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Application Note

# Evaluation of SEC-MALS Data Quality with Premier Protein SEC Columns

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## Abstract

Size Exclusion Chromatography (SEC) coupled with multiangle light scattering (MALS) and UV/RI detection has been increasingly used for characterizing biophysical properties of large molecules including molecular weight, aggregation, size, and conformation. Data quality is essential for the accurate characterization of these properties. We show here that the SEC-MALS noise level is low on the XBridge™ Premier Protein SEC 250 Å, 2.5 µm Column and the ACQUITY™ Premier Protein SEC 250 Å, 1.7 µm Column. The molecular weight of trastuzumab is determined using SEC-UV-MALS data on the Premier Protein 250 Å columns as well as other commercially available Waters SEC columns.

## Benefits

- Low MALS noise levels on the XBridge Premier Protein SEC 250 Å, 2.5 µm Column and the ACQUITY Premier Protein SEC 250 Å, 1.7 µm Column
- Determination of MW of proteins using Premier Protein SEC columns followed by UV and MALS detection

## Introduction

SEC has been widely used for size variant characterization of large molecules. It separates monomers from high molecular weight aggregates, as well as low molecular weight species and other impurities. Coupling with multiangle light scattering (MALS) and UV detector greatly enhances the utility of SEC, since many biophysical properties such as molecular weight, aggregation, size, and conformation of protein biotherapeutics can be characterized.[1,2] The data quality of SEC-MALS experiments is of great importance for accurate characterization.

Waters MaxPeak™ Premier Protein SEC columns are packed with BEH™ particles that are bonded with terminally hydroxylated polyethylene oxide while the column hardware is stainless steel processed using MaxPeak Premier High Performance Surface (HPS) technology. In this application note, we compare the SEC-MALS noise level obtained from the Premier Protein SEC columns with that obtained from Waters diol-bonded ethylene bridge hybrid (BEH) organo-silica based SEC columns as well as a diol-bonded silica-based SEC column. Molecular weight determination of trastuzumab and its size variants were also performed.

## Experimental

### Sample Description

Trastuzumab (21 mg/mL) was injected neat onto the LC system.

### Method Conditions

LC Conditions

| | |
|---|---|
| LC system: | ACQUITY UPLC H-Class PLUS Bio System (Ave. system dispersion: 4 σ < 22 µL |
| Detection: | ACQUITY UPLC TUV Detector with 5 mm titanium flow cell, wavelength: 280 nm |

| | |
|---|---|
| | MALS detector: Wyatt µDAWN |
| Vials: | Polypropylene 12 x 32 mm Screw Neck Vial, with Cap and Pre-slit PTFE/Silicone Septum, 300 µL Volume, 100/pk (p/n: 186002639) |
| Column(s): | XBridge Premier Protein SEC 250 Å, 2.5 µm, 7.8 x 300 mm with mAb Size Variant Standard (p/n: 176005070)<br>ACQUITY Premier Protein SEC 250 Å, 1.7 µm, 4.6 x 300 mm with mAb Size Variant Standard (p/n: 176005072)<br>ACQUITY UPLC Protein BEH SEC Column, 200 Å, 1.7 µm, 4.6 mm x 300 mm (p/n: 186005226)<br>BioResolve SEC mAb Column, 200 Å, 2.5 µm, 7.8 x 300 mm with mAb Size Variant Standard (p/n: 176004595)<br>Diol-bonded, silica-based SEC, 250 Å, 5 µm, 7.8 x 300 mm |
| Column temp.: | Ambient |
| Sample temp.: | 10 °C |
| Injection volume: | 10 µL for 7.8 mm i.d. column<br>3.5 µL for 4.6 mm i.d. column |
| Flow rate: | 0.75 mL/min for 7.8 mm i.d. column |

| | |
|---|---|
| | 0.38 mL/min for 4.6 mm i.d. column |
| Mobile phase: | 2 x DPBS (Phosphate-buffered saline, Dulbecco's formula) |
| Data Management | |
| Chromatography software: | Empower™ 3 (FR 4) |

