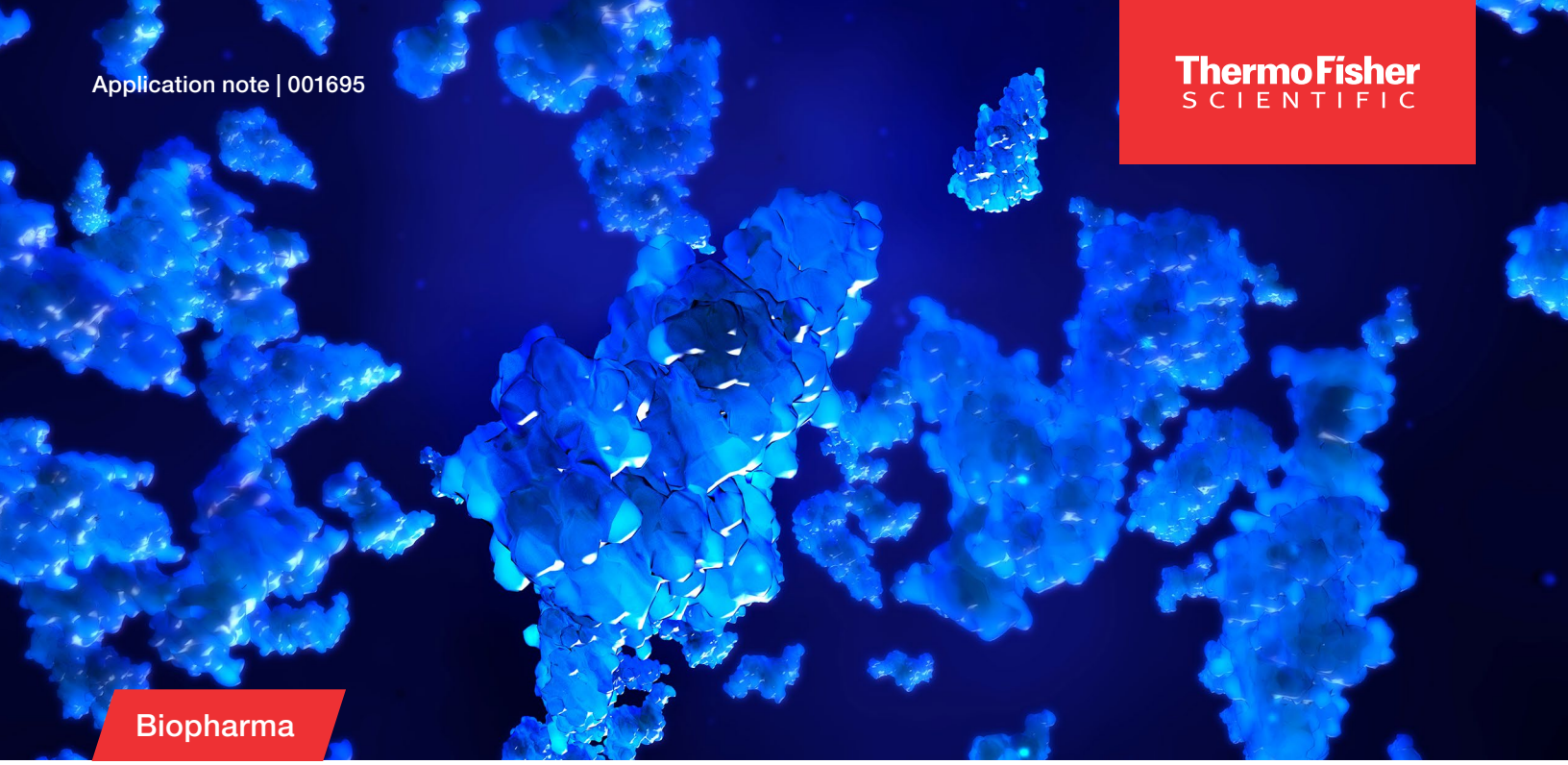


Application note | 001695

Biopharma

Salt gradient analysis of Protein G using a 3 µm monodisperse SAX chromatography column

Authors

Ke Ma, Shane Bechler

Thermo Fisher Scientific, Sunnyvale, CA

Keywords

Biopharma, variants, strong anion exchange, SAX, Protein G, salt gradient, ProPac 3R SAX 3µm column, monodisperse, HPLC, liquid chromatography

Application benefits

- High-resolution separation of protein charge variants
- Easy, straightforward method development
- Consistent lot-to-lot and column-to-column performance

Goal

Detail basic method development and demonstrate high-resolution analysis of Protein G using a salt gradient with a 4 × 100 mm, 3 µm monodisperse SAX column

Introduction

Proteins have been used as a major class of therapeutics for the treatment of various diseases including cancer, cardiovascular diseases, and autoimmune disorders. The market for proteins as therapeutics is expected to continue growing for the foreseeable future. Proteins typically have an isoelectric point (pI) ranging from 4.0 to 12.0, based on their amino acid composition, glycosylation profile, and other post-translational modifications. Ion exchange chromatography (IEX) is a standard technique for analyzing proteins and their associated variants based on their accessible surface charge. Strong anion exchange (SAX) columns in particular are used for the evaluation of acidic proteins (e.g., pI ≤ 7.0). Many acidic proteins are often heavily glycosylated resulting in

complicated variant profiles. Because of this complexity, high-resolution chromatography separations are required to analyze these therapeutics both in the research and QC environments.

The Thermo Scientific™ ProPac™ 3R SAX column has a unique monodisperse resin containing a hydrophilic layer and quaternary ammonium groups. The stationary phase is composed of a 3 µm, nonporous divinylbenzene polymer resin to provide exceptionally high resolving power. Compared to traditional polydisperse particles (right image, Figure 1), the monodisperse particles have a consistent size distribution (left image, Figure 1) resulting in improved column packing and lot-to-lot reproducibility. The thin, hydrophilic layer grafted to the particle core and precisely controlled quaternary amine chemistry reduce secondary interactions between the stationary phase and sample to minimize band broadening. The quaternary ammonium functionality grafted to the hydrophilic layer introduces permanently charged cationic sites to provide the strong anion exchange character required for promoting protein binding when using a low ionic strength mobile phase at an appropriate pH (e.g., 20 mM Tris, pH 8). The reproducible resin chemistry and manufacturing processes eliminate column variability as a concern in method development and data analysis. The ProPac 3R SAX column hardware is polyether ether ketone (PEEK), which has well-established bioinert properties to minimize nonspecific adsorption of protein samples compared to metal-based hardwares.[1] Together, these design choices make the ProPac 3R SAX column capable of analyzing complex proteins with high resolution and excellent reproducibility.

In this application note, we provide practical examples of method design and discuss the development of both a fast QC method and a longer high-resolution analytical method for Protein G and associated variants. Protein G is used as a model protein in this case because it has a pI value of 4.5 and a complicated variant/impurity profile that can be separated using an SAX column. With a well-developed method, we demonstrate the capability of the ProPac 3R SAX column to differentiate samples by comparing Protein G that has been temperature stressed against the standard sample. The reproducibility of the method and column is evaluated by comparing three different synthetic lots of ProPac 3R SAX media made on three different batches of 3 µm monodisperse base resin. Lastly, the performance of the ProPac 3R SAX column is compared against our current ProPac SAX-10 column to demonstrate significant performance improvements.

