Calibrating your Agilent AdvanceBio SEC Columns

Technical Overview

Author

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Introduction

Agilent AdvanceBio SEC columns are specifically designed for aqueous size exclusion chromatography (SEC) of biomolecules. The particles have been manufactured using proprietary technology to combine optimum pore size and pore volume for separating molecules such as proteins, polysaccharides, or aqueous soluble polymers.

Even if the intention is to use the AdvanceBio SEC column for quantification of monomer and dimer content, it is still good practice to regularly perform a calibration using appropriate molecular weight standards. Regular calibration ensures better reproducibility, leading to improved accuracy, and enables earlier detection of potential problems, reducing system downtime and troubleshooting.

For protein separations, the standards should be a range of well characterized proteins covering the entire operating range of the column. For polysaccharide analysis, pullulan polysaccharide molecular weight standards should be used, and for synthetic polyethylene glycol or polyethylene oxide, PEG/PEO standards are suitable.

SEC is a relatively straightforward technique. It relies on simple diffusion into the pore structure of the stationary phase; larger molecules cannot permeate the particles, and elute first, while smaller molecules diffuse readily into the pores, and elute later. This provides two key aspects for the successful use of SEC:

- There should be no secondary interactions between the analyte and the stationary phase
- The pore size should be chosen to match the size of molecules being analyzed

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Proteins are complex molecules containing numerous side chain functionalities: acid, basic, neutral, and hydrophobic. Finding the optimum conditions to avoid secondary interactions can be challenging, however the AdvanceBio SEC product range has a polymeric surface coating applied to the silica particle that overcomes many of these issues.

The mechanism of separation relies on differences in size of molecules in solution. Protein structures are often compact and globular in nature, and proteins often aggregate under stress conditions such as extremes of temperature, pH, or salt composition and for dimers and larger units [1]. This is a particular issue for molecules such as monoclonal antibodies, where the presence of aggregated proteins can lead to adverse effects if administered. SEC provides the ideal tool for quantifying and monitoring protein aggregation. In comparison, polysaccharides and synthetic polymers such as polyethylene oxide do not possess a wide range of functional groups, but are often made up of a series of closely related oligomers. In this case, SEC can provide the analysis of molecular weight, molecular weight distribution, and branching information [2].

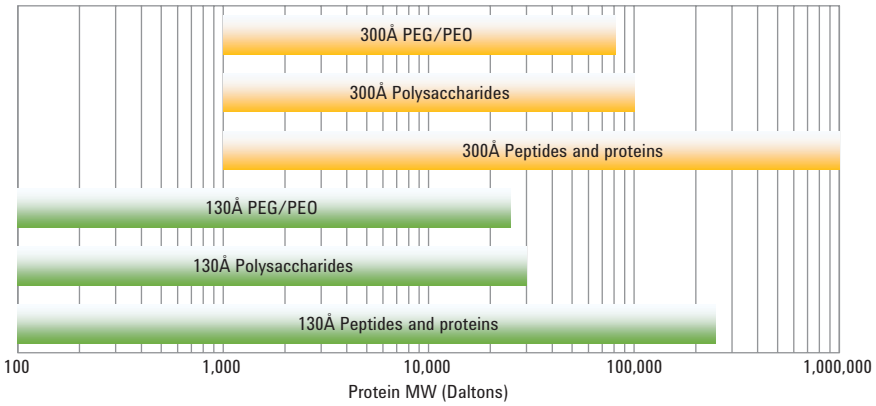

Figure 1. Resolving ranges for Agilent AdvanceBio SEC columns.

Calibrating your Agilent AdvanceBio SEC column

Choose the eluent for analysis. The samples need to be soluble in the mobile phase, and there should be no secondary interactions that lead to molecules eluting earlier or later than expected. For proteins, we recommend 150 mM sodium phosphate, pH 7.0 as this eliminates sodium chloride, which may lead to corrosion, from your HPLC system. However, other mobile phases including phosphate buffered saline (PBS), or high ionic strength mobile phases such as 0.1 M sodium phosphate/0.1 M sodium sulphate may still be used (see Pages 10–11).

