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# Mobile Phase Optimization in SEC Method Development

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## Abstract

Aggregation of monoclonal antibody (mAb) products has an adverse effect on their efficacy as therapeutic agents. Size exclusion chromatography (SEC) is a highly effective method for the detection of aggregates in therapeutic proteins and peptides. This Application Note describes method optimization in the use of Agilent AdvanceBio SEC columns for the characterization of monomers, dimers, and aggregates in standard protein mixtures.

# Introduction

Monoclonal antibody products such as mAbs, antibody drug conjugates (ADCs), Fc-fusion proteins, antibody fragments, and other therapeutic proteins and peptides treat many diseases and medical conditions. mAb products have rapidly been growing in use in the biopharmaceutical industry. Worldwide sales are predicted to reach 125 billion US dollars by 2020[1].

The manufacturing and production of therapeutic proteins is a highly complex process that may include more than 5,000 critical process steps[2]. Aggregation in the production of mAb products negatively affects their efficacy and safety, and results in drug approval failures[3,4,5]. Therefore, the amount of aggregation present in a protein or peptide is a critical quality attribute (CQA) in the biopharmaceutical industry. SEC is considered the gold standard analytical method for the detection of monomers, dimers, aggregates, and degradation products in therapeutic proteins and peptides[3].

AdvanceBio SEC columns are packed with highly uniform 2.7 µm particles, containing a low-binding, polymer-coated, silica stationary phase that provides efficient separations and minimal nonspecific interactions. These columns can be used with many mobile phases containing various concentrations of buffers and salts.

Optimizing buffer and salt concentrations is a key part of maximizing the quality of separations performed on the column to prevent secondary interactions that produce peak tailing, distorted peak shapes, poor resolution, and mobile phase-induced aggregation of proteins[3]. The columns can also be used in tandem for increased resolution. This Application Note demonstrates the effects of different mobile phase compositions in the chromatography of proteins of various sizes and chemistries. Isopropyl alcohol was not used in the mobile phase compositions to decrease secondary interactions, as it is unnecessary to use this with AdvanceBio SEC columns.

# Materials and Methods

## Samples

- IgG from human serum, Sigma, I4506-10MG

- SigmaMAb Antibody Drug Conjugate (ADC) Mimic, Sigma, MSQC8-0.5MG

- Insulin from bovine pancreas, Sigma, I5500

- Cytochrome c from bovine heart, Sigma, C3131-50MG

- Gel filtration standard, Bio-Rad, 1511901

- IgG from bovine serum, Sigma, I9640

- Myoglobin from equine heart, Sigma, M1882

- Pullulan polysaccharide standards kit SAC-10, Agilent, p/n PL2090-0100

- Agilent vial, screw top, amber, certified, 2 mL, 100/pk. Vial size: 12 × 32 mm (12 mm cap) (p/n 5188-6535)

- Agilent screw cap, bonded, blue, PTFE/white silicone septa, 100/pk. Cap size: 12 mm (p/n 5190-7021)

## Columns

- Agilent AdvanceBio SEC 300 Å, 7.8 × 300 mm, 2.7 µm (p/n PL1180-5301)

- Agilent AdvanceBio SEC 130 Å, 7.8 × 300 mm, 2.7 µm (p/n PL1180-5350)

## LC system

- Agilent 1260 Infinity bio-inert quaternary pump (G5611A)

- Agilent 1260 Infinity bio-inert autosampler (G5667A)

- Agilent thermostat for 1200 ALS/fraction collector (G1330B)

- Agilent 1290 Infinity thermostatted column compartment (G1316C)

- Agilent 1260 Infinity DAD detector (G1315C)

## Instrument conditions

Buffers to be specified at a flow rate of 1 mL/min, 25 °C column temperature, chiller set at 4 °C, 220 nm wavelength.

# Results and discussion

## Optimizing phosphate buffer concentration

To examine the effects that differing sodium phosphate buffer concentration has on the chromatography of mAb products and other proteins, a series of experiments was conducted. Various sodium phosphate concentrations at pH 7.0, without sodium chloride, were conducted on the following samples: human IgG, an ADC mimic compound, insulin, and cytochrome c. The following concentrations of phosphate buffer were used: 50, 100, 150, 200, 400, and 600 mM. Ten-microliter injections of 1 mg/mL solutions were performed. Also used was 1,000 mM phosphate buffer, which resulted in overpressuring the system with damage to the column, and is not recommended. Figure 1 contains chromatograms for human IgG. Using 400 or 600 mM phosphate buffer concentrations resulted in poor chromatography, as the IgG monomer peak sharpened with decreasing phosphate buffer concentration (Figure 1). Larger aggregate peaks are evident at 400 and 600 mM phosphate concentrations, suggesting mobile phase-induced aggregation of the IgG sample (see the peak at approximately 4.25 minutes in Figure 1), leading to erroneous results. The tailing factors for the monomer decreased from 600 to 100 mM phosphate buffer (Table 1). Peak resolution between the IgG monomer and dimer increased when decreasing the phosphate buffer concentration (Table 2). The lowest tailing factor and highest resolution were achieved at 150 mM phosphate buffer. This is the recommended condition for the IgG sample (Tables 1 and 2).
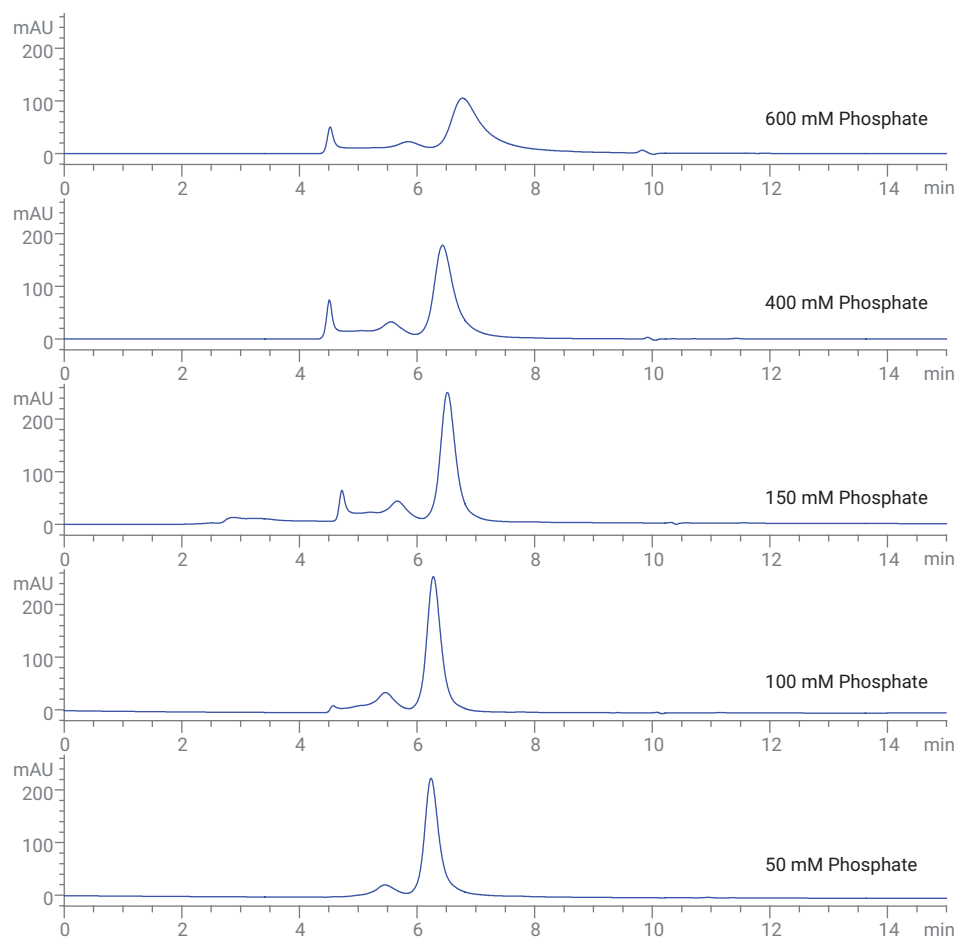


