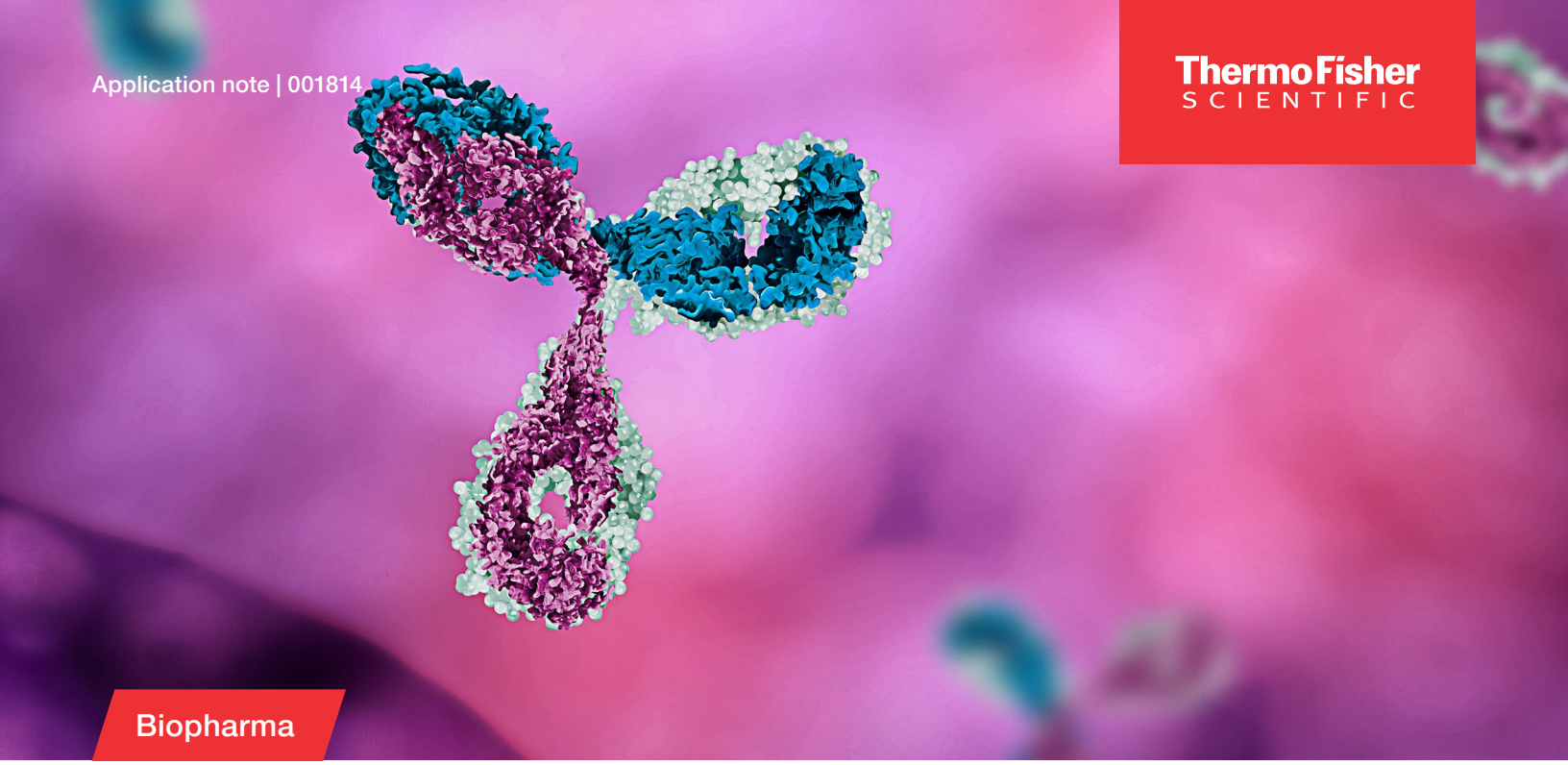
Application note | 001814



Biopharma

Method development for pH gradient analysis of monoclonal antibodies using a 3 µm monodisperse particle strong cation exchange chromatography column

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Keywords

Biopharma, strong cation exchange, SCX, monoclonal antibody, pH gradient, CX-1 pH gradient buffer, ProPac 3R SCX 3µm column, monodisperse, high performance liquid chromatography, NISTmAb, Infliximab, Pertuzumab, Vedolizumab, Secukinumab

Application benefits

- The combination of the Thermo Scientific™ ProPac™ 3R SCX 3µm 2 × 50 mm column and Thermo Scientific™ CX-1 pH gradient buffers provide a high-resolution, fast, easy to optimize, and reproducible platform method for charge variant characterization of mAbs
- Consistent lot-to-lot performance

Goal

Development of high-resolution methods for the analysis of monoclonal antibodies using CX-1 pH gradient buffers with a ProPac 3R SCX 3µm 2 × 50 mm column

Introduction

Monoclonal antibodies (mAbs) are a preferred class of protein therapeutics used for the treatment of various diseases because of their ability to target specific tissues for drug delivery or the modulation of cellular activities. Cellular production and downstream manufacturing processes commonly introduce heterogeneity to the mAb structure by way of post-translational or chemical modifications that can have potential effects on product efficacy, safety, and stability. As such, thorough characterization of mAbs is required to fulfill regulatory requirements to bring new therapeutics to market.