These exercises show that the ProPac 3R SAX column allows for easy method optimization with excellent sensitivity and performance under a broad range of pH, temperature, and mobile phase compositions. The performance gives the user confidence in the detection and identification of acidic or basic variants of existing and novel protein therapeutics during late-stage development, cellular production, downstream purification, and storage and shipping. The ProPac 3R SAX column provides the required performance to meet characterization and regulatory requirements of current and future therapeutics as they continue to increase in complexity.

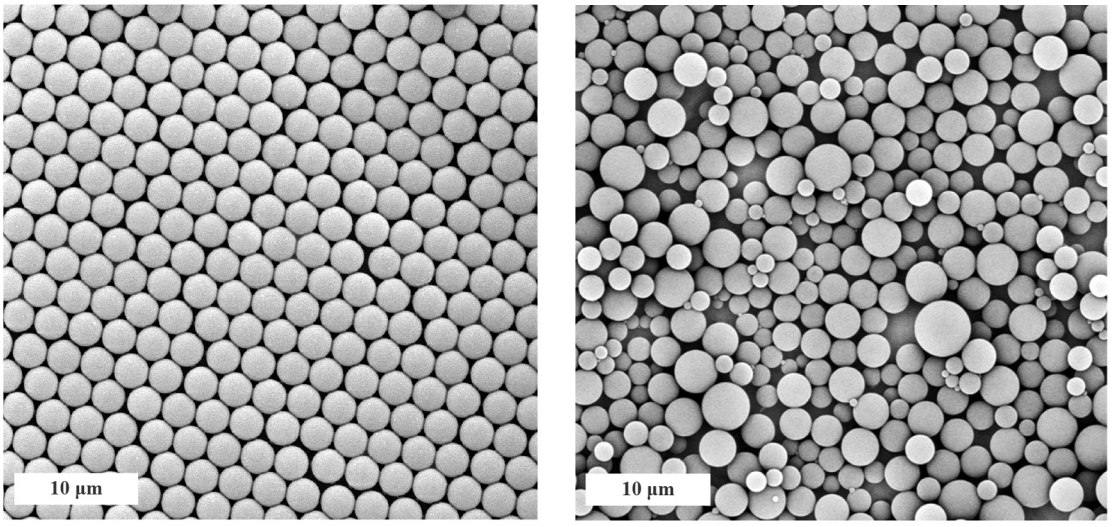


Figure 1. SEM image of monodisperse ProPac 3R (left) vs. polydisperse particles (right). White scale bars are 10 µm in length.

Experimental

Reagents and consumables

- Deionized water, 18.2 MΩ·cm resistivity
- Protein G, Thermo Scientific™ Pierce™ (Fisher Scientific, P/N PI77675)
- Trizma™ Pre-set crystals, pH 8.0 (Sigma-Aldrich, P/N T8443)
- Sodium chloride, Fisher Chemical™ (Fisher Scientific, P/N S271)
- 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

Protein G sample was diluted to a final concentration of 5 mg/mL or 1 mg/mL using DI water

Separation conditions

Thermo Scientific™ Vanquish™ Flex Quaternary UHPLC system, including:

- System Base Vanquish Flex (P/N VF-S01-A)
- Quaternary Pump (P/N VF-P20-A)
- Column Compartment H (P/N VH-C10-A)
- Split Sampler FT (P/N VF-A10-A) with 25 µL (V = 50 µL) sample loop
- Thermo Scientific™ Vanquish™ Variable Wavelength Detector (P/N VF-D40-A) with Thermo Scientific™ Vanquish™ Variable Wavelength Detector Flow cell (P/N 6077.0300)

Column

- ProPac 3R SAX, 3 µm, 4 × 100 mm, P/N 43203-104068

For mobile phase compositions and gradient conditions including flow rate, column temperature, 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.

Data processing

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

Results and discussion

To obtain reproducible results with high resolution and confidence in the peaks being detected, it is critical to establish an optimized method for analysis of the protein and associated variants. In this section, we demonstrate a straightforward approach to develop and optimize a salt gradient method for the analysis of Protein G. A set of parameters in the order of importance, including mobile phase pH, gradient salt concentration, gradient time and flow rate, are optimized. A fast QC method and a longer high-resolution analytical method are shown to demonstrate the separation capability of the column. Columns from different media lots are used after method development to show the method robustness.

Buffer pH effect on chromatography

It is important to select an appropriate mobile phase buffer and pH for the specific protein separation since the charge of the protein of interest can be influenced by the mobile phase pH. With regards to the stationary phase, the quaternary ammonium groups always have a positive charge; as such, buffers of any pH can be used so long as the pH is sufficiently greater than the protein isoelectric point (pI, the pH at which the overall charge of

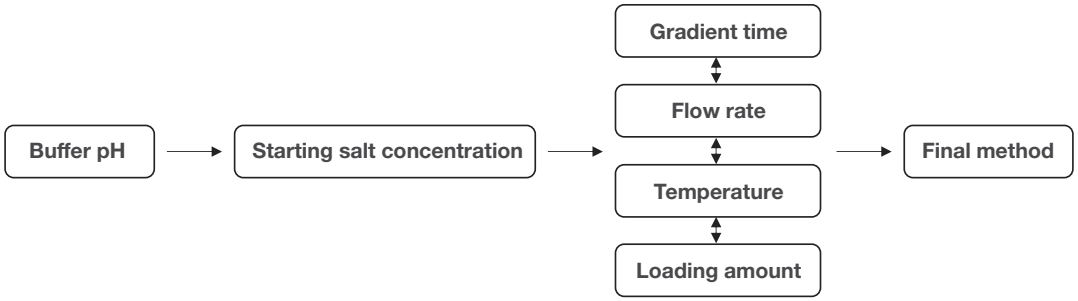


Figure 2. Method development flow chart

the protein is neutral) to promote binding of the anionic protein. We recommend using Good's buffers due to their compatibility with biological molecules, good water solubility, and ability to buffer across the physiological pH range.[2] The effect of buffer pH on sample binding and elution can be seen in Figure 3 showing the analysis of Protein G using Tris buffers at pH 7.5, 8.0, and 8.5. As the pH of the mobile phase increases, the protein elutes later in the chromatogram as acidic groups are more likely to be deprotonated, resulting in increased charge of the protein and/or other neutral groups becoming deprotonated increasing the number of anionic sites. The small increase in retention time from pH 8.0 to 8.5 may be due to a relatively small change in Protein G charge as the pH increases further from the sample pI. At pH 8.0, better basic and proximal acidic variant resolution from the main peak is observed relative to the separation at pH 7.5. Only minor differences are observed between pH 8.0 and 8.5 suggesting that the differences in interactions of the sample with the stationary phase is not significantly changed from pH 8.0 to 8.5. However, we did observe decreased resolution of the proximal acidic peaks at pH 8.5 compared with pH 8.0. For this reason, further method optimization was performed at pH 8.0.

