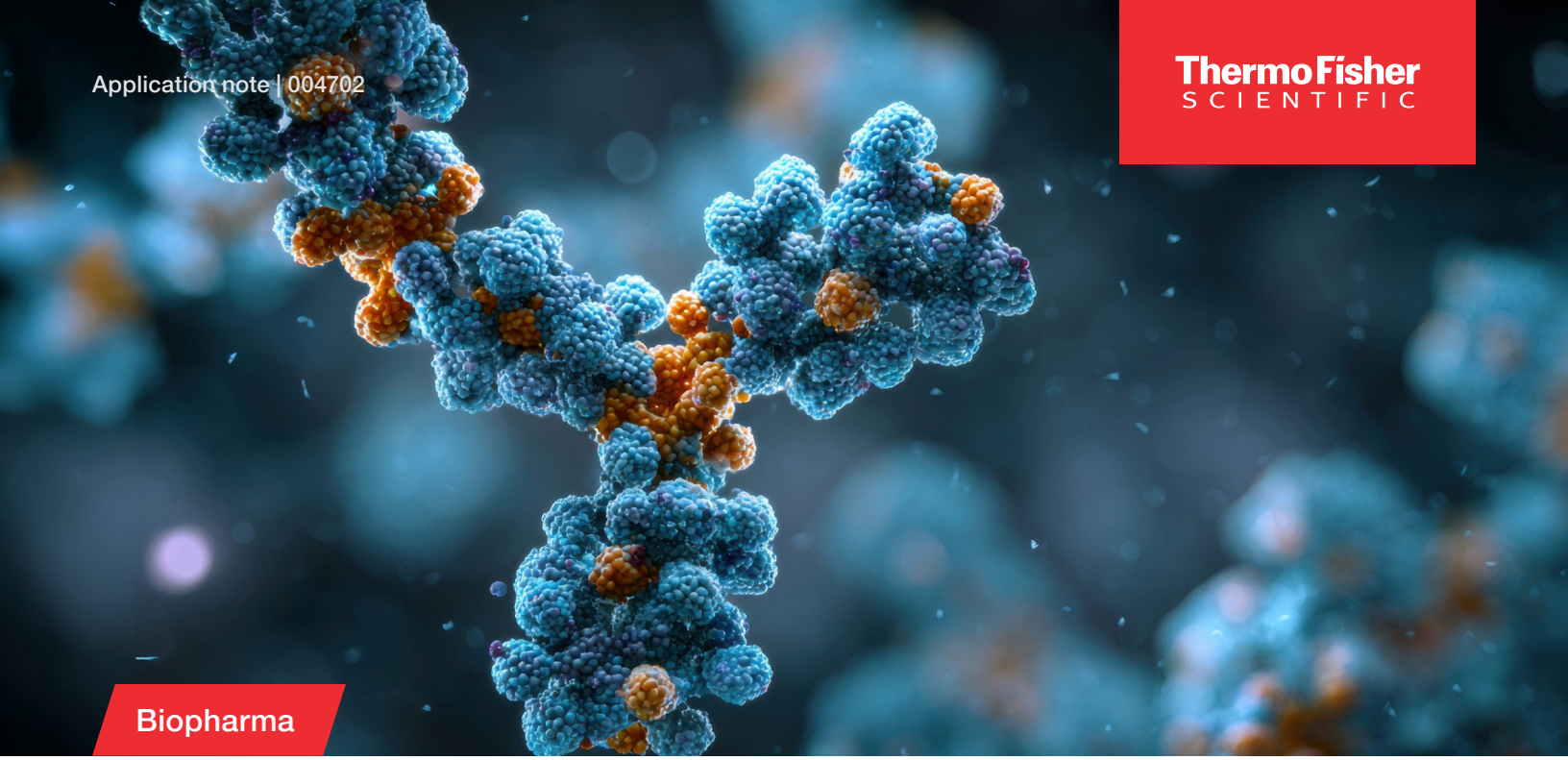


Method development for intact monoclonal antibody separation using a reversed phase supermacroporous particle column

Author

Romina Mae Olivares,
Thermo Fisher Scientific, Sunnyvale, CA

Keywords

Monoclonal antibody, mAb, IgG1, IgG2, IgG4, protein, reversed phase, monodisperse, inert, NISTmAb, secukinumab, pertuzumab, Avastin™, biosimilar, nivolumab, denosumab, HPLC, liquid chromatography, biopharma, SurePac Protein RP MDi Column

Application benefits

- High-resolution reversed phase protein separations including monoclonal antibodies and therapeutics
- Easy straightforward method development

Goal

Demonstrate basic reversed phase method development for high-resolution analysis of monoclonal antibodies using a 2.1 × 50 mm, 2.5 µm monodisperse, supermacroporous particle RP column

Introduction

Monoclonal antibodies (mAbs) represent one of the most important classes of biotherapeutics in modern medicine. Throughout biopharmaceutical development and manufacturing, comprehensive analytical characterization of intact mAbs is essential to ensure product quality, safety, and regulatory compliance. Critical quality attributes (CQAs), including hydrophobic variants, aggregation, truncation, and post-translational modifications, must be accurately detected, resolved, and quantified. High-resolution protein separation methods are therefore foundational tools in early development, process optimization, lot release testing, and stability assessment.