**Figure 1.** IgG separation at different phosphate concentrations.

**Table 1.** Tailing factors versus phosphate concentration.

| Compound | MW (kDa) | pI | 50 | 100 | 150 | 200 | 400 | 600 |
|---|---|---|---|---|---|---|---|---|
| IgG | 150 | 6.8 | 1.33 | 1.12 | 0.73 | 1.25 | 1.31 | 1.94 |
| ADC | 164 | n/a | 1.55 | 1.37 | n/a | 1.56 | 2.48 | n/a |
| Insulin | 5.7 | 5.3 | 2.48 | 2.36 | 2.44 | 2.51 | 1.73 | 1.09 |
| Cytochrome c | 12.3 | 9.3 | 2.1 | n/a | 1.7 | 1.3 | 1.10 | 1.06 |

**Table 2.** Monomer-dimer resolution versus phosphate concentration.

| Compound | MW (kDa) | pI | 50 | 100 | 150 | 200 | 400 | 600 |
|---|---|---|---|---|---|---|---|---|
| IgG | 150 | 6.8 | 1.33 | 1.18 | 1.41 | 1.31 | 1.14 | 0.79 |

**Table 3.** Retention times at different phosphate concentrations.

| Compound | MW (kDa) | pI | 50 | 100 | 150 | 200 | 400 | 600 |
|---|---|---|---|---|---|---|---|---|
| IgG | 150 | 6.8 | 6.24 | 6.28 | 6.29 | 7.55 | 6.44 | 6.77 |
| ADC | 164 | n/a | 6.36 | 6.52 | n/a | 6.51 | 6.96 | n/a |
| Insulin | 5.7 | 9.3 | 10.74 | 10.12 | 9.52 | 9.62 | 11.46 | 12.02 |
| Cytochrome c | 12.3 | 5.3 | 9.18 | 10.8 | 9.4 | 8.55 | 8.53 | 8.58 |

The ADC peak split at 600 mM phosphate buffer was wide and tailing, and had a slightly longer retention time due to secondary interactions (Table 3, Figure 2). Decreased tailing factors and improved peak shapes and sizes were measured and observed with decreasing phosphate buffer concentration (Table 1, Figure 2), making the range of 150 to 50 mM phosphate the preferred buffer range.
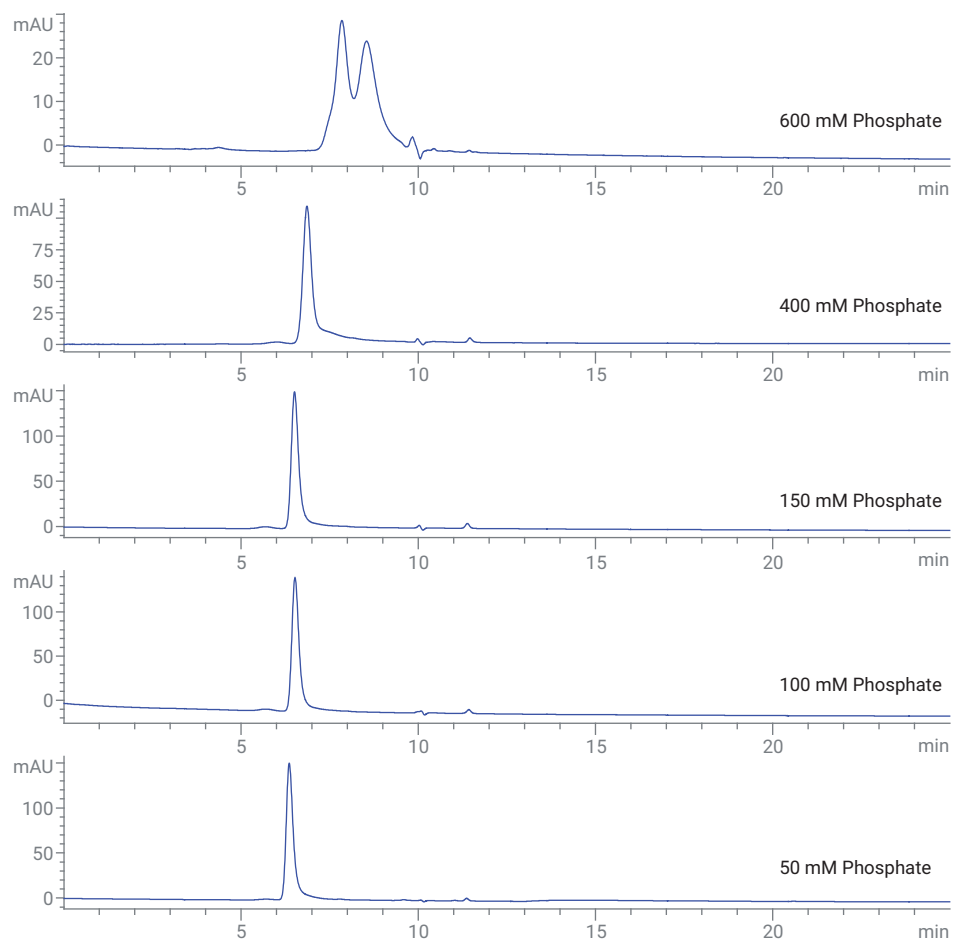


