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Applikationsbericht

Impact of LC System Dispersion on the Size-Exclusion Chromatography Analysis of Monoclonal IgG Antibody Aggregates and Fragments: Selecting the Optimal Column for Your Method

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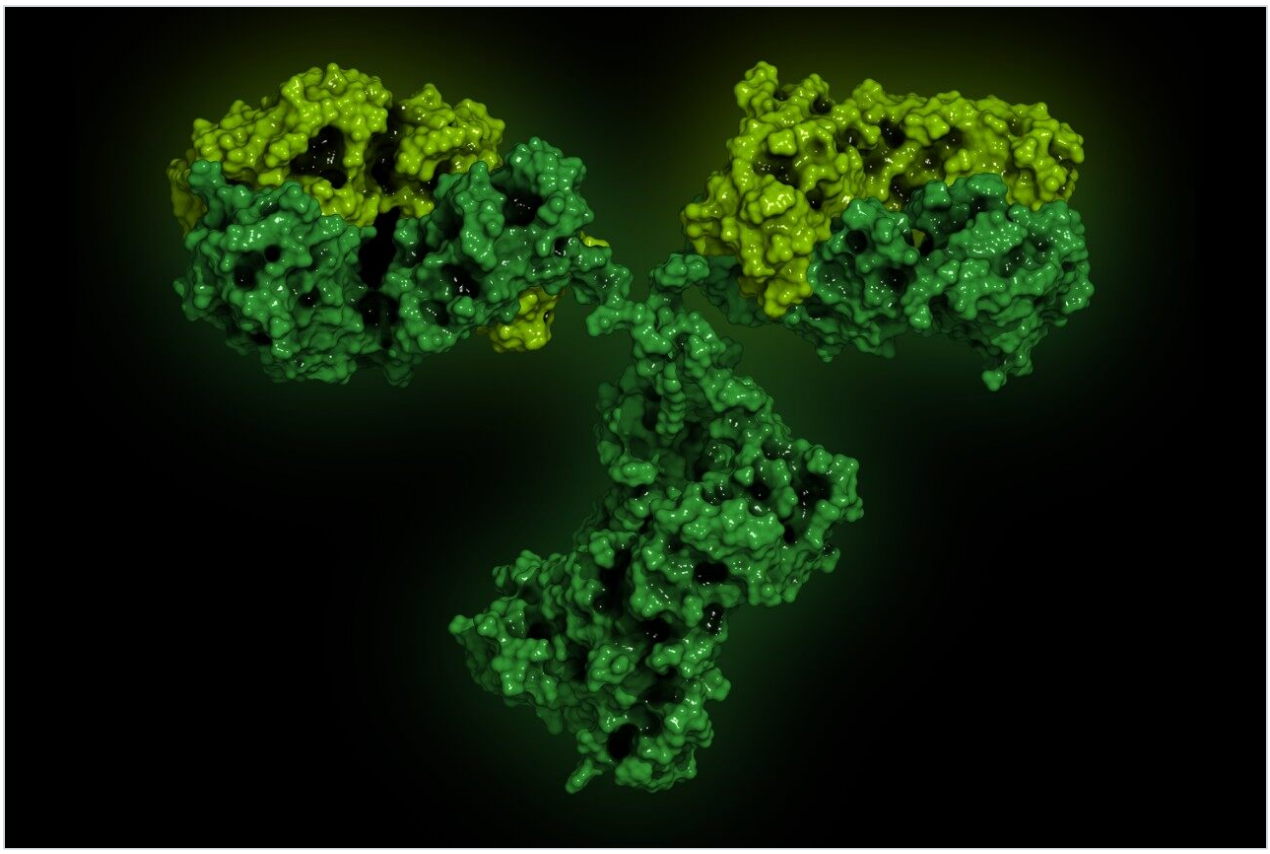
Waters Corporation



Abstract

This application note systematically evaluates the impact that SEC particle size, column length and internal diameter, and LC system extra-column dispersion has on the SEC resolution of HMWS and LMWS of a purified mAb noting that the basic illustrated principles are applicable to SEC of other protein classes. Additionally, the deleterious effect that system dispersion has on the lower limits of detection and the reliability of quantitative results for LMWS will also be demonstrated.

Benefits

- An educational and systematic demonstration of the impact of LC system dispersion on SEC-based mAb separations
- Guidance for selecting the optimal SEC column configuration based on the LC systems to be used and the analytical method requirements including resolution, sensitivity, reproducibility, and transferability
- A comparison of the SEC separation performance of the ACQUITY UPLC H-Class Bio (UPLC) and ACQUITY Arc Bio (UHPLC) Systems

Introduction

Historically, native size-exclusion chromatography (SEC) has been the most widely used methodology for the assessment of non-covalent protein aggregation (high molecular weight species [HMWS]) in recombinant protein based biotherapeutic products.[1] However, in recent years due to the improved capabilities of SEC columns and LC systems, there has also been a greater interest in using SEC for the non-denatured analysis of protein fragments (low molecular weight species [LMWS]) in these samples. Most notably, the analysis of IgG monoclonal antibody (mAb) fragments resulting from the hydrolytic degradation of the hinge region has been targeted.[2] In comparison to the more traditional separation of the dimer and higher molecular weight forms of HMWS (≥300 KDa) from the monomer (~150 KDa), the separation of LMWS fragments, a predominant form of which for a mAb is two-thirds the molecular weight of the mAb monomer (~100 KDa), can be more challenging. This is due to the LMWS and monomer being more similar in size (hydrodynamic radius) versus the size comparison between the monomer and HMWS protein forms. An additional difficulty is presented by the elution order of the proteins in which the low-abundance LMWS peak elutes as a trailing shoulder on the main (monomer) peak.

While the use of higher efficiency SEC columns with particle diameters of 2 µm and smaller has enabled improved efficiencies resulting in higher throughput analyses of HMWS and LMWS forms, due to column hardware and packing constraints, these high efficiency SEC particles have only been made available in columns with internal diameters of 4.6 mm and smaller. Whereas, 7.8 mm internal diameter (I.D.) columns are typically employed for 3 µm and larger SEC particle sizes when using an HPLC chromatographic system. While many HPLC systems are physically capable of operating at the flow rates and back pressures required for many of these smaller particle size 4.6 mm I.D. SEC columns, what is frequently not considered is the fact that extra-column dispersion in typical HPLC configurations is sufficiently large relative to the peak volumes generated by the sub-2-µm columns. The result of this is that the peak resolutions observed are significantly reduced.[3] Extra-column dispersion can be considered as the increase in the volume of an injected sample that occurs as it travel through the flow path of an LC system without a column in place.

The goal of this study was to systematically evaluate the impact that the SEC particle size, column length, and internal diameter, and LC system extra-column dispersion has on the SEC resolution of HMWS and LMWS impurities of a purified mAb noting that the basic illustrated principles are applicable to SEC of other protein classes. Additionally, the deleterious effect that system dispersion has on the lower limits of detection and the reliability of quantitative results for LMWS will also be demonstrated. In summary, recommendations for SEC column selections that are compatible with Waters LC systems and for the analysis of mAbs are provided.

Experimental

Sample description

The mAb samples of infliximab (Remicade) and rituximab (Rituxan) were used past expiry at the original concentration of ~21 mg/mL. Trastuzumab (Herceptin) was used past expiry at a diluted (in water) concentration of 2.0 mg/mL.

Method conditions (unless noted otherwise)

LC conditions

Systems:	ACQUITY UPLC H-Class Bio, unless otherwise

	noted
Detection:	ACQUITY UPLC TUV Detector with 5 mm titanium flow cell
Wavelength:	280 nm, unless otherwise noted
Columns:	ACQUITY UPLC Protein BEH SEC,200 Å, 1.7 µm, 2.1 × 150 mm (p/n: 186008471) ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 × 150 mm (p/n: 186005225) ACQUITY UPLC Protein BEH SEC, 200 Å, 1.7 µm, 4.6 × 300 mm (p/n: 186005226) XBridge Protein BEH SEC, 200 Å, 2.5 µm, 7.8 × 300 mm (p/n: 186009164) XBridge Protein BEH SEC, 200 Å, 2.5 µm, 7.8 × 150 mm (p/n: 186009163) XBridge Protein BEH SEC, 200 Å, 2.5 µm, 4.6 × 150 mm (p/n: 176004335) XBridge Protein BEH SEC, 200 Å, 2.5 µm, 4.6 × 300 mm (p/n: 176004336) XBridge Protein BEH SEC, 200 Å, 3.5 µm, 7.8 × 150 mm (p/n: 176003595) XBridge Protein BEH SEC, 200 Å, 3.5 µm, 7.8 × 300 mm (p/n: 176003593) BEH SEC, 200 Å, 3.5 µm, 4.5 × 300 mm (custom packed)
Column temp.:	Ambient, ~22 °C
Sample temp.:	10 °C

Mobile phase A:	100 mM NaH_2PO_4
Mobile phase B:	100 mm Na_2PO_4
Mobile phase C:	1.00 M NaCl
Mobile phase D:	H_2O

Flow rates and injection volumes, unless otherwise noted:

Column dimension	Flow rate	Injection volume
(mm I.D. × mm L)	(mL/min)	(µL)
4.6 × 150	0.35	1
4.6 × 300	0.35	2
7.8 × 150	1	5.8
7.8 × 300	1	10

All 0.2 µm sterile filtered and Auto•Blend Plus blended at 7.4% A, 12.6 % B, 35% C, and 45% D to yield 20 mm sodium phosphate, 350 mM NaCl, pH 6.8, unless otherwise noted

Sample vials: Polypropylene 12 × 32 mm Screw Neck Vial, with Cap and PTFE/silicone Septum, 300 µL volume (p/n: 186002640)

Data management

Chromatography software: Empower 3

Results and Discussion

Measuring System Dispersion

One chromatography fundamental is that extra-column dispersion, or broadening of a chromatographic peak or band, that does not occur within the packed chromatographic bed of the column always has a deleterious effect on the resolution of a separation. In many protein and peptide gradient separations, such as reversed phase and ion exchange, the analyte binds strongly to the stationary phase under loading conditions and will re-concentrate at the head of the packed bed of the column until the gradient begins. As a result, for these gradient-based separations, the deleterious effect of pre-column band dispersion will be minimized or even eliminated to the point that the principal concern will be dispersion that occurs to the peaks once they are eluted from the column. Conversely, in ideal SEC separations, there is no binding that occurs between the protein sample and the packed SEC particle surface. The practical result of this is that for SEC separations pre-column dispersion will equally degrade the quality of a separation as compared to post-column dispersion. Consequently, evaluating the changes in an injected sample volume and profile that occur after it travels through an LC system without a column in place can be very instructive.

