



HPLC columns

# ProPac Elite WCX 5 µm columns

Product manual

# Safety and special notices

Make sure you follow the precautionary statements presented in this guide.  
The safety and other special notices appear in boxes.

## Safety and special notices include the following



### **Safety**

Indicates a potentially hazardous situation which, if not avoided, could result in death or serious injury



### **Warning**

Indicates a potentially hazardous situation which, if not avoided, could result in damage to equipment



### **Caution**

Indicates a potentially hazardous situation which, if not avoided, may result in minor or moderate injury. Also used to identify a situation or practice that may seriously damage the instrument, but will not cause injury.



### **Note**

Indicates information of general interest



### **Important**

Highlights information necessary to prevent damage to software, loss of data, or invalid test results; or might contain information that is critical for optimal performance of the system



### **Tip**

Highlights helpful information that can make a task easier

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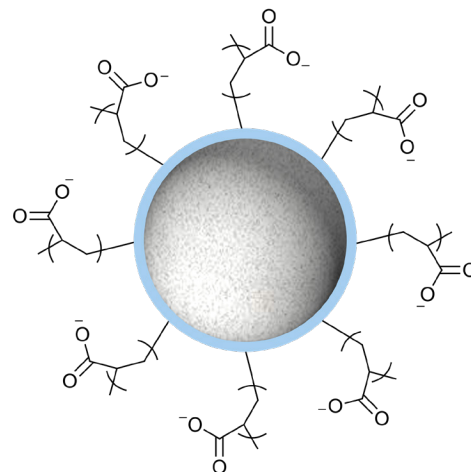
# Introduction

## Weak cation exchange technology

The Thermo Scientific™ ProPac™ Elite WCX (Weak Cation Exchange) 5 µm columns are designed to provide fast, high efficiency, high resolution separations of proteins and glycoproteins based on their accessible surface charge. The 5 µm, non-porous particle is based on a solvent compatible divinylbenzene resin coated with a hydrophilic polymer layer to exclude the protein from the surface of the resin and thus minimize secondary interactions. Grafting of acrylates to this hydrophilic surface introduces carboxylate groups that provide the weak cation exchange character required for promoting protein binding using a low ionic strength mobile phase at an appropriate pH. Running a gradient from low to high ionic strength mobile phase or from low to high pH disrupts the ionic protein-surface interactions resulting in protein and variant elution based on their relative strength of interaction with the surface.

### WCX chromatography applications

WCX column technology is widely used in biopharmaceutical development and qualification of protein-based therapeutics and their associated variants. Most commonly, they are used for the analysis of monoclonal antibodies (mAbs), which are a class of therapeutics widely applied to the treatment of cardiovascular diseases, autoimmune disorders, and cancers. MABs are of particular interest for their ability to target specific cells for drug delivery or modulation of cellular activity with reduced side effects compared to other systemic delivery methods. Because of this specificity, mAbs have been investigated for, and applied to, a wide range of treatments with strong growth expected for the foreseeable future. As the complexity of therapeutics increases, continued improvements in analytical technologies will be required to characterize these proteins and fulfill regulatory requirements to bring these therapeutics to market.



**Figure 1. Representation of DVB particle coated with a hydrophilic layer (blue) and grafted with acrylate groups to provide WCX carboxylate functionality**

### ProPac Elite WCX 5 µm column

The ProPac Elite WCX 5 µm column has been designed upon the technology that made the ProPac WCX-10 column the industry leader in mAb and variant analysis. To provide faster, higher resolution separations, the chemistry has been scaled down to a 5 µm particle to take advantage of shorter diffusion distances resulting in better mass transfer and narrower peaks. Due to increased capacity associated with smaller particles, shorter columns can also be used for faster run times with improved separation relative to larger particle media that require longer column lengths to achieve the same separation. The improved separation abilities of the ProPac Elite WCX 5 µm column provide the advancements required from analytical technologies to continue driving the development of protein therapeutics forward.

# Operation requirements and specifications

Prior to using the ProPac Elite WCX 5 µm column, review all the information in this section on system requirements and column operation. Following these specifications for your system and column will help to ensure the column performs as it is intended and maximize its lifetime.

## System requirements

The ProPac Elite WCX 5 µm columns are designed to be used with a standard bore inert HPLC or UHPLC system having a gradient pump module, autosampler, injection valve, and a detector appropriate for your application (UV, DAD, Fluorescence, MS). A totally inert Thermo Scientific™ Vanquish™ Flex or a Thermo Scientific™ Vanquish™ Horizon UHPLC system is recommended for best results. A properly set up system is required to ensure good chromatographic performance and to extend the lifetime of your column.

A bio-inert system is highly recommended for mobile phases containing halide salts that can cause corrosion of metallic components. Metal leaching from the system components including the pump and connection tubing can contaminate the column and compromise chromatographic performance. Avoid using stainless steel tubing, ferrules, and bolt assemblies and replace them with MP35N tubing, PEEK, or other equivalent inert alternatives. For column-to-tubing connections, Thermo Scientific™ Viper™ capillary or Thermo Scientific™ nanoViper™ fused-silica-lined PEEK tubing equipped with Thermo Scientific™ Viper™ connection fittings are strongly recommended.

## System void volume

Tubing between the injection valve and detector should be ≤ 0.130 mm (0.0050" ID) tubing to minimize dispersion. Minimize the length of all liquid lines, especially the tubing between the column and the detector. The use of larger diameter and/or longer tubing may decrease peak efficiency and peak resolution.

## Column use and physical specifications

### Operational guidelines

To ensure that you do not damage the column hardware or packed bed, take care to operate within the limits of the column. Table 1 indicates the operational limits for each column format in terms of flow rate, maximum column pressure from inlet to outlet, temperature, and mobile phase pH.

**Table 1. Recommended column operating conditions for optimal performance and extending column lifetime**

| Column                  | Recommended flow rate <sup>1</sup> mL/min | Max column pressure <sup>2</sup> psi (bar) | Temperature °C | pH <sup>3</sup>        |
|-------------------------|-------------------------------------------|--------------------------------------------|----------------|------------------------|
| 4 × 250 mm              | 0.4-0.8                                   | 4500 (310)                                 | 10-60 °C       | 2 (5) <sup>3</sup> -12 |
| 4 × 150 mm              | 0.4-1.0                                   |                                            |                |                        |
| 4 × 50 mm               |                                           |                                            |                |                        |
| 2 × 250 mm              |                                           |                                            |                |                        |
| 2 × 150 mm              | 0.1-0.25                                  |                                            |                |                        |
| 2 × 50 mm               | 0.1-0.8                                   |                                            |                |                        |
| 9 × 150 mm <sup>4</sup> | 1.0-4.0                                   | 3300 (230) <sup>4</sup>                    |                |                        |

<sup>1</sup> For any given flow rate, the maximum pressure across the column body should not exceed the maximum recommended pressure to avoid damaging the packed bed.

<sup>2</sup> The column pressure for a given flow rate is calculated as the pressure of the system with column minus the pressure of system with union in place of column.

<sup>3</sup> The column materials are stable from pH 2-12; however, the carboxylate groups will become protonated below pH 5 (pKa carboxylate ~ 4.5) resulting in reduced column capacity and loss of cation exchange functionality.

<sup>4</sup> For the 9 × 150 mm column, 0.18 mm ID tubing is strongly recommended to minimize post-column system backpressure. An 11 µL UV flow cell is also recommended to minimize backpressure. Ramping the column to the desired flow rate over 5 minutes will extend the column lifetime.

# Operation requirements and specifications (continued)

- Operate the column within the operating parameters and specifications described in Table 1. See the footnotes for column format specific directions
- Use the column in the direction of flow marked on the column label
- Slowly increase and decrease the flow rate using a ramp rate of ½ the max recommended flow rate
- Column conditioning: Before the first use or after storage, flush the column with 10 column volumes of mobile phase followed by 2 blank runs with the desired mobile phase before running the sample
- Use a minimum of 20 mM NaCl during operation to avoid high pressures that can damage the column
- Flowing pure DI water on the column will cause high pressure and irreversible column damage
- Adjust mobile phase, temperature, flow rate and gradient slope for best resolution and fast separation. Refer to section 3 for further details on method development for salt and pH gradients



## Caution

Never use pure DI water on the column as this will result in irreversible column damage.



Organic solvents can increase column backpressure and damage the solid phase. Column backpressure increase compared to aqueous solvents can be as much as:

10% acetonitrile – 1.5x

10% isopropyl alcohol – 2x

10% methanol – 1.3x

When using organic solvents, determine the column backpressure at a low flow rate before increasing the flow rate.

## Recommended buffers for salt and pH gradient separations

Salt and pH gradient separations are a standard approach for analyzing proteins, mAbs and their charge variants. Please consult Table 2 below for recommended buffer conditions to achieve optimal separations and maintain good column performance throughout its lifetime.

**Table 2. Recommended buffers and mobile phase requirements**

| Parameter                          | Recommendation                                                                                                                                                                                                                                                                                                     |
|------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Salt gradient buffers <sup>1</sup> | <ul style="list-style-type: none"><li>• MES, MOPS, or other Good's buffers</li></ul>                                                                                                                                                                                                                               |
| pH gradient buffers <sup>1</sup>   | <ul style="list-style-type: none"><li>• Thermo Scientific CX-1 pH gradient buffers</li></ul>                                                                                                                                                                                                                       |
| Minimum salt concentration         | <ul style="list-style-type: none"><li>• 20 mM NaCl to avoid high pressure that can damage the column solid phase;</li><li>• Never use pure deionized water on the column as this will result in irreversible damage</li></ul>                                                                                      |
| Detergent additives                | <ul style="list-style-type: none"><li>• Nonionic, anionic or zwitterionic detergents</li><li>• Do not use cationic detergents as they will irreversibly bind to the column and reduce the separation power</li></ul>                                                                                               |
| Organic solvent compatibility      | <ul style="list-style-type: none"><li>• Up to 10% acetonitrile, methanol, or isopropyl alcohol</li><li>• Organic solvents can significantly increase the column backpressure</li></ul>                                                                                                                             |
| Cleaning agents                    | <ul style="list-style-type: none"><li>• For metal contamination (Fe, Cu, etc.) removal flush the column for 12 hours with 10 mM EDTA + 50 mM NaCl adjusted to pH 8</li><li>• For sample fouling flush the column with 1 M NaCl in your buffer of choice followed by injections (10-50 µL) of 100 mM NaOH</li></ul> |
| Storage solution                   | <ul style="list-style-type: none"><li>• Short term: ≥ 20 mM NaCl and your application buffer</li><li>• Long term: ≥ 20 mM NaCl and your application buffer with 0.1% sodium azide added</li></ul>                                                                                                                  |

<sup>1</sup> Phosphate based buffers are not recommended as they are unable to buffer the column solid phase.

# Operation requirements and specifications (continued)

## Recommended buffers for pH gradient separations

For pH gradient separations, the Thermo Scientific™ CX-1 pH gradient buffers (see Table 3) are recommended, which can be used to run a linear, highly reproducible pH gradient from pH 5.6 to 10.2. As the majority of mAbs have a pI between 6 and 10, these buffers can be used to separate the mAbs from their associated charge variants with little to no method development. Because of this, the CX-1 pH gradient buffers present a simple platform method for mAb analysis that can easily be tailored to the user's own mAb by simple adjustment of the gradient. For examples of mAb analysis and method development using the pH gradient buffers, please reference section 3 on applications.

**Table 3. Thermo Scientific CX-1 pH gradient buffers**

| Description          | Size    | pH      | Cat. no.               |
|----------------------|---------|---------|------------------------|
| pH gradient buffer A | 125 mL  | pH 5.6  | <a href="#">083273</a> |
|                      | 250 mL  |         | <a href="#">085346</a> |
|                      | 500 mL  |         | <a href="#">302779</a> |
|                      | 1000 mL |         | <a href="#">303274</a> |
| pH gradient buffer B | 125 mL  | pH 10.2 | <a href="#">083275</a> |
|                      | 250 mL  |         | <a href="#">085348</a> |
|                      | 500 mL  |         | <a href="#">302780</a> |
|                      | 1000 mL |         | <a href="#">303275</a> |

## Minimum equilibration volumes for buffers

Prior to sample loading and start of the gradient, the column solid phase must be properly equilibrated to promote binding of the analytes. Salt and pH gradient buffers require different volumes of buffer at sample loading conditions to equilibrate the column. Table 4 provides the minimum volumes for salt and pH gradient buffers for each format. If using a buffer system other than the ones recommended above, it is strongly advised that the user evaluate the required equilibration volume by examining the UV, pH, or conductivity of the mobile phase when making a step change to their preferred loading conditions. The volume of mobile phase eluted from the time of the step change to when it is observed for the mobile phase UV, pH, or conductivity trace represents the minimum volume required for equilibration of the stationary phase. Conductivity and pH can be monitored using the Thermo Scientific™ PCM-3000 pH and conductivity monitors.

**Table 4. Minimum recommended volume of buffer to equilibrate the column solid phase before sample analysis**

| Column format | Salt gradient buffer (mL) | pH gradient buffer (mL) |
|---------------|---------------------------|-------------------------|
| 4 × 250 mm    | 7.0                       | 10.5                    |
| 4 × 150 mm    | 4.5                       | 6.5                     |
| 4 × 50 mm     | 2.0                       | 3.0                     |
| 2 × 250 mm    | 2.5                       | 4.0                     |
| 2 × 150 mm    | 2.0                       | 3.0                     |
| 2 × 50 mm     | 1.5                       | 2.0                     |
| 9 × 150 mm    | 23                        | 53                      |

# Operation requirements and specifications (continued)

## Column storage and extended care

To maintain the performance of your column between uses, always store the column filled with the recommended buffers as detailed in Table 2. Use the plugs the column was shipped with to seal the ends of the column to prevent evaporation of the buffer and drying of the solid phase. The formation of salt crystals in a dried bed may result in column clogging or reduced column performance in subsequent uses.

Depending on the length of time between column uses, the storage buffers should be used as follows:

- **Short term storage ( $\leq 1$  day):** Fill the column with buffer with  $\geq 20$  mM NaCl (e.g., 20 mM MES + 20 mM NaCl)
- **Long term storage ( $> 1$  day):** Fill the column with a low ionic strength buffer with  $\geq 20$  mM NaCl and supplemented with 0.1% sodium azide

Sodium azide serves as a preservative to prevent bacterial growth that can damage the column phase, result in column clogging, and decrease column performance. When using the column after storage with a sodium azide containing buffer, flush the column with  $\geq 10$  column volumes of low ionic strength buffer to remove all of the sodium azide before starting any sample runs. At least 2 blank runs with a gradient from low to high salt concentration prior to any sample runs are also recommended for best performance for the initial sample runs.



### Caution

Never use pure deionized water on the column as this will result in irreversible column damage.

## Column performance verification

Each column is shipped with two Certificates of Analysis (CoA) verifying the resin performance and one Quality Assurance Report (QAR) verifying the column performance. The CoAs include a salt gradient separation of a three protein mix (NISTmAb, equine cytochrome C, and ribonuclease A; 2, 4, and 8 mg/mL, respectively) and a pH gradient separation of NISTmAb and variants on a 4 × 150 mm column. These tests are not performed for every column but are used to qualify the resin performance prior to manufacturing columns. The QAR test is an isocratic analysis of equine cytochrome C. Each CoA and QAR provides the test conditions used. These tests can be reproduced to check the performance of your column. The lot qualification tests are performed using a 4 × 150 mm column and the gradient and flow rate should be scaled based on column length and diameter, respectively. The isocratic cytochrome C test conditions can be used as described to verify column performance. Note that differences in system configuration may result in differences in retention time and chromatographic performance. Table 5 provides purchasing information for the proteins used in the CoA and QAR tests.

**Table 5. Proteins used for resin lot qualification and column performance testing**

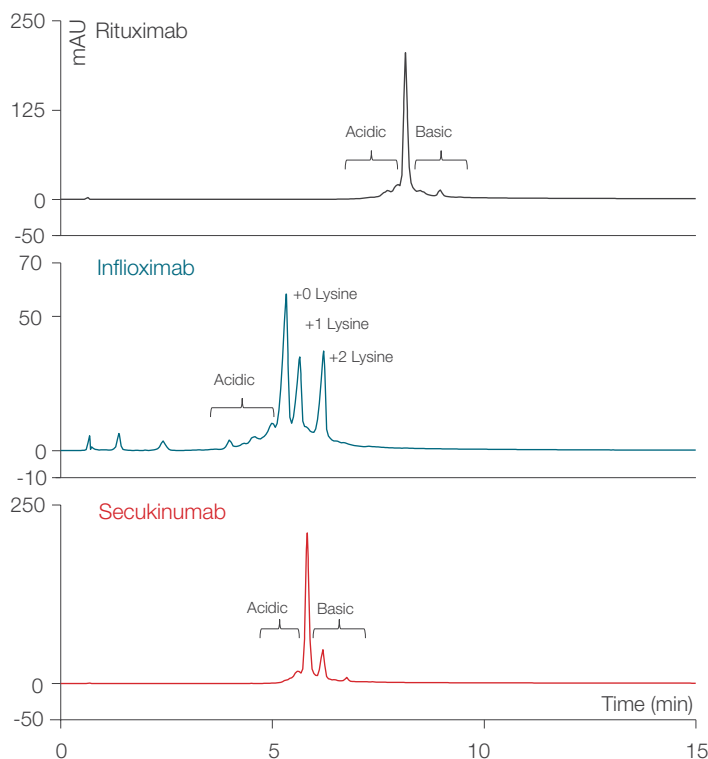
| Protein             | Supplier                                              | Cat. no. |
|---------------------|-------------------------------------------------------|----------|
| NISTmAb             | NIST (National Institute of Standards and Technology) | 8671     |
| Equine Cytochrome C | Sigma-Aldrich                                         | C2506    |
| Ribonuclease A      | Sigma-Aldrich                                         | R5500    |

# WCX chromatography methods

Two approaches are typically used for protein and variant analysis on WCX columns: 1) salt gradient separation and 2) pH gradient separation. For both approaches, the protein is loaded onto the column solid phase using a low ionic strength buffer. The salt gradient approach relies on increasing the solution ionic strength to elute the protein; whereas the pH gradient approach shifts the net charge of the protein from cationic to anionic to disrupt protein-surface interactions. Salt gradients can provide excellent separation of proteins and their associated variants; however, these methods typically require considerable development time investigating different buffer components, pH, salts, gradient slopes, temperatures, etc. The pH gradient approach is a simple platform method for facile separation of proteins and their variants based on their respective accessible isoelectric points (accessible pI).

Figures 2 and 3 show example separations of three pharmaceutical mAbs using a salt and pH gradient, respectively. For both the salt and pH gradients, the more anionic charge (acidic) variants are observed to elute earlier than the main mAb peak whereas the more cationic charge (basic) variants are observed to elute later than the main peak.

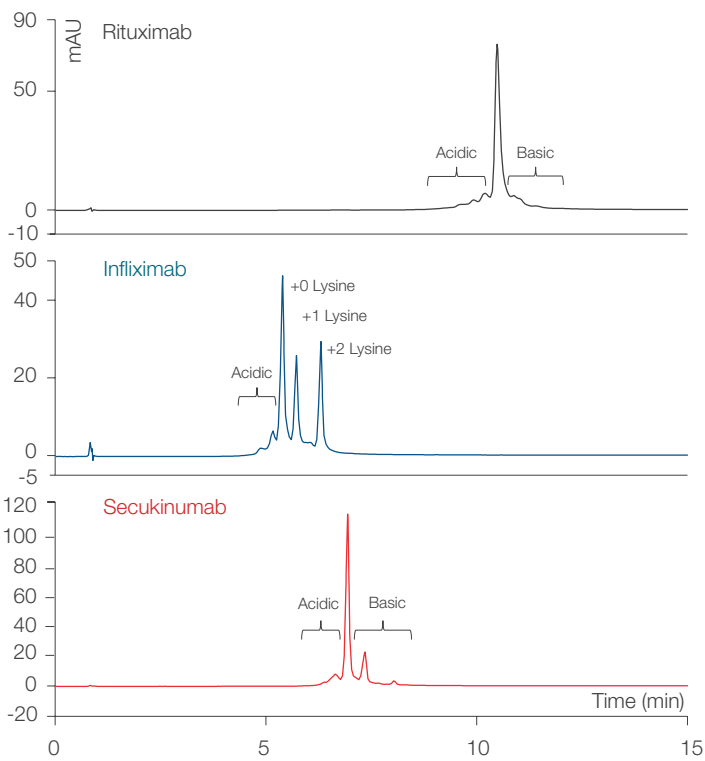
The sections that follow discuss the importance of chromatographic parameters including pH, temperature, flow rate, gradient time, protein loading, and column format on both salt and pH gradient analysis. For each parameter, relevant examples are provided using current pharmaceutical mAb therapeutics to illustrate the effects on the chromatography. These examples can be used as a guide when developing chromatographic methods for the analysis of protein charge variants. Following these discussions, examples of both salt and pH gradient separations of pharmaceutically relevant proteins including IgG1, IgG2, and IgG4 mAbs are provided.



