Technical Overview

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Chromatographic Comparison of Wide Pore Size Exclusion Columns from Different Vendors

Calibration using polyethylene glycol and polyethylene oxide molecular weight standards

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Abstract

As biotherapeutic molecules evolve, the introduction of larger and larger molecules must undergo the same rigorous quality control to ensure product quality and safety. Critical quality attributes for molecules such as adeno-associated viruses (AAVs) and virus-like particles (VLPs) include the level of aggregation present. Size exclusion chromatography (SEC) is an ideal technique for determining protein aggregation, but for these larger molecules and particles, wide pore stationary phases are required.

This application note compares the pore size, pore size distribution, pore volume, and exclusion limit of several commercially available wide pore size exclusion columns from different vendors, so that end users can better understand the differences between the different products.

Introduction

SEC has become the method of choice for aggregate analysis of biotherapeutic molecules, enabling accurate quantification of dimeric or higher-order aggregates. Typically, aggregate analysis of a monoclonal antibody (with hydrodynamic radii of approximately 5 to 6 nm) is performed using an SEC column with a pore size of approximately 200 to 300 Å. For very large biomolecules such as AAVs and VLPs that range in hydrodynamic radius from 20 to 100 nm, SEC is still a viable technique, provided a column with a large enough pore size is used. HPLC column vendors have developed new stationary phase materials that are specifically designed to analyze these molecules, but the columns available are not the same. It is difficult to understand how they compare unless a chromatographic separation is performed.

This application note uses SEC of polyethylene glycol (PEG) and polyethylene oxide (PEO) molecular weight standards, available in a wide range of molecular weights and corresponding to differing sizes in solution, that cover the entire resolving range of the columns used in the study. This allows a direct comparison of the chromatographic properties of each column, such as exclusion limit (i.e. pore size), pore volume, and pore size distribution.

Experimental

Reagents and chemicals

All reagents were HPLC grade or higher.

Instrumentation

Data acquisition was performed on an Agilent 1260 Infinity II bio-inert LC system using Agilent OpenLAB CDS.

Calibration with individual PEG and PEO standards (Table 3) required the use of a refractive index (RI) detector, Agilent 1260 Infinity II refractive index detector (G7162A).

Sample preparation

Samples were dissolved in mobile phase and stored frozen until needed.

Mobile phase preparation

The mobile phase was prepared by dissolving 0.02% w/v sodium azide in Milli-Q water, then filtering through a 0.22 µm membrane filter.

Method conditions

Table 1. HPLC conditions.

Parameter	Value
Column	See Table 2
Mobile Phase	Water (0.02% sodium azide)
Flow Rate	0.35 mL/min
Column Temperature	30 °C
Injection Volume	5 µL
Total Run Time	15 minutes per injection

Table 2. Columns tested.

Column	Description
A	Column A 450 Å, 2.5 µm, 4.6 × 300 mm
B	Agilent AdvanceBio SEC 500 Å, 2.7 µm, 4.6 × 300 mm
C	Agilent Bio SEC-5 500 Å, 5 µm, 4.6 × 300 mm
D	Column D, 700 Å, 3 µm, 4.6 × 300 mm
E	Column E, 750 Å, 3 µm, 4.6 × 300 mm
F	Agilent AdvanceBio SEC 1,000 Å, 2.7 µm, 4.6 × 300 mm
G	Agilent Bio SEC-5, 1,000 Å, 5 µm, 4.6 × 300 mm
H	Column H, 1,000 Å, 3 µm, 4.6 × 300 mm
I	Agilent Bio SEC-5, 2,000 Å, 5 µm, 4.6 × 300 mm

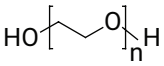
Table 3. PEG/PEO standards used.

