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Charge Variant Method Design for Analysis of Monoclonal Antibodies

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ABSTRACT

Purpose: To detail the effects of chromatography parameters on chromatographic separation of monoclonal antibodies and associated variants on a weak cation exchange chromatography phase. The examples provide guidance for method development for user's individual protein analyses.

Methods: Standard chromatographic parameters including mobile phase composition, temperature, flow rate, gradient time, protein loading, and column format selection are explored for both salt gradient and pH gradient separations on the ProPac Elite WCX 5 µm particle chromatography column.

Results: The work here illustrates how chromatographic parameters can be adjusted to alter chromatographic separation for improved analysis of proteins and associated variants.

INTRODUCTION

Proteins as therapeutics are used to treat a wide range of diseases including cardiovascular disease, autoimmune disorders, and cancers owing to their ability to perform specific biological functions. Cellular manufacturing and downstream processing of the protein products typically results in a range of variant structures including protein glycosylation, lysine truncation, oxidation, and isomerization. These variants can have an adverse effect on protein performance by reducing the efficacy or causing other unintended effects such as autoimmune responses. For these reasons, it is important to understand the structure and relative quantities of these variants during development and production and in the final product.

Weak cation exchange chromatography is a standard technique for separating protein charge variants based on their relative affinities for the column solid phase. Cationic proteins readily adsorb to the anionic stationary phase in a low ionic strength mobile phase. An increase in the ionic strength and/or the pH of the mobile phase results in disruption of the ionic interactions causing the variants to desorb and elute from the column for detection. Chromatographic parameters including mobile phase composition, temperature, flow rate, gradient time, protein loading, and column format selection determine the extent to which variants are separation from the main mAb peak and each other. Proper method design is critical to the development of a robust procedure that provides reproducible separations of mAbs and their variants. In this poster we look at basic chromatography parameters and demonstrate their affect on mAb-variant separation.

MATERIALS AND METHODS

Sample Preparation

All samples were diluted to their final concentration using deionized water.

Test Method(s)

All samples were analyzed on a 4x150mm ProPac Elite WCX 5 µm column (302972) using either a salt gradient or pH gradient buffers (Thermo Scientific CX-1 pH Gradient Buffers). Specific details on each chromatographic separation are provided in the associated figure.

Vanquish Flex Quaternary UHPLC system, including:

- System Base Vanquish Flex (P/N VF-S01-A)
- Quaternary Pump (P/N VF-P20-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A) with 25 µL (V=50 µL) sample loop
- Diode Array Detector HL (P/N VH-D10-A) with Thermo Scientific™ LightPipe™ 10 mm Standard Flow Cell (P/N 6083.0100)
- VWD-3400RS Rapid Separation Variable Wavelength Detector equipped with a PCM-3000 pH and Conductivity Monitor

Buffers

- Salt Gradient:
  - MES Buffer - 2-(N-morpholino)ethanesulfonic acid
  - MOPS Buffer - 3-(N-morpholino)propanesulfonic acid
- pH Gradient:
  - CX-1 pH Gradient Buffers (Buffer A – pH 5.6; Buffer B – pH 10.2)

Data Analysis

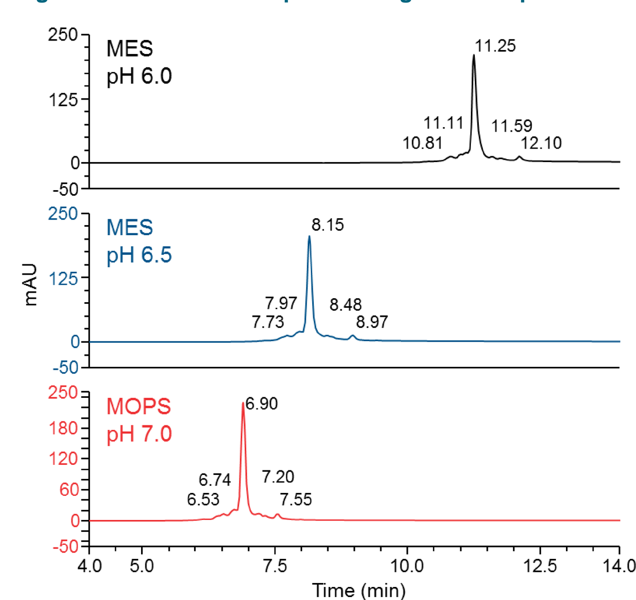
The Thermo Scientific™ Dionex™ Chromeleon™ 7.2.7 Chromatography Data System was used for data acquisition and analysis.

