METHOD DEVELOPMENT OF IONIC-STRENGTH GRADIENT CATION EXCHANGE CHROMATOGRAPHY FOR MONOCLONAL ANTIBODY CHARGE VARIANT ANALYSIS

Authors: Hua Yang, Bill Warren and Stephan M. Koza
Affiliations: Waters Corporation, Milford, MA 01757

Waters
THE SCIENCE OF WHAT'S POSSIBLE.®

To learn about BioResolve columns, please visit www.waters.com/bioresolve

INTRODUCTION

Charge heterogeneity in therapeutic proteins including monoclonal antibodies (mAbs) needs to be characterized and monitored, since it can potentially affect biological activity and safety of the biotherapeutics.[1] Ion exchange chromatography (IEX) has been widely used for the purification, characterization, and routine monitoring of protein charge variants. In selecting between cation-exchange or anion-exchange separations, cation-exchange chromatography (CEX) is the most suitable mode for mAb charge variant characterization, due to the comparatively high isoelectric point (pI) of mAbs. In discussions with development labs we have noted that constant pH with ionic-strength (salt) gradient methods, pH gradient methods, and hybrid methods including both ionic-strength and pH gradients are all commonly employed. This study will focus on ionic-strength gradient method development and the parameters that are often optimized for individual mAbs including pH, salt concentration, gradient time, flow rate, organic modifiers, and temperature, among others. Parameters may be optimized simultaneously using a factorial design or by linearly optimizing a single variable at a time. We have elected the latter approach in order to better demonstrate the impact of each parameter.

METHODS

Sample preparation:

Trastuzumab, adalimumab and bevacizumab were diluted in water to 5 mg/mL. Cetuximab was diluted in water to 1 mg/mL. Drug products were analyzed post expiry.

LC Conditions:

LC system: ACQUITY UPLC H-Class Bio
Sample temp.: 10 ˚C
Column temp.: 30 ˚C
Flow Rate: 0.8 mL/min, unless specifically noted
Injection volume: 1 - 2 µL for 4.6 mm i.d. column;
0.2 µL for 2.1 mm i.d. column
Detection: 280 nm

Mobile Phase A: 100 mM MES monohydrate
Mobile Phase B: 100 mM MES sodium salt
Mobile Phase C: 1 M NaCl
Mobile Phase D: water

Typical gradient for 4.6 x 50 mm column (an AutoBlend Plus method):
The system is pre-defined to deliver 20 mM MES buffer.

Time	Flow Rate (mL/min)	pH*	Salt	Salt Curve
0.00	0.8	6.7	0	
1.00	0.8	6.7	0	11
2.00	0.8	6.7	50*	6
4.00	0.8	6.7	50*	6
9.00	0.8	6.7	85*	6
10.00	0.8	6.7	700	6
10.10	0.8	6.7	0	11
25.00	0.0	6.7	0	11

* pH as well as starting and ending salt concentration vary with different mAbs. A typical optimized gradient has changes in salt concentration of 35 - 40 mM in 5 minutes.

Column:
BioResolve SCX mAb column 4.6 x 50 mm (P/N 186009058)
BioResolve SCX mAb column 4.6 x 100 mm (P/N 186009060)
BioResolve SCX mAb column 2.1 x 50 mm (P/N 186009054)

RESULTS AND DISCUSSION

I. Effect of Mobile Phase pH

There is a compromise that must be generally made between acidic peak resolution and basic peak resolution when the pH is varied.