PEG/PEO	Mp	Part Number
1,511 K	1,511,000	PL2084-2001
1,039 K	1,039,000	PL2084-1001
689 K	689,500	PL2084-0001
538 K	538,000	PL2083-9001
272 K	272,400	PL2083-8001
191 K	191,000	PL2083-7001
117 K	117,900	PL2083-6001
85 K	85,200	PL2083-5001
73 K	73,850	PL2083-4001
49 K	49,650	PL2083-3001
28 K	28,480	PL2083-2001
20 K	20,180	PL2071-1001
15 K	15,190	PL2071-0001
10 K	10,530	PL2070-9001
8 K	8,160	PL2070-8001
3,860	3,860	PL2070-7001
1,470	1,470	PL2070-6001
1,010	1,010	PL2070-5001
610	610	PL2070-4001
410	410	PL2070-3001
106	106	PL2070-1001

Results and discussion

The wide molecular weight range of PEG and PEO standards makes these molecules ideal for determining the chromatographic characteristics of SEC columns. Although other physical characterization techniques are available (including mercury porosimetry and nitrogen adsorption), chromatography provides the best insight as it is performed under the normal operating conditions of the stationary phase material packed in an LC column.

PEG and PEO polymers are hydrophilic and neutral (Figure 1), meaning they are unlikely to interact with SEC stationary phases in any way that might impact the results.



Mw < 20,000 Da = polyethylene glycol (PEG)
Mw > 20,000 Da = polyethylene oxide (PEO)

Figure 1. Chemical structure of PEG and PEO standards.

The absence of a UV chromophore means the use of an RI detector is necessary. Even though the standards were dissolved in the mobile phase, it is common to see imbalance peaks with RI detection.

By including PEG and PEO standards that are too large to fit into the pores of the stationary phase, these will be excluded and elute at the point corresponding to the interstitial, or interparticle, volume.

The smallest molecules will not only travel through the interstitial volume, they will also permeate the pore or intraparticle volume, allowing the pore volume to be determined.

By plotting a chart of the retention time (X-axis) against PEG and PEO molecular weight (Y-axis, logarithmic scale), the exact pore size distribution can be observed.