RESULTS

For all examples provided, current pharmaceutical mAbs were selected for analysis as these are well known class of protein therapeutics that commonly posses multiple charge variants. The salt gradient mobile phase pH and the starting pH for a pH gradient were evaluated for their effects on mAb-variant separation. For both salt and pH gradients, the effects of temperature, protein loading, and flow rate and gradient time were evaluated as well. The examples provided here demonstrate the importance of evaluating each of these parameters when developing new methods for separating and detecting charge variants of mAbs and other proteins. As the examples provided here are specific to the samples analyzed, chromatographers should systematically evaluate each parameter for their own samples in order to achieve the best separation.

Salt Gradient Separation Principles

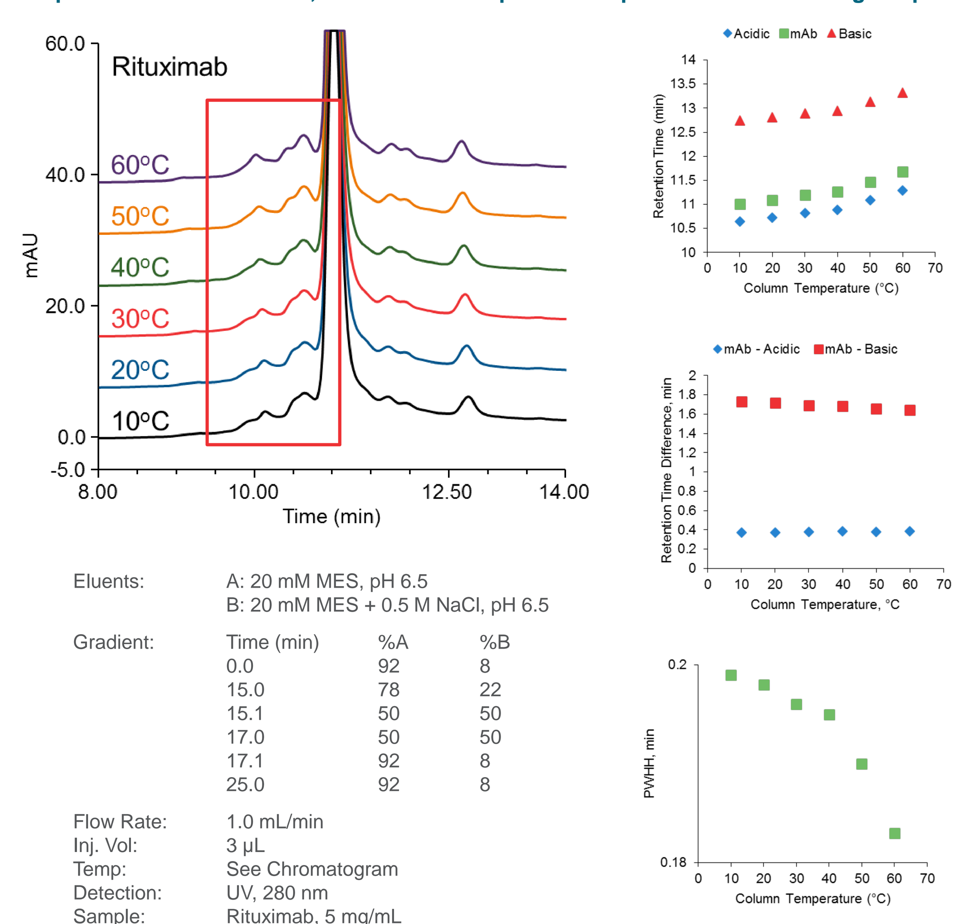
Figure 1. Effect of buffer pH on salt gradient separation of Rituximab charge variants.



Column:	ProPac Elite WCX 5µm		
Format:	4×150mm		
Eluents:	See chromatogram for specific buffer and pH A: 20 mM Buffer* B: 20 mM Buffer* + 0.5 M NaCl		
Gradient:	Time (min)	%A	%B
	0.0	95	5
	15.0	65	35
	15.1	50	50
	16.0	50	50
	16.1	95	5
	25.0	95	5
Flow Rate:	1.0 mL/min		
Inj. Vol:	3 µL		
Temp:	30 °C		
Detection:	UV, 280 nm		
Sample:	Rituximab, 5 mg/mL		
Peak Label:	Retention time		

*Buffers for salt gradients should be able to interact with the solid phase as a buffer. For anionic WCX phases a neutral or cationic zwitterionic buffer or cationic buffer should be selected.

