Evaluation of a New 2 µm Silica-Based Size Exclusion Chromatography Column for the Analysis of Proteins, mAb Fragments and Peptides

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Introduction

- Size Exclusion Chromatography (SEC) columns with 12.5 nm pore size are widely used for the separation of small proteins, peptides and oligos.
- In these separations, higher resolution and sensitivity is important.
- The smaller the particle size, the greater the resolution.
- Previously we reported the development of a 4 µm particle size, 12.5 nm TSKgel SuperSW2000 column (4.6 mm ID × 30 cm) with improved sensitivity and resolution over the 5 µm, 12.5 nm TSKgel G2000SWXL column[1].
- Here we present study results of a newly developed silica based 2 µm, 12.5 nm SEC column in the separation of small proteins, peptides and oligos.
- This new column is also compared with the 4 µm, 12.5 nm, 4.6 mm ID × 30 cm TSKgel SuperSW2000 SEC column.

[1] Figure 3, Separation Report 95; https://www.separations.us.tosohbioscience.com/HPLC_Columns/id-8346/TSKgel_SuperSW2000

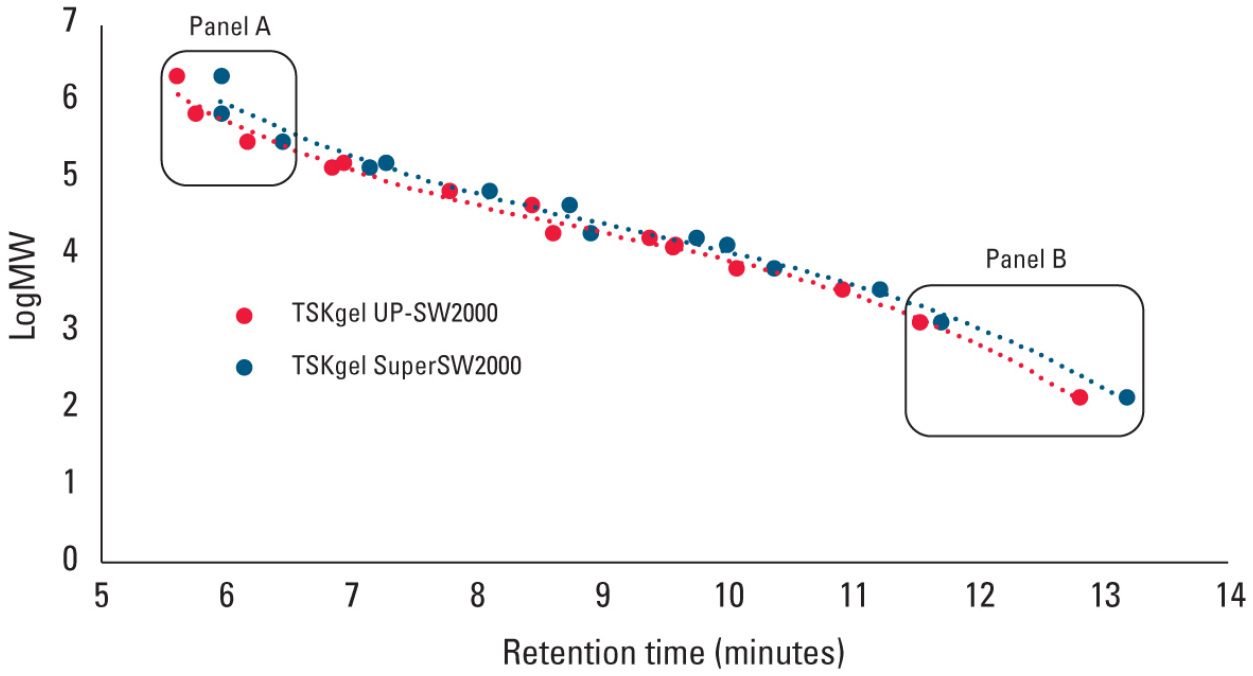
Introduction

SEC Columns	Pore Size	Particle Size	Dimension
TSKgel SuperSW2000	12.5 nm	4 µm	4.6 mm ID × 30 cm
TSKgel UP-SW2000	12.5 nm	2 µm	4.6 mm ID × 30 cm

Chromatographic Conditions (HPLC)

