

Application Note

## High Resolution Size-Exclusion Chromatography Separations of mAb Aggregates, Monomers, and Fragments using BioResolve SEC mAb Columns on UPLC, UHPLC, and HPLC Chromatography Systems

Pamela C. Iraneta, Stephan M. Koza

Waters Corporation



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## Abstract

Waters BioResolve SEC mAb, 200 Å, 2.5 µm Columns are specifically optimized and quality tested for high resolution separations of monoclonal antibody (mAb) aggregates, monomers, and fragments. A mAb Size Variant Standard is used for column performance certification and to guide system and column selection for the robust separation of required mAb size variant quantification. While developed and tested for mAbs, this column can also be equally considered for the SEC separation of protein size variants ranging in molecular weight from approximately 450 kDa to 17 kDa.

The 7.8 mm I.D. columns are shown to provide maximum separation efficiency of the mAb monomer (~150 kDa) from the fragment (~100 kDa) on UPLC, UHPLC, and HPLC platforms. The 4.6 mm I.D. columns provide more economical yet comparable separation performance with accurate quantification, to that of the 7.8 mm I.D. columns, on low dispersion UPLC systems as well as for the less demanding separations of mAb aggregates from monomers on UHPLC or HPLC chromatographic platforms with higher system dispersions.

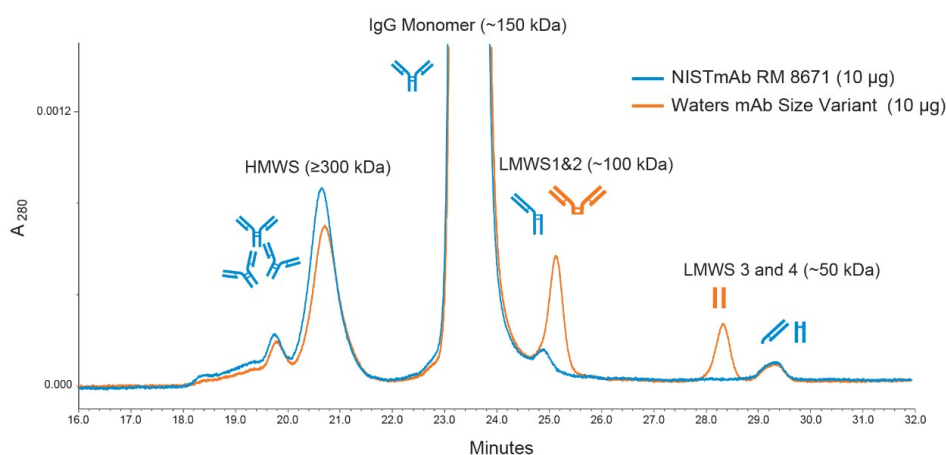
Each manufactured BioResolve SEC mAb Column is performance tested with Waters NIST mAb-based size variant standard that has been supplemented with IdeS digested NISTmAb fragments. These column performance confirmatory tests are obtained using an ACQUITY UPLC H-Class Bio System. This application note will show how LC system dispersion volume affects mAb separations based on the selected SEC column I.D. and length.

## Benefits

- BioResolve SEC mAb Columns are individually performance tested using the Waters mAb Size Variant Standard to help ensure out-of-the-box performance for mAb analyses
- High resolution separations can be achieved on UPLC, UHPLC, and HPLC systems using appropriately selected I.D. and length columns
- The implementation of individual SEC column performance testing with the Waters mAb Size Variant Standard helps increase the confidence in obtaining the needed resolution for reliable mAb size variant quantification

## Introduction

The historical importance of aqueous size-exclusion chromatography (SEC) is well established for the relative quantification of protein aggregates and self-associated forms (high molecular weight species, HMWS) to ensure the efficacy and safety of recombinant protein-based biotherapeutic products.<sup>1</sup> Recently, interest in quantifying monoclonal antibody (mAb) fragments (low molecular weight species, LMWS) in non-denaturing SEC has increased. The cause of the fragmentation appears not to be enzymatically or host cell protein driven, but rather a kinetic metal ion induced hydrolytic cleavage in the upper heavy chain hinge region resulting in the generation of Fab fragment (~50 kDa), Fc with a single Fab domain (Fab/c, ~100 kDa), and low levels of Fc fragment when both Fab domains are hydrolyzed.<sup>2</sup>



*Figure 1. Separation of mAb aggregates, monomers, and fragments of NISTmAb RM 8671 and Waters mAb Size Variant Standard using a BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 x 300 mm Column. LMWS: F(ab')<sub>2</sub> and (Fc/2)<sub>2</sub> IdeS fragments (orange); Fab/c, Fab, Fc hydrolytic degradation fragments (blue). Conditions: Ambient temperature and 0.3 mL/min.*

