Comparison of HPLC and UHPLC Methods to Optimize Monoclonal Antibody Separation by Size Exclusion Chromatography

Daniel Shollenberger, Stacy Shollenberger, Atis Chakrabarti

Tosoh Bioscience LLC

Introduction

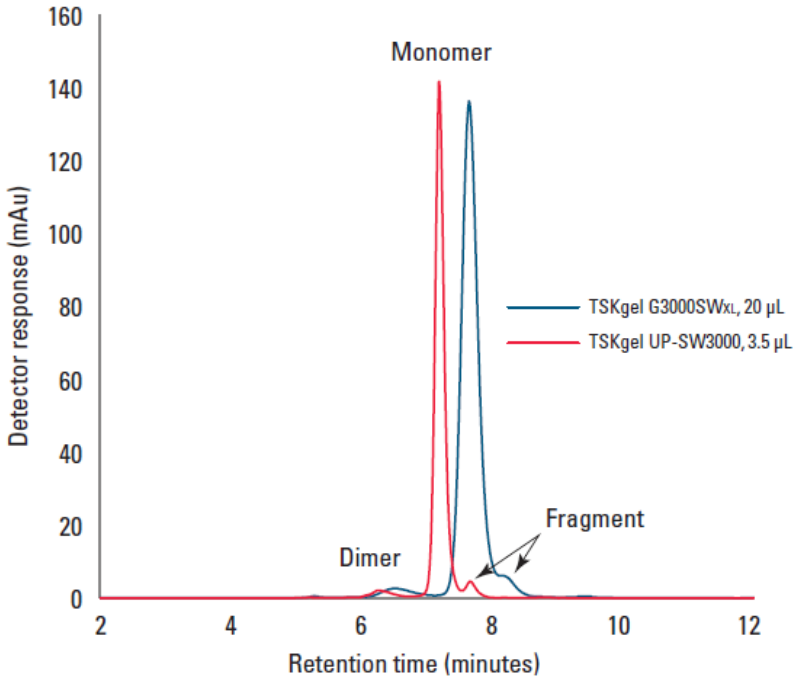
- Size exclusion chromatography (SEC) using polar, silica based stationary phases and high pressure liquid chromatography (HPLC) is widely used for monoclonal antibody (mAb) analysis.
- Recent advances in ultra-high pressure liquid chromatography (UHPLC) instrumentation, column manufacturing, and particle technology have facilitated faster, more efficient SEC separations.
- Smaller particle size, reduced column dimension, and minimal extra column volume while working at higher pressures facilitates improved analyte sensitivity and chromatographic resolution.[1]
- In previous studies, we have shown that standard SEC methods for monoclonal antibody analysis using a TSKgel® G3000SW$_{XL}$, 5 µm, 7.8 mm ID × 30 cm column on a traditional HPLC instrument could be easily transferred to UHPLC instrumentation using a TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm column.[2] The effect of method transfer on analyte recovery, however, was not investigated in this study.
- It is essential to ensure that monomer, aggregate and fragment recovery is maintained when transferring methods from HPLC to UHPLC.
- In this study, we compare analyte recovery as a function of percentage peak area between the previously described HPLC and UHPLC SEC methods.
- Comparisons between columns and instruments were made to isolate and understand the impacts of each variable on the chromatographic separation.

Materials and Methods

Columns:	1. TSKgel UP-SW3000, 2 µm, 4.6 mm ID × 30 cm 2. TSKgel G3000SW$_{XL}$, 5 µm, 7.8 mm ID × 30 cm
Instruments:	Thermo Fisher Dionex Ultimate® 3000 with Chromeleon® v. 6.8 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
Flow rate:	Column 1: 0.35 mL/min Column 2: 1.0 mL/min
Pressure:	Column 1: 28.5 MPa at 0.35 mL/min Column 2: 8.3 MPa at 1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 ºC
Injection vol.:	Column 1: 3.5 µL Column 2: 20 µL
Sample:	TBL mAb 01, 3 mg/mL in mobile phase, 4 ºC