Common modifications to the mAb structure, including lysine truncation, asparagine deamidation, and glycosylation, alter the charge of the biomolecule by addition or elimination of cationic or anionic sites resulting in increased charge heterogeneity. Ion exchange (IEX) chromatography separates components based on their charge and is a standard technique for analyzing monoclonal antibodies and associated variants. Salt gradients are often used for IEX analysis; however, pH gradient methods have been used as an alternative approach for separating proteins from their associated variants based on the isoelectric point (pI, pH at which the analyte charge is neutral) of each analyte. When using a pH gradient, the cationic protein is adsorbed to the stationary phase at low pH conditions followed by a gradient of increasing mobile phase pH. As the pH of the buffer increases, the charge of the protein shifts from cationic to neutral 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. To easily facilitate these types of separations, Thermo Scientific CX-1 pH gradient buffers generate a linear pH gradient that simplifies method optimization for high resolution separations of monoclonal antibodies and their charge variants. To achieve the best separations with these buffers, an appropriate cation exchange column must be used.

For the work presented here, the Thermo Scientific ProPac 3R SCX column was selected based on its ability to achieve high-efficiency protein separations. The packing material is based on a monodisperse 3 µm, nonporous divinylbenzene polymer particle to provide exceptionally high resolving power. A thin, hydrophilic layer is grafted to the particle to reduce secondary interactions of protein samples with the hydrophobic core. This paired with precisely controlled grafting of SCX functionality minimizes band broadening for maximal resolution. The column chemistry has been developed to be compatible with the CX-1 pH gradient buffers. The reproducible resin chemistry and manufacturing processes eliminate column variability as a concern in method development and data analysis. Combined, these design choices make the ProPac 3R SCX column capable of analyzing complex proteins with high resolution and excellent reproducibility.

In this application note, practical examples of method design are shown and discussed for the development of an optimized method for NISTmAb and associated variants using the ProPac 3R SCX 2 × 50 mm column in combination with CX-1 pH gradient buffers. Having established a clear protocol for creating robust chromatography methods, we apply this approach to four additional mAb therapeutics and present the final optimized methods.

Experimental

Reagents and consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Thermo Scientific™ CX-1 Buffer A, 500 mL (P/N 302779)
- Thermo Scientific™ CX-1 Buffer B, 500 mL (P/N 302780)
- NISTmAb (NIST, 8671)
- Thermo Scientific™ SureSTART™ 2 mL Polypropylene Screw Top Microvials (P/N 6ESV9-04PP)
- Thermo Scientific™ SureSTART™ 2 mL Screw Caps (P/N 6ASC9ST1)

Sample preparation

All mAb samples except NISTmAb were diluted to 5 mg/mL using DI water. NISTmAb was used as received from the supplier at 10 mg/mL.

Separation conditions

10× CX-1 gradient buffers were diluted to 1× concentration using deionized water. For mobile phase compositions and gradient conditions including flow rate and injection volume, reference the text and figures in the results and discussion section. Absorbance at 280 nm was used for detection of all samples.

Instrumentation

- Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system, including:
  - System Base Vanquish Flex (P/N VF-S01-A)
  - Quaternary Pump (P/N VF-P20-A) with a 75 µL static mixer (P/N 6044.5100)
  - Column Compartment H (P/N VH-C10-A)
  - Split Sampler FT (P/N VF-A10-A) with 25 µL (V = 50 µL) sample loop
  - Diode Array Detector FG (P/N VF-D11-A) with Thermo Scientific™ Vanquish™ Flow cell (P/N 6083.0550)
  - Thermo Scientific™ UltiMate™ PCM-3000 pH and Conductivity Monitor connected using a Thermo Scientific™ UltiMate™ VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) (used for pH measurement only)

Column

- ProPac 3R SCX 3 µm, 2 × 50 mm (P/N 43103-052068)

Data processing

Thermo Scientific™ Chromeleon™ 7.2.10 Chromatography Data System (CDS) was used for data acquisition and analysis.

Results and discussion

Optimization of chromatographic parameters is critical for designing methods that provide high resolution separations with good reproducibility for proteins. For the work presented here, we use a 2 × 50 mm ProPac 3R SCX column for two reasons: 1) the narrow i.d. will provide good detection sensitivity with low mass loading, and 2) the short column length minimizes method time for analysis. Using this column, we demonstrate a straightforward approach to develop and optimize a fast 5-minute pH gradient using the CX-1 pH gradient buffer. The full optimization approach is first applied to the separation of NISTmAb using a linear gradient ranging from 100% Buffer A to 100% Buffer B (pH 5.6–10.2) to evaluate where NISTmAb and its associated variants elute. The gradient is then optimized by adjusting the pH range over which the separation occurs followed by evaluating the gradient time and flow rate to produce a finalized method. The loading capacity is also investigated to demonstrate the change in separation observed at different mass loading levels. This process was repeated, and the final methods were provided for four other therapeutically relevant mAbs. For all method development, a 75 µL static mixer was used with the pump to minimize system volume and gradient delay.