## Results and Discussion

Blank sample was injected onto brand new SEC columns after 40 to 50 minute equilibration to assess the SEC-MALS noise with 2X DPBS (Phosphate Buffered Saline, Dulbecco's formula) mobile phase. Such noise could be impacted by the pressure pulse during the injection as well as particle shedding from the column. Figure 1 shows the MALS data obtained from five SEC columns. The data collection rate is 5 Hz for the two 4.6 x 300 mm columns, while it is 2 Hz for the three 7.8 x 300 mm columns. The y-axis is set so that the y-value range (the difference between the maximum and the minimum) is the same, except for the diol-bonded silica-based SEC column in Figure 1a. This makes it easy to compare the noise level visually among the columns. Figure 1a shows the data from the entire run, while figure 1b shows the data from a two minute portion of the run and the noise level was estimated based on the two minute portion. In general, the noise level of the Premier Protein SEC columns is 1.5–2 times higher than the corresponding ACQUITY BEH 200 SEC 1.7 µm Column and BioResolve SEC 2.5 µm Column. The MALS noise of the diol-bonded silica-based SEC, 250 Å, 5 µm column is 2.5 and 4 times larger than the ACQUITY Premier Protein SEC Column and the BioResolve SEC Column, respectively. In addition, the diol-bonded silica-based SEC, 250 Å, 5 µm, 7.8 x 300 mm column resulted in two early eluting peaks at around two minutes and six minutes which might be due to the injection pressure pulse causing the release of particulates or fines from the column. No upward spikes are observed on the columns tested, indicating minimal particle shedding from the columns. These results are consistent with previous findings.[3]

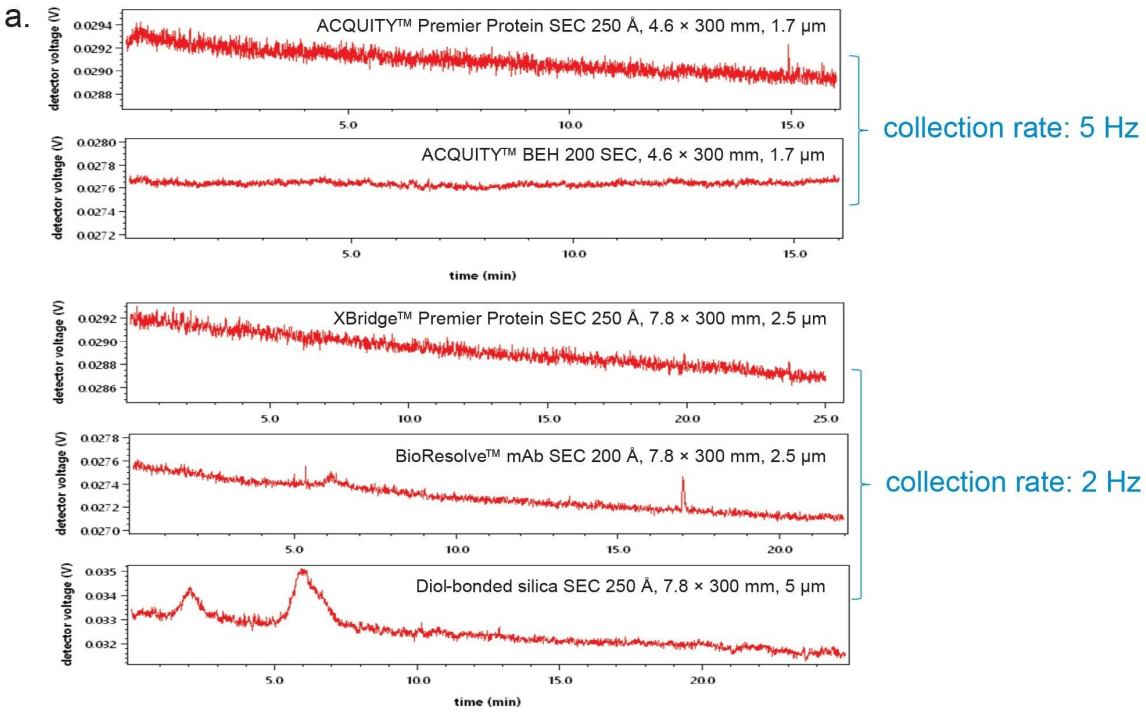
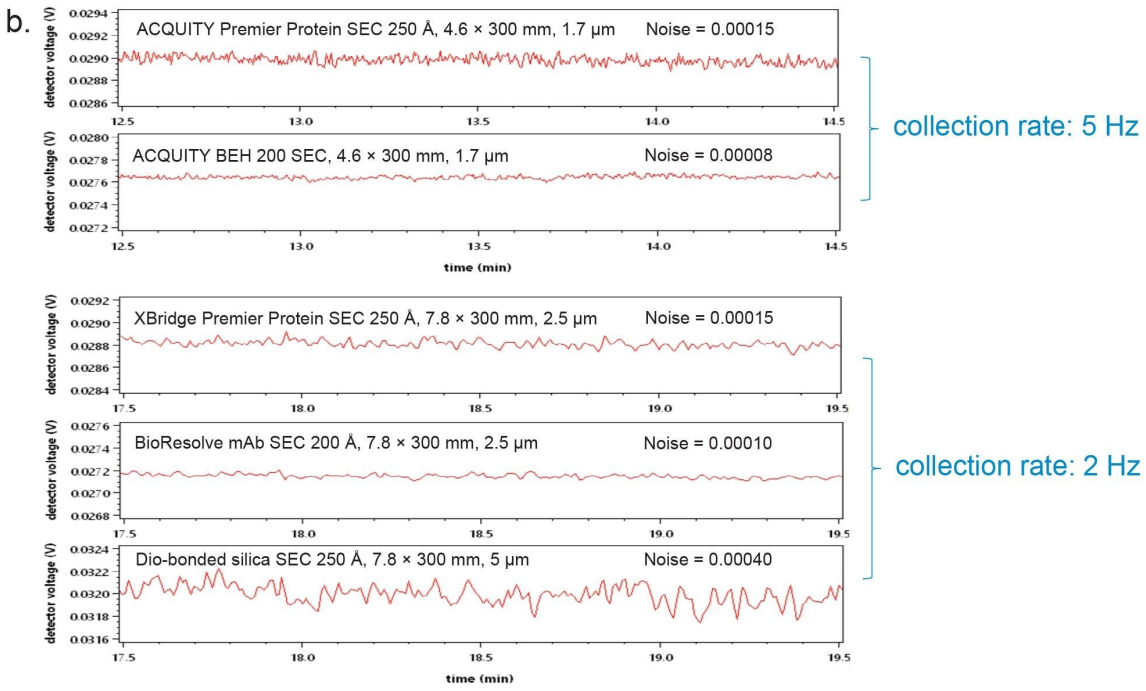