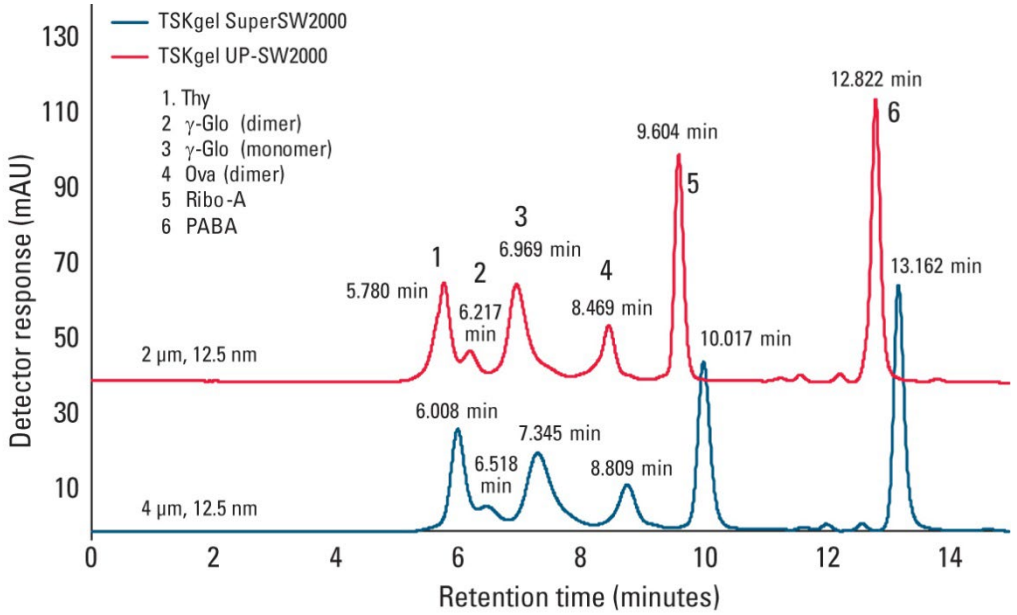
Columns:	TSKgel UP-SW2000 and SuperSW2000
Instruments:	Agilent 1100 with Chemstation v. 2.18.18 Thermo Fisher Ultimate® 3000 UHPLC with Chromeleon® v. 7.0
Mobile phase:	100 mmol/L phosphate buffer, pH 6.7, 100 mmol/L Na_2SO_4, 0.05% NaN_3
Flow rate:	0.35 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	5 µL
Samples:	1. protein standard mixture: thyroglobulin (660 kDa) γ-globulin monomer (150 kDa) ovalbumin (42.7 kDa) ribonuclease-A (13.7 kDa) para amino benzoic acid (137 Da) 2. TBL mAb-01 (Herceptin® biosimilar), 4 mg/mL 3. oligos 4. small proteins 5. mAb fragments

Calibration Curves (HPLC)



- Panels A and B highlight the difference between the columns' near total exclusion and total inclusion limits in terms of particle size and pore size distributions.
- The TSKgel UP-SW2000 column has a slightly higher exclusion limit compared to TSKgel SuperSW2000.

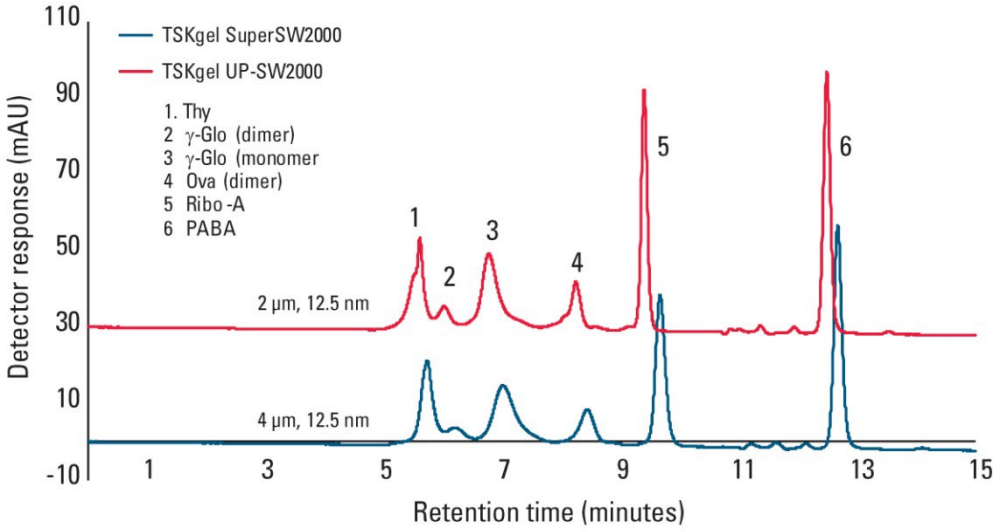
Analysis of Protein Standard Mixture: HPLC



Resolution	TSKgel SuperSW2000 4 µm	TSKgel UP-SW2000 2 µm
Thy - γ Glo Dimer	0.82	0.86
γ Glo Dimer - γ Glo Monomer	1.12	1.45
γ Glo Monomer – Ova	2.17	3.21
Ova – Ribo A	2.65	3.47
Ribo A - PABA	9.26	11.63

The TSKgel UP-SW2000 column yielded significantly higher resolution on the LMW (low molecular weight) side of the 150 kDa molecular weight marker (γ-globulin).

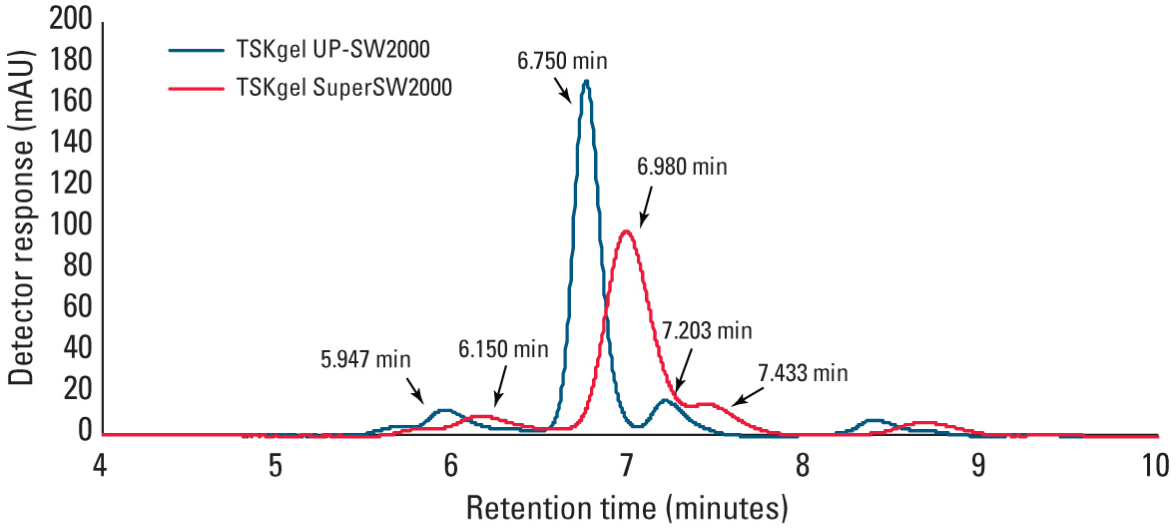
Analysis of Protein Standard Mixture: UHPLC