Figure 2. Effect of column temperature on salt gradient separation of Rituximab charge variants. Top, middle and bottom plots show effect on main peak retention time, variant-mAb separation, and main peak PWHH, respectively, as a function of temperature. For Rituximab, acidic variant separation improves with increasing temperature.



Eluents:	A: 20 mM MES, pH 6.5 B: 20 mM MES + 0.5 M NaCl, pH 6.5		
Gradient:	Time (min)	%A	%B
	0.0	92	8
	15.0	78	22
	15.1	50	50
	17.0	50	50
	17.1	92	8
	25.0	92	8
Flow Rate:	1.0 mL/min		
Inj. Vol:	3 µL		
Temp:	See Chromatogram		
Detection:	UV, 280 nm		
Sample:	Rituximab, 5 mg/mL		

Figure 3. Dynamic loading analysis and sample carryover for a trastuzumab biosimilar on a 4×150mm column. (Left) Chromatograms showing the change in charge variant separation with increasing loading amounts corresponding the data in the right plot. (Right) Red – increase in PWHH with increased mAb loading; Blue – Sample carryover in subsequent blank.

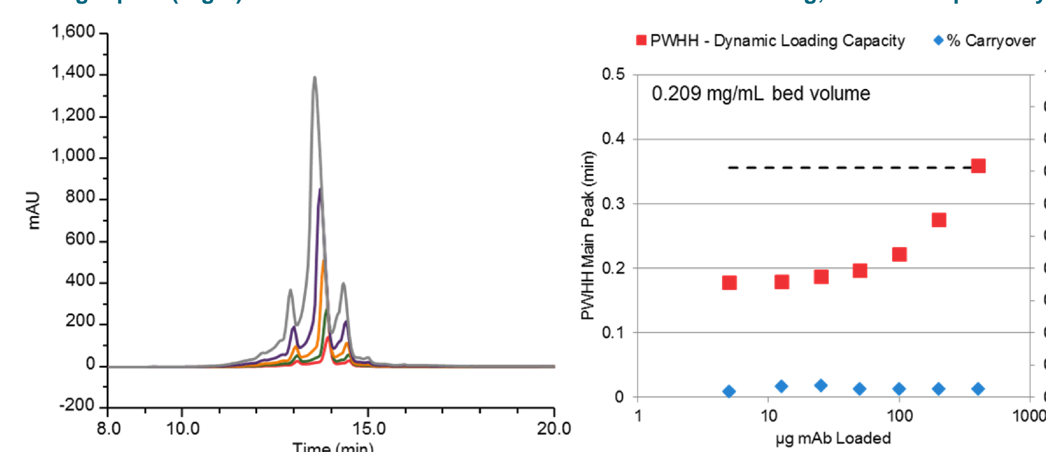
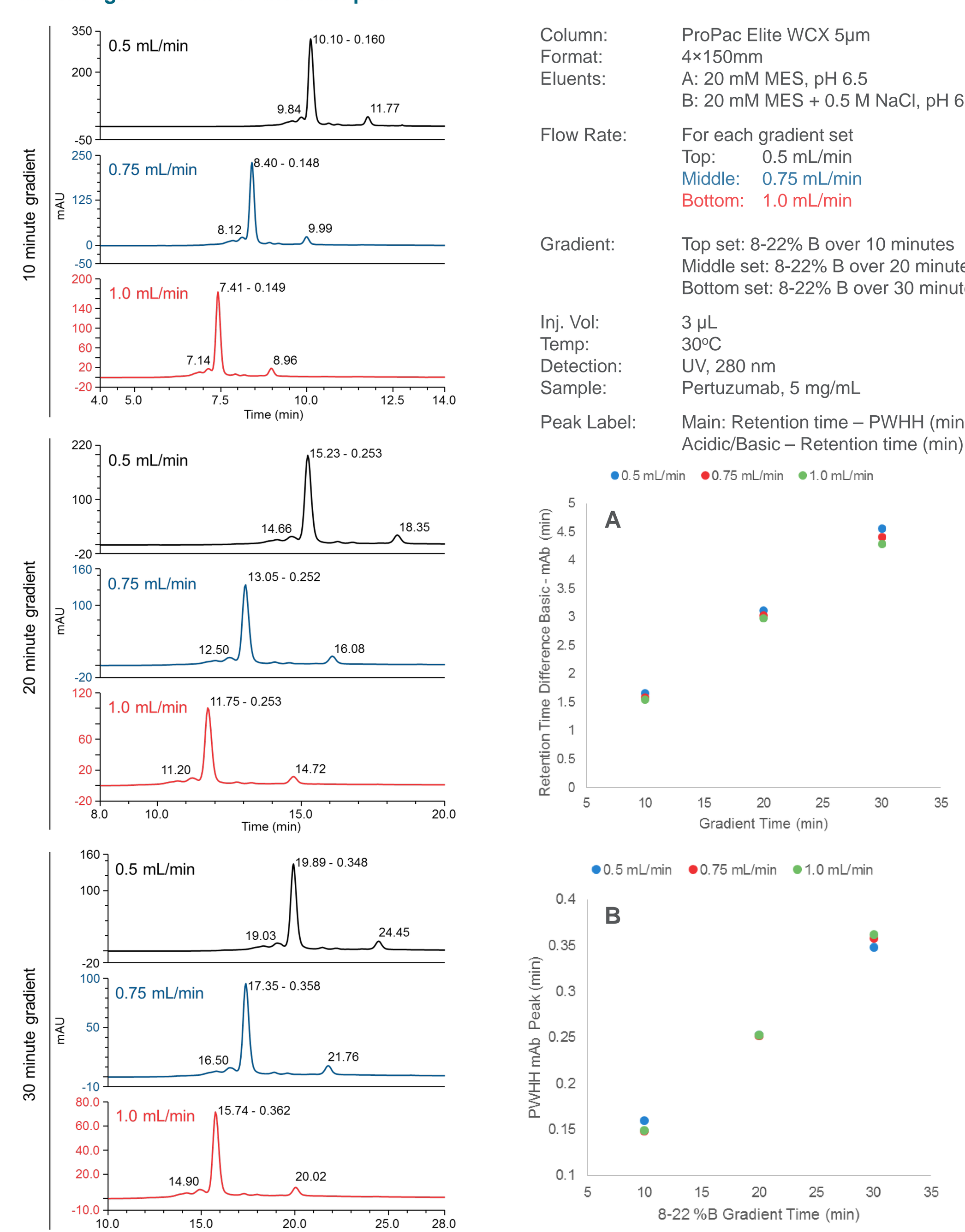


