Guest Author – Genevieve Hodson, Technical Specialist

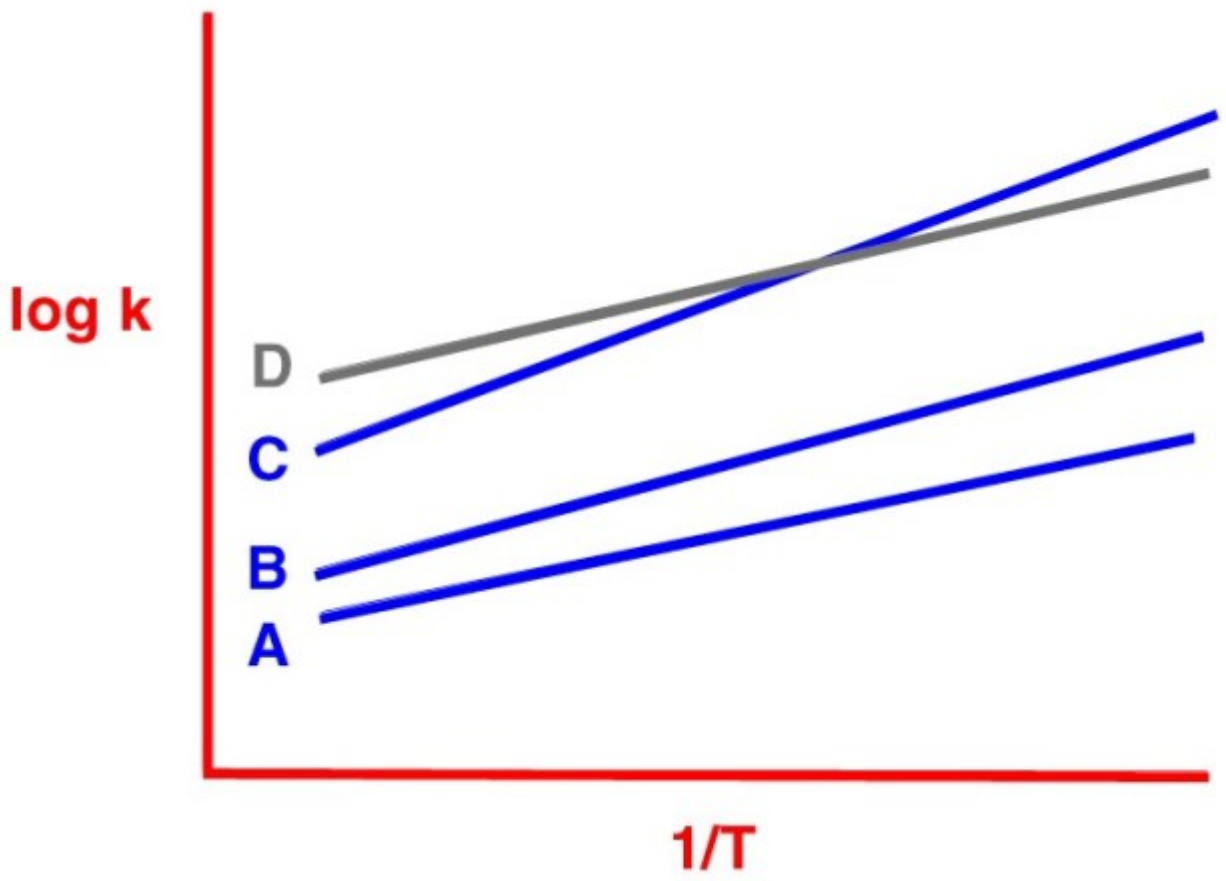
It is common practice to leave LC columns at ‘room temperature’, or ambient temperature conditions, when running routine analytical methods. So why do chromatography companies tell you not to do this? While putting the column heater cover on and setting the methods column temperature to 25°C, which most scientists agree is room temperature, can seem like more work than leaving the column exposed to the ambient lab conditions. And if you are looking for reproducible results, this is a must.

For example, room temperature in a Quality Lab in Texas during the summer when the AC is on full, can cause the DMSO to freeze solid and chemists to wear sweatshirts under their lab coats, is very different than when the AC goes out and you are now doing stability oven studies on pharmaceuticals sitting in vials on the bench top! That would be a range of 19°C to 37°C, for those of you who do not speak Texan. A change of 15°C can have big implications towards both the methods reproducibility and the columns overall lifetime. But why? I know you are asking yourself that very question right now, so let’s talk about it!