The separation of HMWS (≥300 kDa) from the main mAb monomer (~150 kDa) generally presents much less of a challenge compared to the separation of the Fab/c fragment, which is closer in size to the monomer and elutes at the tail of the monomer, and is typically present at low abundance (Figure 1). However, LC system improvements, higher efficiency SEC columns, and a better understanding of how LC system dispersion affects component resolution have increased the ability to obtain reliable separations of these different mAb forms.<sup>3</sup>

Each BioResolve SEC mAb Column is performance tested using a mAb Size Variant Standard on a low dispersion LC system to show its capabilities. The observed chromatographic efficiency of a

separation is affected by each of the components in the chromatographic system: the injector, tubing, column, and detector. In this application note, a series of chromatograms are shown for a variety of LC systems demonstrating the relationship between column dimensions (internal diameter [I.D.] and length) and system dispersion on the observed chromatographic efficiency of a separation. The 7.8 mm I.D. x 300 mm length columns provided high resolution separations and accurate quantification of aggregates, monomers, and fragments, independent of modern LC system dispersions.

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## Experimental

System dispersion was measured using 0.16 mg/mL caffeine diluted in 50/50 acetonitrile/water (UPLC Absorbance Test Solutions Kit, p/n: [700002642](#), Solution 7). The mobile phase was 50/50 acetonitrile/water (v/v) and the flow rate was 0.5 mL/min. A zero dead volume union (p/n: [700002636](#)) was used in place of the column. After a 10-min equilibration time, three blank mobile phase injections were followed by five consecutive 0.5  $\mu$ L injections of caffeine using a run time of 1-2 min. The caffeine peak widths at 4.4% (5-sigma peak width) were averaged. The average was multiplied by 500  $\mu$ L/min to obtain the reported 5-sigma extra-column dispersion ( $5\sigma_{ec}$ ) volumes. The dispersion data on the three systems (four configurations) are reported in Table 1.

LC Systems	System Volume ( $\mu$ L)	5-Sigma W at 4.4% ( $\mu$ L)	USP Tailing
1) ACQUITY UPLC H-Class Bio with CH-A	16.8	10	1.25
2) ACQUITY UPLC H-Class Bio with CH-30A	22.6	13	1.33
3) ACQUITY Arc Bio with 30-cm CH	43.4	30	1.37
4) Alliance HPLC with CH	61.6	49	1.66

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*Table 1. System dispersion characteristics for LC systems.*

### Sample Description:

Waters mAb Size Variant Standard (p/n: [186009284](#)) contains 160  $\mu$ g of stabilized and lyophilized NISTmAb RM8671 which has been supplemented with 2  $\mu$ g of nonreduced IdeS digested NISTmAb fragments ( $(F(ab')_2)$  and  $(Fc/2)_2$ ). The lyophilized contents of each vial was solublizes using 70  $\mu$ L of MilliQ water.

## Method Conditions

### LC Conditions

Systems: ACQUITY UPLC H-Class Bio, 5-Sigma system dispersion = 10  $\mu$ L, 13  $\mu$ L  
ACQUITY Arc Bio, 5-Sigma system dispersion = 30  $\mu$ L  
Alliance HPLC, 5-Sigma system dispersion = 49  $\mu$ L

Detectors: Tunable Ultraviolet (TUV) with a 5 mm Ti Flow Cell for ACQUITY UPLC H-Class Bio, 2489 UV/Vis with 10 mm Bio Inert Flow Cell for the ACQUITY Arc Bio and Alliance

Detection: 280 nm, 10 Hz, fast filter

Vials: Max Recovery Sample vials (p/n: 186000327C)

Column(s): BioResolve SEC mAb, 200  $\text{\AA}$ , 2.5  $\mu$ m,

4.6 x 150 mm (p/n: 176004592\*)

4.6 x 300 mm (p/n: 176004593\*)

7.8 x 150 mm (p/n: 176004594\*)

7.8 x 300 mm (p/n: 176004595\*)

\* Includes column and one complimentary vial of mAb Size Variant Standard

Column temp.:	35 °C active preheaters CH-A (ACQUITY UPLC H-Class), CH-30A (ACQUITY UPLC H-Class), and convection heaters 30-cm CH (ACQUITY Arc), CH (Alliance)
Sample temp.:	8 °C
Sample:	2.28 mg/mL Waters mAb Size Variant Standard
Injection volume:	Varies: 1.8, 3.5, 5, or 10 µL depending on column configuration (I.D. and length)
Flow rate:	0.200 mL/min (for 4.6 mm I.D.)/0.575 mL/min (for 7.8 mm I.D.)
Seal wash:	10% HPLC-grade methanol/90% 18.2 MΩ water v/v (Seal wash interval set to 0.5 min)
Sample manager washes:	18.2 MΩ water
Mobile phase A:	50 mM sodium phosphate pH 7.0, 200 mM KCl
Mobile phase B and C:	18.2 MΩ water
Mobile phase D:	10% acetonitrile/90% 25 mM sodium phosphate pH 7.0 + 100 mM potassium chloride
Syringe draw rate:	30 µL/min
Needle placement:	1.0 mm
Air gaps:	None