The measurement of extra-column dispersion (system band broadening) has been studied extensively and for the interested reader a far more thorough discussion of system dispersion and its measurement can be found in the companion publication to this application note (720006337EN). For this discussion the $5\sigma_{ec}$ dispersion volumes (based on peak width at 4.4% peak height) were determined as diagrammed in Figure 1. As can be seen in the dispersion profiles shown in Figure 2, the extra-column dispersion peaks are asymmetrical with a noticeable tailing or skewed profile, and by making a peak width measurement closer to the baseline more of the influence of peak tailing can potentially be accounted for. Therefore, using peak width at 4.4% peak height to determine 5σPeak values, while somewhat arbitrary, was selected due to it being the peak width at the lowest percentage of a peak height that is provided by most common chromatography data systems such as Waters Empower 3 and Agilent ChemStation. The varied extra-column dispersions shown in Figure 2 were created on an ACQUITY UPLC H-Class Bio System by adding sample loops at the inlet side of the column.

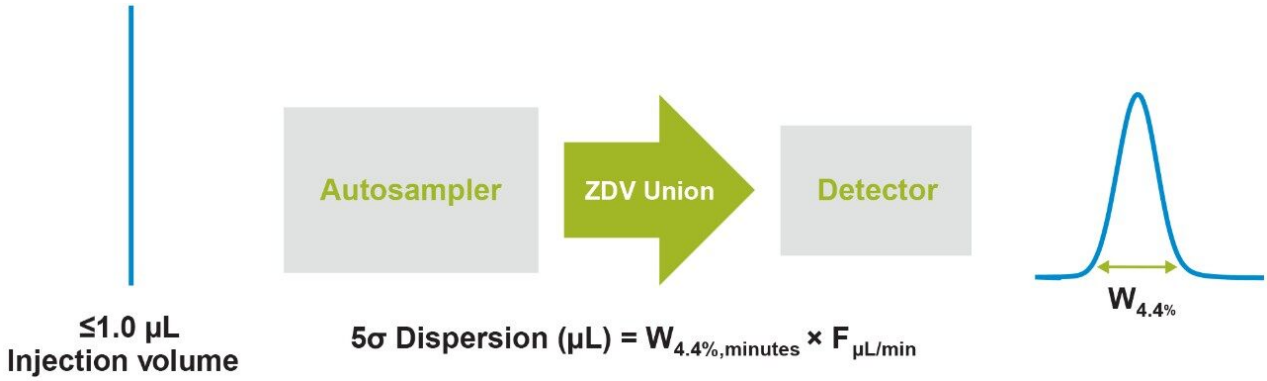


Figure 1. Measurements of extra-column dispersion were carried out using 3:7 water:acetonitrile as a mobile phase at a 0.3mL/min flow rate. The sample was 1 µL of 0.16 mg/mL caffeine in 1:9 water:acetonitrile. The UV absorbance was monitored at 273 nm at a sampling rate of 40 Hz.

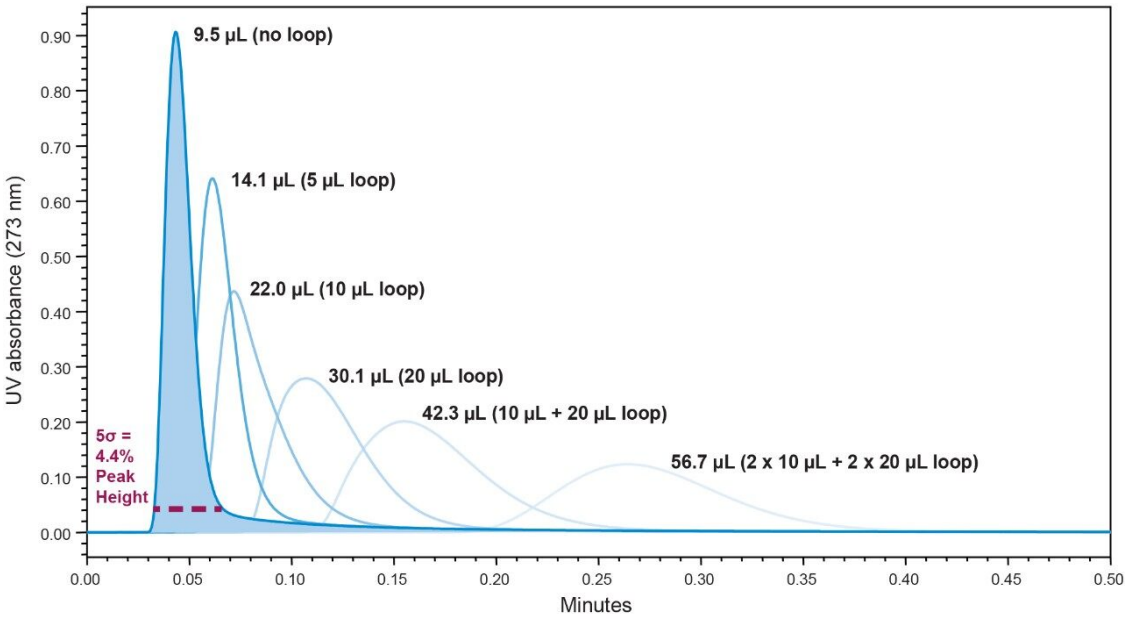


Figure 2. Shown are the measurements of 5-sigma extra-column dispersion volumes ($5\sigma_{ec}$) for this study based on peak width at 4.4% peak height. Experiments were carried out as described in the caption of Figure 1. Sample loops were connected pre-column to generate the larger dispersion volumes.

Impact Of System Dispersion On Separation And Quantification Of Biotherapeutic Igg HMWS And LMWS

Shown in Figure 3 is a well resolved SEC chromatogram of infliximab, a biotherapeutic chimeric human-

mouse IgG monoclonal antibody. This sample was analyzed well past expiry and the HMWS, presumed to be predominantly dimer (~300 KDa), is present at a level of approximately 0.75% in the samples tested. The LMWS1 fragment is the result of one of the FAb domains of the antibody being hydrolytically cleaved from the protein at a site within the hinge region of the antibody and has a molecular weight of ~100 KDa while the LMWS2 fragment is a mixture of FAb and Fc domains (both ~50 KDa).[4] The free Fc domain being the result of both FAb arms being cleaved. Of these product related impurities, the most challenging to resolve is LMWS1 due to this form not being as proportionally different in size from the monomer fraction as the HMWS is from the IgG monomer, and by its elution position within the tailing segment of the far more abundant IgG monomer. A low abundance species that trails the main peak in a separation becomes more problematic to resolve and quantify since any increase in the tailing of the main peak will have a significant impact on the resolution and integrated area of that low abundance peak. To address the extent of this impact we evaluated several mixtures of the mAb that contained levels of LMWS1 ranging from approximately 0.4 to 4.1% peak area. These samples were evaluated on several different SEC columns using an LC system where the $5\sigma_{ec}$ volumes were purposely modified to be between 9.5 µL and 56.7 µL.