Resolution	TSKgel SuperSW2000 4 µm	TSKgel UP-SW2000 2 µm
Thy - γ Glo Dimer	-	1.01
γ Glo Dimer - γ Glo Monomer	0.82	1.54
γ Glo Monomer – Ova	2.43	3.70
Ova – Ribo A	3.16	4.73
Ribo A - PABA	10.78	15.22

The TSKgel UP-SW2000 SEC column yielded higher sensitivity and higher resolution in the separation of protein standards as shown in the table below.

Analysis of Herceptin Biosimilar: UHPLC

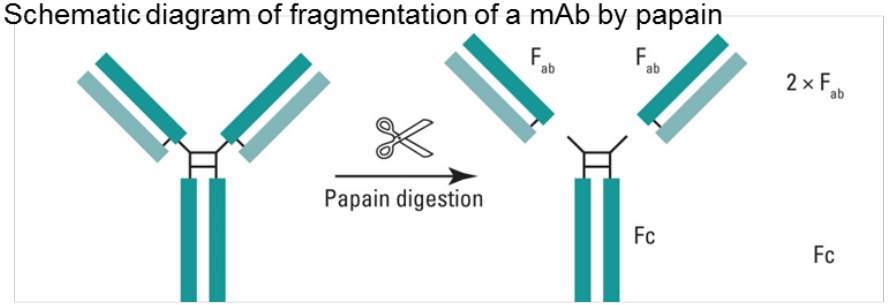


Resolution	TSKgel SuperSW2000 4 µm	TSKgel UP-SW2000 2 µm
Monomer - Dimer	1.4	2.1
Monomer - Fragment	-	1.4

The TSKgel UP-SW2000 column yielded higher sensitivity and resolution in the separation of the dimer and the fragments compared to the TSKgel SuperSW2000 column (see table below).

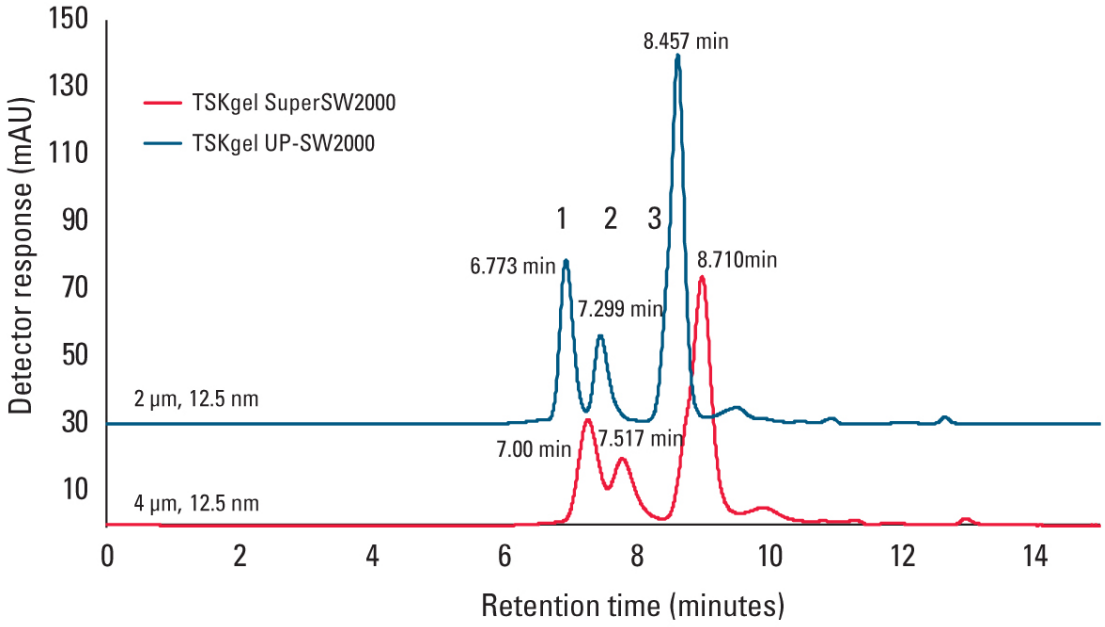
Analysis of Papain Digest with TSKgel UP-SW2000