Determining gradient salt concentrations

It is necessary to design the gradient method so that the protein is separated by the change in salt concentration without isocratic elution occurring during loading. If the sample is already eluting isocratically during loading, the user will observe reduced lot-to-lot and column-to-column reproducibility for a given method. This can be tested for by comparing the separation when using a gradient with and without an isocratic hold. Figure 4 compares the elution of Protein G using gradients at two different initial salt concentrations of 80 mM (16% B) and 60 mM NaCl (12% B). For each starting salt concentration, the separation is evaluated with and without a 5-minute isocratic hold (the change in time between the injection and start of the gradient) to determine if isocratic elution occurs during loading. At 80 mM NaCl loading, the separation of the variants from the main peak increases with the isocratic hold and the PWHH of the main peak increases indicating isocratic elution. At 60 mM NaCl loading, the separation of the variants relative to the main peak are consistent with and without the isocratic hold and the main peak PWHH is unchanged. Based on these results, isocratic elution is observed at 80 mM NaCl loading but not 60 mM NaCl.

Column:	ProPac 3R SAX, 3 µm		
Format:	4 × 100 mm		
Eluents:	A: 20 mM Tris		
	B: 20 mM Tris + 500 mM NaCl		
Eluents pH:	Top:	7.5	
	Middle:	8.0	
	Bottom:	8.5	
Gradient:	Time (min)	%A	%B
	0.0	88	12
	1.0	88	12
	16.0	58	42
	16.1	0	100
	18.0	0	100
	18.1	88	12
	30.0	88	12
Flow rate:	0.5 mL/min		
Inj. volume:	1 µL		
Temp.:	30 °C		
Detection:	UV, 280 nm		
Sample:	Protein G – 5 mg/mL		
Peak label:	Retention time		

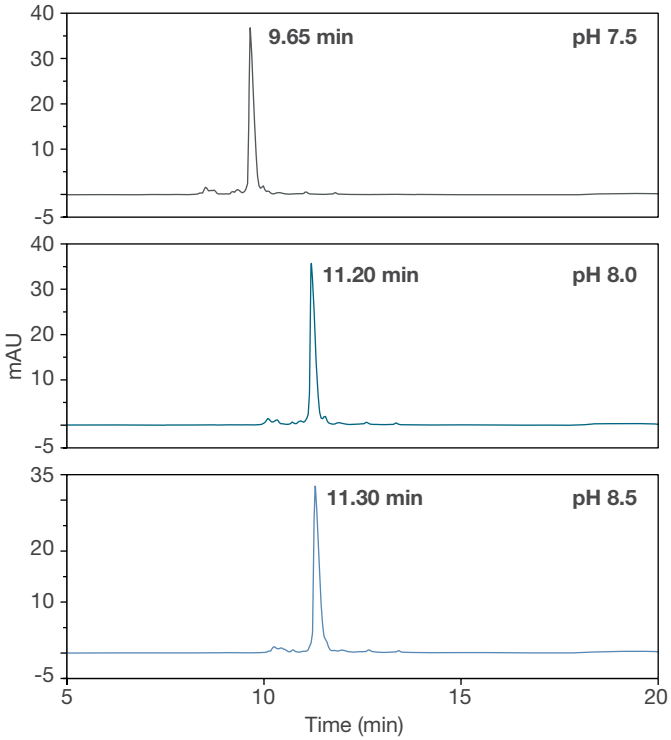


Figure 3. Effect of buffer pH on the separation of Protein G and associated charge variants

Column: ProPac 3R SAX, 3 µm
Format: 4 × 100 mm
Eluents: A: 20 mM Tris, pH 8.0
B: 20 mM Tris + 500 mM NaCl, pH 8.0

Gradient: 1st: 16–46% B, 0 min isocratic hold
2nd: 16–46% B, 5 min isocratic hold
3rd: 12–42% B, 0 min isocratic hold
4th: 12–42% B, 5 min isocratic hold

Flow rate: 0.5 mL/min
Inj. volume: 1 µL
Temp.: 30 °C
Detection: UV, 280 nm
Sample: Protein G – 5 mg/mL

Peak label: Retention time - PWHH

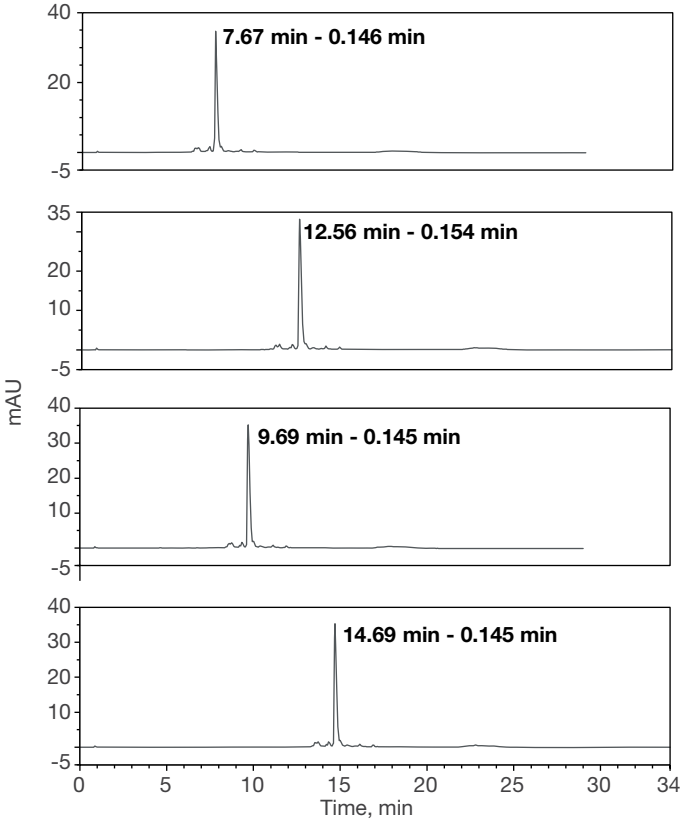


Figure 4. Effect of starting salt concentration on Protein G separation with and without a 5-minute isocratic hold: 16–46 %B (top two) and 12–42 %B (bottom two)

Further confirmation of isocratic elution is evidenced by comparing the retention times of each peak with and without the isocratic hold. Isocratic elution occurs when the retention time difference for the protein and variant peaks with and without the hold decreases below 5 minutes and when the PWHH ratio (no-hold/hold) becomes less than 1. Based on the results in Figure 4, 60 mM NaCl would be the recommended salt loading concentration for Protein G with Tris buffer pH 8.0 as it is the maximum salt concentration observed to meet both criteria. Choosing this starting condition will provide better column-to-column and lot-to-lot consistency for a given method.

Determining salt gradient slope

After determining the appropriate mobile phase pH and starting salt concentration, the rate of salt concentration change or gradient slope should be determined next. Figure 5 shows the analysis of Protein G using a salt gradient from 60 to 210 mM NaCl over 10, 15, 20, and 30 minutes. These data illustrate the effect of gradient time on the retention time and separation of variant peaks from the main protein peak. Comparison of chromatograms at different gradient times shows that the gradient is primarily responsible for separation of the variant peaks from the main peak. These results clearly show that the resolution of the variant peaks increases with increasing gradient time. The appropriate gradient for a given analysis is determined by the user's goals. Those looking to do rapid separation with fast quantitation in a QC environment may choose a 10-minute gradient, while those in early-stage R&D may look to utilize a longer gradient to maximally separate each peak for additional analyses.

