

Advantages of Agilent AdvanceBio SEC Columns for Biopharmaceutical Analysis

Comparing Columns from Different Vendors to Improve Data Quality

Technical Overview

Introduction

Size exclusion chromatography (SEC) is a standard technique for determining molar mass averages, and is often used to describe size-based separation of biomolecules. In SEC, separation efficiency comes only from the stationary phase, while the mobile phase should have no effect. Therefore, column selection for a given biomolecule is crucial for the successful application of SEC. There are different types of SEC columns commercially available that come in various dimensions, porosities, and particle sizes. Agilent AdvanceBio SEC columns incorporate breakthrough technology for SEC analysis. The columns have been designed and manufactured by Agilent to give maximum efficiency without risk of shear degradation of samples, or clogging between particles. The unique method of manufacture controlling pore size, structure, and volume, and then applying a hydrophilic polymeric coating ensures that protein peaks are sharp and well resolved.

This Technical Note compares SEC columns from different vendors using a mobile phase commonly used for a separation with protein standards, monoclonal antibodies, and an antibody drug conjugate (ADC). We wanted to identify differences in column performance that could impact the quality of the data being generated. We showcase the unique advantage of AdvanceBio SEC columns for high-resolution separation of biomolecules for biopharmaceutical applications.



Agilent Technologies

Materials and Methods

Columns

7.8 × 300 mm

- Agilent AdvanceBio SEC 300Å, 2.7 μm
- Other vendor A 200Å, 3.5 μm
- Other vendor B 450Å, 3.5 μm
- Other vendor C 250Å, 5 μm
- Other vendor D 250Å, 4 μm
- Other vendor E 300Å, 3 μm

4.6 × 300 mm

- Agilent AdvanceBio SEC 300Å, 2.7 μm
- Other vendor F 450Å, 2.5 μm
- Other vendor G 200Å, 1.7 μm

Results and Discussion

Comparison of 7.8 × 300 mm SEC columns for robust SEC-DAD methods

Separation of protein standards

Figure 1 compares the resolution of a six-protein reference standard on columns with different pore sizes, and clearly shows the effect of pore size on resolution. The largest proteins, thyroglobulin and its monomer, have good resolution with 450Å and 300Å pores. However, as the pore size decreases, the largest proteins are excluded and resolution is poor. While the largest proteins were better resolved on the 450Å column B and 300Å column E, both columns delivered broader peak widths, which makes quantitation of low levels of aggregation more difficult and less accurate. The AdvanceBio SEC column improved resolution of high, medium, and low weight biomolecules, which is critical for separation of many of them (Figure 1). AdvanceBio SEC also provided improved sensitivity, narrow peak width, and lower elution volume.

Table 1. Chromatographic conditions.

Parameter	7.8 × 300 mm SEC columns	4.6 × 300 mm SEC columns
Flow rate	0.8 mL/min	0.4 mL/min
Mobile phase	150 mM phosphate buffer, pH 7.0	150 mM phosphate buffer, pH 7.0
Instrument	Agilent 1260 Infinity Bio-inert Quaternary LC	Agilent 1290 Infinity Binary LC with G1315D DAD and Bio-inert flow cell (1200 bar)
Injection volume	5 μL	5 μL
TCC	Ambient	Ambient
Detection	UV, 220 nm	UV, 220 nm
Samples	<ul style="list-style-type: none"> • Protein molecular weight markers (thyroglobulin aggregate, thyroglobulin, IgG, bovine serum albumin, myoglobin), and uracil total permeation marker • Therapeutic monoclonal antibodies (rituximab and trastuzumab) • Therapeutic antibody drug conjugate 	