Schematic diagram of fragmentation of a mAb by papain



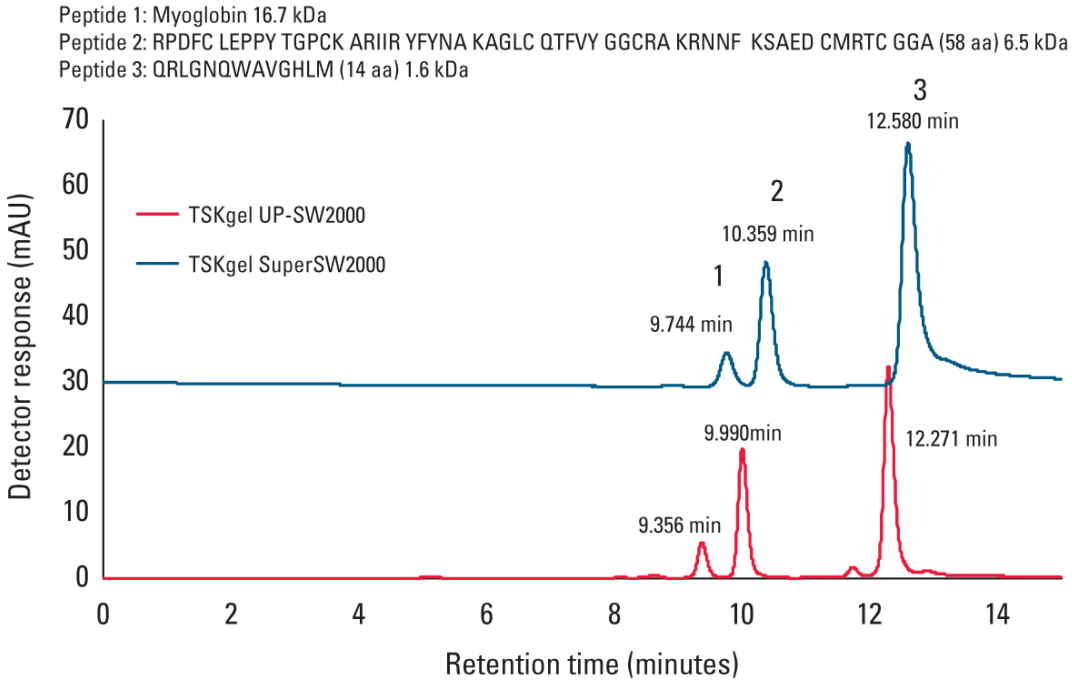
- **Preparation of 2x digestion buffer:** 0.485 g of Tris, 0.060 g of EDTA, and 0.024 g of L-cysteine was dissolved in 15 mL of DI water. The pH was adjusted to 7.6 using HCl following the final volume adjustment to 20 mL with DI water.
- **Digestion of mAb with papain:** 2 mg/mL papain solution was prepared in DI water. 20 µL of mAb was mixed with 50 µL of 2x digestion buffer, 26 µL of DI water and 4 µL of papain solution in water (2 mg/mL). The mixture was incubated in water bath at 37 ºC for 4 hours.

Analysis of Papain Digest of mAb: HPLC



Resolution	TSKgel SuperSW2000 4 µm	TSKgel UP-SW2000 2 µm
Peak 1 – Peak 2	0.77	1.3
Peak 2 – Peak 3	1.79	2.83

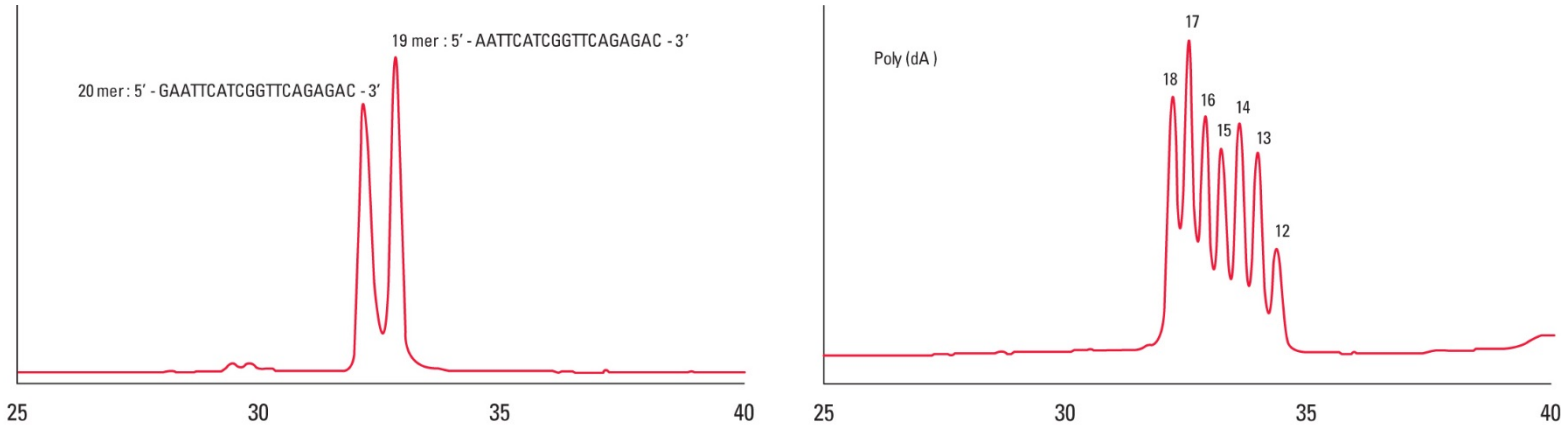
Analysis of Small Protein and Peptide Mixture