**Figure 2.** ADC chromatography at different phosphate concentrations.

The challenges of obtaining quality chromatography with insulin have been documented, particularly the problems associated with peak tailing and fronting in SEC chromatography of insulin[6]. Distorted peak shapes and poor recovery were observed with insulin at 600 and 400 mM phosphate buffer. Taller, sharper peaks were observed at lower phosphate concentrations, suggesting that these are better buffer concentrations (Figure 3). Tailing factors remained constant along the range of 50 to 150 mM phosphate buffer (Table 1), but shifts in retention time were also measured at 50 and 100 mM phosphate buffer (Table 3). This indicated secondary interactions, suggesting 150 mM phosphate is the preferred mobile phase.
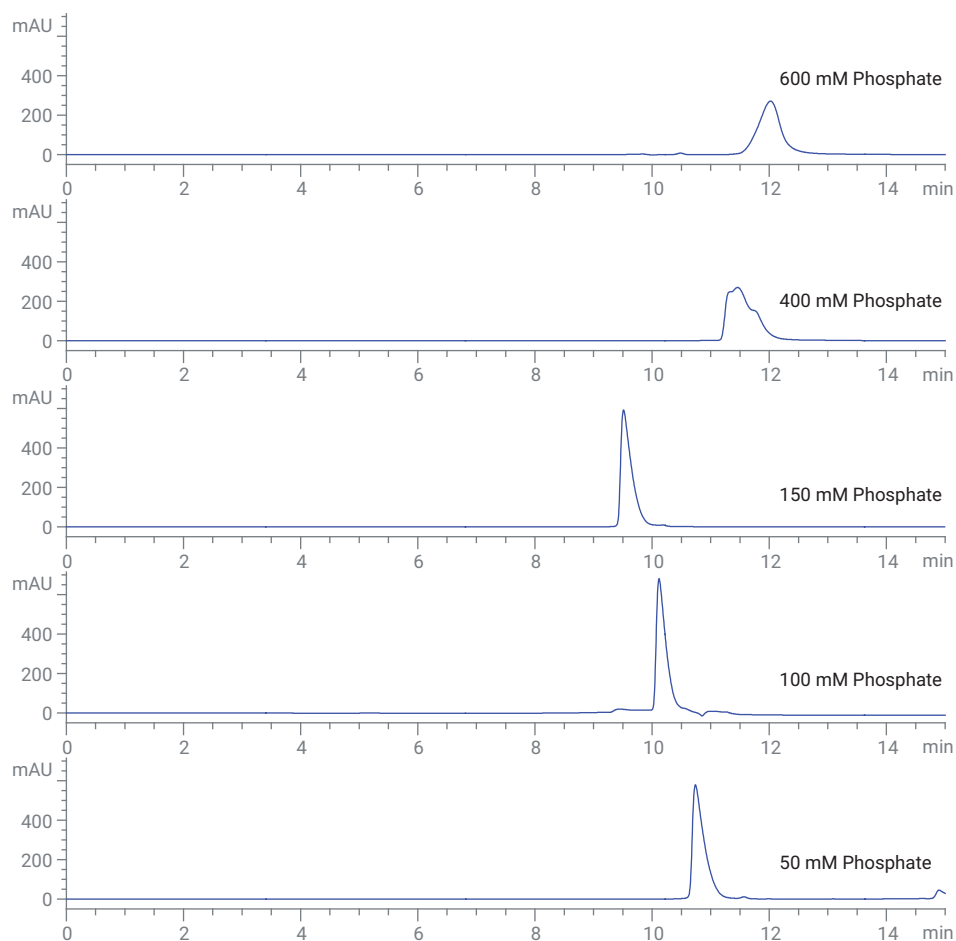


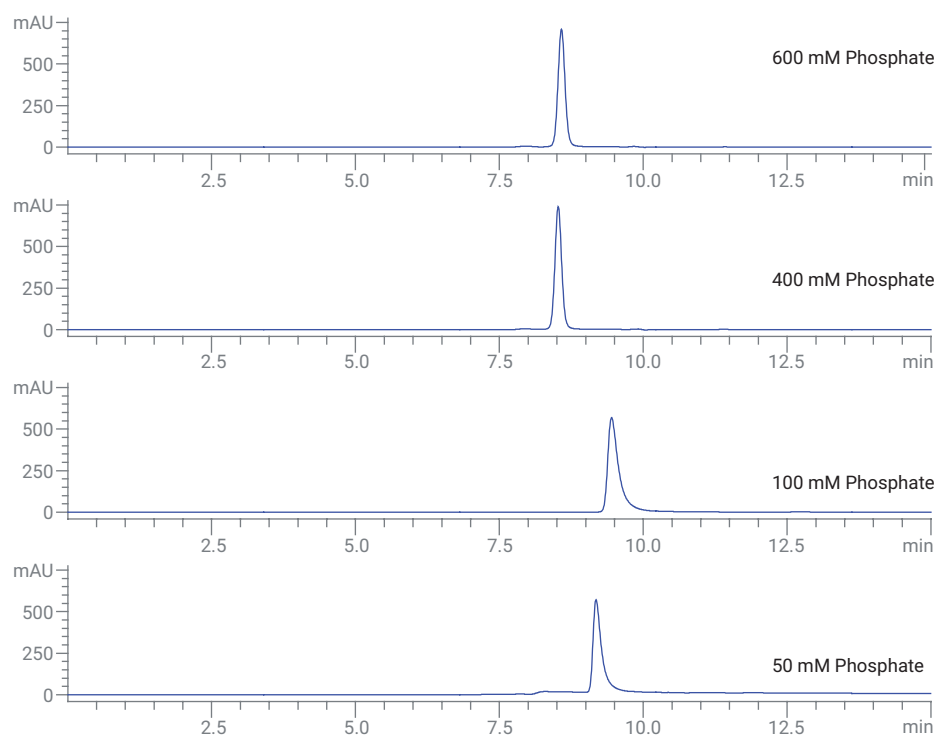**Figure 3.** Insulin chromatography at different phosphate concentrations.

