Three novel prototype SEC columns for the separation of an antibody monomer from its dimer, higher aggregates, and antibody fragments

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Objective

To evaluate a set of prototype SEC columns designed for the analysis of monoclonal antibodies

Introduction

- Size exclusion chromatography (SEC) performed under aqueous conditions, also known as Gel Filtration Chromatography (GFC), is popular for the isolation and quality control of monoclonal antibodies and other therapeutic proteins and peptides.
- Monoclonal antibodies (mAbs) are widely used as biopharmaceuticals and new mAbs are still being developed by modifying the complementarity determining regions.
- mAbs easily undergo structural and chemical changes during preparation and storage processes and such denaturation may cause loss of therapeutic efficacy or manifestations of toxicity.
- Therefore, therapeutic mAbs must be subject to strict quality control.
- Size exclusion chromatography (SEC) is a powerful and convenient tool for determining mAb monomers and their impurities, including aggregates, oligomers, and mAb fragments.

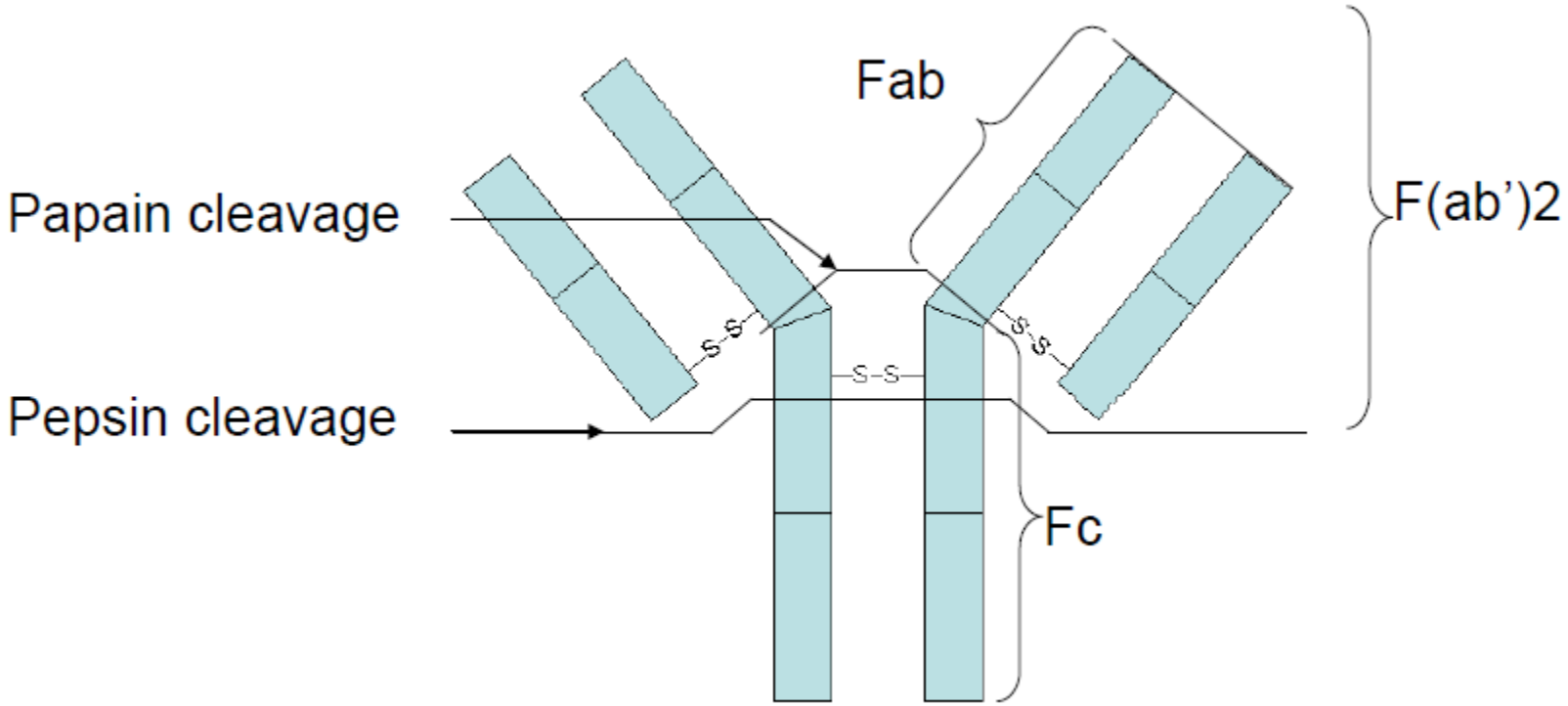
Introduction continued

We have developed three silica-based prototype SEC columns designed especially for mAb analysis:

1. A 4.6 mm ID × 15 cm semi-micro column packed with 25 nm pore size, 4 µm particles, which is designed for high throughput analysis of mAbs.
2. A 7.8 mm ID × 30 cm analytical column packed with the same particles as mentioned above. The column dimension is compatible with conventional LC systems with relatively large extra-column dead volume and is suitable for high resolution analysis of mAb monomers and dimers.
3. A 7.8 mm ID × 30 cm analytical column packed with newly developed 30 nm pore size, 3 µm particles. Larger pore size with the estimated exclusion limit of ~4 $\times 10^6$ Da provides improved separation and quantitation of mAb aggregates and oligomers.

Here we report the features of these new SEC prototype columns and their superior performance of mAb separation in comparison to conventional columns.

Structure of IgG



IgG is a relatively large molecule (approx. 150 kDa) and in order to improve the penetration to the tissue , fragmentation is carried out. Digestion with papain or pepsin is commonly applied to obtain antibody fragments without the loss of activity.

When papain is used for the antibody digestion, 2 Fab and 1 Fc are obtained from 1 antibody. When pepsin is used, a F(ab')2 is obtained.