Reversed-phase liquid chromatography (RP-LC) is widely used for intact mAb analysis due to its ability to separate proteins based on hydrophobicity while maintaining compatibility with UV detection and mass spectrometry (MS). When properly optimized, RP-LC provides sharp peak shapes, reproducible retention, and effective resolution of low-level variants. However, intact antibody separations require columns that can withstand elevated temperatures, support efficient mass transfer for large biomolecules, and deliver consistent lot-to-lot performance.

Thermo Scientific™ SurePac™ Protein RP MDi™ Columns are designed to meet these requirements. They are packed with supermacroporous, 2.5 µm monodisperse polymer particles with a uniform particle size distribution and a broad pore size range. The pore structure provides accessible surface area for the separation of low and high molecular weight biomolecules, such as antibody fragments and intact monoclonal antibodies. The polymeric nature of the SurePac Protein RP MDi Column media provides both pH and temperature stability, making it an ideal column for mAb analysis.

In this application note, we demonstrate a systematic method development approach for reversed-phase separation of intact monoclonal antibodies using a 2.1 x 50 mm SurePac Protein RP MDi Column. Separation is highlighted by achieving high resolution of variants with varying hydrophobicity and ensuring robust, reproducible performance suitable for biopharmaceutical development and quality control workflows. NISTmAb is used as a model protein for this case study as a commercially available reference standard. After establishing a structured protocol to create a robust chromatographic method, the same optimization strategy was extended to four additional monoclonal antibody therapeutics and the final optimized method presented.

Experimental

Reagents and consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Fisher Chemical™ Optima™ LC/MS Trifluoroacetic acid (TFA) (Cat. No. A116-50)
- Fisher Chemical™ Optima™ LC/MS Acetonitrile (MeCN) (Cat. No. A955-1)
- NISTmAb (NIST, 8671)
- Reference *Results and discussion* section for additional mAb samples.

Sample handling equipment

Thermo Scientific™ SureSTART™ Glass, 0.3 mL vials (Cat. No. 6PSV903FIVP)

Sample preparation

All mAb samples were diluted to 1 mg/mL using DI water.

Instrumentation

Thermo Scientific™ Vanquish™ Horizon UHPLC System, including:

- Thermo Scientific™ Vanquish™ System Base (Cat. No. VF-S01-A-03)
- Thermo Scientific™ Vanquish™ Binary Pump H (Cat. No. VH-P10-A)
- Thermo Scientific™ Vanquish™ Column Compartment H (Cat. No. VH-C10-A-03) with Thermo Scientific™ Vanquish™ Active Pre-heater MP35N 0.1 x 380 mm (Cat. No. 6732.0110) and Thermo Scientific™ Vanquish™ Post-column 1 µL Cooler MP35N 0.10 x 445 mm (Cat. No. 6732.0510)
- Thermo Scientific™ Vanquish™ Diode Array Detector HL (Cat. No. VH-D10-A)
- Thermo Scientific™ Vanquish™ LightPipe™ HL10 mm Standard Flow Cell (Cat. No. 6083.0100B)

Column

Thermo Scientific™ SurePac™ Protein RP MDi™ Column, 2.1 x 50 mm (Cat. No. 43722-052132)

Chromatographic conditions

Mobile phase compositions and gradient conditions, including flow rate, column temperature, and injection volume, are given in the respective text and figures in the *Results and discussion* section. Absorbance at 280 nm was used for detection of all samples.

Data processing

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

Results and discussion

Optimization of chromatographic parameters is critical to design a method that provides high resolution and highly reproducible protein separations. In this section, we demonstrate a straightforward approach to develop and optimize methods for the analysis of NISTmAb. The full optimization approach was first applied to the separation of NISTmAb using a 10-minute linear gradient ranging from 100% mobile phase A to 100% mobile phase B to evaluate at which composition NISTmAb and associated variants elute. The gradient was then optimized by adjusting first the gradient slope and starting and ending %B, then flow rate. Lastly, temperature was optimized to produce a finalized method. Dynamic loading capacity was also evaluated to demonstrate changes in separation observed at different mass loading levels. This process was repeated, and the final methods were provided for other therapeutic mAbs.

Select initial conditions

Establishing an appropriate initial chromatographic condition is a critical first step in developing a robust reversed-phase method for intact monoclonal antibody analysis. The following starting parameters are recommended for evaluating protein separations using the SurePac Protein RP MDi Column. The 2.1 × 50 mm analytical format was selected to provide high resolution while maintaining practical run times and manageable system pressure.

Mobile phase A H_2O with 0.1% trifluoroacetic acid (TFA) and mobile phase B 90% acetonitrile and 9.9% water with 0.1% TFA were used as common reversed-phase mobile phases for protein separations. TFA controls the mobile phase pH and serves as an ion-pairing agent to improve peak shape by increasing the strength of hydrophobic interactions and reducing secondary interactions that can lead to fronting or tailing. This composition provides strong chromatographic performance for LC/UV applications and serves as a reliable baseline for further optimization.