Reproducibility

For those chemists out there reading, we are going back to good old thermodynamics (if you thought you were done with P-Chem, you thought wrong!). As the temperature changes, the thermodynamics inside the column between the stationary phase and the mobile phase is

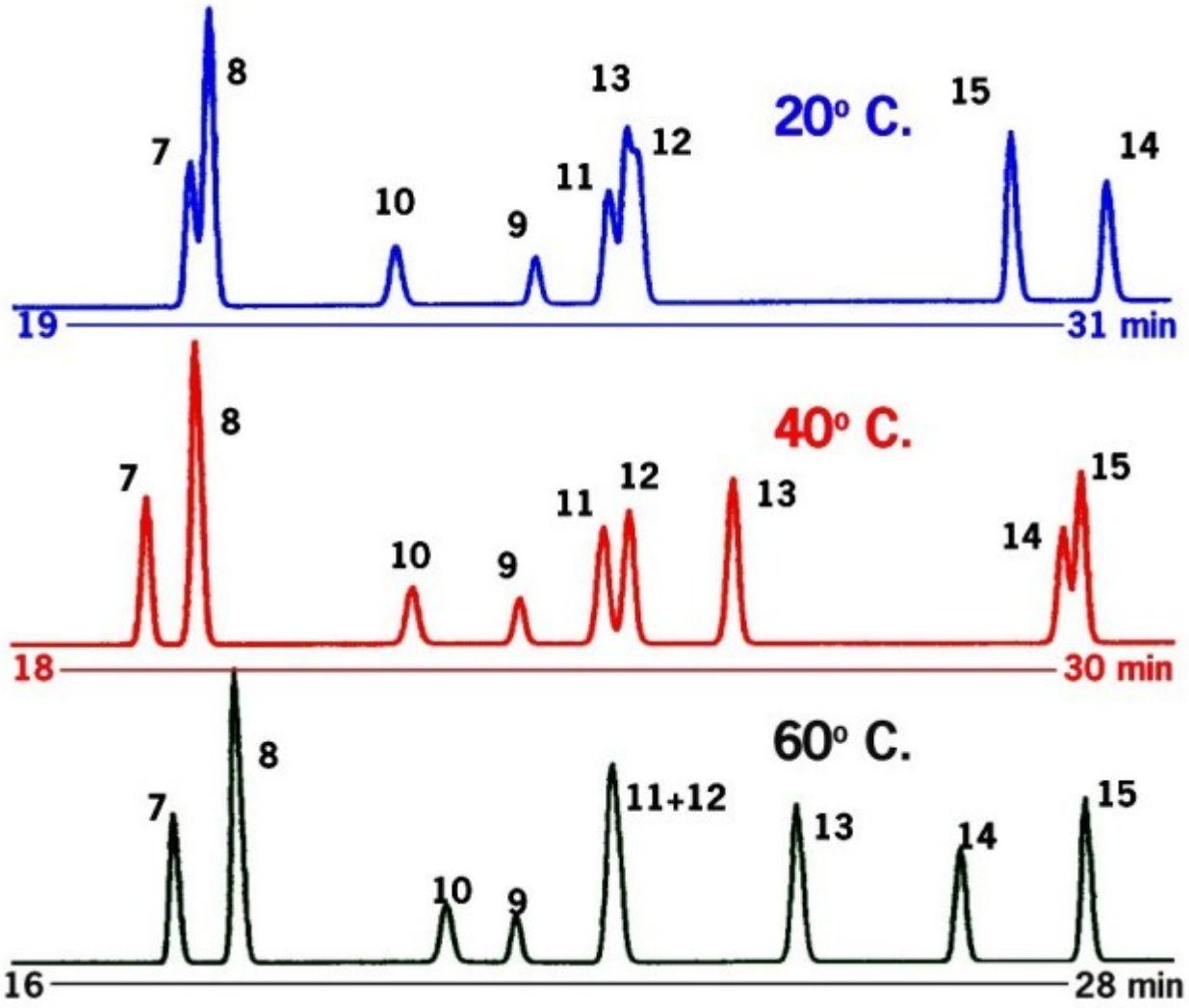
directly affected. Running a method at varying temperatures, may impact analytes capacity factor (k) or how long the analytes are retained on the column. A plot of the apparent capacity factor vs temperature is shown below for some theoretical analytes.



Plot of apparent capacity factor (k) vs temperature

At elevated temperatures, there is an increase in the kinetic energy of both the mobile phase and the analytes. The increase in kinetic energy of the analytes will cause them to be

heated and could disrupt the intermolecular binding that is the separation mechanism between the analytes and the stationary phase. Many times this causes all the analytes to come out sooner from the column, causing a reduction in the retention time. Usually this is most noticeable with large temperature jumps, example below with temperature increases in 20°C intervals.