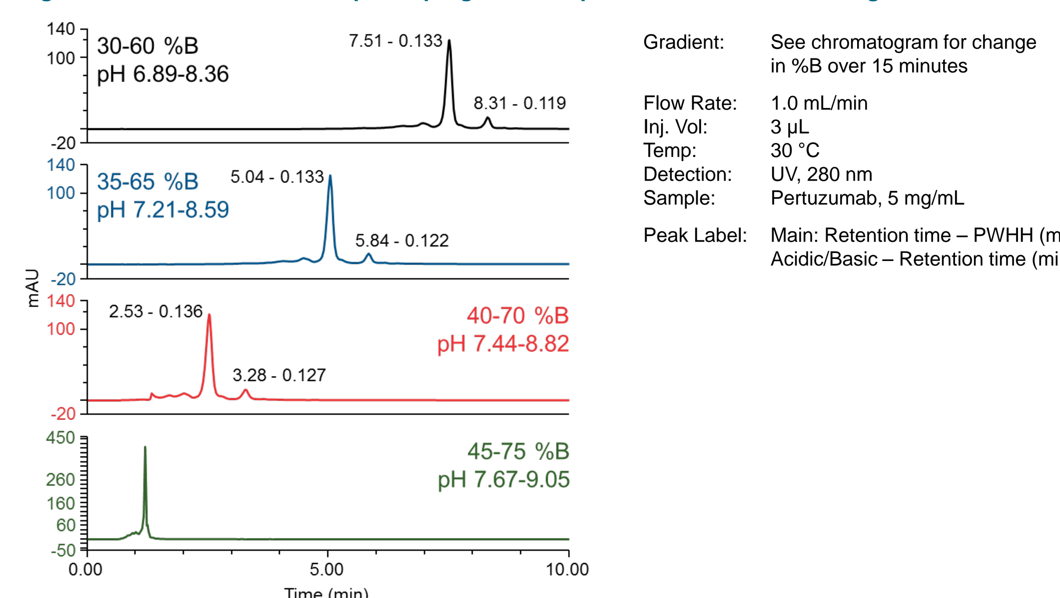
Figure 4. Chromatograms showing the effect of flow rate and gradient time on the separation of charge variants of Pertuzumab. Plot A shows the retention time difference between the main mAb peak and the largest basic peak. Plot B shows the change in PWHH for the main peak.