Determining gradient starting conditions

The first step to method design is to determine the initial or loading pH to bind the sample and all associated variants. To determine the loading pH, an initial gradient method covering a broad pH range was used to evaluate at which points in the gradient NISTmAb and its charge variants elute. Figure 2 shows the analysis of NISTmAb using a gradient from pH 5.6 to 10.2 (0% to 100% B) over 5 minutes on a 2 × 50 mm column.

This rapid change in pH does not provide any meaningful separation of the acidic and basic variants for NISTmAb; however, fine tuning of the initial and final pH for the gradient can provide an optimized separation of the mAb. The gradient times in Figure 2 are used for all subsequent analyses with the initial gradient composition (at time points -0.2, 0.0, 7.1, and 14) and final gradient composition (at time points 5.0 and 6.0) adjusted as indicated in each figure. For each gradient, the mobile phase is set to 100% A (pH 5.6) from 6.1 to 7.0 minutes to re-equilibrate the phase faster after completion of the run.

The starting pH for the gradient must be sufficiently lower than the pI of the acidic variants to promote binding of the sample. Figure 3 illustrates the effect on NISTmAb and associated variant separation on a 2 × 50 mm column using a simple pH gradient. The mAb is loaded at 45, 50, and 55% B (pH 7.67, 7.90, and 8.13, respectively) and eluted using an increase of 10% B over 5 minutes. When loading at pH 8.13, NISTmAb elutes closer to the void at 1.15 minutes with relatively poor resolution of the proximal acidic peak (see middle chromatogram for acidic/basic peak labeling) indicating the starting pH is too high for effective binding and separation. Comparison of the separation when loading at 45% B and 50% B (pH 7.67 and 7.90, respectively) shows the relative separation of the proximal variants are consistent; however, the latent basic peaks were not observed to elute when using the gradient from pH 7.67 to 8.13. For this reason, the gradient from 50% to 60% B (pH 7.90 to 8.36) was chosen for further optimization. These results demonstrate the importance of determining the loading and final gradient pH to ensure both acidic and basic peaks are eluted within the gradient.

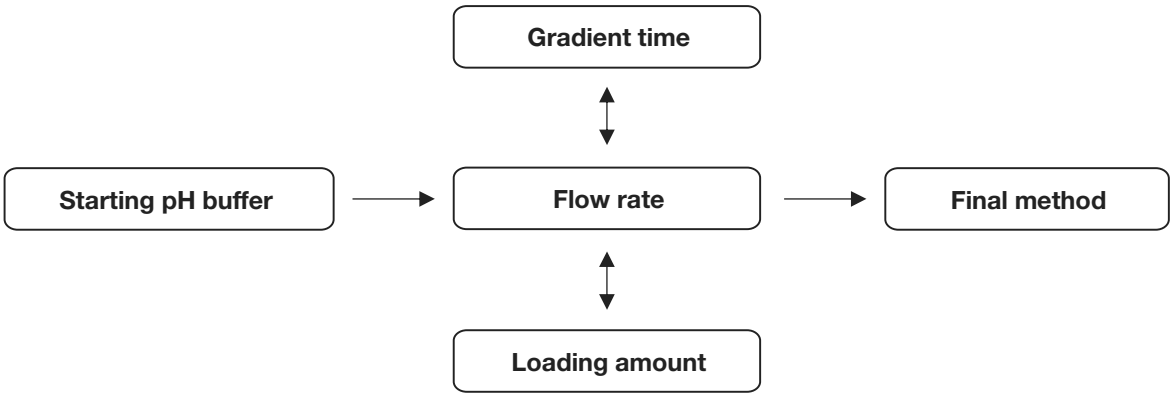
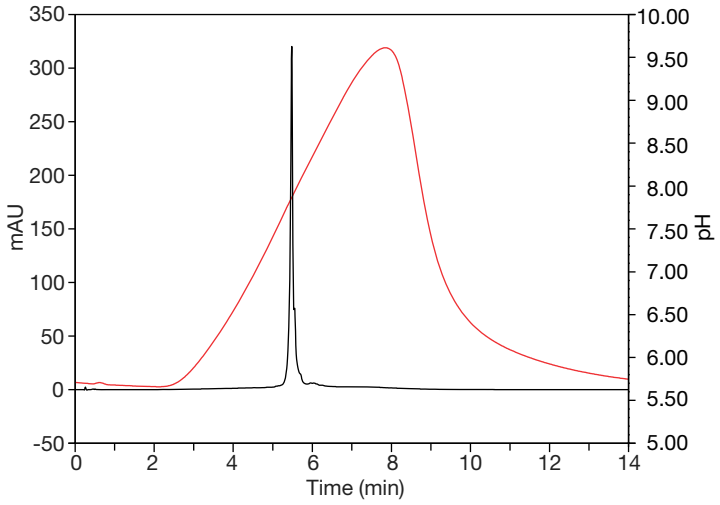