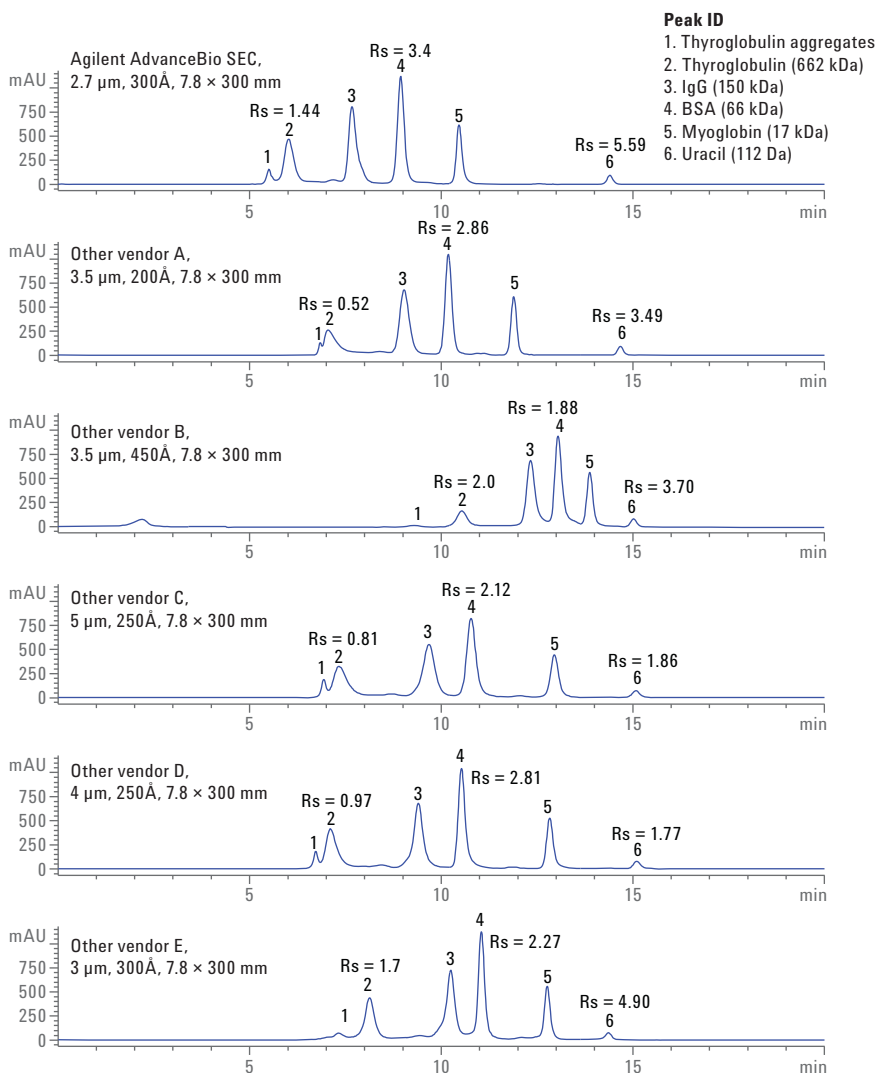


Figure 1. Comparison of SEC profiles of protein standards on 7.8 × 300 mm SEC columns. The resolution between peaks 1 and 2, 3 and 4, and 5 and 6 were calculated.

To evaluate the effect of pore size on the separation of a range of protein markers, calibration curves for each column were plotted (Figure 2). Molecules larger than the pore size are excluded, and smaller molecules penetrate the pores to define the exclusion limit and permeation range of a column. An ideal SEC column is one that separates biomolecules in the most linear part of the calibration curve. When we evaluated columns for the separation of the same protein range, AdvanceBio SEC provided a linear and shallow slope for most of the resolution curve. This performance resulted in improved separation of protein markers within the molecular weight range 662 kDa to 112 Da, the range of most protein-based biotherapeutics.

Comparing pore volumes

Peak capacity and resolution in SEC are primarily determined by pore volume. Larger pore volume provides better resolution across a wider molecular weight range. Table 2 shows the pore volumes calculated from the total permeation point (uracil) and exclusion limit (thyroglobulin aggregate) for the columns. AdvanceBio SEC provided higher pore volume (7.11 mL) compared to the other columns, and thus provided better resolution and higher peak capacity.