**Table 4.** Tailing factors versus 150 mM phosphate buffer and varying NaCl concentrations.

| Compound | MW (kDa) | pI | 0 | 50 | 100 | 150 | 200 | 500 |
|---|---|---|---|---|---|---|---|---|
| IgG | 150 | 6.8 | 0.73 | 1.16 | 1.19 | 1.19 | 1.21 | 1.62 |
| ADC | 164 | n/a | n/a | 1.51 | n/a | 1.43 | 1.43 | 1.52 |
| Insulin | 5.7 | 5.3 | 2.55 | 2.61 | 2.55 | 2.53 | 2.39 | 2.18 |
| Cytochrome c | 12.3 | 9.3 | 1.7 | 3.32 | 1.27 | 1.11 | 1.07 | 1.02 |

**Table 5.** Resolution of IgG between monomer and dimer at 150 mM phosphate, pH 7.0, 50 to 500 mM NaCl.

| Compound | MW (kDa) | pI | 0 | 50 | 100 | 150 | 200 | 500 |
|---|---|---|---|---|---|---|---|---|
| IgG | 150 | 6.8 | 1.41 | 1.37 | 1.36 | 1.43 | 1.36 | 1.37 |

The experiments were repeated using cytochrome c. The chromatography of cytochrome c, unlike the other proteins studied, had decreasing tailing factors, better peak shapes, and higher peak areas at higher phosphate concentrations (Figure 4, Table 1), indicative of fewer secondary interactions at these concentrations. This suggests that 400 or 600 mM phosphate buffers are the preferred mobile phases for cytochrome c.



**Figure 4.** Cytochrome c chromatography at different phosphate buffer concentrations.

## Optimizing sodium chloride concentrations with phosphate buffer

Another set of experiments was conducted with a constant concentration of 150 mM phosphate buffer (pH 7.0), selected as a concentration and pH of phosphate buffer that historically worked for most proteins, and 50, 100, 150, 200, and 500 mM concentrations of sodium chloride with the same compounds from the previous set of experiments.

IgG had aggregates present in every condition, with the size of the aggregate peaks increasing from 50 to 500 mM NaCl, suggesting that the increased salt concentration caused IgG aggregation (Figure 5). Adding salt to the mobile phase is not recommended with 150 mM phosphate buffer.
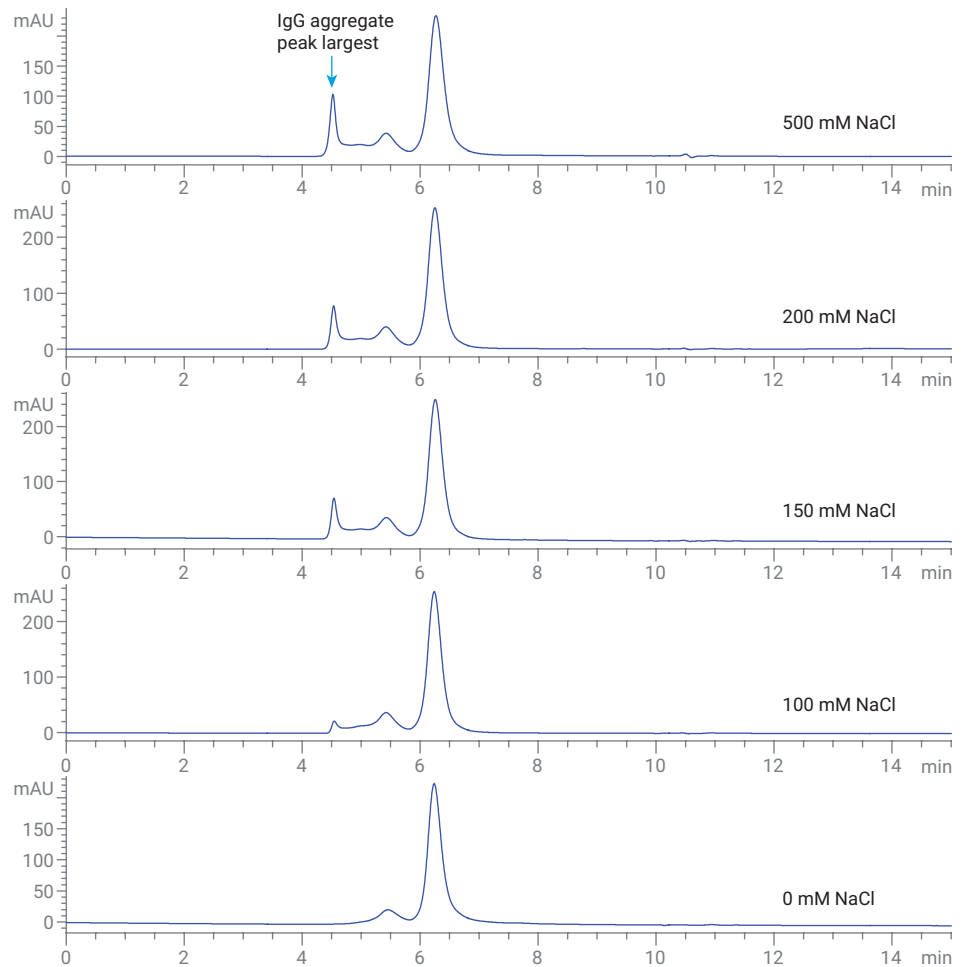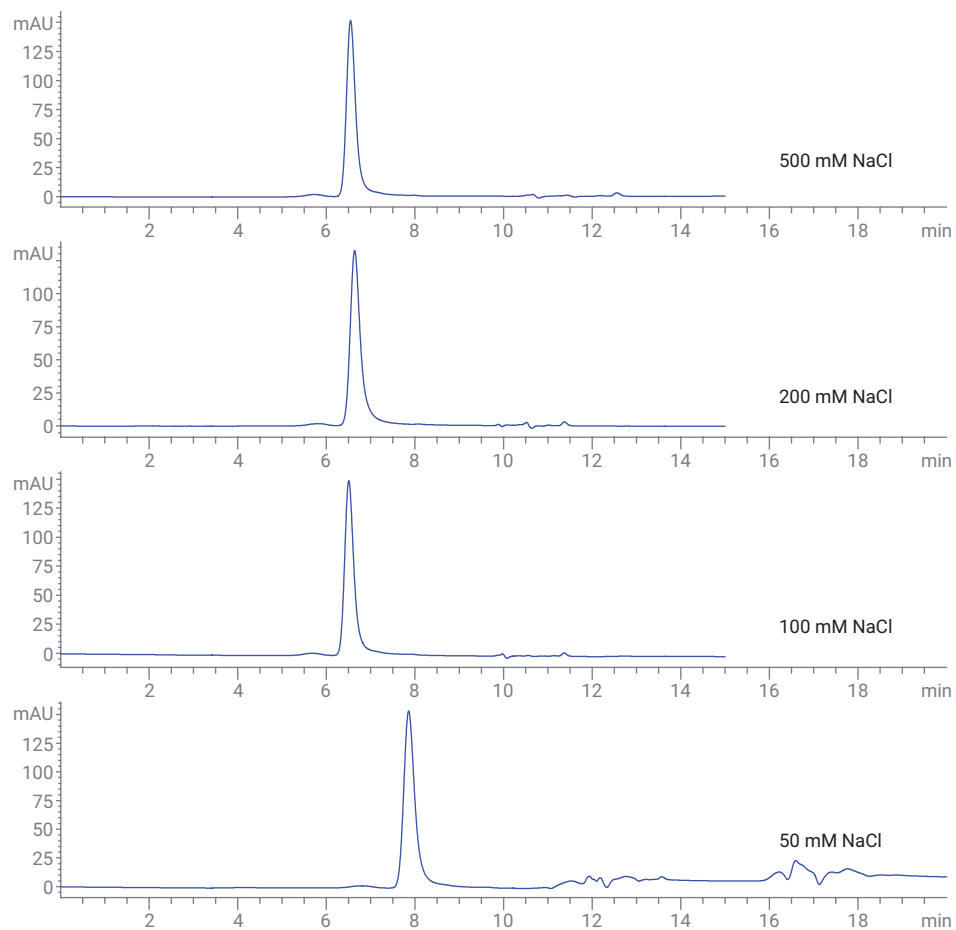