Data channels: ACQUITY TUV ChA 280 nm; System pressure, room temperature

Mobile phase A: Prepare by mixing 2.66 g of anhydrous dibasic sodium phosphate, 4.36 g of monobasic potassium phosphate monohydrate, and 14.91 g of potassium chloride per L of water followed by filtration using sterile 0.2 µm nylon filter units (filtered mobile phase pH 6.9)

Chromatography software: Empower 3 FR 3.0



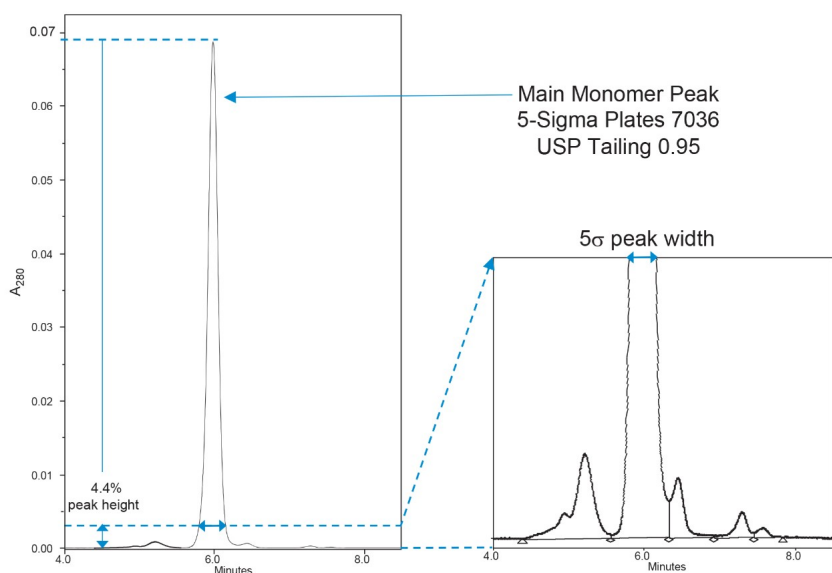
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## Results and Discussion

SEC is a unique and challenging form of chromatography. Under ideal conditions (method development required), proteins migrate through the column with minimal-to-no interaction with the stationary phase. No retention, only diffusion driven separations achieved by the size hindrance provided by the specifically designed pore sizes in the packing's porous structure. As a result, the amount of band spreading that occurs to the analyte zone as it travels from the injector to the detector will have a significant impact on SEC resolutions. An in-depth evaluation of the effect of system dispersion on the SEC analysis of mAb aggregates (HMWS) and fragments (LMWS), and the effect that extra-column dispersion has on that separation can be found in previously published application notes.<sup>4,5</sup>

### Measuring System Dispersion

When conducting band spreading experiments to evaluate system dispersion, it is important to measure the band broadening at a peak width of 4.4% of its height when SEC is our intended analysis. Many of the impurities that we intend to separate and quantify have peak heights well below 4.4% of the peak height of the main peak (Figure 2). The effect of LC system dispersion on the 5-sigma efficiency and USP tailing factor for the main mAb peak plays a key role in achieving resolution for the ~100 kDa fragment. There is benefit in documenting these parameters during method development since these data are potential indicators of column failure or a system dispersion problem. Consequently, the data from this analysis can be incorporated in standard procedures to help ensure the column and system are fit-for-purpose prior to the analysis of valuable samples.



*Figure 2. Separation of Waters mAb Size Variant Standard on a BioResolve SEC mAb, 4.6 x 150 mm Column using an ACQUITY UPLC H-Class Bio System. Conditions provided in the experimental section.*

The Waters mAb Size Variant Standard was used to systematically investigate the effect of the various system dispersions on the chromatographic performance of each of the four columns listed below. The same columns were evaluated on each system. Columns were checked before and after all evaluations and found to maintain their performance. The individual quality report chromatogram, that is provided in the BioResolve SEC mAb Column box, is essentially the same as that generated on the ACQUITY UPLC H-Class Bio System, in the examples below.

In these experiments, four BioResolve SEC mAb Columns (4.6 x 150 mm, 4.6 x 300 mm, 7.8 x 150 mm, and 7.8 x 300 mm) are used on three different relatively modern LC Systems:

- The lowest dispersion (UPLC) system is the ACQUITY UPLC H-Class Bio having a dispersion of 10  $\mu\text{L } 5\sigma_{\text{ec}}$  for the 150 mm length column heater (CH-A) and 13  $\mu\text{L } 5/\text{sec}$  for the 300 mm length column heater (CH-30A): both having active pre-heating.
- The intermediate dispersion (UHPLC) system is the ACQUITY Arc Bio having a dispersion of 30  $\mu\text{L } 5\sigma_{\text{ec}}$ . For all columns, the forced air convection 30-cm column heater (30-cm CH) was used.
- The largest dispersion (HPLC) system is the Alliance having a dispersion of 49  $\mu\text{L } 5\sigma_{\text{ec}}$ . For all columns, the forced air convection column heater box was used.