Column:	ProPac Elite WCX 5µm
Format:	4×150mm
Eluents:	A: 20 mM MES, pH 6.5 B: 20 mM MES + 0.5 M NaCl, pH 6.5
Flow Rate:	For each gradient set Top: 0.5 mL/min Middle: 0.75 mL/min Bottom: 1.0 mL/min
Gradient:	Top set: 8-22% B over 10 minutes Middle set: 8-22% B over 20 minutes Bottom set: 8-22% B over 30 minutes
Inj. Vol:	3 µL
Temp:	30°C
Detection:	UV, 280 nm
Sample:	Pertuzumab, 5 mg/mL
Peak Label:	Main: Retention time – PWHH (min) Acidic/Basic – Retention time (min)

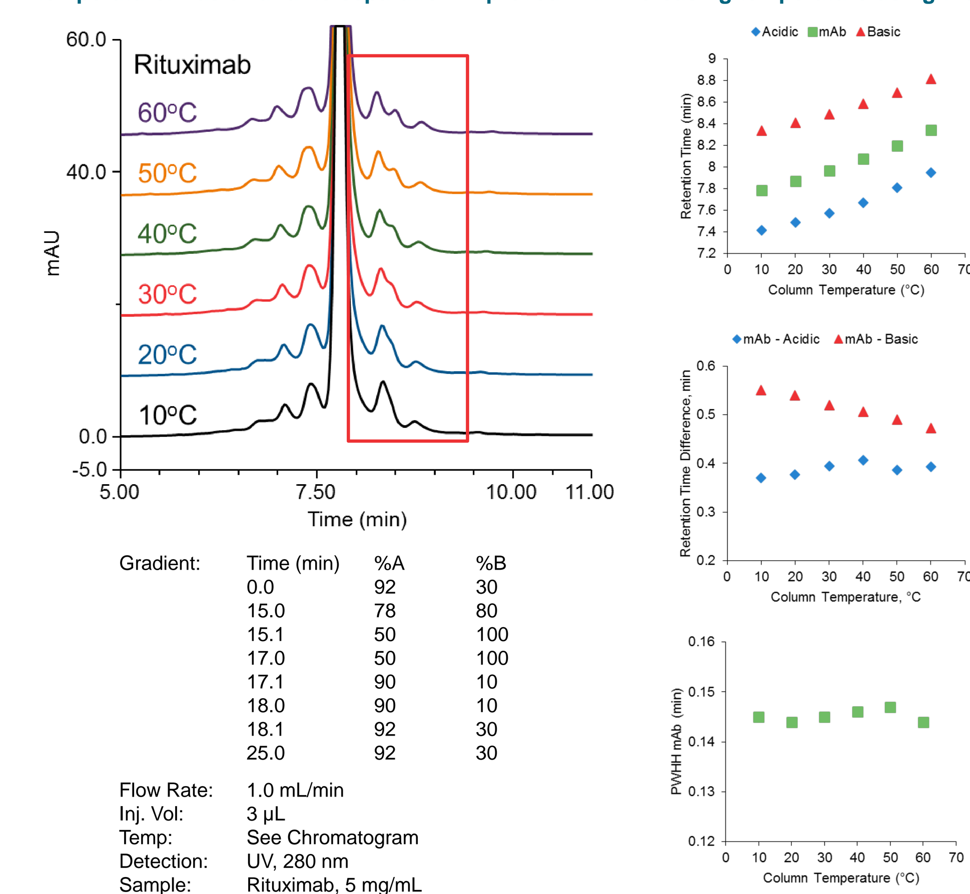
pH Gradient Separation Principles

Figure 5. Effect of initial buffer pH on pH gradient separation of Rituximab charge variants.