Eluents: A: 20 mM Tris, pH 8.0
B: 20 mM Tris + 500 mM NaCl, pH 8.0

Gradient: Top: 12–42% B in 10 min
Middle: 12–42% B in 20 min
Bottom: 12–42% B in 30 min

Flow rate: 0.5 mL/min
Inj. volume: 1 µL
Temp.: 30 °C
Detection: UV, 280 nm
Sample: Protein G – 5 mg/mL

Peak label: Retention time

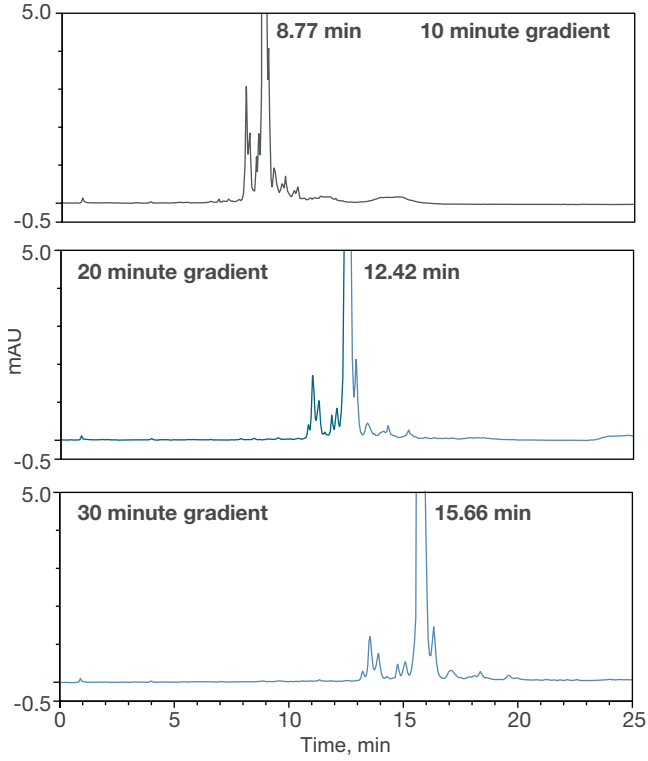


Figure 5. Effect of gradient time on the separation of Protein G and associated charge variants. Time scale is zoomed-in to 25 minutes to aid visualization of variant peaks.

Determining flow rate

After determining an appropriate gradient, other parameters with smaller but important effects on the separation can be optimized. Figure 6 shows the analysis of Protein G using a flow rate of 0.3, 0.4, and 0.5 mL/min. 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 basic (left) and acidic (right) peaks from the main protein peak decreases slightly.

Column: ProPac 3R SAX, 3 µm
Format: 4 × 100 mm
Eluents: A: 20 mM Tris, pH 8.0
B: 20 mM Tris + 500 mM NaCl, pH 8.0

Gradient:

Time (min)	%A	%B
0.0	88	12
1.0	88	12
16.0	58	42
16.1	0	100
18.0	0	100
18.1	88	12
30.0	88	12

Flow rate: Top: 0.3 mL/min
Middle: 0.4 mL/min
Bottom: 0.5 mL/min
Inj. volume: 1 µL
Temp.: 30 °C
Detection: UV, 280 nm
Sample: Protein G – 5 mg/mL

Peak label: Retention time

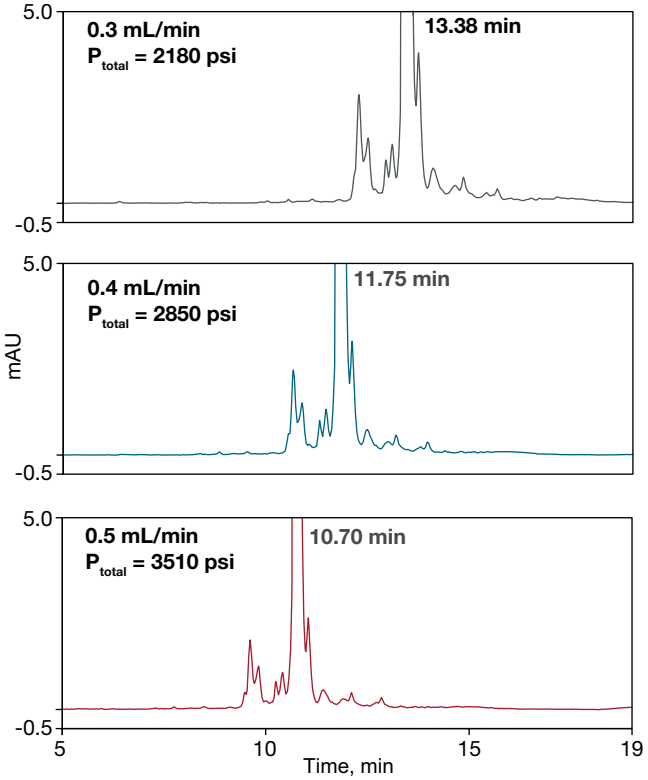


Figure 6. Effect of flow rate on the separation of Protein G and associated charge variants

For protein G, the PWHH of the main protein peak showed minor broadening from 0.25 min at 0.5 mL/min to 0.28 min at 0.3 mL/min. for the gradient time and loading masses tested. At higher flow rates, the peak-to-valley is slightly maximized at 0.5 mL/min; however, overall peak spread is greatest at 0.3 mL/min. Comparison of the signal strength on the y-axis of the chromatograms in Figure 6 shows that using lower flow rates results in increased signal strength due to a higher sample concentration in the detector. Because of this, lower sample mass loading may be used with lower flow rates while still detecting and quantifying the sample peaks. Lastly, the overall column pressure is an important consideration for some users. The total system pressure decreases from ~3,500 psi to ~2,200 psi when going from 0.5 to 0.3 mL/min. As with gradient slope, the conditions selected are dependent on the user's needs. High flow rates would benefit rapid testing QC environments, whereas R&D groups with limited sample may benefit from using a lower flow rate to maximize peak signal especially for low abundance variants.

Temperature effect on chromatography

Protein retention conditions can be altered by changing the column temperature used during separation. For ion exchange chromatography, higher temperatures typically result in elution of proteins later in the gradient compared with lower temperature separations. This contrasts with reverse-phase methods, which typically display weakened interactions with the stationary phase under higher temperature conditions and thus earlier elution times for the same gradient. This effect is largely due to reduced waters of hydration for ionic groups on both the stationary phase and the protein surface. As such, the ionic interactions between the protein and stationary phase are stronger, and higher concentrations of salt are required to disrupt the interactions to elute the protein. Figure 7 illustrates the effects of temperature on the analysis of Protein G, showing elution of Protein G and its variants at higher salt concentrations, with increased temperature and reduced column pressure. Generally, changing the temperature does not significantly alter the selectivity of the separation for protein variants; however, some minor differences in peak separation are commonly observed. This could be due to a minor change in selectivity or possibly changes in protein conformation that provide exposure of typically obscured ionic groups. A benefit of increasing the temperature is reduced column pressure from ~3,000 psi at 30 °C to ~2,260 psi at 60 °C due to reduced viscosity of the mobile phase. However, higher temperatures may also alter the properties of the sample itself, e.g., due to on column sample oxidation. In the absence of knowledge about how temperature may adversely affect one's sample, it is recommended to use 30 °C. The user should take these factors into consideration when designing the method.