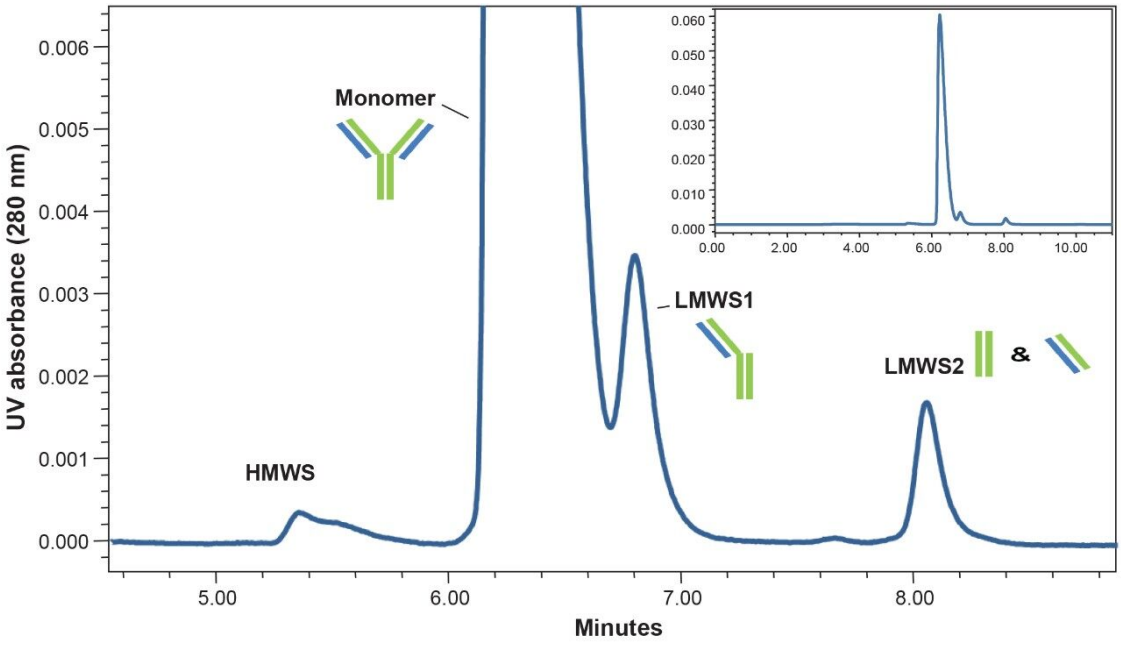


Figure 3. Sample chromatogram demonstrating the optimal separation obtained for a degraded infliximab sample using a 1.7 µm particle size SEC column with an internal diameter of 4.6 mm and length of 300 mm. The 5-sigma extracolumn dispersion (σ_{ec}) of the LC system was 9.5 µL. Peak identifications are: high molecular weight (HMWS, ~300 KDa), mAB monomer (~150 KDa), 2/3 mAb fragment comprised of one Fab and one Fc domain (LMWS1, ~100 KDa), and co-eluting Fc and Fab domains (LMWS2, ~50 KDa). The measured peak area percent for HMWS in this sample is 0.75%, that of the LMWS1 fragment is a 4.1%, and that of the LMWS2 fragment is 1.7%.

Examples of the chromatographic results for the infliximab samples evaluated on the 4.6 mm I.D. (1.7 µm SEC particles), 150 mm and 300 mm length columns are shown in Figures 4 and 5, and those for the 7.8 mm I.D. (3.5 µm SEC particles), 300 mm length column are shown in Figure 6. The chromatograms for the 7.8 mm I.D. (3.5 µm), 300 mm column are not shown as the LMWS1 fragment was not resolved on this column. A summary of the quantitative results for HMWS determination using all four columns are presented in Figure 7. We observe that the resolutions between the HMWS and the mAb monomer, and the integrated HMWS percent peak areas for both 7.8 mm I.D. (3.5 µm) columns and for the 1.7 µm, 4.6 × 300 mm column is minimally impacted by increases in extra-column dispersion. This is due in part to the high degree of separation that is achieved between the two peaks (R_s>1.6), the large peak volumes generated by these three columns, and the order of elution such that peak tailing of the significantly more abundant monomer has less impact on resolution. Additionally, upon closer inspection of the chromatographic profiles, we observe that the HMWS peak is somewhat polydisperse and as a result, the resolution values will appear to be more

consistent as they will be strongly dictated by the size distribution of self-associated forms eluting in the HMWS peak.

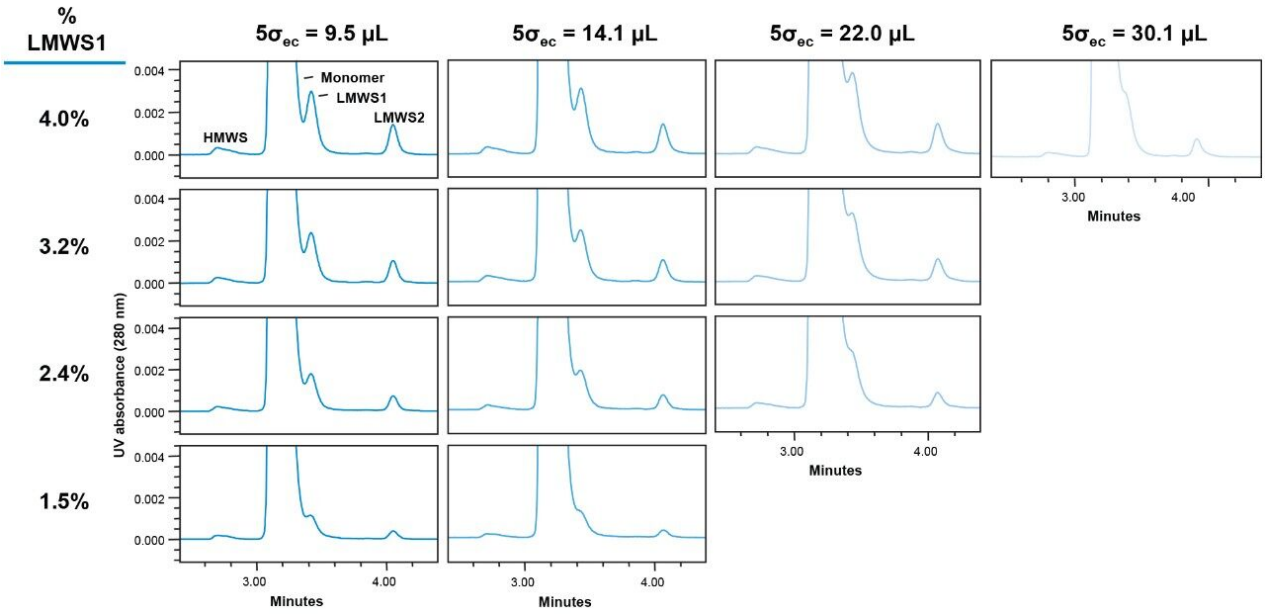


Figure 4. Zoomed view chromatograms demonstrating the impact of extra-column dispersion (σ_{ec}) on the separation of LMW1 mAb fragment with 1.7 µm particle size SEC column with an internal diameter of 4.6 mm and length of 150 mm. The predicted peak area percents for the LMWS1 fragment in each of the samples evaluated at the σec conditions tested are shown in the left column and range from 4.1% to 1.5%. Peak identifications are provided in Figure 8.

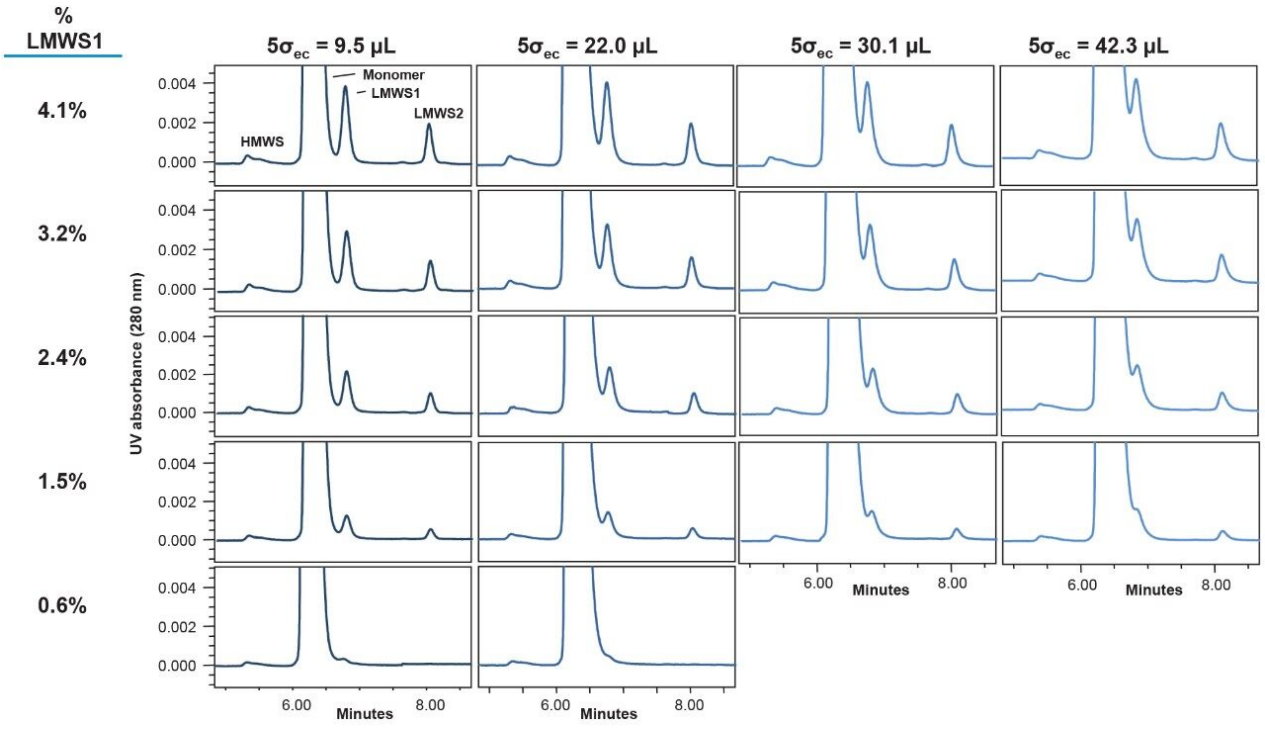


Figure 5. Zoomed view chromatograms demonstrating the impact of extra-column dispersion (σec) on the separation of LMWS1 mAb fragment with 1.7 µm particle size SEC column with an internal diameter of 4.6 mm and length of 300 mm. The predicted peak area percents for the LMWS1 fragment in each of the samples evaluated at the σec conditions tested are shown in the left column and range from 4.1% to 0.6%. Peak identifications are provided in Figure 8.