**Figure 5.** IgG, 150 mM phosphate buffer with differing NaCl concentrations.

The ADC mimic peaks were similar at every salt concentration. The tailing factors remained nearly constant across the range of salt concentrations (Figure 6, Table 4). The peak shapes and tailing factors were similar across sodium chloride concentrations, indicating that adding sodium chloride to the mobile phase has negligible effects.



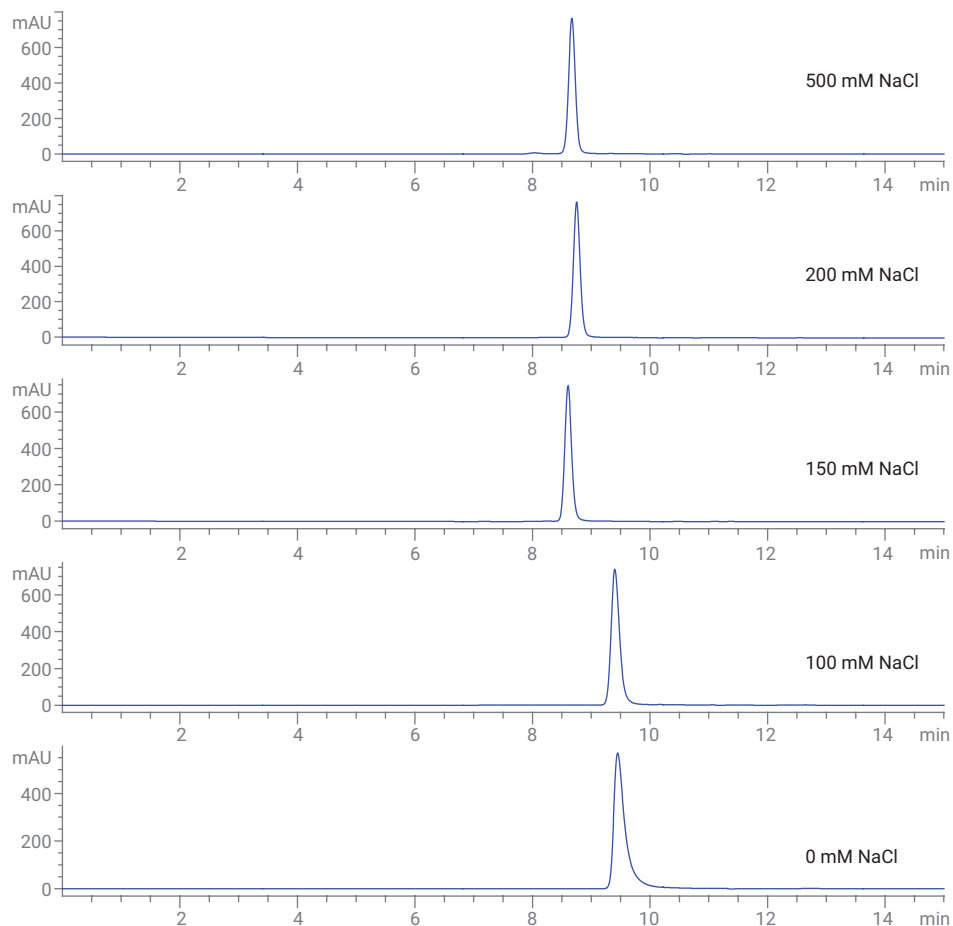**Figure 6.** ADC, 150 mM phosphate buffer with differing NaCl concentrations.