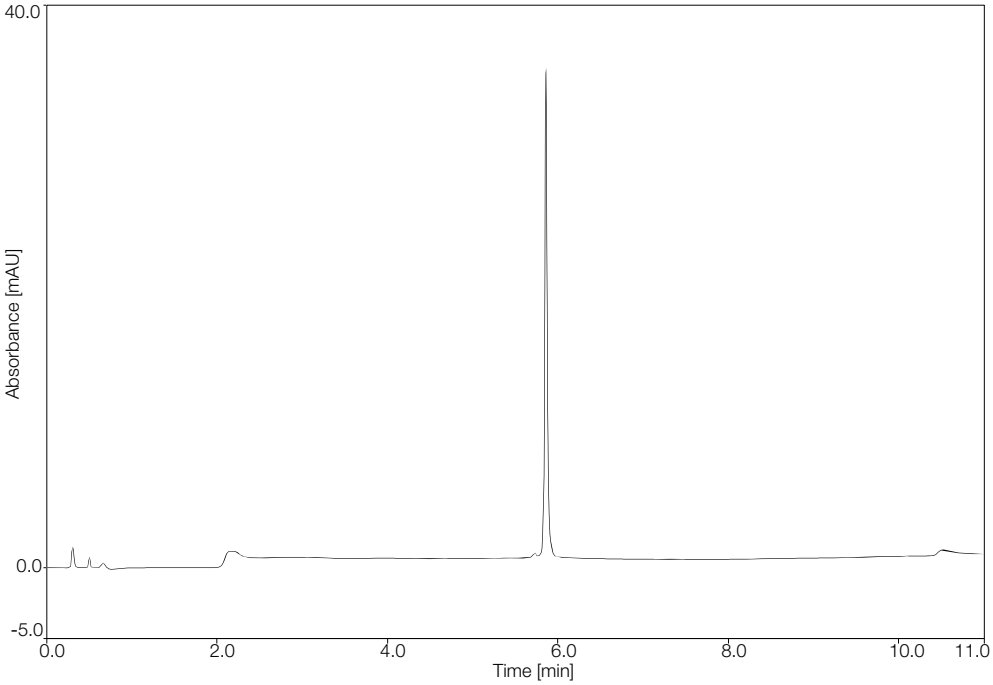
An initial flow rate of 0.4 mL/min was used to provide a balance between efficiency and throughput for the 2.1 mm format. Accurate thermal management is particularly important for intact protein separations at elevated temperatures. Protein separations were performed at 80 °C to enhance mass transfer and improve peak shape for large biomolecules. To ensure optimal thermal control, an active mobile phase pre-heater was used. A pre-heater is used to ensure thermal equilibrium between the mobile phase and the column, preventing efficiency loss caused by temperature differences between the heated column and the incoming solvent.

Additionally, a post-column cooler was used prior to the Vanquish Diode Array Detector (DAD) to reduce the eluent temperature before entering the detector. This is recommended when operating at column temperature of 60 °C or higher to protect the detector flow cell and ensure stable detector performance. An initial injection volume of 0.5 µL was used for sample evaluation to avoid possible overloading and allow accurate evaluation of column performance. These conditions serve as a baseline for gradient optimization.

Initial gradient screening

An initial gradient from 0% to 100% B over 10 minutes was used to determine the approximate elution window of the target protein and evaluate overall chromatographic behavior as shown in Figure 1. This approach allows identification of the elution region of the intact monoclonal antibody and any associated variants, impurities, or degradation products. The gradient times in Figure 1 were used for all subsequent analyses with the initial (time points 0, 1, and 13.1 min) and final (time points 11 and 13 min) gradient conditions adjusted as indicated in each figure. For each gradient, the mobile phase was set to initial gradient condition at 13.1 minutes to re-equilibrate the phase after completion of the run.

Resolution of closely eluting variants should also be assessed qualitatively at this stage. Although the initial gradient may not provide optimal separation, it enables identification of the appropriate %B range for later refinement. Once the primary elution window is established, the gradient range can be narrowed and slope adjusted to improve resolution and throughput.



Column	SurePac Protein RP MDi	
Format	2.1 x 50 mm	
Mobile phase buffers		
Buffer A	H_2O/TFA (99.9:0.1 v/v)	
Buffer B	ACN/H_2O/TFA (90:9.9:0.1 v/v)	
Time	%A	%B
0.0	100.0	0.0
1.0	100.0	0.0
11.0	0.0	100.0
13.0	0.0	100.0
13.1	100.0	0.0
18.0	100.0	0.0
Flow rate	0.4 mL/min	
Temperature		
	80 °C Pre-heater	
	80 °C Column chamber	
	45 °C Post-column cooler	
Detection	UV 280 nm	
Inj. volume	0.5 µL	
Sample	NISTmAb 1 mg/mL	

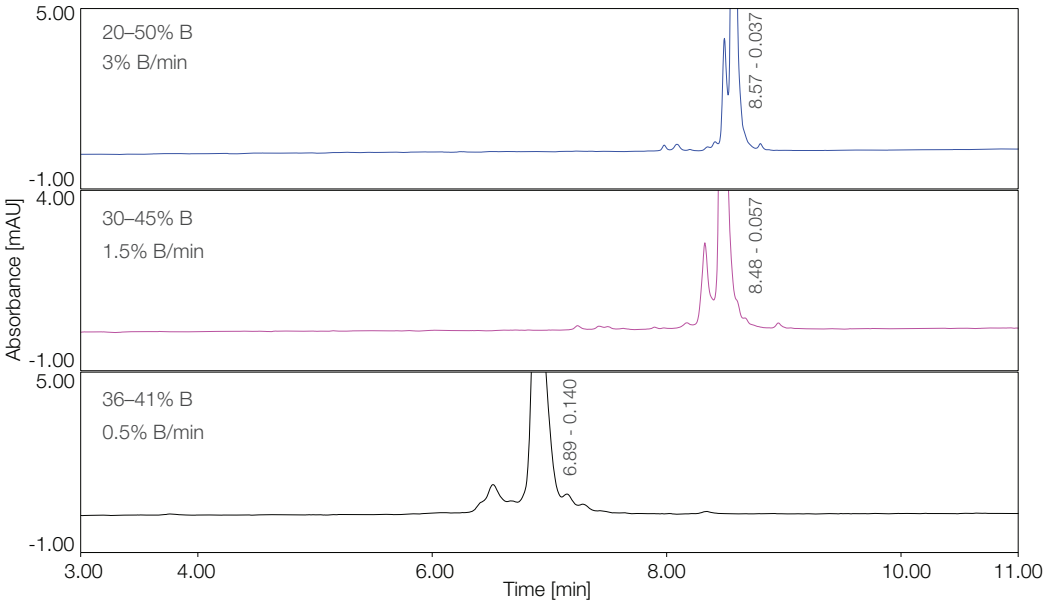
Figure 1. Analysis of NISTmAb and associated variants on a 2.1 x 50 mm SurePac Protein RP MDi Column using a gradient over the total organic range.