Resolution	TSKgel SuperSW2000 4 µm	TSKgel UP-SW2000 2 µm
Peak 1 – Peak 2	1.7	2.39
Peak 2 – Peak 3	5.81	8.21

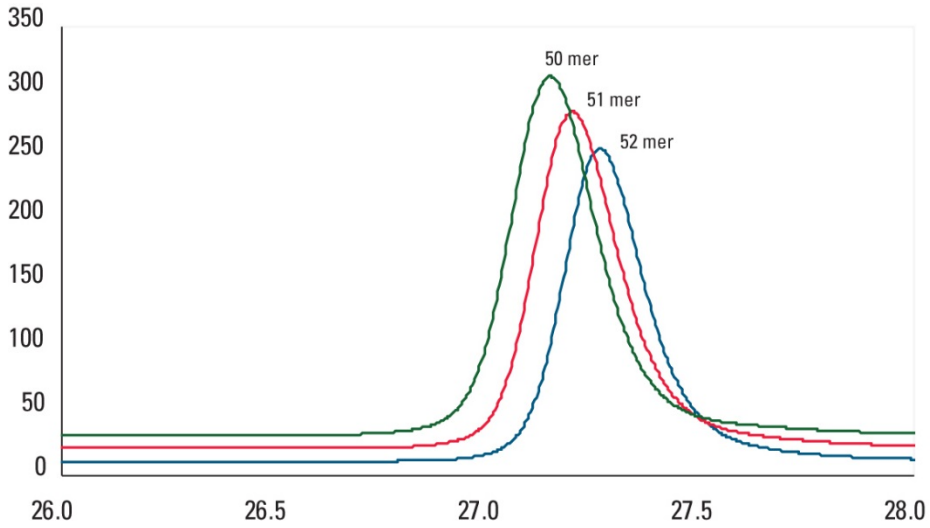
This study shows that TSKgel UP-SW2000 separated these small proteins and peptides with greater sensitivity and resolution.

Analysis of Oligonucleotides with TSKgel UP-SW2000



Column:	TSKgel UP-SW2000, 4.6 mm ID × 30 cm × 2
Mobile phase:	0.05% NaN_3 and 0.3 mol/L NaCl in 0.05 mol/L phosphate, pH 6.7
Flow rate:	0.2 mL/min
Temperature:	25 °C
Detection:	UV @ 260 nm

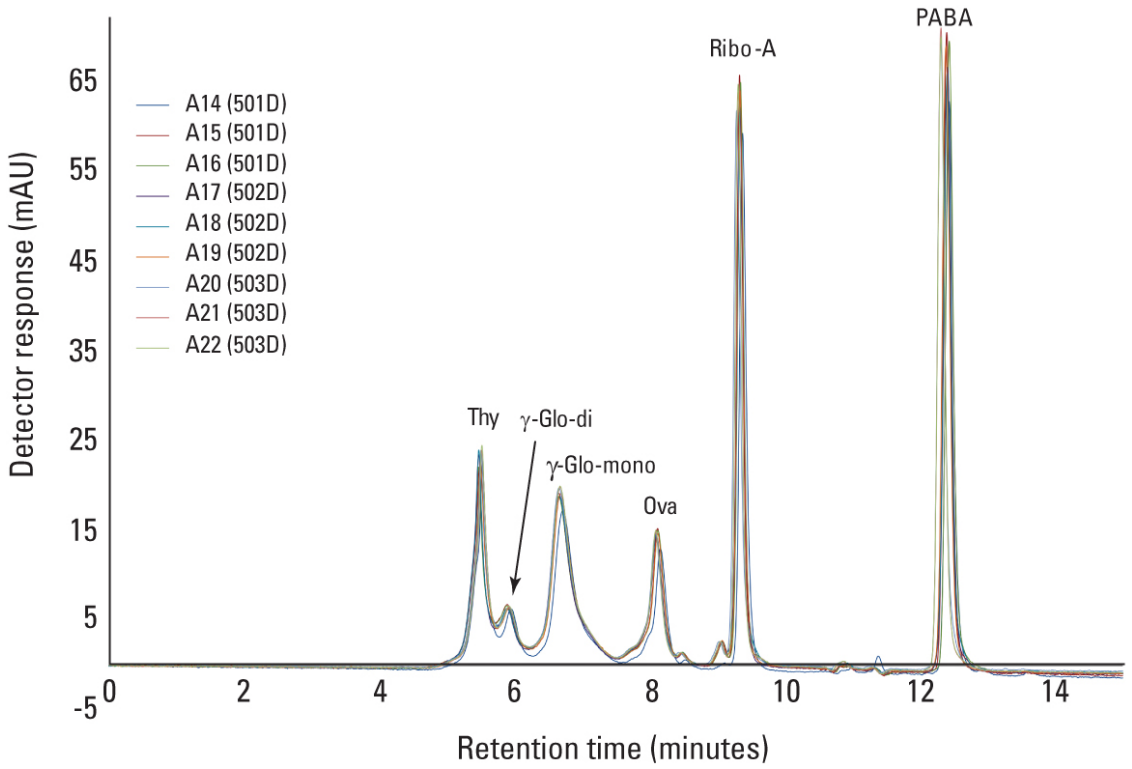
Analysis of Oligonucleotides with TSKgel UP-SW2000



Column:	TSKgel UP-SW2000, 4.6 mm ID × 30 cm × 2
Mobile phase:	0.05% NaN_3 and 0.3 mol/L NaCl in 0.05 mol/L phosphate, pH 6.7
Flow rate:	0.2 mL/min
Temperature:	25 °C
Detection:	UV @ 260 nm

This study shows that the TSKgel UP-SW2000 can separate oligomers with one mer difference.