The chromatographic results of these experiments for the 7.8 mm I.D. columns are shown in Figure 3, and those for the 4.6 mm I.D. column are shown in Figure 4.

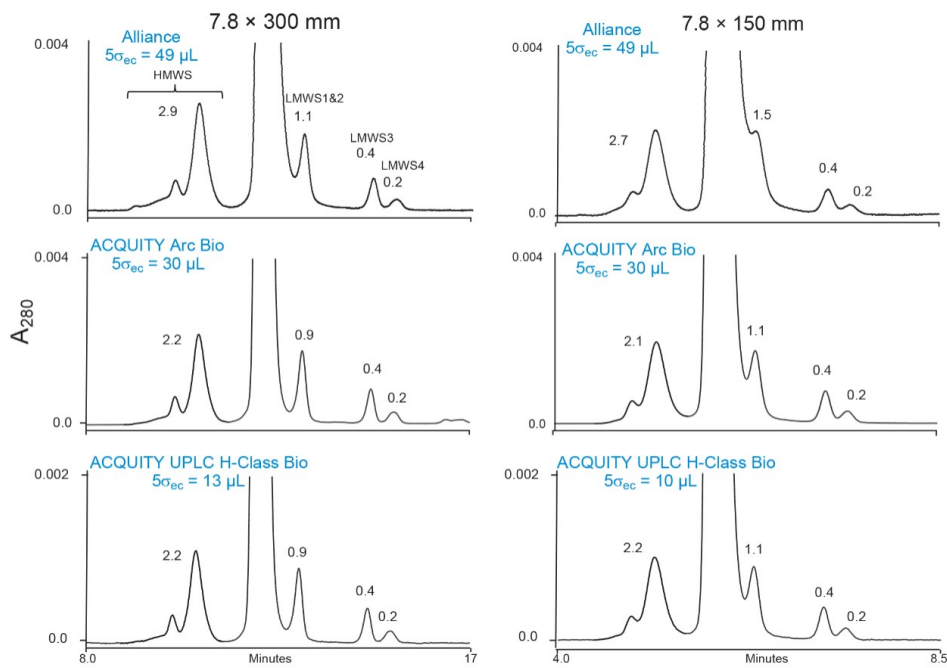


Figure 3. Separation of Waters mAb Size Variant Standard on BioResolve SEC mAb, 7.8 x 300 mm and 7.8 x 150 mm Columns on LC systems with 49 µL (Alliance), 30 µL (ACQUITY Arc), and 10 or 13 µL (ACQUITY UPLC H-Class) system dispersions. Percent areas are reported for each chromatogram. Conditions provided in the experimental section.

