TOSOH

Application Note

Comparison of HPLC and UHPLC Methods in the Quality Control of mAb Separations by SEC

Introduction

Accounting for the intrinsic heterogeneity of monoclonal antibodies (mAbs) is essential to ensure production of consistent and safe biotherapeutics. Size exclusion chromatography (SEC) is the standard method for aggregate and fragment analysis of mAbs in biopharmaceutical quality control (QC). This analysis has traditionally relied on large-pore, silica based solid supports with larger particle sizes and column geometries.

Recent trends in HPLC column and particle technology have facilitated faster, more efficient separations by utilizing smaller particle size solid supports and reducing column geometry. Optimization of these column parameters yields improvements in sensitivity and chromatographic resolution, which results in more accurate quantitation, identification and characterization of analytes. This application note compares analyte recovery and resolution between a traditional QC HPLC-SEC method and an updated QC UHPLC-SEC method. Comparisons between columns and instruments were made in order to isolate and understand the impact of each variable on the chromatographic separation.

Experimental HPLC Conditions

Quality Control Conditions

Column:	1. TSKgel® UP-SW3000, 2 µm, 4.6 mm ID × 30 cm 2. TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm
Instruments:	1. Thermo Fisher Dionex Ultimate® 3000 with Chromeleon® v. 6.8 UHPLC 2. Agilent 1200 HPLC
Mobile phase:	100 mmol/L KH_2PO_4/Na_2HPO_4 pH 6.7, 100 mmol/L, Na_2SO_4, 0.05% NaN_3
Gradient:	isocratic
Flow rate:	Column 1: 0.35 mL/min Column 2: 1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	Column 1: 3.5 µL Column 2: 20 µL
Sample:	TBL mAb 01, 3 mg/mL in mobile phase, 4 °C

Instrument Dispersion Conditions

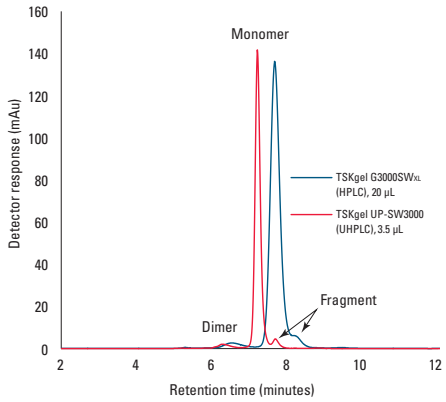
Mobile phase:	60/40 water/acetonitrile
Instruments:	1. Thermo Fisher Dionex Ultimate 3000 with Chromeleon v. 6.8 UHPLC 2. Agilent 1200 HPLC
Gradient:	isocratic
Flow rate:	0.1 mL/min
Detection:	UV @ 215 nm, >10 Hz sampling rate
Temperature:	25 °C
Injection vol.:	0.5 µL
Sample:	1% acetone in mobile phase

	Tubing (ID × Length)	
Instruments	**Injector to Column**	**Column to Detector**
Agilent 1200	0.18 mm × 280 mm	0.18 mm × 360 mm
Dionex 3000	0.18 mm × 450 mm	0.13 mm × 250 mm

Results and Discussion

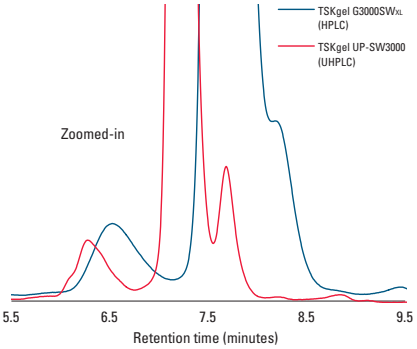
TBL mAb 01 was injected onto a TSKgel G3000SWXL, 5 µm SEC column coupled to an Agilent 1200 HPLC instrument and a TSKgel UP-SW3000, 2 µm column attached to a Thermo Fisher Dionex Ultimate 3000 UHPLC system in order to compare analyte recovery as a function of relative peak area. *Figure 1a* shows that no loss or difference in recovery is observed when the same SEC method is transferred from an HPLC instrument using a 5 µm TSKgel G3000SWXL column to a UHPLC instrument using a 2 µm TSKgel UP-SW3000 column. Additionally, the decreased internal diameter (ID) of the UHPLC column results in enhanced sensitivity requiring less volume (3.5 µL) be injected on the column to obtain results comparable to the HPLC-SEC method (20 µL).