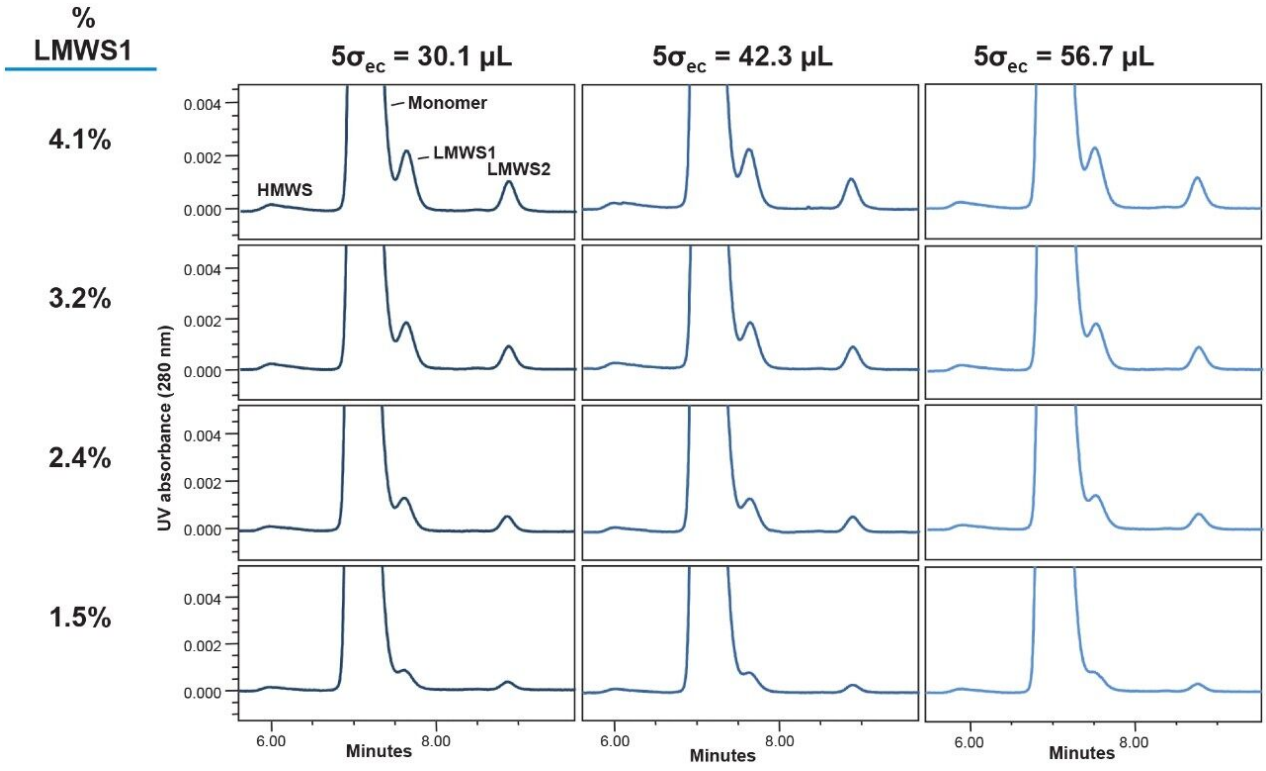


Figure 6. Zoomed view chromatograms demonstrating the impact of extra-column dispersion (σec) on the separation of LMWS1 mAb fragment with 3.5 µm particle size SEC column having an internal diameter of 7.8 mm and length of 300 mm. The predicted peak area percents for the LMWS1 fragment in each of the samples evaluated at the σec conditions tested are shown in the left column and range from 4.1% to 1.5%. Peak identifications are provided in Figure 8.

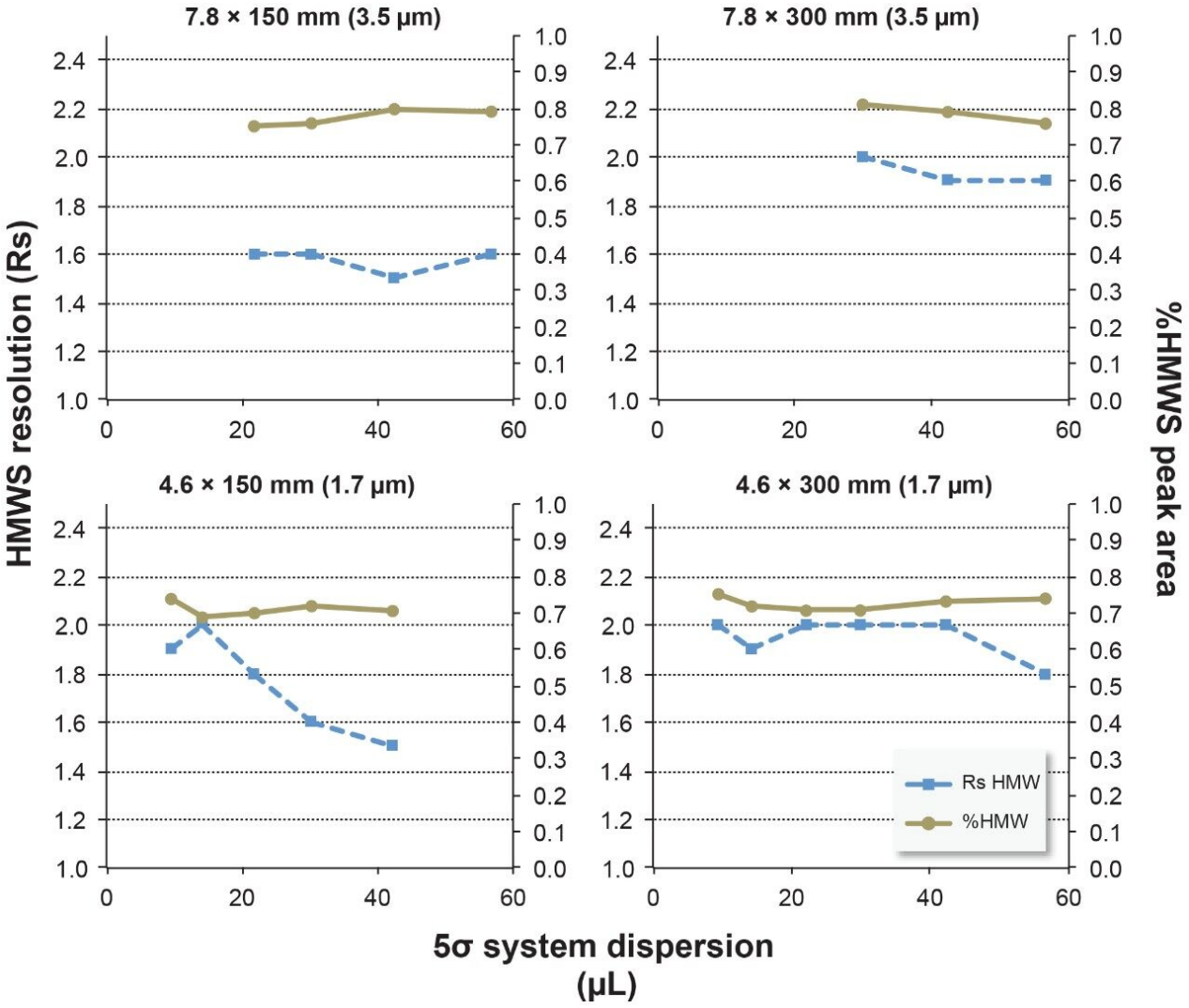


Figure 7. Shown are quantitative comparisons for the HMWS peak from the chromatograms shown in Figures 10 through 13. In addition, the results from the evaluation of HMW for the 3.5 µm particle size SEC column with an internal diameter of 4.6 mm and length of 150 mm are included. The USP resolution between the monomer and HMWS peak (dashed blue line with square markers) and the HMWS integrated percent peak areas (brown lines with round markers) are plotted against the system dispersions tested as described in the text. Sample injection volumes and flow rates were proportional to column internal diameter.

Despite this factor, we still observe a significant decrease in resolution for the 1.7 µm (4.6 × 150 mm) column as system dispersion increases. This is a result of the smaller peak volumes generated by this column (Figure 8). However, the integrated HMWS percent peak area is still consistent for this column. We see a similar trend when comparing the 300 mm columns although the 1.7 µm (4.6 × 300 mm) column is not outperformed until we operate at a $5\sigma_{ec}$ of greater than 40 µL. When we consider the IgG peak volumes based on 5σ peak

widths generated by these columns (Figure 8), we can see why the longer length, larger internal diameter, and larger particle size columns are not as greatly impacted by extra-column dispersion. For example, in comparing the peak volumes generated by the 3.5 µm, 7.8 mm I.D. columns to those of the 4.6 mm I.D. (1.7 µm) columns, nearly a 4-fold increase in peak volume is observed. We also observe an approximate 50% increase in peak volume as column lengths are increased from 150 mm to 300 mm. These results indicate that for the SEC analysis of HMWS, system dispersion volumes will likely need to be minimized and controlled to derive a significant resolution benefit from 4.6 mm I.D. columns packed with 1.7 µm particles. In this example, 5σec dispersion volumes should be lower than 15 µL for the 150 mm column, and below 40 µL for the 300 mm column.