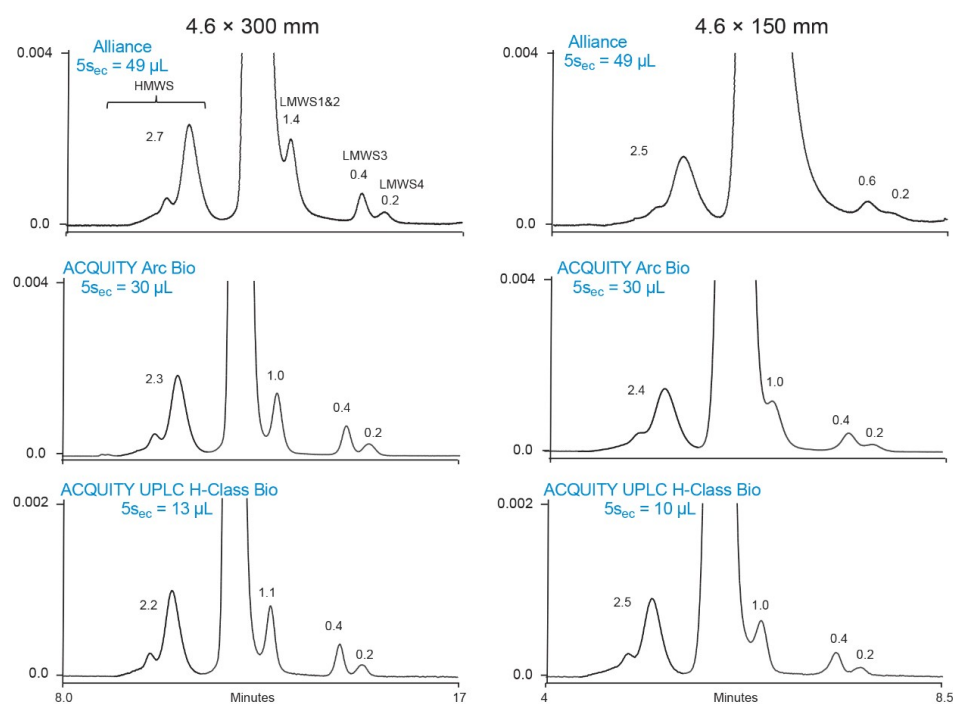


Figure 4. Separation of Waters mAb Size Variant Standard on BioResolve SEC mAb, 4.6 x 300 mm and 4.6 x 150 mm Columns on LC systems with 49 µL (Alliance), 30 µL (ACQUITY Arc), and 10 or 13 µL (ACQUITY UPLC H-Class) system dispersions. Percent areas are reported for each chromatogram. Conditions provided in the experimental section.

Before reviewing the chromatographic results, it is important to point out the various components in the Waters mAb Size Variant Standard. The percent composition of the lower molecular weight species in commercial mAb drug products are generally very low and variable among product batches, making the utility of such samples as standards undesirable. To address this shortcoming, Waters created a mAb Size Variant Standard. Each vial of the standard contains 160 µg of National Institute of Standards and Technology (NIST) mAb Reference Material (RM) 8671 that has been supplemented with 2 µg of the IdeS (FabRICATOR®) digested NISTmAb. Figure 5 elucidates the source of the various components in the mAb Size Variant Standard. As seen in the chromatogram (Figure 5b) the ~100 kDa Fab/c fragment endogenous to the NISTmAb elutes slightly earlier than the IdeS generated ~100 kDa F(ab')<sub>2</sub> fragment. Since the order of elution is taken as a difference in hydrodynamic volume, Fab/c will be more difficult to separate from the monomer as compared to F(ab')<sub>2</sub>. The BioResolve SEC mAb, 7.8 x 300 mm Column was used at a lower flow rate (0.3 mL/min) in order to achieve the resolution shown for Fab/c in the NISTmAb RM 8671. More details can be found in the mAb Size Variant Standard care and use manual (p/n: [720006811EN](#)). More information on NISTmAb RM 8671 can be found at [nist.gov](#).<sup>6</sup>

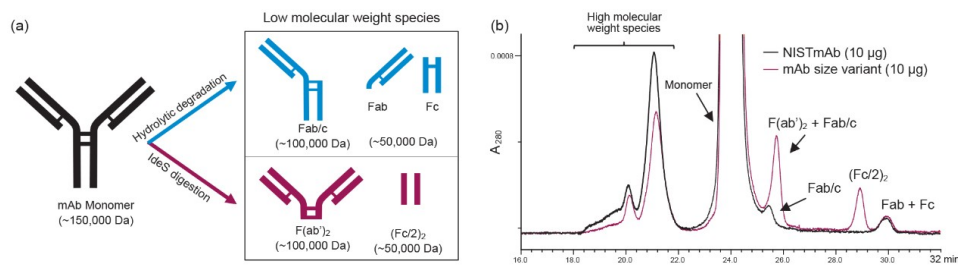


Figure 5. (a) and (b) mAb graphic illustrating the difference between the intact monomer and fragments found in NISTmAb and mAb Size Variant Standard. A representative A<sub>280</sub> SEC chromatogram of NISTmAb (black trace) and modified mAb Size Variant Standard (red trace) cropped to show both high and low molecular weight species, in addition to the monomer. Due to similarity in hydrodynamic volume, F(ab')<sub>2</sub> and Fab/c are not resolved. Data were collected with a BioResolve SEC mAb, 200 Å, 2.5 µm, 7.8 x 300 mm Column with absorbance measured at 280 nm using a flow rate of 0.3 mL/min at ambient temperature.

As shown in Figure 3, the separation of the mAb Size Variant Standard on 7.8 mm I.D. columns, we see that the chromatograms display very similar separation performance for the HMWS, main, and fragment peaks on the 7.8 x 300 mm column run on the HPLC, UHPLC, and UPLC systems. The shorter 7.8 x 150 mm column provides, as predicted, lower resolution separations, but also shows a visible degree of performance loss for LMWS1&2 on the HPLC system. HMWS are adequately separated on both 7.8 mm I.D. columns on the three LC systems.

Although the 7.8 x 150 mm column in Figure 3 provides what appears to be adequate resolution for LMWS1&2 on the UPLC system, it is important to understand that most of the LMWS1&2 peak area is due to the IdeS supplemented fragment, F(ab')<sub>2</sub>. As previously mentioned, this fragment appears to be slightly smaller (therefore slightly better resolved) than the naturally occurring Fab/c fragment (Figure 5).