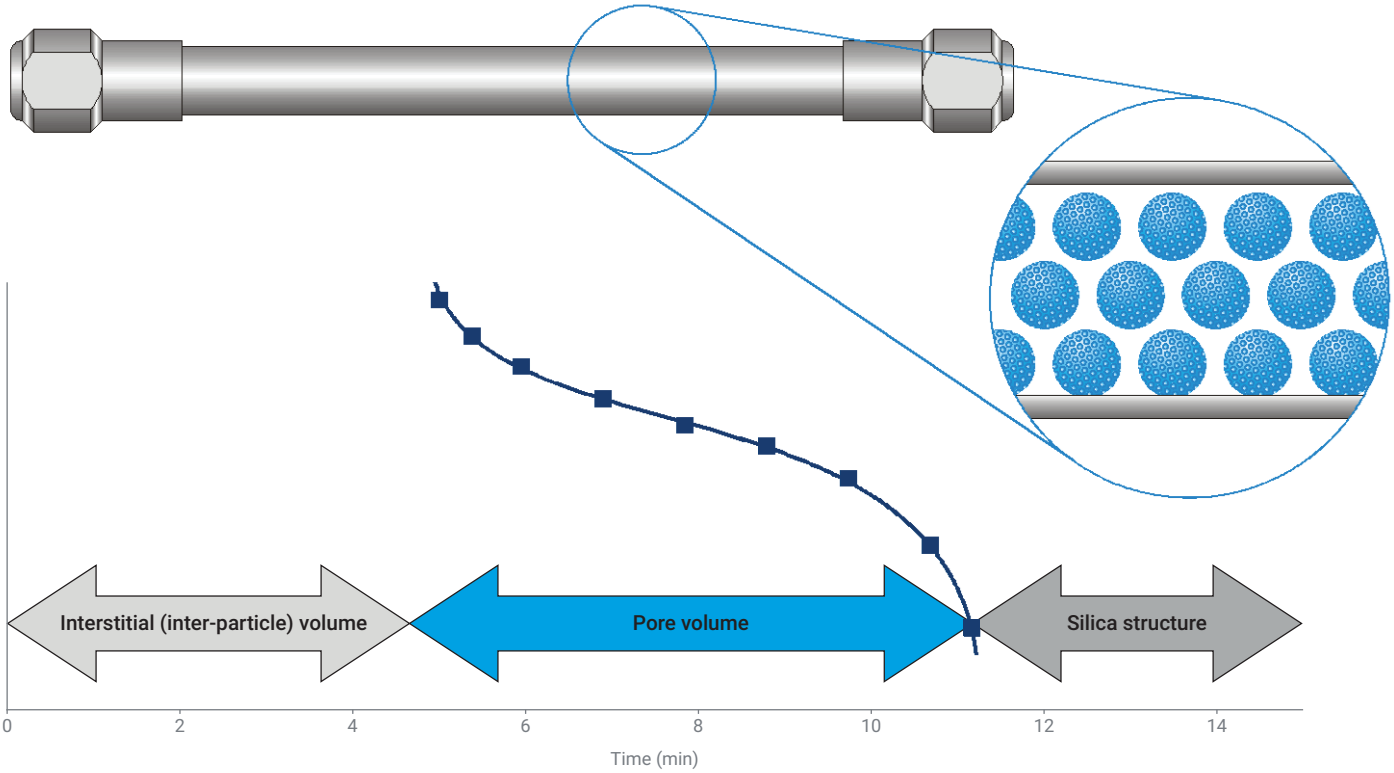


Figure 2. Illustration of an SEC column with cutaway showing interstitial (interparticle) volume and pore (intraparticle volume).

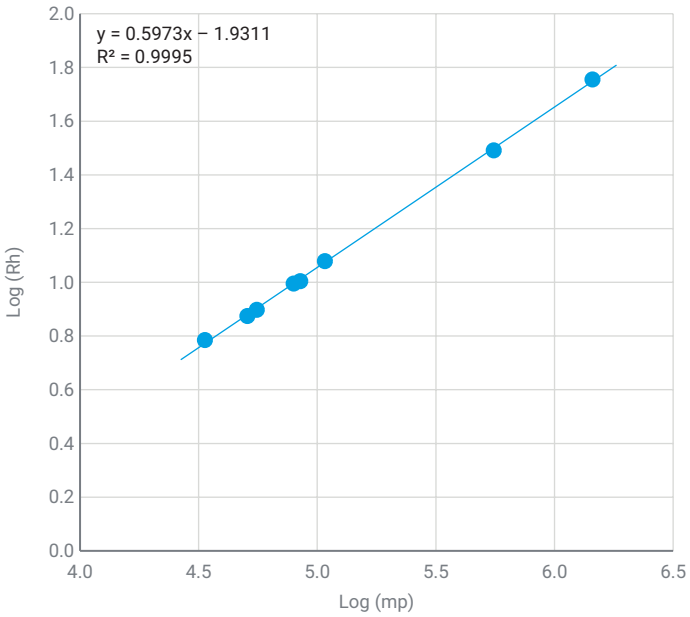


Figure 3. Peak average molecular weight (Mp) versus hydrodynamic radius of a range of polyethylene oxide standards using triple detection.

Table 4. PEO peak average MW versus hydrodynamic radius (Rh).

Mp	Rh (nm)
1,444,836	57.0
554,248	31.0
107,606	12.0
84,769	10.1
79,353	9.9
55,590	7.9
50,680	7.5
33,582	6.1

Figure 3 illustrates the linear relationship between PEO molecular weight versus hydrodynamic radius (Rh), obtained for a selection of standards and analyzed using triple detection. Triple detection includes refractive index, light scattering, and viscometer detectors, and therefore enables the molecular weight and size of molecules to be analyzed simultaneously (summarized in Table 4).

Most notably, the highest molecular weight sample (~ 1.5 MDa) has a hydrodynamic radius of nearly 60 nm. This means the overall size (i.e. diameter) will be 120 nm, or 1,200 Å, which will be excluded from virtually all of the columns being tested. A PEO molecule with a molecular weight of ~ 500 kDa will have a hydrodynamic radius of approximately 30 nm (a diameter of 600 Å).

The range of standards used in the testing of the individual SEC columns (Table 3) ensures that the entire pore structure is investigated.