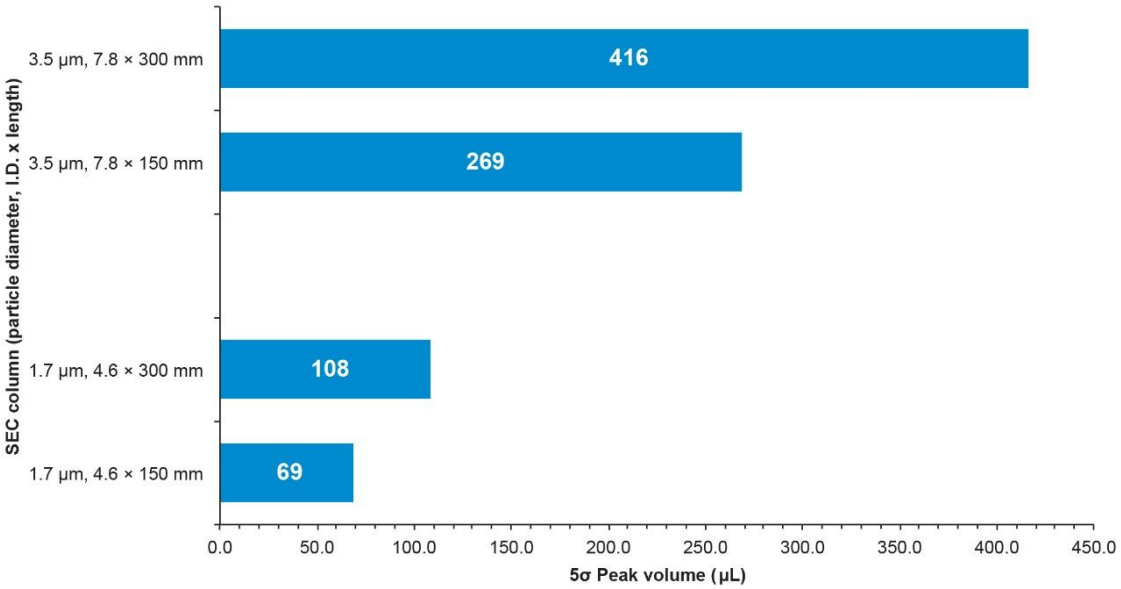


Figure 8. Shown are the estimated $5\sigma_{ec}$ peak volumes (based on peak width at 4.4% height) for an IgG peaks in the Waters BEH200 SEC Protein Standard Mix. Chromatograms are not shown. Peak volumes were corrected for 5-sigma system dispersion volumes.

We will next examine the effect that extra-column dispersion has on the separation of LMWS and mAb monomer. Based on the chromatographic results presented in Figures 4 through 6, a series of graphs are presented (Figure 9) showing the effect of extra-column dispersion on the peakto- valley ratio between the monomer and the LMWS1 peaks (left axes) and percent areas of the LMWS1 peaks (right axes). The results for the 3.5 µm (7.8 × 150 mm) column have been omitted as the LMWS1 fragment was not resolved on that column.

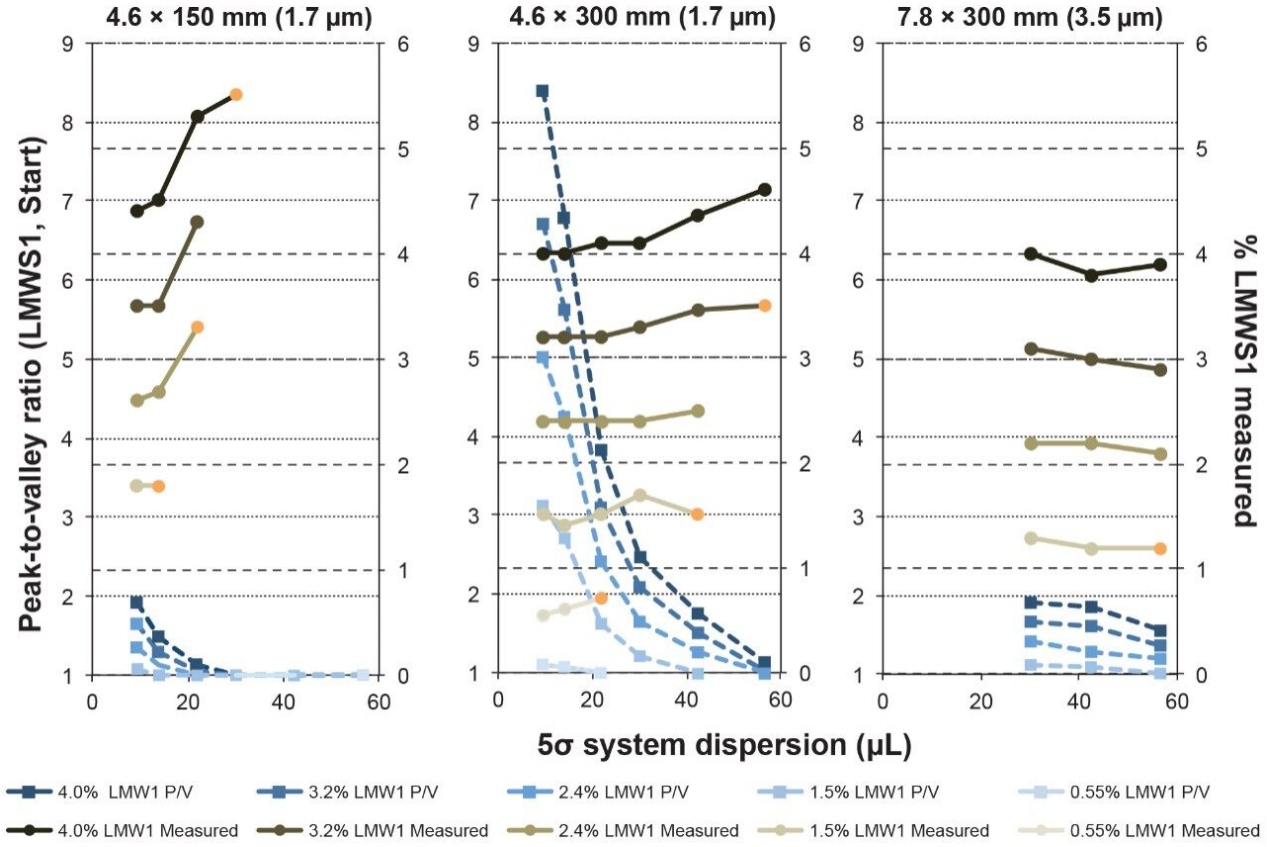


Figure 9. Quantitative comparisons for the determination of LMWS1 from the chromatograms shown in Figures 4 through 6. The peak-to-valley ratio for the LMWS1 mAB fragment (blue dashed lines with square markers) and the LMWS1 integrated percent peak areas (brown solid lines with round markers) are plotted against the system dispersions tested as described in the text. Orange circles indicate measured % LMWS1 data points where the P/V ratio for the LMWS1 peak was ≤1.01. Sample injection volumes and flow rates were proportional to column internal diameter.

In comparing the resolution (P/V) results between the two 1.7 µm, 4.6 mm I.D. columns, we can immediately see that the use of the 300 mm length column provides significantly greater resolution of the mAb monomer and LMWS1 peaks. This is primarily the result of approximately doubling the plate count or efficiency of the separation, however, as observed in Figure 8 the increased peak volume produced by the longer column also reduces the impact of extra-column volume. This greater resolution also provides for a lower limit of detection, which in this case will be arbitrarily defined as a P/V ratio greater than 1.01. Additionally, the 300 mm column can resolve the LMWS1 peak at larger $5\sigma_{ec}$ levels. When we compare the quantitative results (% LMWS1), we can see that throughout the range of $5\sigma_{ec}$ levels tested that the percent of the LMWS1 fragment is never consistent with respect to $5\sigma_{ec}$ for the 150 mm length column, while for the 300 mm length column the measured percent LMWS1 is more consistent although it does begin to deviate more significantly as $5\sigma_{ec}$

levels exceed 30 µL. It should be noted that the quantitative results for the more well resolved LMWS2 peak were consistent for these three column configurations and for the 3.5 µm (7.8 × 150 mm) column throughout the range of $5\sigma_{ec}$ volumes evaluated.

In comparing the performance of the 3.5 µm (7.8 × 300 mm) column to that of the 1.7 µm (4.6 × 300 mm) column, we observe that the P/V ratio was on average only 7 to 20% lower at $5\sigma_{ec}$ levels of 30 µL and that at $5\sigma_{ec}$ levels of 42 µL and greater the P/V ratios for the 3.5 µm column were reproducibly higher. However, unlike the 1.7 µm column, this larger column format produced more consistent quantitative results for LMWS1 as $5\sigma_{ec}$ increased above 22 µL. To summarize, these results for the SEC analysis of the LMWS1, the use of a 1.7 µm (4.6 × 150 mm) column is not recommended for a validated method due to the significant variation in quantification observed with changes in system dispersion. The 1.7 µm (4.6 × 300 mm) column produced the highest resolutions and reliable LMWS1 quantification when $5\sigma_{ec}$ levels of approximately 25 µL and lower are maintained. The 3.5 µm (7.8 × 300 mm) column produced significantly lower resolutions than what could be achieved using the 1.7 µm (4.6 × 300 mm) column on a low dispersion LC, however, the quantitative results were consistent with respect to system dispersion. In addition, for these mAb separations at $5\sigma_{ec}$ levels greater than 40 µL, the 3.5 µm (7.8 × 300 mm) column provided the highest resolution.