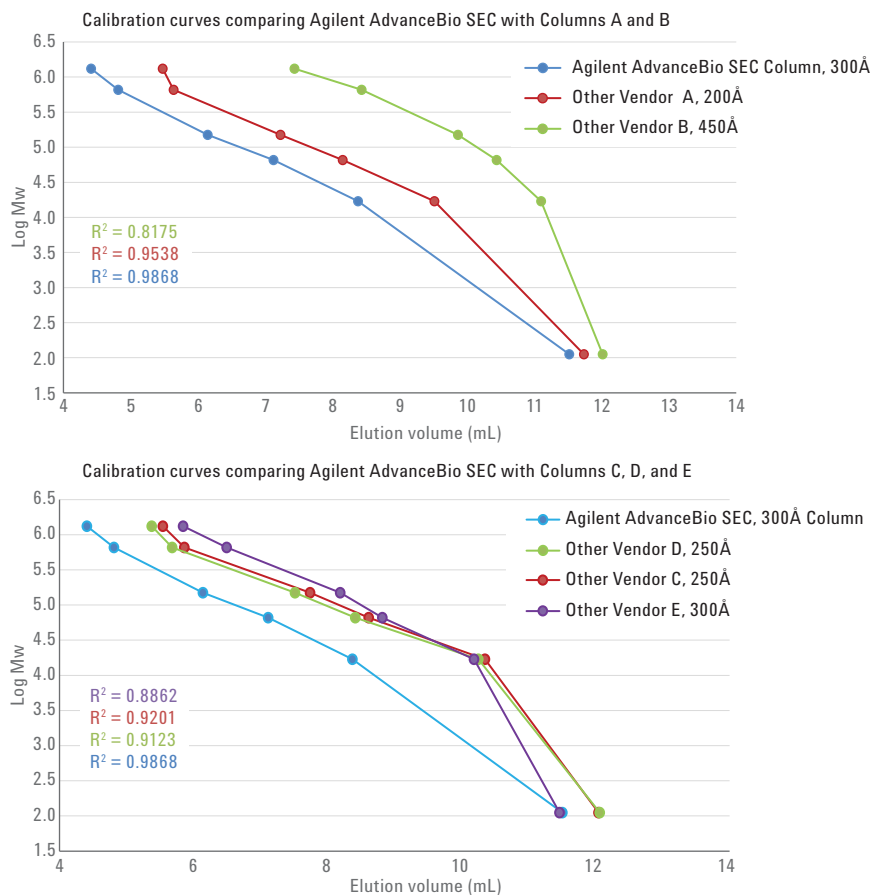


Figure 2. Calibration curves for protein standards on various 7.8 × 300 mm SEC columns.

Table 2. Total column pore volume comparison.

Column	Pore volume (mL)
7.8 × 300 mm	
Agilent AdvanceBio SEC 300Å, 2.7 μm	7.11
Other vendor A 200Å, 3.5 μm	6.25
Other vendor B 450Å, 3.5 μm	4.57
Other vendor C 250Å, 5 μm	6.51
Other vendor D 250Å, 4 μm	6.70
Other vendor E 300Å, 3 μm	5.62
4.6 × 300 mm	
Agilent AdvanceBio SEC 300Å, 2.7 μm	2.36
Other vendor F 450Å, 2.5 μm	2.06
Other vendor G 200Å, 1.7 μm	2.20

Separation of therapeutic monoclonal antibodies

Figure 3 shows the improvement in data quality for the separation of therapeutic trastuzumab on the AdvanceBio SEC 2.7 μm column. The chromatogram displays the resolution values of various columns for the separation of monomer and aggregates. AdvanceBio SEC and

column D showed the highest resolution factors of 2.37 and 2.41, respectively, for trastuzumab monomer and its aggregate. Column E, which claims to separate larger proteins or antibody dimers and higher aggregates, failed to resolve the aggregate, and it was not possible to integrate the monomer and aggregate to determine its resolution.