Material and methods

Columns

- TSKgel® SuperSW mAb HTP, 4.6 mm ID x 15 cm, 4 µm particle*
- TSKgel SuperSW mAb HR, 7.8 mm ID x 30 cm, 4 µm particle*
- TSKgel UltraSW Aggregate, 7.8 mm ID x 30 cm, 3 µm particle*
- TSKgel G3000SWXL, 7.8 mm ID x 30 cm, 5 µm particle
- All TSKgel columns were manufactured by Tosoh (Tokyo, Japan).
  *** prototype columns**

Instrumentation

- The HPLC system was a Tosoh liquid chromatograph equipped with pump (DP-8020), column oven (CO-8020), UV detector (UV-8020), and data processor (LC-8020 model II).
- Agilent 1200 (Chemstation - Rev B.04.01)

Material and methods continued

Samples

- Standard TSKgel SWXL test mixture: thyroglobulin, γ-globulin, ovalbumin, ribonuclease A, PABA
- Pullulan standards were obtained from Showa Denko (Tokyo, Japan).
- Monoclonal antibodies:
  - Kaketsuken (Kumamoto, Japan) *(Figures 3, 5-9)*
  - Monoclonal antibody: BI-mAb-2 from Boehringer-Ingelheim (gift from Tosoh Bioscience GmbH); concentration: 4.5 g/L in glycine/Na phosphate, pH 6.0
  - BI-mAb-01 from Boehringer-Ingelheim (gift from Tosoh Bioscience GmbH); in 0.1 mol/L citrate buffer, pH 6.0; concentration: 28 g/L
  - Human IgG (Sigma I8640-10MG; Tech grade >80% SDS-PAGE)
  - Mouse IgG (Tech grade from serum, Sigma I8765-10MG, Lot #95H8845)

Material and methods continued

Papain Digestion

Mouse IgG_1
(5 g/L, 10 mmol/L phosphate buffer, pH 7.3 + 0.15 mol/L NaCl
+ 1 mmol/L EDTA • 2Na + 25 mmol/L β-mercaptoethanol)
↓
Addition of Papain solution,10 vol%
(1 g/L, 10 mmol/L phosphate buffer, pH 7.3 + 0.15 mol/L NaCl
+ 1 mmol/L EDTA • 2Na + 25 mmol/L β-mercaptoethanol)
↓
37°C
↓
Sampling 48.5 µL
↓
Addition of 1.5 µL 1 mol/L iodoacetamide
↓
40°C, 15 min
↓
Addition of 950 µL 20 mmol/L phosphate buffer + 0.3 mol/L NaCl, pH 7.0
↓
Analysis by SEC

Papain: from papaya latex
Sigma P4762, 14 units/mg protein

Material and methods continued

Aggregate formation by heat denaturation was carried out by adjusting the pH of the antibody solution from pH 6.0 to 5.5 using dilute phosphoric acid followed by incubation at 60 °C over time.

Chromatographic conditions

- **Mobile Phase:** 100 mmol/L potassium phosphate buffer, 100 mmol/L sodium sulfate, pH 6.7 + 0.05% $NaN_{3;}$ unless mentioned otherwise
- **Flow rate:** 1.0 mL/min (0.35 mL/min for 15 cm column)
- **Detection:** UV @ 280 nm
- **Temperature:** ambient / 25 °C except during heat denaturation study
- **Injection vol.:** 10 µL

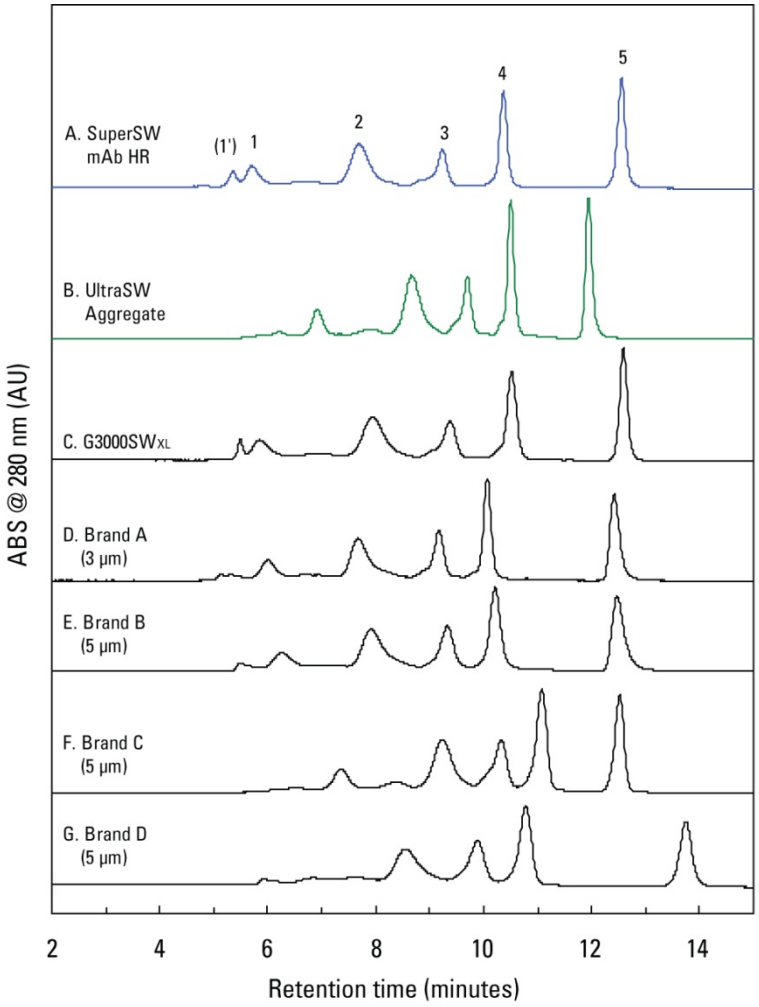
Material and methods

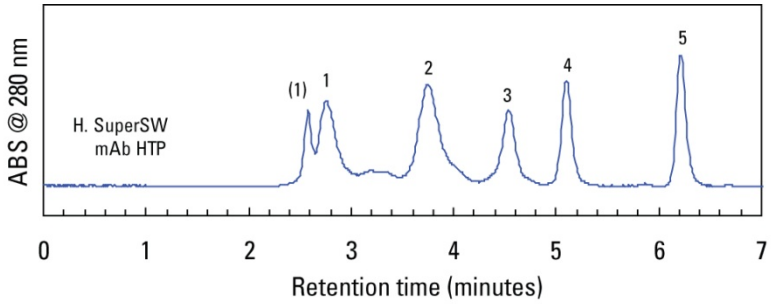
- High purity HPLC grade Sigma Aldrich chemicals were used in this study.
- High purity 18.2 m.Ohm-cm quality water was used to make buffer and samples.