Gradient slope

Gradient slope is a critical parameter in reversed-phase separation of intact monoclonal antibodies and their variants. The rate of increase in organic modifier (%B per minute) directly influences retention time, peak width, and the resolution of closely related species. A shallower gradient (smaller change in %B/min) increases the interaction time between the analyte and the stationary phase, enhancing separation of structurally similar variants. However, this extended interaction typically results in broader peaks. In contrast, a steeper gradient produces sharper peaks but may compromise resolution of closely eluting variants. Figure 2 illustrates this effect. NISTmAb was analyzed under identical flow rate (0.4 mL/min) and temperature (80 °C) conditions using different gradient slopes. After the initial gradient screening, a gradient window was selected to position the main monoclonal antibody peak near the middle of the chromatogram while maintaining adequate retention of both early- and late-eluting variants. For this study, a 10-minute gradient window was selected based on the initial screening results. Once the gradient window was established, the gradient slope was optimized by adjusting the %B change per minute while maintaining complete analyte elution within the selected time window. This sequential approach enabled the method to be readily adapted to different monoclonal antibodies by first defining an appropriate elution window and then optimizing the gradient slope to achieve the desired balance of resolution, analysis time, and peak width. At 3% B/min (20%–50% B range), the main NISTmAb peak eluted rapidly with the narrowest peak width; however, separation of low-level variants was limited. Reducing the slope to 1.5% B/min (30%–45% B range) improved separation of adjacent variants while maintaining acceptable peak width. Further decreasing the slope to 0.5% B/min (36%–41% B range) maximized separation of minor species from the main peak. Although peak width increased, the increased variant separation improved detection of these minor variants for quantitation. The gradient slope should be carefully optimized according to analytical objectives and separation performance. In this study, 0.5% B/min was selected for further evaluation, as it provided the best separation of the proximal minor variants.

Flow rate

Figure 3 evaluates the analysis of NISTmAb at three different flow rates (0.1, 0.2, and 0.4 mL/min) using a gradient from 37%–42 %B gradient over 10 minutes. The impact of flow rate on retention time, peak width, and backpressure was assessed. Column backpressure increased proportionally with flow rate. Increasing the flow rate resulted in shorter retention times for both the main NISTmAb peak and its associated variants, primarily due to reduced gradient delay time. The peak width at half height (PWHH) of the main peak decreased as flow rate increased from 0.1 to 0.4 mL/min, resulting in sharper peaks. Resolution of minor variants also improved at higher flow rates. Flow rate should be optimized according to the intended application. In this instance, 0.4 mL/min was selected for further evaluation as it provided the best separation of the proximal variants while operating at a safe column pressure.



Column	SurePac Protein RP MDi
Format	2.1 x 50 mm
Mobile phase buffers	
Buffer A	H_2O/TFA (99.9:0.1 v/v)
Buffer B	ACN/H_2O/TFA (90:9.9:0.1 v/v)

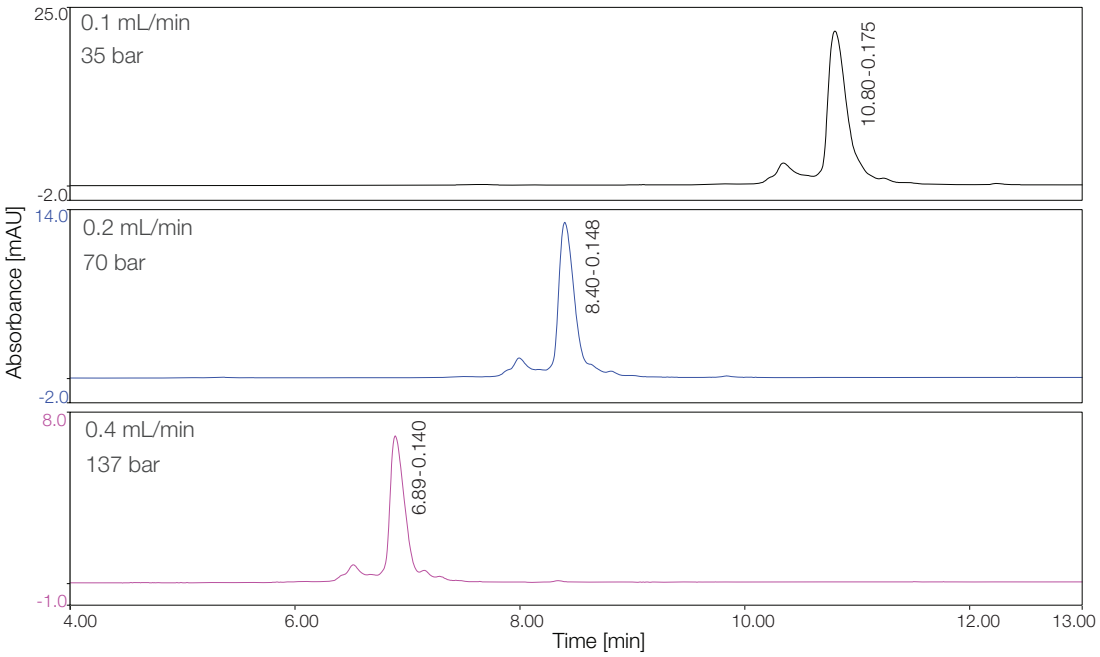
Reference chromatogram for initial and final %B