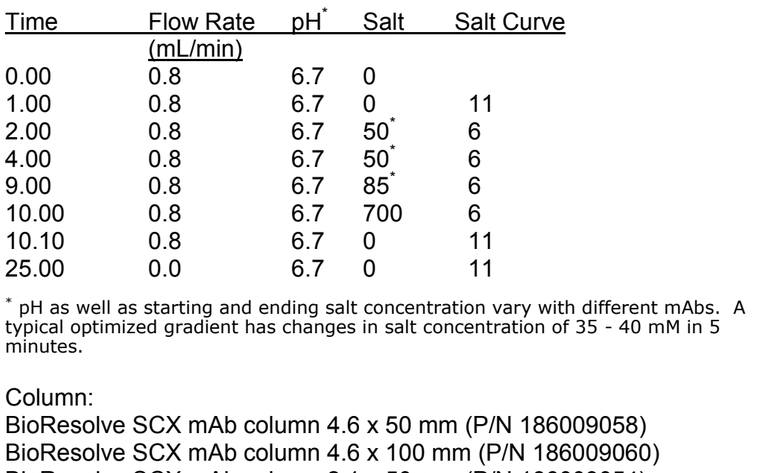


Figure 1. Effect of pH on trastuzumab charge variant separation. 20 mM MES, 50 – 155 mM NaCl in 15 mins. The arrows point to the rescaled figures that show details of low abundance peak separation.

II. Effect of Gradient Slope

a. Salt Concentration

As the starting and ending salt concentration is narrowed down, the gradient slope becomes shallower, and the separation of the charge variants improves, as indicated in peak to valley (p/v) ratio.

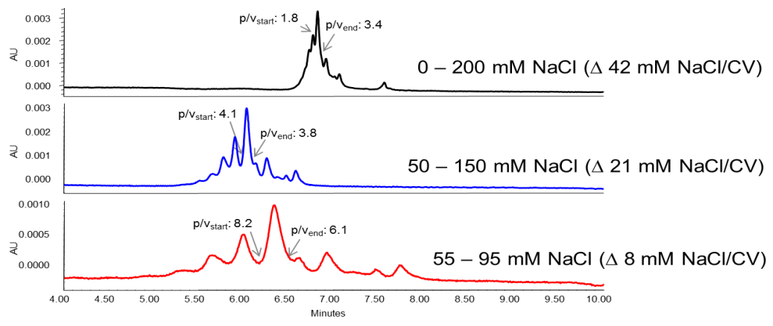


Figure 2. Effect of salt concentration on cetuximab charge variant separation. 20 mM MES pH 6.0. Gradient time: 5 mins

b. Gradient Time

Longer gradient time results in shallower gradient slope. However, there is a practical limit to decreasing gradient slope in order to increase the resolution of these separations. At a certain point, longer gradient time will not improve the separation further.

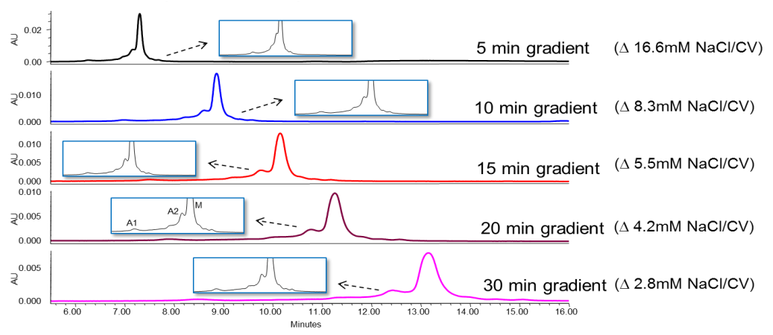
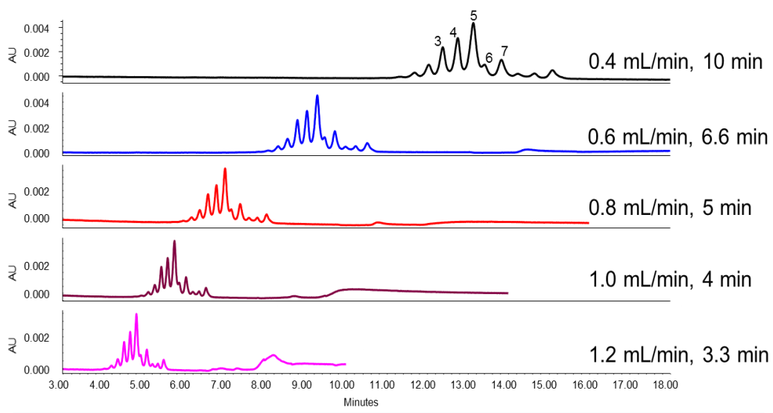