Table 1: Specifications of the columns

Column	TSKgel SuperSW mAb HTP	TSKgel SuperSW mAb HR	TSKgel UltraSW Aggregate
Column dimension	4.6 mm ID × 15 cm	7.8 mm ID × 30 cm	7.8 mm ID × 30 cm
Base material	Silica gel		Silica gel
Functional group	Diol		Diol
Particle size	4 µm		3 µm
Pore size	25 nm		30 nm
Separation range (for globular proteins)	10,000 - 500,00 Da		10,000 - 2,000,000 Da
Applications	Fast separation of mAb monomer and dimer (UHPLC compatible)	Separation of mAb monomer and dimer (conventional LC compatible)	Separation of mAb aggregates

Figure 1: Analysis of standard proteins

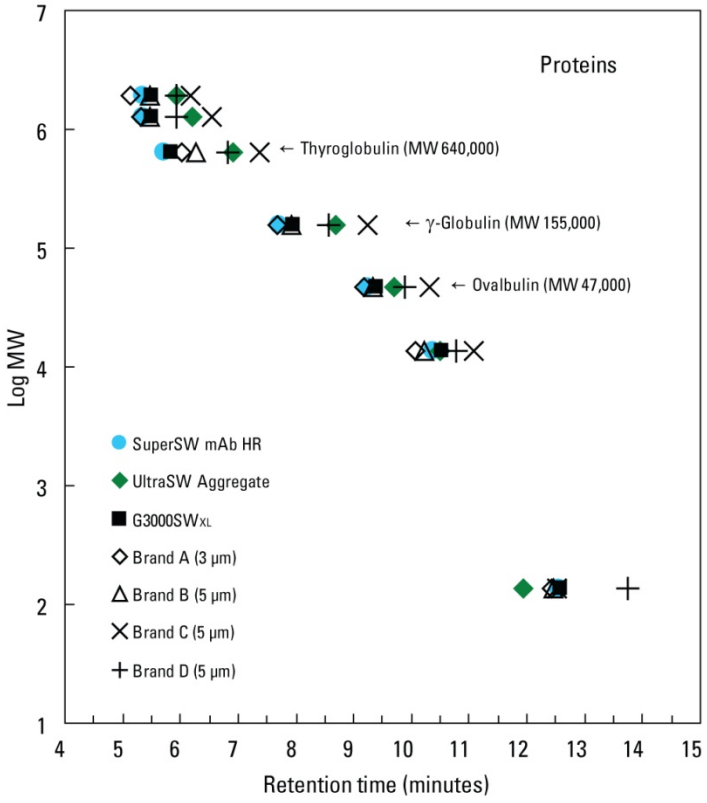


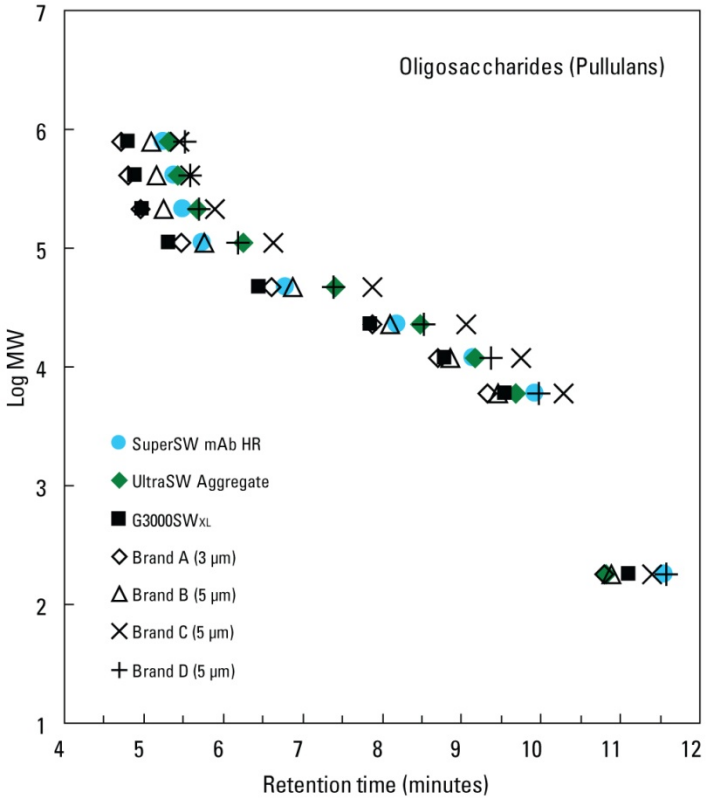


Column dimension: A-E: 7.8 mm ID × 30 cm
F&G: 8.0 mm ID × 30 cm
H: 4.6 mm ID × 15 cm
Mobile phase: 200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate: A-G: 1.0 mL/min H: 0.35 mL/min
Detection: UV @ 280 nm
Temperature: 25 °C
Injection vol.: A-G: 10 µL H: 3.5 µL
Samples: 1 thyroglobulin (MW 640,000) (A-G: 0.5 g/L H: 2.0 g/L)
(1) thyroglobulin oligomer
2 γ-globulin (MW 155,000) (A-G: 1.0 g/L H: 1.5 g/L)
3 ovalbumin (MW 47,000) (A-G: 1.0 g/L H: 1.5 g/L)
4 ribonuclease A (MW 13,700) (1.5 g/L)
5 p-aminobenzoic acid (MW 137) (0.01 g/L)

New TSKgel SEC prototype columns show their superior performance over other columns.