The insulin peak was deformed at 500 mM salt, and had better peak shapes from 200 to 50 mM salt (Figure 7). No improvement in peak shape was obtained by adding salt to the phosphate buffer, suggesting that adding salt to 150 mM phosphate was unnecessary.
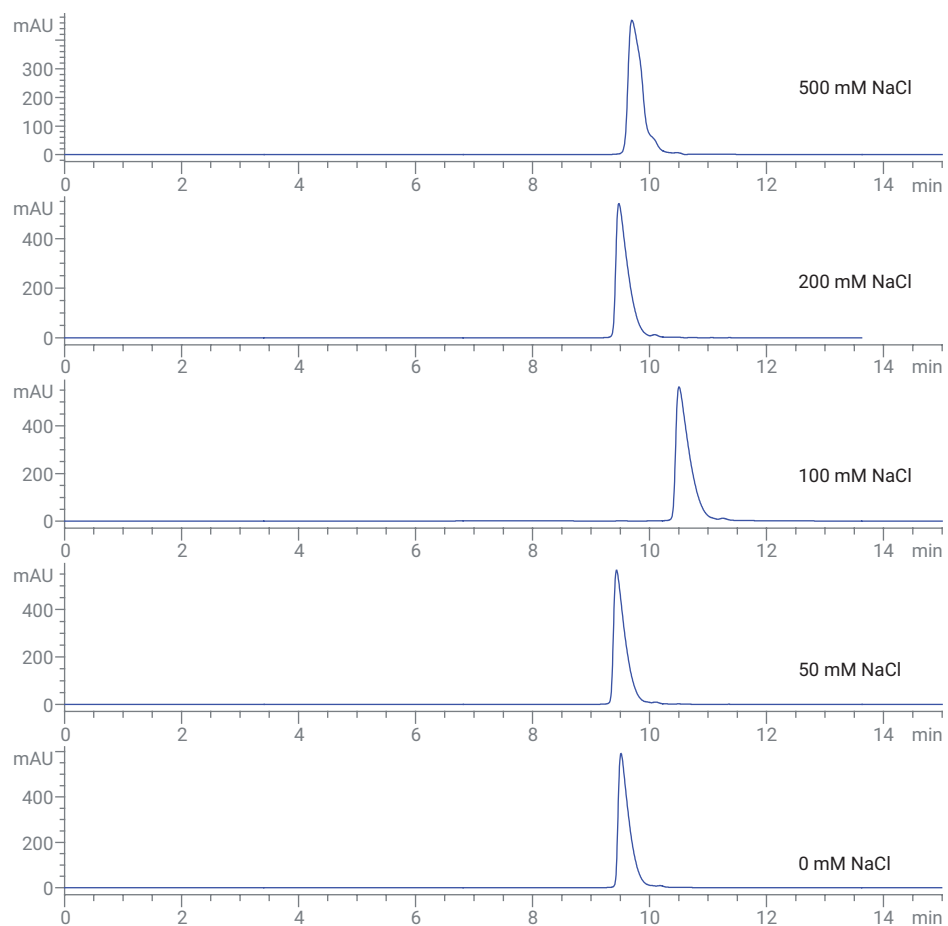


**Figure 7.** Insulin, 150 mM phosphate with differing NaCl concentrations.

The cytochrome c peak was similar in shape and size from 500 to 100 mM NaCl (Figure 8). Tailing factors increased with decreasing salt concentrations (Table 4), and retention times started to increase from 100 to 0 mM salt (Table 6), indicative of secondary interactions. This suggested that 150 mM phosphate with 150 to 500 mM NaCl was the preferred mobile phase composition.



**Figure 8.** Cytochrome c, 150 mM phosphate buffer with differing NaCl concentrations.

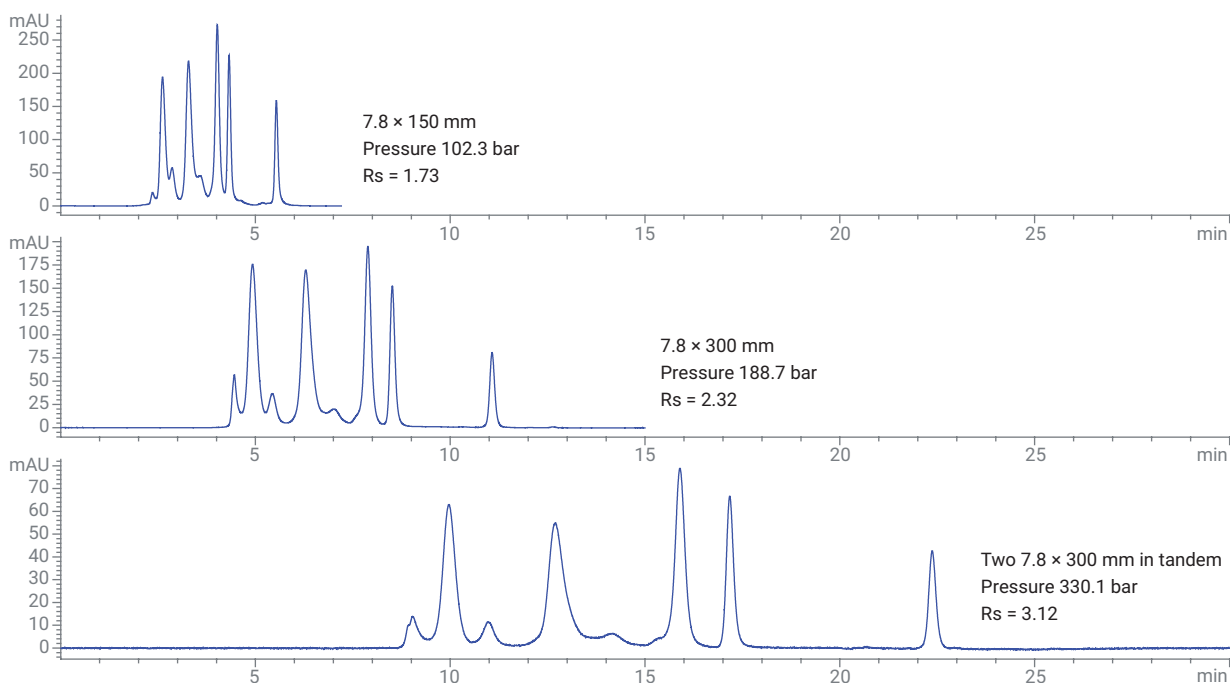**Table 6.** Retention times at 150 mM phosphate and different NaCl concentrations.

| Compound | MW (kDa) | pI | 0 | 50 | 100 | 150 | 200 | 500 |
|---|---|---|---|---|---|---|---|---|
| IgG | 150 | 6.8 | 6.77 | 6.27 | 6.31 | 6.28 | 6.29 | 6.32 |
| ADC | 164 | n/a | n/a | 7.56 | 6.51 | n/a | 6.55 | 6.64 |
| Insulin | 5.7 | 5.3 | 9.52 | 10.29 | 9.43 | 10.51 | 9.48 | 9.7 |
| Cytochrome c | 12.3 | 9.3 | 9.4 | 8.72 | 9.4 | 8.61 | 8.75 | 8.67 |

## Columns in tandem: column length

Column lengths should be based on resolution and throughput needs. Figure 9 shows separation of the Bio-Rad gel filtration standard with increasing AdvanceBio SEC 300 Å column lengths: 7.8 × 150 mm, 7.8 × 300 mm, and two 7.8 × 300 mm columns connected in tandem. The degree of separation increased with column length, which also extended analysis time and increased system pressure.