Figure 1. Method development flow chart



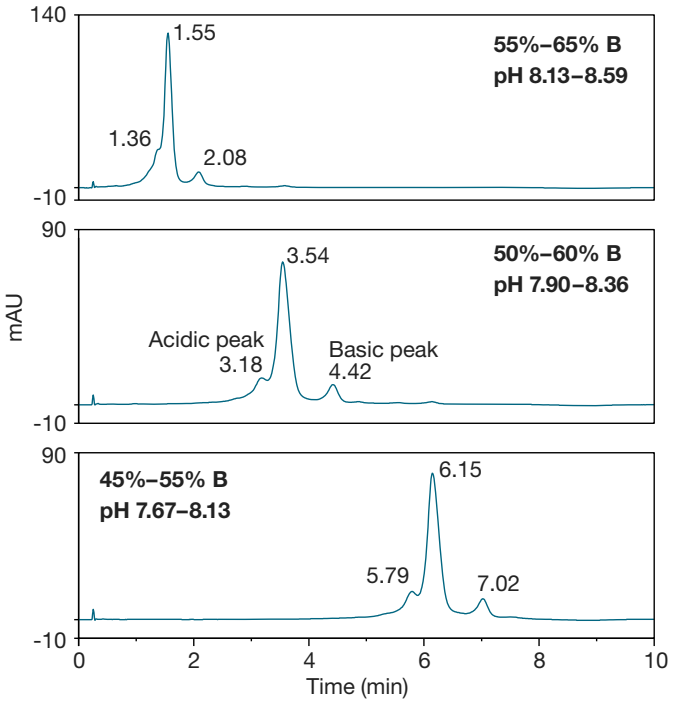
Column:	ProPac 3R SCX, 3µm
Format:	2 × 50 mm
Mobile phase buffer:	Buffer A: 1x, CX-1 Buffer A pH 5.6 Buffer B: 1x, CX-1 Buffer B pH 10.2
Flow rate:	0.3 mL/min

Gradient:

Time (min)	%A	%B
-0.2	100	0
0.0	100	0
5.0	0	100
6.0	0	100
6.1	100	0
7.0	100	0
7.1	100	0
14.0	100	0

Temperature:	30 °C
Detection:	280 nm
Inj. volume:	1 µL
Sample:	NISTmAb 10 mg/mL

Figure 2. pH gradient analysis of NISTmAb and associated variants on a 2 × 50 mm ProPac 3R SCX column using a gradient over the total buffer pH range, pH 5.6–10.2. The red trace indicates mobile phase pH (right axis) as measured by the PCM-3000 pH and conductivity monitor.



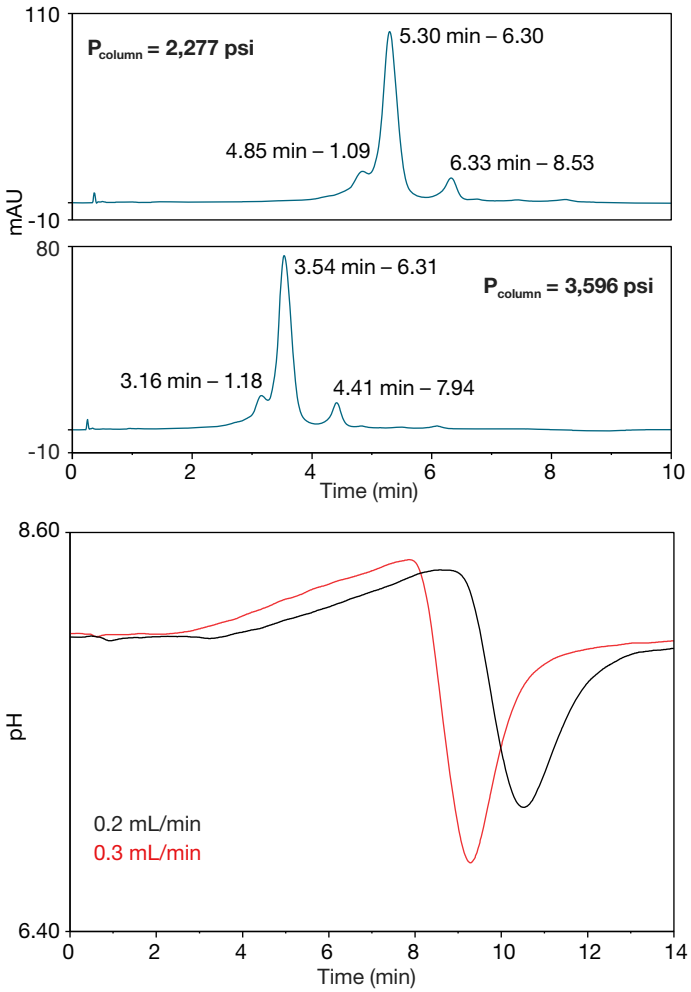
Column:	ProPac 3R SCX, 3µm
Format:	2 × 50 mm
Mobile phase buffer:	Buffer A: 1x, CX-1 Buffer A pH 5.6 Buffer B: 1x, CX-1 Buffer B pH 10.2
Flow rate:	0.3 mL/min
Gradient:	See chromatogram for change in %B over 5 minutes
Temperature:	30 °C
Detection:	280 nm
Inj. volume:	1 µL
Sample:	NISTmAb 10 mg/mL
Peak label:	Retention time

Figure 3. Effect of gradient starting conditions on NISTmAb and associated variants separation

Flow rate effect on pH gradient separation

Figure 4 shows the analysis of NISTmAb at a flow rate of 0.2 and 0.3 mL/minute using a gradient from pH 7.90–8.36 over 5 minutes. 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 from the main protein peak of the acidic peak (left) increases slightly while that of the basic peaks (right) decrease. For NISTmAb, the increased peak-to-valley ratio of the proximal acidic peak provides greater resolution at 0.3 mL/min. The basic peaks have slightly better retention time separation at lower flow rates; however, the peaks are practically baseline resolved in each case. The peak width half height (PWHH) of the main protein peak decreases from 0.2 mL/min to 0.3 mL/min.

