Effect of Temperature on Reversed Phase Separations of Monoclonal Antibodies

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The analysis of intact monoclonal antibodies (mAbs) by reversed phase HPLC is a common technique for assessing protein variation, such as clipping and heterogeneity due to post-translational modifications. Whether for LC-UV impurity analysis or for confirming primary sequence using LC-MS, the analysis of intact mAbs can provide useful insight on a protein therapeutic.

As with small molecule analysis, proteins run by reversed phase will elute in order of hydrophobicity; i.e. more hydrophobic proteins will retain longer. However, unlike small molecules, the secondary and tertiary structure of the protein impact the separation and the so-called "native" structure of proteins is disrupted by typical reversed phase conditions. Acidic modifier and organic solvent partially dissociate the protein, leading to the exposure of hydrophobic side chains, and potentially contributing to peak broadening.
To overcome peak broadening, a bioZen™ 2.6 µm WidePore C4 LC Column was utilized in combination with method parameter enhancements.

Although these denaturing conditions are inherent to the detect, one particularly useful method parameter to adjust is column temperature. Modulation in temperature modulates the protein secondary and tertiary structure, which can be utilized to ultimately effect peak shape and even selectivity; presumably, slight changes in protein conformation affect the interaction of hydrophobic amino acid residues in the primary sequence with the moderately hydrophobic stationary phase.

Figure 1 shows improvements in peak shape with for intact infliximab, when comparing 70 °C to 80 °C column temperature. Interestingly, we observe a decrease in performance with further increases to 90 °C. Upon further investigation, this increase in peak width is due to partial separation of another variant, observed as a later eluting shoulder in **Figure 2.**

The effect of temperature is more clearly observed in subunit analysis of mAbs (**Figure 3**). Increase in temperature improves peak width at half height when comparing 70 °C and 80 °C, with improvements in peak widths for light chain. However, peak widths are slightly worse when comparing 80 °C and 90 °C.

More interesting are the impurity profiles for the heavy chain, which vary between each method column temperature (**Figure 4**). Depending on the importance in characterizing each impurity, one profile may be more desirable than another.

In summary, increases in temperature can lead to improvements in peak shape in large molecule reversed phase LC methods, as seen with the bioZen WidePore C4. Further, selectivity needs to be assessed separately as the increases in temperature can be detrimental to the separation. At the intact level, one may observe partial separation of another impurity, which could lead to overall peak broadening but a more detailed impurity profile. At the subunit level, differences in impurity profile can be observed and as such, temperature should be implemented as part of the design of experiment.

LC Conditions

Column:	bioZen 2.6 µm WidePore C4
Catalog No.:	00D-4786-AN
Dimensions:	100 x 2.1 mm
Mobile Phase A :	0.1 % TFA in Water
Mobile Phase B:	0.1 % TFA in Acetonitrile
Gradient:	20-55 % B in 6 minutes *(Fig 1,2)* 25-45 % B in 6 minutes *(Fig 3,4)*
Flow Rate:	0.5 mL/min
Detection:	280 nm *(Fig 1,2)* 214 nm *(Fig 3,4)*
Temperature:	*As Indicated*
Sample:	Infliximab (1 mg/mL) NIST mAb (1 mg/mL)

Figure 1:

Stacked chromatogram showing the effect of temperature on peak widths at half height for intact infliximab. Optimal peak widths are observed at 80 °C.

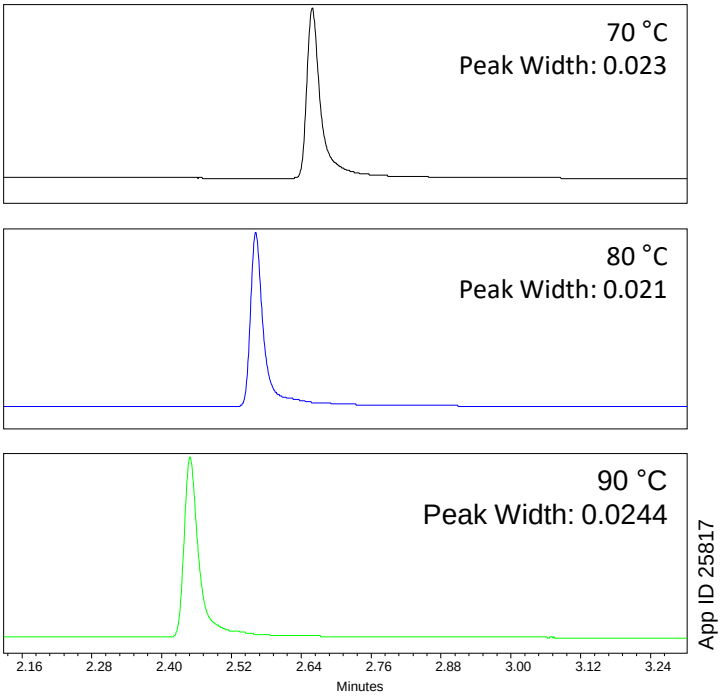


Figure 2:

Detailed overlay of intact infliximab, showing a nominal improvement in impurity profile at 90 °C.