Figure 2: Calibration curves

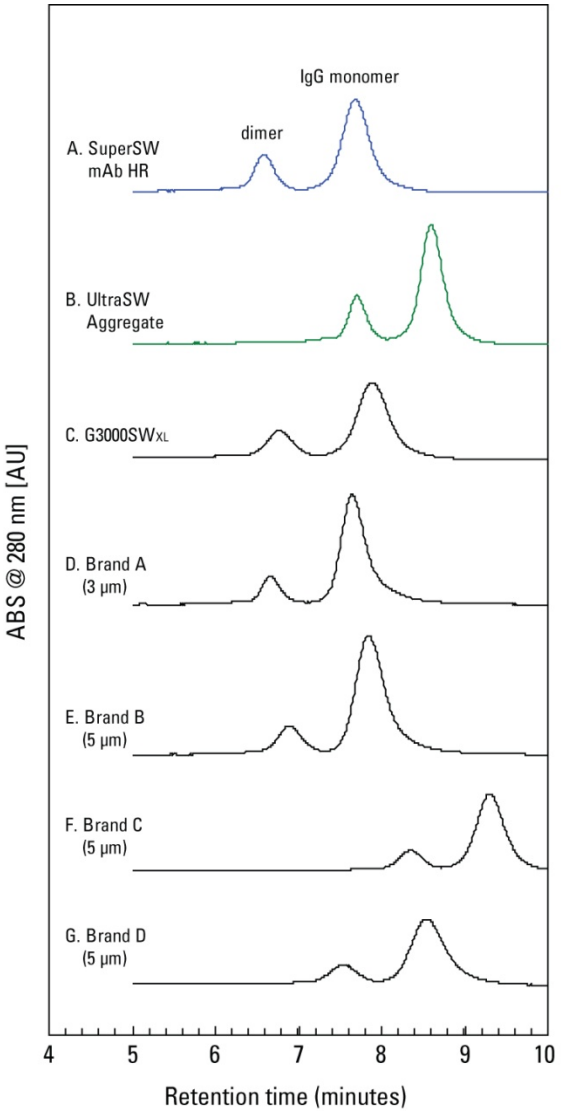


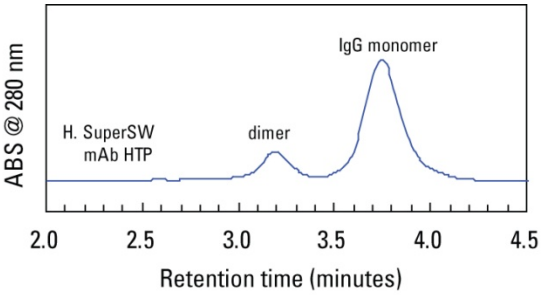


Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Samples:	thyroglobulin, γ-globulin, ovalbumin, ribonuclease A, p-aminobenzoic acid

Mobile phase:	50 mmol/L $NaNO_3$
Flow rate:	1.0 mL/min
Detection:	RI
Temperature:	25 °C
Injection vol.:	10 µL
Samples:	pullulans (MW 788,000/ 404,000/ 212,000/ 112,000/ 47,300/ 22,800/ 11,800/ 5,900), glucose

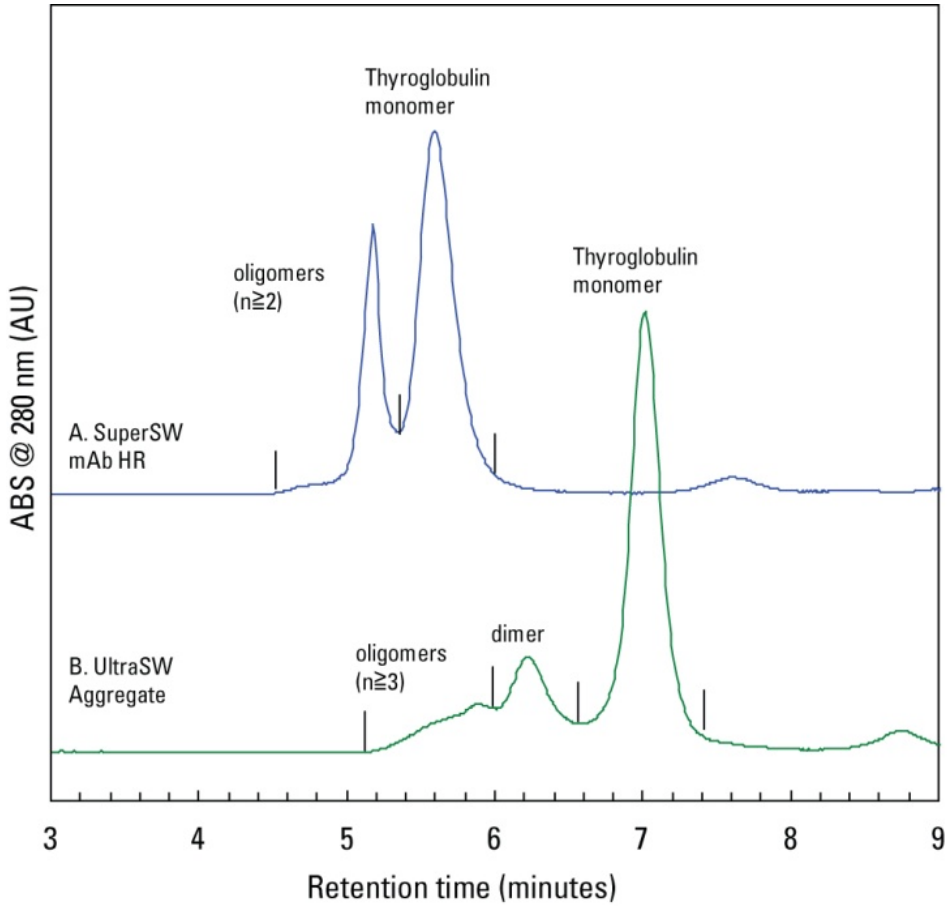
Figure 3: Separation of IgG monomer and dimer





Column dimension: A-E: 7.8 mm ID × 30 cm
F&G: 8.0 mm ID × 30 cm
H: 4.6 mm ID × 15 cm
Mobile phase: 200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate: A-G: 1.0 mL/min H: 0.35 mL/min
Detection: UV @ 280 nm
Temperature: 25 °C
Injection vol. : A-G: 10 µL H: 3.5 µL
Sample: IgG (A-G: 1.0 g/L H: 4.5 g/L)

Figure 4: Analysis of thyroglobulin monomer, dimer and aggregates



Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	10 µL
Sample:	thyroglobulin (2.0 g/L)