Comparison of the signal strength on the y-axis of the chromatograms in Figure 4 shows that using lower flow rates results in increased signal strength due to a higher concentration of 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. Lower flow rates also have the benefit of reduced pressure on the column. The selected flow rate to use will depend on the user's objective. In this instance, 0.3 mL/min was selected for further evaluation as it provided the best separation of the proximal acidic variant based on the peak-to-valley ratio while still operating at a safe column pressure and without significant loss to basic peak resolution. If improved basic variant separation, lower operating pressure, or improved detection sensitivity are required, the 0.2 mL/min method may be a more suitable option for the user.

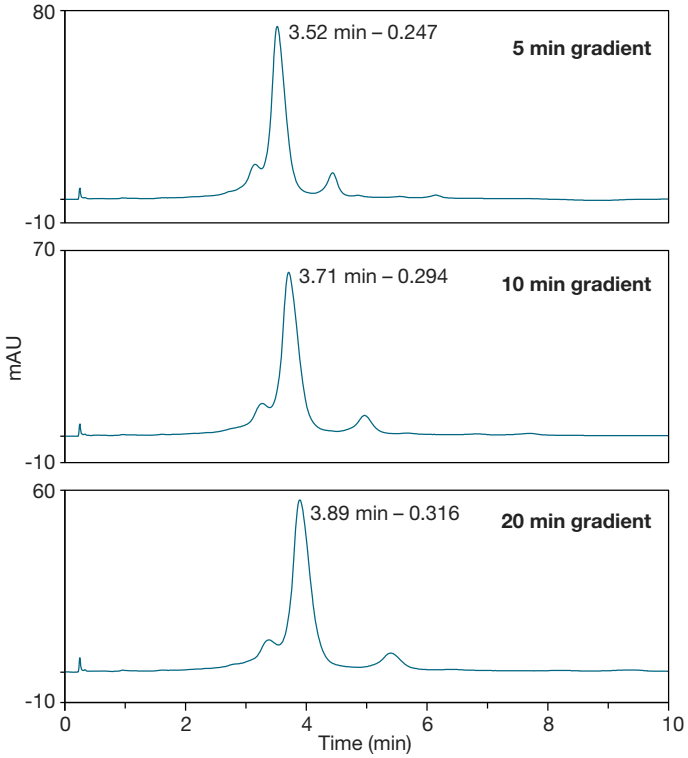


Column:	ProPac 3R SCX, 3µm
Format:	2 × 50 mm
Mobile phase buffer:	Buffer A: 1x, CX-1 Buffer A pH 5.6 Buffer B: 1x, CX-1 Buffer B pH 10.2
Flow rate:	Black – 0.2 mL/min Red – 0.3 mL/min
Gradient:	50–60% B over 5 minutes
Temperature:	30 °C
Detection:	280 nm
Inj. volume:	1 µL
Sample:	NISTmAb 10 mg/mL
Peak label:	Retention time–Peak-to-Valley ratio

Figure 4. Effect of flow rate on the separation of NISTmAb and associated charge variants (top) and associated pH traces (bottom)

Gradient time effect on pH gradient separation

After determining an appropriate flow rate, the gradient slope was evaluated to determine if the separation could be further optimized. The slope of the pH gradient influences the time required for analysis and the resolution of the different mAb variants. Figure 5 shows the analysis of NISTmAb using a gradient from 50% to 60% B over 5, 10, and 20 minutes. The relative retention time separation of all peaks is observed to increase with increasingly long gradients; however, this also adds considerable time to the method, specifically with regards to the latest eluting basic peaks which also become weaker in signal as the gradient time is extended. For this note, we are aiming to achieve fast separations; as such, the 5-minute gradient provides a fast analysis time while maintaining good separation of both acidic and basic variants. For each application, the user can tune the gradient slope/time to balance their specific needs for analysis time versus peak resolution.



Column:	ProPac 3R SCX, 3µm
Format:	2 × 50 mm
Mobile phase buffer:	Buffer A: 1x, CX-1 Buffer A pH 5.6 Buffer B: 1x, CX-1 Buffer B pH 10.2
Flow rate:	0.3 mL/min
Gradient:	50–60% B, see chromatogram for gradient time
Temperature:	30 °C
Detection:	280 nm
Inj. volume:	1 µL
Sample:	NISTmAb 10 mg/mL
Peak label:	Retention time–PWHH

Figure 5. Effect of gradient time on the separation of NISTmAb and associated variants

Sample loading and carryover analysis

For typical protein loading levels, sample concentration and injection volume do not significantly influence the separation of the protein and associated variants for a given loading mass. However, the total mass of protein loaded can have a significant effect on peak resolution. To evaluate the loading capacity of the phase using a pH gradient, dynamic loading analysis was performed on the 2 × 50 mm column as shown in Figure 6. The chromatograms show loading masses of 1–50 µg of NISTmAb. As the sample loading amount increases above 30 µg, 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 plot in Figure 6 shows the PWHH of the main peak versus the mass of protein loaded. This type of experiment is commonly referred to as dynamic loading capacity with overloading here defined as the loading mass of twice the PWHH of lowest mass loaded (indicated by the dashed line). The dynamic loading capacity is calculated as 0.171 mg/mL bed volume which is ~1.3× the loading capacity of the same mAb using a salt gradient.[1] 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 a salt gradient separation method, which is dependent on salt disruption of mAb-stationary phase ionic interactions. This higher mass loading with pH gradients may enable detection of minor peaks without loss of resolution.