**Figure 2. Separation of charge variants for three pharmaceutical mAbs using a salt gradient**

| ProPac Elite WCX 5 $\mu$ m column, 4 $\times$ 150 mm |                                                         |       |
|------------------------------------------------------|---------------------------------------------------------|-------|
| Cat. no.                                             | <a href="#">302972</a>                                  |       |
| Mobile phase                                         | A: 20 mM MES pH 6.5<br>B: 20 mM MES pH 6.5 + 0.5 M NaCl |       |
| Flow rate                                            | 1.0 mL/min                                              |       |
| Inj. volume                                          | 3 $\mu$ L                                               |       |
| Temperature                                          | 30 $^{\circ}$ C                                         |       |
| Detection                                            | UV, 280 nm                                              |       |
| Analytes                                             | Top: Rituximab, 5 mg/mL                                 |       |
|                                                      | Middle: Infliximab, 5 mg/mL                             |       |
|                                                      | Bottom: Secukinumab, 5 mg/mL                            |       |
|                                                      | Time (min)                                              | %A %B |
| Gradient                                             | 0.0                                                     | 95 5  |
|                                                      | 15.0                                                    | 65 35 |
|                                                      | 15.1                                                    | 50 50 |
|                                                      | 16.0                                                    | 50 50 |
|                                                      | 16.1                                                    | 95 5  |
|                                                      | 25.0                                                    | 95 5  |

# WCX chromatography methods (continued)



| ProPac Elite WCX 5 $\mu$ m column, 4 $\times$ 150 mm |                                                                                        |       |
|------------------------------------------------------|----------------------------------------------------------------------------------------|-------|
| Cat. no.                                             | 302972                                                                                 |       |
| Mobile phase                                         | A: CX-1 pH gradient buffer A<br>B: CX-1 pH gradient buffer B                           |       |
| Flow rate                                            | 1.0 mL/min                                                                             |       |
| Inj. volume                                          | 2 $\mu$ L                                                                              |       |
| Temperature                                          | 30 $^{\circ}$ C                                                                        |       |
| Detection                                            | UV, 280 nm                                                                             |       |
| Analytes                                             | Top: Rituximab, 5 mg/mL<br>Middle: Infliximab, 5 mg/mL<br>Bottom: Secukinumab, 5 mg/mL |       |
| Gradient                                             | Time (min)                                                                             | %A %B |
|                                                      | 0.0                                                                                    | 80 20 |
|                                                      | 15.0                                                                                   | 20 80 |
|                                                      | 15.1                                                                                   | 0 100 |
|                                                      | 17.0                                                                                   | 0 100 |
|                                                      | 17.1                                                                                   | 80 20 |
|                                                      | 25.0                                                                                   | 80 20 |

Figure 3. Separation of charge variants for three pharmaceutical mAbs using CX-1 pH gradient buffers

# WCX chromatography methods (continued)

## Salt gradient separation principles

When using a salt gradient, the ionic strength of the solution is increased over time typically using a salt such as NaCl. At higher salt concentrations, the cationic salt component will exclude the interactions of the cationic protein species from the carboxylate groups of the solid phase leading to protein desorption and elution from the column. Proteins with a greater number of cationic groups and/or fewer anionic groups will tend to elute at higher salt concentrations relative to those with fewer cationic groups and/or more anionic groups.

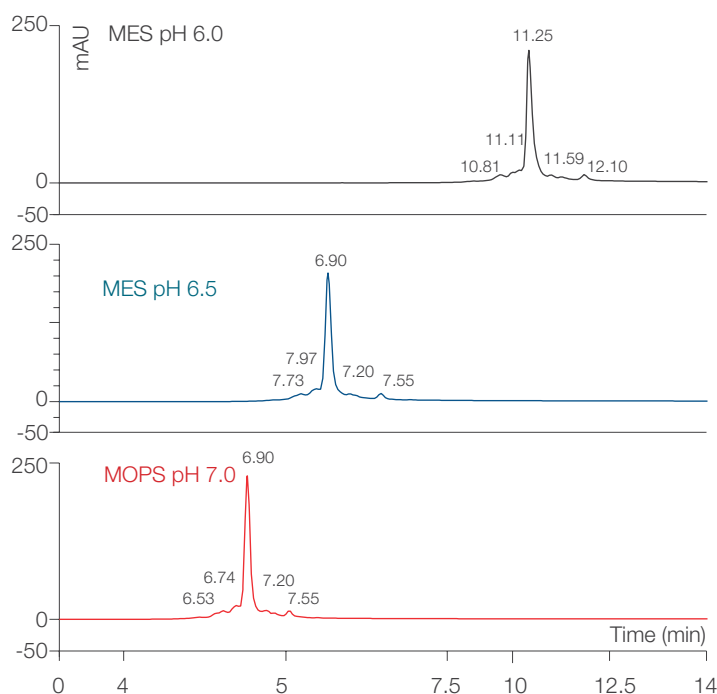
## Mobile phase pH selection

The charge of the column solid phase and the protein of interest can both be influenced by the mobile phase pH. For this reason, it is important to select an appropriate mobile phase buffer and pH for your specific protein separation. With regards to the stationary phase, the carboxylate groups of the mobile phase have a pKa of ~4.5; as such, buffers with a pH  $\geq 5.0$  will provide a suitably charged stationary phase for adsorption of cationic proteins.

The mobile phase pH should also be less than the surface isoelectric point (pI, the pH at which the overall charge of the protein is neutral) of the protein and all variants of interest; otherwise, the analytes will have an overall negative charge and fail to adsorb to the stationary phase. This effect can be observed in Figure 4 showing the analysis of the mAb Pertuzumab using MES buffers at pH 6.0 and 6.5 and MOPS buffer at pH 7.0.

The properties of the buffer may also influence selectivity of the separation; however, for MES and MOPS buffers, the relative separation of the proteins and mAb are not significantly affected. As the pH of the mobile phase increases, the proteins elute earlier in the chromatogram as cationic groups are less likely to be protonated resulting in reduced charge of the protein and/or other neutral groups become deprotonated increasing the number of anionic sites. The peak width half height (PWHH) and the relative separation of acidic and basic variants from the main peak decrease with increasing pH.

| ProPac Elite WCX 5 $\mu$ m column, 4 $\times$ 150 mm |                                                 |       |
|------------------------------------------------------|-------------------------------------------------|-------|
| Cat. no.                                             | <a href="#">302972</a>                          |       |
| Mobile phase                                         | A: 20 mM buffer<br>B: 20 mM buffer + 0.5 M NaCl |       |
| Flow rate                                            | 1.0 mL/min                                      |       |
| Inj. volume                                          | 3 $\mu$ L                                       |       |
| Temperature                                          | 30 $^{\circ}$ C                                 |       |
| Detection                                            | UV, 280 nm                                      |       |
| Analytes                                             | Pertuzumab, 5 mg/mL                             |       |
| 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  |

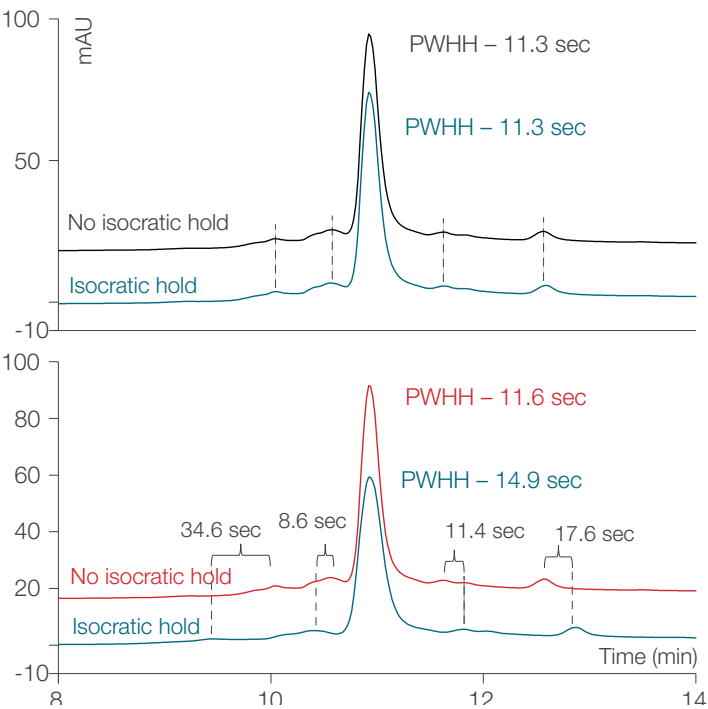


**Figure 4. Zoomed in time range showing the effect of buffer pH on the separation of Pertuzumab and associated charge variants.** The sample was analyzed using 20 mM MES pH 6.0 and 6.5 and 20 mM MOPS pH 7.0.

# WCX chromatography methods (continued)

## Determining salt gradient loading and elution concentrations

For run-to-run consistency of mAb separation, gradient methods should be designed so the mAb is separated by the change in salt concentration, without isocratic elution occurring during loading. Figure 5 compares the elution of a mAb using gradients at two different initial salt concentrations of 40 mM and 60 mM NaCl. For each starting salt concentration, the separation is evaluated with and without an isocratic hold (the change in time between the injection and start of the gradient) to determine if isocratic elution occurs during loading. The retention times of the mAb peak with and without the isocratic hold are normalized to compare the relative separation of the variants. At 40 mM NaCl loading, the separation of the variants relative to the main peak are consistent with and without the isocratic hold (dashed lines), and the main peak PWHH is unchanged. At 60 mM NaCl loading, the separation of the variants from the main peak increases with the isocratic hold (brackets indicate increase in separation), and the PWHH of the main peak increases. Based on these results, isocratic elution is observed at 60 mM NaCl loading but not 40 mM NaCl.

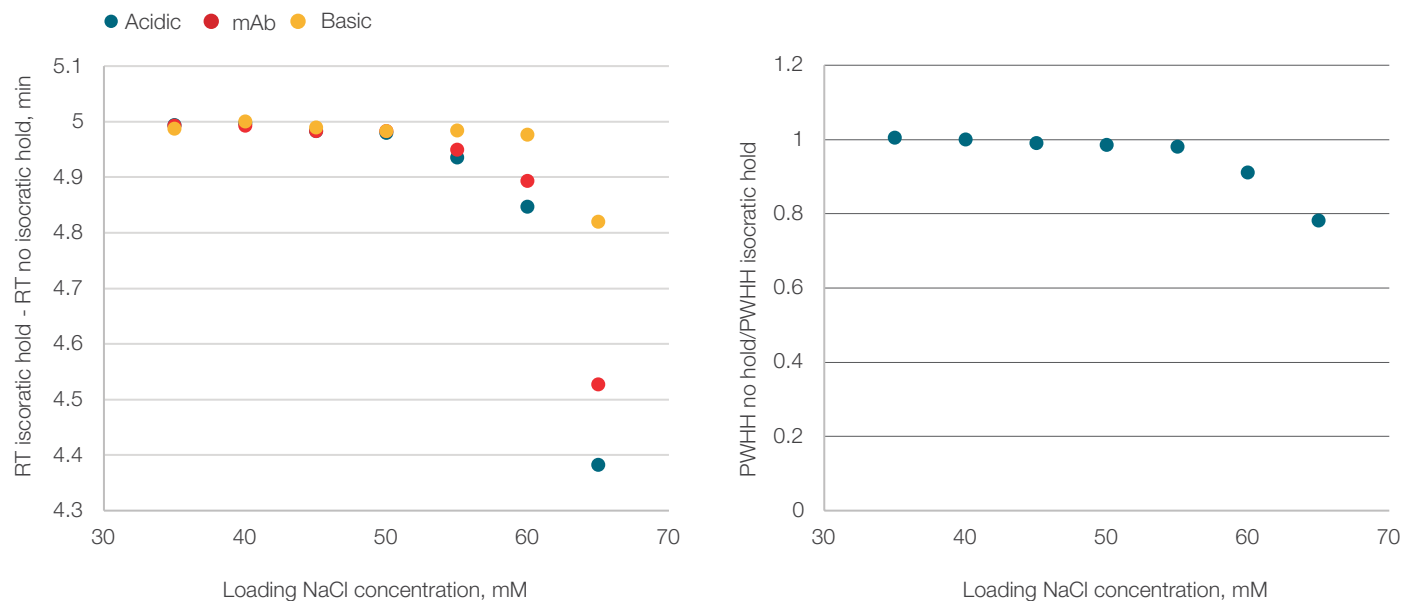


**Figure 5. Effect of starting salt concentration on rituximab variant separation with and without a 5 minute isocratic hold: (top) 8-22 %B and (bottom) 12-26 %B.** Main peak retention times are normalized to compare relative separation of variants. Dashed lines and brackets assist in comparing the separation of the same variants.

Comparison of the retention times with and without the 5 minute isocratic hold was evaluated for rituximab from a starting salt concentration of 35 mM up to 65 mM NaCl. Figure 6 plots (left) the retention time difference of the peaks with and without the isocratic hold versus salt concentration and (right) the ratio of PWHH of the mAb peak with and without the isocratic hold versus salt concentration. Isocratic elution occurs when the retention time difference for the mAb and variant peaks with and without the hold decreases below 5 minutes and when the PWHH ratio becomes less than 1. Based on the plots in Figure 6, 40 mM NaCl would be the recommended salt loading concentration for Rituximab with MES buffer pH 6.5 as it is the maximum salt concentration observed to meet both of these criteria.

| ProPac Elite WCX 5 $\mu$ m column, 4 $\times$ 150 mm |                                                         |                 |
|------------------------------------------------------|---------------------------------------------------------|-----------------|
| Cat. no.                                             | 302972                                                  |                 |
| Mobile phase                                         | A: 20 mM MES pH 6.5<br>B: 20 mM MES pH 6.5 + 0.5 M NaCl |                 |
| Flow rate                                            | 1.0 mL/min                                              |                 |
| Inj. volume                                          | 3 $\mu$ L                                               |                 |
| Temperature                                          | 30 $^{\circ}$ C                                         |                 |
| Detection                                            | UV, 280 nm                                              |                 |
| Analytes                                             | Rituximab, 5 mg/mL                                      |                 |
| Gradient                                             | Time (min)                                              | %A %B           |
|                                                      | 0.0                                                     | 92(88) 8 (12)   |
|                                                      | 15.0                                                    | 78 (74) 22 (26) |
|                                                      | 15.1                                                    | 60 40           |
|                                                      | 16.0                                                    | 60 40           |
|                                                      | 16.1                                                    | 92(88) 8 (12)   |
|                                                      | 25.0                                                    | 92(88) 8 (12)   |

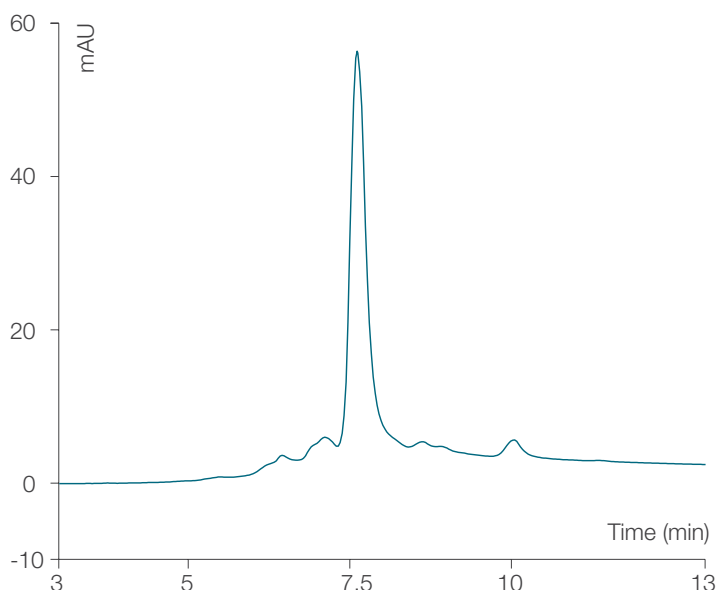
# WCX chromatography methods (continued)



**Figure 6. Effect of loading NaCl concentration on sample analysis: (left) retention time difference for mAb peaks and major acidic and basic variants of rituximab when evaluated with a 5 minute isocratic hold and without the isocratic hold and Right the ratio of PWHH without an isocratic hold/PWHH with a 5 minute isocratic hold.**

Gradients with lower changes in salt concentration per minute usually give the best separation of the protein from its variants. However, the salt concentration required to prevent isocratic elution of the protein is typically much lower than the concentration required for elution. This can result in long method times due to the difference in loading and eluting salt concentrations. To minimize the time required for analysis, the gradient can be stepped to a higher salt concentration after loading to elute the protein while using a shallow gradient to achieve good separation without adding significant time to the method. Figure 7 shows the separation of rituximab using 40 mM NaCl to load the protein, stepping the salt concentration to 65 mM NaCl and increasing to 110 mM NaCl over 15 minutes to elute the mAb. Stepping the salt concentration in this example reduces the analysis time by over 7 minutes and enables the use of a gradient designed for excellent resolution of acidic and basic variants.

# WCX chromatography methods (continued)



**Figure 7. Zoomed in time range showing the analysis of rituximab using a gradient optimized for loading followed by a gradient step and elution for acidic and basic variant separation**

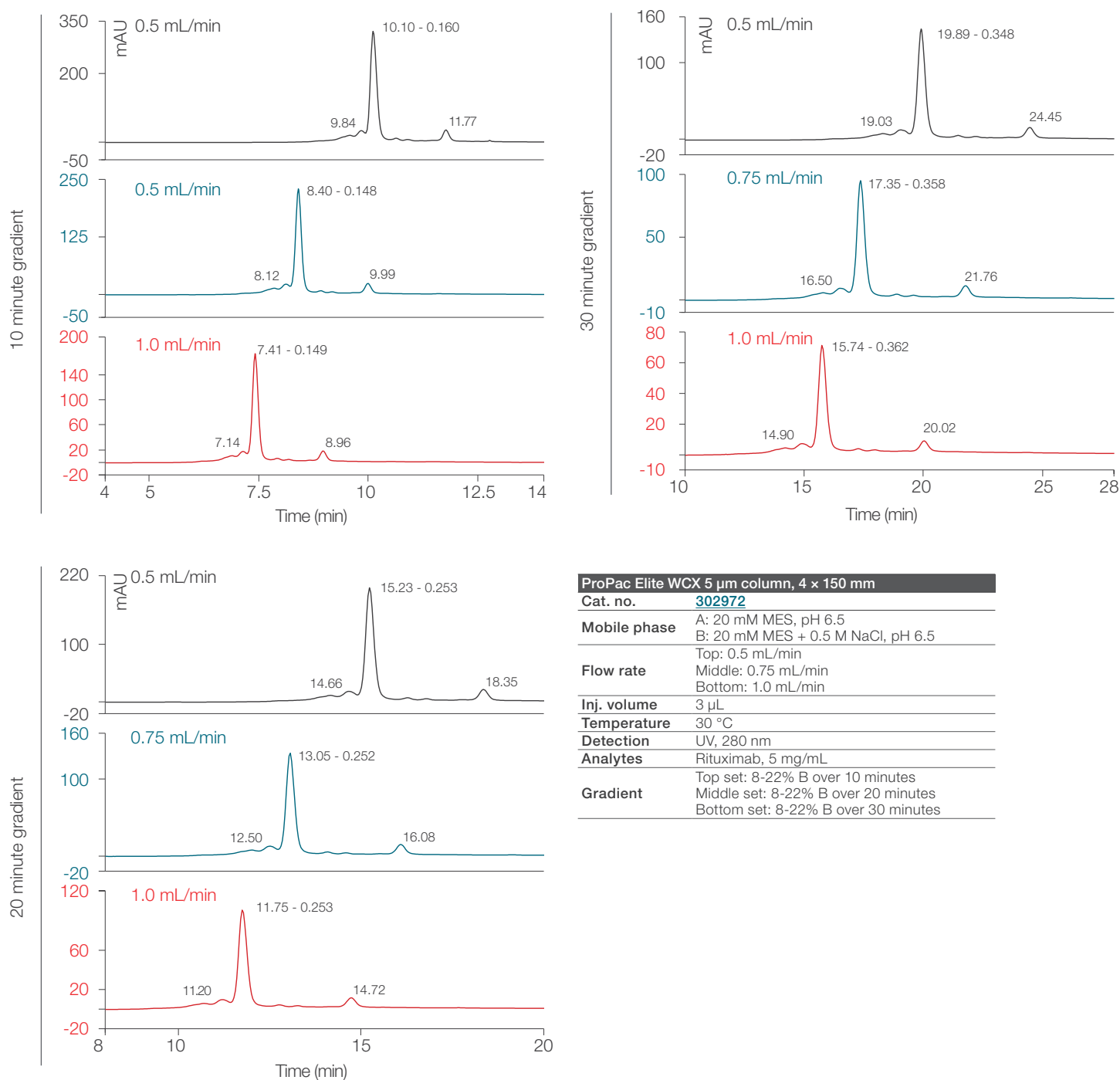
| ProPac Elite WCX 5 $\mu$ m column, 4 $\times$ 150 mm |                                                           |       |
|------------------------------------------------------|-----------------------------------------------------------|-------|
| Cat. no.                                             | <a href="#">302972</a>                                    |       |
| Mobile phase                                         | A: 20 mM MES, pH 6.5<br>B: 20 mM MES + 0.5 M NaCl, pH 6.5 |       |
| Flow rate                                            | 1.0 mL/min                                                |       |
| Inj. volume                                          | 3 $\mu$ L                                                 |       |
| Temperature                                          | 30 $^{\circ}$ C                                           |       |
| Detection                                            | UV, 280 nm                                                |       |
| Analytes                                             | Rituximab, 5 mg/mL                                        |       |
|                                                      | Time (min)                                                | %A %B |
|                                                      | 0.0                                                       | 92 8  |
|                                                      | 0.8                                                       | 92 8  |
|                                                      | 1.0                                                       | 87 13 |
| Gradient                                             | 16.0                                                      | 78 22 |
|                                                      | 16.1                                                      | 50 50 |
|                                                      | 18.0                                                      | 50 50 |
|                                                      | 18.1                                                      | 92 8  |
|                                                      | 25.0                                                      | 92 8  |

## Flow rate and gradient slope effects on salt gradient separation

Figure 8 shows the analysis of pertuzumab using a flow rate of 0.5, 0.75, and 1.0 mL/min (top, middle, and bottom chromatograms, respectively of each set) with a salt gradient from 40-110 mM NaCl over 10, 20, and 30 minutes (top, middle, and bottom sets, respectively). For each set at a specific gradient length, the retention time of the main peak and associated variants decreases with increasing flow rate primarily due to a decrease in gradient delay. With increasing flow rate the separation of the (left) acidic and (right) basic peaks from the main mAb peak decreases as shown in the plots in Figure 9. The PWHH of the main mAb peak is constant for a given time gradient indicating that PWHH is largely independent of flow rate under the gradient times and loading masses tested. Comparison of the signal strength on the y-axis of the chromatograms in Figure 8 shows that using lower flow rates results in increased signal strength due to a higher concentration of the sample in the detector. Because of this, lower sample mass loading may be used with lower flow rates while still being able to detect and quantify the sample peaks.