Time	%B		
	3% B/min	1.5% B/min	0.5% B/min
0.0	20	30	36
1.0	20	30	36
11.0	50	45	41
13.0	50	45	41
13.1	20	30	36
18.0	20	30	36

Flow rate	0.4 mL/min
Temperature	80 °C Pre-heater 80 °C Column chamber 45 °C Post-column cooler
Detection	UV 280 nm
Inj. volume	0.5 µL
Sample	NISTmAb 1 mg/mL
Legend	RT - PWHH

RT- Retention time; PWHH - peak width at half height

Figure 2. Effect of gradient slope on NISTmAb and associated variants separation.

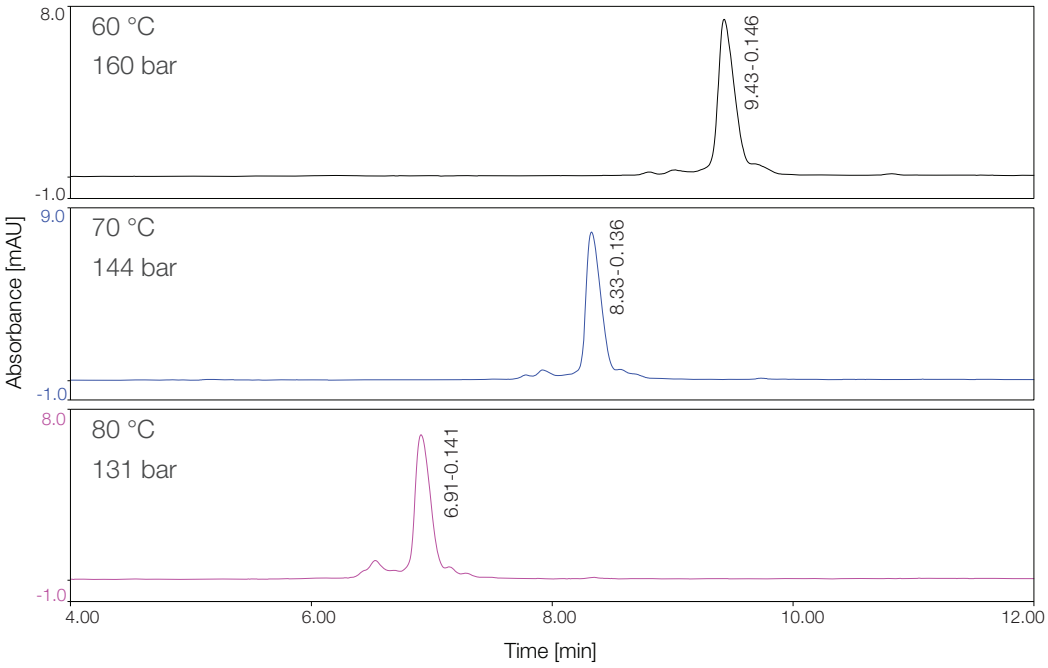


Column	SurePac Protein RP MDi
Format	2.1 x 50 mm
Mobile phase buffers	
Buffer A	H_2O/TFA (99.9:0.1 v/v)
Buffer B	ACN/H_2O/TFA (90:9.9:0.1 v/v)

Time	%A	%B
0.0	63.0	37.0
1.0	63.0	37.0
11.0	58.0	42.0
13.0	58.0	42.0
13.1	63.0	37.0
18.0	63.0	37.0

Flow rate	See chromatogram
Temperature	80 °C Pre-heater 80 °C Column chamber 45 °C Post-column cooler
Detection	UV 280 nm
Inj. volume	0.5 µL
Sample	NISTmAb 1 mg/mL
Legend	RT - PWHH

Figure 3. Effect of flow rate on the separation of NISTmAb and associated variants.



Column	SurePac Protein RP MDi
Format	2.1 x 50 mm
Mobile phase buffers	
Buffer A	H_2O/TFA (99.9:0.1 v/v)
Buffer B	ACN/H_2O/TFA (90:9.9:0.1 v/v)

Time	%A	%B
0.0	63.0	37.0
1.0	63.0	37.0
11.0	58.0	42.0
13.0	58.0	42.0
13.1	63.0	37.0
18.0	63.0	37.0

Flow rate	0.4 mL/min
Temperature	See chromatogram 45 °C Post-column cooler
Detection	UV 280 nm
Inj. volume	0.5 µL
Sample	NISTmAb 1 mg/mL
Legend	RT - PWHH

Figure 4. Effect of temperature on NISTmAb separation and column pressure.