The effect of system dispersion on the 4.6 mm I.D. columns in Figure 4 presents a different picture than that for the 7.8 mm I.D. columns discussed above. The LMWS1&2 are separated with the 4.6 x 300 mm column on all three systems with a noticeable loss of resolution observed as system dispersion increases. This trend is even more abrupt for the 150 mm column length. No significant change in HMWS resolution is observed for the 4.6 x 300 mm column on the different LC systems, however, a slight loss of HMWS resolution is observed for the 150 mm column length, as system dispersion increases.

## Resolution of Aggregates

The trends visually observed in the chromatograms of Figures 3 and 4 were quantified using the

Empower system suitability parameters. For aggregate resolution, the USP resolution at half height (HH) was used to assess the quality of the separations between the main monomer peak and the dimer across the three systems for these columns. The frequently proclaimed value of 1.5 for baseline resolution is not relevant for most real world chromatographic separations. A criterion adopted for acceptable resolution for real world samples is 1.75-2.0.<sup>7</sup> This shift to higher values is due to effects of peak asymmetries and the dissimilar areas encountered in SEC separations.<sup>7</sup> In Figure 6, for the dimer-main peak resolution values we observe that for all, but the 4.6 x 150 mm column, the resolution remains essentially the same across the three LC systems. The 26% loss in resolution for the 4.6 x 150 mm column across the three LC systems remains above acceptable levels for many aggregate analyses.

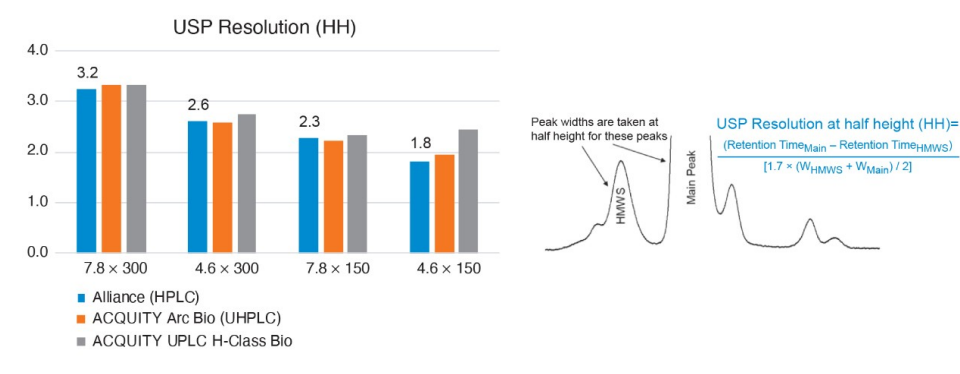


Figure 6. Dimer-Main peak USP resolution (HH) data for the four-column set across the three LC systems. Resolution equation shown. Conditions provided in the experimental section.

## Resolution of Fragments

In order to quantitatively measure and compare the resolutions for LMWS1&2, another type of resolution metric is needed, the peak-to-valley ratio (p/v). The p/v parameter, illustrated in Figure 7, is typically used to quantify the quality of a separation between peaks that have disparate areas and are too closely eluting (partially coeluting) to obtain a resolution value from the traditionally used resolution equations. The value of p/v is used to allow quantitative assessment of very difficult separations such as those encountered for fragment analysis. An example, of the use of the  $p/v \geq 2.0$  criterion for quantification of closely eluting peaks in SEC, can be found in the USP Pharmacopoeia Monograph for Insulin.<sup>8</sup> The USP Insulin monograph calls for an end  $p/v \geq 2.0$  when quantifying the relative amount of high molecular weight protein (HMWP) in the insulin sample.<sup>8</sup> Because USP resolution (HH) values were not available for all the column configuration/system combinations in this study, the start p/v parameter was used for these comparisons.

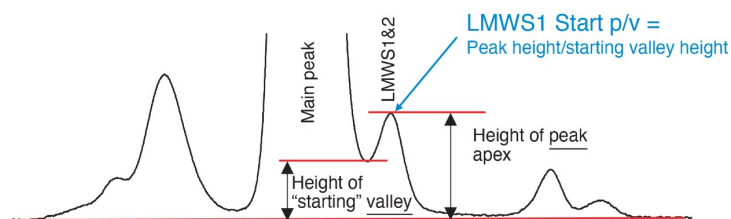


Figure 7. Chromatographic example and calculation for LMWS1&2 start p/v.

In Figure 8, the p/v resolution values are maintained consistently across the UPLC and UHPLC systems for all the configurations. Generally, p/v ratios are less reproducible as they increase above three (data not shown). As predicted, the resolutions on the 300 mm length columns are higher than on the 150 mm length columns. The 4.6 x 150 mm column was not included in this figure because it failed to resolve LMWS1&2 on the Alliance, and on the other systems the p/v was 1.6 and lower. As p/v resolutions increase above 1, the % areas for LMWS1&2 gradually decrease until they start to level off as they approach p/v > 2. This trend is visible for all the columns on the Alliance.

