

# APPLICATIONS

## Optimization of a Gradient Profile 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.

The utility of this analytical technique becomes even more apparent when analyzing intact mAbs with UHPLC columns packed with core-shell materials, which minimize peak broadening inherent in the chromatographic separation of any large molecule. Utilizing a [bioZen™ 2.6 µm WidePore C4](#) LC column allows for better resolution and reproducibility through a wider pore core-shell particle combined with bioinert hardware.

In order to adjust method parameters using bioZen WidePore C4 for intact protein separation, the gradient program is evaluated. Typical approaches include adjusting the gradient program to an appropriate  $k'$  value between 2 and 10. Further shallowing of the gradient can be implemented to nominally improve selectivity and separation of minor variants. This can be as shallow as 1% B/column volume. In general, this shallowing of the gradient slope can change selectivity and thus improve resolution.

**Figure 1** shows the effect of shallowing the gradient for intact NIST mAb, from 20-55% B to 25-45% B. There is a marked increase in peak widths with the shallower gradient, in addition to decrease in peak heights, as one runs the shallower gradient. Depending on the intended target method attributes, the shallowing of the gradient might be interpreted as a decrease in chromatographic performance. However, **Figure 2** shows an earlier eluting impurity and as such, if the intent is to obtain more detail in impurity profiling, especially in the context of intact, large molecule analysis, shallowing of the gradient might be considered a viable strategy.

That said, a decrease in peak height and broadening of peaks may be detrimental to the impurity profile. As observed in **Figure 3**, NIST mAb heavy chain and light chain show a decrease in peak heights when shallowing the gradient; light chain elutes earlier while heavy chain elutes later. There is a nominal improvement in resolution between some of the close eluters. However in observing the impurity profile for the light chain in **Figure 4**, the increase in resolution adversely effects peak heights and as such, there may be difficulty in quantitation.

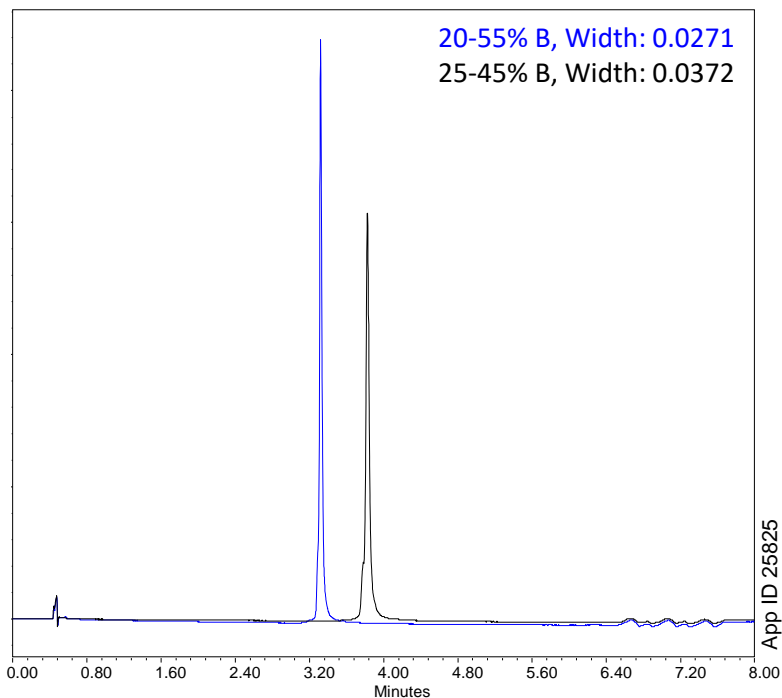
In summary, a common approach to improving resolution in intact and subunit analysis of mAbs by reversed phase using [bioZen WidePore C4](#) is to shallow the gradient. This can improve resolution and thus enable characterization of closely eluting impurities. However, resulting changes in resolution can lead to peak broadening; further method parameters to optimize could include adjustments to temperature and flow-rate.

### LC Conditions

<b>Column:</b>	<a href="#">bioZen 2.6 µm WidePore C4</a>
<b>Catalog No.:</b>	<a href="#">00D-4786-AN</a>
<b>Dimensions:</b>	100 x 2.1 mm
<b>Mobile Phase A :</b>	0.1 % TFA in Water
<b>Mobile Phase B:</b>	0.1 % TFA in Acetonitrile
<b>Gradient:</b>	<i>As indicated</i> , 6 minutes
<b>Flow Rate:</b>	0.5 mL/min
<b>Temperature:</b>	90°C
<b>Detection:</b>	UV-Vis @ 280 nm (Fig 1,2) UV-Vis @ 214 nm (Fig 3,4)
<b>Sample:</b>	NIST mAb (1 mg/mL)
<b>Injection:</b>	2 µL