Dissolve the standards in the mobile phase. Ensure that the solution is clear and tha there is no insoluble material present. If necessary, filter the sample. Consider changing the mobile phase to ensure that the samples fully dissolve.

Record the chromatogram for each of the standards, and plot the retention time (x-axis) versus the molecular weight (y-axis) to create the calibration curve. Notice that the y-axis is plotted on a logarithmic scale.

Conditions

Parameter	Value
Column:	Agilent AdvanceBio SEC 130Å, 2.7 µm, 7.8 × 300 mm (p/n PL1180-5350) Agilent AdvanceBio SEC 300Å, 2.7 µm, 7.8 × 300 mm (p/n PL1180-5301)
Samples:	Agilent polyethylene glycol calibration kit, PEG-10, 10 × 0.2 g (p/n PL2070-0100) Agilent polyethylene oxide calibration kit, PEO-10, 10 × 0.2 g (p/n PL2080-0101) AdvanceBio SEC 130Å Protein Standard, lyophilized, 1.5 mL (p/n 5190-9416) AdvanceBio SEC 300Å Protein Standard, lyophilized, 1.5 mL (p/n 5190-9417) Protein standards (Sigma-Aldrich) Samples prepare at 0.5 – 1.0 mg/mL in mobile phase
Eluent:	150 mM Sodium phosphate buffer, pH 7.0, PBS, pH 7.4 (10 mM phosphate, 140 mM NaCl) or 100 mM Sodium phosphate + 100 mM sodium sulfate, pH 7.0
Flow rate:	1.0 mL/min
Detector:	RI for polysaccharides and PEG / PEO standards UV, 220 nm for peptides and proteins
System:	Agilent 1260 Infinity Bio-inert LC (with additional refractive index detector)

Peptide and Protein Calibration

Proteins and peptides are generally very compact molecules containing multiple hydrogen bonds, electrostatic interactions, or covalent bonds such as disulphide bridges. Choosing a range of standards covering the operating range of the column for calibration is preferred. It is also possible to use prepared standard mixtures, as peaks are likely to be clearly defined and well resolved (Figures 3A and 3B).

The presence of a diverse range of side chain functionality from different amino acids may mean undesirable secondary interactions can occur, and so different mobile phase compositions may need to be tested.

The peaks shown are generally very sharp and well defined since they represent a single molecular species. It is quite common for proteins to contain aggregates, and these too are typically well resolved – evident as an earlier eluting peak or series of peaks.

		Retention time (min)	
Protein/Peptide	**MW**	**130Å**	**300Å**
Thyroglobulin	670,000	4.60	5.14
γ-Globulin	150,000	4.90	6.53
BSA	66,000	5.53	7.57
Ovalbumin	44,300	6.04	8.13
Myoglobin	17,600	6.77	8.79
Cytochrome C	12,327	6.95	8.92
Aprotinin	6,511	7.56	9.38
Neurotensin	1,672	9.42	10.54
Angiotensin-II	1,040	9.94	10.82

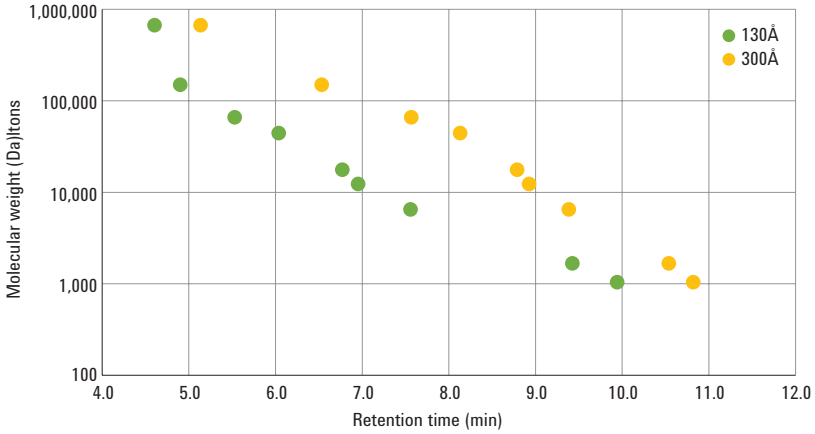

Figure 2. Peptide and protein calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for protein and peptide standards