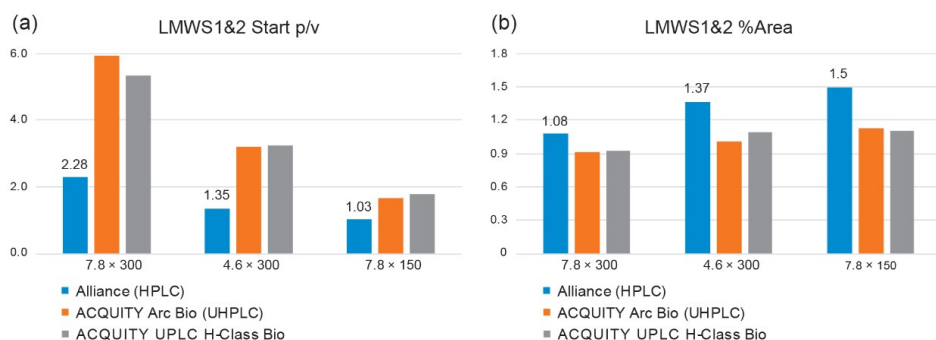


Figure 8. (a) LMWS1&2 start p/v and (b) LMWS1&2 % areas for the three columns used across three systems. Conditions provided in the experimental section.

## Main Peak

Central to all SEC mAb chromatograms is the main peak. It is apparent from the chromatograms in Figures 3 and 4 that the efficiencies for the main peak are degraded by system dispersion. As separations lose efficiency, analyte peak heights decrease. Not often discussed is the effect of a system's dispersion on the sensitivity of the analysis due to this decrease in peak height which directly impacts the signal-to-noise ratio of the impurities that we are intending to quantify in these separations.

In Figure 9a, the correlation coefficient squared ( $R^2$ ) values between system dispersion volume and 5-sigma plates show increasingly stronger correlations as column volume decreases.  $R^2$  values

represent the percent of the variation in plates that can be explained by the difference in system dispersion volumes; meaning that 84-100% of the decrease in main peak 5-sigma plates can be explained by the increasing system dispersion volumes. The slopes of the linear correlations are steeper on the 4.6 mm I.D. columns compared to those on the 7.8 mm I.D. columns, indicating a greater impact of *dispersion volume* on the 4.6 mm I.D. columns. It is also noticeable that the efficiency even on the 4.6 x 300 mm column was not as high as that obtained on the 7.8 x 300 mm, suggesting that system dispersion is limiting its performance even on the UPLC system.

The dispersion volume and its tailing factor are not independent variables. However, comparing the  $R^2$  values between the dispersion peak volumes (Figure 9a) and tailing factors (Figure 9b) to main peak plates suggests that it is the system dispersion tailing factor that plays a stronger role in influencing changes in the main peak plates on the 7.8 mm I.D. columns than on the 4.6 mm I.D. columns. It is not known at this time how universally applicable these observations are to all systems but does suggest that reducing tailing for the dispersion peak may lead to even better performance for 7.8 mm I.D. configurations.

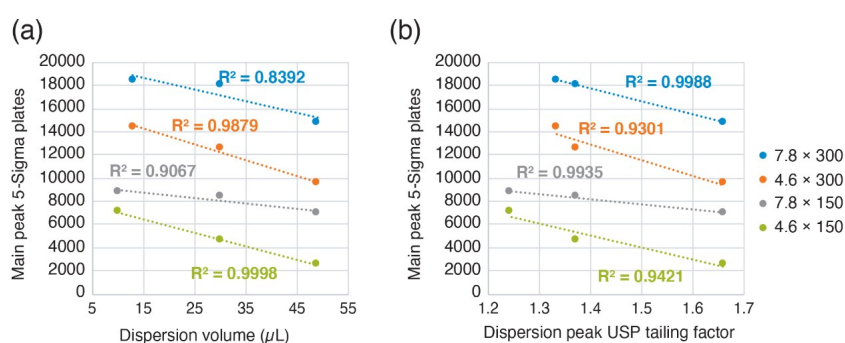


Figure 9. Effect of system dispersion on main peak 5-sigma plates; (a) Effect of dispersion volume; (b) Effect of dispersion peak USP tailing factor. Conditions provided in the experimental section.

Focusing on the 300 mm length columns, it was observed that the LMWS1&2 p/v was most strongly influenced by the *tailing factors* of both the main peak and the dispersion peak. The p/v values were still found to be influenced by the main peak plates ( $R^2$  values of 0.88 for 4.6 mm I.D. and 0.95 for 7.8 mm I.D.) and system dispersion volume ( $R^2$  values of 0.80 for 4.6 mm I.D. and 0.64 for 7.8 mm I.D.), just not as strongly as with those described in Figure 10. Since the tailing factor for the main peak is also strongly correlated with the tail factor for the dispersion peak (Figure 9b), minimization of tailing in the dispersion peak should be investigated as a means of improving the resolution for fragment analyses.