If more sensitive LMWS1 analysis is required, and when using an LC system that cannot take advantage of a 1.7 µm (4.6 × 300 mm) column, a reasonable option will be to operate two 3.5 µm (7.8 × 300 mm) columns in series resulting in a total column length of 600 mm. Although this will increase analysis time, the resultant method will be more reliable and easily transferrable. The use of multiple SEC columns in series is demonstrated by a comparison of the separations obtained for LMWS1 of trastuzumab on the 1.7 µm (4.6 × 300 mm) column, the 3.5 µm (7.8 × 300 mm) column, and on two 3.5 µm (7.8 × 300 mm) columns run in series (Figure 10). We did not rigorously transfer our method between these columns but instead elected to use the commonly used flow rates of 0.4 mL/min for the 1.7 µm (4.6 × 300 mm) column, and 1.0 mL/min for the 3.5 µm (7.8 × 300 mm) column. The 1.7 µm (4.6 × 300 mm) column was evaluated on both an ACQUITY UPLC H-Class Bio System ($5\sigma_{ec}$ = 20 µL) and on an ACQUITY Arc Bio System ($5\sigma_{ec}$ = 34 µL). We can see from these comparisons that the LMWS1 and the mAb monomer, which is estimated to be present at a ~0.3% relative abundance, are effectively separated on the 1.7 µm (4.6 × 300 mm) column when using the ACQUITY UPLC H-Class Bio System (P/V=1.32), whereas the higher dispersion of the ACQUITY Arc Bio System compromises the separation (P/V = 1.03) to a level that would likely result in unreliable quantification of LMWS1. The HMWS and LMWS2 are both adequately separated on both LC systems. When the method was run using a single 3.5 µm (7.8 × 300 mm) column run at a flow rate of 1.0 mL/min with the ACQUITY Arc Bio System, lower component resolution was observed in comparison to a 1.7 µm (4.6 × 300 mm) column run on the ACQUITY Arc Bio System, as would be predicted. However, when using two 3.5 µm (7.8 × 300 mm) columns in series at 1 mL/min the resolution (P/V = 2.1) was significantly improved over that observed for

the 1.7 µm (4.6 × 300 mm) column configured to the ACQUITY UPLC H-Class Bio System (P/V = 1.32). This is because even with a $5\sigma_{ec}$ value of 20 µL, the ACQUITY UPLC H-Class Bio System degrades the resolution (P/V) of the separation between the monomer and LMWS1 from the theoretical maximum. We can observe this clearly based on the P/V ratios for the LMWS1 of infliximab on this column (See Figure 5). While this tandem column method requires an approximately two-fold increase in analysis time in addition to using more sample and mobile phase, it provides a reliable and sensitive method. Given the immense peak volumes generated (~550 µL), the separation efficiencies obtained are not predicted to be significantly impacted by $5\sigma_{ec}$ dispersions of even greater than 60 µL.

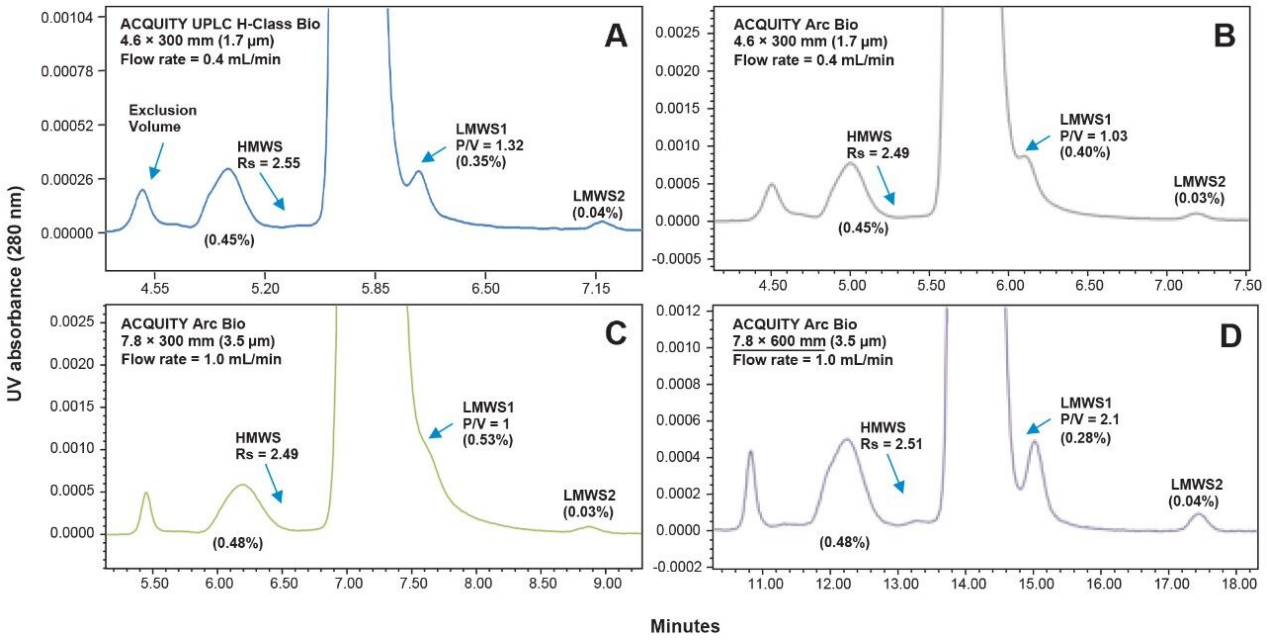


Figure 10. Zoomed view chromatograms demonstrating the impact of extra-column dispersion (σ_{ec}) of an ACQUITY UPLC H-Class Bio System (30 cm column heater, $5\sigma_{ec}$ = 20 µL) and an ACQUITY Arc Bio System (5 σ_{ec} = 34 µL) on the SEC separation of HMWS, and the mAb fragments LMWS1 and LMWS2 for trastuzumab. The top two chromatograms (A and B) compare the results obtained using a 4.6 × 300 mm (1.7 µm) column on the ACQUITY UPLC H-Class Bio System (A) and the ACQUITY Arc Bio (B) at a flow rate of 0.4 mL/min. The bottom left chromatogram (C) was generated using a 7.8 × 300 mm (3.5 µm) column, while the bottom right chromatogram (D) was produced using two 7.8 × 300 mm (3.5 µm) columns in series, both at a flow rate of 1.0 mL/min. The mobile phase was 25 mM sodium phosphate, 400 mM NaCl, pH 7.2. Injection volumes were 5 µL (A and B), 15 µL (C) and 21 µL (D). The UV flow cell pathlength was 5 mm for the ACQUITY UPLC H-Class Bio and 10 mm for the ACQUITY Arc Bio.

2.5 µm BEH SEC Columns for Igg HMWS And LMWS Analysis

Following the publication of the original version of this application note, Waters produced an intermediate 2.5 µm particle size series of BEH SEC columns. Based in part on some of the studies presented in this study, the goal of this product was to provide higher resolution separations versus the 3.5 µm particle size and resolutions approaching those that can be observed on 4.6 mm ID, 1.7 µm particle size, columns while using LC systems with 20 µL or larger $5\sigma_{ec}$ dispersion volumes. The data presented in this section represent an entirely different set of experiments as the samples used in the previous sections of this application note were no longer available. For additional information on the comparative performance of the 2.5 µm particle size BEH SEC columns, reference "High Resolution and High Throughput Size-Exclusion Chromatography Separations of IgG Antibody Aggregates and Fragments on UHPLC and HPLC Systems with 2.5 µm BEH Particles" (Waters Application Note, p/n: 720006522EN).

A comparison of the chromatograms produced using three 200 Å BEH SEC particles for the separation of rituximab, a chimeric (mouse/human) anti-CD20 IgG1 antibody, is shown in Figure 11. Only the 300 mm column lengths are compared in this study as it was already shown that the use of 1.7 µm (4.6 × 150 mm) columns significantly decreases the resolution and limit of quantification for the LMWS1 fragment. In Figure 11, the impact of system dispersion was evaluated on 1.7 µm (4.6 × 300 mm), 2.5 µm (4.6 × 300 mm), and 2.5 µm (7.8 × 300 mm) columns. The 3.5 µm (7.8 × 300 mm) column was only evaluated at a $5\sigma_{ec}$ system dispersion volume of 38.8 µL.