Table 2: Summary of column performance

Column	Slopes of calibration curve		MW exclusion limit for proteins (Da)	Separation of IgG		Separation of thyroglobulin	
	thyroglobulin /γ-globulin	γ-globulin /ovalbumin		TP (monomer)	Rs (dimer/monomer)	TP (monomer)	Rs (dimer/monomer)
4.6 mm ID × 15 cm column:							
TSKgel SuperSW mAb HTP	-0.618	-0.659	8.4×10^5	1,765	1.69	1,238	0.76
7.8 mm ID × 30 cm columns:							
TSKgel SuperSW mAb HR	-0.309	-0.335	8.4×10^5	2,338	1.97	2,463	1.00
TSKgel UltraSW Aggregate	-0.351	-0.506	$3.6 \times 10^{6(*)}$	4,446	1.91	5,155	1.34
TSKgel G3000SW$_{XL}$	-0.294	-0.360	8.6×10^5	1,835	1.64	1,421	0.87
Brand A (3 µm)	-0.370	-0.345	$3.0 \times 10^{6(*)}$	2,888	1.95	2,165	n.d.
Brand B (5 µm)	-0.370	-0.368	1.2×10^6	2,270	1.56	1,133	1.15
8.0 mm ID × 30 cm columns:							
Brand C (5 µm)	-0.328	-0.474	$3.6 \times 10^{6(*)}$	3,304	1.59	2,894	n.d.
Brand D (5 µm)	-0.353	-0.390	1.4×10^6	1,868	1.33	n.d.	n.d.

(*) V_0 was estimated by elution times of pullulans

Summary of column performance

- The TSKgel SuperSW mAb HTP prototype column exhibited equal separation between IgG monomer and dimer in half the analysis time compared to the conventional SEC column, TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm.
- The TSKgel SuperSW mAb HR prototype column exhibited superior resolving power for IgG monomer and dimer compared to other SEC columns.
- The TSKgel UltraSW Aggregate prototype column, which possesses a larger MW exclusion limit, exhibited superior resolving power for thyroglobulin oligomers with high molecular weight.

Figure 5: Separation of papain-digested IgG by TSKgel SuperSW mAb HTP prototype column

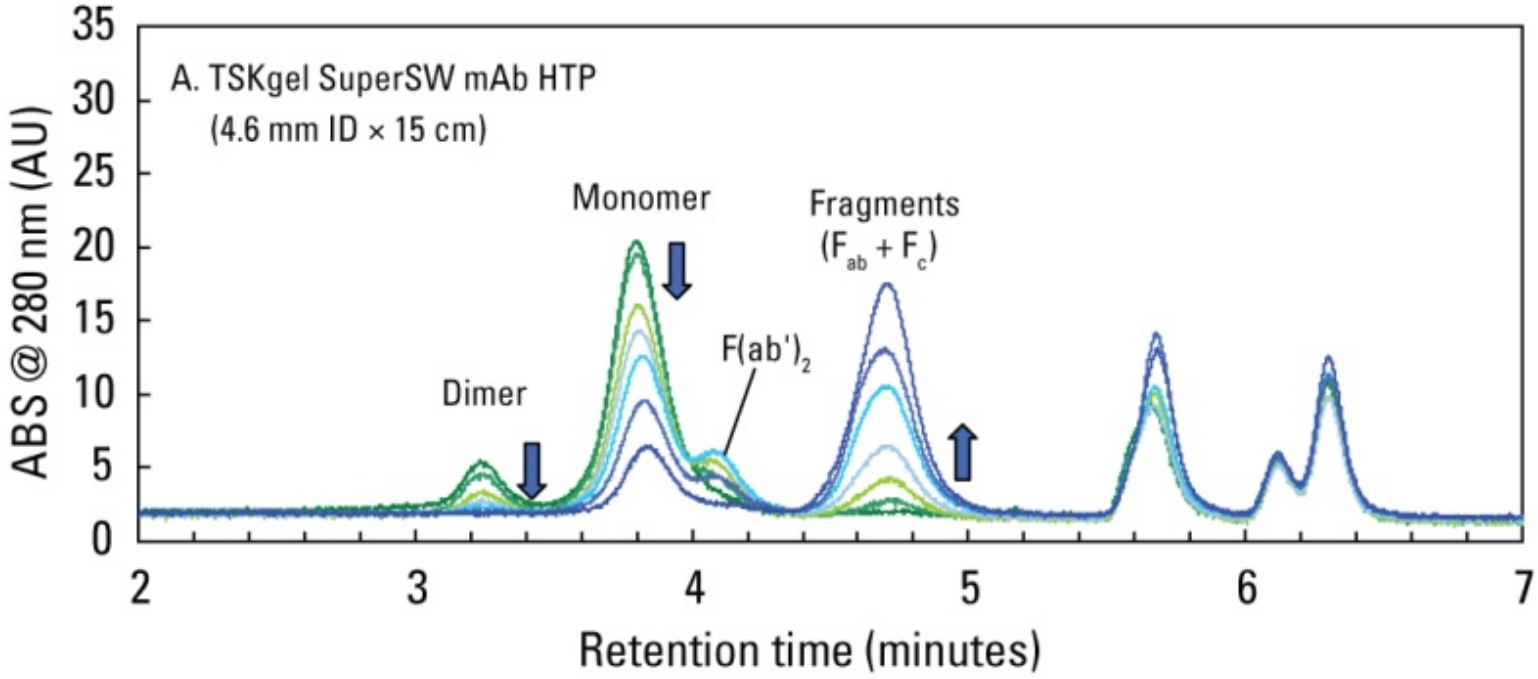


Figure 6: Separation of papain-digested IgG by TSKgel SuperSW mAb HR prototype column