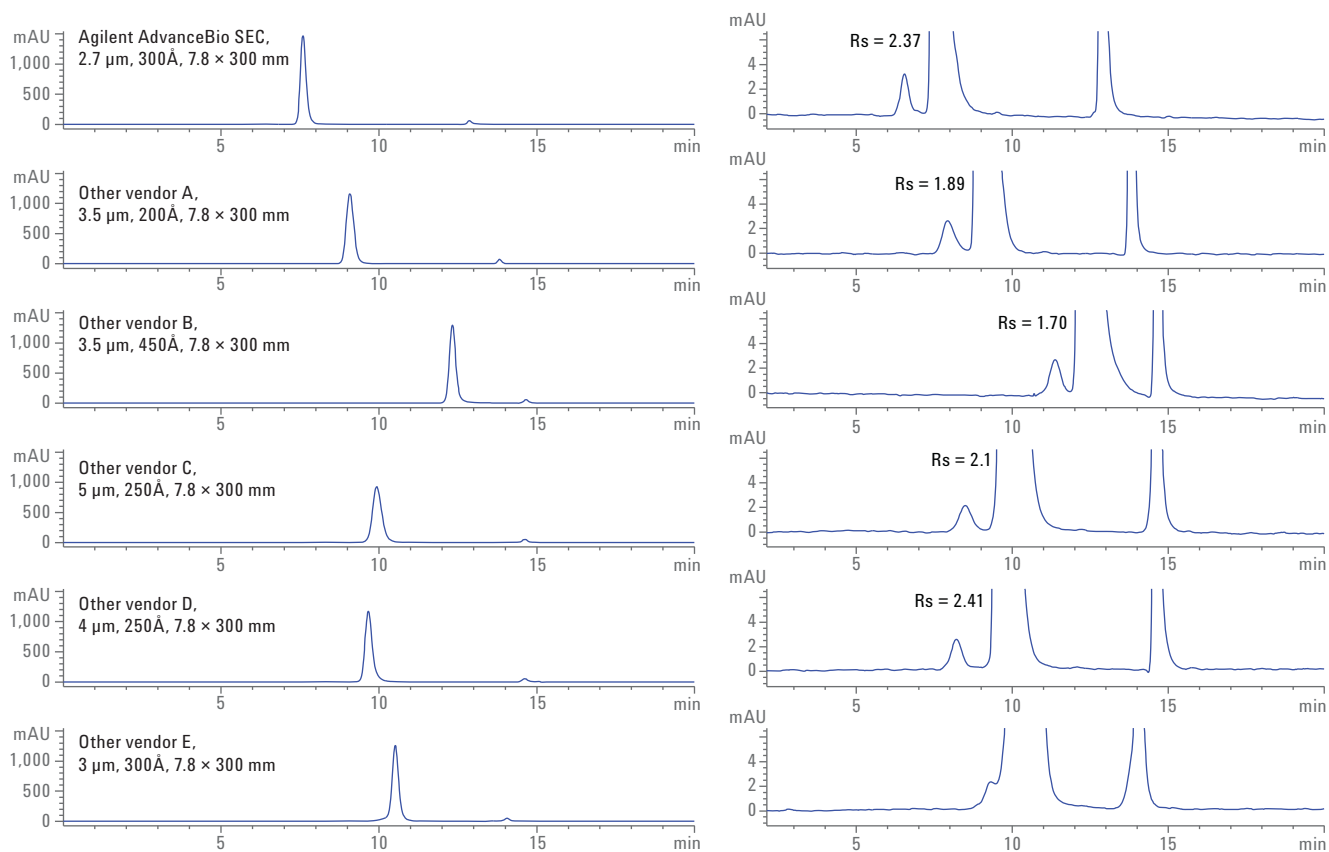


Figure 3. Analysis of trastuzumab monomer and aggregate using 7.8 x 300 mm SEC columns.

Comparisons were also made between the columns for the separation of the commercial therapeutic monoclonal antibody rituximab, to show how the columns performed in real situations. The area highlighted in Figure 4 reveals the capability of AdvanceBio SEC to resolve

aggregates and fragments compared to columns A to E. The optimal pore size and pore volume of AdvanceBio SEC offers the higher resolution necessary for the separation of therapeutic monoclonals, their aggregates, and degradation products.

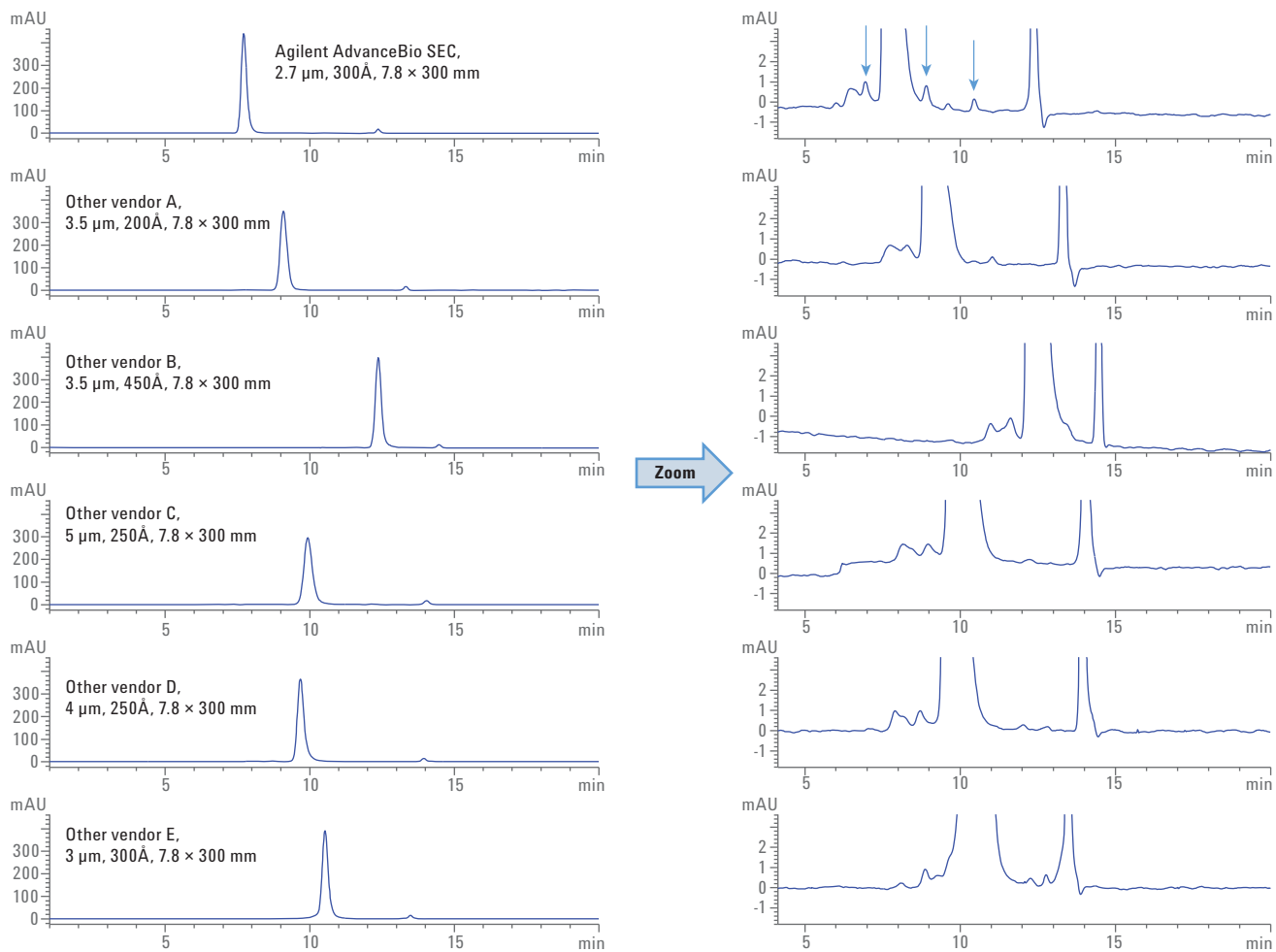


Figure 4. Comparison of profiles of rituximab on various 7.8 × 300 mm SEC columns.