*Figure 1. SEC-MALS baseline noise collected with five brand new SEC Columns upon a blank injection. The mobile phase is 2 x DPBS.*

*1a. baseline shown with the full chromatogram.*




*1b. 2-min portion of the chromatogram where noise is determined and noted at upper right corner.*

Trastuzumab was separated on all five SEC columns for molecular weight (MW) determination with 2x DPBS mobile phase. Figures 2a and 2b show the separation with both MALS (upper trace, red) and UV 280 nm (lower trace, blue) detection. Consistent with previous results,[3–7] high molecular weight species (HMWS2, HMWS1) and low molecular weight species (LMWS2) were well separated from the monomer peak. Partial separation of LMWS1 was observed on all the columns except for the diol-bonded silica-based column where LMSW1 was embedded underneath the monomer peak. The resolution between LMWS1 and monomer is slightly better for the ACQUITY BEH 200 SEC, 4.6 x 300 mm 1.7 µm Column than for the ACQUITY Premier SEC 250 Å, 4.6 x 300 mm 1.7 µm Column (Figure 2a), possibly because the peak volume is slightly smaller for the Premier SEC column so the performance of the Premier SEC column is more negatively impacted by the system dispersion. Extra peaks appeared around six to – seven minutes on the diol-bonded silica-based column (broken circle), which again could be due to the impact of the pressure pulse during injection.

The average signal to noise ratio of MALS detection is shown on the upper right corner in Figure 2. It is obtained by dividing the trastuzumab monomer peak height by the noise level where the baseline is relatively stable (n=4 or 5). The signal to noise ratio of the ACQUITY Premier Protein SEC columns is 1.5–2 times lower than the

corresponding ACQUITY BEH 200 SEC 1.7 µm Column and BioResolve SEC 2.5 µm Column, largely due to the slightly higher noise level. The signal to noise ratio of the diol-bonded silica-based column is about one order of magnitude lower than the other four columns, due to both lower signal and higher noise.