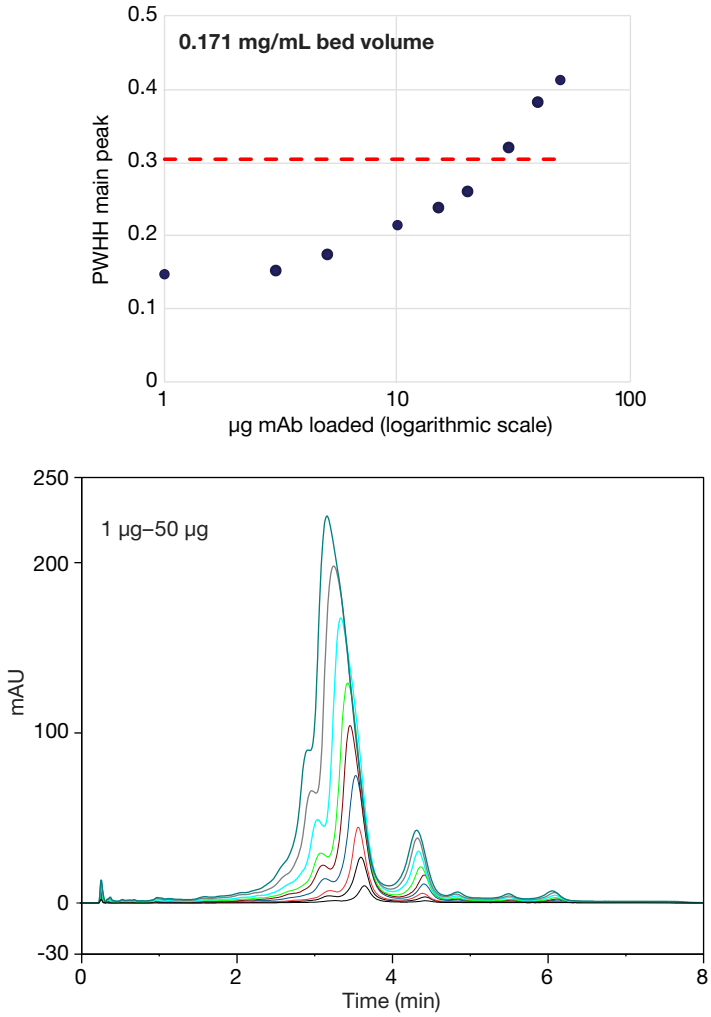


Figure 6. Chromatogram overlays showing the dynamic loading analysis of NISTmAb using the 0.3 mL/min pH gradient method in Figure 4. The plot shows the corresponding PWHH of the main mAb peak against the masses of mAb loaded in the column.

Lastly, we evaluated the ProPac 3R SCX 3 µm stationary phase for carryover at high mass loading levels. Figure 7 shows the overlaid chromatograms for a 20 µg injection run using 10 mg/mL NISTmAb and the following blank run with no injection. No carryover was detected in the blank run, demonstrating the low carryover properties of the stationary phase even at high mass loading levels, which enables consecutive protein injection runs without interference due to carryover from previous injections.

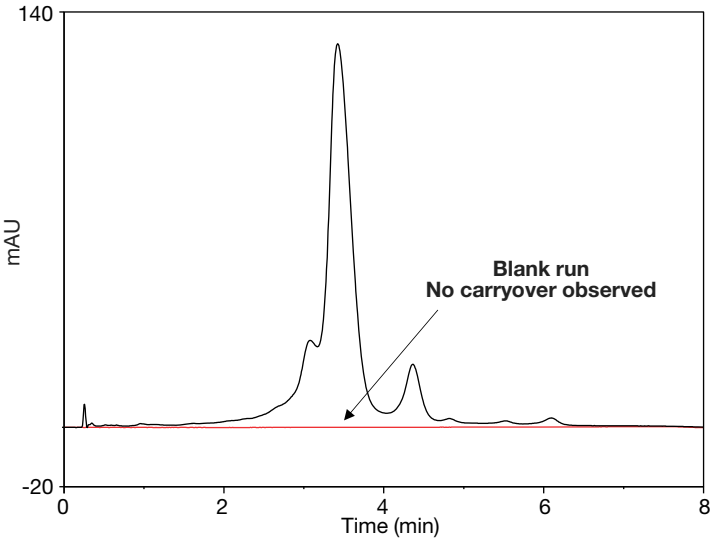
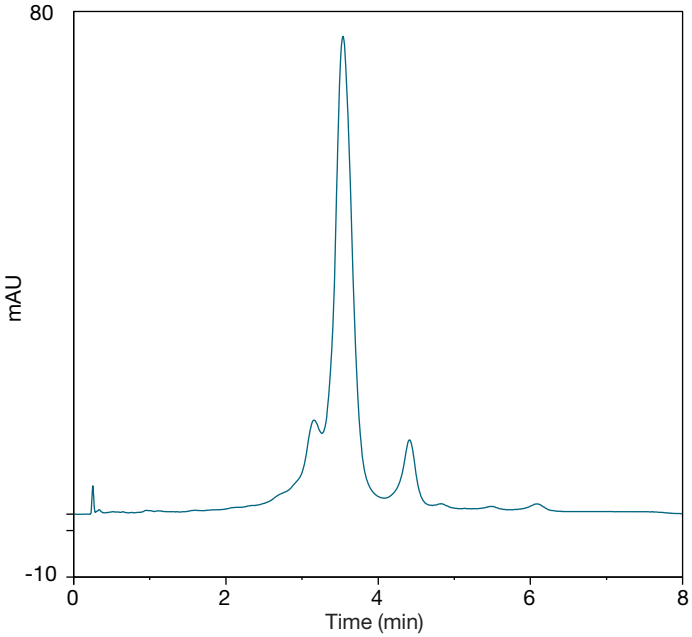