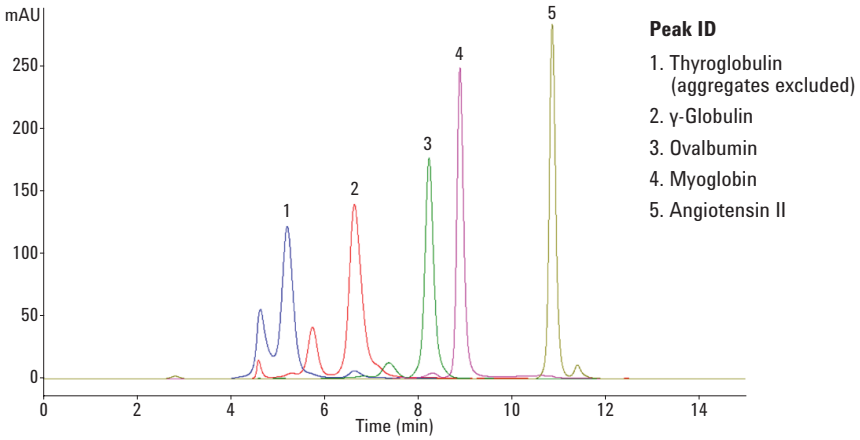

Figure 3A. Individual peptide and proteins contained in Agilent AdvanceBio SEC 300Å Protein Standard (p/n 5190–9417) on an Agilent AdvanceBio SEC 300Å column.

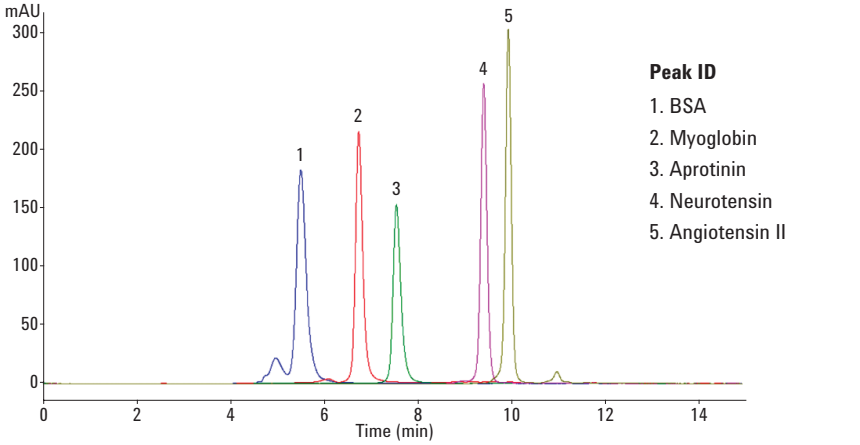

Figure 3B. Individual peptides and proteins contained in Agilent AdvanceBio SEC 130Å Protein Standard (p/n 5190–9416) on an Agilent AdvanceBio SEC 130Å column.

Polysaccharide Calibration

Calibration with polysaccharide standards should be used when your analyte is also a polysaccharide, for example starch or cellulose. There are many types of polysaccharide, including linear, branched, or sulfated molecules. Unlike peptides or proteins, they comprise of a range of chain lengths and can extend to very large sizes. Therefore, it is important to ensure that the column you are using is capable of resolving the molecular weight range of polysaccharide you are investigating.

The mass difference of 162 for each additional hexose means that separating individual oligomers is very difficult unless the sample is sufficiently small (Figure 5C), achieved using two columns in series for additional resolution.

The combination of two columns run in series can increase resolution to the extent that individual oligomers are becoming visible in the 5,800 Da polysaccharide sample.

This sample contains oligomers of approximately 30–40 units, with a mass difference of 162 Da each.