Separation of antibody drug conjugates
 SEC analysis of antibody drug conjugates (ADCs) requires columns with reduced secondary interaction of hydrophobic payloads with the column stationary phase. Most published methods for SEC analysis of ADCs performed using aqueous phases led to poor peak shape and incomplete resolution of aggregate from the monomeric conjugate. This effect was due to nonspecific interaction of hydrophobic payload with the stationary phase. Adding organic modifiers has been shown to overcome

this effect, but the increased mobile phase viscosity, and hence pressure, can compromise flow rate or column life time. Sample integrity can also be compromised as the organic can affect sample aggregation.

Analysis of ADC using columns A to E with aqueous mobile phase led to poor peak shape due to nonspecific interaction of the hydrophobic payload with the stationary phase, as shown in Figure 5. However, peaks were symmetrical when the ADC was analyzed using an AdvanceBio SEC with an aqueous

mobile phase, 150 mM phosphate buffer. This method also gave the highest resolution of monomer and aggregate ($R_s = 1.75$), indicating no nonspecific interaction of the hydrophobic drug with the stationary phase (Figure 5). In addition, the AdvanceBio SEC was able to separate and resolve ADC aggregates better than the other columns, indicating its suitability for the analysis of ADC and aggregates. The broader peaks produced by columns from other vendors not only impacted sensitivity and limited the detection of low levels of aggregates, but also made peak integration difficult.