Gradient:	See chromatogram for change in %B over 15 minutes
Flow Rate:	1.0 mL/min
Inj. Vol:	3 µL
Temp:	30 °C
Detection:	UV, 280 nm
Sample:	Pertuzumab, 5 mg/mL
Peak Label:	Main: Retention time – PWHH (min) Acidic/Basic – Retention time (min)

Figure 6. Effect of column temperature on pH gradient separation of Rituximab charge variants. Top, middle and bottom plots show effect on main peak retention time, variant-mAb separation, and main peak PWHH, respectively, as a function of temperature. Basic variants separation improved with increasing temperature using the pH gradient.



Gradient:	Time (min)	%A	%B
	0.0	92	30
	15.0	78	80
	15.1	50	100
	17.0	50	100
	17.1	90	10
	18.0	90	10
	18.1	92	30
	25.0	92	30
Flow Rate:	1.0 mL/min		
Inj. Vol:	3 µL		
Temp:	See Chromatogram		
Detection:	UV, 280 nm		
Sample:	Rituximab, 5 mg/mL		

Figure 7. Dynamic loading analysis and sample carryover for a trastuzumab biosimilar on a 4x150mm column. (Left) Chromatograms showing the change in charge variant separation with increasing loading amounts corresponding the data in the right plot. (Right) Red – increase in PWHH with increased mAb loading; Blue – Sample carryover in subsequent blank.

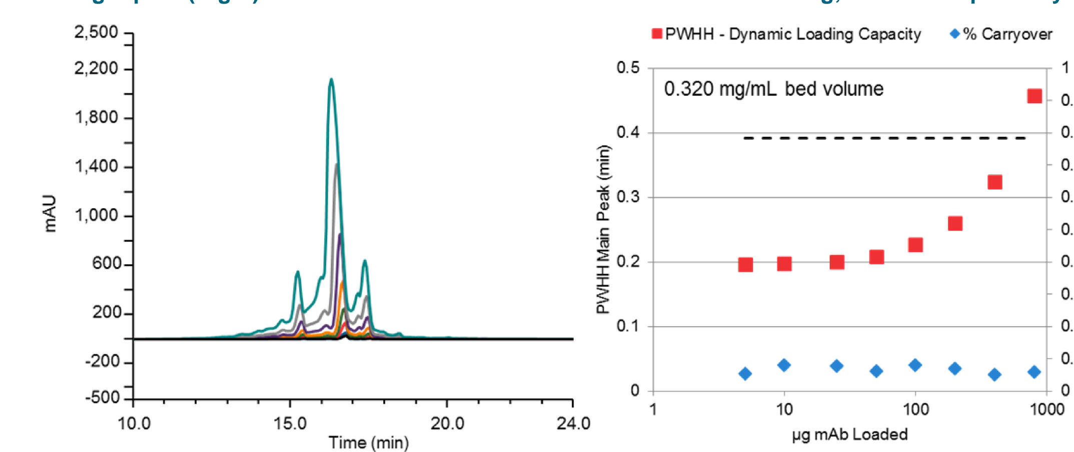
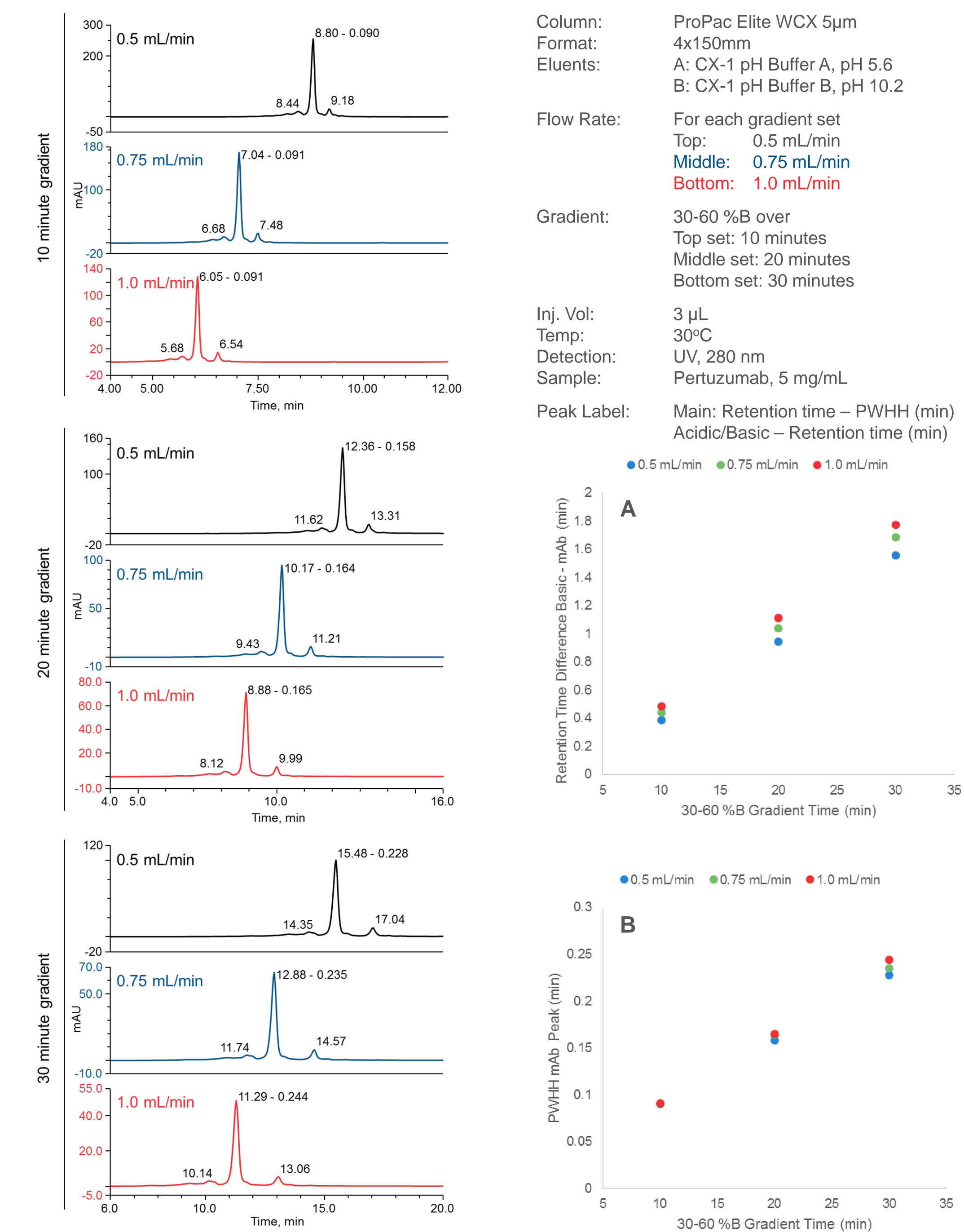