Figure 8 also illustrates the effect of gradient time on the retention time and separation of variant peaks from the main mAb peak. Comparison of chromatograms at the same flow rate but at different gradient times show that the gradient is primarily responsible for separation of the variant peaks from the main peak. The plots in Figure 10 show the linear increase in gradient time results in a linear increase in the separation of the (left) acidic and (right) basic variants from the main mAb peak. The tight clustering of the peaks at the different flow rates indicates that flow rate has a much weaker effect on the separation of variants from the main peak.

# WCX chromatography methods (continued)



**Figure 8. Effect of flow rate (0.5, 0.75, and 1.0 mL/min; top, middle, and bottom chromatogram of each set) and gradient time (10, 20, and 30 minutes; top, middle, and bottom sets of 3) on salt gradient separation of Pertuzumab and associated variants.** The main peak is labeled with retention time and PWHH. Acidic and basic peaks are labeled with retention time only.

# WCX chromatography methods (continued)

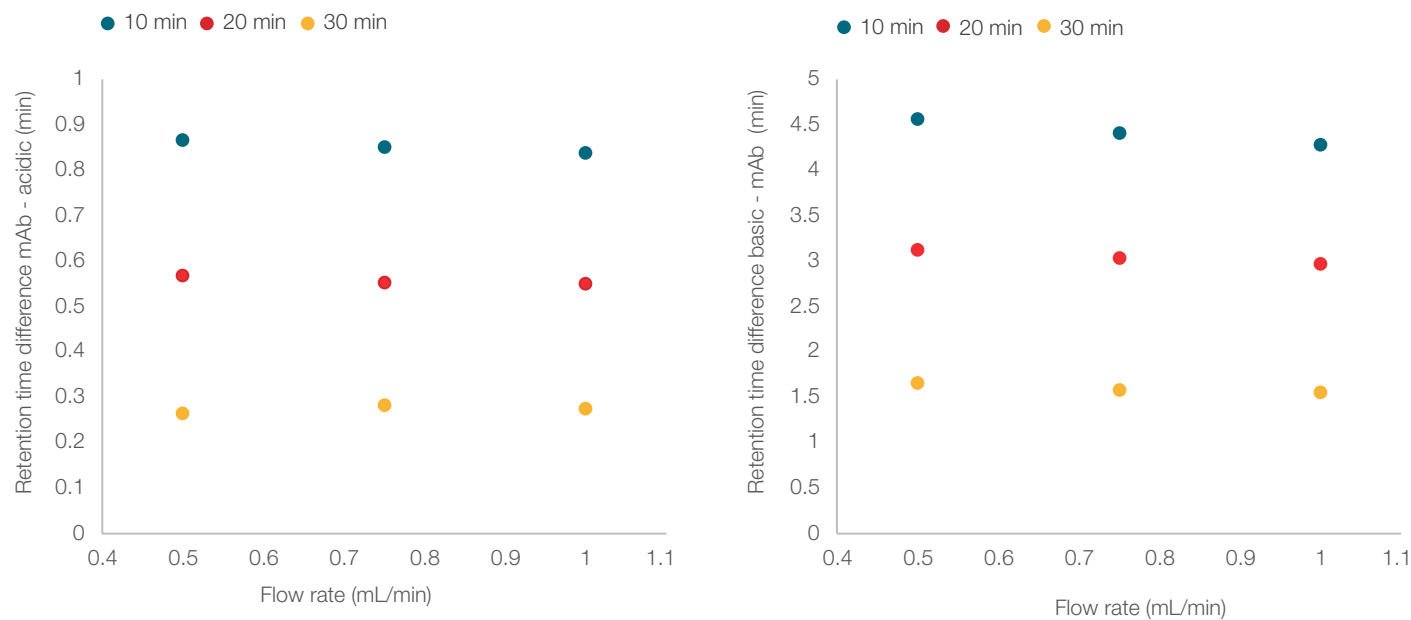


Figure 9. Plots showing the effect of flow rate on the separation of the proximal acidic variant peak (left) and largest basic variant peak (right) from the main pertuzumab mAb peak for a gradient from 40-110 mM NaCl over 10, 20, and 30 minutes. The data corresponds to the chromatograms shown in Figure 8.

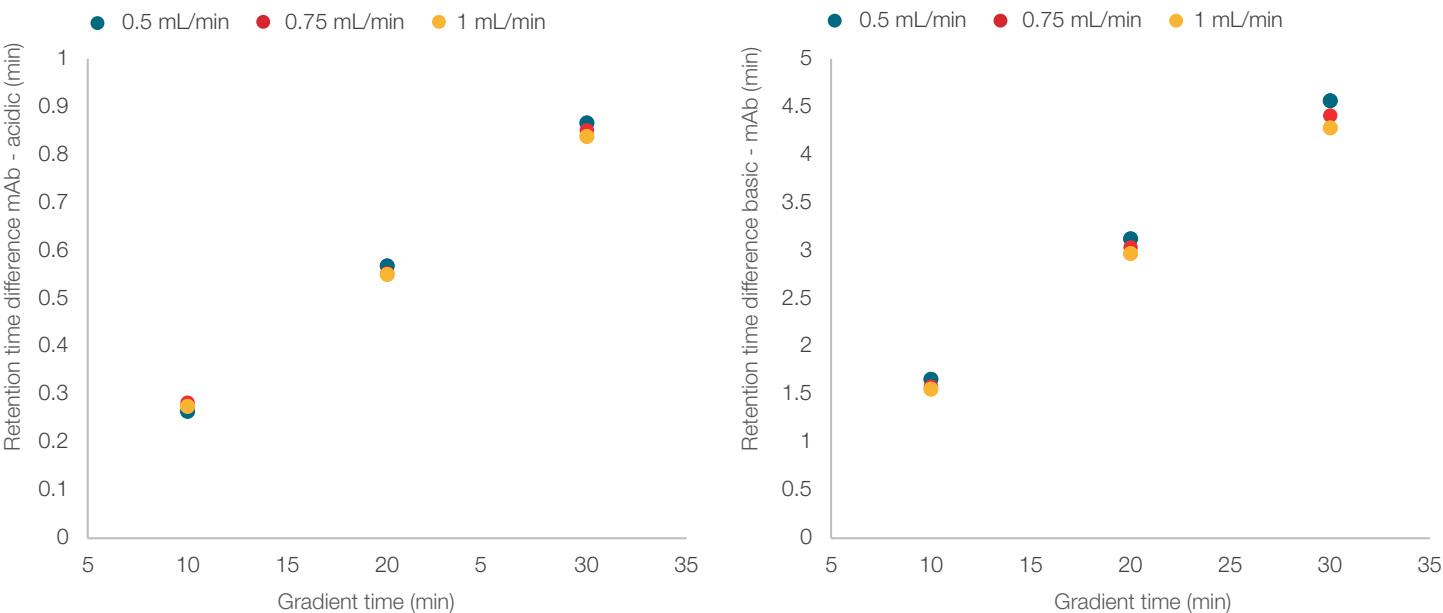


Figure 10. Plots showing the effect of gradient time for a gradient from 40-100 mM NaCl on the separation of the proximal acidic variant peak (left) and largest basic variant peak (right) from the main Pertuzumab mAb peak at 0.5, 0.75, and 1.0 mL/min. The data corresponds to the chromatograms shown in Figure 8.

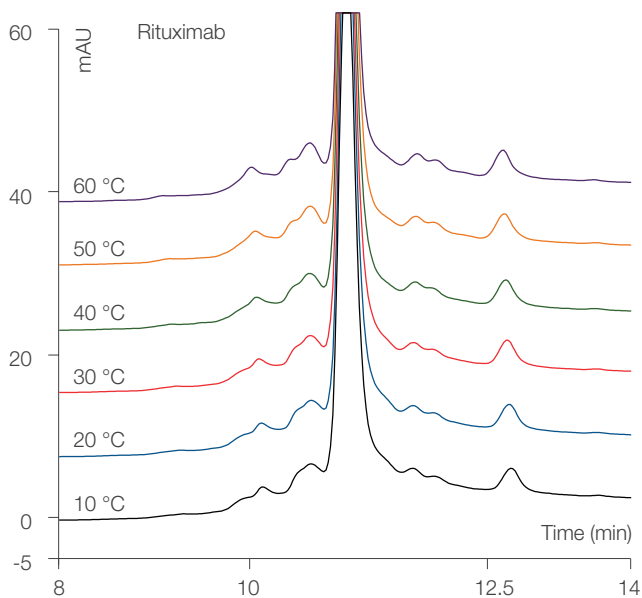
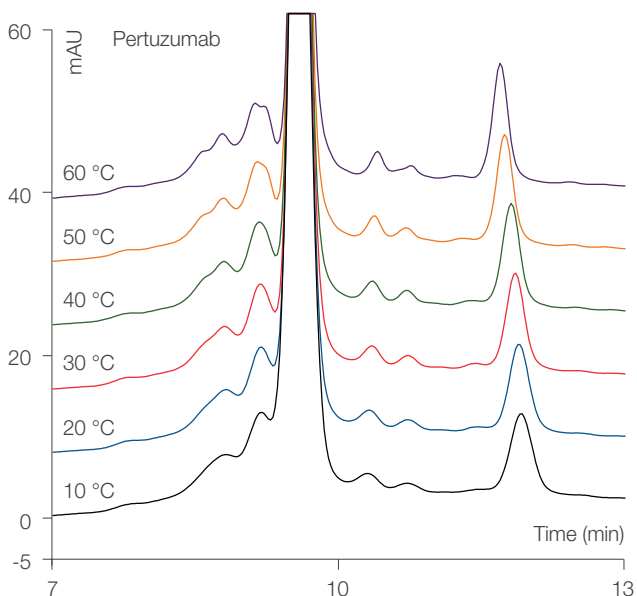
# WCX chromatography methods (continued)

## Temperature effect on salt gradients

Column temperature is a parameter that can be used to alter the separation of a protein or mAb and their variants. Typically for separations based on ionic interactions, the elution time of the protein increases with increasing column temperature. This is primarily due to the removal of waters of hydration from ionic sites resulting in improved interactions between the cationic protein and anionic stationary phase.

The effects of temperature on mAb and variant elution is demonstrated by the analysis of pertuzumab (left) and Rituximab (right) shown in Figure 11 (note retention time of the main peak is normalized to aid comparison of variant separations). In general, the separation of charge variants relative to the main peak did not vary significantly with temperature; however, small improvements in basic and acidic variant separation for both pertuzumab and rituximab were observed with increasing temperature.

| ProPac Elite WCX 5 $\mu$ m column, 4 $\times$ 150 mm |                                                         |       |
|------------------------------------------------------|---------------------------------------------------------|-------|
| Cat. no.                                             | 302972                                                  |       |
| Mobile phase                                         | A: 20 mM MES pH 6.5<br>B: 20 mM MES pH 6.5 + 0.5 M NaCl |       |
| Flow rate                                            | 1.0 mL/min                                              |       |
| Inj. volume                                          | 3 $\mu$ L                                               |       |
| Temperature                                          | See chromatogram                                        |       |
| Detection                                            | UV, 280 nm                                              |       |
| Analytes                                             | Left: pertuzumab, 5 mg/mL                               |       |
|                                                      | Right: rituximab, 5 mg/mL                               |       |
| Gradient                                             | Time (min)                                              | %A %B |
|                                                      | 0.0                                                     | 92 8  |
|                                                      | 15.0                                                    | 78 22 |
|                                                      | 15.1                                                    | 50 50 |
|                                                      | 16.0                                                    | 50 50 |
|                                                      | 16.1                                                    | 92 8  |
|                                                      | 25.0                                                    | 92 8  |

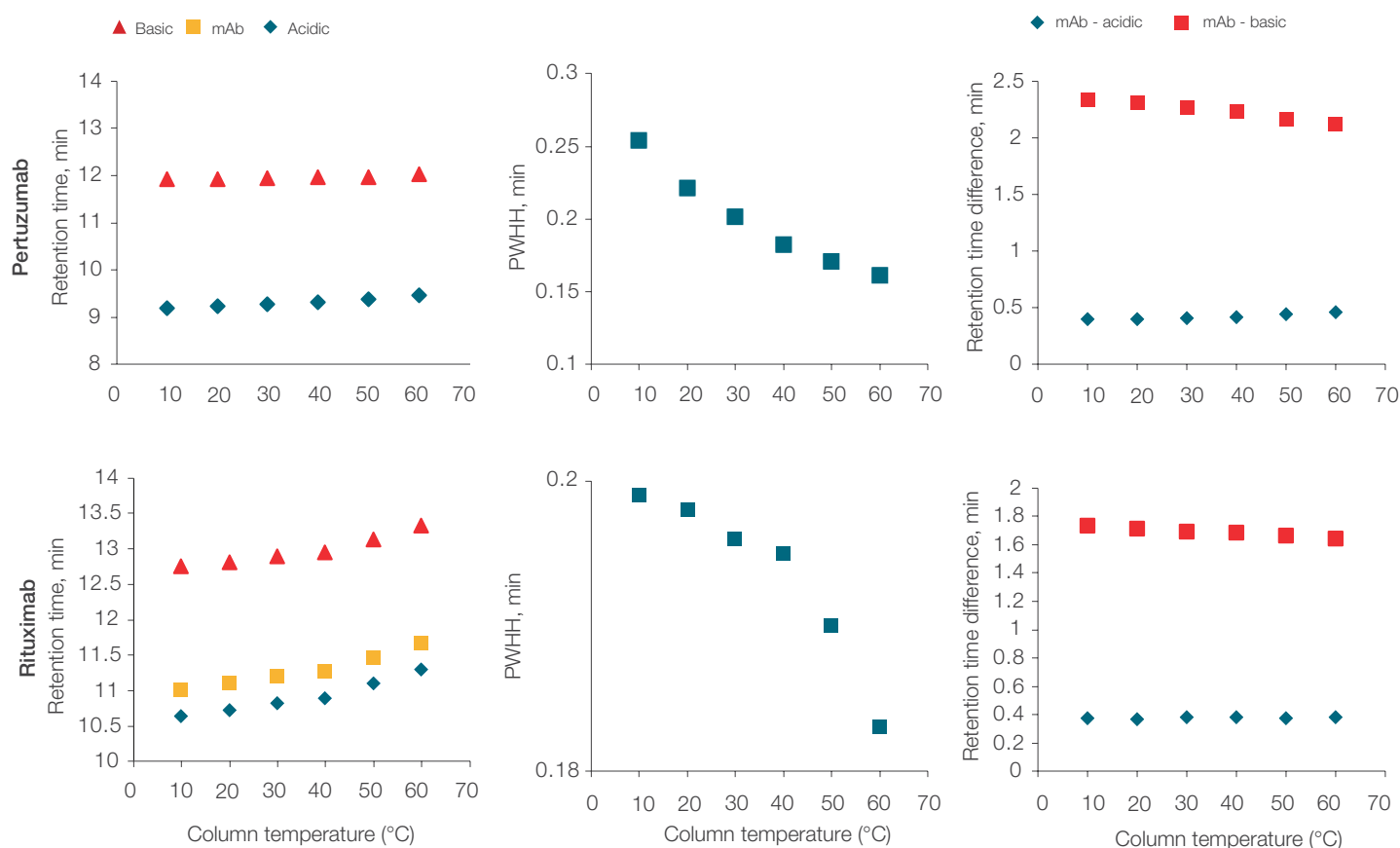


**Figure 11. Effect of temperature on Pertuzumab and Rituximab analysis using a salt gradient.** The retention time of the main mAb peak is normalized to aid comparison of the relative variants separations.

# WCX chromatography methods (continued)

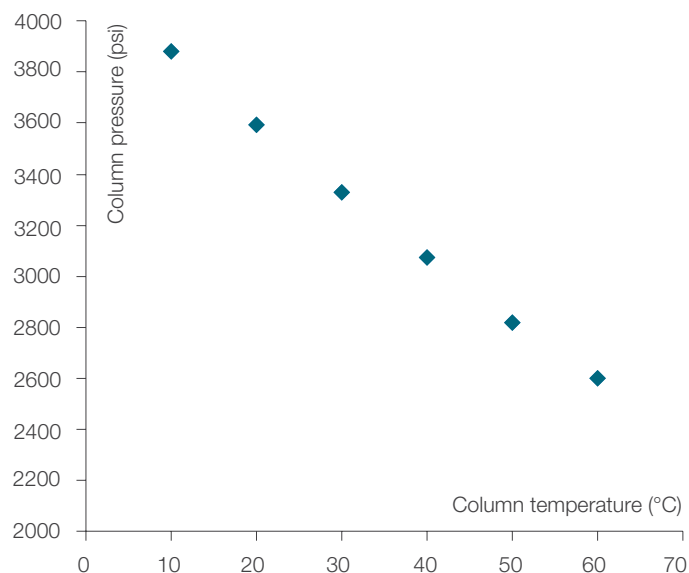
Figure 12 shows plots of the temperature effect on retention time, PWHH, and variant separation from the main peak for both mAbs in Figure 11. For pertuzumab, the main peak retention time increased and the PWHH decreased with increasing temperature. The relative separation of the acidic variants increased whereas the basic peak variant separation decreased relative to the main peak with increasing temperature. For rituximab, the main peak retention time increased with increasing temperature and the PWHH decreased. The relative separation of the acidic

variants from the main peak increased whereas the basic variant separation decreased. Based on these results, the separation of variants can be improved for both mAb tested by operating at 50-60 °C. Do not operate at temperatures above 60 °C as temperature induced denaturation may occur and adversely affect the protein structure and chromatographic separation. Figure 13 shows that the column backpressure decreases linearly with increasing temperature offering an additional point of control for column operation.



**Figure 12. Effect of temperature on (left) mAb and acidic and basic variant retention time, (middle) mAb PWHH, and (right) retention time difference between acidic variant - mAb and basic variant - mAb. Data are from the chromatograms in Figure 8.**

# WCX chromatography methods (continued)

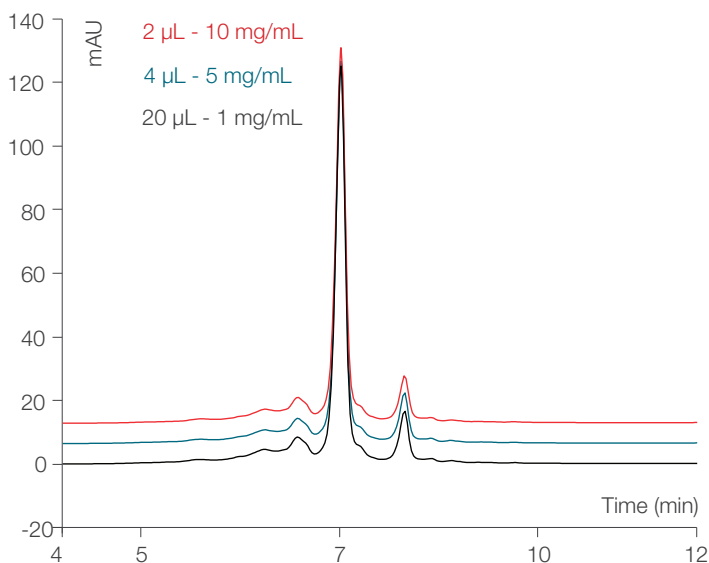


**Figure 13.** Effect of temperature on column back pressure at 1 mL/min flow rate with 20 mM MES pH 6.5 + 40 mM NaCl

## Protein loading and carryover analysis

For typical protein loading levels, sample concentration and injection volume do not significantly influence the separation of the mAb and associated variants. The chromatograms in Figure 14 show 20 µg loading of pertuzumab on a 4 × 150 mm column using 10, 5, and 1 mg/mL samples with 2, 4, and 20 µL injections, respectively.

Comparison of each chromatogram shows that the variant separations from the main peak and the PWHH is constant regardless of the sample concentration or the amount of sample loaded.