Column:	ProPac 3R SAX, 3 µm		
Format:	4 × 100 mm		
Eluents:	A: 20 mM Tris, pH 8.0		
	B: 20 mM Tris + 500 mM NaCl, pH 8.0		
Gradient:	Time (min)	%A	%B
	0.0	88	12
	1.0	88	12
	16.0	58	42
	16.1	0	100
	18.0	0	100
	18.1	88	12
	30.0	88	12
Flow rate:	0.5 mL/min		
Inj. volume:	1 µL		
Temp.:	See chromatograms		
Detection:	UV, 280 nm		
Sample:	Protein G – 5 mg/mL		
Peak label:	Retention time		

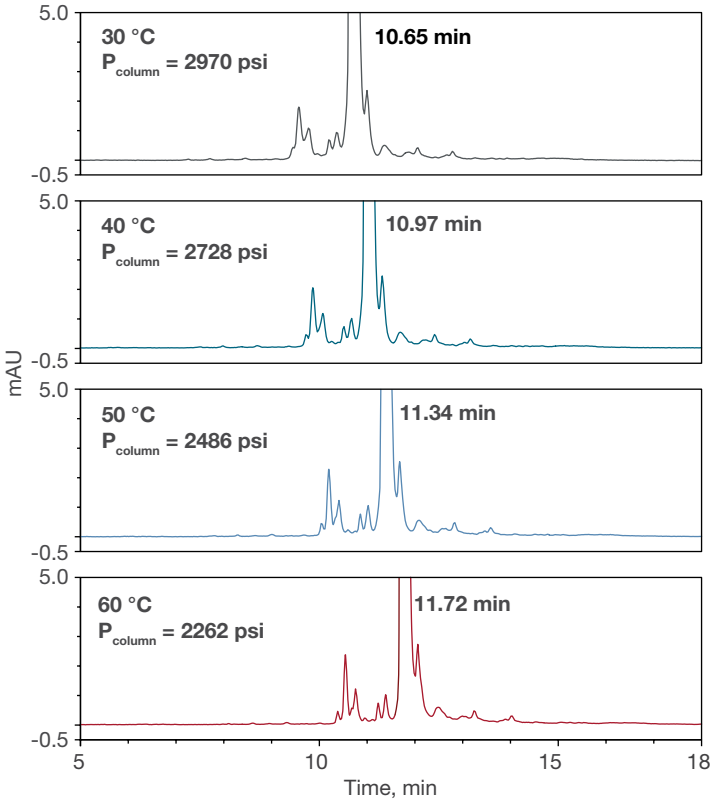


Figure 7. Effect of temperature on the separation of Protein G and associated charge variants and column pressure

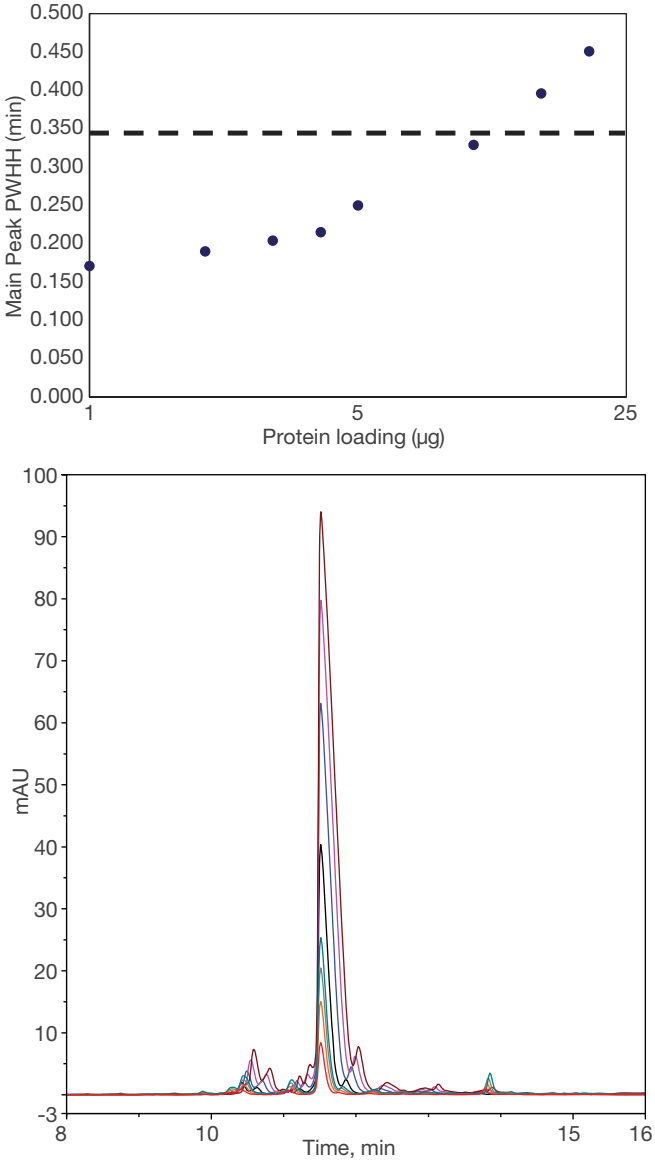


Figure 8. Chromatogram overlays showing the dynamic loading analysis of Protein G using a salt gradient (bottom). The plot on the top shows the corresponding PWHH of the main protein peak versus the masses of Protein G loaded in the chromatogram, dashed line represents 2X PWHH of the lowest mass loaded. Peak retention time is normalized for ease of comparison.

Sample loading and carryover analysis

By themselves, sample concentration and injection volume do not typically influence the gradient separation of a protein and associated variants; however, the total protein loading level can have significant effects on the separation. The chromatograms in the lower part of Figure 8 show 1–20 µg loading of Protein G using sample concentrations of either 1 or 5 mg/mL. As the sample loading amount increases above 5 µg, the stationary phase becomes overloaded, and the analysis of the sample separation 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 top plot of Figure 8 shows the PWHH of the main peak versus the mass of protein loaded. The dashed line in the plot indicates 2X 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. The dynamic loading capacity is protein dependent and can vary depending on protein molecular weight and structure, with lower molecular weight proteins generally having lower dynamic loading capacities due to their high surface area relative to mass. The example provided here can generally be applied to other proteins.

The ProPac 3R SAX 3 µm stationary phase is designed for very low carryover even at high mass loading levels. Figure 9 shows the overlaid chromatograms for a 50 µg injection run using 5 mg/mL Protein G and the following blank run with no injection. The measured carryover in the blank run was only 0.06%. These results demonstrate 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.