Temperature

Temperature is another parameter that can be adjusted to optimize separation of proteins and their variants. Figure 4 evaluates the impact of column temperature on the separation of NISTmAb. Column temperature was evaluated at 60 °C, 70 °C, and 80 °C while maintaining a constant flow rate of 0.4 mL/min over 10 minutes. Increasing the column temperature resulted in a systematic decrease in both retention time and column pressure. Retention time decreased from approximately 9.6 min at 60 °C to 7.3 min at 80 °C, consistent with reduced strength of hydrophobic interactions with the media. Column pressure was reduced from 160 bar to 131 bar due to reduced viscosity of the mobile phase at elevated temperatures. Peak widths also decreased slightly with increasing temperature. Minor impurity

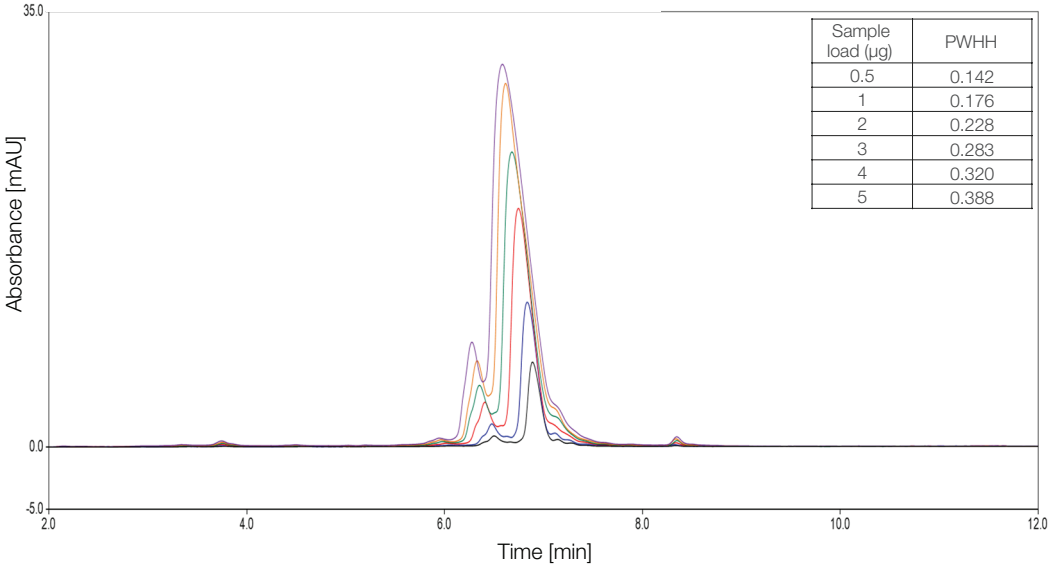
peaks were detectable under all temperature conditions but appeared most clearly defined at 80 °C, resulting in improved resolution of proximal variants. Improvements in peak width and variation resolution are likely attributable to improved mass transfer and a reduction in secondary interactions with increasing temperature. The user should consider these effects and any possible adverse effects on their sample when selecting the temperature for their respective analysis.

Sample loading

The total mass of protein loaded into the column can have a significant effect on peak width and resolution. To evaluate the loading capacity of the phase, dynamic loading analysis was performed on the 2.1 x 50 mm column shown in Figure 5. The chromatograms show loading masses of 0.5–5.0 µg of NISTmAb. The loaded mass was varied by adjusting the injection volume while maintaining a constant sample concentration. At lower sample load (0.5–1 µg), the main NISTmAb peak remained sharp and symmetrical, indicating operation within the optimal dynamic loading range of the column. At higher sample load (≥2 µg), peak width increased more significantly, consistent with the onset of column overloading effects, which may reduce resolution of closely eluting variants. Dynamic loading capacity is influenced by protein size, hydrophobicity, and structural characteristics. Therefore, loading behavior should be evaluated individually for each protein. The example provided may be used as a general reference for other protein analytes.

Optimized method

Based on the approach provided in the previous sections, we developed an optimized method for NISTmAb analysis that delivers high-resolution method with a 10-minute gradient at 0.4 mL/min as shown in Figure 6. The high-resolution gradient provided greater separation of associated variants and the main peak, enabling improved separation and quantification of each variant. Figure 7 demonstrates NISTmAb and its associated variants can also be separated using a 4-minute gradient, supporting high-throughput analytical workflows. Although resolution of closely eluting variants was reduced compared to the 10-minute gradient method, the fast gradient still maintained acceptable separation between the main peak and associated variants. This approach is suitable when analytical turnaround time is prioritized, such as in high-volume QC environments where rapid screening is required. Both methods can be further optimized as needed to improve for time and separation by adjusting the gradient starting and ending mobile phase conditions.