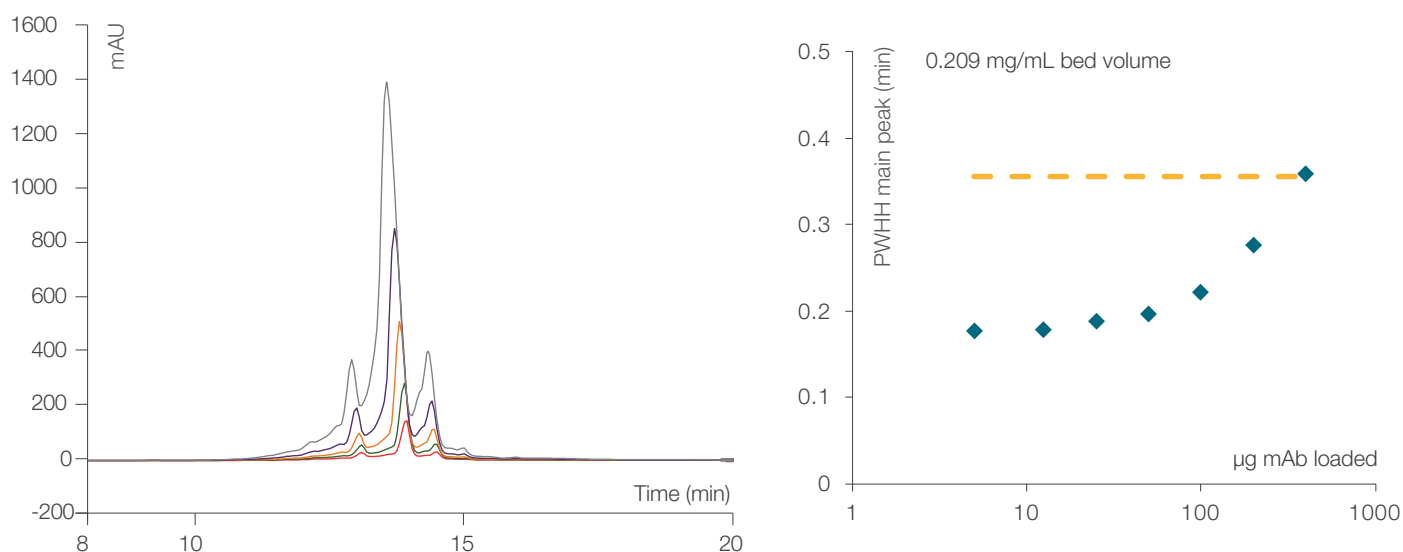
**Figure 14.** Chromatograms of the separation of 20 µg pertuzumab loading using a 2 µL, 4 µL, and 20 µL injections with 10 mg/mL, 5 mg/mL and 1 mg/mL samples, respectively

| ProPac Elite WCX 5 µm column, 4 × 150 mm |                                                         |    |    |
|------------------------------------------|---------------------------------------------------------|----|----|
| Cat. no.                                 | <a href="#">302972</a>                                  |    |    |
| Mobile phase                             | A: 20 mM MES pH 6.5<br>B: 20 mM MES pH 6.5 + 0.5 M NaCl |    |    |
| Flow rate                                | 1.0 mL/min                                              |    |    |
| Inj. volume                              | 3 µL                                                    |    |    |
| Temperature                              | See chromatogram                                        |    |    |
| Detection                                | UV, 280 nm                                              |    |    |
| Analytes                                 | Pertuzumab                                              |    |    |
| Gradient                                 | Time (min)                                              | %A | %B |
|                                          | 0.0                                                     | 92 | 8  |
|                                          | 30.0                                                    | 78 | 22 |
|                                          | 30.1                                                    | 50 | 50 |
|                                          | 32.0                                                    | 50 | 50 |
|                                          | 32.1                                                    | 92 | 8  |
|                                          | 40.0                                                    | 92 | 8  |

# WCX chromatography methods (continued)

As sample loading amount increases above those shown in Figure 14, the stationary phase becomes overloaded and the analysis of the sample begins to degrade due to peak broadening and shifting of peaks to earlier in the chromatogram as they are excluded from the stationary phase by adsorbed protein. The overlay of chromatograms in Figure 15 shows this effect for a biosimilar of Trastuzumab (Herceptin™). The plot on the side of Figure 15 shows the PWHH of the main peak versus the mass of protein loaded. The dashed line in the plot indicates 2× the PWHH of the lowest mass loaded.

This type of experiment is commonly referred to as dynamic loading analysis with overloading here defined as the loading mass of twice the PWHH of lowest mass loaded. For the ProPac Elite WCX 5  $\mu\text{m}$  stationary phase, the dynamic loading capacity was calculated as 0.209 mg/mL bed volume, where the bed volume is the total column volume as calculated by the column body dimensions. The dynamic loading mass is important in selecting an appropriate column format for achieving the desired separation. The dynamic loading capacity is protein dependent and can vary depending on protein molecular weight and structure. The example provided here can generally be applied to other mAbs.

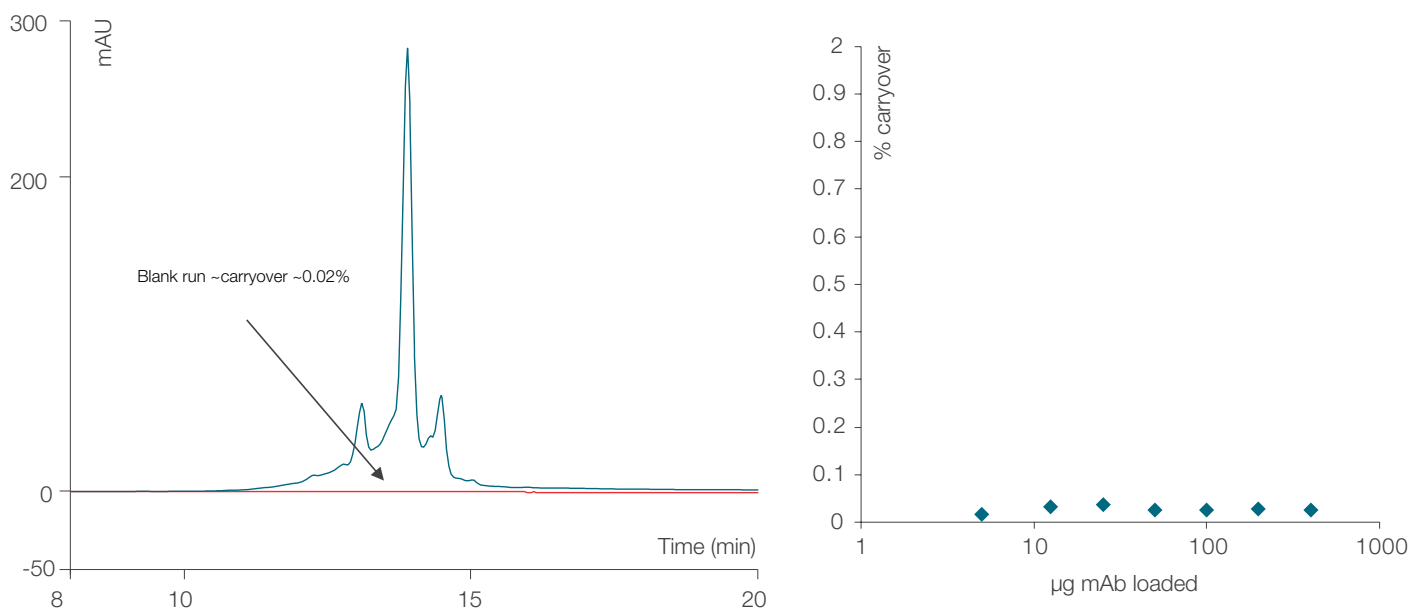


**Figure 15. Chromatogram overlays showing the dynamic loading analysis of a Trastuzumab biosimilar on a 4 × 150 mm ProPac Elite WCX 5  $\mu\text{m}$  column using a salt gradient.** The plot shows the corresponding PWHH of the main mAb peak versus the masses of mAb loaded in the chromatogram.

# WCX chromatography methods (continued)

The ProPac Elite WCX 5  $\mu\text{m}$  stationary phase is designed for very low carryover even at high mass loading levels. Figure 16 shows the overlaid chromatograms for a 50  $\mu\text{g}$  injection run on a  $4 \times 150$  mm column using the Trastuzumab biosimilar from the dynamic loading analysis and the following blank run with no injection. The measured carryover in the blank run was only 0.02%.

The plot on the right of Figure 16 shows the percent carryover for loading of the same mAb from 5  $\mu\text{g}$  up to 400  $\mu\text{g}$  total protein loading. These results demonstrate the low carryover properties of the stationary phase, which enables protein injections from run to run without interference due to carryover from previous injections.



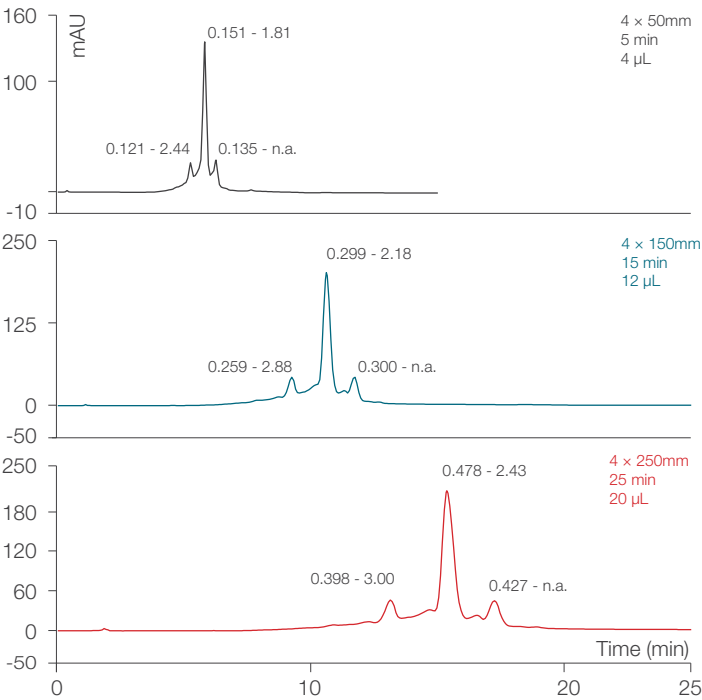
**Figure 16. (Left) Overlaid chromatograms showing a 50  $\mu\text{g}$  injection and elution of a Trastuzumab biosimilar on a  $4 \times 150$  mm column using a salt gradient and the following blank run to measure carryover. (Right) A plot of the percent carryover for the mAb at loading levels ranging from 5  $\mu\text{g}$  up to 400  $\mu\text{g}$  total loading.**

# WCX chromatography methods (continued)

## Column format selection

Due to greater capacity, longer columns provide improved separation of variant peaks from the main mAb peak relative to shorter columns. Figure 17 demonstrates the improved separation achieved by increasing the length of a 4 × 50 mm column to 150 mm and 250 mm long columns with the gradient

time and sample mass loading scaled to the column length. As expected, the resolution value for each peak improves with increasing column length.



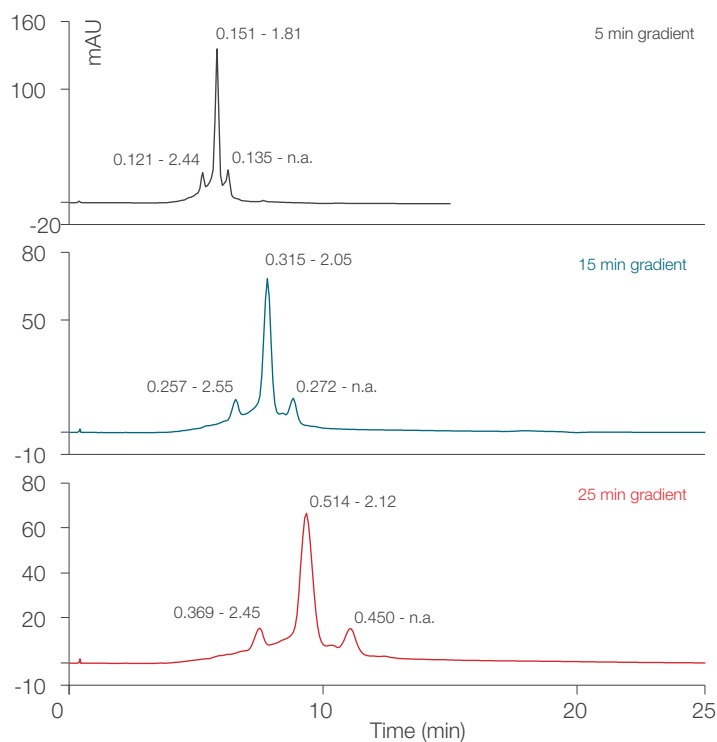
| ProPac Elite WCX 5 µm column |                                                                   |
|------------------------------|-------------------------------------------------------------------|
| Mobile phase                 | A: 20 mM MES, pH 6.5<br>B: 20 mM MES + 0.5 M NaCl, pH 6.5         |
| Flow rate                    | 0.6 mL/min                                                        |
| Inj. volume                  | See chromatogram                                                  |
| Temperature                  | 30 °C                                                             |
| Detection                    | UV, 280 nm                                                        |
| Analytes                     | Trastuzumab biosimilar, 5 mg/mL                                   |
| Gradient                     | Loading – 9% B 12-22% B;<br>see chromatogram for time of gradient |

**Figure 17. The effect of column length on resolution of acidic and basic variants for a Trastuzumab biosimilar.** The gradient length and sample mass loading are scaled to the column length.

# WCX chromatography methods (continued)

To demonstrate that the improvement is not solely due to the increase in gradient length, the extended 15 minute and 25 minute gradients were run using a 4 × 50 mm and a 4 × 150 mm column with 20 µg loading for each column. The chromatograms for the 4 × 50 mm column are shown in Figure 18 and the resolutions of the acidic and mAb peaks for all formats and

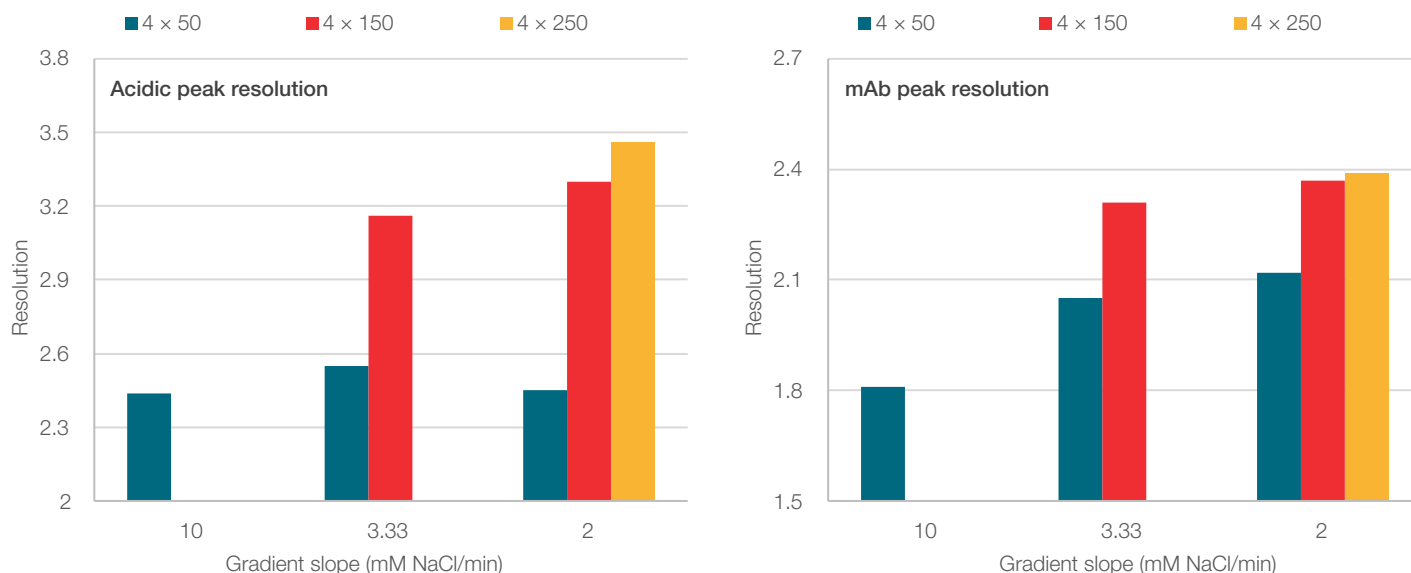
gradients tested are summarized in the column charts in Figure 19. At these low loading levels, the 4 × 50 mm column achieves good resolution of the acidic and basic peaks. However, the longer 150 mm and 250 mm column formats still provide better resolution when using longer gradients compared to the shorter 4 × 50 mm column.



| ProPac Elite WCX 5 µm column, 4 × 50 mm |                                                                   |
|-----------------------------------------|-------------------------------------------------------------------|
| Cat. no.                                | 302972                                                            |
| Mobile phase                            | A: 20 mM MES pH 6.5<br>B: 20 mM MES pH 6.5 + 0.5 M NaCl           |
| Flow rate                               | 0.6 mL/min                                                        |
| Inj. volume                             | 3 µL                                                              |
| Temperature                             | 30 °C                                                             |
| Detection                               | UV, 280 nm                                                        |
| Analytes                                | Trastuzumab biosimilar, 5 mg/mL                                   |
| Gradient                                | Loading – 9% B 12-22% B;<br>see chromatogram for time of gradient |

**Figure 18.** Chromatograms showing the effect of gradient time on the separation of variants for a Trastuzumab biosimilar using a 4 × 50 mm column

# WCX chromatography methods (continued)



**Figure 19. Column charts comparing the resolution of the (left) acidic to mAb peak and the (right) mAb to basic peak for a Trastuzumab biosimilar using different column formats and gradient slopes.** 10, 3.33, and 2 mM NaCl/min gradient slopes correspond to the 5, 15, and 25 minute gradients in Figure 15.

The protein loading levels are quite low for these analyses and the dynamic loading capacity of 0.209 mg/mL bed volume for this mAb suggests that significantly higher levels of protein loading can be achieved while maintain good separation. Specifically, the 4 x 50 mm, 4 x 150 mm and 4 x 250 mm columns are expected to become dynamically overloaded at approximately 130 µg, 390 µg, and 655 µg loading, respectively. To more clearly demonstrate the improvement gained by using a longer column, the performance of the 4 x 50 mm, 4 x 150 mm, and 4 x 250 mm formats using a 25 minute gradient at 100 µg total loading was evaluated.

This loading mass is near the limit of the 4 x 50 mm format's dynamic loading but still within the mass loading limit of the longer columns. The chromatograms for the three column lengths are shown in Figure 20 and the resolution values for the acidic and main peak are summarized in the column chart in Figure 21. These results show the benefit of using the longer column format as the 4 x 50 mm and 4 x 150 mm columns both suffer from peak broadening and consequently reduced resolution as shown in the column chart. To analyze higher masses without compromising the separation of variants, use longer column formats.

# WCX chromatography methods (continued)

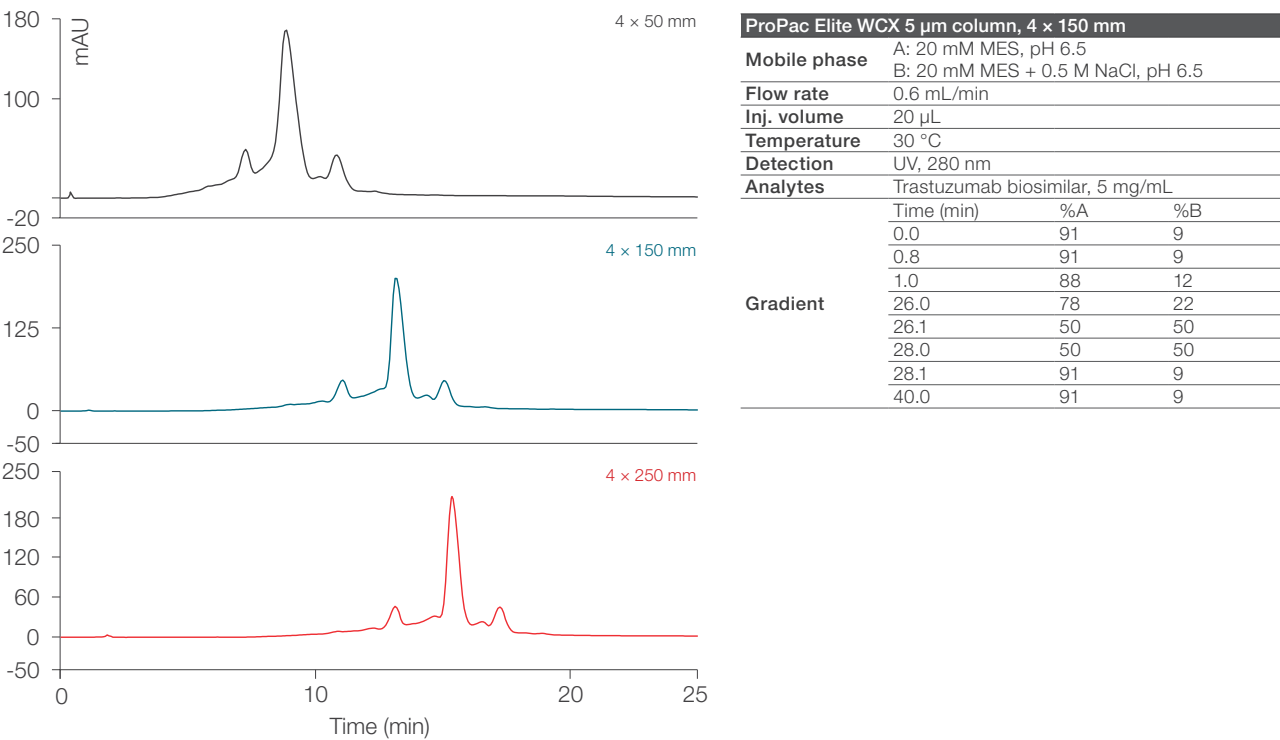


Figure 20. Chromatograms showing the effect of column length (4 x 50 mm, 4 x 150 mm, and 4 x 250 mm) on PWHH and variant resolution for 100 µg loading of a Trastuzumab biosimilar with a constant gradient time of 25 minutes

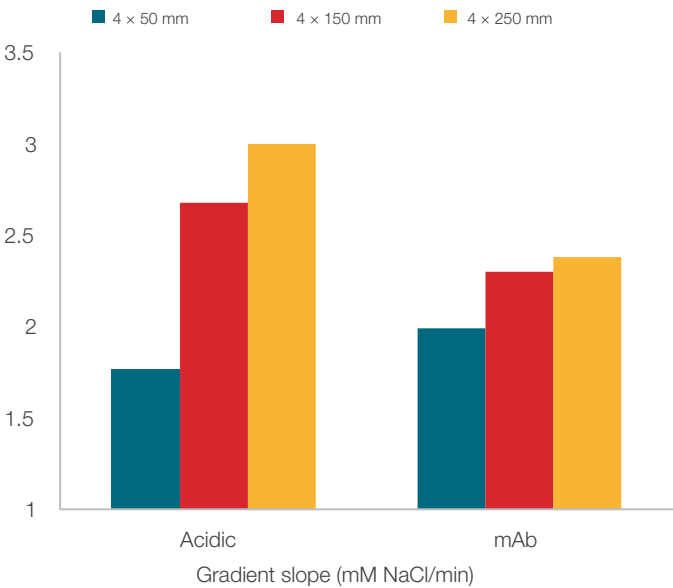


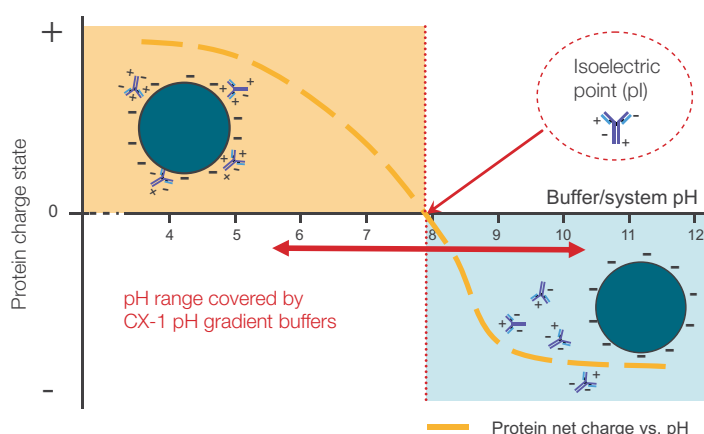
Figure 21. Column chart of the acidic-mAb peak and mAb-basic peak resolution for the separation of Trastuzumab biosimilar variants in Figure 18

# WCX chromatography methods (continued)

## pH gradient separation principles

The mechanism of protein elution for a pH gradient is different from the salt gradient. Under salt gradient conditions, salt ions disrupt the interactions of the cationic protein with the anionic solid phase resulting in desorption and elution. For a pH gradient the cationic protein is adsorbed to the stationary phase at a pH less than the protein isoelectric point (pI). As the pH of the buffer increases, the charge of the protein shifts from cationic to neutral at the pI and then anionic at higher buffer pH values. The change in protein charge results in desorption from the anionic surface and elution from the column.