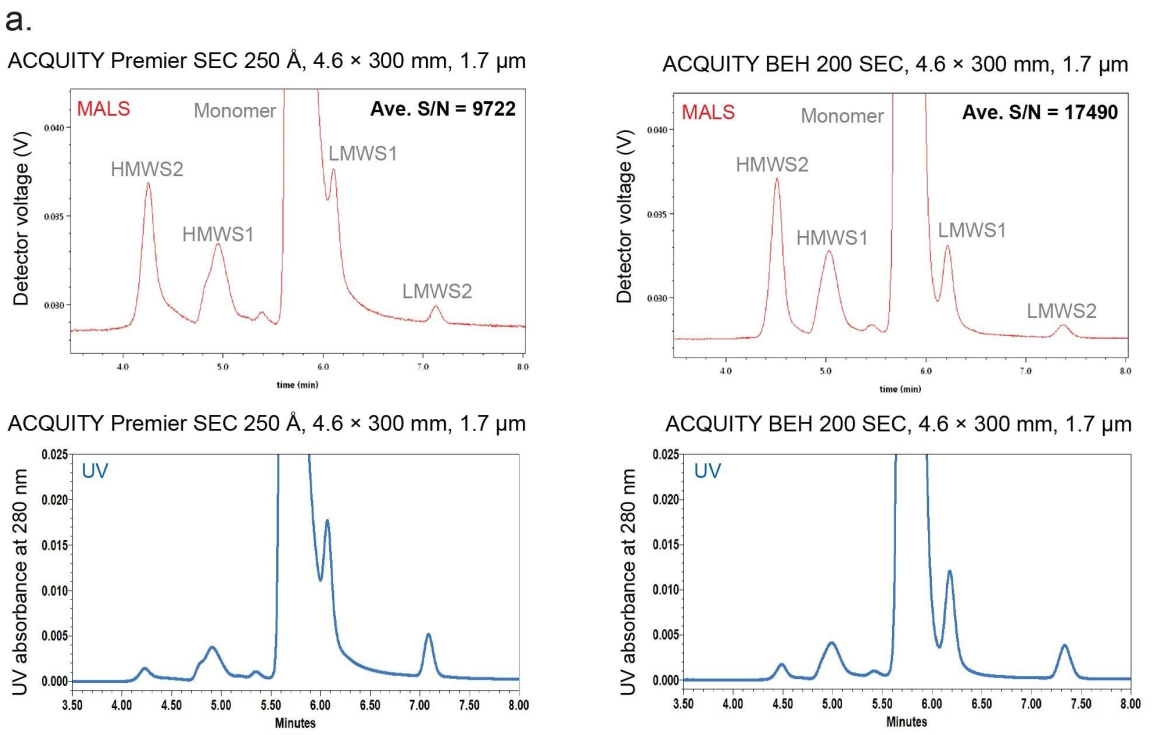
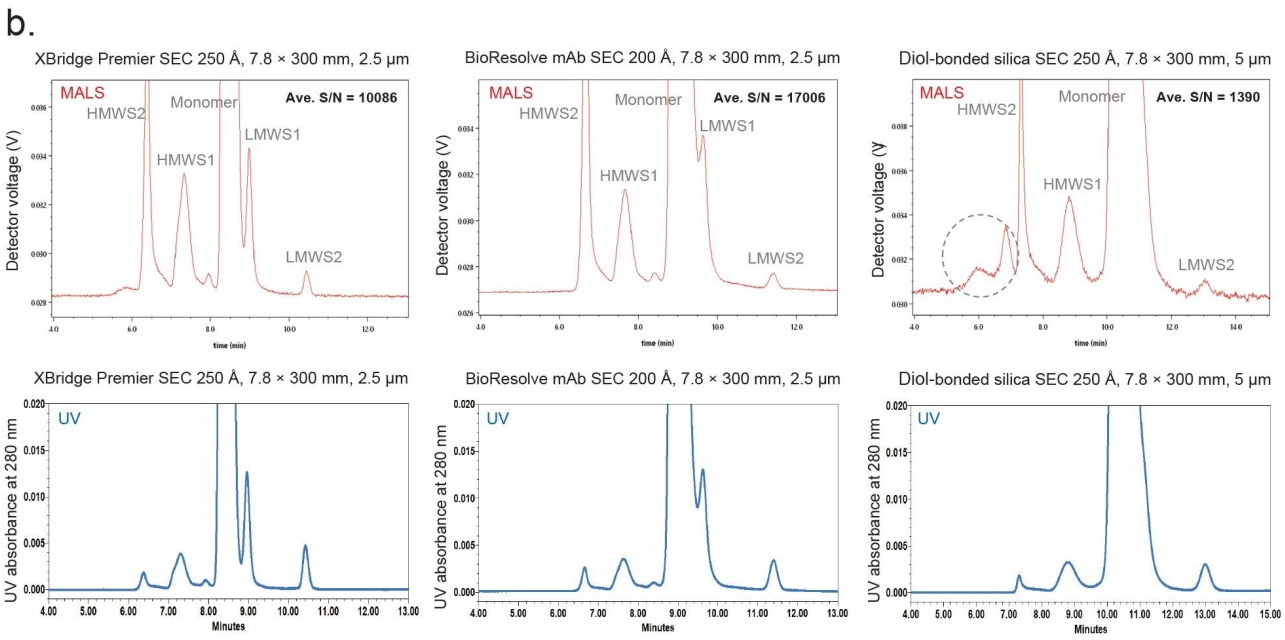