Figure 7. Overlaid chromatograms showing a 20 µg injection and elution of NISTmAb and the following blank run to measure carryover using the 0.3 mL/min pH gradient method in Figure 4

Final pH gradient method

Based on the analyses in the previous sections, we provide here in Figure 8 a final method using a 2 × 50 mm column format with a 5 min gradient at 0.3 mL/min for fast separation of mAb charge variants. The method was optimized for fast analysis by using a low volume pump static mixer (75 µL) on the system and a high flow rate (0.3 mL/min). The high flow rate reduces gradient delay and column re-equilibration time while the fast 5-minute gradient time elutes the protein and associated variants over a short time-period. With this optimized method, the high resolution and capacity of the ProPac 3R SCX column provides narrow peaks with sufficient retention time separation to detect the variants associated with NISTmAb.



Column:	ProPac 3R SCX, 3µm
Format:	2 × 50 mm
Mobile phase buffer:	Buffer A: 1x, CX-1 Buffer A pH 5.6 Buffer B: 1x, CX-1 Buffer B pH 10.2
Flow rate:	0.3 mL/min

Gradient:

Time (min)	%A	%B
-0.2	50	50
0.0	50	50
5.0	40	60
6.0	40	60
6.1	100	0
7.0	100	0
7.1	50	50
14.0	50	50

Temperature:	30 °C
Detection:	280 nm
Inj. volume:	1 µL
Sample:	NISTmAb 10 mg/mL

Figure 8. Zoomed in chromatogram of NISTmAb analysis using 5-minute gradient at 0.3 mL/min flow rate

Using the optimized 5-minute gradient method shown in Figure 8, we compared the performance of three different lots of media to evaluate the column-to-column and lot-to-lot reproducibility of the ProPac 3R SCX columns. Figure 9 shows excellent reproducibility observed in this analysis. The ProPac 3R technology platform made using monodisperse particles and precision-controlled chemistry makes this possible, giving the user confidence in their separation with different lots of media.