This mechanism is depicted schematically in Figure 22 with the dashed line indicating the charge of a theoretical protein. The red arrows indicate the pH operating range from 5.6 to 10.2 for the CX-1 pH gradient buffers used in this manual. Many cationic proteins including mAbs have a pI in this range allowing them to be analyzed using this buffer system. The sections that follow discuss the technical aspects of designing pH gradient methods.



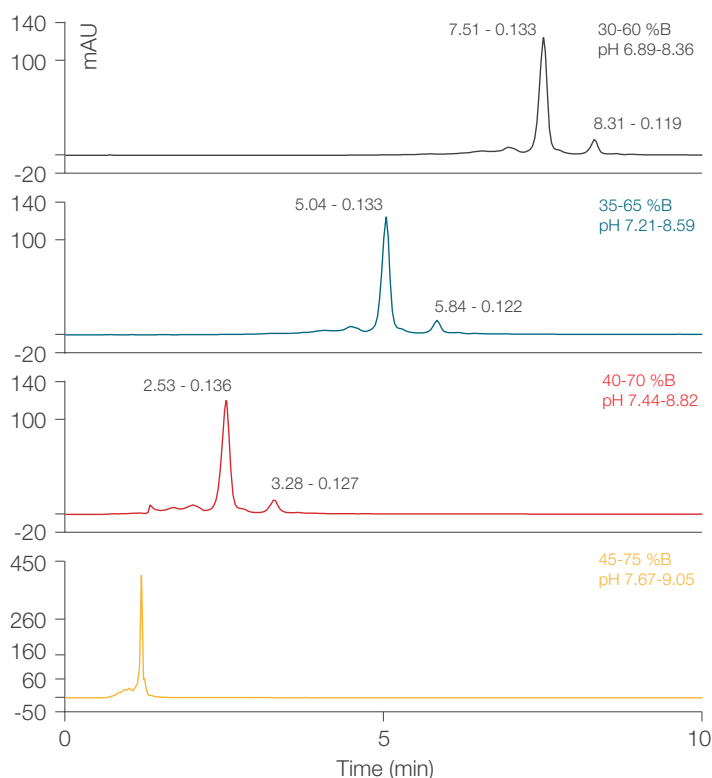
**Figure 22. Schematic showing the adsorption and desorption mechanism of cationic proteins on anionic stationary phases when using pH gradient buffers**

### Determining gradient starting conditions

Due to the low ionic strength of the CX-1 pH gradient buffers, isocratic elution during loading does not typically occur for pH gradients in contrast to salt gradient methods. As such, it is only important to determine the pH loading conditions to bind all the sample and associated variants of interest to the solid phase. Specifically, care should be taken to ensure the starting pH is lower than the acidic variant with the lowest pI. Figure 23 shows the separation of Pertuzumab on a 4 × 150 mm column. The mAb is loaded at 30, 35, 40, and 45 %B (pH 6.98, 7.21, 7.44, and 7.67, respectively) and eluted using an increase of 30 %B (1.38 pH units) over 15 minutes. When loading at pH 7.67 Pertuzumab elutes in the void at 1 min. When loading at pH 7.44, the acidic variants elute just as the gradient reaches the column indicating the starting pH is too high for effective separation.

The relative separation of the variants to the main peak is unchanged when loading at pH 6.98 and 7.21 with nominal differences observed at pH 7.44 where partial elution of acidic variants at the start of the gradient is observed. This data indicates that a maximum pH of ~7.21 can be used to analyze the sample without loss of variant separation. The loading pH for a user's sample should be carefully chosen to avoid elution of the mAb and variants in the void.

# WCX chromatography methods (continued)



|                                                      |                                                              |
|------------------------------------------------------|--------------------------------------------------------------|
| ProPac Elite WCX 5 $\mu$ m column, 4 $\times$ 150 mm |                                                              |
| Cat. no.                                             | 302972                                                       |
| Mobile phase                                         | A: CX-1 pH gradient buffer A<br>B: CX-1 pH gradient buffer B |
| Flow rate                                            | 1 mL/min                                                     |
| Inj. volume                                          | 3 $\mu$ L                                                    |
| Temperature                                          | 30 $^{\circ}$ C                                              |
| Detection                                            | UV, 280 nm                                                   |
| Analytes                                             | Pertuzumab, 5 mg/mL                                          |
| Gradient                                             | See chromatogram for change in %B over 15 minutes            |

**Figure 23. Effect of loading buffer pH on pertuzumab and associated variant separation**

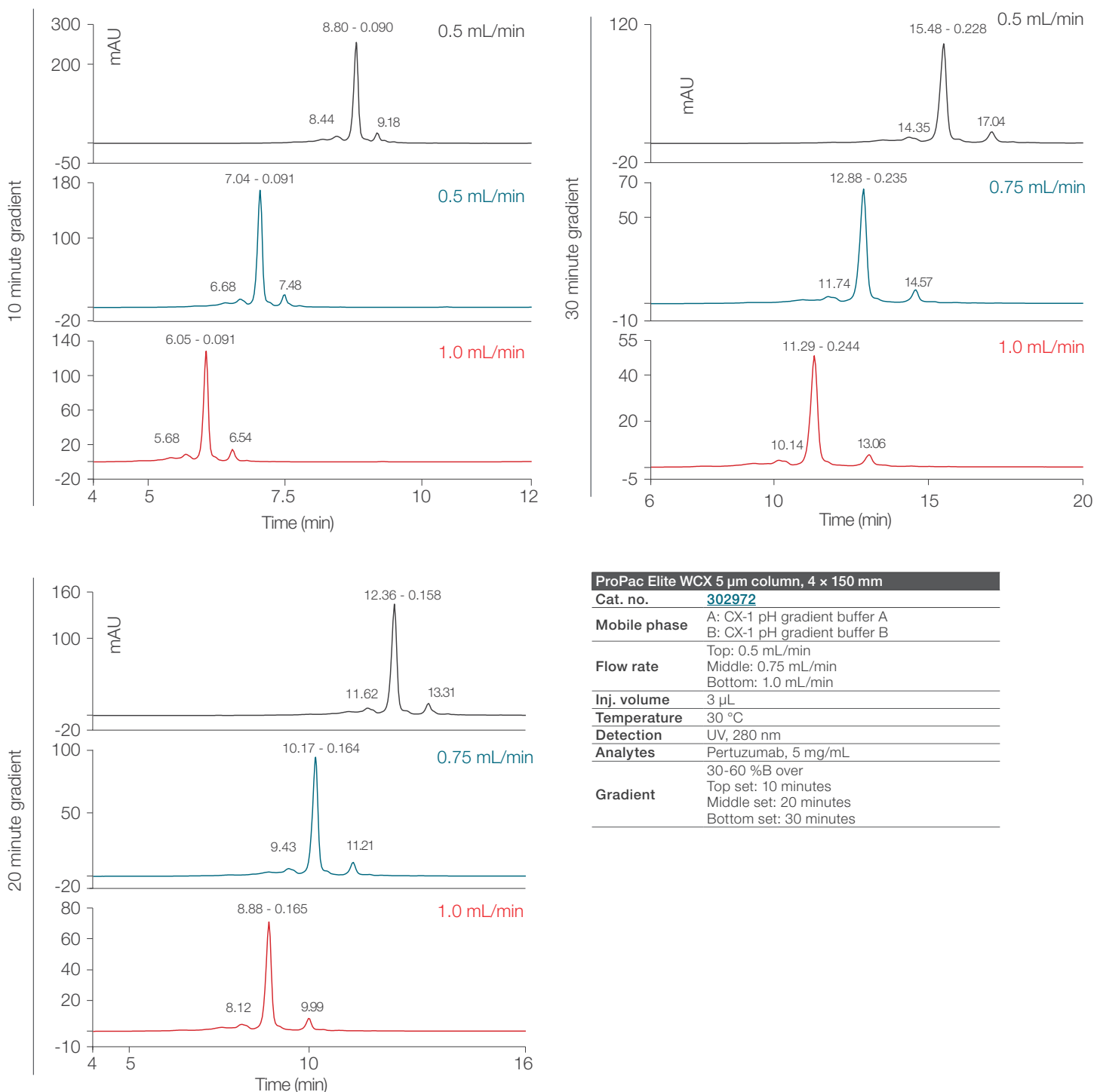
## Flow rate and gradient slope effects on pH gradient separation

Figure 24 shows the analysis of Pertuzumab using a flow rate of 0.5, 0.75, and 1.0 mL/min (top, middle, and bottom chromatograms, respectively of each set) with a pH gradient from pH 6.98 to 8.36 B over 10, 20, and 30 minutes (top, middle, and bottom sets, respectively). For each set at a specific gradient length, the retention time of the main peak and associated variants decreases with increasing flow rate due largely to a decrease in the gradient delay. The separation of the acidic and basic variants relative to the main peak decreases with increasing flow rate as shown in the plots in Figure 25. For longer gradients, (e.g., 30 minutes) the PWHH of the main mAb peak decreased with decreasing flow rate. At shorter gradient times (e.g. 10 minutes) the PWHH is observed to be independent of flow rate for the loading masses tested. A benefit to using lower flow rates is the increase in signal strength. Comparison of the signal strength on the y-axis of the chromatograms in Figure 24 demonstrates this effect. As a result lower sample mass loading may be used with lower flow rates while still being able to detect and quantify the mAb and associated variants.

Figure 24 also illustrates the effect of gradient time on the retention time and separation of variant peaks from the main mAb peak. Comparison of chromatograms at the same flow rate but at different gradient times shows that the gradient is primarily responsible for the extent to which the variant peaks are separated from the main peak. The plot in Figure 26 shows this effect as the linear increase in gradient time results in a linear increase in the separation of variant peaks based on retention time differences. The tight clustering of the peaks at the different flow rates indicates that flow rate has a much weaker effect on the separation of variants from the main peak. However, the 30 minute gradient data shows that for a pH gradient increased flow rates result in an increase in the separation of variants from the main mAb peak, specifically for the basic peak.

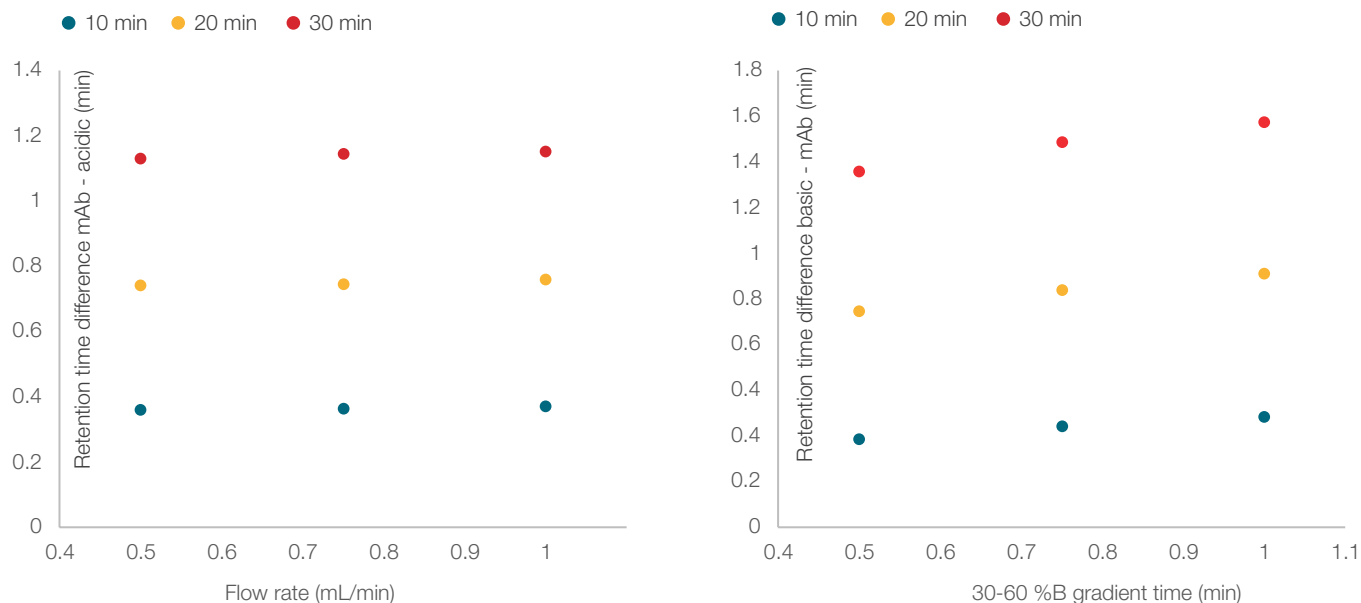
Taking the data in Figures 24-26 together, the separation of variants for Pertuzumab under these conditions is dependent on the flow rate with lower flow rates giving narrower peaks and faster flow rates giving better variant separation. As such, the flow rate should be carefully selected to maximize variant separation without excessive peak broadening.

# WCX chromatography methods (continued)

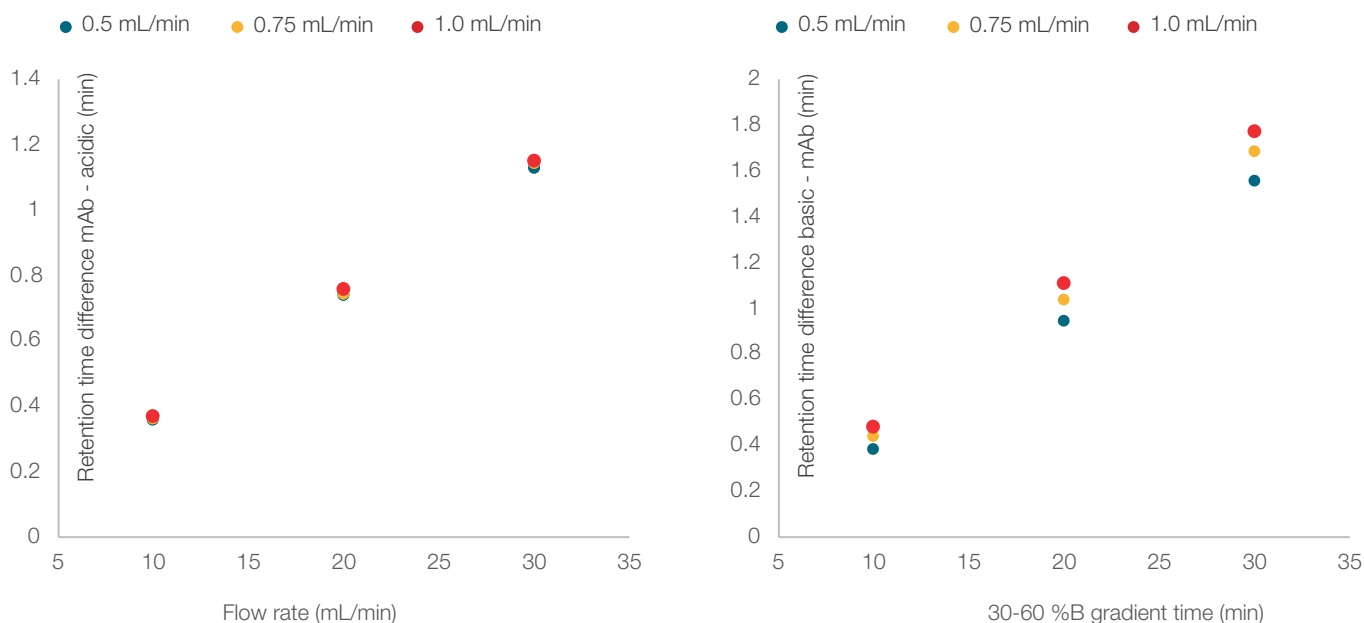


**Figure 24. Effect of flow rate (0.5, 0.75, and 1.0 mL /min; top, middle, and bottom chromatogram of each set) and gradient time (10, 20, and 30 minutes; top, middle, and bottom sets of 3) on the pH gradient separation of Pertuzumab and associated variants.** The main peak is labeled with retention time and PWHH. Acidic and basic peaks are labeled with retention time only.

# WCX chromatography methods (continued)



**Figure 25.** Plots showing the effect of flow rate on the separation of the proximal acidic variant peak (left) and largest basic variant peak (right) from the main Pertuzumab mAb peak using a pH gradient from 6.98-8.36 over 10, 20, and 30 minutes. The data corresponds to the chromatograms shown in Figure 27.



**Figure 26.** Plots showing the effect of gradient time for a pH gradient from 6.98-8.36 on the separation of the proximal acidic variant peak (left) and largest basic variant peak (right) from the main Pertuzumab mAb peak at 0.5, 0.75, and 1.0 mL/min. The data corresponds to the chromatograms shown in Figure 27.

# WCX chromatography methods (continued)

## Temperature effect on pH gradient separation

When using a pH gradient, column temperature can be used to alter the selectivity and separation of a protein or mAb. The effects of temperature on mAb and variant elution for the pH gradient are demonstrated by the analysis of Pertuzumab (left) and Rituximab (right) shown in Figure 27. The separation of charge variants relative to the main peak did not vary significantly with temperature; however, small improvements in basic and acidic variant separation for both Pertuzumab and Rituximab were observed with increasing temperature.

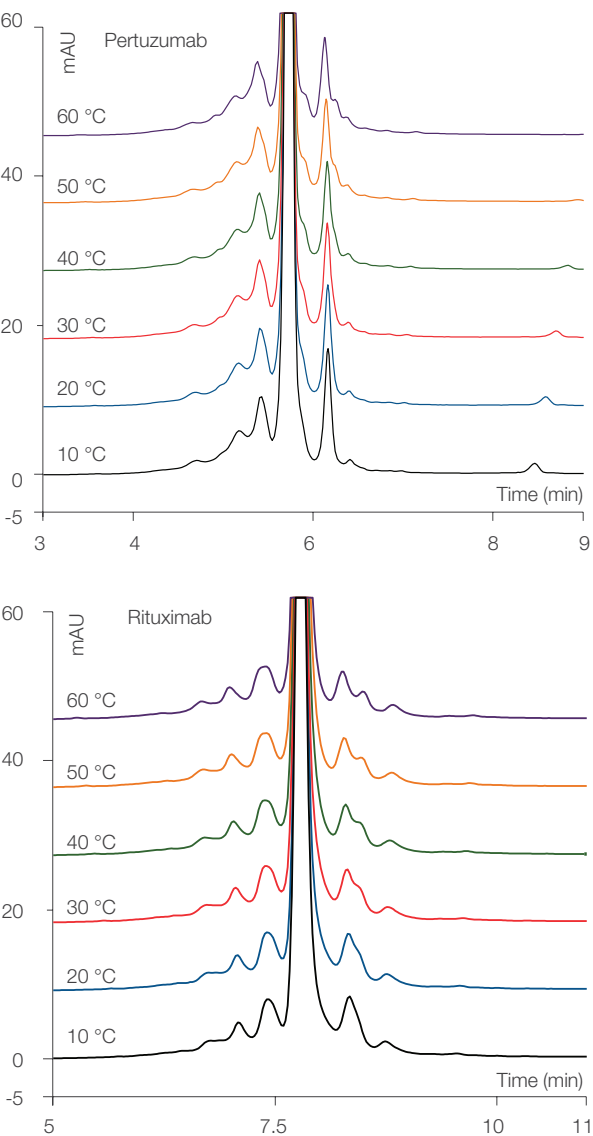


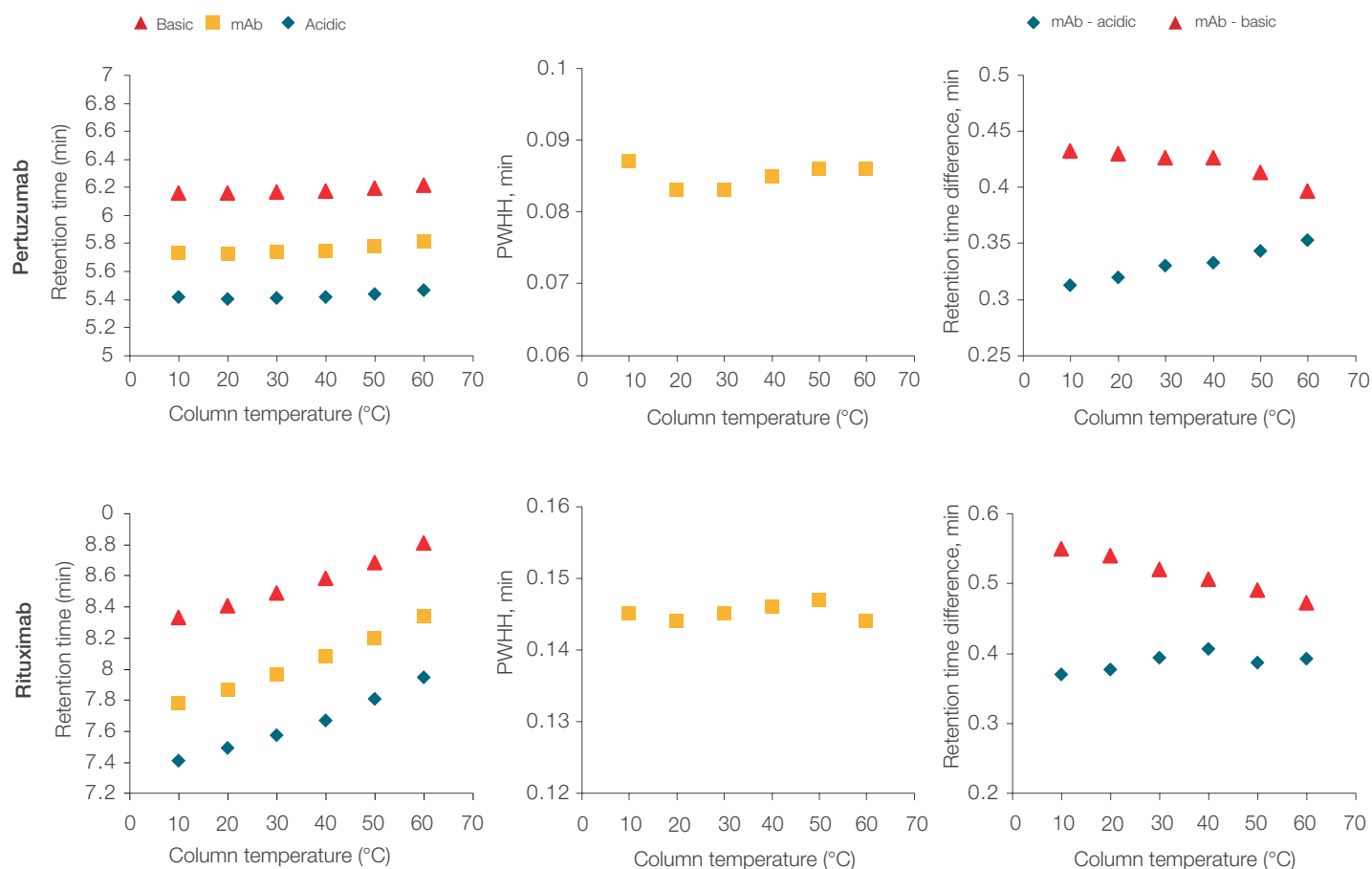
Figure 27. Chromatograms showing the effect of temperature on mAb and variant separation for Pertuzumab (top) and Rituximab (bottom) using a pH gradient

Figure 28 shows plots of the temperature effect on retention time, PWHH, and variant separation from the main peak for both mAbs in Figure 27. For Pertuzumab, the main peak and variant retention times were changed only slightly with increasing temperature, and the PWHH was relatively constant but increased at 50-60 °C.