*Figure 2. Trastuzumab and its size variants are separated on the five SEC Columns tested with 2 x DPBS as the mobile phase. Red traces (top) are MALS signals, and the blue traces (bottom) are the UV 280 nm signals.*

*2a. Chromatograms obtained from two 4.6 x 300 mm, 1.7 µm Columns.*




*2b. Chromatograms obtained from three 7.8 x 300 mm, 2.5 or 5 µm Columns. HMWS: high molecular weight species; LMWS: low molecular weight species.*

Data from SEC-UV-MALS experiments were processed by Wyatt's Astra software (Figure 3 and Table 1). Consistent results were obtained among all the columns for the molecular weight of the monomer and HMWS1 which is likely the dimer. Variations of LMWS1 MW is observed among the columns tested, possibly because the peak is mostly eluting underneath the tail of the monomer peak. Variations for LMWS2 MW among columns is likely due to low abundance of the peak. Large MW variation occurs across the LMWS2 peak on the diol-bonded silica-based column (Figure 3e), likely due to the combination of low signal and high noise.

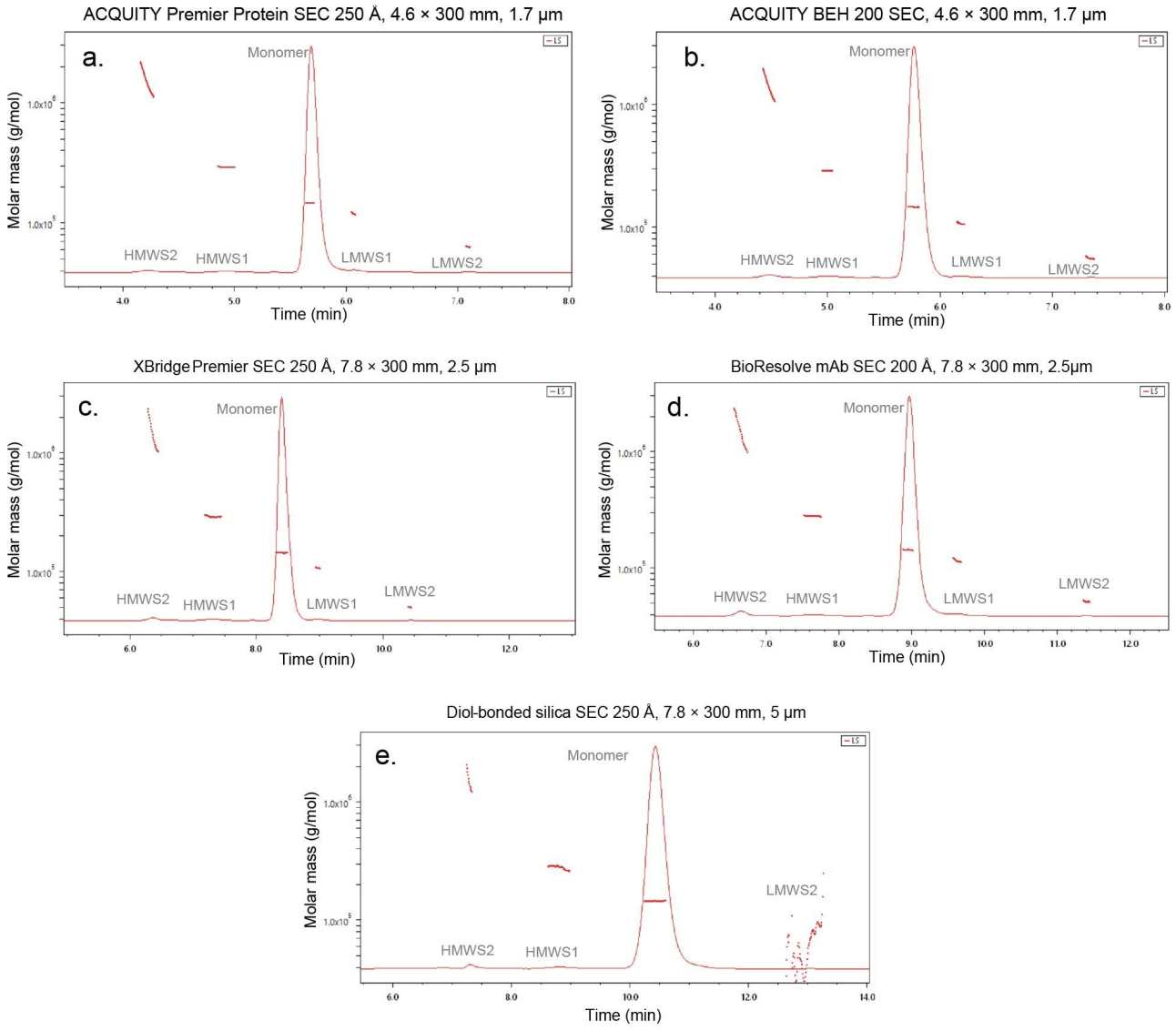



*Figure 3. Molecular weight determination of trastuzumab and its size variants from data obtained from the five SEC Columns tested with 2 x DPBS as the mobile phase. Data is processed by Astra software from Wyatt Technology. The results are listed in Table 1.*

| Molecular weight X1000 (g/mol) | Premier SEC 4.6 × 300 mm, 1.7 µm | BEH200 SEC 4.6 × 300 mm, 1.7 µm | Premier SEC 7.8 × 300 mm, 2.5 µm | BioResolve SEC 7.8 × 300 mm, 2.5 µm | Diol-bonded silica SEC 7.8 × 300 mm, 2.5 µm |
|---|---|---|---|---|---|
| **HMWS2** | 1444.3 | 1355.7 | 1392.1 | 1425.9 | 1452.7 |