Results: *SEC Column Comparison Using UHPLC*

Figure 1a: UHPLC Analysis of mAb on TSKgel G3000SWXL and TSKgel UP-SW3000; Comparison of Analyte Recovery

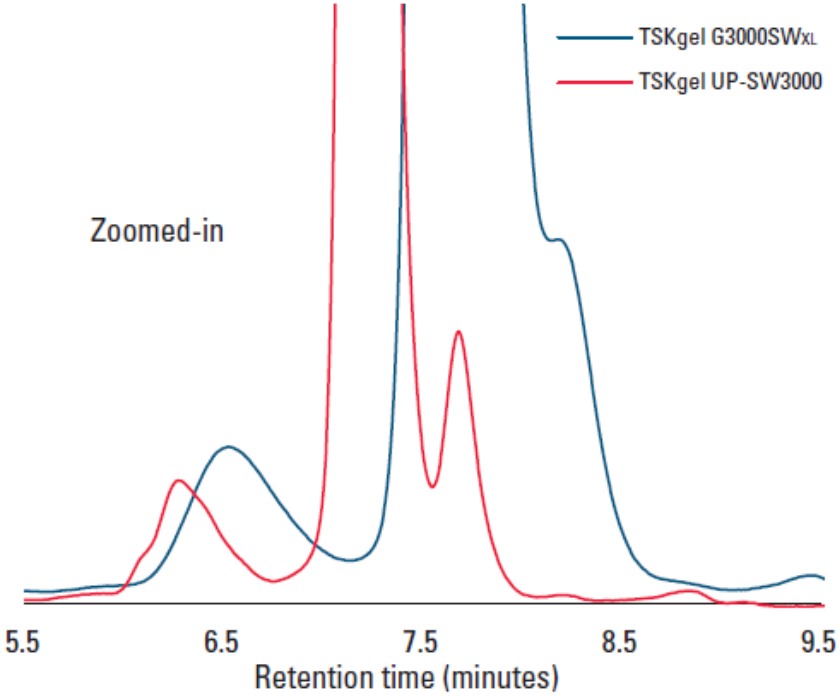


Column	% Dimer	% Monomer	% Fragment
TSKgel G3000SWXL	3.08	93.00	3.54
TSKgel UP-SW3000	3.03	92.95	3.51

- No loss or difference in recovery is observed when the same SEC method is transferred from a TSKgel G30000SWXL, 5 µm column to a TSKgel UP-SW3000, 2 µm column analyzed on the same UHPLC system.
- The smaller particle size of the TSKgel UP-SW3000 column yielded sharper peaks and greater sensitivity.

Results: *SEC Column Comparison Using UHPLC, Con't*

Figure 1b: UHPLC Analysis of mAb on TSKgel G3000SWXL and TSKgel UP-SW3000; Comparison of Peak Resolution