For Rituximab, the main peak and variant retention times increased with increasing temperature while the PWHH in general decreased. For both mAbs, the separation of the proximal acidic peak from the main peak increased with temperature while separation of the basic from the main peak decreased. Based on these results, the separation of variants for these mAbs can be improved for both samples by operating at higher temperatures. Figure 29 shows that the column backpressure decreases linearly with increasing temperature offering an additional point of control for column operation. From these results, the user should determine the optimal temperature for analysis of their particular mAb sample.

| ProPac Elite WCX 5 µm column, 4 × 150 mm |                                                              |    |     |
|------------------------------------------|--------------------------------------------------------------|----|-----|
| Cat. no.                                 | 302972                                                       |    |     |
| Mobile phase                             | A: CX-1 pH gradient buffer A<br>B: CX-1 pH gradient buffer B |    |     |
| Flow rate                                | 1.0 mL/min                                                   |    |     |
| Inj. volume                              | 3 µL                                                         |    |     |
| Temperature                              | See chromatogram                                             |    |     |
| Detection                                | UV, 280 nm                                                   |    |     |
| Analytes                                 | Left: pertuzumab, 5 mg/mL                                    |    |     |
|                                          | Right: rituximab, 5 mg/mL                                    |    |     |
| 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  |
|                                          |                                                              |    |     |

# WCX chromatography methods (continued)



**Figure 28.** Effect of temperature on pH gradient analysis of Pertuzumab and Rituximab showing (left) mAb and acidic and basic variant retention time, (middle) mAb PWHH, and (right) retention time difference between acidic variant – mAb and basic variant – mAb. Data are from the chromatograms in Figure 28.

# WCX chromatography methods (continued)

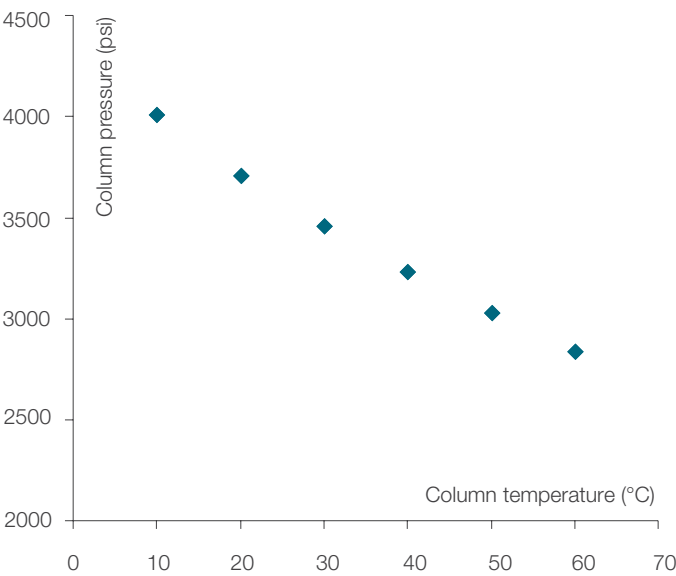
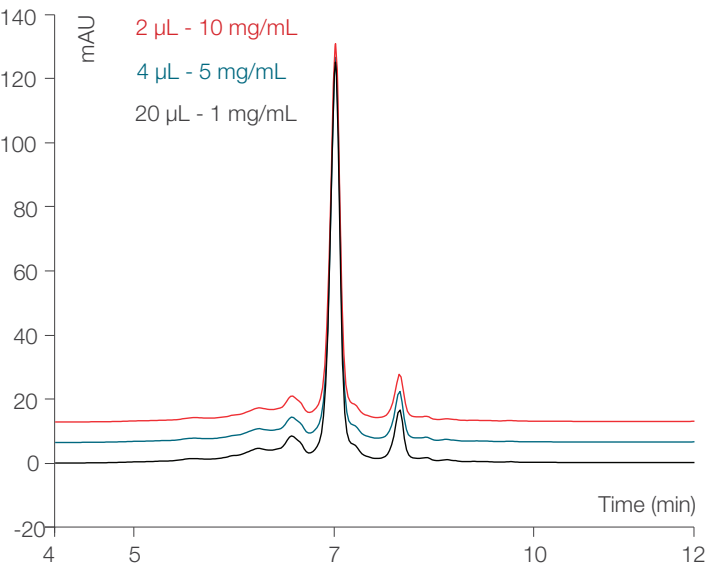


Figure 29. Effect of temperature on column back pressure for a 4 × 150 mm column at 1 mL/min flow rate with CX-1 pH gradient buffers

### Protein loading and carryover analysis

As shown for salt gradients, sample concentration and injection volume do not significantly influence the separation of the mAb and associated variants for typical protein loading levels when using a pH gradient. The chromatograms in Figure 30 show 20 µg loading on a 4 × 150 mm column using 10, 5, and 1 mg/mL

samples with 2, 4, and 20 µL injections, respectively. Comparison of each chromatogram shows that the variant separations from the main peak and the PWHH is constant regardless of the sample concentration or the amount of sample loaded.



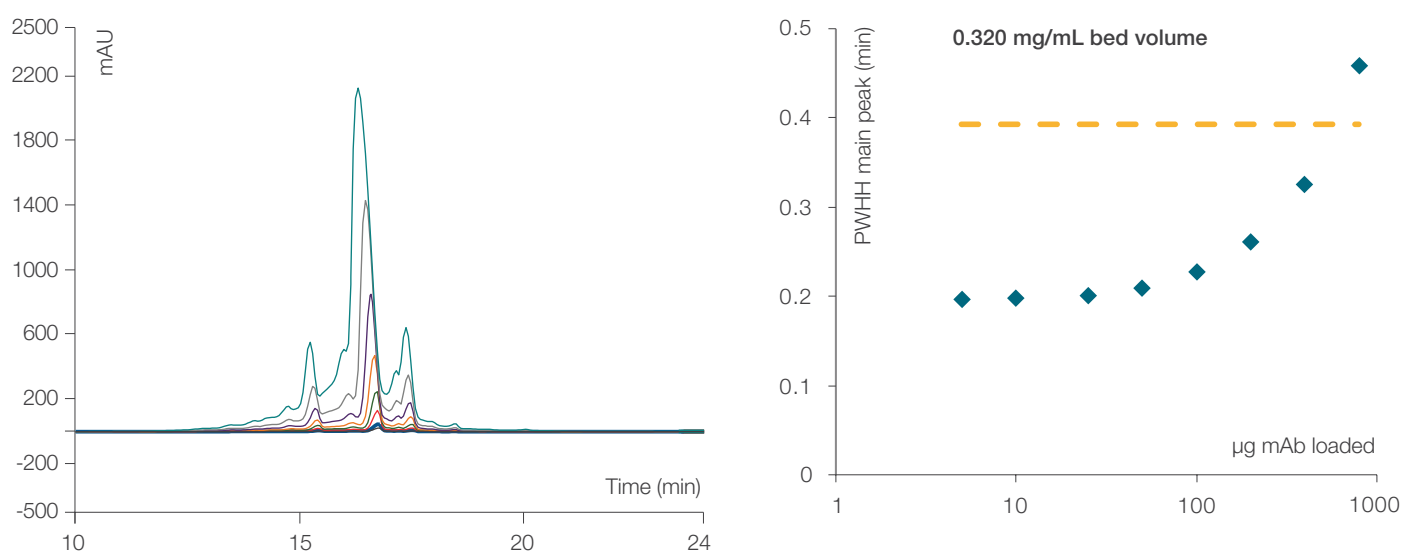
| ProPac Elite WCX 5 µm column, 4 × 150 mm |                                                              |    |     |
|------------------------------------------|--------------------------------------------------------------|----|-----|
| Cat. no.                                 | 302972                                                       |    |     |
| Mobile phase                             | A: CX-1 pH gradient buffer A<br>B: CX-1 pH gradient buffer B |    |     |
| Flow rate                                | 1.0 mL/min                                                   |    |     |
| Inj. volume                              | See chromatogram                                             |    |     |
| Temperature                              | 30 °C                                                        |    |     |
| Detection                                | UV, 280 nm                                                   |    |     |
| Analytes                                 | Pertuzumab                                                   |    |     |
|                                          | Time (min)                                                   | %A | %B  |
|                                          | 0.0                                                          | 70 | 30  |
|                                          | 10.0                                                         | 40 | 60  |
|                                          | 10.1                                                         | 0  | 100 |
|                                          | 12.0                                                         | 0  | 100 |
|                                          | 12.1                                                         | 90 | 10  |
|                                          | 13.0                                                         | 90 | 10  |
|                                          | 13.1                                                         | 70 | 30  |
|                                          | 20.0                                                         | 70 | 30  |
| Gradient                                 |                                                              |    |     |
|                                          |                                                              |    |     |
|                                          |                                                              |    |     |
|                                          |                                                              |    |     |
|                                          |                                                              |    |     |
|                                          |                                                              |    |     |
|                                          |                                                              |    |     |
|                                          |                                                              |    |     |
|                                          |                                                              |    |     |
|                                          |                                                              |    |     |

Figure 30. Consistent chromatographic separation of variants for 20 µg Pertuzumab loading using a 2 µL, 4 µL, and 20 µL injection with 10 mg/mL, 5 mg/mL and 1 mg/mL samples, respectively

# WCX chromatography methods (continued)

The same dynamic loading analysis for the salt gradient was performed on the 4 × 150 mm column using a pH gradient. The overlay of chromatograms in Figure 31 shows the results using the biosimilar of Trastuzumab (Herceptin). The plot on the right shows the PWHH of the main peak versus the mass of protein loaded with the dashed line indicating 2× the PWHH of the lowest mass loaded. The calculated dynamic loading capacity of 0.320 mg/mL bed volume is approximately 1.5 times the loading capacity for the same mAb when using a pH gradient instead of a

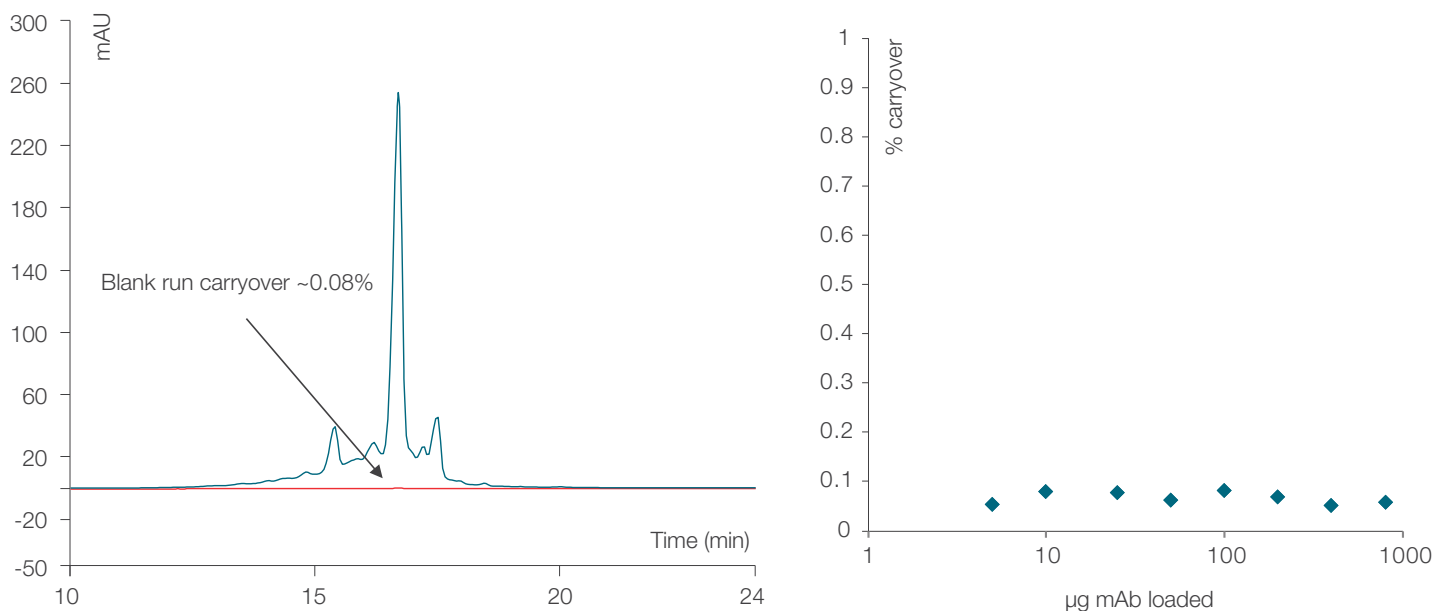
salt gradient. The higher loading capacity is due to the difference in mechanisms for pH gradient separation which is dependent on the change in mAb charge compared to the salt gradient separation method, which is dependent on salt disruption of mAb-stationary phase ionic interactions. Because of the high loading capacity when using the pH gradient method, it is not necessary to use longer columns to obtain a good separation of the mAb and its associated variants.



**Figure 31. Chromatogram showing the dynamic loading analysis of a Trastuzumab biosimilar on a 4 × 150 mm ProPac Elite WCX 5  $\mu\text{m}$  column using a pH gradient.** The plot shows the corresponding PWHH of the main mAb peak versus the masses of mAb loaded in the chromatogram.

# WCX chromatography methods (continued)

Figure 32 shows (left) the carryover for a 50  $\mu\text{g}$  injection of the Trastuzumab biosimilar and (right) the percent carryover for protein mass loading ranging from 5-800  $\mu\text{g}$ . The carryover of proteins analyzed using the pH gradient is very low with the percent carryover less than 0.2% up to 800  $\mu\text{g}$  mAb loading. The low carryover enables run-to-run protein injections without protein from previous runs interfering with sample characterization.



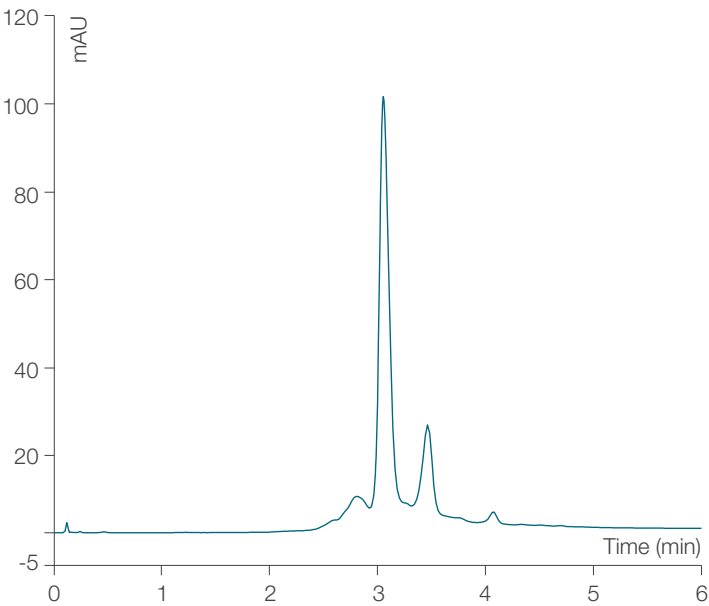
**Figure 32. (Left) overlaid chromatograms showing a 50  $\mu\text{g}$  injection and elution of a Trastuzumab biosimilar on a 4  $\times$  150 mm column using a pH gradient and the following blank run to measure carryover. (Right) A plot of the percent carryover for the mAb at loading levels ranging from 5  $\mu\text{g}$  up to 800  $\mu\text{g}$  total loading.**

# WCX chromatography methods (continued)

## Fast pH gradient analysis

The high dynamic loading capacity of the ProPac Elite WCX 5 µm columns allows the use of smaller column formats for pH gradient analysis without the risk of overloading the column resulting in poor chromatography. Figure 33 shows a fast 10 minute pH gradient separation of Secukinumab. For this method, a 2 × 50 mm column was chosen since its small column volume adds minimal gradient delay. This format is specifically designed to perform at higher pressures enabling the use of flow rates

up to 0.8 mL/min making it the preferred column format for fast method analysis. For pH methods with a starting pH greater than pH 5.6, to speed up column re-equilibration set the pH to 100% buffer A briefly at the end of the run then step the pH back to the starting gradient conditions as detailed in Figure 33. This approach returns the pH of the mobile phase to the initial conditions faster than simply stepping down from high pH to the initial pH.



| ProPac Elite WCX 5 µm column, 2 × 50 mm |                                                              |     |
|-----------------------------------------|--------------------------------------------------------------|-----|
| Cat. no.                                | 303028                                                       |     |
| Mobile phase                            | A: CX-1 pH gradient buffer A<br>B: CX-1 pH gradient buffer B |     |
| Flow rate                               | 0.8 mL/min                                                   |     |
| Inj. volume                             | 2 µL                                                         |     |
| Temperature                             | 30 °C                                                        |     |
| Detection                               | UV, 280 nm                                                   |     |
| Analytes                                | Secukinumab, 5 mg/mL                                         |     |
| Gradient                                | Time (min)                                                   | %A  |
|                                         | -0.2                                                         | 78  |
|                                         | 0.0                                                          | 78  |
|                                         | 5.0                                                          | 53  |
|                                         | 6.0                                                          | 53  |
|                                         | 6.1                                                          | 100 |
|                                         | 7.0                                                          | 100 |
|                                         | 7.1                                                          | 78  |
|                                         | 10.0                                                         | 78  |
|                                         |                                                              | %B  |
|                                         |                                                              | 22  |
|                                         |                                                              | 22  |
|                                         |                                                              | 47  |
|                                         |                                                              | 47  |
|                                         |                                                              | 0   |
|                                         |                                                              | 0   |
|                                         |                                                              | 22  |
|                                         |                                                              | 22  |