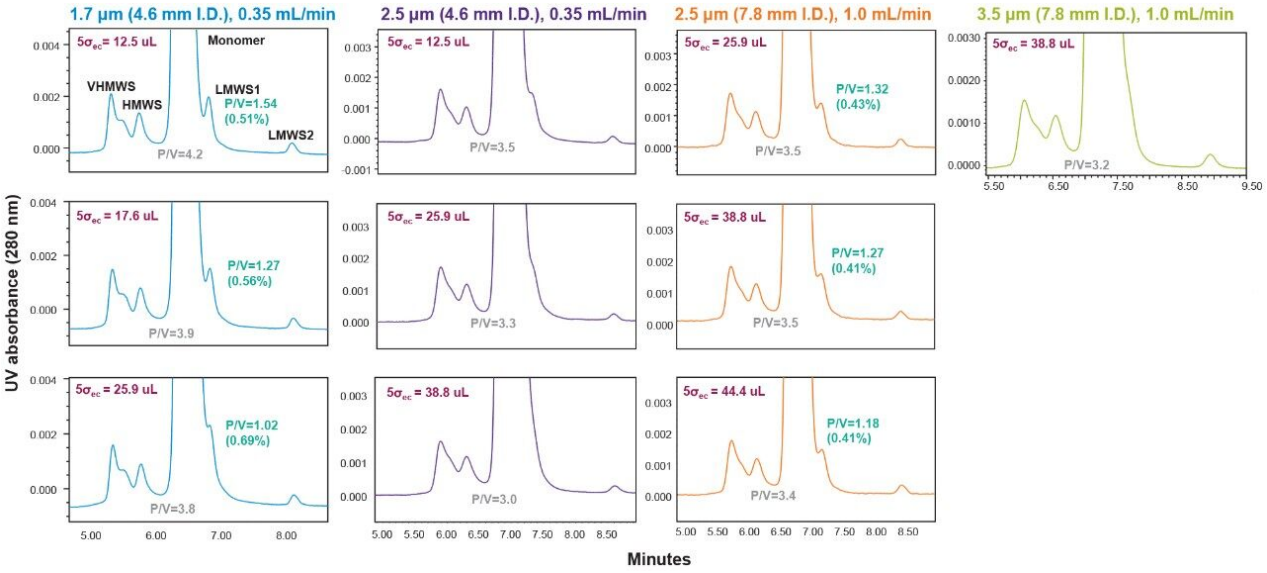


Figure 11. A comparison of the separation of rituximab on 200 Å pore size BEH SEC particles with diameters of 1.7 µm, 2.5 µm, and 3.5 µm. All columns were 300 mm in length, and sample loads and flow rates were proportional to the square of the column I.D. For the 1.7 µm and 2.5 µm (4.6 mm I.D.) columns, the flow rate was held constant, and system dispersion was increased. For the 2.5 µm and 3.5 µm (7.8 mm I.D.) columns, the system dispersion was constant, and flow rates were decreased. Mobile phase was 20 mm sodium phosphate, 400 mM NaCl, pH 7.2. Percent peak areas were determined by drop-baseline peak integration.

The first consideration is the critical pair separation between the HMWS and the monomer. HMWS is presumed to be predominantly a dimer (~300 KDa) that is present at a level of approximately 0.5% in the sample tested. By comparing the top row of chromatograms, it is observed that the HMWS-monomer separation improves (higher P/V values) as particle size is decreased when the columns are operated at equal linear velocities and on systems with appropriate dispersion volumes. Throughout the range of $5\sigma_{ec,}$ system dispersion volumes show that the 1.7 µm particle size column provided better resolution than the 7.8 mm I.D. (2.5 µm or 3.5 µm) columns, although this advantage diminishes as system dispersion is increased. It also shows that for the 2.5 µm particles, the performance of the 4.6 mm I.D. column matches that of the 7.8 mm I.D. column at only the lowest dispersion volume tested ($5\sigma_{ec}$ = 12.5 µL); and, increases in system dispersion do not significantly alter the HMWS P/V values for the 7.8 mm I.D. (2.5 µm) column. Similar comparisons can be made for HMWS separations using 150 mm length columns. Although, as we would predict, the impact of increasing system dispersion is proportionally greater.

The other consideration is the separation of the IgG LMWS1 fragment on these columns when operated at equivalent linear velocities. As noted previously, this separation is further complicated by the elution position

of the LMWS peak within the tailing segment of the far more abundant monomer and by the low abundance (~0.4%) of LMWS1 in the sample. The effect that system dispersion has on P/V ratio and percent peak area for 1.7 µm (4.6 × 300 mm) and 2.5 µm (7.8 × 300 mm) columns are also presented in Figure 12. For the 1.7 µm (4.6 × 300 mm) column, we observe a precipitous decrease in P/V as $5\sigma_{ec}$ system dispersion volume is increased from 12.5 µL to 25.9 µL. This loss in resolution also resulted in an increase in the integrated relative peak area of LMWS1 from 0.5% up to 0.7%, which is consistent with previous results (Figure 9).

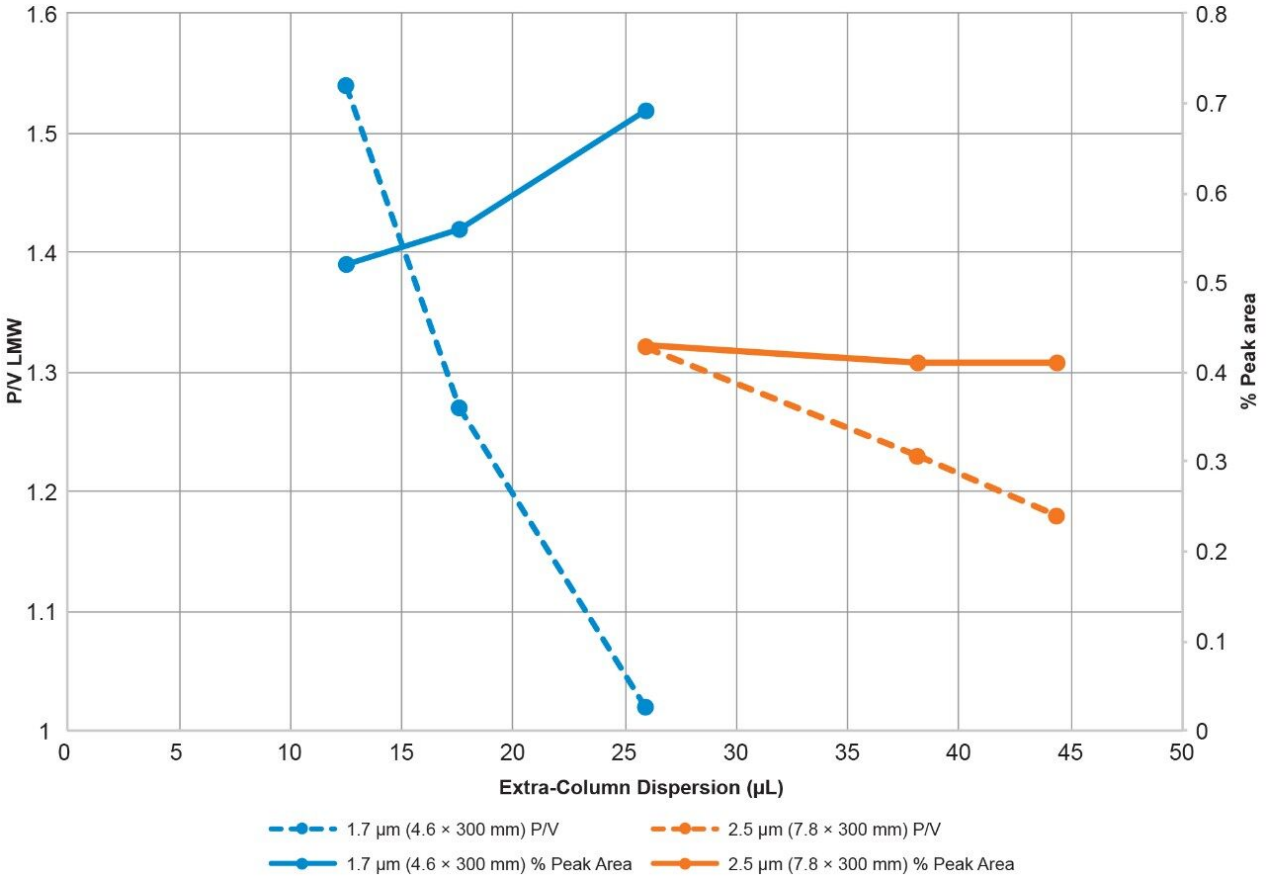


Figure 12. Shown are quantitative comparisons for the determination of LMWS1 from the chromatograms shown in Figure 11 for the 1.7 µm (4.6 × 300 mm) and 2.5 µm (7.8 × 300 mm) columns run at equivalent analysis times. The peak-to-valley ratio for the LMWS1 mAb fragment (dashed lines) and the LMWS1 integrated percent peak areas (solid lines) are plotted against the system dispersions tested (as described in the text). Sample injection volumes and flow rates were proportional to column internal diameter.

In comparison, the 2.5 µm (7.8 × 300 mm) column, while providing the same sample throughput, generated a comparable or greatly improved separation to that observed for the 1.7 µm column at a $5\sigma_{ec}$ system dispersion volume of 17.6 µL to 25.9 µL, performance that is more typical of UHPLC and some UPLC system

configurations capable of using 30 cm columns. As further evidence of the impact that extra-column dispersion can have when using 4.6 mm I.D. SEC columns, it is also observed that there is a significant loss of LMWS1 resolution for the 4.6 mm I.D. (2.5 µm) column versus the 7.8 mm I.D. column. In addition, there is a modest decrease in LMWS1 resolution for the 2.5 µm (7.8 × 300 mm) column as $5\sigma_{ec}$ system dispersion volume is increased from 25.9 µL to 44.4 µL, however, this loss of resolution does not have a significant impact on the percent peak areas of LMWS1. As predicted, there is a significant increase in LMWS1 resolution for the 2.5 µm (7.8 × 300 mm) column versus the 3.5 µm (7.8 × 300 mm) column.

To match the LMWS1 resolution obtained on the 1.7 µm (4.6 × 300 mm) column ($5\sigma_{ec}$ = 12.5 µL) with the 2.5 µm and 3.5 µm particle size columns we need to either increase column length, as previously shown for the 3.5 µm (7.8 × 300 mm) column (Figure 10); or, decrease flow rate. As shown in Figure 13, the P/V achieved at a 0.75 mL/min flow rate when using the 2.5 µm (7.8 × 300 mm) column and 0.25 mL/min on the 3.5 µm (7.8 × 300 mm) column is comparable to that observed for the 1.7 µm (4.6 × 300 mm) column. This corresponds to a 33% longer analysis time for the 2.5 µm (7.8 × 300 mm) column and a 4-fold increase in analysis time for the 3.5 µm (7.8 × 300 mm) column.