		Retention time (min)	
Polysaccharide	**MW**	**130Å**	**300Å**
788K	788,000	4.48	4.74
380K	380,000	4.58	4.84
100K	100,000	4.73	5.33
48K	48,000	4.89	6.19
23.7K	23,700	5.39	7.42
5.8K	5,800	7.16	9.22
Maltotriose	504	9.46	10.72
Maltose	342	9.73	10.82
Glucose	180	10.01	11.09

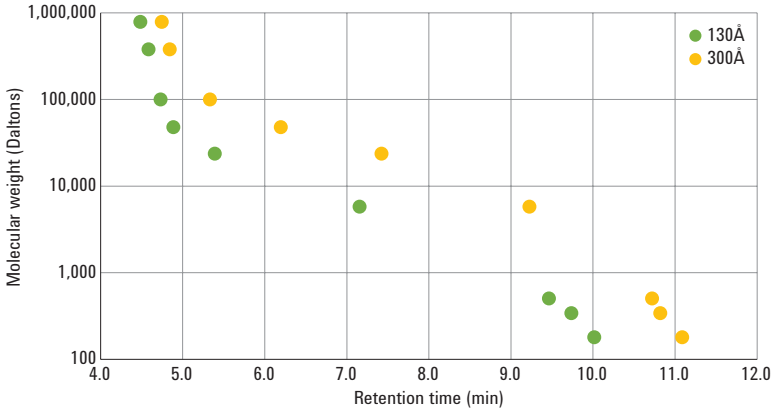

Figure 4. Polysaccharide calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for polysaccharide standards

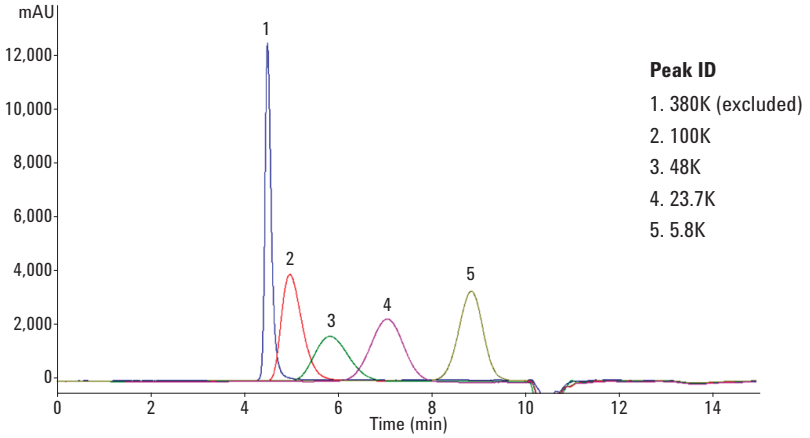

Figure 5A. Polysaccharide standards on an Agilent AdvanceBio SEC 300Å column.

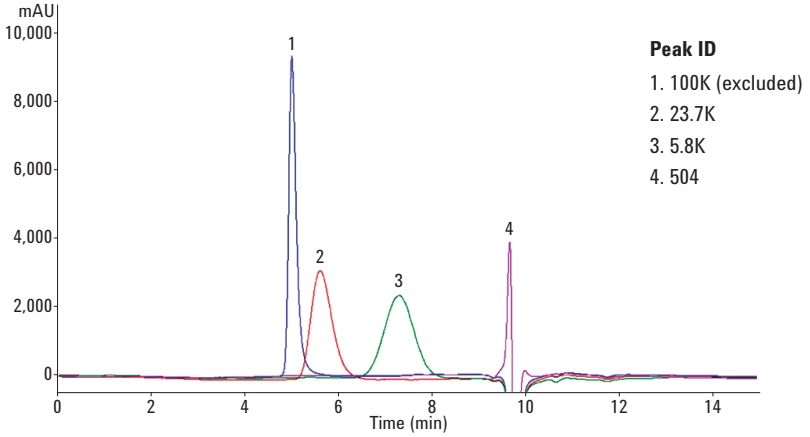

Figure 5B. Polysaccharide standards on an Agilent AdvanceBio SEC 130Å column.

