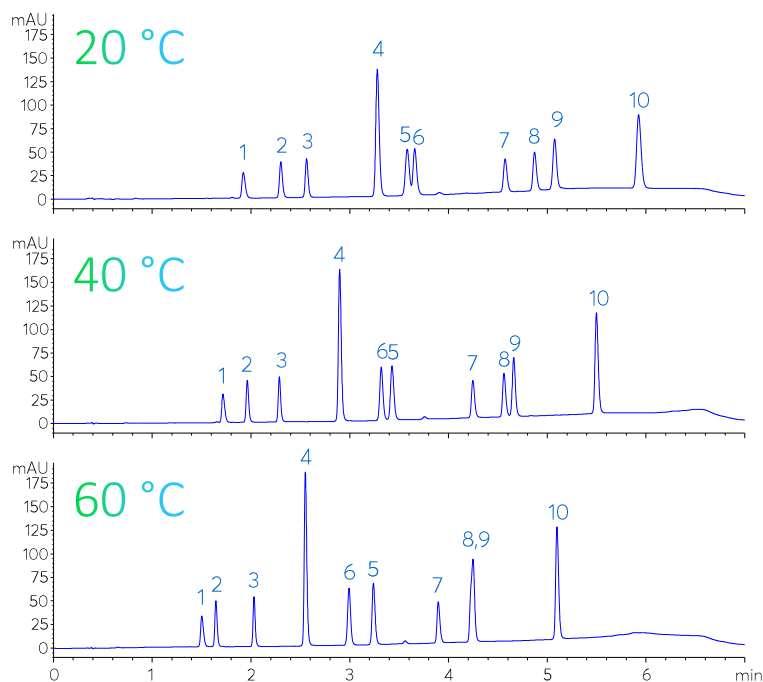| Flow rate:	0.4 mL/min
Injection:	1 µL
Detection:	UV, 214 nm
Sample:	1. Norephedrine
	2. Terbutaline
	3. N-acetylprocainamide
	4. Pindolol
	5. Quinine
	6. Quinoxaline
	7. Proprietary compound 1
	8. Diphenhydramine
	9. Proprietary compound 2
	10. Nortriptyline

Figure 4: Gradient separation of a range of basic compounds on an Avantor® ACE® Excel 3 C18-PFP at three different temperatures. The mobile phases, gradient profile and flow rate were kept constant to demonstrate the effect of changing column temperature.

EFFECT ON BAND BROADENING (PEAK WIDTH)

The Van Deemter equation (equation 5 in its simplified form)[4,5] relates the dispersion (peak width) on the column to three key parameters, named as the A, B and C terms (further information on the Van Deemter equation and band broadening can be found in reference 5). The B and C terms are both dependant on the diffusion co-efficient and are therefore temperature dependant.

$$HETP = A + \frac{B}{u} + Cu \qquad (5)$$

Where:
HETP = Height equivalent to a theoretical plate
u = Mobile phase linear velocity

B term
The B term refers to longitudinal diffusion. Increasing column temperature will increase the degree of Brownian motion, which will increase the amount of longitudinal diffusion (peak gets wider with all other parameters being equal); in fact the rate of diffusion in all directions is increased.

C term
The C term relates to the resistance to mass transfer and is dominant at higher flow rates. The diffusion rate affects this term inversely; opposite to the effect that it has on the B term. For the C term, the increase in diffusional rate means that there is less resistance to mass transfer; since an equilibrium concentration is reached quicker than at lower temperatures and so the peak width will decrease. The enhanced mass transfer at higher temperatures means that band broadening due to the C term is greatly reduced at higher temperature, leading to much narrower chromatographic peaks at high flow rates.

Figure 5 presents experimentally determined Van Deemter plots generated on a 5 µm column at two different temperatures. Although the absolute HETP minimum (i.e. maximum efficiency) does not change with temperature, the linear velocity at which this minimum occurs increases. The impact of the C term is reduced, so the curve becomes significantly flatter at higher temperatures; allowing the column to be operated at a higher flow rate without as sharp a drop-off in column performance. Operating the column at a higher temperature therefore means that narrower, higher efficiency peaks can be obtained at higher flow rates; enabling faster, higher throughput separations.
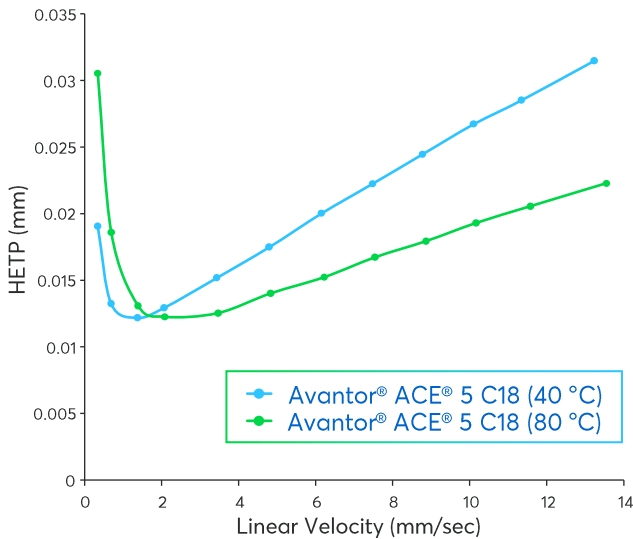
Figure 5: Experimentally determined Van Deemter curves for the Avantor® ACE® 5 C18 at 40 and 80 °C.

Column dimensions: 50 x 2.1 mm; Mobile phase: MeCN/H$_2$O (33:67 v/v @ 40 °C, 27.5:72.5 v/v @ 80 °C) + 0.1% TFA; Sample: Ketoprofen (k = 9).

CONSIDERATIONS WHEN USING ELEVATED TEMPERATURE

Column stability can be a concern with some columns, so it is important to refer to the manufacturers specifications before operating a column at elevated temperature. In general, for most silica-based columns, lifetime will be reduced when operated for extended periods of time at elevated temperatures, particularly at pH extremes.[6] At low pH, the stationary phase will be gradually stripped which will result in the silane activity of the phase increasing, whilst at high pH, silica dissolution is accelerated by temperature. Hybrid phases, where an organic molecule is incorporated into the bulk silica structure, tend to be more stable at high temperature. More exotic phases, such as carbon or zirconia, are also more stable; however their disadvantage tends to be too much retention or lower efficiency.

It is also important to consider compound stability at elevated temperatures and ensure that thermal degradation of analytes of interest does not occur on the column. Analyte stability is dependent on two factors: the duration that the compound is at an elevated temperature and the temperature it is elevated to. In general, as the temperature is increased, analyte residence time on column is reduced and therefore so is analyte exposure to the elevated temperature. The ability to operate at higher flow rates may also be used to further reduce exposure. In general, many compounds are stable in the timescales involved in LC, particularly fast LC and UHPLC, therefore elevated column temperatures tend not to be problematic.[7] Compound stability can be relatively easily determined and is worth assessing on an application dependant basis where elevated column temperatures are used.

CONCLUSION

The temperature that an LC separation is carried out at can have a profound effect on the chromatographic results obtained. This article has demonstrated that changes in column temperature can impact both analyte retention and selectivity. It is therefore essential that a constant temperature is maintained to achieve reproducible results.

Equally, it is also highly valuable to investigate column temperature during method development to help optimise separation selectivity. In addition, the use of elevated column temperatures can help to achieve faster separations at higher flow rates and increase sample throughput and laboratory efficiency.

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