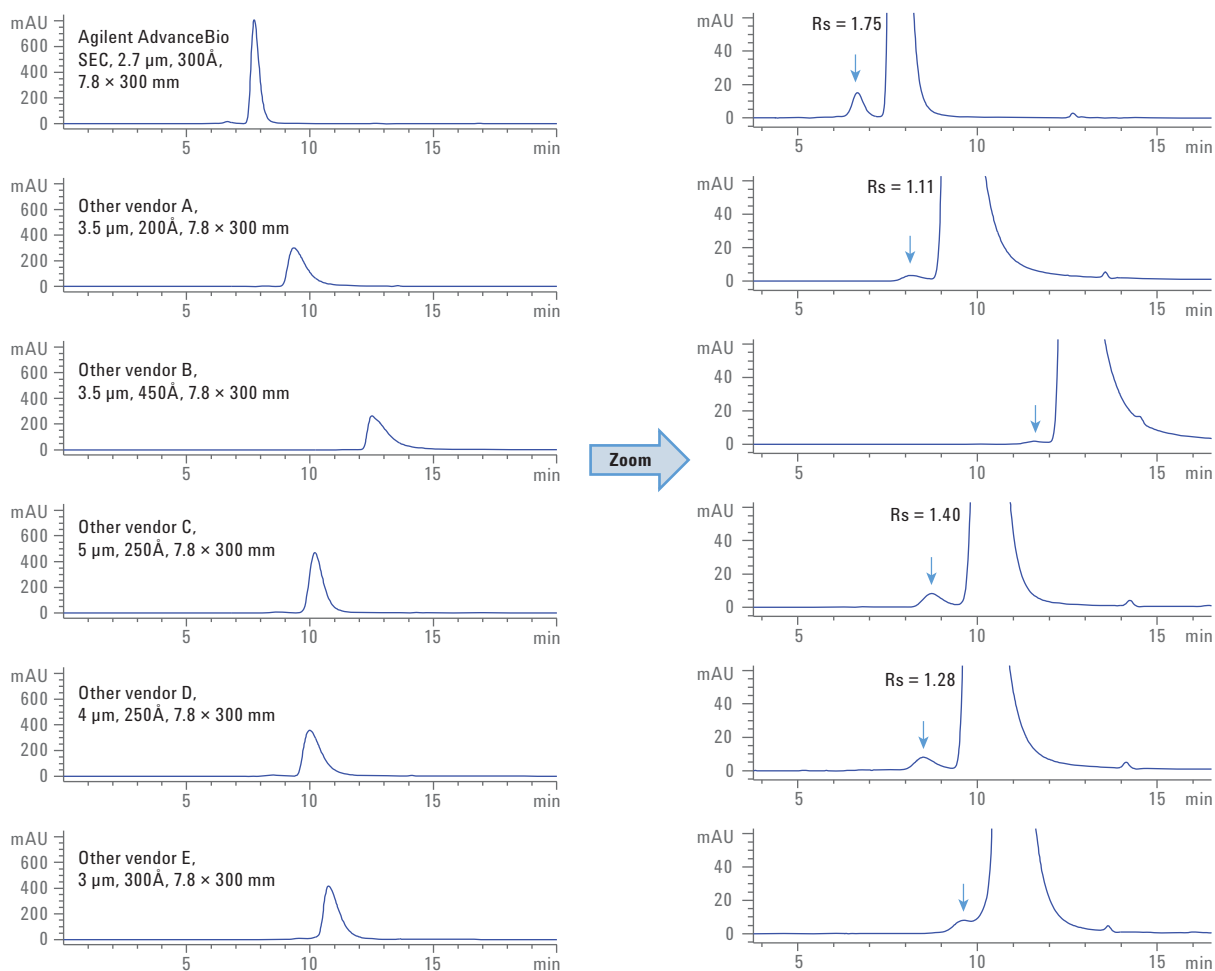


Figure 5. Comparison of profiles of therapeutic ADC on various 7.8 x 300 mm SEC columns.

Comparison of 4.6 × 300 SEC mm columns for sensitive methods

Separation of protein standards

Figure 6 compares the resolution of a five-protein reference standard on AdvanceBio SEC 300Å, 4.6 × 300 mm, 2.7 μm and columns F and G. The largest protein, thyroglobulin aggregate (peak 1), was well resolved, with improved sensitivity and narrow peaks compared to other columns. Overall, AdvanceBio SEC offers better resolution for high, medium, and low molecular weight markers than column F, and is comparable to the 1.7 μm column G.

The AdvanceBio SEC calibration curve has less slope, with the curve showing an excellent coefficient value for more accurate molecular weight (Figure 7). AdvanceBio SEC also gives higher pore volume (2.36 mL) compared to column F (2.06 mL) and column G (2.20 mL), and thus provides better resolution and higher peak capacity. In addition, column F requires a higher run time to resolve the same range of markers. The permeation range of the column (5.48 mL) also exceeds the column volume (4.9 mL), suggesting some nonspecific interaction during elution.

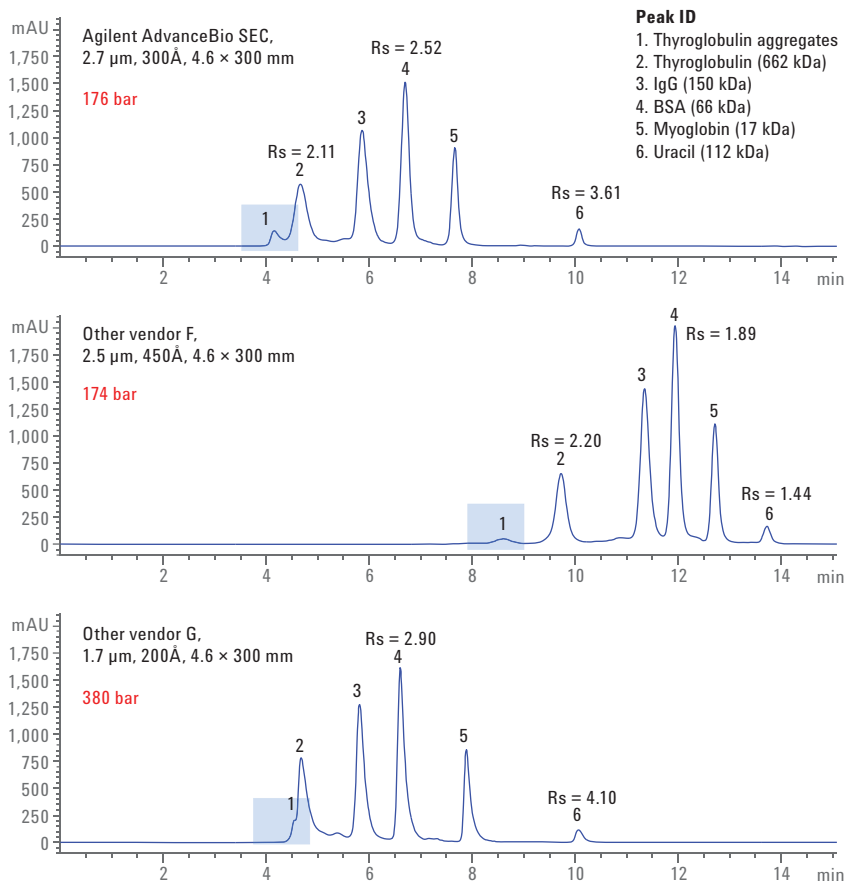


Figure 6. Comparison of profiles of protein standards on various 4.6 × 300 mm SEC columns. The resolution between peaks 1 and 2, 3 and 4, and 5 and 6 were calculated.