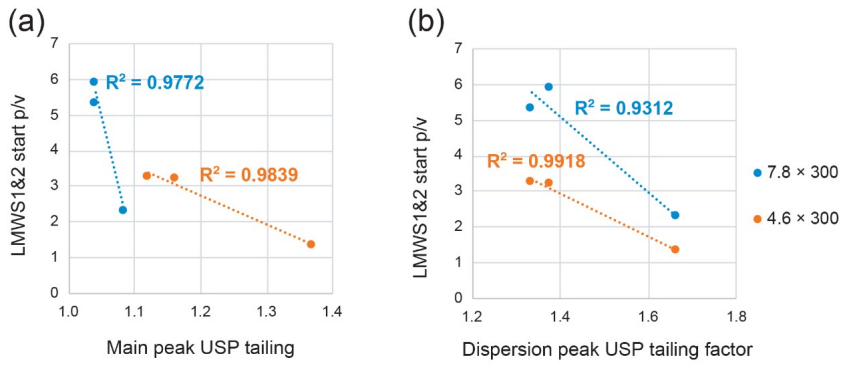
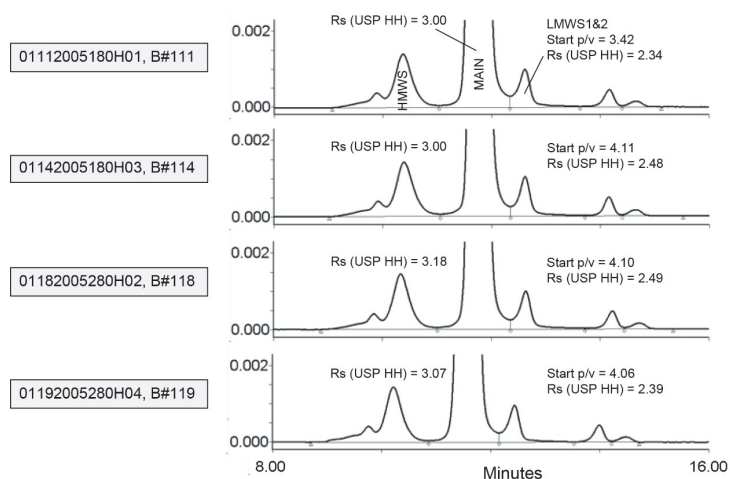


Figure 10. Factors influencing LMWS1&2 p/v: (a) Main peak USP tailing factor; (b) Dispersion peak USP tailing factor for 300 mm length columns across the three LC systems. Conditions provided in the experimental section.

## Conclusion

Waters BioResolve SEC mAb Columns can provide high resolution and reproducible separations of mAb HMWS, monomers, and LMWS. The 7.8 mm column I.D. configurations provide better chromatographic performance than the 4.6 mm I.D. columns on HPLC, UHPLC, and UPLC systems, and are therefore the most recommended configurations when high resolution separations, particularly those requiring analysis of partially resolved LMWS1 (Fab/c). The 4.6 mm I.D. columns can be nearly as effective as the 7.8 mm I.D. columns on UPLC systems and for the analysis of HMWS on UHPLCs and in some cases HPLCs. The advantages offered by the 4.6 mm I.D. columns are reduced sample demands and mobile phase consumption, along with being more economical.

In these experiments, the LC systems used were not modified to further reduce dispersion from that of their typically configured components. For example, it is well known that reducing the length and I.D. of the connection tubing may further reduce dispersion.<sup>9</sup> The “out-of-the-box” performance of BioResolve SEC mAb Columns is tested by Waters for each column using the Waters mAb Size Variant Standard on low dispersion LC systems and chromatograms should appear similar to those presented on the ACQUITY UPLC H-Class System in Figures 3 and 4. Representative quality testing results for four different batches of BioResolve SEC mAb are presented in Figure 11 and demonstrate a high degree of reproducibility.



*Figure 11. Resolution comparison using the Waters mAb Size Variant Standard on four different BioResolve SEC mAb batches in 7.8 x 300 mm columns run on the same ACQUITY UPLC H-Class Bio System. Conditions provided in the experimental section.*

Successful implementation of SEC in the research, development, and manufacture of mAb

biotherapeutics is dependent on understanding and selecting a column that is compatible with the dispersion levels of the utilized LC system(s) to help ensure the generation of reliable data especially in a validated method. Development of separation conditions that provide robust separations while minimizing secondary interactions with both the column and LC system flow path are critical to the success of any SEC method. Finally, it is important to consider establishing and using a reliable mAb-based reference sample to help ensure column, LC system, mobile phase, and method readiness prior to the analysis of desired mAbs.

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