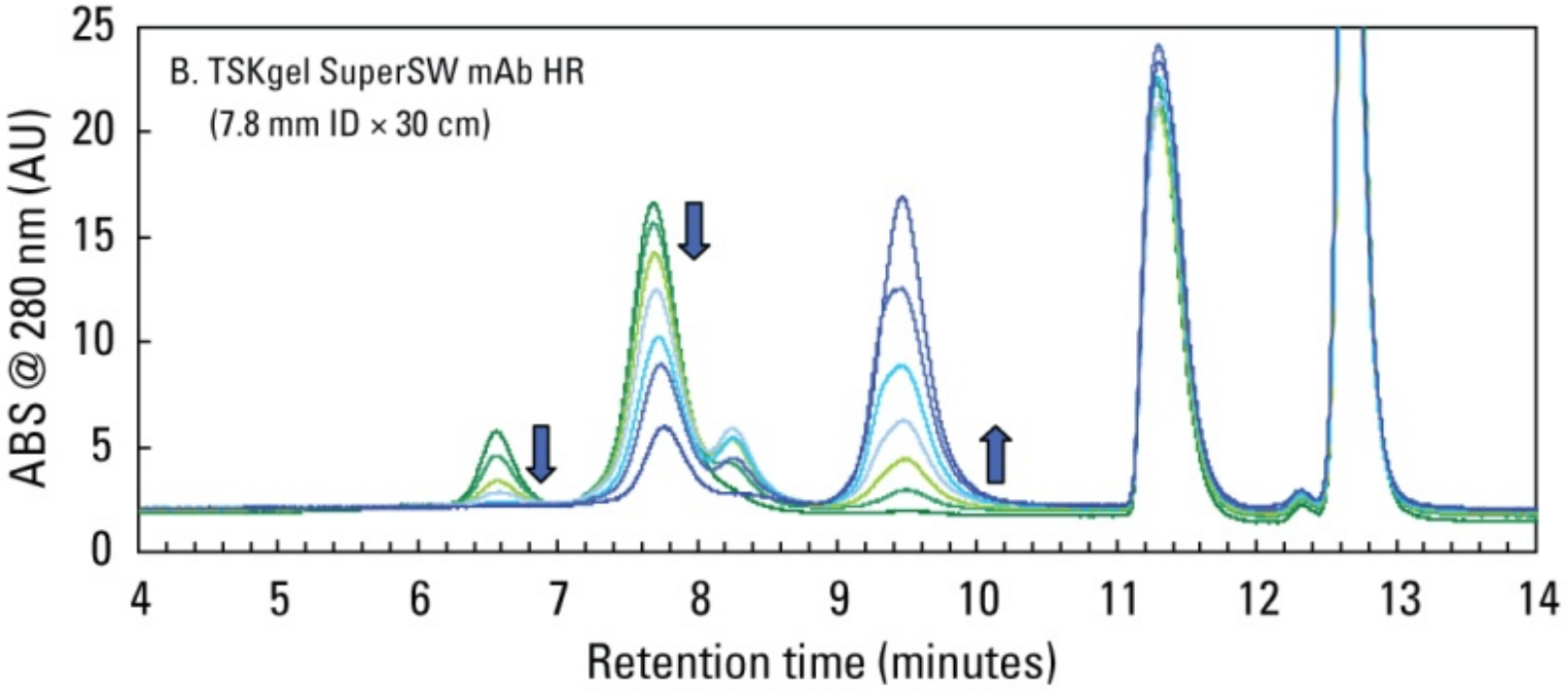


Figure 7: Separation of papain-digested IgG by TSKgel UltraSW Aggregate prototype column