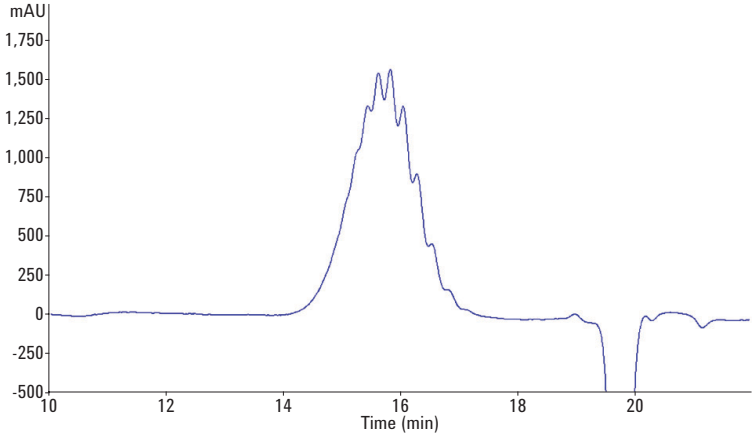

Figure 5C. Polysaccharide 5.8K run on Agilent AdvanceBio SEC columns 300Å + 130Å in series.

PEG/PEO Calibration

Polyethylene glycol (PEG) and polyethylene oxide (PEO) are synthetic polyethers, available in a range of sizes covering a diverse molecular weight range. Similar to polysaccharides, they comprise of a range of chain lengths, however the mass difference is just 44 Da.

		Retention time (min)	
PEG/PEO	**MW**	**130Å**	**300Å**
905K	905,000	4.41	4.73
692K	692,000	4.41	4.73
498.6K	498,600	4.58	4.83
305.5K	305,500	4.55	4.85
135.8K	135,800	4.65	4.93
77.35K	77,350	4.71	5.20
46.47K	46,470	4.77	5.67
21.3K	21,300	5.11	6.81
12.14K	12,140	5.65	7.74
8.73K	8,730	6.10	8.28
3.87K	3,870	7.26	9.32
1.48K	1,480	8.59	10.26
420	420	9.79	11.03
106	106	10.64	11.44

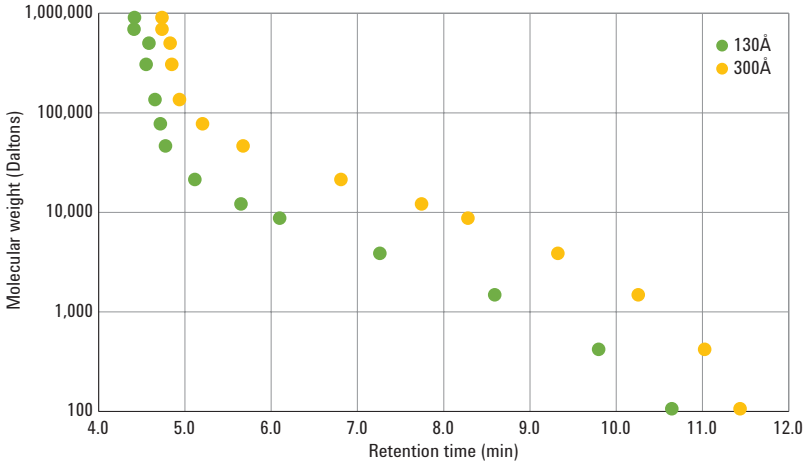

Figure 6. PEG/PEO Calibration on Agilent AdvanceBio SEC columns.