PEG and PEO standards are not compact, globular molecules like proteins. Instead, they are elongated random coils, so their size in solution is much larger compared to their molecular weight. Plotting a calibration curve with retention time along the X-axis and Log(MW) along the Y-axis results in a classical s-shaped curve (Figures 4A to 4I).

Some of the very high molecular weight PEO standards are excluded and show very little difference in retention time, with only hydrodynamic forces driving the separation.

The optimum separation performance comes from the extended linear region between the exclusion limit and the total permeation point. This should be as long as possible (primarily driven by the pore volume of the stationary phase), but should also have the shallowest slope possible so that the maximum resolution is achieved between molecules that fall into this region. Finally, the lower molecular weight PEG standards below the optimum range for the column should show little separation (meaning pore volume is not being wasted).

Linear fits for these three different regions allows the intersection points to be determined, allowing a more useful comparison between columns from different vendors.

Table 5 summarizes the retention times for the different standards, and highlights which data points are used for the excluded region, the linear region, and the total permeation points.

Table 6 contains the linear fit results, and Table 7 contains the intersection points for each column tested, together with estimated pore dimensions based on the molecular weight values.

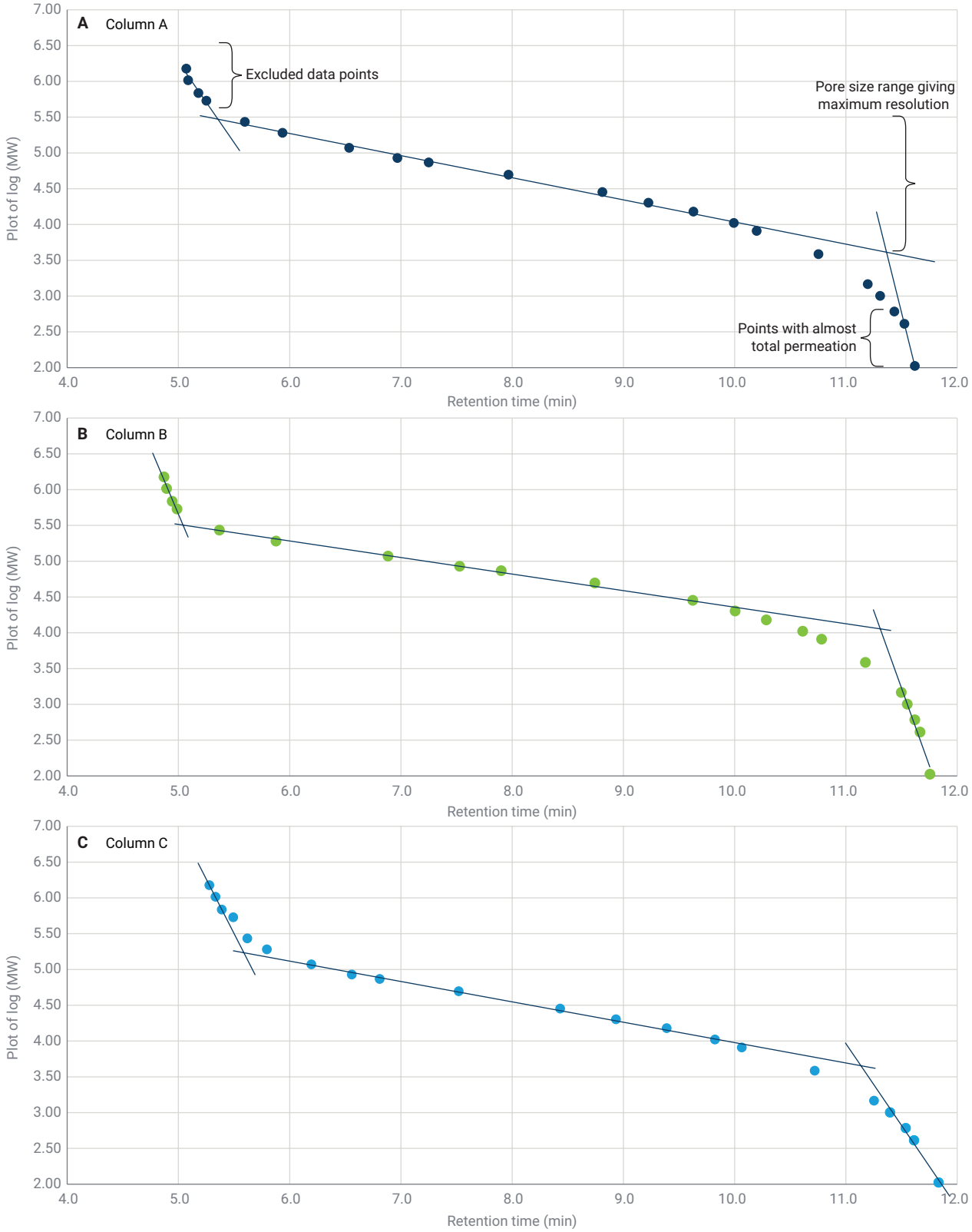
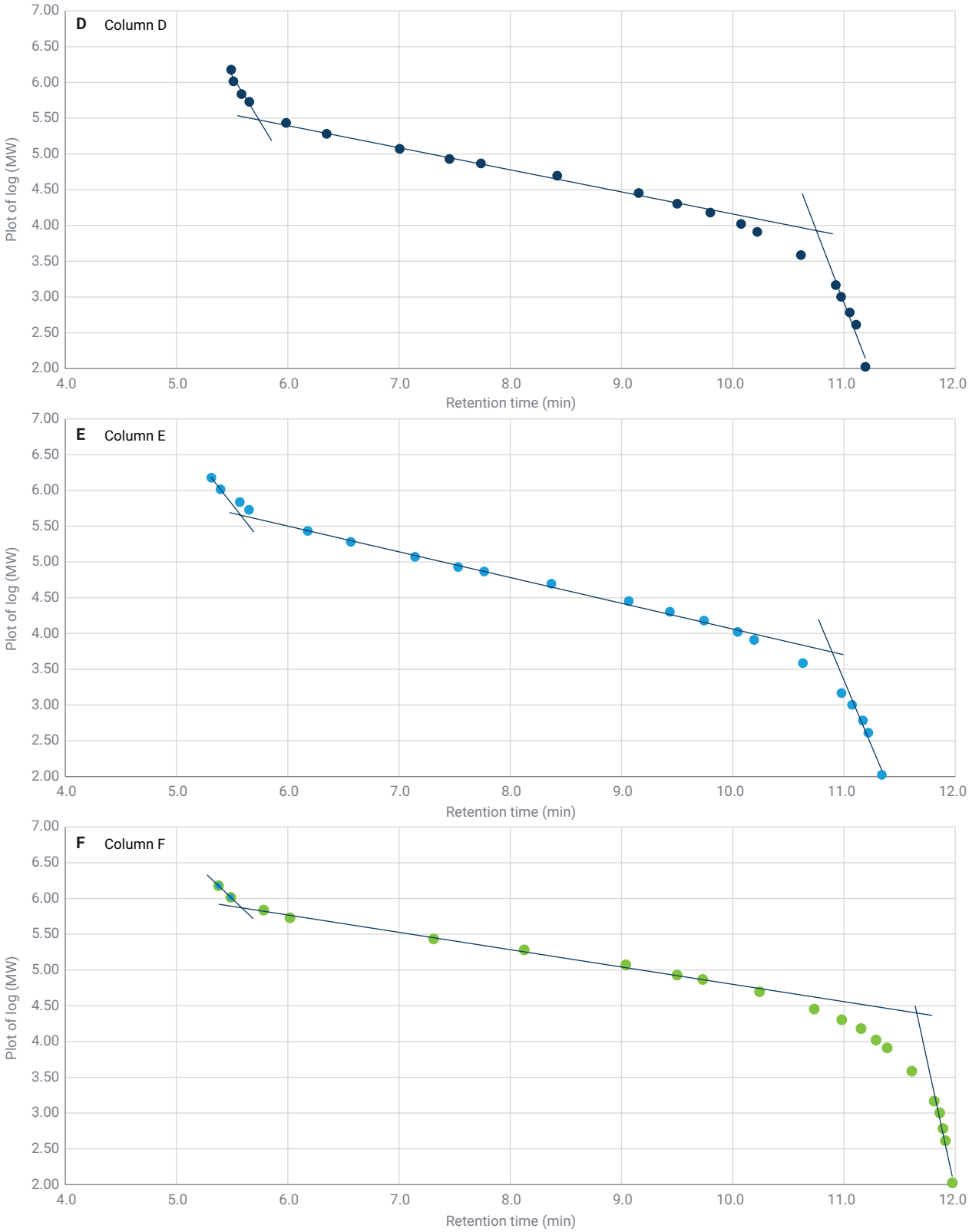