Figure 3. Effect of gradient time on bevacizumab charge variant separation. 20 mM MES pH 5.4, 120 - 200 mM NaCl.

c. Flow Rate

1). When both the flow rate and gradient time are varied and the total gradient volume is kept constant, the gradient slope is kept constant. The p/v ratio showed a slight decrease at higher flow rates. This may be the result of increased post column dispersion at higher flow rates or minor changes of the protein conformation due to the higher hydrostatic pressures imposed.[2]



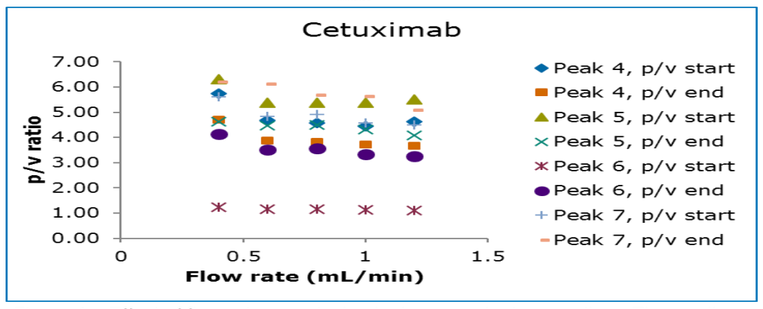


Figure 4a. Effect of flow rate on cetuximab charge variant separation. The gradient volume is kept constant.

2). When the flow rate is varied while the gradient time is kept constant, the gradient slope is shallower with higher flow rate. As a result, the p/v ratio increases for most of the peaks with the increase of flow rate.

Figure 4b. Effect of flow rate on cetuximab charge variant separation. The gradient time is kept constant.

III. Effect of Temperature

Temperature may impact the overall retention, but it does not generally have significant impact on the selectivity differences and ultimately the resolution between mAb charge variants.

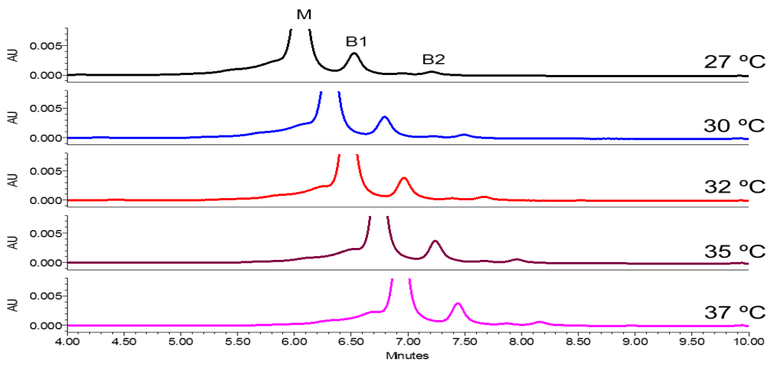


Figure 5. Effect of temperature on adalimumab charge variant separation. 20 mM MES pH 5.7, 140 – 175 mM NaCl in 5 mins.

IV. Effect of Organic Additives

Addition of a small amount of organic solvent in the mobile phases results in almost identical chromatograms except for a slight increases of the retention time, indicating minimal hydrophobic interaction between the mAb and the stationary phase.

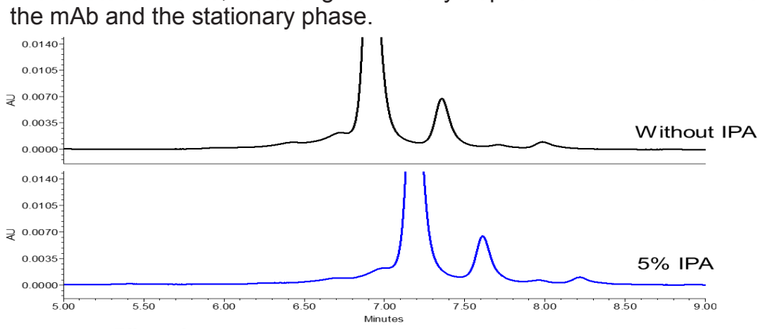


Figure 6. Effect of isopropanol as a mobile phase additive on adalimumab charge variant separation. 20 mM MES pH 6.0, 115 – 155 mM NaCl in 5 mins.