Representative chromatograms for PEG/PEO standards

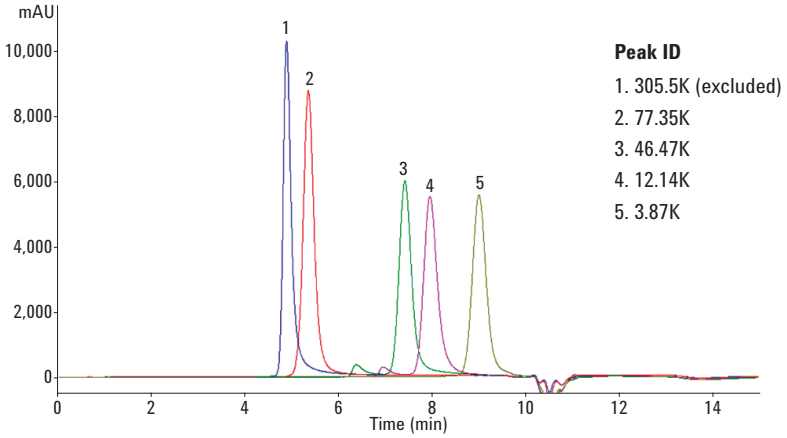

Figure 7A. PEG/PEO standards on an Agilent AdvanceBio SEC 300Å column.

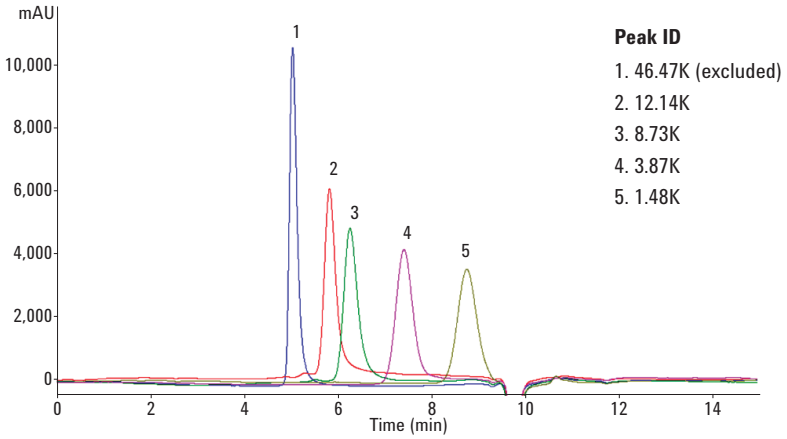

Figure 7B. PEG/PEO standards on an Agilent AdvanceBio SEC 130Å column.

Effect of Mobile Phase Composition

The impact of mobile phase on the size of the molecule in solution, or in overcoming secondary interactions needs to be taken into consideration, as this will influence the retention time and alter the shape of the calibration curve. This is clearly visible in the comparison of three different mobile phases: 150 mM sodium phosphate, pH 7.0; PBS, pH 7.4 (approximately 10 mM sodium phosphate, 140 mM NaCl); 0.1 M sodium phosphate + 0.1 M sodium sulfate, pH 7.0 (Figure 8).

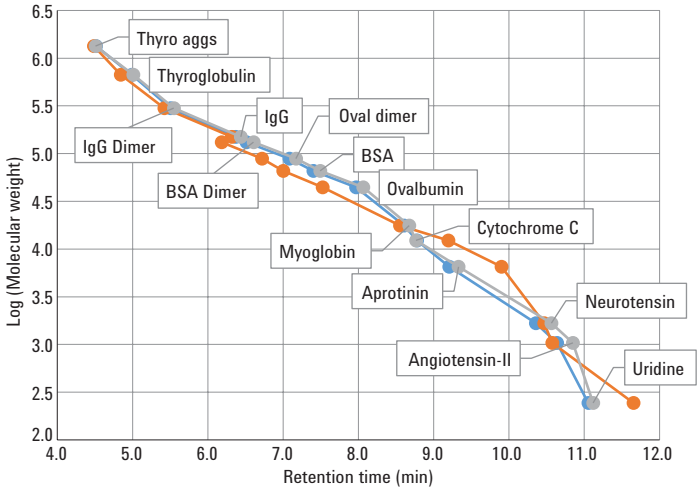

Figure 8. Effect of mobile phase composition on column calibration with protein standards.

For molecules that do not present secondary interactions, such as polysaccharides, the effect of mobile phase composition is minimal (Figure 9).