Column: ProPac 3R SAX, 3 µm
Format: 4 × 100 mm
Eluents: A: 20 mM Tris, pH 8.0
B: 20 mM Tris + 500 mM NaCl, pH 8.0

Gradient:	Time (min)	%A	%B
	0.0	88	12
	1.0	88	12
	16.0	58	42
	16.1	0	100
	18.0	0	100
	18.1	88	12
	30.0	88	12

Flow rate: 0.5 mL/min
Inj. volume: 10 µL vs. blank
Temp.: 30 °C
Detection: UV, 280 nm
Sample: Protein G – 5 mg/mL

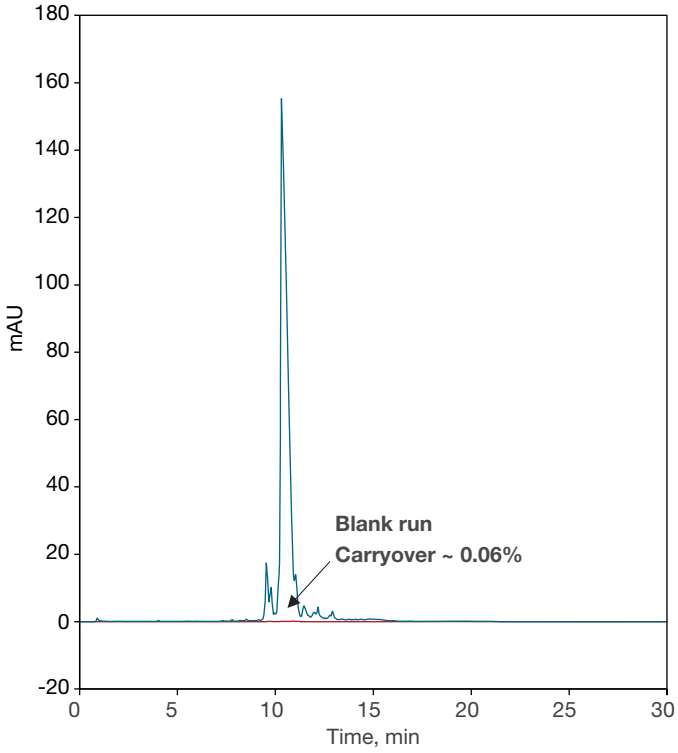


Figure 9. Overlaid chromatograms showing a 50 µg injection and elution of Protein G using a salt gradient and the following blank run without injection to measure carryover

Optimized salt gradient methods – Short vs. long

After determining the appropriate pH and starting salt conditions for gradient analysis, the user can perform iterative analyses of their sample with different gradient slopes, flow rates, temperatures, and loading amounts to achieve the best separation for their particular sample. Using this approach with the analyses provided in the previous sections, we provide here two methods: a fast method with a 6 min gradient at 0.5 mL/min flow rate (Figure 10) and a longer high-resolution

Column: ProPac 3R SAX, 3 µm
Format: 4 × 100 mm
Eluents: A: 20 mM Tris, pH 8.0
B: 20 mM Tris + 500 mM NaCl, pH 8.0

Gradient:	Time (min)	%A	%B
	0.0	88	2
	6.0	58	42
	6.1	0	100
	8.0	0	100
	8.1	88	12
	20.0	88	2

Flow rate: 0.5 mL/min
Inj. volume: 1 µL
Temp.: 30 °C
Detection: UV, 280 nm
Sample: Protein G – 5 mg/mL

Peak label: Retention time

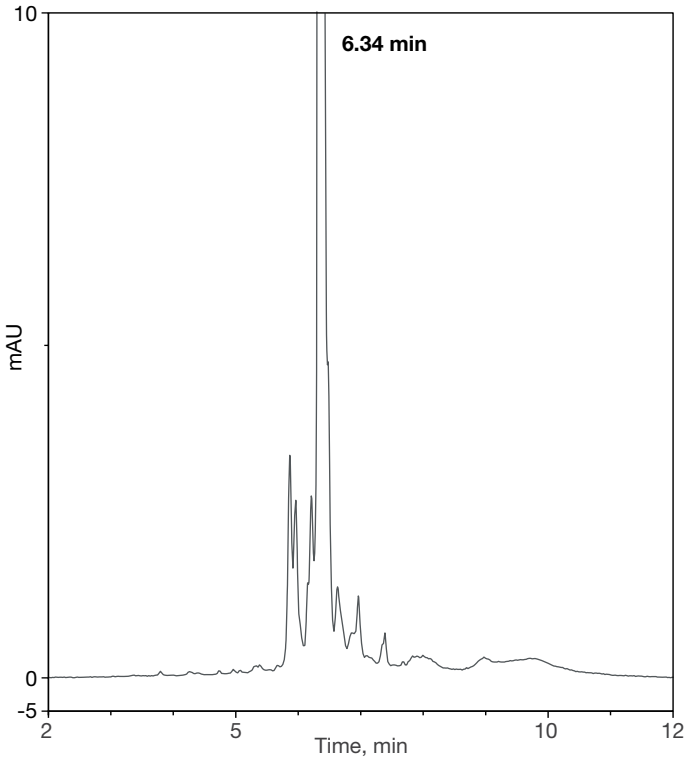


Figure 10. Chromatogram of a fast method with a 6-minute gradient at 0.5 mL/min flow rate

analytical method with a 30 min gradient at 0.3 mL/min flow rate (Figure 11). The fast method would be suitable in a rapid QC environment in which the expected peak profile and peak identities are already known, and the user wants to confirm the quality of their product both qualitatively and quantitatively. The high flow rate reduces time due to gradient delay, while the relatively fast gradient over 6 minutes quickly elutes the protein and associated variants. The high resolution and capacity of the ProPac 3R SAX column provides narrow peaks with sufficient retention time separation to detect the large number of variants associated with Protein G in a short amount of time. For labs looking to achieve high-resolution QC or explicitly identify individual peaks such as in an early development research lab, using the longer gradient method to achieve greater baseline separation of charge variants and the main peak would be preferred. In this approach, the lower flow rate was used to maximize peak signal especially if the user is sample limited. The high resolution and improved column fluidics achieved using the monodisperse particle do not limit the user to using only high flow rates to obtain narrow peaks. If needed, the gradient time could be extended further to achieve even greater peak separation. The longer gradient approach is suitable for explicit quantitation of peaks and/or fraction collection experiments in which the user wishes to analyze the variants in greater detail using orthogonal chromatography methods or assays. The flexibility of use and robust range of operating conditions for the ProPac 3R SAX column enables the user to design methods for a wide range of applications.

Having designed sound, optimized methods as described above for these columns, it is important that the user be able to realize the same performance column-to-column and lot-to-lot. The ProPac 3R technology platform made using monodisperse particles and precision-controlled chemistry makes this possible. Figure 12A shows the analysis of Protein G using the 30-minute gradient optimized method with columns from three different lots of media, with Figure 12B providing an enlarged view at 15 to 25 min. Here we can see robust performance providing excellent reproducibility lot-to-lot to give the same, reliable separation profile for chromatographers.