Figure 8. Chromatograms showing the effect of flow rate and gradient time on the separation of charge variants of Pertuzumab. Plot A shows the retention time difference between the main mAb peak and the largest basic peak. Plot B shows the change in PWHH for the main peak.



Column:	ProPac Elite WCX 5µm
Format:	4x150mm
Eluents:	A: CX-1 pH Buffer A, pH 5.6 B: CX-1 pH Buffer B, pH 10.2
Flow Rate:	For each gradient set Top: 0.5 mL/min Middle: 0.75 mL/min Bottom: 1.0 mL/min
Gradient:	30-60 %B over Top set: 10 minutes Middle set: 20 minutes Bottom set: 30 minutes
Inj. Vol:	3 µL
Temp:	30°C
Detection:	UV, 280 nm
Sample:	Pertuzumab, 5 mg/mL
Peak Label:	Main: Retention time – PWHH (min) Acidic/Basic – Retention time (min)

CONCLUSIONS

For the mAbs tested here, the following conclusions were observed; however, these results are sample dependent and should be evaluated for the user's specific sample.

- Buffer pH for salt gradients and temperature for salt and pH gradients can influences the relative separation of charge variants. Temperature had a greater effect on PWHH for salt gradient methods.
- Protein loading determines the ability to resolve variants for a specific column format. Column formats should be selected based on the mass of protein required to be analyzed. pH gradients showed higher levels of protein loading for all samples tested than salt gradient methods.
- Gradient time is the primary determinant of variant separation while flow rate may result in minor adjustments to peak separation for salt and pH gradients.

TRADEMARKS/LICENSING

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