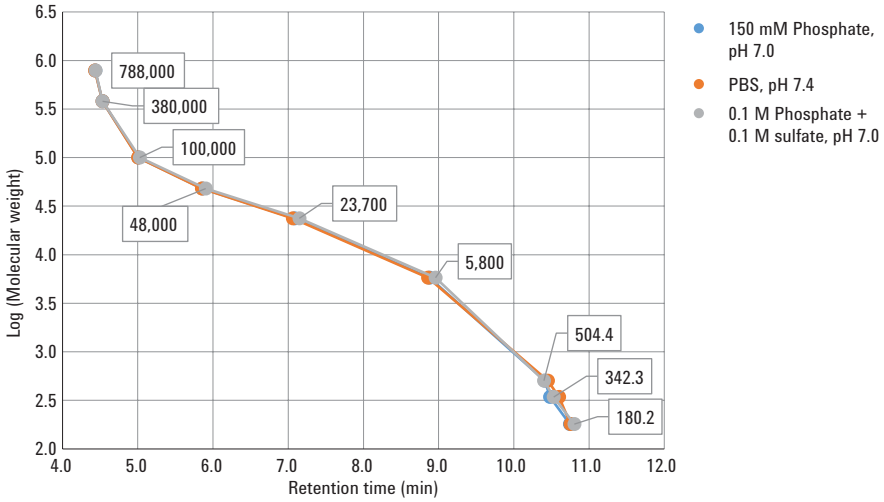

Figure 9. Effect of mobile phase composition on column calibration with polysaccharide standards.

When choosing a mobile phase for peptide or protein analysis by SEC, it is important to determine the effect that differences in pH or ionic strength may have on the sample of interest. Figures 10A, 10B, and 10C show a noticeable shift in the retention time of BSA under different mobile phase conditions. Resolution factors between dimer and monomer peaks may also be affected, so method optimization and method robustness should be fully explored.

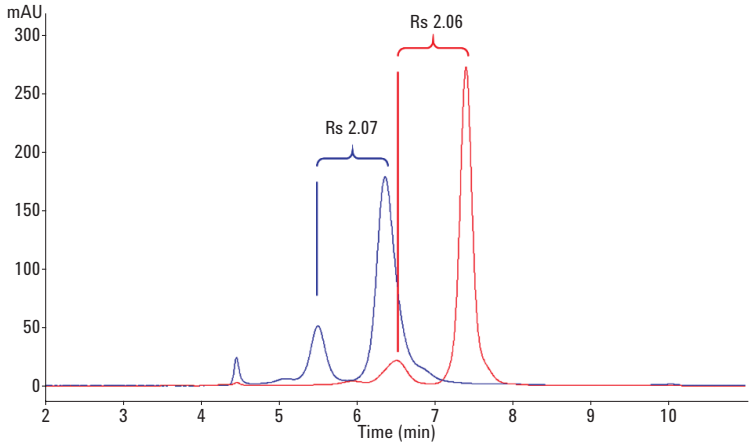

Figure 10A. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300Å column using 150 mM sodium phosphate, pH 7.0.

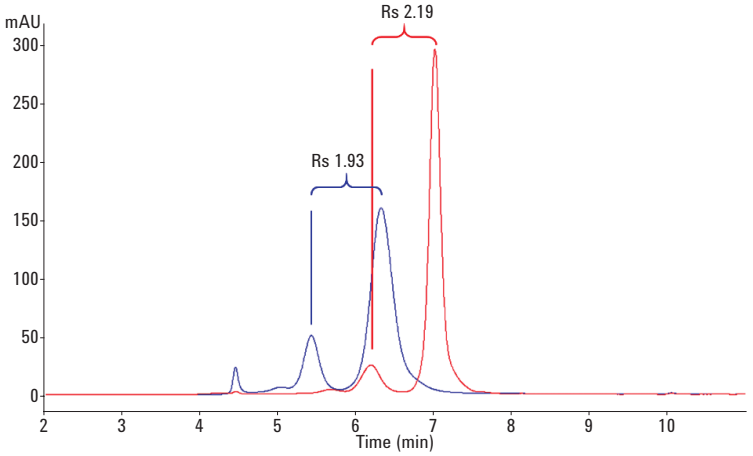

Figure 10B. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300Å column using PBS, pH 7.4.