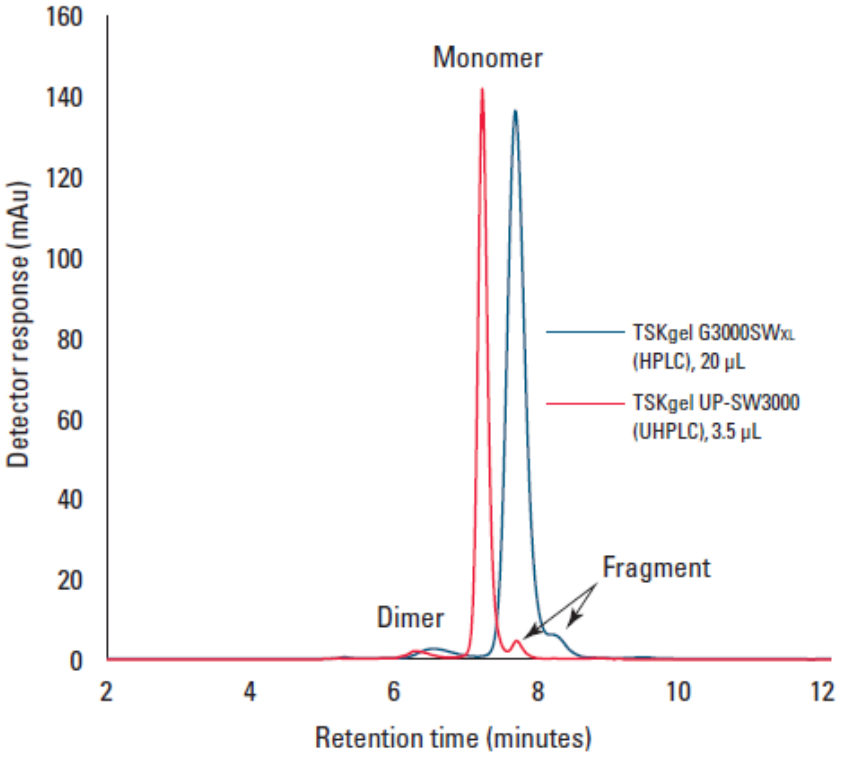


Column	Rs (Agg./Mon.)	Rs (Mon./Frag.)
TSKgel G3000SWXL	1.64	Not resolved
TSKgel UP-SW3000	2.24	1.63

Smaller particle size and narrower internal diameter of the TSKgel UP-SW3000 column leads to an increase in column efficiency and peak resolution.

Results: *Comparison of HPLC and UHPLC SEC Methods*

Figure 2a: UHPLC Analysis of mAb on TSKgel UP-SW3000, 2 µm Column versus HPLC Analysis of mAb on TSKgel G3000SWXL, 5 µm Column; Comparison of Analyte Recovery

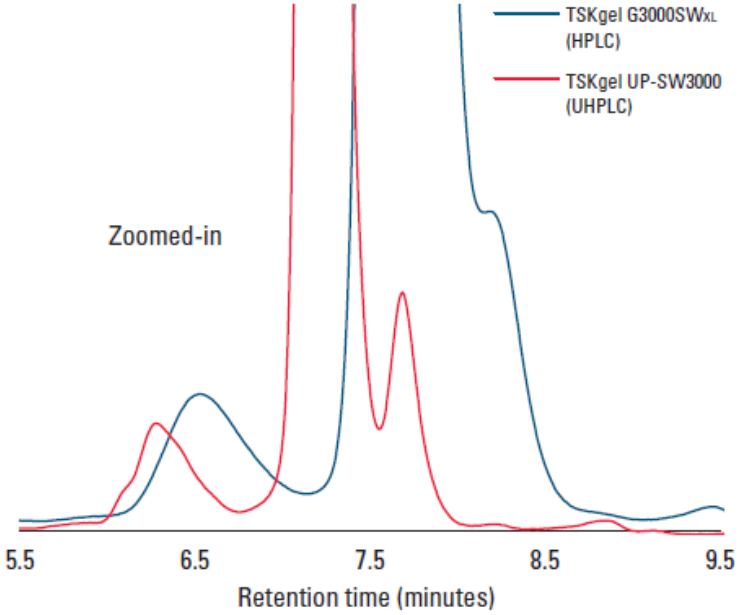


Column	% Dimer	% Monomer	% Fragment
TSKgel G3000SWXL	2.95	92.88	3.58
TSKgel UP-SW3000	3.03	92.95	3.51

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.

Results: *Comparison of HPLC and UHPLC SEC Methods, Con't*

Figure 2b: UHPLC Analysis of mAb on TSKgel UP-SW3000, 2 µm Column Versus HPLC Analysis of mAb on TSKgel G3000SWXL, 5 µm Column; Comparison of Peak Resolution



Column	Instrument	Rs (Agg./Mon.)	Rs (Mon./Frag.)	N
TSKgel G3000SWXL	UHPLC	1.64	Not resolved	4154
TSKgel UP-SW3000	UHPLC	2.24	1.63	12399
TSKgel G3000SWXL	HPLC	1.34	Not resolved	3779

- Smaller particle size and narrower internal diameter of the TSKgel UP-SW3000 column leads to an increase in column efficiency and peak resolution.
- Optimized UHPLC instrumentation yields gains in peak resolution, improving the confidence for peak integration.