7.8 × 150 mm
Pressure 102.3 bar
Rs = 1.73

7.8 × 300 mm
Pressure 188.7 bar
Rs = 2.32

Two 7.8 × 300 mm in tandem
Pressure 330.1 bar
Rs = 3.12

**Figure 9.** Chromatographic separation of Bio-Rad gel filtration standard with different lengths of AdvanceBio SEC 300 Å columns.

## Columns in tandem: pore size

The Bio-Rad gel filtration standard was also separated by the AdvanceBio SEC 130 Å and AdvanceBio SEC 300 Å columns individually, and connected in tandem. The resolution between ovalbumin and myoglobin was increased when the columns were used in tandem (Figure 10), likely due to the increase in overall column length. The largest proteins in the mixture are excluded from the 130 Å column, as the pores are too small for these proteins. IgG from bovine serum and myoglobin was also analyzed using the 130 and 300 Å AdvanceBio SEC columns individually, and connected in tandem. The larger molecular weight IgG (150 kDa) and the dimer are not resolved by the AdvanceBio SEC 130 Å, but are resolved by the AdvanceBio SEC 300 Å. No improvement in resolution between the monomer and dimer was achieved by analyzing the sample on the two columns connected in tandem (Figure 11).
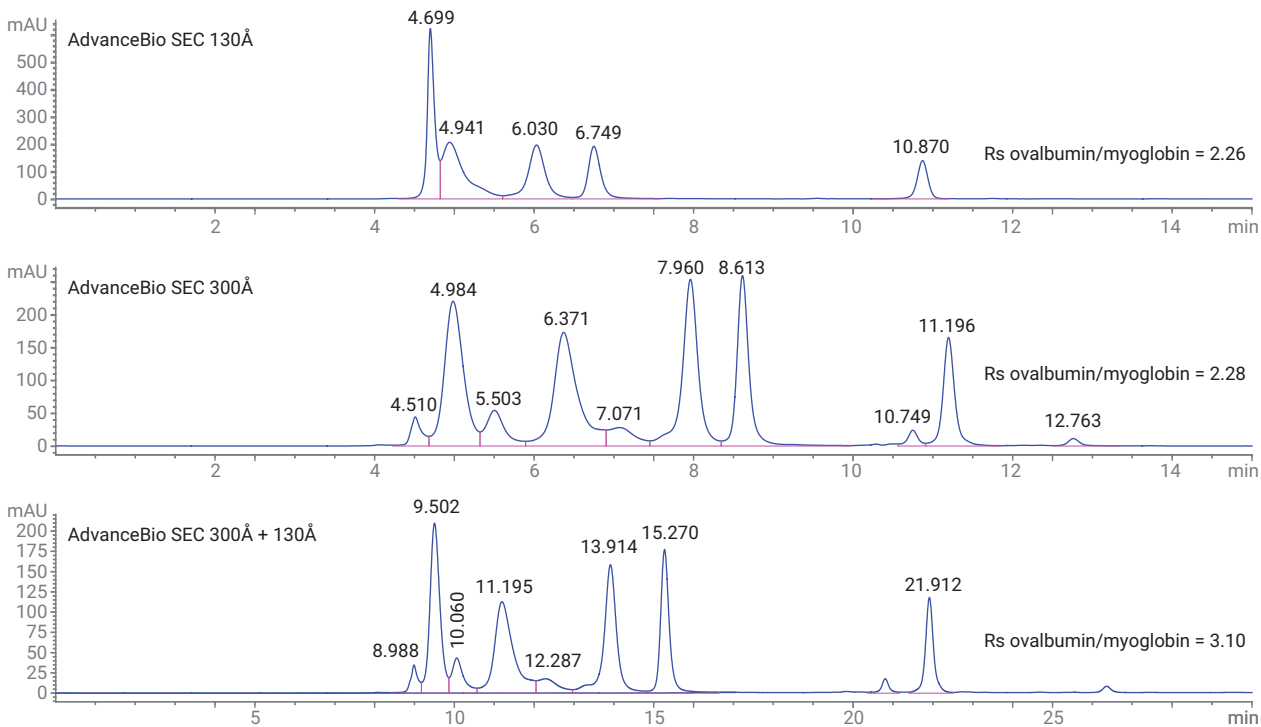


**Figure 10.** Separation of Bio-Rad gel filtration standard using 130 Å and 300 Å columns individually and in tandem.

Both columns also separated the smaller myoglobin molecule individually, and the resolution was improved by running the sample on the two columns connected in tandem (Figure 12), though this improvement may be likely due to increased column length. One final set of experiments was conducted with polysaccharides using AdvanceBio SEC 130 Å and 300 Å columns run individually, and connected in tandem. The larger polysaccharides only showed some resolution when the two columns were connected in tandem (Figure 13).
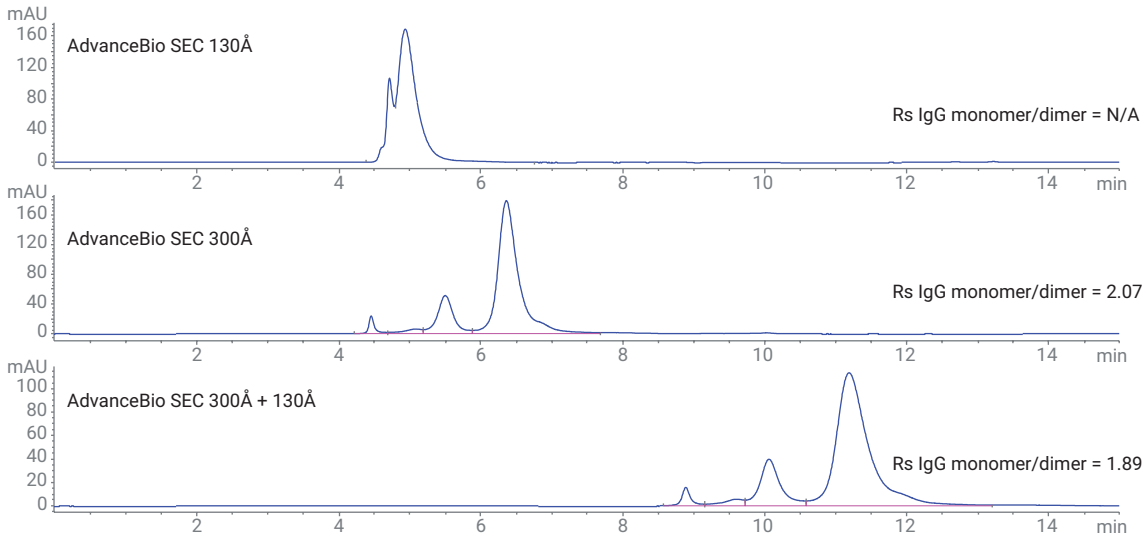