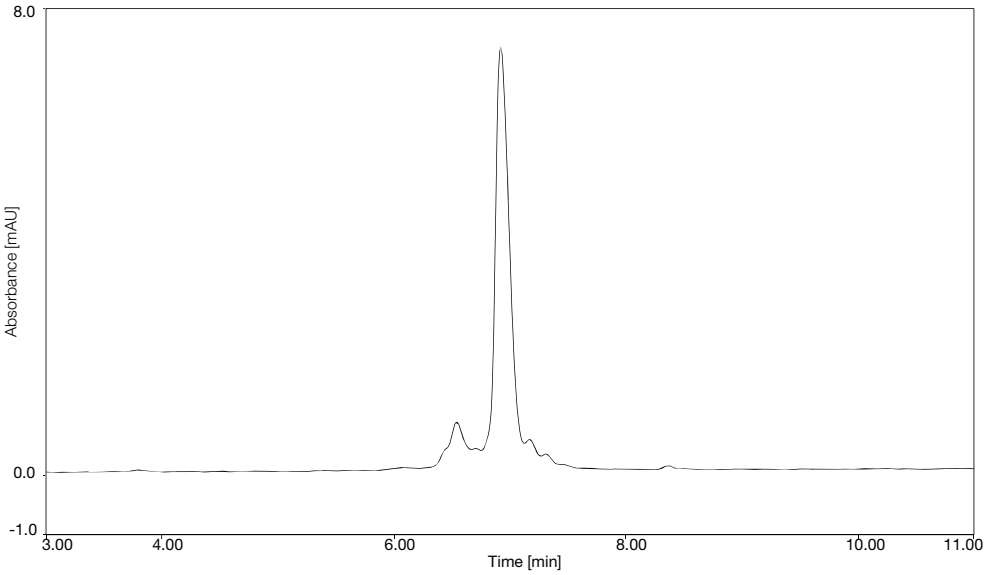
Sample load (µg)	PWHH
0.5	0.142
1	0.176
2	0.228
3	0.283
4	0.320
5	0.388

Column	SurePac Protein RP MDi
Format	2.1 x 50 mm
Mobile phase buffers	
Buffer A	H_2O/TFA (99.9:0.1 v/v)
Buffer B	ACN/H_2O/TFA (90:9.9:0.1 v/v)

Time	%A	%B
0.0	63.0	37.0
1.0	63.0	37.0
11.0	58.0	42.0
13.0	58.0	42.0
13.1	63.0	37.0
18.0	63.0	37.0

Flow rate	0.4 mL/min
Temperature	80 °C Pre-Heater 80 °C Column chamber 45 °C Post-column cooler
Detection	UV 280 nm
Inj. volume	0.5 µL - 5 µL
Sample	NISTmAb 1 mg/mL

Figure 5. Chromatogram overlays showing the dynamic loading analysis of NISTmAb. The table shows the effect of mass and volume of mAb loaded on PWHH.

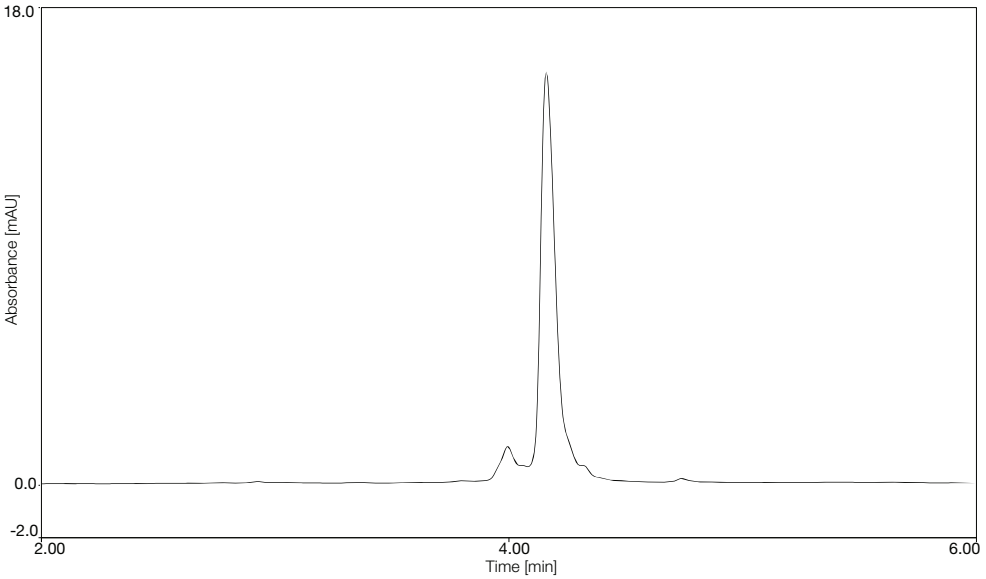


Column	SurePac Protein RP MDi
Format	2.1 x 50 mm
Mobile phase buffers	
Buffer A	H_2O/TFA (99.9:0.1 v/v)
Buffer B	ACN/H_2O/TFA (90:9.9:0.1 v/v)

Time	%A	%B
0.0	63.0	37.0
1.0	63.0	37.0
11.0	58.0	42.0
13.0	58.0	42.0
13.1	63.0	37.0
18.0	63.0	37.0

Flow rate	0.4 mL/min
Temperature	80 °C Pre-heater 80 °C Column chamber 45 °C Post-column cooler
Detection	UV 280 nm
Inj. volume	0.5 µL
Sample	NISTmAb 1 mg/mL

Figure 6. Chromatogram of 10-minute gradient at 0.4mL/min flow rate.



Column	SurePac Protein RP MDi
Format	2.1 x 50 mm
Mobile phase buffers	
Buffer A	H_2O/TFA (99.9:0.1 v/v)
Buffer B	ACN/H_2O/TFA (90:9.9:0.1 v/v)

Time	**%A**	**%B**
0.0	63.0	37.0
1.0	63.0	37.0
5.0	58.0	42.0
7.0	58.0	42.0
7.0	63.0	37.0
12.0	63.0	37.0

Flow rate	0.4 mL/min
Temperature	80 °C Pre-Heater 80 °C Column chamber 45 °C Post-column cooler
Detection	UV 280 nm
Inj. volume	0.5 µL
Sample	NISTmAb 1 mg/mL

Figure 7. Chromatogram of 4-minute gradient at 0.4 mL/min flow rate.