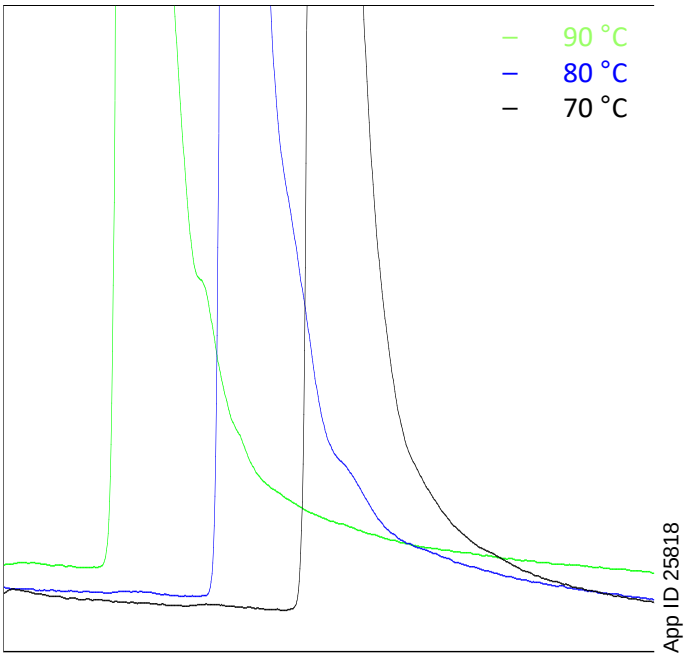


Figure 3:

Chromatogram stack of reduced NIST mAb. Light chain show improved peak width at half height run at the 80 °C when compared to 70 °C, though no improvement as temperature is increased to 90 °C.

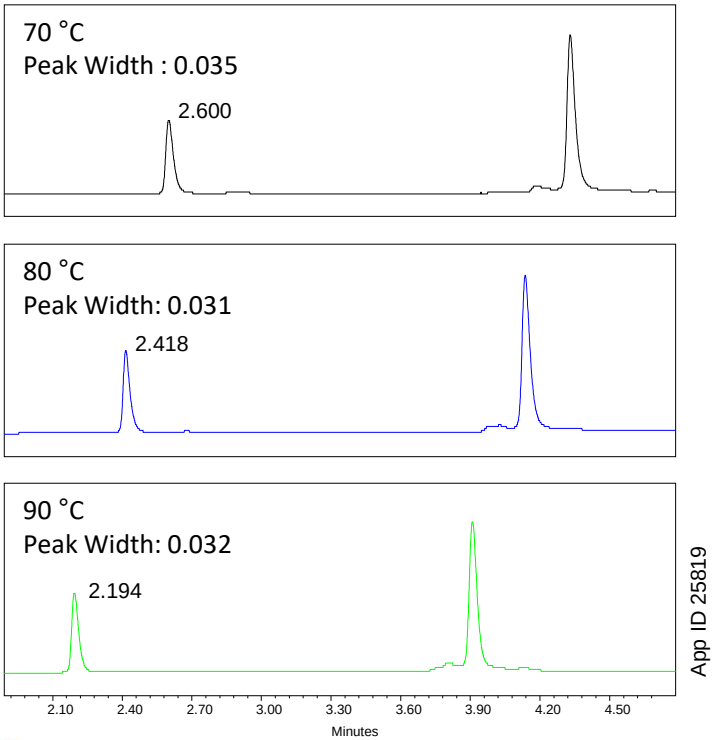
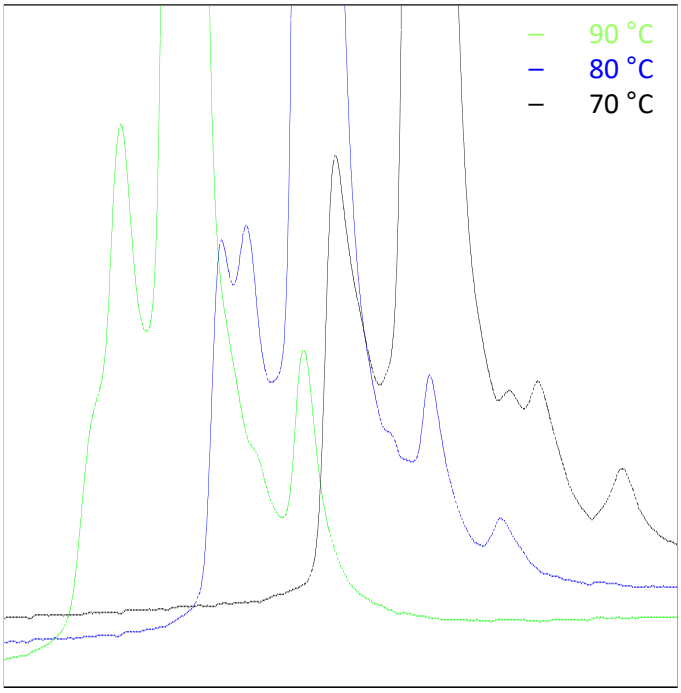


Figure 4:

Chromatogram overlay of heavy chain of NIST mAb, showing differences in impurity profile. Both 70 °C and 80°C show improved separation of later eluting shoulder impurities.



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