Lot-to-Lot Analysis of Standard Protein Mixture with TSKgel UP-SW2000: UHPLC



- Lot-to-lot reproducibility establishes robust and tightly controlled specifications and production procedures.
- The analysis shows that the analysis was reproducible over 3 different lots.
- %RSD analysis of the peak parameters such as retention time, % relative peak area, resolution between the peaks and column efficiency are low, as shown below.

Lot-to-Lot Analysis of Standard Protein Mixture with TSKgel UP-SW2000: UHPLC

QC STD

Retention time (minutes)

Column #	Lot #	Thy	γ-Glo-mono	Ova	Riba-A	PABA
A14	501D	5.54	6.727	8.17	9.383	12.433
A15	501D	5.549	6.667	8.127	9.337	12.4
A16	501D	5.48	6.667	8.133	9.353	12.437
A17	502D	5.483	6.67	8.107	9.343	12.41
A18	502D	5.487	6.673	8.103	9.35	12.403
A19	502D	5.57	6.693	8.13	9.343	12.387
A20	503D	5.52	6.67	8.093	9.297	12.31
A21	503D	5.53	6.687	8.1	9.317	12.313
A22	503D	5.553	6.703	8.123	9.323	12.32
	Average	5.524	6.684	8.121	9.338	12.379
	STDEV	0.033	0.021	0.023	0.024	0.051
	%RSD	**0.603**	**0.038**	**0.289**	**0.262**	**0.413**

QC STD

Resolution (Rs)

Column #	Lot #	Thy-g-Glo	γ-Glo-mono	Ova-RiboA	Ribo-A-PABA
A14	501D	3	3.59	5.13	15
A15	501D	3.05	3.68	5.29	15.95
A16	501D	3.06	3.68	5.27	15.86
A17	502D	3.03	3.51	5.09	14.99
A18	502D	3.11	3.5	5.29	15.41
A19	502D	3.01	3.54	5.13	15.32
A20	503D	3.01	3.56	5.12	15.52
A21	503D	3.06	3.65	5.37	15.52
A22	503D	3.03	3.63	5.25	15.66
	Average	3.04	3.59	5.22	15.47
	STDEV	0.03	0.07	0.10	0.34
	%RSD	**1.13**	**1.95**	**1.90**	**2.17**

- %RSD deviation in retention times was less than 1% across 9 columns; 3 different lots, 3 columns from each lot.
- %RSD of the resolution between the peaks were low and reproducible, across 9 columns; 3 different lots, 3 columns from each lot.

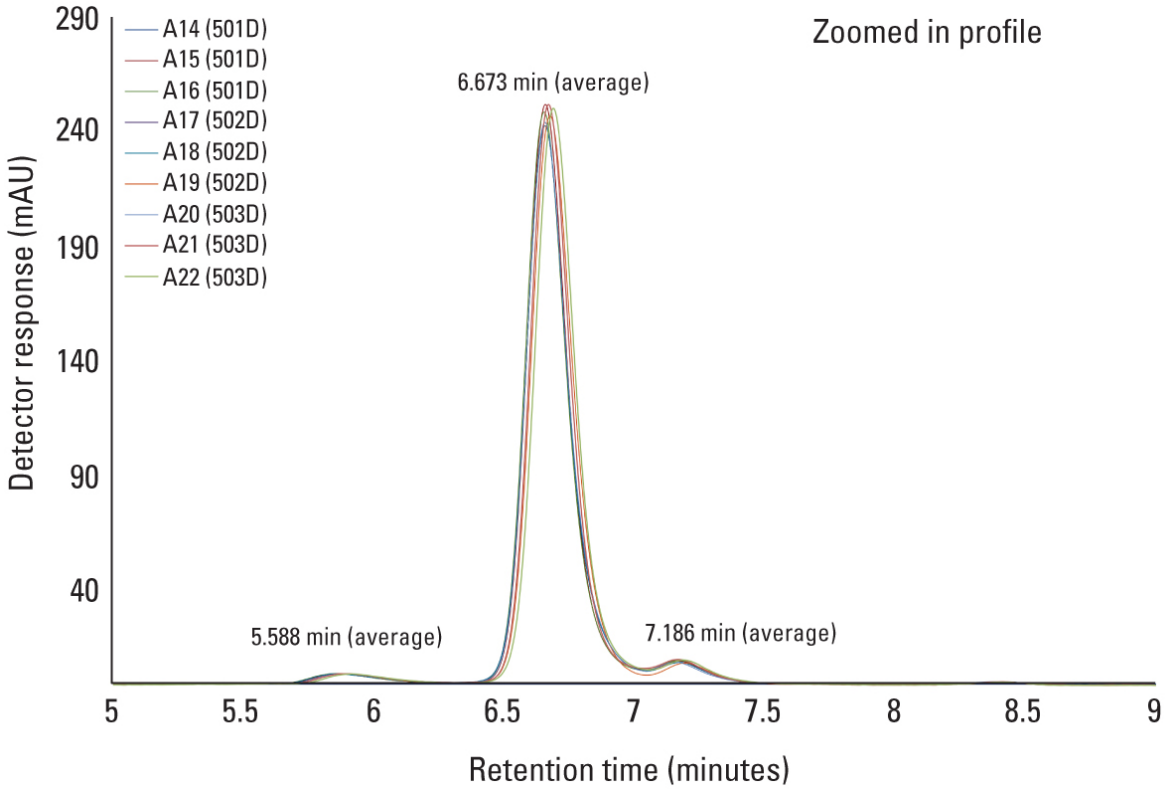
Lot-to-Lot Analysis of Standard Protein Mixture with TSKgel UP-SW2000: UHPLC

