Choosing the Right Calibration for the Agilent Bio SEC-3 Column

Application Note

Biotherapeutics & Biosimilars

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Introduction

Size exclusion chromatography (SEC) is a technique for separating molecules by size using aqueous eluents. This typically includes molecules such as proteins, oligonucleotides, and other complex biopolymers. For molecules of discreet molecular weight, such as proteins, SEC can be used to detect and quantitate monomers, dimers, aggregates, and fragments. The Agilent Bio SEC-3 300Å column is ideally suited to the analysis of globular proteins.

Calibration choice

One of the factors that influences column choice in SEC is the column's resolving range. This can be identified by running a calibration, using standards appropriate to the analyte type, molecular weight range, and size in solution. Thus, for biopolymers such as polysaccharides, we used pullulan polysaccharide calibrants, for proteins we used protein standards, and for synthetic polydispersed polymers we used polyethylene glycol and polyethylene oxide standards.

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Conditions

Column:	Agilent Bio SEC-3, 300Å, 4.6 × 300 mm, 3 µm (p/n 5190-2513)
Samples:	Agilent pullulan polysaccharide standards kit SAC-10 (p/n PL2090-0100) Agilent polyethylene glycol standards kit PEG-10 (p/n PL2070-0100) Agilent polyethylene oxide standards kit PEO-10 (p/n PL2080-0101) Protein standards (Sigma-Aldrich Corp.)
Eluent:	150 mM Sodium phosphate buffer, pH 7
Flow rate:	0.35 mL/min
Detector:	RI for pullulan polysaccharides, PEGs and PEOs UV, 220 nm for proteins
System:	Agilent 1260 Infinity LC

Results and Discussion

Pullulan polysaccharide calibration

Figure 1 shows a typical calibration curve using pullulan polysaccharide standards on a Bio SEC-3, 3 µm column. The pullulan polysaccharide calibrant kit contains mono-, di-, tri-, and tetra-saccharides to define the bottom end of the calibration. The pullulan polysaccharides are well characterized with a narrow polydispersity. These are linear macromolecules of maltotriose units. The molecular weight range of the kit is 180, glucose to 788,000.

The suggested resolving range, the shallowest part of the calibration curve for this type of biopolymer, is 1,000 to 120,000 molecular weight. Table1 shows the pullulan polysaccharide calibration data.

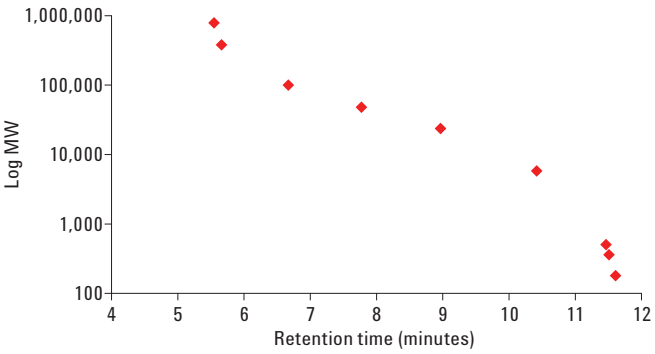

Figure 1. Pullulan polysaccharide calibration curve using an Agilent Bio SEC-3, 300Å column.

Table1. Pullulan polysaccharide calibration data for an Agilent Bio SEC-3, 300Å column.

Pullulan or polysaccharide	Nominal Mp (g/mol)	Retention time (min)
788 K	788,000	5.55
380 K	380,000	5.66
100 K	100,000	6.67
48 K	48,000	7.77
23.7 K	23,700	8.97
5.8 K	5,800	10.42
Maltotriose	504	11.46
Maltose	360	11.51
Glucose	180	11.61

Protein calibration

The molecular weight range used for protein calibration was 931 to 670,000. Figure 2 shows the resulting plot achieved using the Bio SEC-3, 300Å column. In solution, low molecular weight proteins can deviate from linear elution behavior. This is due to the characteristics of proteins in solution and the type of structure they form, for example, how they are folded. Therefore, the elution is influenced by their actual size in solution and not just the molecular weight.

The suggested protein molecular weight resolving range for the column is approximately 5,000 to 1,250,000 molecular weight. Table 2 shows the protein calibration data.