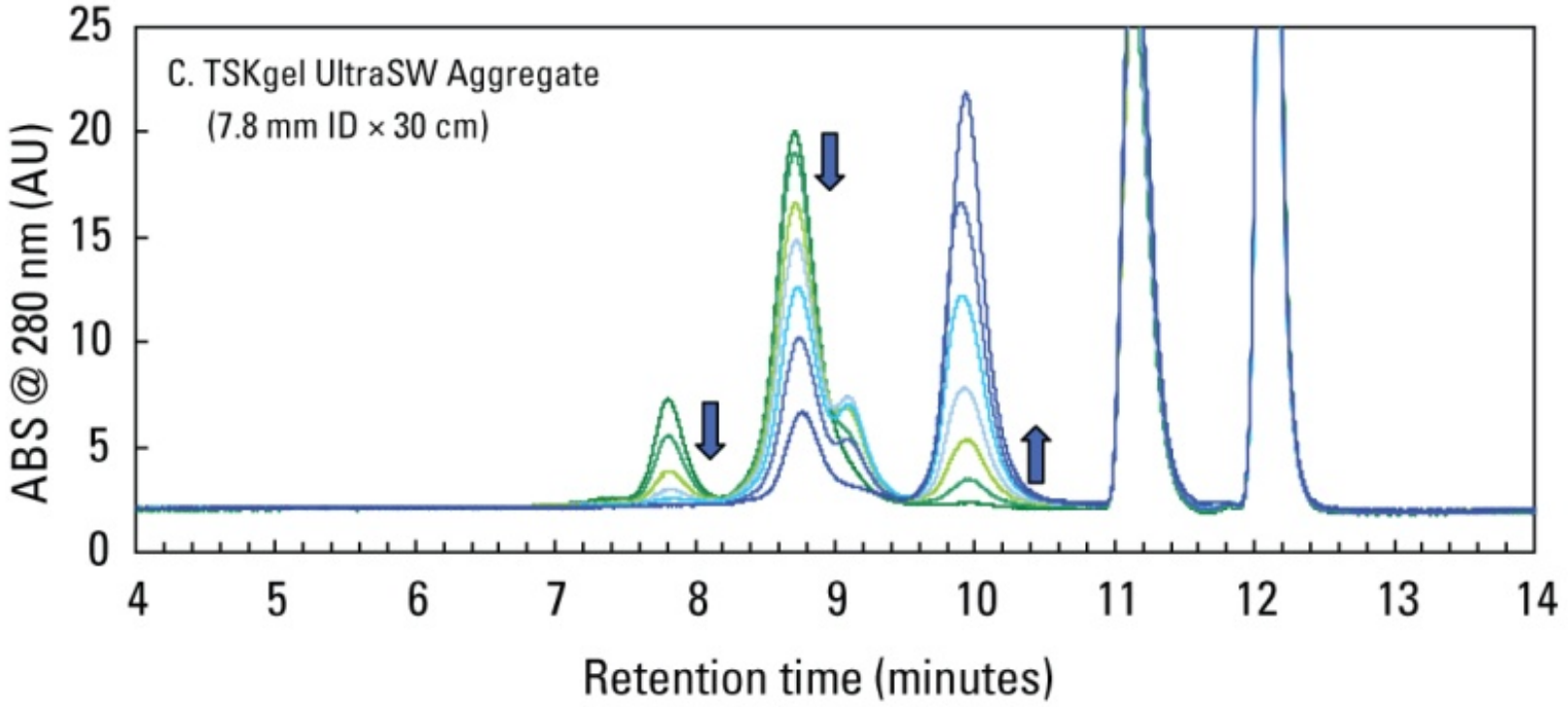


Figure 8: Separation of papain-digested IgG by TSKgel G300SWXL column

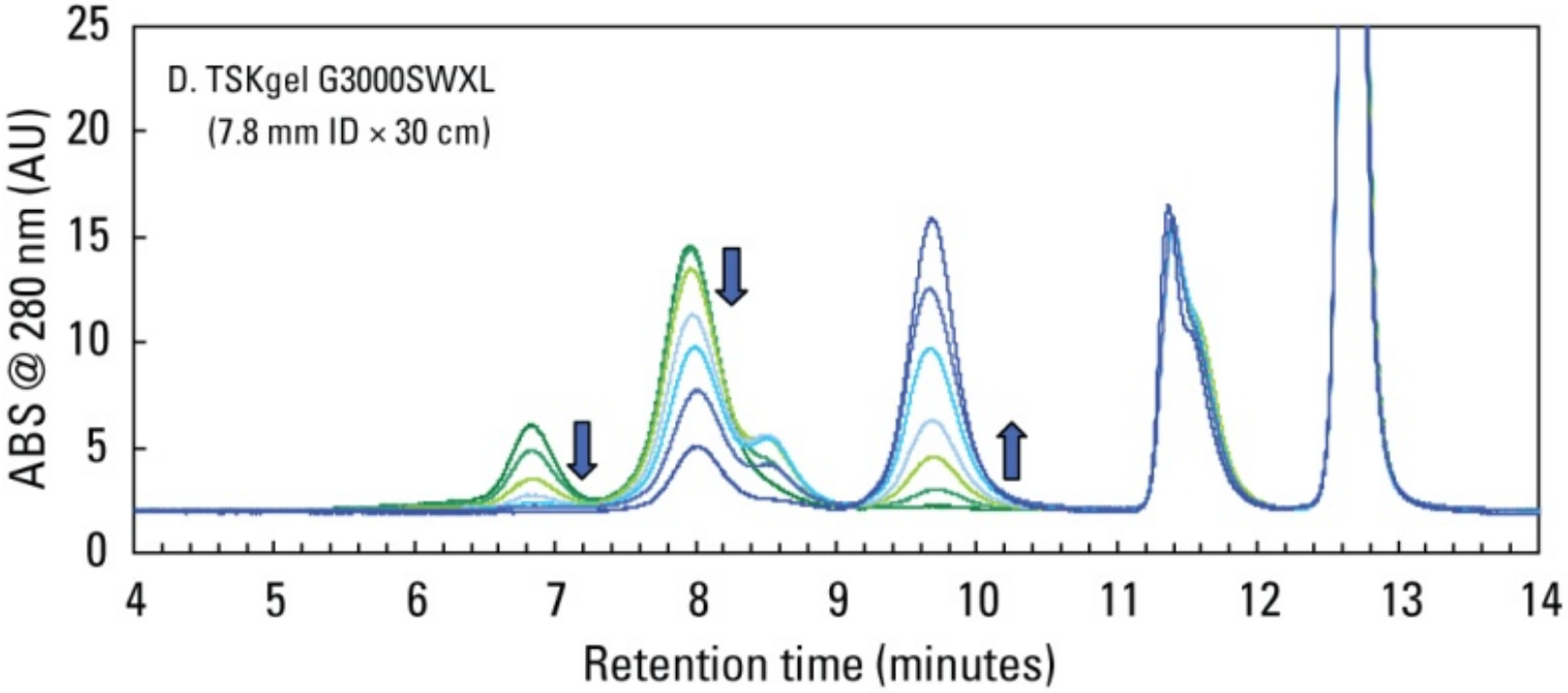
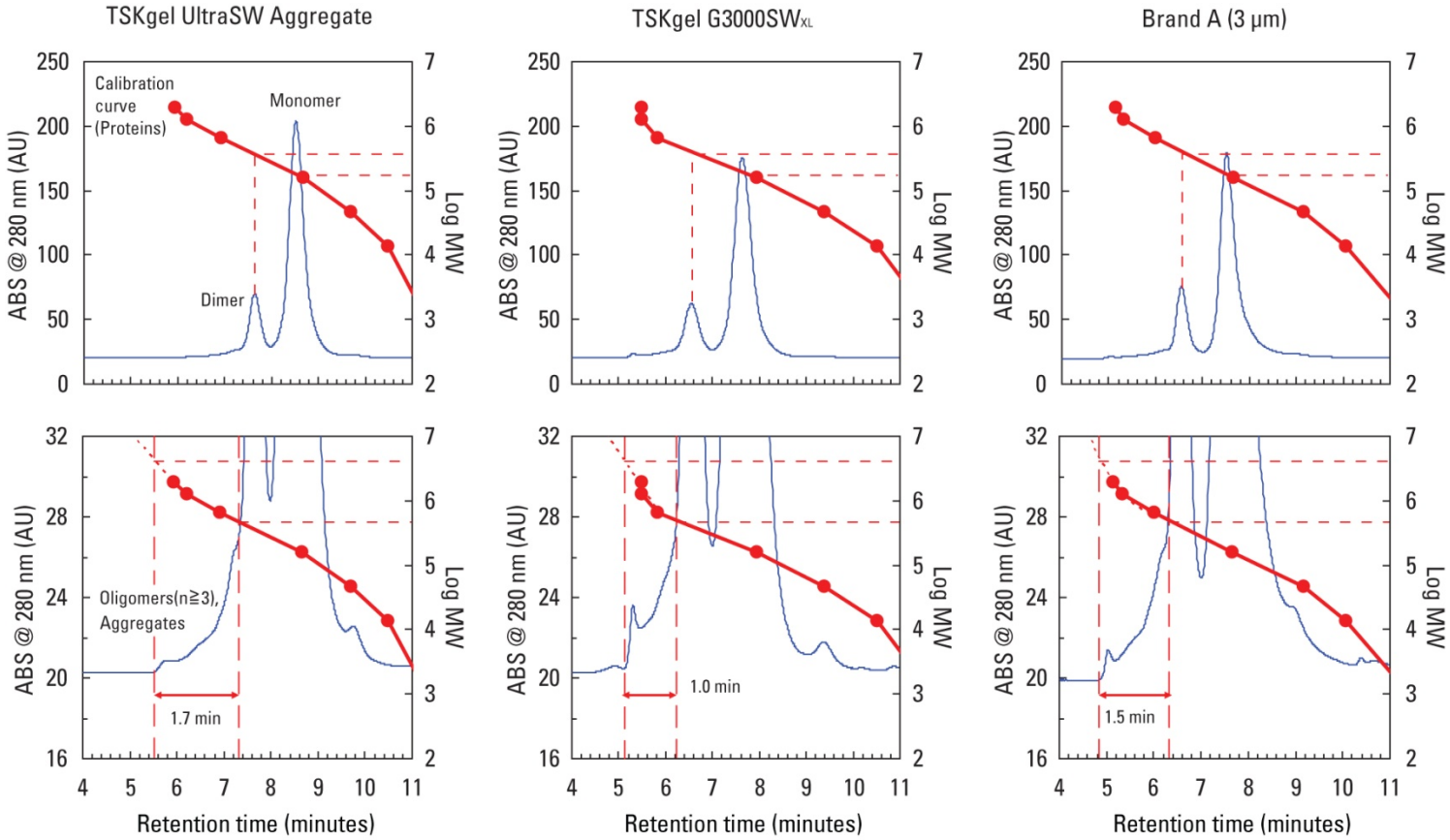


