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).

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 to isolate and understand the impact of each variable on the chromatographic separation.

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

Table 1

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: UHPLC: 0.35 mL/min; HPLC: 1.0 mL/min

Detection: UV @ 280 nm

Temp.: 25 °C

Injection vol.: UHPLC: 3.5 μL; HPLC: 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

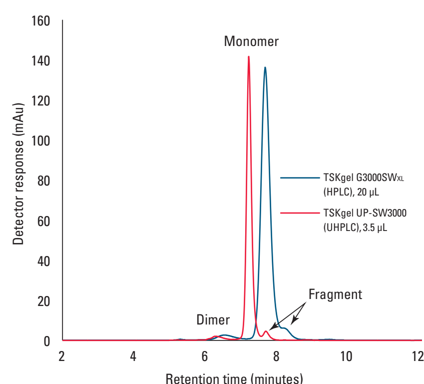
Detection: UV @ 215 nm, >10 Hz sampling rate

Temp.: 25 °C

Injection vol.: 0.5 μL

Sample: 1% acetone in mobile phase

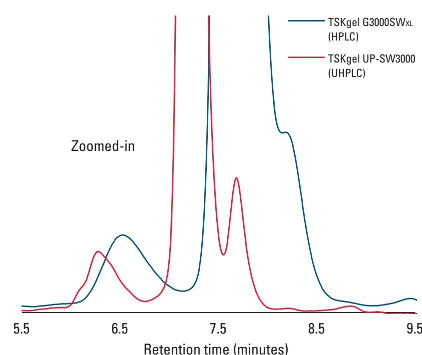
COMPARISON OF HPLC AND UHPLC SEC METHODS



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

Figure 1a

ZOOMED-IN COMPARISON OF HPLC AND UHPLC SEC METHODS



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

Figure 1b

RESULTS AND DISCUSSION

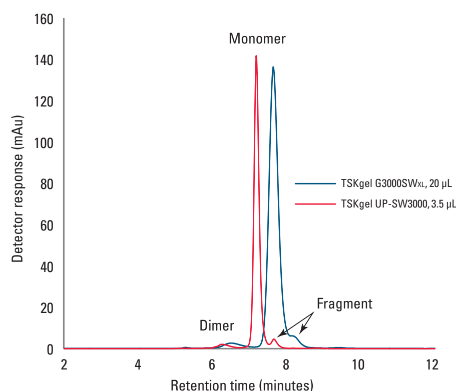
There is no loss or difference in recovery when the same SEC method is transferred from an HPLC instrument using a 5 µm TSKgel G3000SWXL column to a UHPLC instrument using a 2 µm TSKgel UP-SW3000 column (Figure 1a). Additionally, the decreased internal diameter (ID) of the UHPLC column results in enhanced sensitivity, requiring less volume (3.5 µL) injected on the column to obtain results comparable to the HPLC-SEC method (20 µL).

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

Comparisons between columns and instruments were then made 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 offer sharper peaks, higher sensitivity, increased efficiency, and improved resolution compared to the traditional 5 µm column (Figure 2b).

When the separation takes place in one column volume, as it is the case in SEC, instrument dispersion plays a critical role in separation efficiency. To compare the dispersion of each system independently of the column, acetone was analyzed using a zero dead volume fitting in place of the HPLC or UHPLC column.

SEC COLUMN COMPARISON USING UHPLC

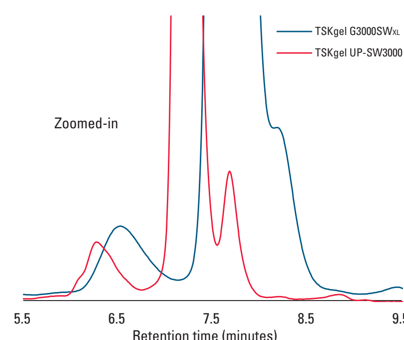


Column	% Dimer	% Monomer	% Fragment
TSKgel G3000SWXL	3.08	93.00	3.54
TSKgel UP-SW3000	3.03	92.95	3.51

Figure 2a

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 UHPLC system has a 2.5 fold lower dispersion volume, impacting the chromatographic performance positively.

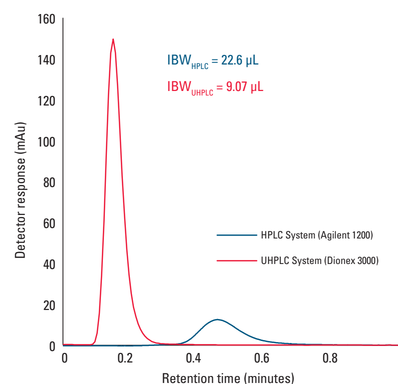
ZOOMED-IN SEC COLUMN COMPARISON USING UHPLC



Column	Rs (Agg./Mon.)	Rs (Agg./Frag.)
TSKgel G3000SWXL	1.34	not resolved
TSKgel UP-SW3000	2.24	1.63

Figure 2b

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

Figure 3

CONCLUSIONS

The TSKgel G3000SWXL, 5 µm, and the TSKgel UP-SW3000, 2 µm column offer similar results for mAb recovery regardless of the utilized instrumentation. Smaller particle size and narrower column ID increase efficiency values resulting in sharper, taller peaks, which translates to a 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 provides the best quality separation, yielding to higher resolutions and sensitivities.