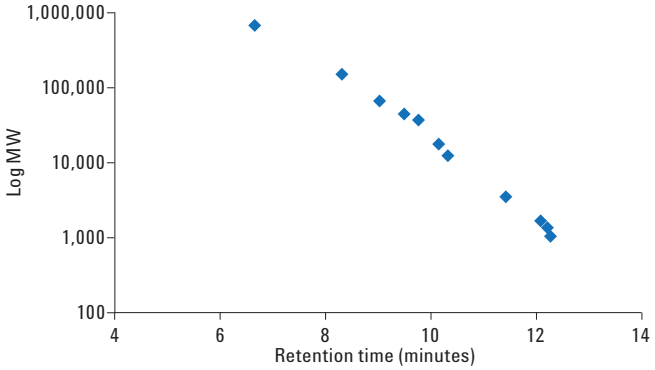

Figure 2. Protein calibration curve using an Agilent Bio SEC-3, 300Å column.

Table 2. Protein calibration data using an Agilent Bio SEC-3, 300Å column.

Protein or peptide	Nominal Mp (g/mol)	Retention time (min)
Thyroglobulin	670,000	6.66
γ-globulin	150,000	8.31
BSA	66,000	9.03
Ovalbumin	44,300	9.50
B-lactoglobulin	36,800	9.77
Myoglobin	17,600	10.15
Cytochrome C	12,327	10.33
Insulin B chain	3,496	11.43
Neurotensin	1,672	12.09
Vitamin B12	1,355	12.22
Angiotensin II	1,040	12.28

Polyethylene glycol and polyethylene oxide calibration

Figure 3 shows the Bio SEC-3, 300Å column calibration curve obtained when calibrated with PEG/PEO calibration standards. The glycols cover the lower molecular weight range and the oxides the higher MW range. The two types are chemically similar, differing only in the end group. The PEGs have a dihydroxyl end group and the PEOs have a single hydroxyl end group.

If they are used together, they can span a very wide molecular weight range. In this instance, the resolving range is 1,500 to 1,250,000 molecular weight. Table 3 shows the polyethylene glycol and polyethylene oxide calibration data.

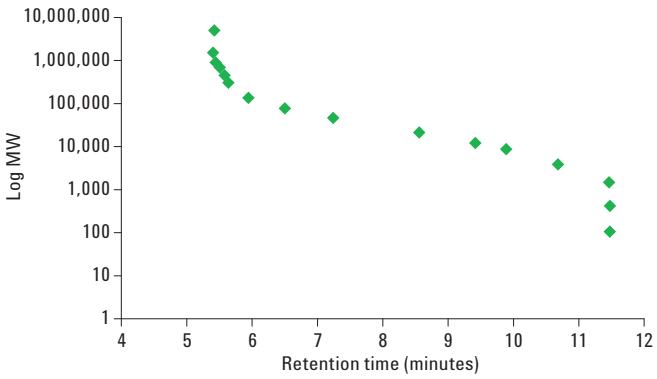

Figure 3. Polyethylene glycol and polyethylene oxide calibration curve using an Agilent Bio SEC-3, 300Å column.

Table 3. Polyethylene glycol and polyethylene oxide calibration data using an Agilent Bio SEC-3, 300Å column.

PEG or PEO	Nominal Mp (g/mol)	Retention time (min)
> 5M	5,000,000	5.42
1,522,000	1,522,000	5.40
905,000	905,000	5.44
692,000	692,000	5.51
454,000	454,000	5.58
305,500	305,500	5.64
135,800	135,800	5.94
77,350	77,350	6.50
46,470	46,470	7.24
21,300	21,300	8.56
12,140	12,140	9.42
8,730	8,730	9.89
3,870	3,870	10.68
1,480	1,480	11.46
420	420	11.48
106	106	11.47

With proteins, it is important to recognize that the SEC mechanism works by separating solutes dependant on their size in solution and not their molecular weight. This is evident when comparing the calibration plot of the proteins/peptides with the pullulan/polysaccharide and PEG/PEO curves, as shown in Figure 4. The pullulan/polysaccharide and PEG/PEO calibrants provided quite similar calibration curves but the protein/peptide curve was shifted and a different shape.

Proteins are composed of complex polypeptide chains that form three-dimensional structures. These structures are affected by the environment to which they are exposed, for example pH or ionic strength. The chains will form the shape that is most suited to them and so the structure and size may vary.