Figure 4. (A) Plot of log (MW) against retention time for column A, showing regions used to determine intersection points. (B–I) Plot of log (MW) against retention time for columns B through I.



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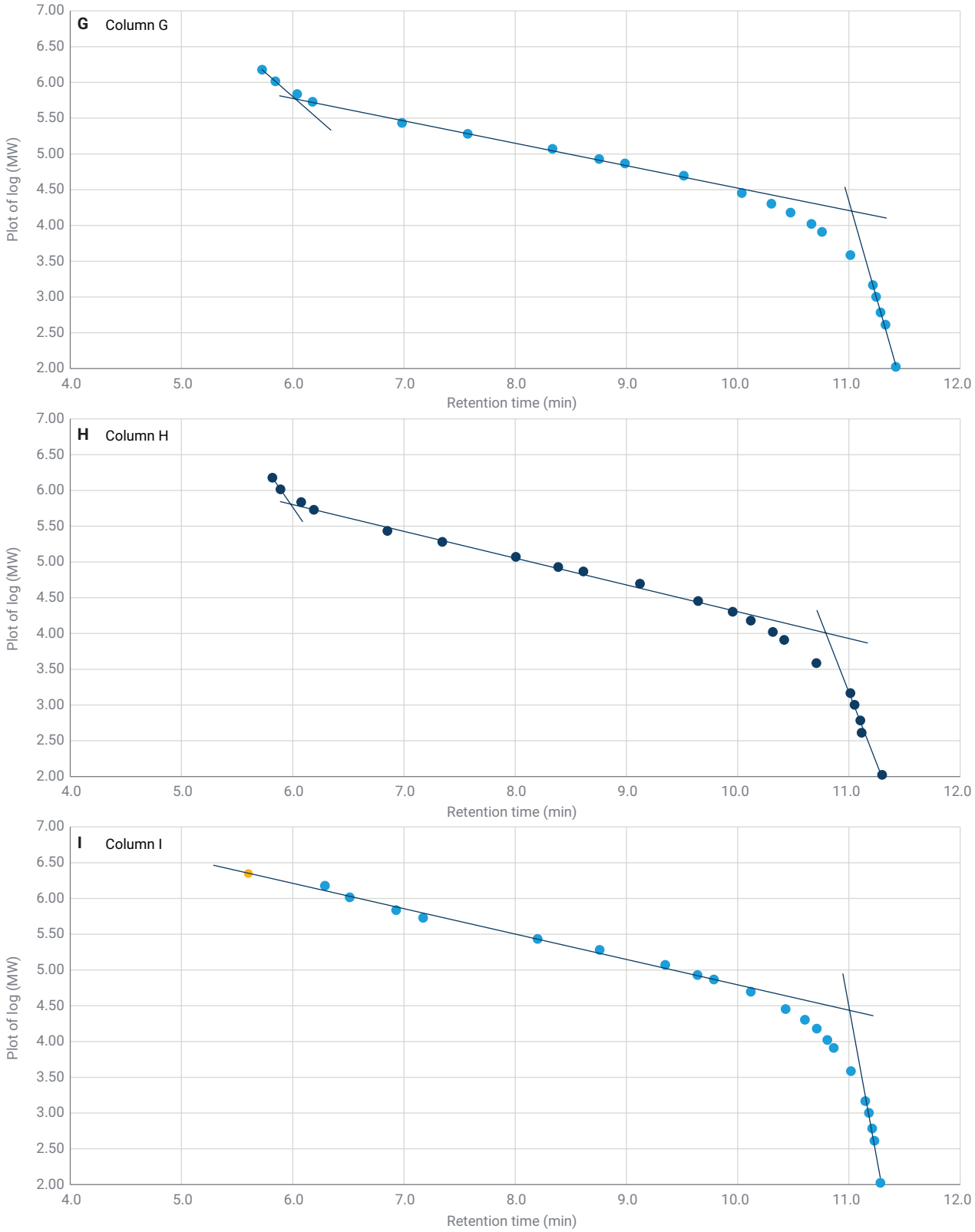