Figure 33. Fast 10 minute analysis of Secukinumab on a 2 × 50 mm column

# WCX chromatography methods (continued)

## Monoclonal antibody analysis

### Salt gradient analysis of mAb variants

#### Analysis of IgG1 mAbs and biosimilars

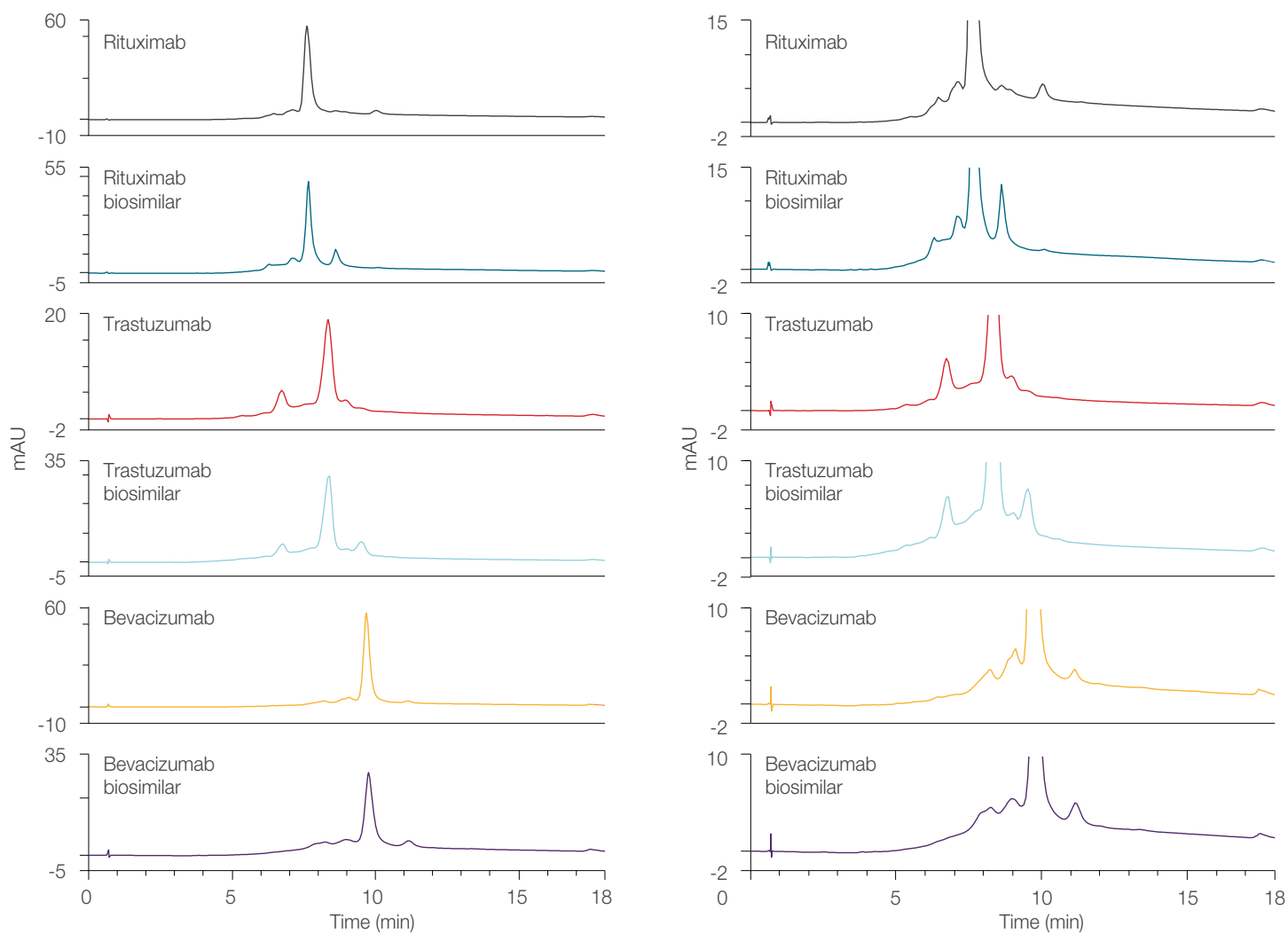
Using the salt gradient approaches discussed above to determine non-isocratic elution loading conditions and a step gradient to minimize analysis time, methods were developed for the IgG1 mAbs Rituximab\*, Trastuzumab\*, Bevacizumab\*, Infliximab, Vedolizumab, Secukinumab, and Pertuzumab (\*and biosimilars). For simplicity, the temperature and mobile phase buffer were set to 30 °C and 20 mM MES pH 6.5. For some mAbs separation of variants may be improved by further optimizing the buffer pH or column temperature. The general gradient used is described below with the loading, initial and final %B gradient values provided in Table 7. Figure 34 shows the comparison of mAbs with available biosimilars using the same gradient method to directly compare the innovator mAb to the biosimilar. Figure 35 shows the analysis of additional mAbs using the same approach. In all cases, separation of acidic and basic variants is achieved as shown in the enlarged view on the right. These methods also enable direct comparison of innovator and biosimilar mAbs to demonstrate the structural differences in the final product.

| General mAb gradient for ProPac Elite WCX 5 µm column, 4 × 150 mm |                                                            |              |
|-------------------------------------------------------------------|------------------------------------------------------------|--------------|
| Cat. no.                                                          | 302972                                                     |              |
| Mobile phase                                                      | A: 20 mM MES, pH 6.5<br>B: 20 mM MES + 500 mM NaCl, pH 6.5 |              |
| Flow rate                                                         | 1 mL/min                                                   |              |
| Temperature                                                       | 30 °C                                                      |              |
| Gradient                                                          | Time (min)                                                 | %B (Table 6) |
|                                                                   | 0.0                                                        | Loading      |
|                                                                   | 0.8                                                        | Loading      |
|                                                                   | 1.0                                                        | Initial      |
|                                                                   | 16.0                                                       | Final        |
|                                                                   | 16.1                                                       | 50           |
|                                                                   | 18.0                                                       | 50           |
|                                                                   | 18.1                                                       | Loading      |
|                                                                   | 25.0                                                       | Loading      |

**Table 7. Gradient parameters for analysis of pharmaceutical mAbs using a general salt gradient**

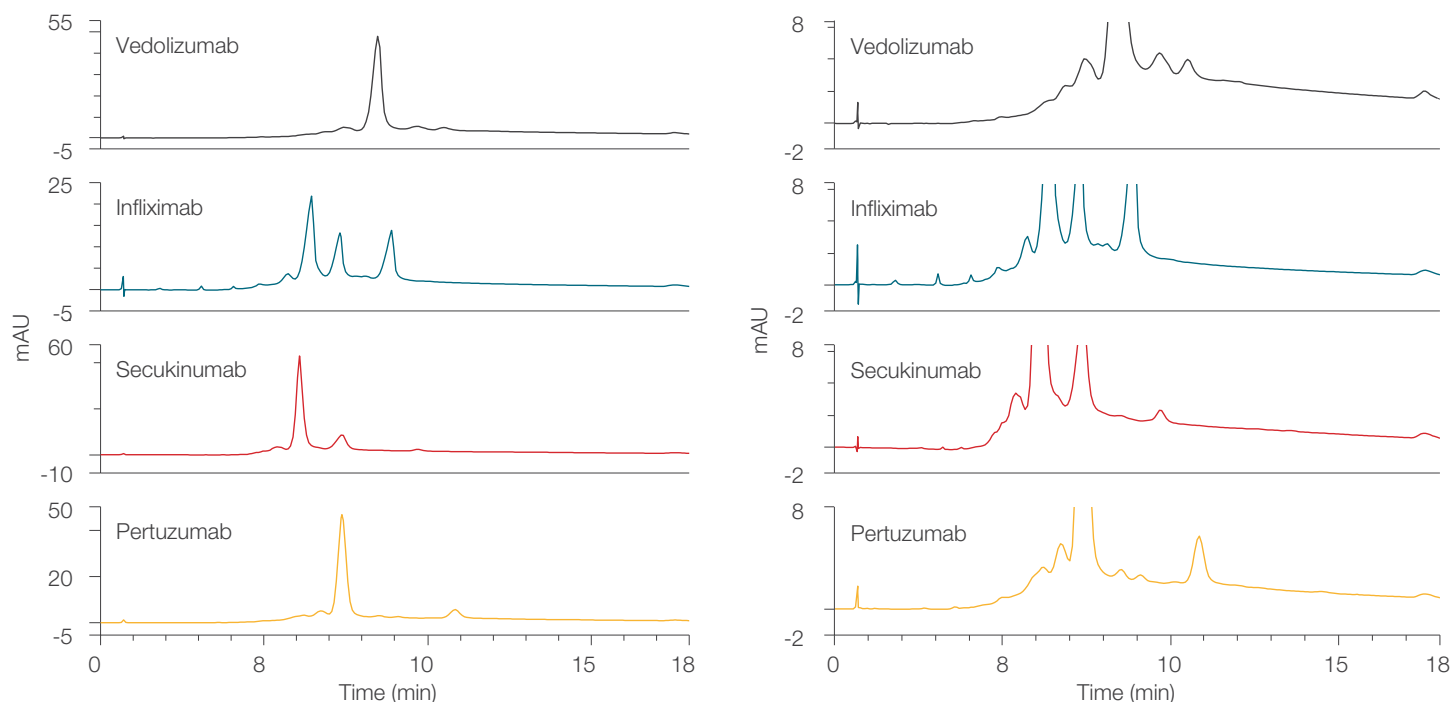
| mAb                    | %B loading | %B initial | %B final | $\Delta B/\Delta t$ %/min | Minutes reduced |
|------------------------|------------|------------|----------|---------------------------|-----------------|
| Rituximab              | 8          | 13         | 22       | 0.60                      | 8.3             |
| Rituximab biosimilar   | 8          | 13         | 22       | 0.60                      | 8.3             |
| Trastuzumab            | 9          | 12         | 20       | 0.53                      | 5.7             |
| Trastuzumab biosimilar | 9          | 12         | 20       | 0.53                      | 5.7             |
| Bevacizumab            | 8          | 10         | 17       | 0.47                      | 4.3             |
| Bevacizumab biosimilar | 8          | 10         | 17       | 0.47                      | 4.3             |
| Vedolizumab            | 5          | 7          | 14       | 0.47                      | 4.3             |
| Infliximab             | 4          | 8          | 18       | 0.67                      | 6.0             |
| Secukinumab            | 5          | 10         | 16       | 0.40                      | 12.5            |
| Pertuzumab             | 9          | 12         | 20       | 0.53                      | 5.7             |

# WCX chromatography methods (continued)



**Figure 34. Analysis of innovator mAbs and respective biosimilars on a 4 × 150 mm column using the general mAb gradient detailed in Table 7.** Left chromatograms show the full signal range with right chromatograms showing a detailed view of the mAb variants.

# WCX chromatography methods (continued)



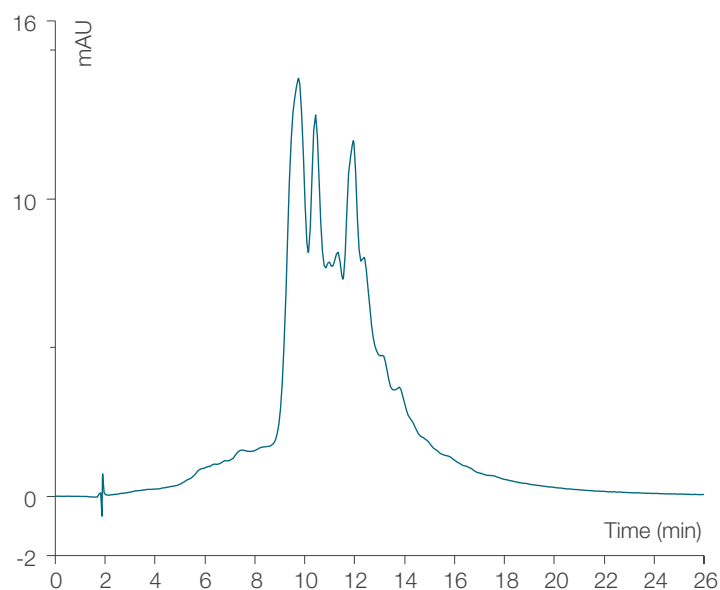
**Figure 35. Analysis of mAbs on a 4 x 150 mm ProPac Elite WCX 5 µm column using the general mAb gradient for 4 x 150 mm detailed in Table 7. Left chromatograms show the full signal range with right chromatograms showing a detailed view of the mAb variants.**

## IgG2 and IgG4 mAb analysis

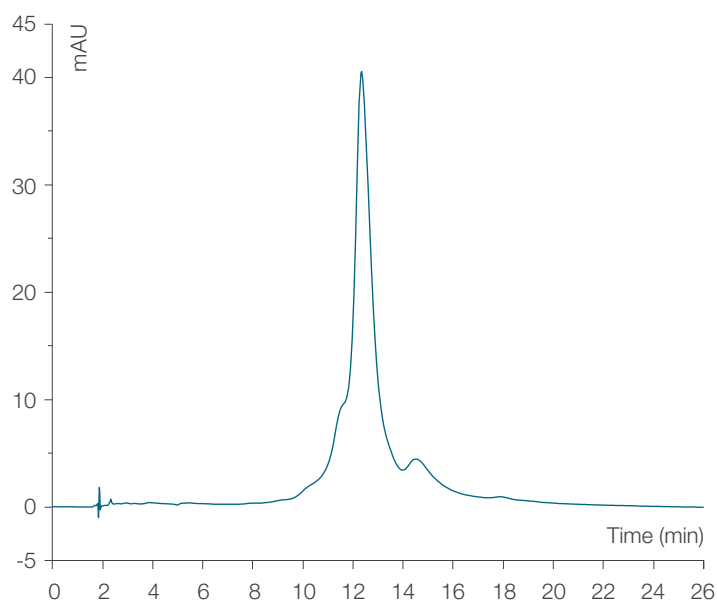
The IgG2 and IgG4 antibody subclasses are capable of targeting antigens but do not activate antibody-dependent cell mediated cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC) common to IgG1 and IgG3 subclasses. For this reason, IgG2 and IgG4 are chosen when the goal is to neutralize soluble antigens without triggering effector responses. Compared to IgG1 and IgG3, these antibodies have different in vivo properties and structural features that need to be considered when developing therapeutic mAbs. Both have a shorter hinge length and different exposed residues on the constant domains compared to the IgG1 subclass. IgG2 has four disulfide bonds

while IgG1 and IgG4 have two. IgG2 can form covalent dimers between identical or different IgG2 molecules with intermolecular disulfide bonds whereas IgG4 has the capability to form a half-molecule with intra-chain disulfide bonds. These properties present a greater challenge for characterization of structural and charge heterogeneity necessary for quality control of the therapeutic mAb products. Charge heterogeneity of two IgG2s and one IgG4 therapeutic mAbs – Panitumumab, Denosumab and Nivolumab – was analyzed using a 4 x 250 mm ProPac Elite WCX 5 µm column and are shown below in Figures 36 and 37.

# WCX chromatography methods (continued)



| ProPac Elite WCX 5 $\mu$ m column, 4 $\times$ 250 mm |                                                          |    |     |
|------------------------------------------------------|----------------------------------------------------------|----|-----|
| Cat. no.                                             | <a href="#">303025</a>                                   |    |     |
| Mobile phase                                         | A: 20 mM MES, pH 6.5<br>B: 20 mM MES, 0.5 M NaCl, pH 6.5 |    |     |
| Flow rate                                            | 0.6 mL/min                                               |    |     |
| Inj. volume                                          | 5 $\mu$ L                                                |    |     |
| Temperature                                          | 30 $^{\circ}$ C                                          |    |     |
| Detection                                            | UV, 280 nm                                               |    |     |
| Analytes                                             | IgG2 denosumab, 5 mg/mL                                  |    |     |
|                                                      | Time (min)                                               | %A | %B  |
|                                                      | 0.0                                                      | 83 | 17  |
|                                                      | 1.0                                                      | 83 | 17  |
| Gradient                                             | 26.0                                                     | 78 | 22  |
|                                                      | 26.1                                                     | 0  | 100 |
|                                                      | 28.0                                                     | 0  | 100 |
|                                                      | 28.1                                                     | 83 | 17  |
|                                                      | 40.0                                                     | 83 | 17  |



| ProPac Elite WCX 5 $\mu$ m column, 4 $\times$ 250 mm |                                                          |    |     |
|------------------------------------------------------|----------------------------------------------------------|----|-----|
| Cat. no.                                             | <a href="#">303025</a>                                   |    |     |
| Mobile phase                                         | A: 20 mM MES, pH 6.5<br>B: 20 mM MES, 0.5 M NaCl, pH 6.5 |    |     |
| Flow rate                                            | 0.6 mL/min                                               |    |     |
| Inj. volume                                          | 5 $\mu$ L                                                |    |     |
| Temperature                                          | 30 $^{\circ}$ C                                          |    |     |
| Detection                                            | UV, 280 nm                                               |    |     |
| Analytes                                             | IgG2 panitumumab, 5 mg/mL                                |    |     |
|                                                      | Time (min)                                               | %A | %B  |
|                                                      | 0.0                                                      | 83 | 17  |
|                                                      | 1.0                                                      | 83 | 17  |
| Gradient                                             | 26.0                                                     | 78 | 22  |
|                                                      | 26.1                                                     | 0  | 100 |
|                                                      | 28.0                                                     | 0  | 100 |
|                                                      | 28.1                                                     | 83 | 17  |
|                                                      | 40.0                                                     | 83 | 17  |

Figure 36. Analysis of IgG2 mAbs Denosumab and Panitumumab on a 4  $\times$  250 mm ProPac Elite WCX 5  $\mu$ m column

# WCX chromatography methods (continued)

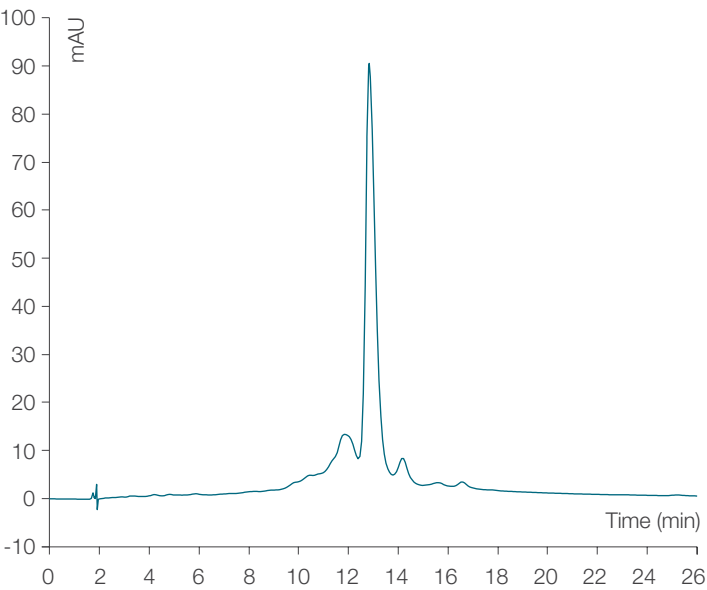


Figure 37. Analysis of the IgG4 mAb Nivolumumab on a 4 × 250 mm ProPac Elite WCX 5 µm column

| ProPac Elite WCX 5 µm column, 4 × 250 mm |                                                          |            |
|------------------------------------------|----------------------------------------------------------|------------|
| Cat. no.                                 | 303025                                                   |            |
| Mobile phase                             | A: 20 mM MES, pH 6.5<br>B: 20 mM MES, 0.5 M NaCl, pH 6.5 |            |
| Flow rate                                | 0.6 mL/min                                               |            |
| Inj. volume                              | 5 µL                                                     |            |
| Temperature                              | 30 °C                                                    |            |
| Detection                                | UV, 280 nm                                               |            |
| Analytes                                 | IgG4 Nivolumab, 5 mg/mL                                  |            |
| Gradient                                 | Time (min)                                               | %A      %B |
|                                          | 0.0                                                      | 90      10 |
|                                          | 1.0                                                      | 90      10 |
|                                          | 26.0                                                     | 85      15 |
|                                          | 26.1                                                     | 0      100 |
|                                          | 28.0                                                     | 0      100 |
|                                          | 28.1                                                     | 90      10 |
|                                          | 40.0                                                     | 90      10 |

The pI of Denosumab and Nivolumab have been reported to be 8.9 and 8.0 respectively, while Panitumumab has a relatively lower pI of 6.8. Due to the pI of the proteins panitumumab was analyzed at pH 5.6 whereas denosumab and nivolumab were analyzed at pH 6.5 in order to ensure loading of the mAbs and associated variants for subsequent separation using a salt gradient.

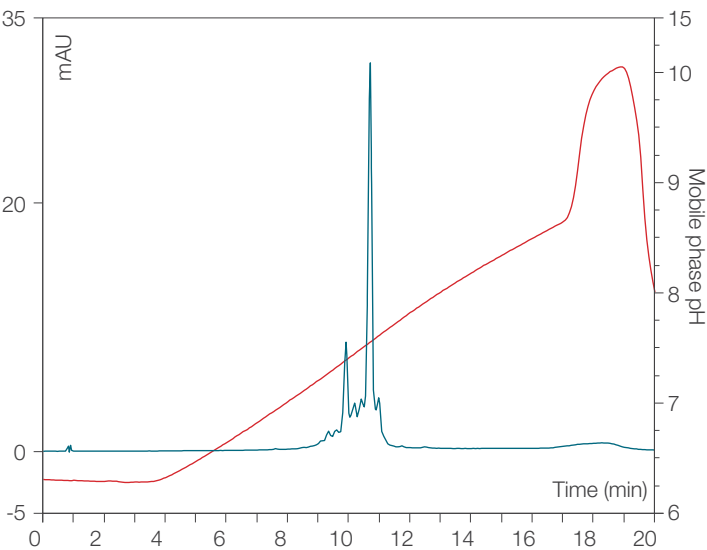
The column was able to separate more than 15 variants of panitumumab. The multiple peaks observed may be a combination of different structural isoforms and charge variants. These results demonstrate the high resolving power of the ProPac Elite WCX column is suitable for analysis of IgG2 and IgG4 mAbs.

# WCX chromatography methods (continued)

## pH gradient analysis of mAb variants

### Analysis of IgG1 mAbs using an optimized gradient

A gradient covering a broad pH range is used when first evaluating the analysis of a mAb and its charge variants. Figure 38 shows the analysis of Trastuzumab using a gradient from pH 6.52 to 8.82 (20 to 70 %B) on a 4 × 150 mm ProPac Elite WCX 5 µm column.



**Figure 38. pH Gradient analysis of IgG1 Trastuzumab on a 4 × 150 mm ProPac Elite WCX 5 µm column using a wide range gradient**

The above approach in Figure 38 was applied to a panel of mAbs to determine gradient conditions optimized for each mAb using a 4 × 150 mm column. The mAbs evaluated, the initial and final gradient values for %B of CX-1 pH gradient buffers, and the %B elution values are provided in Table 8. The standard gradient conditions are given below. Figure 39 shows the analysis of Rituximab, Trastuzumab, and Bevacizumab and their associated biosimilars using the gradient conditions above and detailed in Table 8. Figure 40 shows the analysis of the additional mAbs Infliximab, Secukinimab, Pertuzumab, and Vedoluzimab.

