

Introduction-

Column temperature plays two important roles in reversed-phase high-performance liquid chromatography (RP-HPLC): (1) control of retention (k) and (2) control of selectivity (α). While changes in retention as a function of temperature are ubiquitous, selectivity changes for any given solute pair are more pronounced for ionized samples and samples with more polar substituents.¹ Therefore the effects and control of temperature on peptide separations which are routinely separated at low pH, in an ionized state, by (RP-HPLC) must be taken into consideration to produce reliable and robust peptide maps.

Peptide Sample Preparation-

Bovine cytochrome c and ammonium bicarbonate were obtained from Sigma Chemical Co. (St. Louis, MO). Trypsin was obtained from Promega (Madison, WI). The bovine cytochrome C was suspended in 100 mM ammonium bicarbonate buffer pH 7.8 and digested overnight at 37 °C with trypsin at a ratio of 1:50 (w/w) trypsin:protein.

Chromatography-

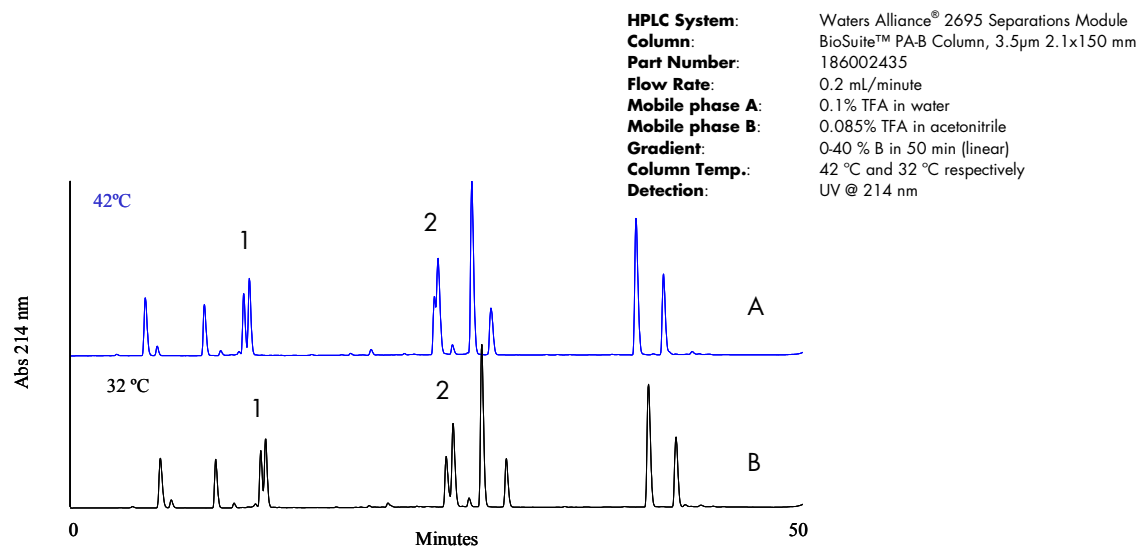


Figure 1. Effect of temperature on the RP-HPLC separation of bovine cytochrome c digest. Chromatogram A was run at 42 °C while chromatogram B was run at 32 °C .

Results and Discussion-

Figure 1 shows the peptide map of bovine cytochrome c. All conditions remain constant with the exception of a change in column temperature. It can be seen from figure 1 that temperature greatly affects the retention and resolution of peptides. Peptides separated at higher temperature (42 °C) show a distinct reduction in the retention when compared to the same peptide fragments separated at 32°C.

Figure 1 also shows the change in resolution between two closely eluting peaks. Resolution was measured using the discrimination factor. The discrimination factor, d_o , which uses the height of the smaller peak in the pair h_p and the height at the valley h_v to measure the extent of the separation between adjacent peaks. It varies between 0 and 1 and increases when the separation improves². It is calculated using the following formula:

$$d_o = (h_p - h_v) / h_p$$

The results using this formula show a change in separation for peaks (1) of 0.71 to 0.85, an increase of 0.14, with an increase of 10 °C. Conversely the results for peaks (2) show a decrease in the separation of 0.58, from 0.78 to 0.20, with an increase of 10 °C (Figure 1).

Since temperature has been shown here to be a variable in the separation of peptides, control of the column temperature is critical for reproducible chromatography of peptides. Figure 2 shows a separation of two closely eluting peptides from a bovine cytochrome c tryptic digest at temperatures differing by only 2 °C. Five replicate injections were made at both temperatures. The average retention time at 38 °C was 33.16 with a RSD of 0.06%. The average retention time at 40 °C was 33.25 with a percent RSD of 0.05% (Table 1).

Injection #	38 °C	40 °C
1	33.21	33.15
2	33.25	33.14
3	33.26	33.18
4	33.25	33.17
5	33.26	33.17
Average	33.25	33.16
stdev	0.019	0.016

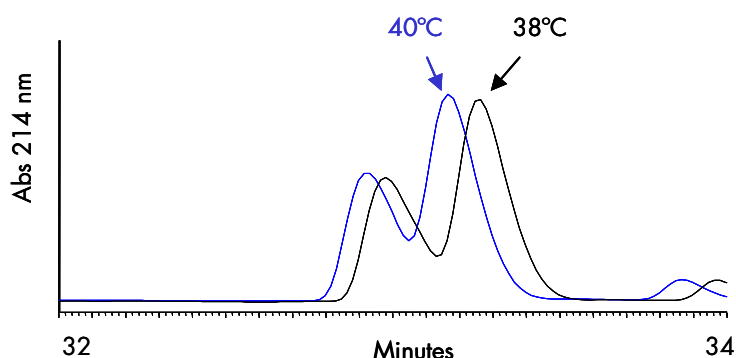


Table 1. Retention time data at 38 °C and 40 °C

Figure 2. Effect of small changes in temperature on peptide retention

Conclusion-

The data shown here indicates that temperature can effect the retention time and separation of peptides. This effect of temperature can either increase or decrease the resolution of closely eluting peaks. The data further shows that at even a 2 °C difference in temperature can impact the retention of peptides. Therefore, it is important to have consistent column temperature control for reproducible peptide mapping. Waters Alliance 2695 separations module has been shown to have excellent column temperature control capability.

References-

1. J. Dolan, *Journal of Chromatography A*, 965 (2002) 195
2. U. Neue, *HPLC Columns: Theory, Technology, and Practice*, (1997) 303