Column: ProPac 3R SAX, 3 µm
Format: 4 × 100 mm
Eluents: A: 20 mM Tris, pH 8.0
B: 20 mM Tris + 500 mM NaCl, pH 8.0

Gradient:

Time (min)	%A	%B
0.0	88	12
1.0	88	2
31.0	58	42
31.1	0	100
33.0	0	100
33.1	88	12
45.0	88	12

Flow rate: 0.3 mL/min
Inj. volume: 1 µL
Temp.: 30 °C
Detection: UV, 280 nm
Sample: Protein G – 5 mg/mL

Peak label: Retention time

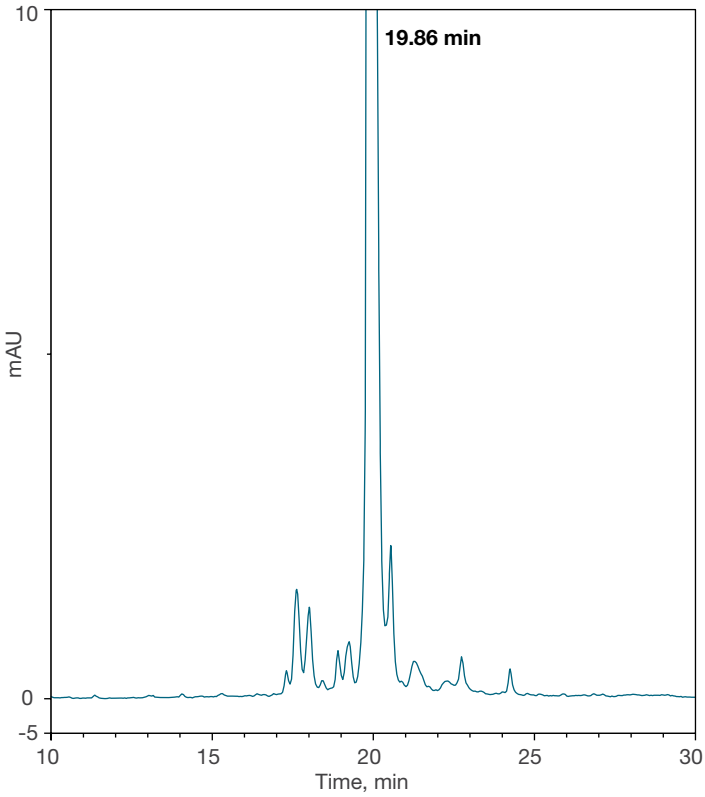
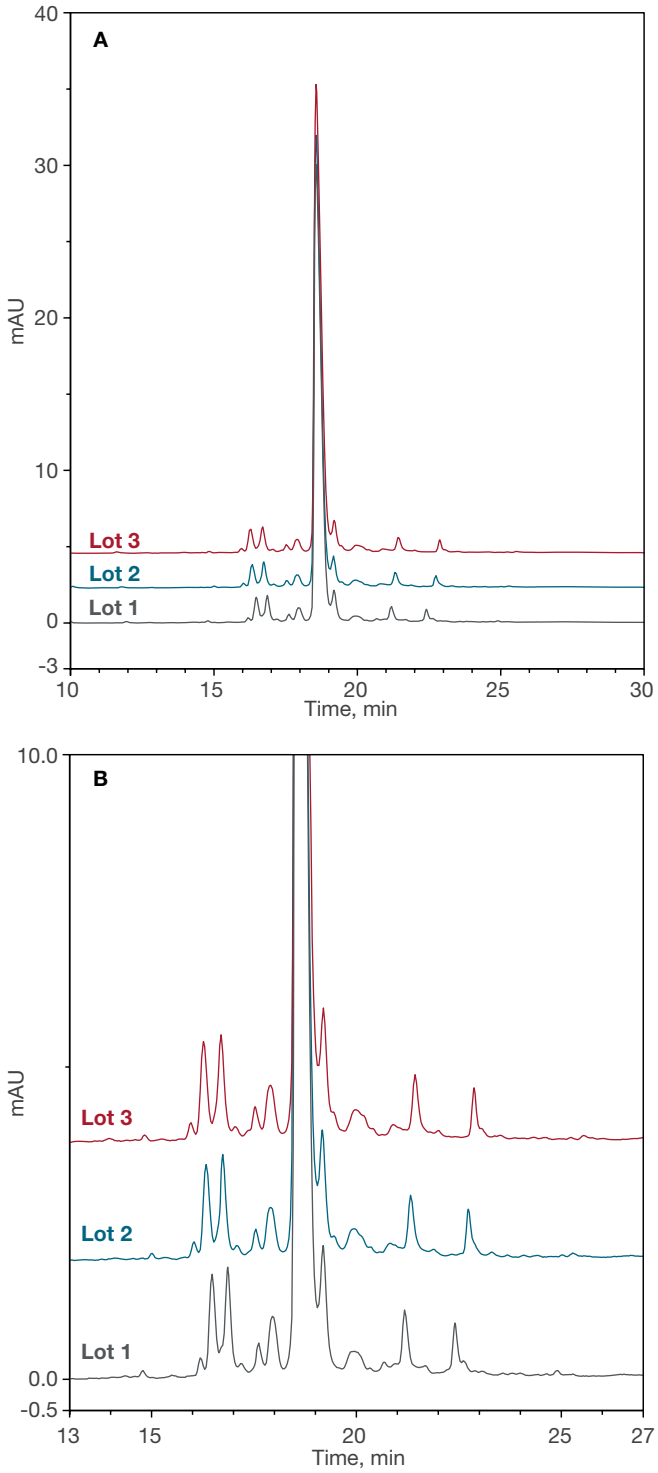


Figure 11. Chromatogram of a longer high-resolution analytical method with a 30-minute gradient at 0.3 mL/min flow rate



Column: ProPac 3R SAX, 3 µm, 3 lots
Format: 4 × 100 mm
Eluents: A: 20 mM Tris, pH 8.0
B: 20 mM Tris + 500 mM NaCl, pH 8.0

Gradient:

Time (min)	%A	%B
0.0	88	12
1.0	88	12
31.0	58	42
31.1	0	100
33.0	0	100
33.1	88	12
45.0	88	12

Flow rate: 0.5 mL/min
Inj. volume: 1 µL
Temp.: 30 °C
Detection: UV, 280 nm
Sample: Protein G – 5 mg/mL

Figure 12. (A) Chromatograms of three different lots of ProPac SAX columns with a 30-minute gradient at 0.3 mL/min flow rate and (B) an enlarged view of the chromatograms. The retention time and signal of the main peak are normalized for ease of comparison of variant separation.

Stressed sample evaluation

To demonstrate the utility of the ProPac 3R SAX column, we provide a practical example evaluating Protein G in its native form against a Protein G sample that has been stressed at 40 °C for 72 hours to induce thermal stress to observe modifications. Figure 13 compares these unstressed and stressed Protein G samples. The temperature treatment of the protein results in the presence of more acidic and basic variants with some of the pre-existing variants significantly increasing in abundance. Despite the increase in variants for the stressed sample, the ProPac 3R SAX column maintains excellent resolution of the peaks, enabling easy comparison against the unstressed sample. This type of study can be used to determine the stability conditions for the user's samples and to help determine the identity of specific peaks based on their change under thermal stress.