**Figure 11.** Separation of IgG from bovine serum using 130 Å and 300 Å columns individually and in tandem.
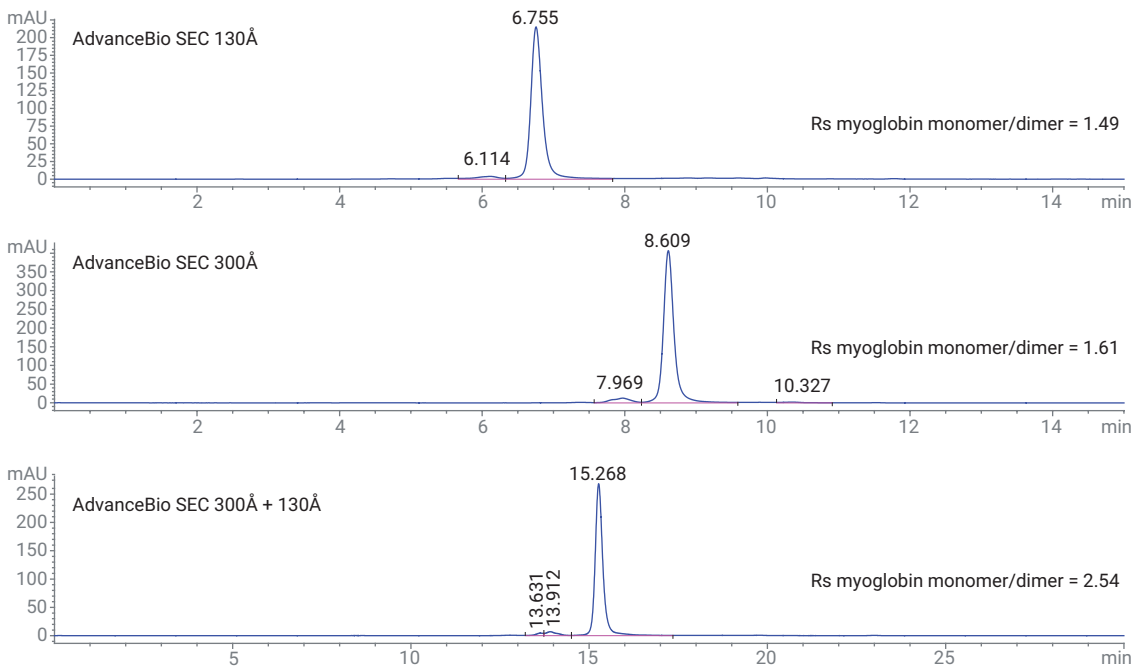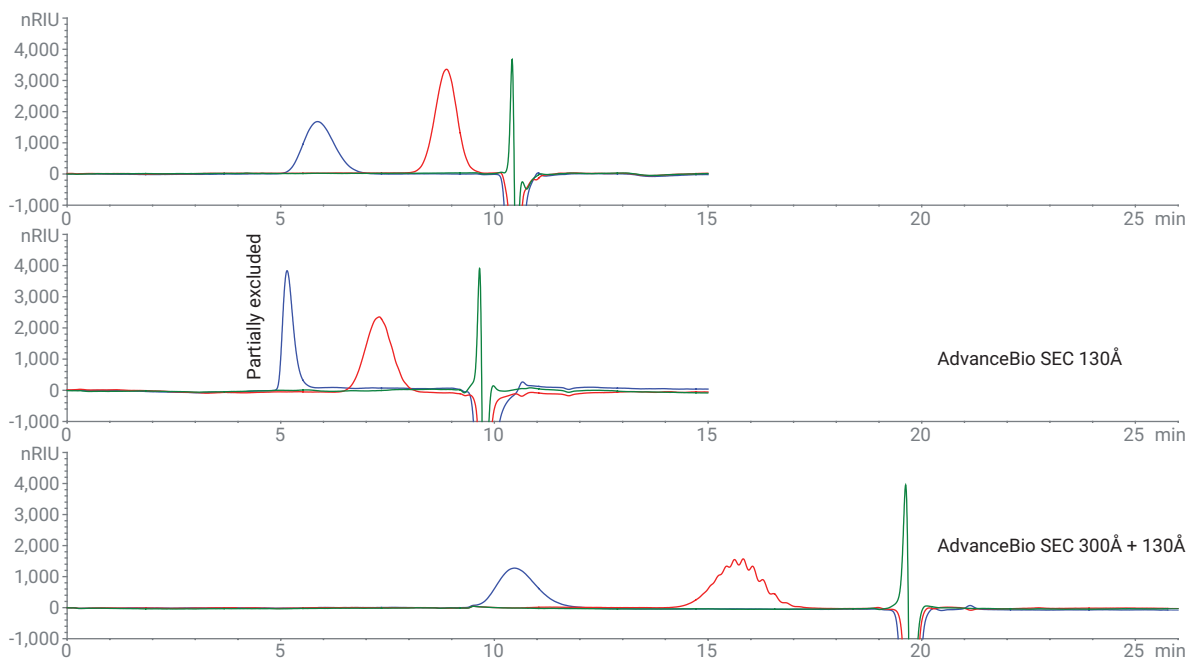


**Figure 12.** Separation of myoglobin using 130 Å and 300 Å columns individually and in tandem.

**Figure 13.** Polysaccharide sample separated by AdvanceBio SEC 300 Å and 130 Å columns individually and in tandem.

## Conclusion

Mobile phase optimization is a fundamental and critical element of method development to create procedures that effectively minimize secondary interactions between proteins and the column, which can cause peak tailing, peak shape distortion, poor sample recovery, and poor resolution. It also minimizes aggregation of the proteins, which causes erroneous results. Buffer selection should also use the least amount of buffer necessary, and salt should only be added if needed. This will reduce risk of corrosion and wear and tear, and extend the lifetime of the column. The use of AdvanceBio SEC columns with optimized methods is an integral tool in the characterization of monomers, dimers, and aggregates, which are critical quality attributes in the biopharma industry.

## References

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