Figure 4. (A) Plot of log (MW) against retention time for column A, showing regions used to determine intersection points. (B–I) Plot of log (MW) against retention time for columns B through I.

Table 5. Data points used for linear fit to determine intersection times: excluded data points (orange), linear data points (green), and total permeation data points (red).

PEG/PEO	MW	Log(MW)	Retention Time (min) by Column								
			A	B	C	D	E	F	G	H	I
1,511 K	1,511,000	6.18	5.07	4.87	5.28	5.49	5.31	5.38	5.72	5.82	6.29
1,039 K	1,039,000	6.02	5.09	4.89	5.33	5.51	5.39	5.49	5.84	5.89	6.51
689 K	689,500	5.84	5.18	4.94	5.39	5.58	5.57	5.78	6.04	6.08	6.93
538 K	538,000	5.73	5.25	4.99	5.49	5.65	5.65	6.02	6.18	6.19	7.17
272 K	272,400	5.44	5.60	5.37	5.62	5.98	6.18	7.31	6.98	6.85	8.20
191 K	191,000	5.28	5.94	5.88	5.79	6.35	6.57	8.13	7.57	7.34	8.76
117 K	117,900	5.07	6.53	6.88	6.19	7.01	7.14	9.04	8.33	8.01	9.35
85 K	85,200	4.93	6.97	7.53	6.56	7.45	7.53	9.50	8.75	8.39	9.64
73 K	73,850	4.87	7.25	7.90	6.81	7.74	7.77	9.73	8.99	8.61	9.79
49 K	49,650	4.70	7.97	8.74	7.52	8.42	8.37	10.24	9.52	9.12	10.12
28 K	28,480	4.45	8.81	9.62	8.43	9.15	9.06	10.73	10.04	9.64	10.43
20 K	20,180	4.30	9.22	10.00	8.93	9.50	9.43	10.98	10.30	9.95	10.60
15 K	15,190	4.18	9.63	10.28	9.39	9.80	9.74	11.15	10.48	10.12	10.71
10 K	10,530	4.02	9.99	10.61	9.82	10.07	10.04	11.29	10.66	10.32	10.81
8 K	8,160	3.91	10.20	10.78	10.06	10.22	10.19	11.39	10.76	10.42	10.86
3,860	3,860	3.59	10.75	11.17	10.72	10.61	10.63	11.61	11.01	10.71	11.02
1,470	1,470	3.17	11.20	11.50	11.25	10.93	10.98	11.81	11.21	11.01	11.15
1,010	1,010	3.00	11.31	11.55	11.40	10.97	11.07	11.86	11.24	11.05	11.18
610	610	2.79	11.43	11.62	11.54	11.05	11.17	11.89	11.28	11.10	11.21
410	410	2.61	11.53	11.67	11.61	11.11	11.22	11.91	11.33	11.11	11.23
106	106	2.03	11.62	11.75	11.83	11.19	11.34	11.97	11.42	11.30	11.28

Table 6. Linear fit statistics by column.