QC STD

% Rel. peak area						
Column #	Lot #	Thy	γ-Glo-mono	Ova	Riba-A	PABA
A14	501D	15.59	21.93	9.29	23.54	29.65
A15	501D	14.4	22.5	9.29	23.35	30.46
A16	501D	14.32	22.46	9.2	23.46	30.56
A17	502D	15.28	22.9	9.57	22.59	29.66
A18	502D	14.5	22.43	9.31	23.21	30.54
A19	502D	15.21	22.64	9.32	22.87	29.97
A20	503D	15.2	22.9	9.13	23.03	29.69
A21	503D	15.65	23.43	9.01	23.14	29.78
A22	503D	15.45	23.25	9.23	22.39	29.68
	Average	15.067	22.716	9.261	23.064	29.999
	STDEV	0.521	0.458	0.153	0.389	0.404
	%RSD	**3.455**	**2.018**	**1.655**	**1.686**	**1.345**

- Peak area reproducibility is a critical criteria for system suitability in size exclusion chromatography.
- %RSD of the % relative peak area between the peaks were low and reproducible, across 9 columns; 3 different lots, 3 columns from each lot.
- Thyroglobulin yielded slightly higher %RSD, this is possibly associated with the fact that it elutes in the total exclusion region.
- All other proteins yielded < 2% RSD.

Lot-to-Lot Analysis of mAb with TSKgel UP-SW2000: UHPLC



Lot-to-Lot Analysis of mAb with TSKgel UP-SW2000: UHPLC

mAb

Retention time (minutes)				
Column #	Lot #	Dimer	Monomer	Fragment
A14	501D	5.937	6.713	7.233
A15	501D	5.87	6.663	7.18
A16	501D	5.857	6.66	7.177
A17	502D	5.863	6.663	7.177
A18	502D	5.87	6.663	7.177
A19	502D	5.883	6.683	7.2
A20	503D	5.883	6.663	7.16
A21	503D	5.907	6.677	7.173
A22	503D	5.923	6.697	7.193
	Average	5.888	6.676	7.186
	STDEV	0.028	0.019	0.021
	%RSD	**0.475**	**0.280**	**0.294**

mAb

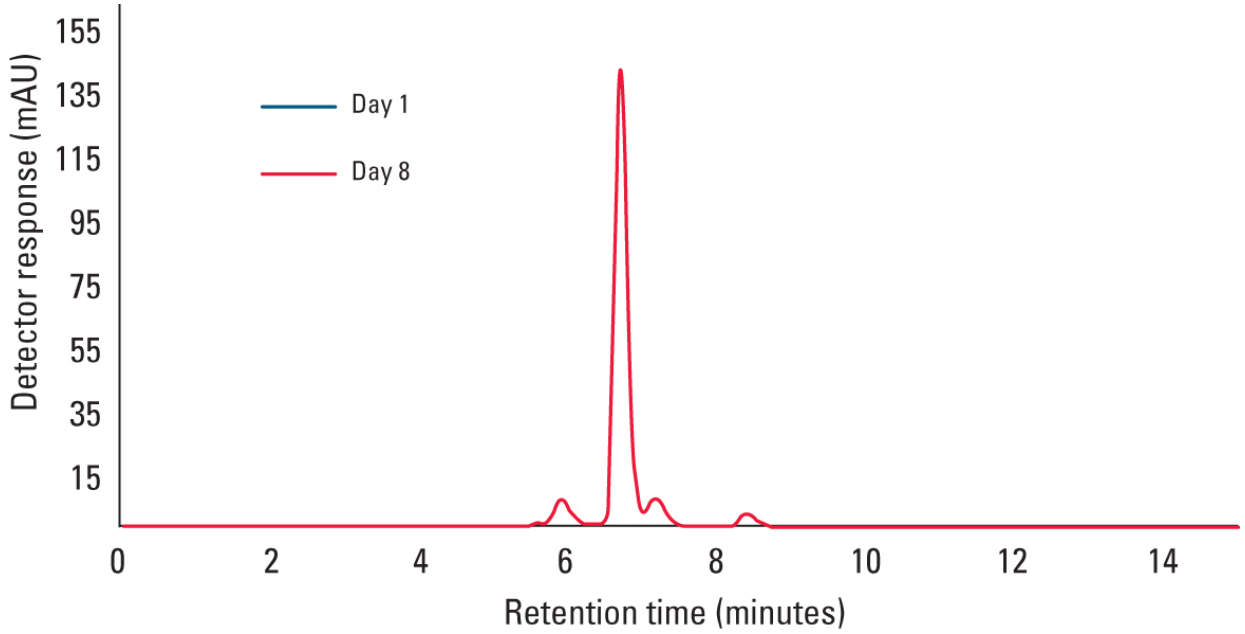
Resolution (Rs)		
Column #	Lot #	Dimer-Monomer
A14	501D	2.05
A15	501D	2
A16	501D	1.99
A17	502D	1.93
A18	502D	1.93
A19	502D	1.98
A20	503D	1.9
A21	503D	1.92
A22	503D	1.94
Average		1.960
STDEV		0.048
%RSD		**2.447**

- Retention times of mAb monomer, dimer and fragment peaks were reproducible from lot-to-lot.
- %RSD for over 9 columns (3 columns from 3 different lots) was very low.
- Resolution between monoclonal antibody monomer and dimer peaks were reproducible from lot-to-lot with low %RSD.