Conditions

Column: RP C8 5µm, 4.6 x 150mm

Elution: 0 – 60% ACN with 0.1%TFA over 60 min at 1 ml/min.

Temperature: As indicated.

Sample: Tryptic digest of human growth hormone.

Reference: Hancock, Chloupek, kirkland and Snyder, J. Chromatography 686, 31-43 (1994)

Changes in temperature of only a few degrees Celsius could be seen impacting only a specific analytes in a chromatogram. In this case, it is likely that the small temperature difference is enough to push a favorable interaction with the stationary phase or with the mobile phase. It can be seen in samples as only one analyte shifting in retention compared to the others, who maintain their retention times. As it is hard to predict which analyte, if any, could be impacted and shift in retention due to the small temperature fluctuations, best practice is to maintain a consistent temperature by setting the column heater on with the column heater cover on.

Oligonucleotides provide a different. Imagining the 3D structure of this class of compounds, lets thinking about what happens when the temperature changes and they might partially unfold. The partial unfolding could expose hydrophobic groups that may have been previously buried causing the oligo to now retain more on the column. Conversely, the unfolding could expose hydrophilic groups causing the opposite retention to happen. A wonderful Technical Note has been written about this and is referenced here:

APPLICATIONS

Effect of Temperature on Single Stranded Oligonucleotide Analysis

Ivan Lebedev, Dani Xing, Brian Rivera
Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501, USA

Overview

Therapeutic oligonucleotides represent a recent breakthrough in the pharmaceutical industry. However, characterization of oligos, specifically by ion-pair reversed phase liquid chromatography (IP-RPLC), can be quite challenging. Oligos are manufactured by solid phase synthesis, where nucleotides are added in a step-wise manner. As such, impurities such as n-1 and n+1 must be characterized, and this may require extensive method development to optimize. Further, it may be necessary for characterization and quantitation of other closely related impurities, such as oxidation of phosphorothioates.

Another challenge is the variation in chromatography seen with oligo analysis. Retention times, peak shapes, and peak area recovery are often variable from injection to injection in any given sequence. One assumed cause of these variations is intramolecular interactions that compromise chromatographic performance. As such, high temperatures, exceeding 60 °C, are often implemented to improve separation. Although this is a requirement when working with double-stranded oligos such as siRNAs, there is still a question of whether this is also necessary for single stranded oligos. Here we present the effect of temperature on two oligos, a 5′ conjugated oligo and a phosphorothioate, and how it might be implemented for method development of single stranded oligonucleotides.

Figure 1 illustrates the effect of increasing the method running temperature in 5 °C increments. Retention time for the 5′-Amino C12 oligonucleotide decreases as temperature increases, which is common for any chromatographic method. Often, efficiency and peak shapes are improved at higher temperatures, though that is not observed in this example. Further, in comparing the 45 °C and 65 °C impurity profiles as shown in the insets show little to no differences. One might conclude then that selectivity is not being effected.

To better confirm selectivity changes, mass spectrometry is necessary to identify and characterize impurity peaks. A 22 mer DNA Phosphorothioate, with an unknown sequence was run by high resolution MS. The measured mass for the thioate was 6772.6 Da. The oligo was then run at different temperatures to observe any changes in selectivity for the impurity profiling.

In comparing methods run at 60 and 70 °C **(Figure 2)**, as expected, there was a marked decrease in retention for the main peak with the higher temperature running conditions. However, both methods gave similar impurity profiles, with the three earlier eluting impurities observed. The deconvoluted mass spectrum confirmed the same elution order; Impurity Peak 1 was 6443.4 Da, Peak 2 was 6468.4 Da, and Peak 3 was 6170.8 Da. Deconvoluted spectra for Peak 1 with both running conditions are shown in **Figure 3**. Interestingly, Peak 1 and 2 are likely both n-1 impurities, with Peak 1 being target sequence minus guanosine, and Peak 2 being target sequence minus thymidine.

In summary, temperature is often utilized for oligonucleotide analysis. However, for single stranded oligos, there may not be a benefit to running at temperatures exceeding 60 °, as increases in temperature may not improve chromatography nor effect selectivity as one might observe with other macromolecules.