V. Effect of Column Length

Longer column length has more impact on the separation quality than running a longer gradient on a shorter column.

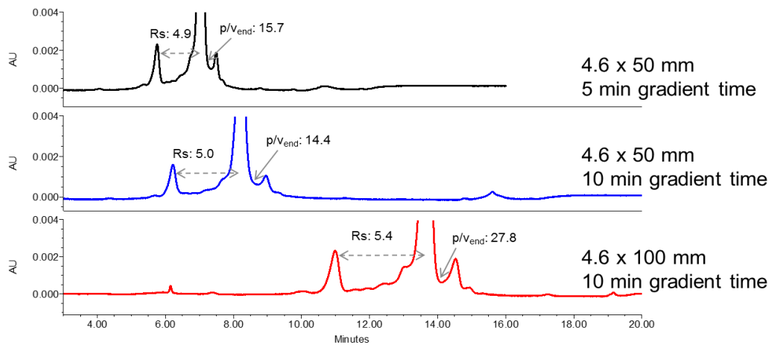


Figure 7. Effect of column length on Trastuzumab charge variant separation. 20 mM MES pH 6.7, 50 -85 mM NaCl.

VI. Effect of Column I.D.

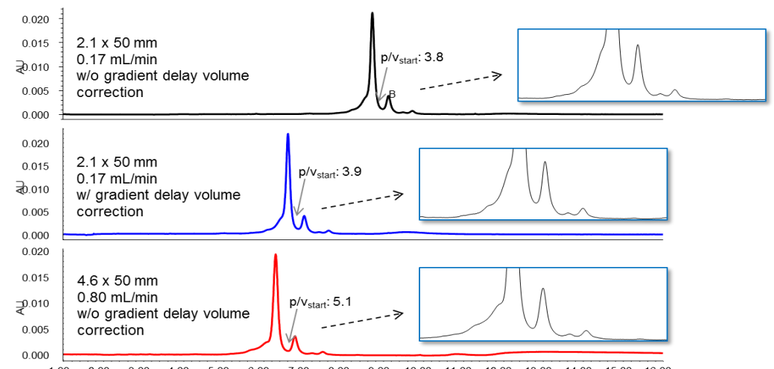


Figure 8. Adalimumab charge variant separation on 2.1 mm I.D. column and 4.6 mm I.D. column, without (top) and with (middle) gradient delay volume correction on the 2.1 mm I.D. column.

CONCLUSION

Method development is an important step in charge variant analysis of biotherapeutic protein by cation exchange chromatography and there are many factors that can be manipulated.

- Mobile phase pH can affect the resolutions of the acidic peaks and basic peaks inversely, so an operating pH needs to be chosen carefully to determine the optimal compromise for these separations.
- Gradient slope affects selectivity and resolution. Gradient slope as defined by column volumes can be altered by changing salt concentration, gradient time or flow rate. A shallower gradient slope increases the resolution, however, at a certain point, further decreasing the gradient slope will not further improve the separation.
- Temperature has minimal impact on the selectivity of mAb charge variants. However, it is recommended that temperature be controlled for improved reproducibility.
- Increasing column length has more impact on resolution than increasing gradient time on a shorter column.
- For Waters BioResolve SCX mAb column, organic additives have minimal impact on the separation and the recovery of mAbs, which indicates that there is little hydrophobic interaction between the analytes and this CEX stationary phase.

References

1. Khawli L.A. et al., Charge variants in IgG1. mAbs 2:6 (2010) 613 - 624.
2. Lauber M.A. et al., Designing a new particle technology for robust charge variant analysis of mAbs. Waters Application Note, 720006475EN (2018).

TO DOWNLOAD A COPY OF THIS POSTER, VISIT WWW.WATERS.COM/POSTERS