Results: *Comparison of Instrument Dispersion*

For a technique like SEC, where the separation takes place in one column volume, instrument dispersion place a critical role in separation efficiency. Therefore, a study was performed to compare instrument dispersion using a zero dead volume union.

Materials and Methods

Instruments: Thermo Fisher Dionex Ultimate 3000 with Chromeleon v. 6.8
Agilent 1200 HPLC

Mobile phase: 60/40 water/acetonitrile

Flow rate: 0.1 mL/min

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

Temperature: 25 ºC

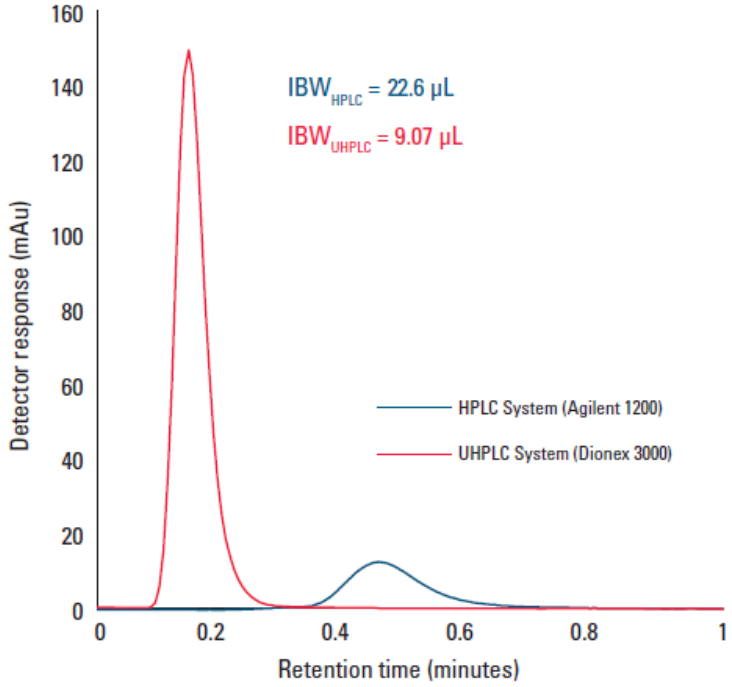
Injection vol.: 0.5 µL

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 m × 450 mm	0.13 mm × 250 mm

Results: *Comparison of Instrument Dispersion, Con't*

Figure 3: UHPLC Versus HPLC Analysis of Acetone, 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

- The UHPLC system produces a much more narrow and taller peak, indicating less volume for the acetone to disperse in the instrument.
- A calculation of IBW for the HPLC and UHPLC systems confirmed that the HPLC system has a 2.5 fold greater dispersion volume, which impacts chromatographic performance.

Calculation of Instrument Bandwidth for the HPLC System

$$\sigma = \frac{T_R * F}{\sqrt{N}} \qquad IBW = 4\sigma$$

Where: T_R = Retention Time
F = Flow Rate
N = Efficiency
σ = Peak Standard Deviation (band spreading)
IBW = Instrument Bandwidth (Dispersion Volume)

$$\sigma_{HPLC} = \frac{0.453\ min \times \frac{0.1\ mL}{min}}{\sqrt{64}}$$

$$\sigma_{HPLC} = 0.00566\ mL \times \frac{1000\ \mu L}{1\ mL} = 5.66$$

$$IBW_{HPLC} = 4\sigma = 4 \times 5.66\ \mu L = 22.6\ \mu L$$

Conclusions

- This study 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 mAb separations.
- 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 potentially sensitivity.

References

1. Modern Size Exclusion Liquid Chromatography, 2nd Ed; John Wiley and Sons, 2009.

2. Characterization of New 2 µm Particle Size, 25 nm Pore Size Analytical Size Exclusion Chromatography Column with Larger Exclusion Limit Useful for the Separation of Biomolecules Using UHPLC and HPLC CRYSTAL BENNER, Atis Chakrabarti, Tosoh Bioscience LLC, PITTCON 2015 Abstract # 120-1

3. R. A. Henry, H. K. Brandes, D. T. Nowlan and J. W. Best, Practical Tips for Operating UHPLC Instruments and Columns, LCGC North America April 2013