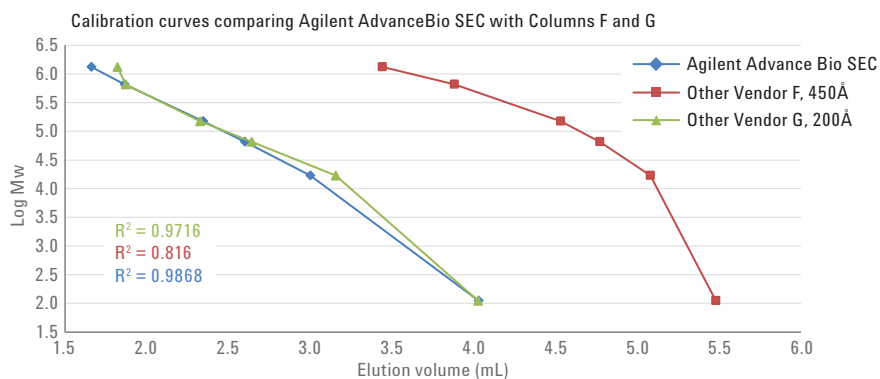


Figure 7. Calibration curves for protein standards on various 4.6 × 300 mm SEC columns.

Separation of therapeutic monoclonal antibody

Figure 8 shows the separation of the mAb trastuzumab on the 4.6×300 mm columns. The chromatogram displays the resolution values in the separation of monomer and aggregates. AdvanceBio SEC shows the resolution of the monomer peak to be comparable to the $2.5 \mu\text{m}$, 450\AA column F, as well as the $1.7 \mu\text{m}$, 200\AA column G. Additionally, AdvanceBio SEC and column F were able to identify degraded trastuzumab, highlighted in blue, which column G could not separate.

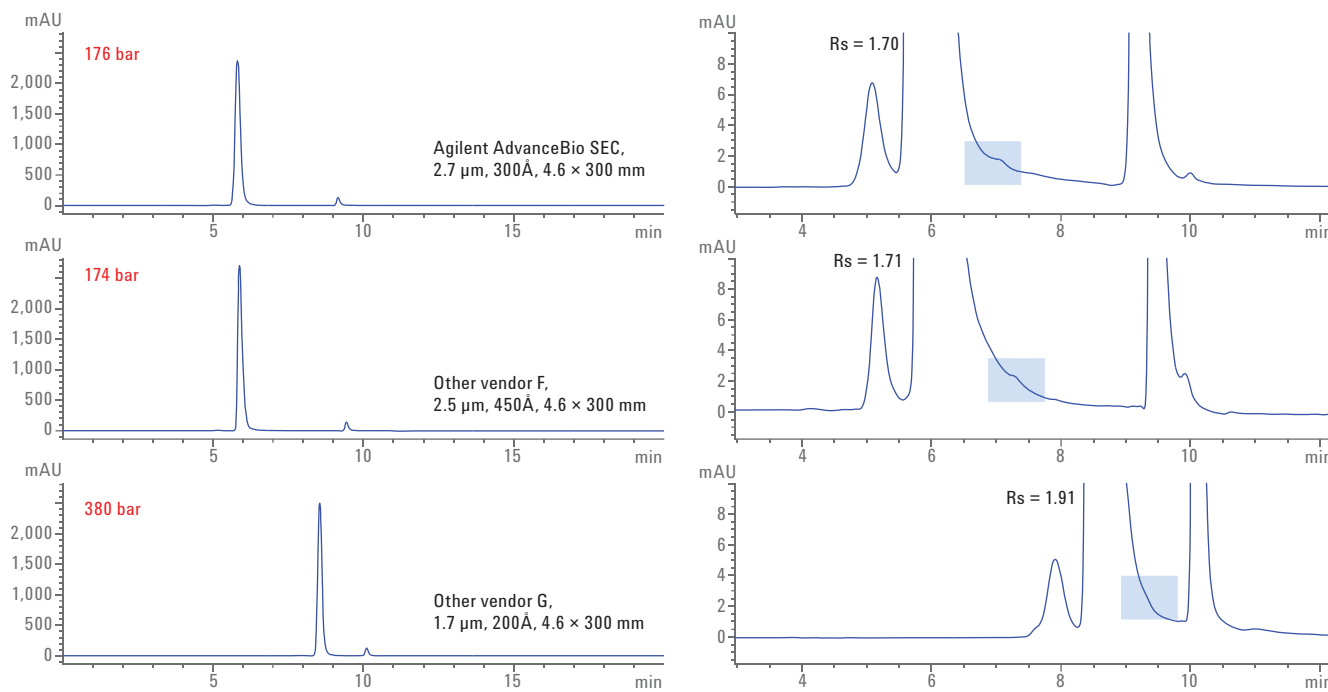


Figure 8. Analysis of trastuzumab monomer and aggregate using 4.6×300 mm SEC columns from different manufacturers.