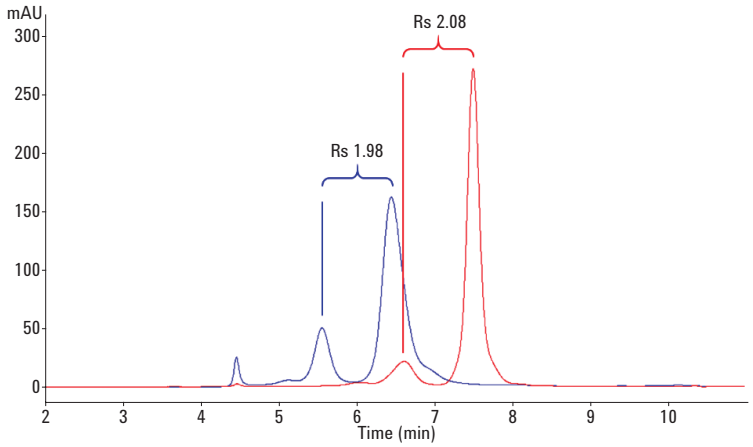

Figure 10C. γ-Globulin and BSA on an Agilent AdvanceBio SEC 300Å column using 100 mM sodium phosphate + 100 mM sodium sulfate, pH 7.0.

Conclusion

Calibrating your Agilent AdvanceBio SEC size exclusion column with appropriate standards ensures you understand the correct working range, and can allow you to use calibration curves to estimate the molecular size of unknown molecules. However, regular calibration with a selection of standards is beneficial, and can be used to monitor column performance over a period of time, allowing early detection of potential problems. In turn, corrective action can be taken, ultimately reducing system downtime and improving productivity.

Agilent offers a range of column dimensions and different pore sizes suitable for differently sized molecules. Together with the extensive range of Agilent LC instrumentation, consumables, and technical support, we can provide the ultimate workflow solution for your needs.

Ordering Information

Part number	Description
PL1180-5301	Agilent AdvanceBio SEC 300Å, 2.7 µm, 7.8 × 300 mm
PL1180-3301	Agilent AdvanceBio SEC 300Å, 2.7 µm, 7.8 × 150 mm
PL1180-1301	Agilent AdvanceBio SEC 300Å, 2.7 µm, 7.8 × 50 mm guard
PL1580-5301	Agilent AdvanceBio SEC 300Å, 2.7 µm, 4.6 × 300 mm
PL1580-3301	Agilent AdvanceBio SEC 300Å, 2.7 µm, 4.6 × 150 mm
PL1580-1301	Agilent AdvanceBio SEC 300Å, 2.7 µm, 4.6 × 50 mm guard
5190-9417	Agilent AdvanceBio SEC 300Å Protein Standard, lyophilized, 1.5 mL
PL1180-5350	Agilent AdvanceBio SEC 130Å, 2.7 µm, 7.8 × 300 mm
PL1180-3350	Agilent AdvanceBio SEC 130Å, 2.7 µm, 7.8 × 150 mm
PL1180-1350	Agilent AdvanceBio SEC 130Å, 2.7 µm, 7.8 × 50 mm guard
PL1580-5350	Agilent AdvanceBio SEC 130Å, 2.7 µm, 4.6 × 300 mm
PL1580-3350	Agilent AdvanceBio SEC 130Å, 2.7 µm, 4.6 × 150 mm
PL1580-1350	Agilent AdvanceBio SEC 130Å, 2.7 µm, 4.6 × 50 mm guard
5190-9416	Agilent AdvanceBio SEC 130Å Protein Standard, lyophilized, 1.5 mL

See Also

1. *Size exclusion chromatography for Biomolecule analysis: A 'How to' Guide*; 5991-3651EN.
2. *GPC/SEC standards: Product guide;* 5990-7996EN.
3. *Calibrating GPC columns: A Guide to Best Practice*; 5991-2720EN.

References

1. Critical Reviews in Therapeutic Drug Carrier Systems **1993**, *10(4)*, 307-377.
2. www.agilent.com/cs/library/slidepresentation/Public/1-Conventional_GPC_-_Polymers_ans_Molecular_Weight.pdf

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