Evaluation of method development workflow for other mAbs

The methodology discussed above has been applied to a range of mAbs. Each mAb was optimized using the 10-minute linear gradient. For each mAb evaluated, initial and final gradient values for %B of organic solvent are provided in Tables 1 and 2. Figure 8 demonstrates excellent mAb-variant separation for each mAb using the SurePac Protein RP MDi Column. The SurePac Protein RP MDi Column provides high resolution of minor variants with a simple linear gradient method.

Table 1. General mAb gradient.

General mAb gradient for 2.1 x 50 mm column		
Eluents:	A: H_2O/TFA (99.9:0.1 v/v) B: ACN/H_2O/TFA (90:9.9:0.1 v/v)	
Flow rate	0.4 mL/min	
Temperature	80 °C Pre-heater 80 °C Column chamber 45 °C Post-column cooler	
Gradient	**Time**	**%B**
	0.0	Initial
	1.0	Initial
	11.0	Final
	13.0	Final
	13.1	Initial
	18.0	Initial

Table 2. Gradient parameters for analysis of mAbs.

mAb	Initial %B	Final %B	Inj. vol. (µL)
Secukinumab	38	43	2.0
Pertuzumab	34	39	2.0
Avastin™	35	40	2.0
Rituximab biosimilar	34	39	2.0
Nivolumab (IgG4)	34	39	2.0
Denosumab (IgG2)	35	40	5.0

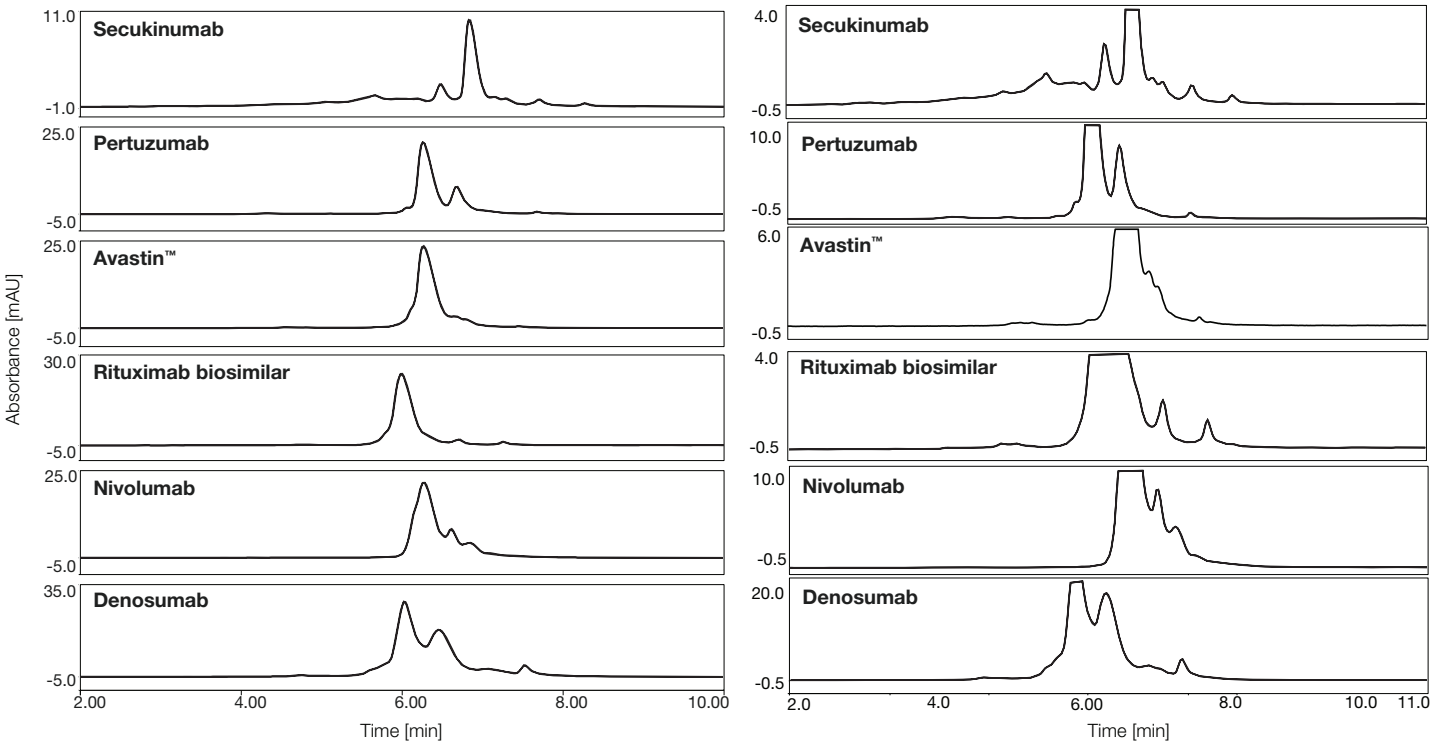


Figure 8. Analysis of mAbs. The left chromatograms show the full signal strength, and the right chromatograms show the zoomed-in detail view of the mAb variants.

Conclusion

The SurePac Protein RP MDi Column enables high-resolution separation of intact monoclonal antibodies and associated variants. By fine-tuning gradient slope, flow rate, and temperature, users can achieve consistent, robust, and high-resolution separations designed for their analytical needs.

Learn more at **thermofisher.com/surepacprotein**