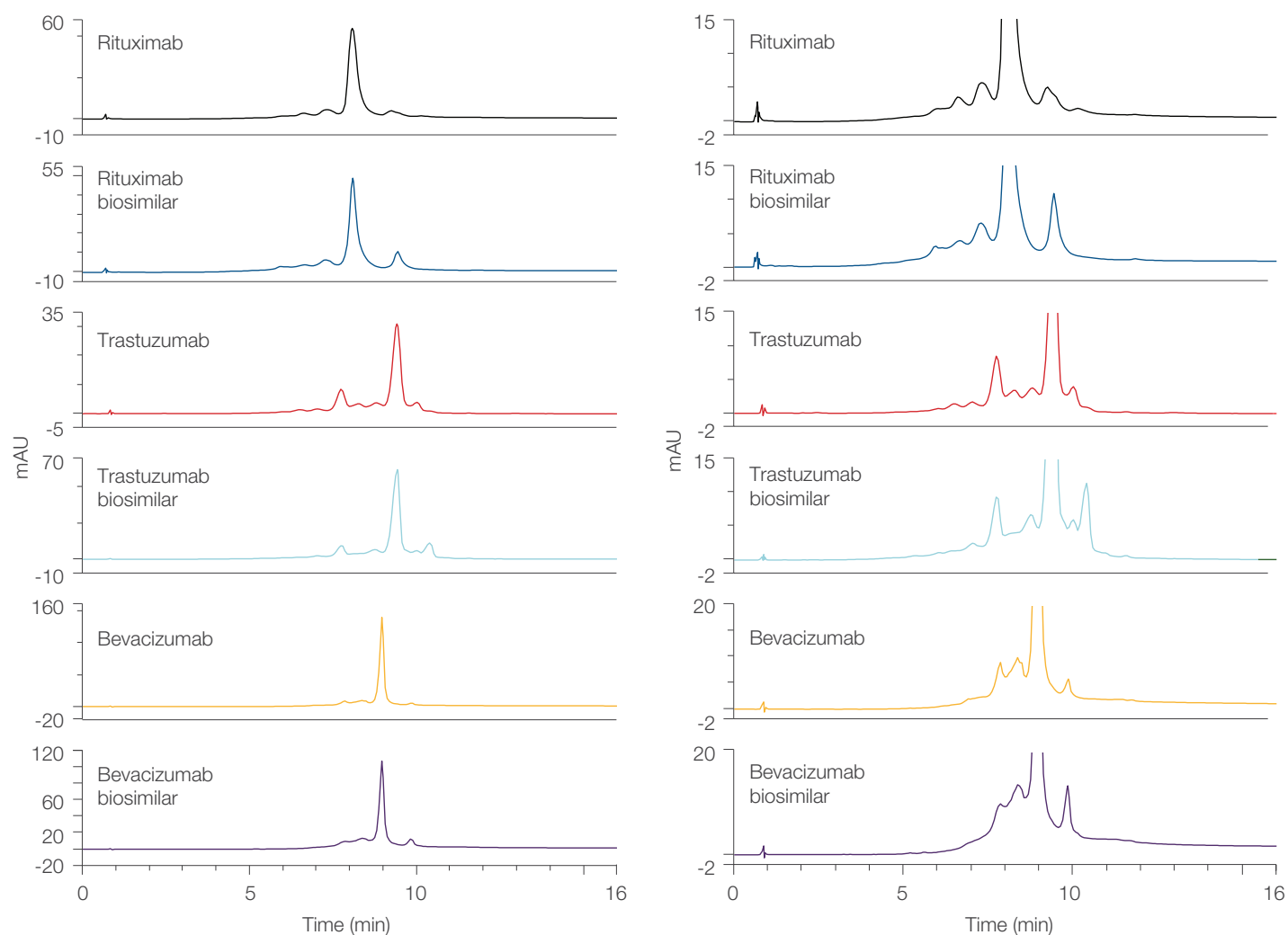
The red trace indicates the pH corresponding to elution of the variant and main peaks and can be used to determine appropriate initial and final pH gradient conditions for optimized separations of the mAb and charge variants.

| General mAb pH gradient for ProPac Elite WCX 5 µm column, 4 × 150 mm |                                                              |              |
|----------------------------------------------------------------------|--------------------------------------------------------------|--------------|
| Cat. no.                                                             | 302972                                                       |              |
| Mobile phase                                                         | A: CX-1 pH gradient buffer A<br>B: CX-1 pH gradient buffer B |              |
| Flow rate                                                            | 0.8 mL/min                                                   |              |
| Inj. volume                                                          | 4 µL                                                         |              |
| Temperature                                                          | 30 °C                                                        |              |
| Gradient                                                             | Time (min)                                                   | %B (Table 7) |
|                                                                      | 0.0                                                          | Initial      |
|                                                                      | 15.0                                                         | Final        |
|                                                                      | 15.1                                                         | 100          |
|                                                                      | 17.0                                                         | 100          |
|                                                                      | 17.1                                                         | Initial      |
|                                                                      | 25.0                                                         | Initial      |

# WCX chromatography methods (continued)

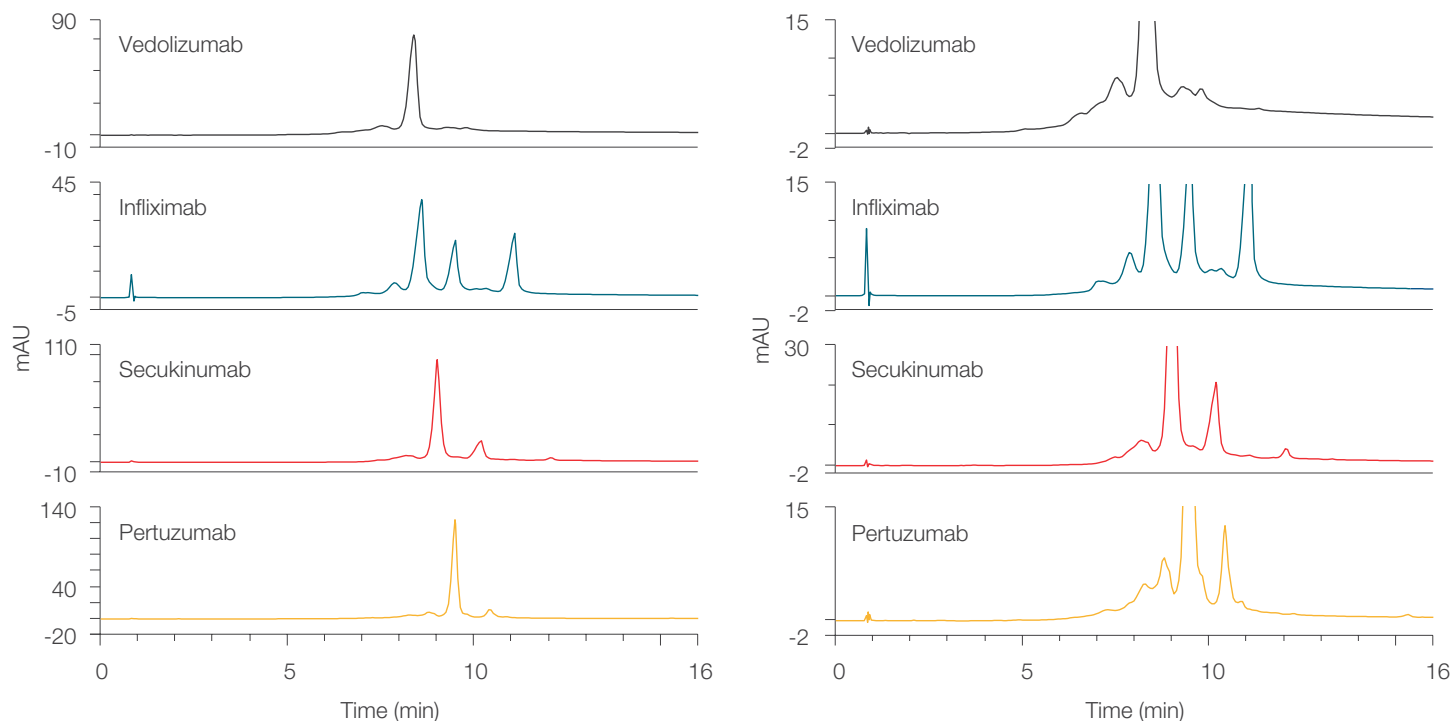
**Table 8. Gradient parameters for analysis of pharmaceutical mAbs using a pH gradient**

| mAb                        | %B initial | %B final | Ret. time (min) | Elution %B* |
|----------------------------|------------|----------|-----------------|-------------|
| Rituximab and biosimilar   | 43         | 68       | 11.94           | 56.6        |
| Trastuzumab and biosimilar | 37         | 62       | 10.32           | 51.0        |
| Bevacizumab and biosimilar | 27         | 52       | 7.06            | 40.9        |
| Vedolizumab                | 24         | 49       | 6.12            | 38.0        |
| Infliximab                 | 22         | 47       | 5.45            | 35.7        |
| Secukinumab                | 28         | 53       | 7.55            | 42.3        |
| Pertuzumab                 | 35         | 60       | 9.72            | 49.5        |



**Figure 39: Analysis of innovator mAbs and respective biosimilars on a 4 x 150 mm column using the general pH gradient detailed in Table 8.** Left chromatograms show the full signal range and right chromatograms showing a detailed view of the mAb variants.

# WCX chromatography methods (continued)



**Figure 40: Analysis of mAbs on a 4 x 150 mm column using the general mAb gradient for 4 x 150 mm detailed in Table 8.** Left chromatograms show the full signal range with right chromatograms showing a detailed view of the mAb variants.

Figure 39 demonstrates the utility of using the pH gradient approach to directly compare the innovator and biosimilar products. Inspection of the right hand enlarged scale chromatograms shows differences in the acidic and basic variant profile for innovators and their respective biosimilars when using the same pH gradient. These structural differences between the products may have adverse or beneficial effects on the performance of the drug and must be accounted for during development and manufacturing. Figure 40 illustrates the broader utility of the pH gradient approach for additional mAbs. The enlarged scale on the right shows good separation of both acidic and basic variants.

# WCX chromatography methods (continued)

## Fast pH gradient analysis

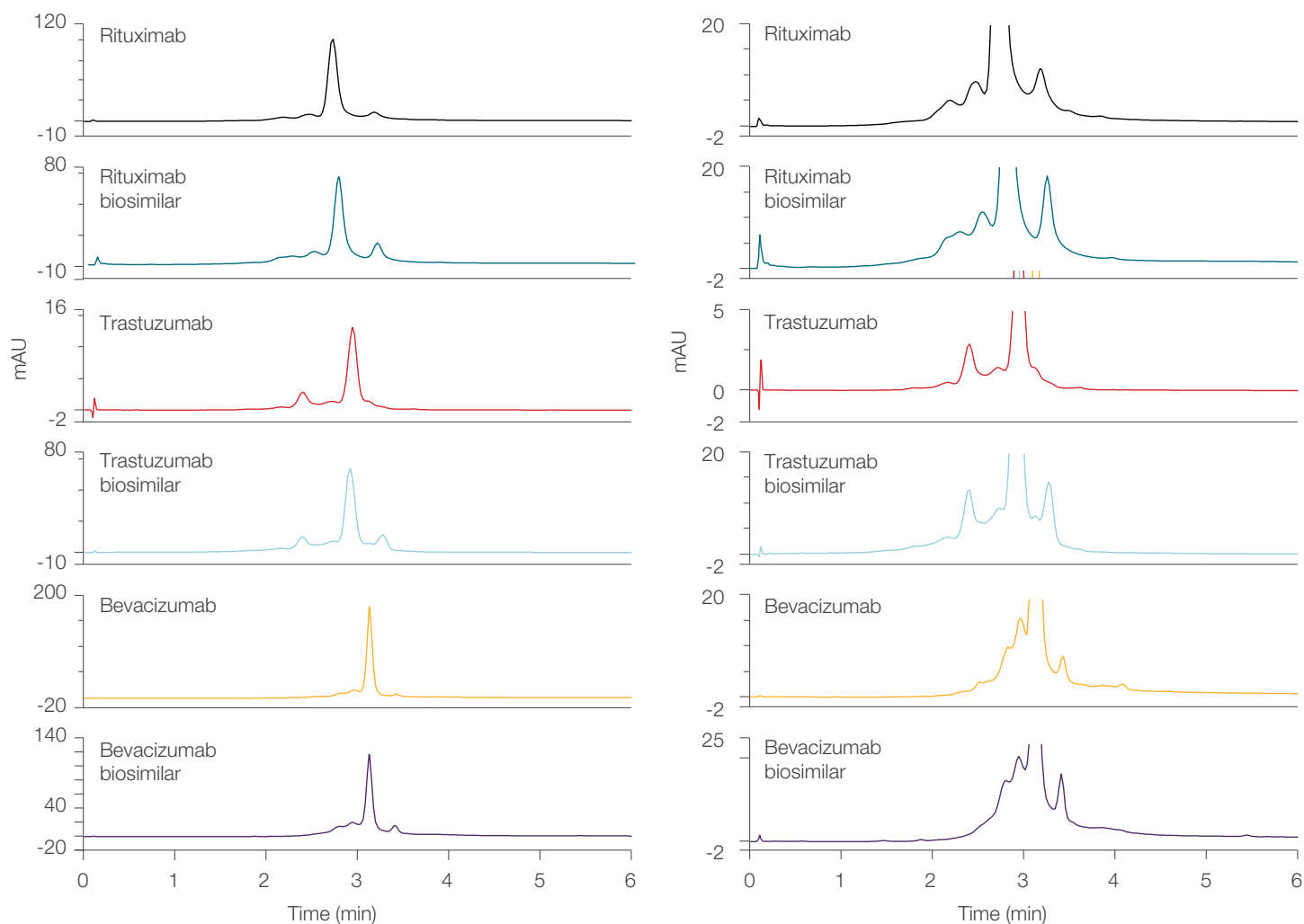
The general approach applied above for the 4 × 150 mm column was used with the 2 × 50 mm column format and a high flow rate of 0.8 mL/min to achieve variant analysis in a minimal amount of time of only 10 minutes using the general gradient below. The mAbs evaluated, the initial and final gradient values for %B of CX-1 pH gradient buffers, and the %B elution values are provided in Table 9.

| General mAb gradient for ProPac Elite WCX 5 µm column, 2 × 50 mm |                                                              |              |
|------------------------------------------------------------------|--------------------------------------------------------------|--------------|
| Cat. no.                                                         | 303028                                                       |              |
| Mobile phase                                                     | A: CX-1 pH gradient buffer A<br>B: CX-1 pH gradient buffer B |              |
| Flow rate                                                        | 0.8 mL/min                                                   |              |
| Inj. volume                                                      | 2 µL                                                         |              |
| Temperature                                                      | 30 °C                                                        |              |
| Gradient                                                         | Time (min)                                                   | %B (Table 8) |
|                                                                  | 0.0                                                          | Initial      |
|                                                                  | 5.0                                                          | Final        |
|                                                                  | 6.0                                                          | Final        |
|                                                                  | 6.1                                                          | 0            |
|                                                                  | 7.0                                                          | 0            |
|                                                                  | 7.1                                                          | Initial      |
|                                                                  | 10.0                                                         | Initial      |

**Table 9. Gradient parameters for fast analysis of pharmaceutical mAbs using a pH gradient**

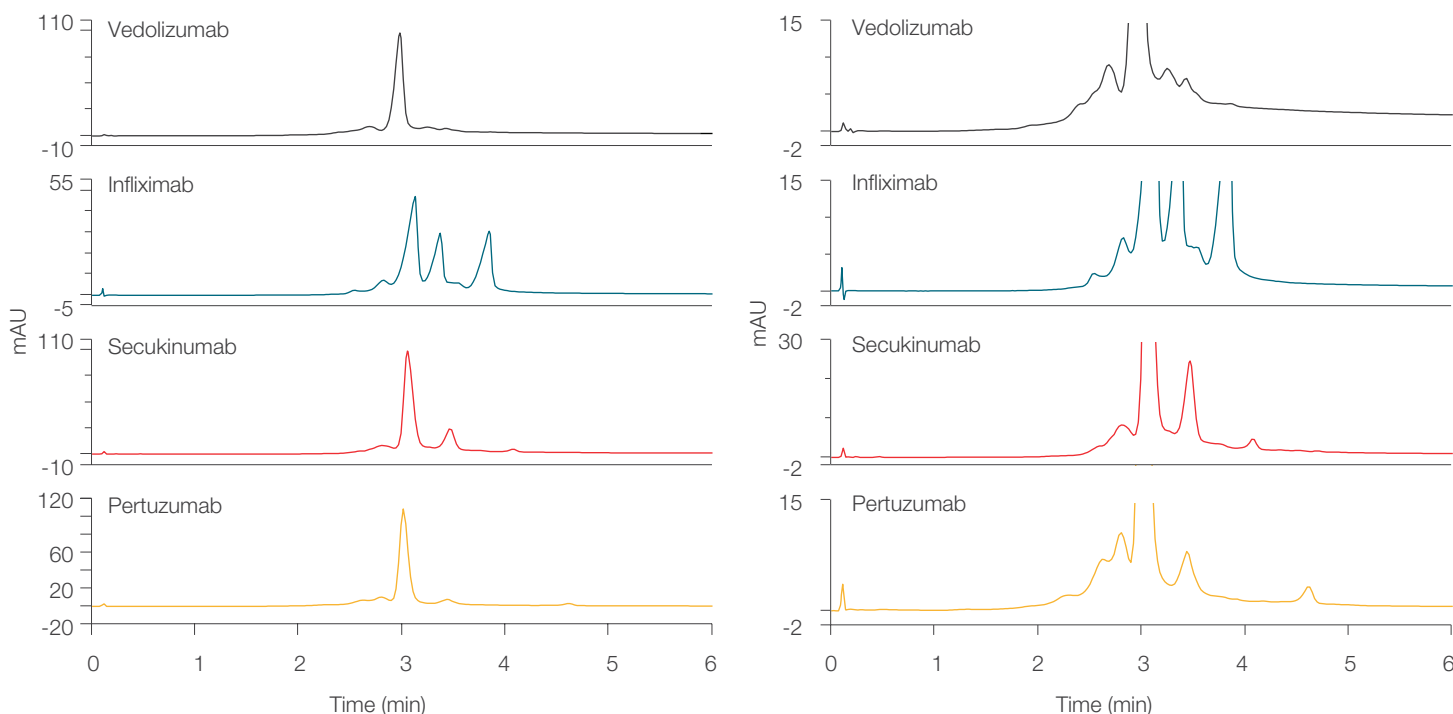
| mAb                        | %B initial | %B final | Ret. time (min) | Elution %B* |
|----------------------------|------------|----------|-----------------|-------------|
| Rituximab and biosimilar   | 37         | 62       | 3.85            | 51.5        |
| Trastuzumab and biosimilar | 32         | 57       | 3.40            | 47.0        |
| Bevacizumab and biosimilar | 22         | 47       | 2.42            | 37.2        |
| Vedolizumab                | 18         | 43       | 1.99            | 32.9        |
| Infliximab                 | 17         | 42       | 1.93            | 32.3        |
| Pertuzumab                 | 30         | 55       | 3.21            | 45.1        |
| Secukinumab                | 22         | 47       | 2.45            | 47.5        |

# WCX chromatography methods (continued)



**Figure 41. Fast gradient analysis of innovator IgG1 mAbs and respective biosimilars on a 2 × 50 mm column using the fast pH gradient detailed in Table 9. Left chromatograms show the full signal range and right chromatograms showing a detailed view of the mAb variants.**

# WCX chromatography methods (continued)



**Figure 42. Fast gradient analysis of additional IgG1 mAbs on a 2 × 50 mm column using the general pH gradient detailed in Table 9.** Left chromatograms show the full signal range with right chromatograms showing a detailed view of the mAb variants.

Analysis on the 2 × 50 mm column achieves good separation of both acidic and basic variants allowing direct comparison of the innovator and biosimilar mAbs shown in Figure 41.

The same performance is also observed for the additional mAbs Vedolizumab, Infliximab, Secukinumab, and Pertuzumab in Figure 42. The enlarged scale chromatograms on the right side of Figures 41 and 42 show that only a small decrease in resolution occurs when going from the 4 × 150 mm format to the 2 × 50 mm format; however, the total analysis time decreases from 25 minutes to only 10 minutes. These results demonstrate the utility of using high flow rates with the 2 × 50 mm column to achieve fast separations for high throughput analysis.

# Ordering information

## ProPac Elite WCX 5 µm columns

| Description                                                                 | Quantity | Cat. no                |
|-----------------------------------------------------------------------------|----------|------------------------|
| ProPac Elite WCX Column, 5 µm, 2 × 50 mm                                    | Each     | <a href="#">303028</a> |
| ProPac Elite WCX Column, 5 µm, 2 × 150 mm                                   | Each     | <a href="#">303027</a> |
| ProPac Elite WCX Column, 5 µm, 2 × 250 mm                                   | Each     | <a href="#">303026</a> |
| ProPac Elite WCX Column, 5 µm, 4 × 50 mm                                    | Each     | <a href="#">302973</a> |
| ProPac Elite WCX Column, 5 µm, 4 × 150 mm                                   | Each     | <a href="#">302972</a> |
| ProPac Elite WCX Column, 5 µm, 4 × 250 mm                                   | Each     | <a href="#">303025</a> |
| ProPac Elite WCX Column, 5 µm, 9 × 150 mm                                   | Each     | <a href="#">303355</a> |
| ProPac Elite WCX Analytical Column, 5 µm, 4 × 250 mm, 3 Columns from 1 Lot  | Each     | <a href="#">303061</a> |
| ProPac Elite WCX Analytical Column, 5 µm, 4 × 250 mm, 3 Columns from 3 Lots | Each     | <a href="#">303062</a> |
| ProPac Elite WCX Analytical Column, 5 µm, 4 × 150 mm, 3 Columns from 1 Lot  | Each     | <a href="#">302976</a> |
| ProPac Elite WCX Analytical Column, 5 µm, 4 × 150 mm, 3 Columns from 3 Lots | Each     | <a href="#">302977</a> |

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