Column: ProPac 3R SAX, 3 µm
Format: 4 × 100 mm
Eluents: A: 20 mM Tris, pH 8.0
B: 20 mM Tris + 500 mM NaCl, pH 8.0

Gradient:

Time (min)	%A	%B
0.0	88	12
1.0	88	12
16.0	58	42
16.1	0	100
18.0	0	100
18.1	88	12
30.0	88	12

Flow rate: 0.5 mL/min
Inj. volume: 1 µL
Temp.: 30 °C
Detection: UV, 280 nm
Sample: Protein G – 5 mg/mL
Top: Non-stressed
Bottom: Thermally stressed

Peak label: Retention time

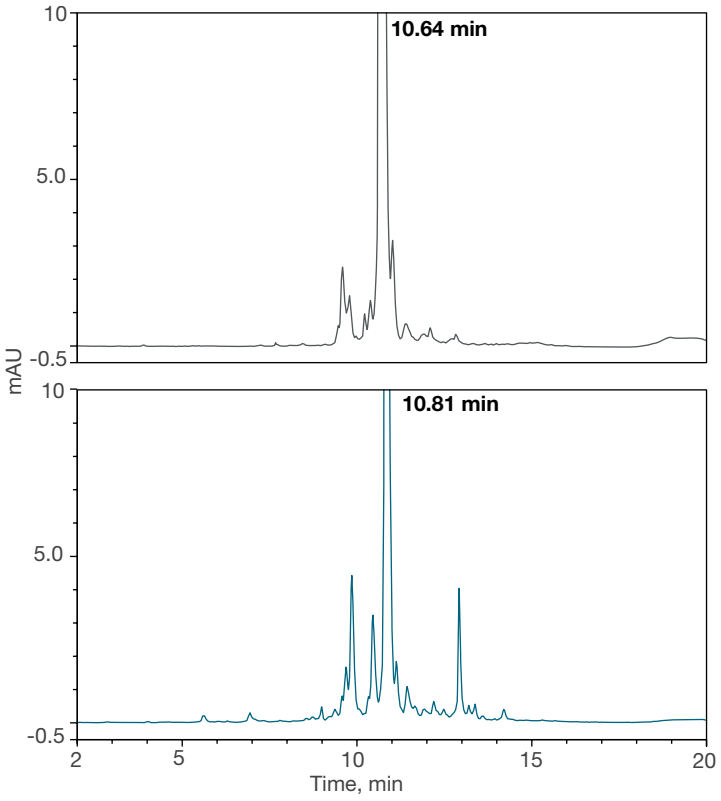


Figure 13. Chromatograms of non-stressed (top) and stressed (bottom) Protein G samples with a 15-minute gradient at a 0.5 mL/min flow rate

Lastly, we provide a comparison with ProPac SAX-10 columns in Figure 14 to demonstrate the superiority of the new ProPac 3R SAX column. For the ProPac SAX-10 column, the flow rate is increased to 1 mL/min to maximize performance and the injection volume is scaled to the column length. For direct comparison of the media for each product, a custom 4 × 100 mm ProPac SAX-10 column (black trace) was packed for this evaluation.

The 4 × 250 mm format (blue trace) is also provided to show the superior performance of the ProPac 3R SAX column despite its shorter length of 100 mm. The results show the significant advantage of the ProPac 3R SAX column (red trace) in the protein and charge variants separation, with narrower peaks and a greater number of basic and acidic variants being detected and resolved.

Columns: Gray: ProPac SAX-10, 10 µm, 4 × 100 mm
Blue: ProPac SAX-10, 10 µm, 4 × 250 mm
Red: ProPac 3R SAX, 3 µm, 4 × 100 mm
Eluents: A: 20 mM Tris, pH 8.0
B: 20 mM Tris + 500 mM NaCl, pH 8.0

Gradient:

Time (min)	%A	%B
0.0	88	12
1.0	88	12
31.0	58	42
31.1	0	100
33.0	0	100
33.1	88	12
45.0	88	12

Flow rate: Gray: 1.0 mL/min
Blue: 1.0 mL/min
Red: 0.3 mL/min
Inj. volume: Gray: 1 µL
Blue: 2.5 µL
Red: 1 µL
Temp.: 30 °C
Detection: UV, 280 nm
Sample: Protein G – 5 mg/mL

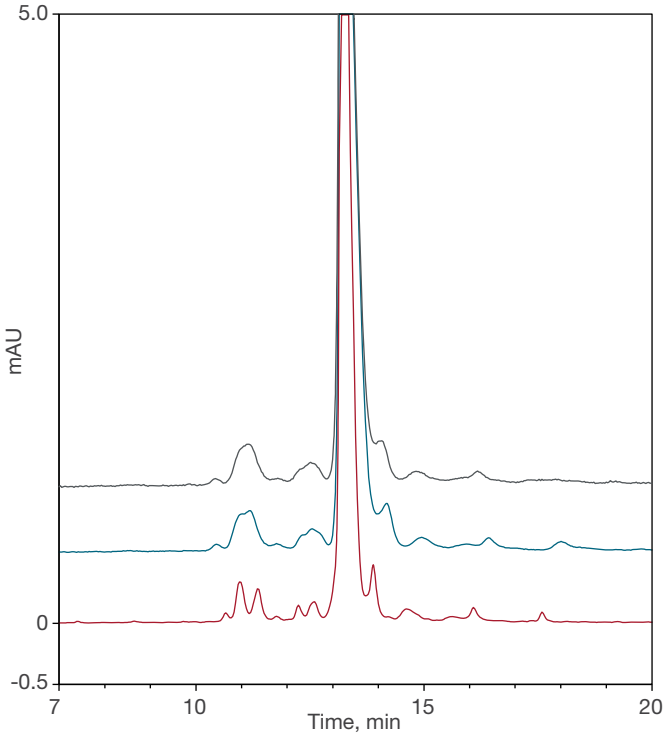


Figure 14. Chromatograms of Protein G using a ProPac SAX-10 4 × 100 mm column (gray), ProPac SAX-10 4 × 250 mm column (blue), and a ProPac 3R SAX 4 × 100 mm column (red) separately. The retention time and signal of the main peak are normalized for ease of comparison of variant separation.

Conclusion

- The ProPac 3R SAX 3 µm column provides excellent separation of Protein G charge variants using a salt gradient. Its unique design provides high resolution, robust performance, and excellent reproducibility needed for charge variant analysis.
- Buffer pH, gradient salt concentration, gradient slope, flow rate, temperature, and sample loading amounts can be optimized to provide consistent, high-resolution variant separation.

References

1. Rivera, B.; Anspach, J.A.; Rao, S. *LCGC Supplements,* **2018,** *36*(6), 24–29.
2. Taha, M.; e Silva, F.; Quental, M.; Ventura, S.M.; Freire. M.; Coutinho, J.P. *Green Chemistry,* **2014,** *16*(6), 3149–3159.

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

AN001695-EN 0323S