LC Conditions

Columns:	bioZen™ 2.6 µm Oligo
Dimension:	100 x 2.1 mm (Figure 1) 00D-4790-AN 150 x 2.1 mm (Figures 2,3) 00F-4790-AN
Mobile Phase:	Figure 1: A: 12.5 mM HFIP, 4 mM TEA in Water B: 12.5 mM HFIP, 4 mM TEA in Methanol Figures 2,3: A: 100 mM HFIP, 4 mM TEA in Water B: 100 mM HFIP, 4 mM TEA in Methanol
Gradient:	5-30 % B in 14 minutes (Figure 1) 25-95 % B in 15 minutes (Figures 2,3)
Flow Rate:	0.3 mL/min
Injection:	1 µL
Temperature:	As noted in Figures
Detection:	UV @ 260 nm (Figure 1,2) TOF-MS (Figure 3)
Sample:	5′-Amino C12 Oligo (Figure 1) DNA 22 mer Phosphorothioate (Figure 2,3)

Column Limitations

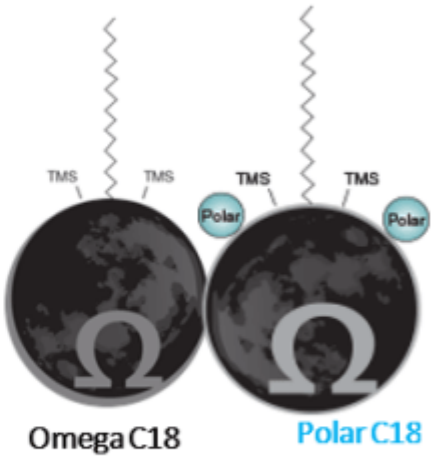
Temperatures can impact a columns overall lifetime as well. The media/phase of the column are what drives it's specification for max temperature.

A columns temperature stability can differ depending on what solvent and buffer are used. Example can be found in the table below for our Luna Omega brand column. There is a noted difference in how the C18 compares to the Polar C18 due to slight differences in the bonding of the phases. For most reverse phase column 60°C to 90°C is a common max temperature range.

Luna Omega Stability

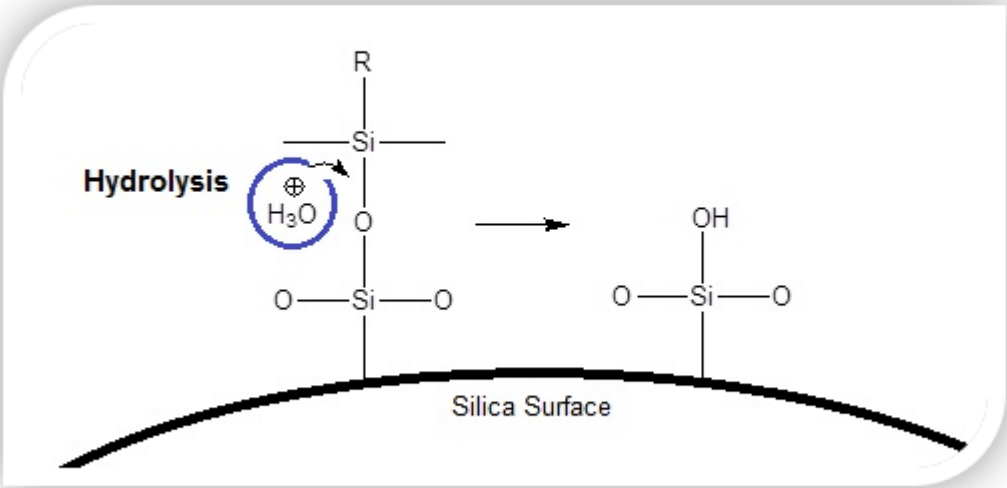
Mobile Phase	Temperature Limit
0.1% Formic acid	90º C
0.1% TFA	80º C
Phosphate at pH 6	50º C
Phosphate at pH 7.5	50º C/30º C*
Ammonium Bicarbonate pH 8.5	50º C/30º C*

*Luna Omega Polar C18



Elevated temperatures under acidic conditions provide favorable thermodynamic conditions for hydrolysis of the ligand bonded to the silica. Hydrolysis is observed as retention times shifting earlier due to there is less phase to provide interactions in the stationary phase for the analytes. A loss of resolution occurs sooner than the overall shift in retention. It is common to see baseline resolution disappear or peaks merging together, when compared to a new column with the same phase. **Best practice is to not leave columns in the oven when turned on and no solvent flow going through the system.** Most mobile phases have some organic in them that will evaporate out faster.

Hydrolysis Mechanism:



If you have any additional questions regarding column temperature, or other

chromatographic inquires, reach out to our Technical Specialists, like Genevieve, through our free online chat service – Chat Now.

You will be able to chat with technical experts nearly 24/7 for any help you might need in the lab from method development, product assistance and recommendations, and so much more. Click the link or image below to start chatting today! www.phenomenex.com/chat

- Click to share on Tumblr (Opens in new window)
- Click to share on Reddit (Opens in new window)