	Linear Fit by Column								
	A	B	C	D	E	F	G	H	I
Excluded Points									
Slope	−2.250	−3.686	−3.043	−2.557	−1.984	−1.479	−1.367	−2.259	N/A
Intercept	17.522	24.087	22.237	20.159	16.716	14.130	14.003	19.320	–
Linear Points									
Slope	−0.309	−0.231	−0.285	−0.309	−0.360	−0.242	−0.313	−0.374	−0.355
Intercept	7.127	6.671	6.824	7.249	7.661	7.222	7.656	8.049	8.340
Total Permeation Points									
Slope	−6.250	−4.328	−2.285	−4.062	−3.705	−7.164	−5.436	−3.989	−8.479
Intercept	74.641	52.998	29.098	47.605	44.097	87.890	64.138	47.057	97.753

Table 7. Intersection points by column.

	Intersection Points by Column								
	A	B	C	D	E	F	G	H	I
Intersection (Exclusion Point)									
Time	5.36	5.04	5.59	5.74	5.58	5.59	6.02	5.98	5.60 (est.)
Log(MW)	5.47	5.50	5.23	5.48	5.65	5.87	5.77	5.81	6.35
MW	296,000	319,500	171,500	299,000	451,000	739,500	586,500	645,000	225,750
Rh (nm)	21.7	22.7	15.7	21.9	27.9	37.5	32.7	34.6	73.1
Size (Å)	434	455	314	437	559	751	654	692	1462
Intersection (Inclusion Point)									
Time	11.36	11.31	11.13	10.75	10.89	11.65	11.03	10.79	11.01
Log(MW)	3.61	4.05	3.66	3.93	3.74	4.40	4.20	4.01	4.44
MW	4,000	11,250	4,500	8,500	5,500	25,000	16,000	10,250	27,250
Rh (nm)	1.7	3.1	1.8	2.6	2.0	5.0	3.8	2.9	5.2
Size (Å)	33	62	36	52	40	99	76	58	105

If the optimum pore volume for each column is estimated as the difference between the inclusion intersection point and the exclusion intersection point, a chart comparing the different columns can be created (Figure 5). Simply comparing pore volume is not sufficient. It is necessary to know if the pore volume is in the right region for the molecules to be analyzed. If the slope of the linear fit portion is compared for each column, the shallowest gradient possible is desirable to have maximum resolving power in the region of interest (Figure 6).

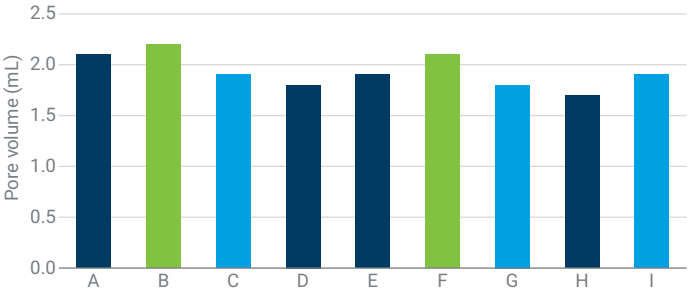

Figure 5. Comparison of pore volume.

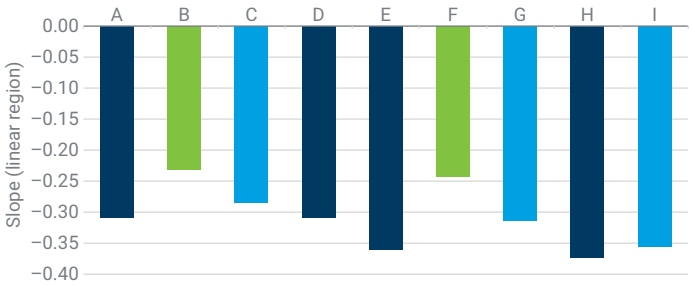

Figure 6. Comparison of linear slope.

Conclusion

By comparing different columns, this application note demonstrates that columns A, B, and F all have excellent pore volume (> 2.0). However, which columns will offer maximum resolution over their intended working size range can only be determined by comparing the slope in the linear region. In this case, both column B and column F will provide the optimum separation performance.

These columns are Agilent AdvanceBio SEC columns in 500 and 1,000 Å pore sizes.

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