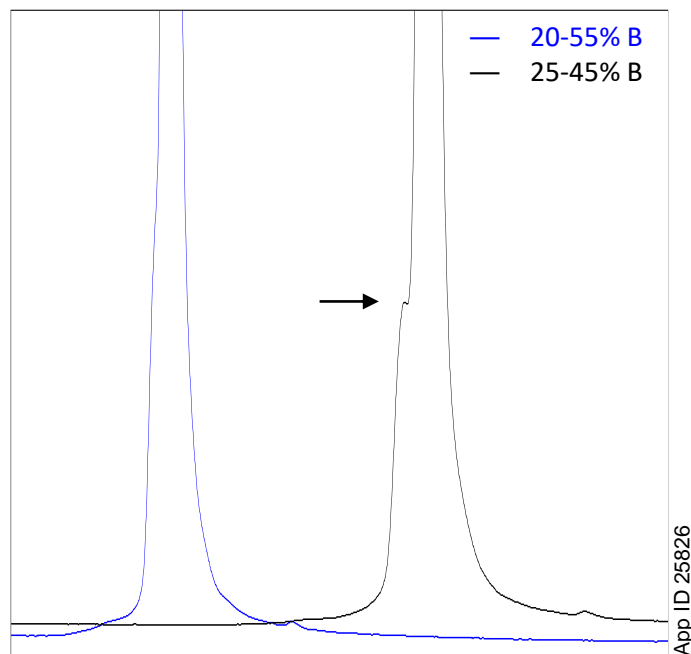
**Figure 1:**

Overlay showing the effect of shallowing gradient. A decrease in peak height and broadening of peak width is observed with the 25-45% B gradient program.



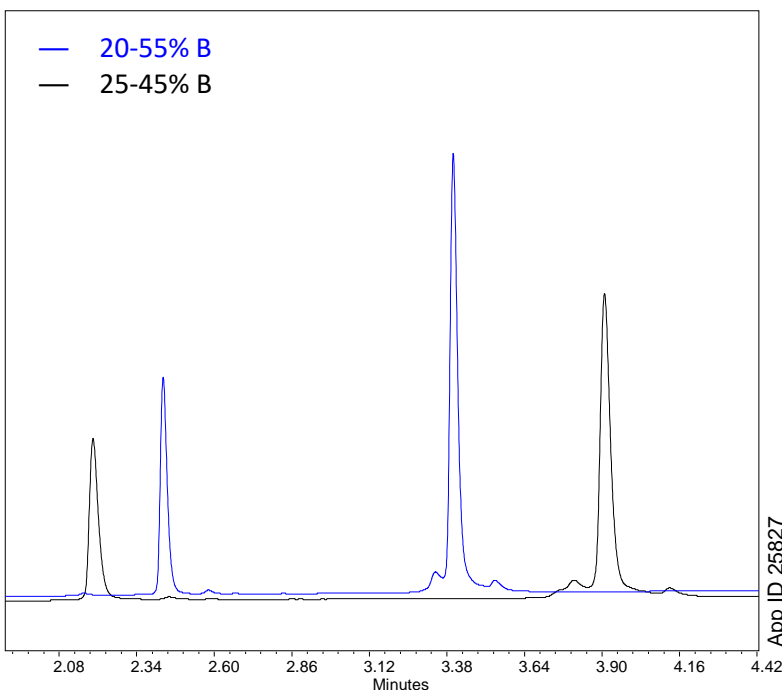
**Figure 2:**

Nominal improvement in variant separation using the shallow gradient program.



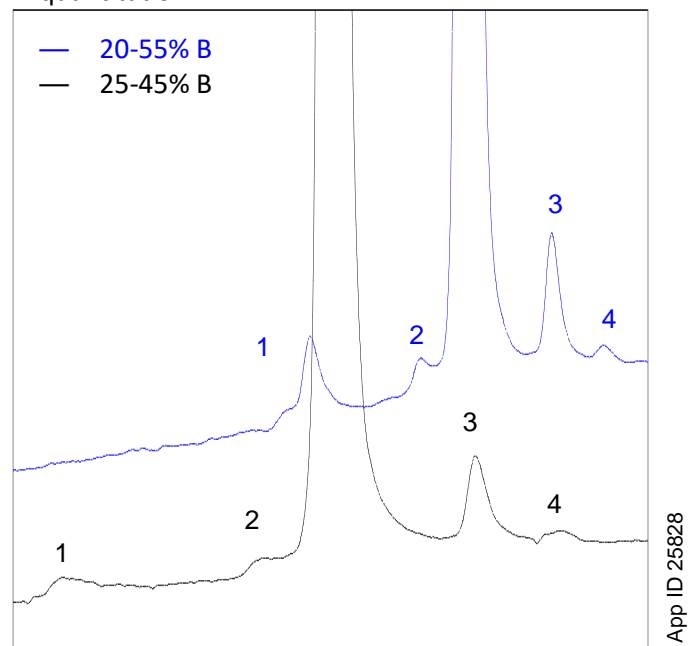
**Figure 3:**

NIST mAb light chain and heavy chain, showing increase in resolution though a drop in overall peak heights.



**Figure 4:**

Although light chain sees an increase in resolution between closely eluting impurities, the drop in peak heights loses some detail in the profile, which could effect quantitation.



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