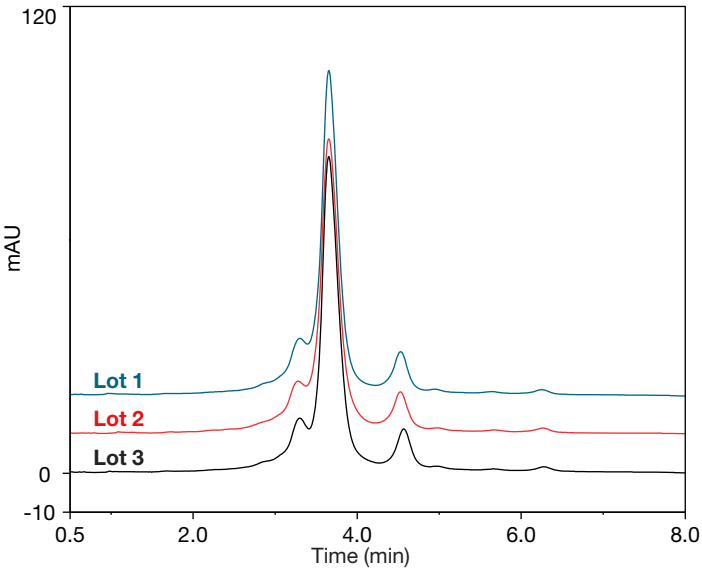


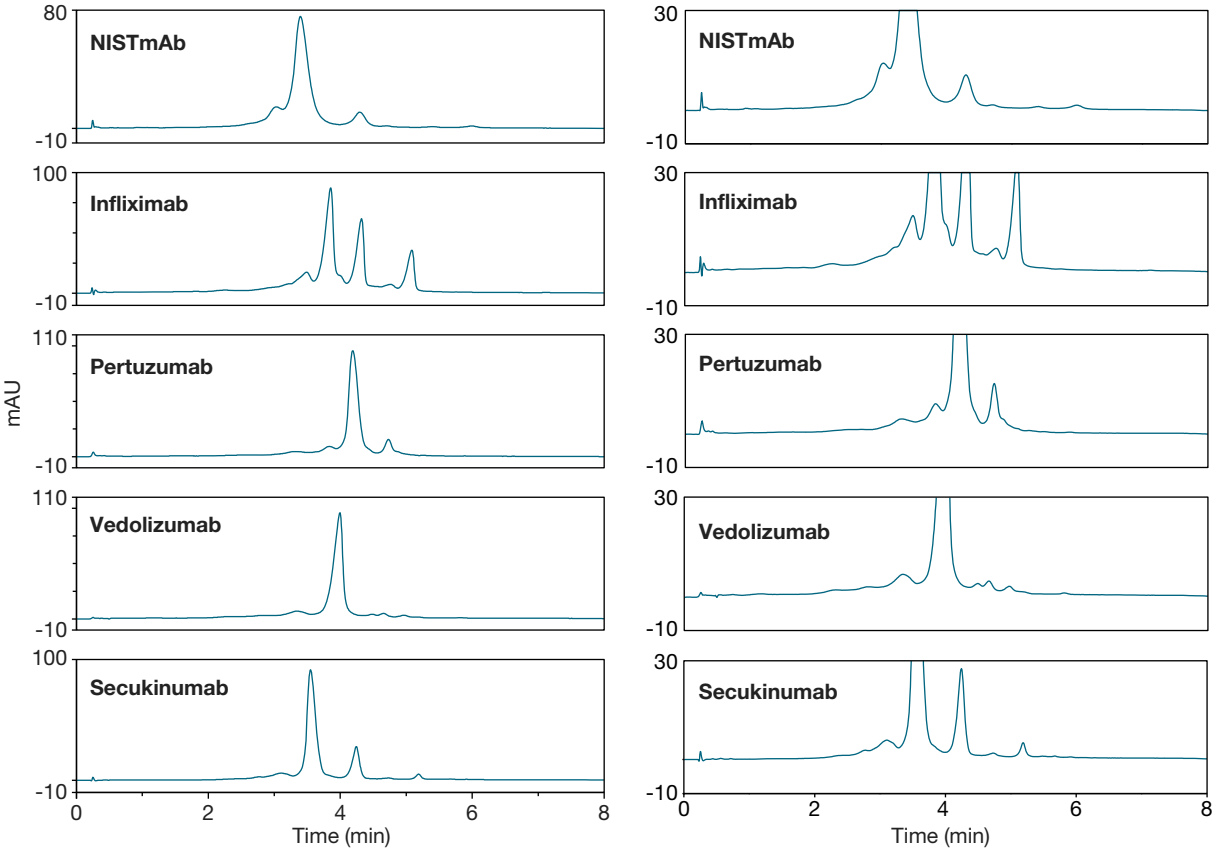
Figure 9. Zoomed-in view of chromatograms of three different lots using optimized method with a 5-minute gradient at 0.3 mL/min flow rate. Retention time of main mAb peak is normalized to aid comparison of variant separation.

Optimized methods for additional mAbs

The process discussed above for pH gradient optimization was applied to four additional mAbs. Each method was optimized for mobile phase composition and sample loading using a flow rate of 0.3 mL/min to achieve fast variant analysis with a total method time of 14 minutes using a linear pH gradient. For each mAb evaluated, initial and final gradient values for %B of CX-1 pH gradient buffers are provided in Table 1. Figure 10 demonstrates excellent mAb-variant separation for each mAb using the ProPac 3R SCX 2 × 50 mm column with a simple pH gradient over 5 minutes using CX-1 Gradient Buffers at 30 °C.

Table 1. Gradient parameters for analysis of mAbs using pH gradient

mAb	Initial %B	Final %B	Concentration	Inj. vol. (µL)
NIST	50	60	10 mg/mL	1.0
Infliximab	24	44	5 mg/mL	3.0
Pertuzumab	36	51	5 mg/mL	1.5
Vedolizumab	23	38	5 mg/mL	1.5
Secukinumab	27	47	5 mg/mL	1.5



General mAb gradient for 2 × 50 mm

Eluents: A: 1X, CX-1 Buffer A pH 5.6
B: 1X, CX-1 Buffer B pH 10.2

Flow rate: 0.3 mL/min
Temperature: 30 °C

Gradient:

Time (min)	%B (Table 1)
-0.2	Initial
0.0	Initial
5.0	Final
6.0	Final
6.1	0
7.0	0
7.1	Initial
14.0	Initial

Figure 10. Analysis of mAbs using pH gradient detailed in Table 1. Left chromatograms show the full signal strength, and the right chromatograms show the zoomed-in detailed view of the mAb variants.

Conclusion

- The combination of ProPac 3R SCX 3µm columns and CX-1 gradient buffers provide fast, reproducible, and excellent separation for monoclonal antibodies and associated charge variants with low carryover.
- Starting pH concentration, flow rate, and gradient time can be optimized to provide consistent, robust, and high-resolution variant separation.

Reference

1. Olivares, R.; Bechler, S., Thermo Fisher Scientific Application Note 001694: Salt gradient analysis of monoclonal antibodies using a 3 µm monodisperse SCX chromatography column, 2023.

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