| **HMWS1** | 289.7 | 285.8 | 289.1 | 277.1 | 277.0 |
| **Monomer** | 145.9 | 145.0 | 143.1 | 142.5 | 144.6 |
| **LMWS1** | 118.9 | 106.1 | 106.4 | 115.4 | Not detected |
| **LMWS2** | 62.9 | 55.4 | 49.8 | 51.3 | 62.0 |

*Table 1. Molecular weight of trastuzumab and its size variants determined based on SEC-UV-MALS experiments by Wyatt's Astra software.*

## Conclusion

Data quality of SEC-MALS is important for accurate and reproducible determinations of molecular weight and other biophysical properties of large molecules. In this application note, we evaluated the SEC-MALS noise level on the MaxPeak Premier Protein SEC columns (ACQUITY Premier Protein SEC 250 Å, 1.7 µm and XBridge Premier Protein SEC 250 Å, 2.5 µm) as well as ACQUITY UPLC Protein BEH SEC 200 Å, 1.7 µm Column, BioResolve SEC mAb 200 Å, 2.5 µm Column and a diol-bonded silica-based column. The data show that the SEC-MALS noise levels on the ACQUITY Premier Protein SEC columns were slightly higher than those on the ACQUITY Protein BEH 200 SEC columns. In comparison to the diol-bonded silica-based 250 Å, 5 µm column the SEC-MALS noise levels were significantly improved for the ACQUITY Premier Protein SEC columns. For trastuzumab size variants, consistent results were obtained among all the columns for the molecular weight of the monomer and HMWS1. Variations occur among the columns for LMWS1 and LMWS2 MW likely due to partial separation and low abundance, respectively. On the diol-bonded silica-based column, large MW variation occurs across the LMWS2 peak, likely due to the combination of low signal and high noise.

## References

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