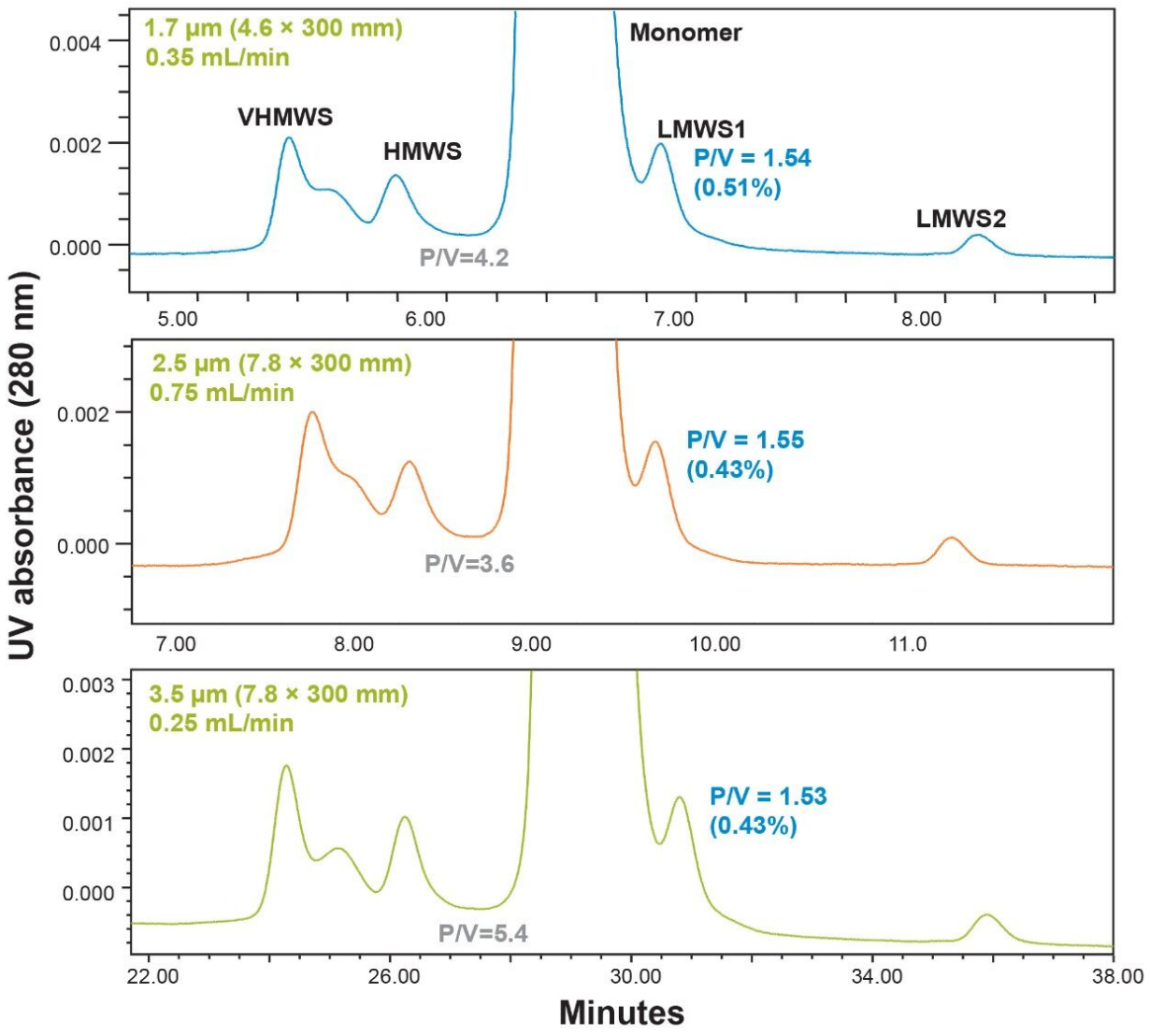


Figure 13. A comparison of the separation of rituximab on 200 Å pore size BEH SEC particles with diameters of 1.7 µm, 2.5 µm, and 3.5 µm. All columns were 300 mm in length and sample loads were proportional to the square of the column I.D. For the 2.5 µm and 3.5 µm columns, the flow rate was reduced to yield comparable resolution of the LMSW1 peak. System dispersion ($5\sigma_{ec}$) was 12.5 µL for the 1.7 µm (4.6 × 300 mm) column, and 38.8 µL for the 2.5 µm (7.8 × 300 mm) and 3.5 µm (7.8 × 300 mm) columns. Mobile phase was 20 mm sodium phosphate, 400 mM NaCl, pH 7.2. Further experimental details are provided in the text. Percent peak areas were determined by drop-baseline peak integration.

In summary, for the analysis of LMWS1 fragments by SEC, the use of a 1.7 µm (4.6 × 300 mm) column can provide shorter analysis times as compared to the 2.5 µm (7.8 × 300 mm) column provided that UPLC

system dispersion is minimized and controlled. Alternatively, comparable HMWS and LMWS resolutions can be realized when using the 2.5 µm (7.8 × 300 mm) column at moderately lower linear velocities and increased analysis times with the added benefit of the methods being far less dependent on the system dispersion and run at lower pressures; thereby, allowing for the use of UHPLC and modern HPLC systems. The 2.5 µm particle size in a 7.8 × 300 mm column configuration will outperform the same particle in a 4.6 × 300 mm column configuration, and that performance increase improves as system dispersion increases. As a result, the 7.8 mm column I.D. is generally recommended unless there is a desire to limit sample or mobile phase volumes, and system dispersion will be controlled. In all cases, a 2.5 µm particle size column will outperform a 3.5 µm particle size column of the same length and I.D. The advantages of the 3.5 µm particle size will be an approximate 50% lower back pressure, enabling its use on some LC systems with low, upper pressure capabilities.

Conclusion

We have attempted to systematically address the interplay between LC system extra-column dispersion with SEC particle size, column I.D., and column length with respect to the analyses of mAb HMWS as well as LMWS impurities. With these relationships in mind, we developed a set of general guidelines for matching Waters BEH SEC columns with three Waters LC systems most recommended for SEC separations (Table 1). In addition, these data suggest that an evaluation of extra-column dispersion might be an important variable to consider in robustness testing for some SEC methods. As a final note, if a developed method must be transferred for use on LC systems with unacceptably large extra-column dispersion, either decreasing the flow rate or increasing the column length of the method may effectively mitigate the impact of greater, extra-column dispersion without fundamentally altering the selectivity of the separation.

The reader is also referred to a companion publication to this application note “Evaluating the Impact of LC System Dispersion on the Size-Exclusion Chromatography Analysis of Proteins” (Waters Application Note, p/n: 720006337EN). This publication captures additional data and theoretical discussion on SEC and system dispersion, in addition to SEC method development advice.

Table 1. SEC column recommendations based on LC system dispersion.

	Separation[1]	ACQUITY UPLC H-Class Bio (15 cm CH)[2] 5σ ≤ 12 µL UPLC	ACQUITY UPLC H-Class Bio[4] (30 cm CH)[2] 12 µL ≤ 5σ ≤ 25 µL UPLC/UHPLC	ACQUITY Arc Bio[4] (30 cm CH)[2] 25 µL ≤ 5σ ≤ 35 µL UHPLC	Alliance[4] (30 cm CH)[2] 35 µL ≤ 5σ ≤ 45 µL HPLC	Alliance[4] (30 cm CH)[2] 5σ ≥ 45 µL HPLC
300 mm Column length	HMWS	1.7>2.5>3.5	1.7[3]>2.5>3.5	$2.5_{7.8}$>1.7[3]>3.5	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5
	LMWS1	1.7>2.5>3.5	1.7[3]>$2.5_{7.8}$>3.5	$2.5_{7.8}$>1.7[3]	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5
	LMWS2	1.7>2.5>3.5	1.7>2.5>3.5	$2.5_{7.8}$>1.7[3]>3.5	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5
150 mm Column length	HMWS	1.7>2.5>3.5	1.7[3]>2.5>3.5	$2.5_{7.8}$>1.7[3]>3.5	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5
	LMWS1	1.7[3]	Not recommended			
	LMWS2	1.7>2.5>3.5	1.7[3]>2.5>3.5	$2.5_{7.8}$>1.7[3]>3.5	$2.5_{7.8}$>3.5	$2.5_{7.8}$>3.5

[1] Particle size (1.7 µm, 2.5 µm, and 3.5 µm) recommendations are provided in the order that is predicted to provide the most resolution for that separation for equivalent analysis times. The symbol (≈) indicates that performance advantage will depend on specific 5σ system dispersion of LC used and analytes. Analyses and LC configurations where only the 2.5 µm, 7.8 mm I.D. column is exclusively recommended are indicated by subscript (2.57.8). Where the 7.8 mm I.D. column is not specified, the 4.6 mm I.D. column may be considered, if reduced consumption of mobile phase or sample is desired; however, the separation on the 4.6 mm I.D. column may be of lower resolution due to LC system dispersion.

[2] CH: Column Heater Configuration. Note: 30 cm SEC columns will not fit into ACQUITY CH-A and comparably sized column heaters, but will fit in ACQUITY CH-30A and comparably sized column heaters.

[3] Control of system dispersion levels may be required to maintain resolution.

[4] Variations in LC configurations (detector, connectors, etc.) can cause variations in LC dispersion.

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