Lot-to-Lot Analysis of mAb with TSKgel UP-SW2000: UHPLC

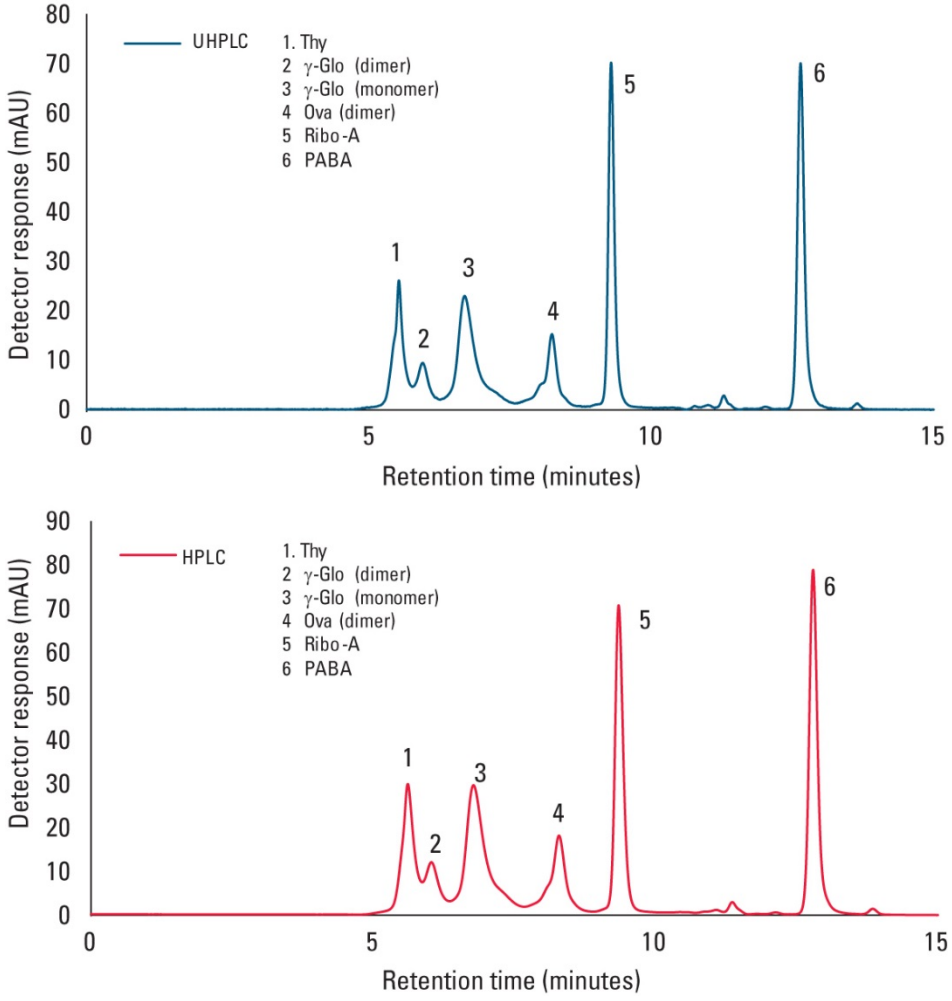
mAb			
% Rel. peak area			
Column #	Lot #	Monomer	Dimer
A14	501D	93.25	3.18
A15	501D	90.97	3.06
A16	501D	90.8	3.12
A17	502D	91.84	3.12
A18	502D	91.59	3.06
A19	502D	91.69	3.07
A20	503D	91.25	3.04
A21	503D	90.69	3.1
A22	503D	90.89	3.03
	Average	91.441	3.087
	STDEV	0.795	0.048
	%RSD	**0.869**	**1.545**

Day-to-Day Reproducibility: UHPLC



The chromatographic profile yielded excellent day-to-day reproducibility – establishing the robust packing of this SEC column.

Method Compatibility between HPLC and UHPLC Instruments



- The chromatographic conditions are the same for both runs.
- Only the flow rate was adjusted to 0.35 mL/min when run in UHPLC.
- This study shows that the TSKgel UP-SW2000 column yielded comparable analysis between HPLC and UHPLC.
- Details of the peak parameters and comparative data analysis are available upon request.

Conclusions

- The new TSKgel UP-SW2000, 2 µm, 12.5 nm silica-based SEC column yielded higher sensitivity and resolution compared to a TSKgel SuperSW2000, 4 µm SEC column of the same pore size in the analysis of proteins, mAb fragments, peptides, and oligos, particularly in the LMW region of a 150 kDa MW marker.
- The diol surface chemistry of the TSKgel UP-SW2000 is similar to that of the 4 µm TSKgel SuperSW2000 column, with a slightly higher exclusion limit.
- This SEC column is capable of separating the impurities in oligos differing even by only one mer.
- The TSKgel UP-SW2000 column is compatible for both HPLC and UHPLC instrumentation, requiring only an adjustment in flow rate.