Figure 1a. Comparison of HPLC and UHPLC SEC Methods



Column	% Dimer	% Monomer	% Fragment
TSKgel G3000SWXL	2.95	92.88	3.58
TSKgel UP-SW3000	3.03	92.95	3.51

Figure 1b. Zoomed-in Comparison of HPLC and UHPLC SEC Methods

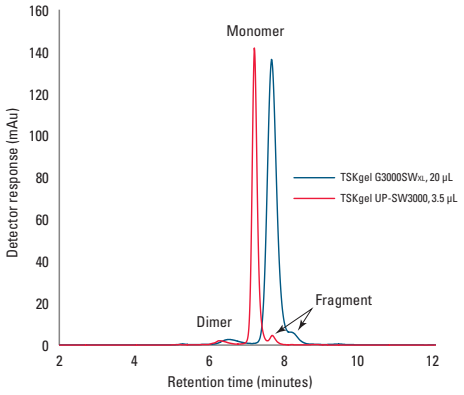


Column	Instrument	Rs (Agg./Mon.)	Rs (Mon./Frag.)	N
TSKgel G3000SWXL	HPLC	1.34	Not resolved	3779
TSKgel UP-SW3000	UHPLC	2.24	1.63	12399

Resolution between aggregate and monomer, as well as monomer and fragment, was then calculated for each QC method. *Figure 1b* shows that the UHPLC-SEC QC method leads to an increase in efficiency and peak resolution. While the resolution between monomer and fragment could not be discerned operating under the conventional HPLC-SEC method, the UHPLC-SEC method yielded separation between the two species, resulting in a calculated resolution of 1.63.

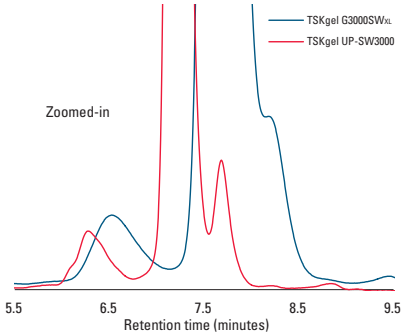
Comparisons between columns and instruments were then made in order to isolate and understand the impact of each variable on the chromatographic separation. For column comparisons, the Thermo Fisher Dionex Ultimate 3000 UHPLC system was used to compare peak area, resolution and efficiency between the TSKgel G3000SWXL and TSKgel UP-SW3000 columns. As shown in *Figure 2a*, no loss or difference in recovery is observed between the two columns analyzed on the same UHPLC system. The smaller particle size and narrower internal diameter of the TSKgel UP-SW3000 column was shown to generate the following benefits compared to the traditional 5 µm column: sharper peaks, greater sensitivity, increased efficiency, and improved resolution *(Figure 2b)*.

Figure 2a. SEC Column Comparison Using UHPLC



Column	% Dimer	% Monomer	% Fragment
TSKgel G3000SWXL	3.08	93.00	3.54
TSKgel UP-SW3000	3.03	92.95	3.51

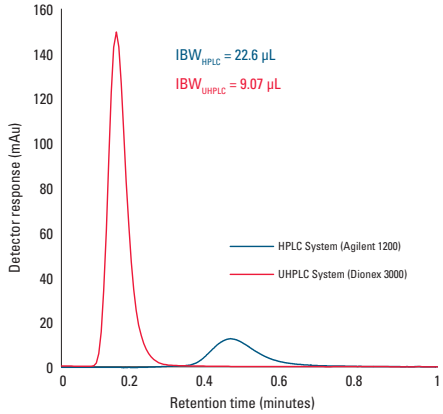
Figure 2b. Zoomed-in SEC Column Comparison Using UHPLC



Column	Rs (Agg./Mon.)	Rs (Mon./Frag.)
TSKgel G3000SWXL	1.64	Not resolved
TSKgel UP-SW3000	2.24	1.63

For a technique like SEC, where the separation takes place in one column volume, instrument dispersion plays a critical role in separation efficiency. In order to compare the dispersion of each system independent of the column, acetone was analyzed using a zero dead volume fitting in place of the HPLC or UHPLC column. *Figure 3* shows that the UHPLC system produces a narrower and taller peak, indicating less volume for the acetone to disperse in the instrument. A calculation of instantaneous bandwidth (IBW) for the HPLC and UHPLC systems confirmed that the HPLC system has a 2.5 fold greater dispersion volume, causing a negative impact on chromatographic performance.

Figure 3. Comparison of Instrument Dispersion



System	T_R Acetone	Flow rate	Efficiency
Agilent 1200 HPLC	0.453 min	0.1 mL/min	64 plates
Thermo Fisher Dionex Ultimate 3000	0.147 min	0.1 mL/min	42 plates

Conclusions

This application note confirms that the TSKgel G3000SWXL, 5 µm column and the TSKgel UP-SW3000, 2 µm column produce similar results for mAb recovery regardless of the utilized instrumentation. Smaller particle size and narrower column internal diameter increase efficiency values resulting in sharper, taller peaks, which translates to better resolution for biopharmaceutical QC. Instrument dispersion volume has a direct effect on column performance in SEC; instrument optimization is key to improving separation quality. An optimized UHPLC method and column provide the best quality separation, yielding gains in resolution and sensitivity.