Separation of antibody drug conjugate

Figure 9 illustrates the SEC separation of ADC on various 4.6×300 mm columns. It is evident that the AdvanceBio SEC showed the highest resolution factor ($R_s = 1.47$) for ADC monomer and its aggregate, compared to columns F and G. Columns F and G gave poor peak shapes due to the interaction of hydrophobic payloads with the column stationary phase. The Advance BioSEC column also provided better peak shape for ADC monomer, and improved resolution of its aggregate. In addition to better separation of protein markers, mABs/ADCs, and their aggregates, AdvanceBio SEC operates at approximately 176 bar, as opposed to the 380 bar of the sub- $2 \mu\text{m}$

column G column. The latter requires LC systems that are capable of tolerating high backpressures.

Conclusions

The potential of size exclusion chromatography for biomolecule characterization is very high. This Technical Overview showcases several benefits of Agilent AdvanceBio SEC columns for biopharmaceutical analysis when compared to SEC columns from other vendors. We first used the 7.8×300 mm columns to separate a range of proteins, mAb monomer, aggregates, and fragments. The ideal pore size and pore volume of AdvanceBio SEC offer excellent separation and peak

capacity for monomers, aggregates, and fragments for reliable and robust separations. To achieve better sensitivity, the method was transferred to a 4.6×300 mm column and compared with $2.5 \mu\text{m}$, 450\AA and $1.7 \mu\text{m}$, 200\AA columns, which also resulted in superior resolution. In addition, resolution and peak capacities can be improved significantly by coupling two columns in series. This is only possible with AdvanceBio SEC columns that can run at lower pressures. Finally, AdvanceBio SEC columns provide better peak shapes and certainty of resolving ADC monomers and their aggregates using aqueous mobile phase, without compromising column life and sample integrity.

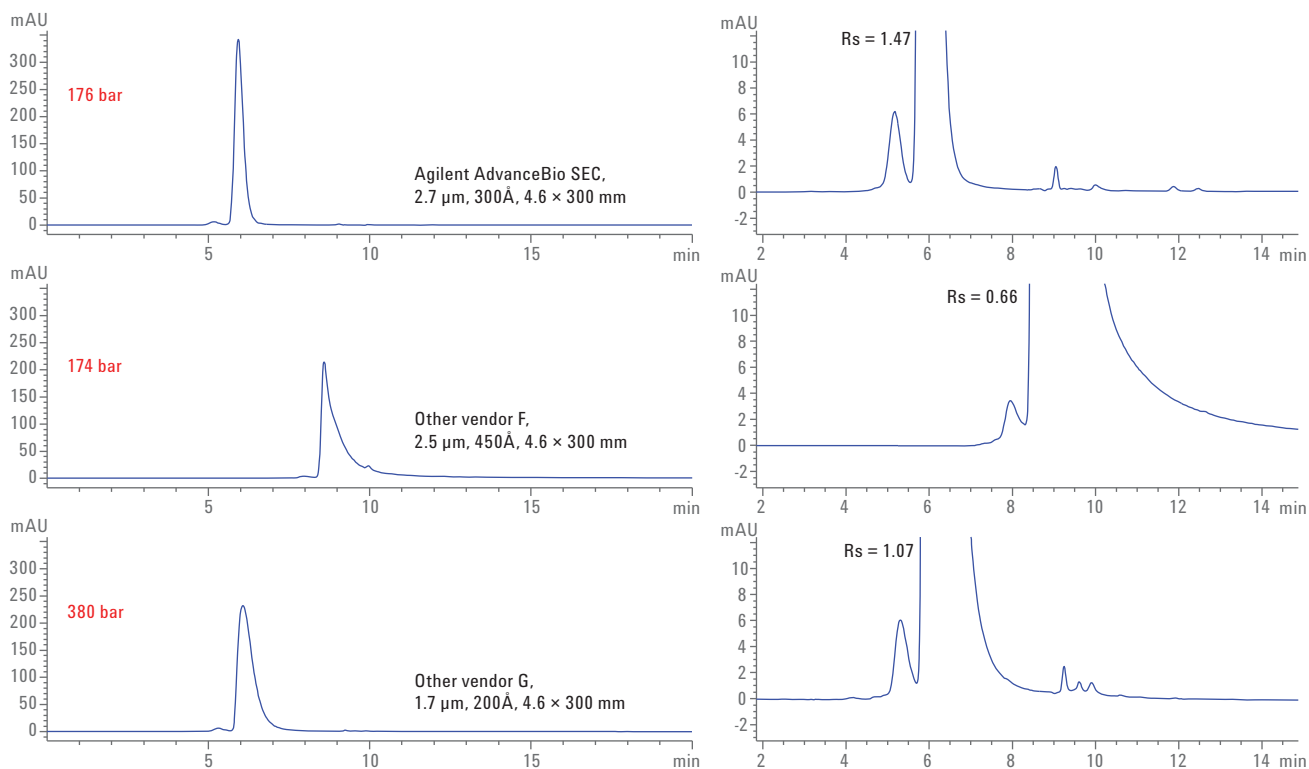


Figure 9. Comparison of ADC separation on various 4.6×300 mm SEC columns.

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