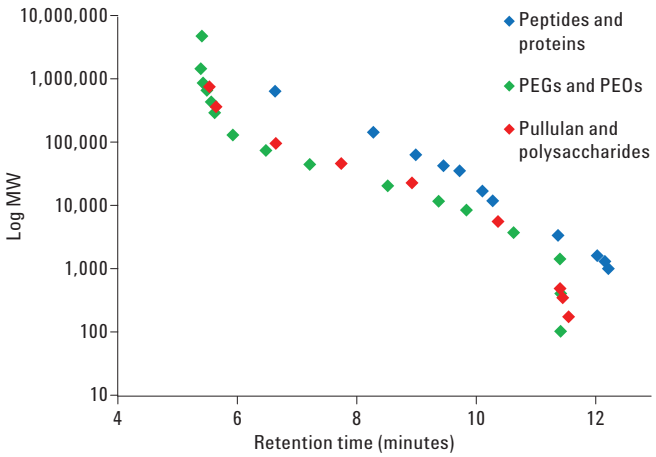

Figure 4. Comparison of calibration plots generated for all three types of calibrant.

To demonstrate that elution time is due to size rather than molecular weight, consider the retention times for calibrants with a molecular weight of approximately 50,000, in which there is a significant difference (Figure 5). The PEG elutes just after 7 minutes, the polysaccharide elutes at just over 7.5 minutes, but the protein elutes at approximately 9.5 minutes.

This clearly demonstrates that the SEC separation mechanism is based on the actual size and not molecular weight. Therefore, when using calibration curves it is important to specify what calibrants have been used. For example, it can be stated that the sample of interest has a pullulan/polysaccharide equivalent molecular weight of 50,000.

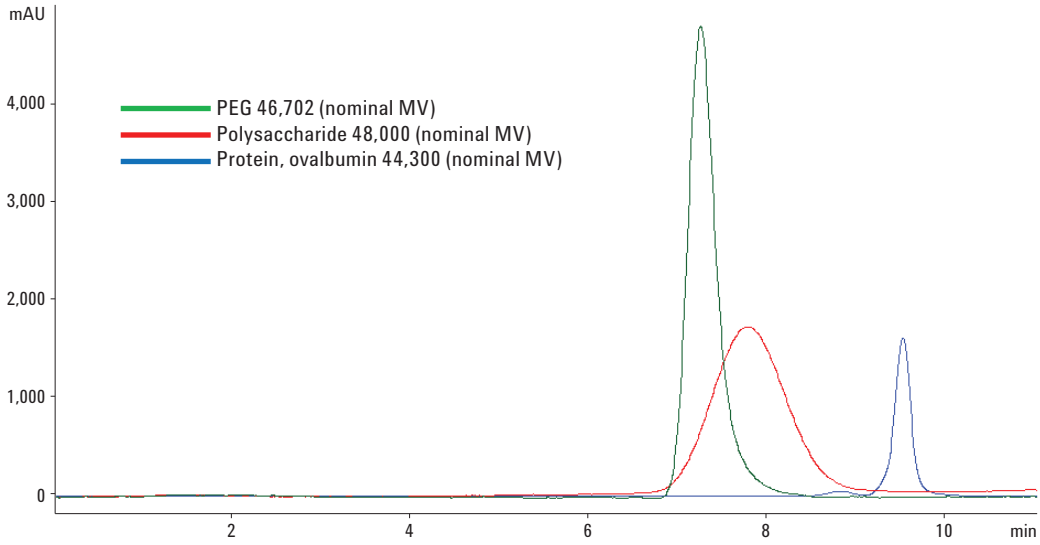

Figure 5. Overlay of chromatograms obtained for calibrants of similar molecular weight.

Conclusions

Calibrating Agilent Bio SEC columns to identify their resolving range is straightforward, provided the calibrant is matched to the analyte. The plots shown in this application note demonstrate that both pullulan/polysaccharide and PEG/PEO standards can be used with the Agilent Bio SEC-3 300Å columns, and that calibration plots are similar and simple to interpret.

Therefore, for maximum resolution in SEC, it is important to work within the resolving range of the column and where the calibration curve is less steep. The linear section of the plots identifies the molecular size range for which optimum resolution can be achieved by using this Bio SEC-3 300Å column.

Agilent manufactures a range of polymer standards that are ideal reference materials for generating accurate and reliable SEC column calibrations. The calibration curves presented here relate to the Agilent Bio SEC-3 300Å columns but the same calibrants can be used to calibrate the full range of Agilent Bio SEC columns, which includes 100Å and 150Å pore sizes, and 5 µm particle size columns in 4.6 × 300 mm and 7.8 × 300 mm dimensions.

For More Information

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Printed in the USA
May 14, 2013
5991-2463EN

Agilent Technologies