Table 3: Summary of separation of papain-digested IgG

Column	Undigested IgG				IgG digested with papain for 1,440 min	
	ET (min) (monomer)	TP (dimer)	TP (monomer)	Rs (d/m)	TP (fragments)	Rs (m/f)
TSKgel SuperSW mAb HTP, 4.6 mm ID × 15 cm	3.798	2,005	1,909	1.78	2,489	2.46
TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm	7.683	2,895	2,320	2.02	3,826	2.87
TSKgel UltraSW Aggregate, 7.8 mm ID × 30 cm	8.710	5,563	4,279	1.90	7,807	2.49
TSKgel G3000SW$_{XL}$, 7.8 mm ID × 30 cm	7.963	1,912	1,781	1.63	3,883	2.50

- **TSKgel SuperSW mAb HTP prototype column reduced the overall analysis time in half compared to that using a conventional TSKgel G3000SW$_{XL}$ SEC column without any compromise in resolution between monomer/dimer in undigested IgG or monomer/fragments.**
- **TSKgel SuperSW mAb HR prototype column exhibited superior resolving power for monomer/dimer and monomer/fragment separation.**

Figure 9: Separation of IgG aggregates

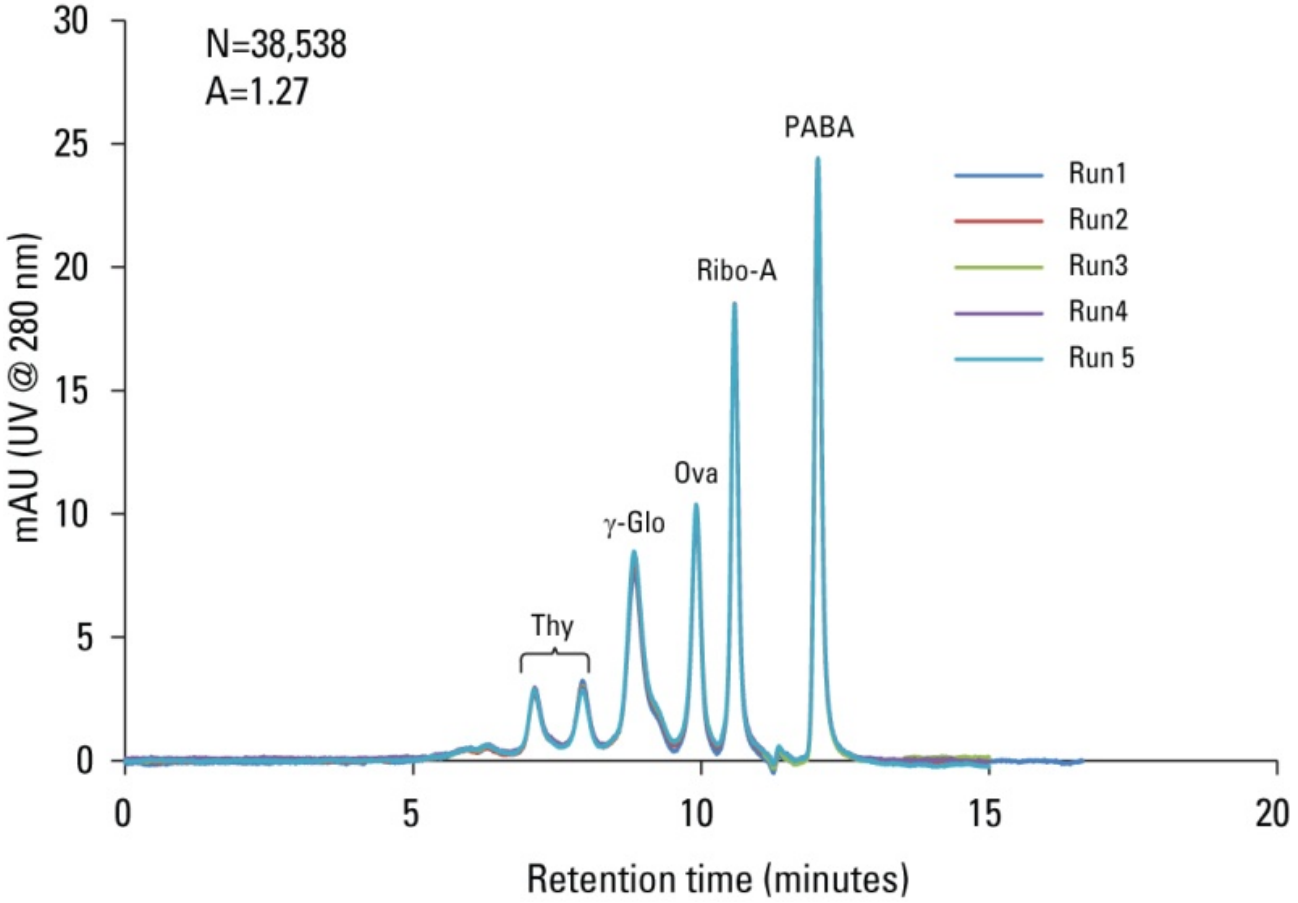


Mobile phase:	200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	50 µL
Sample:	IgG (1.0 g/L)

Figure 9: Results

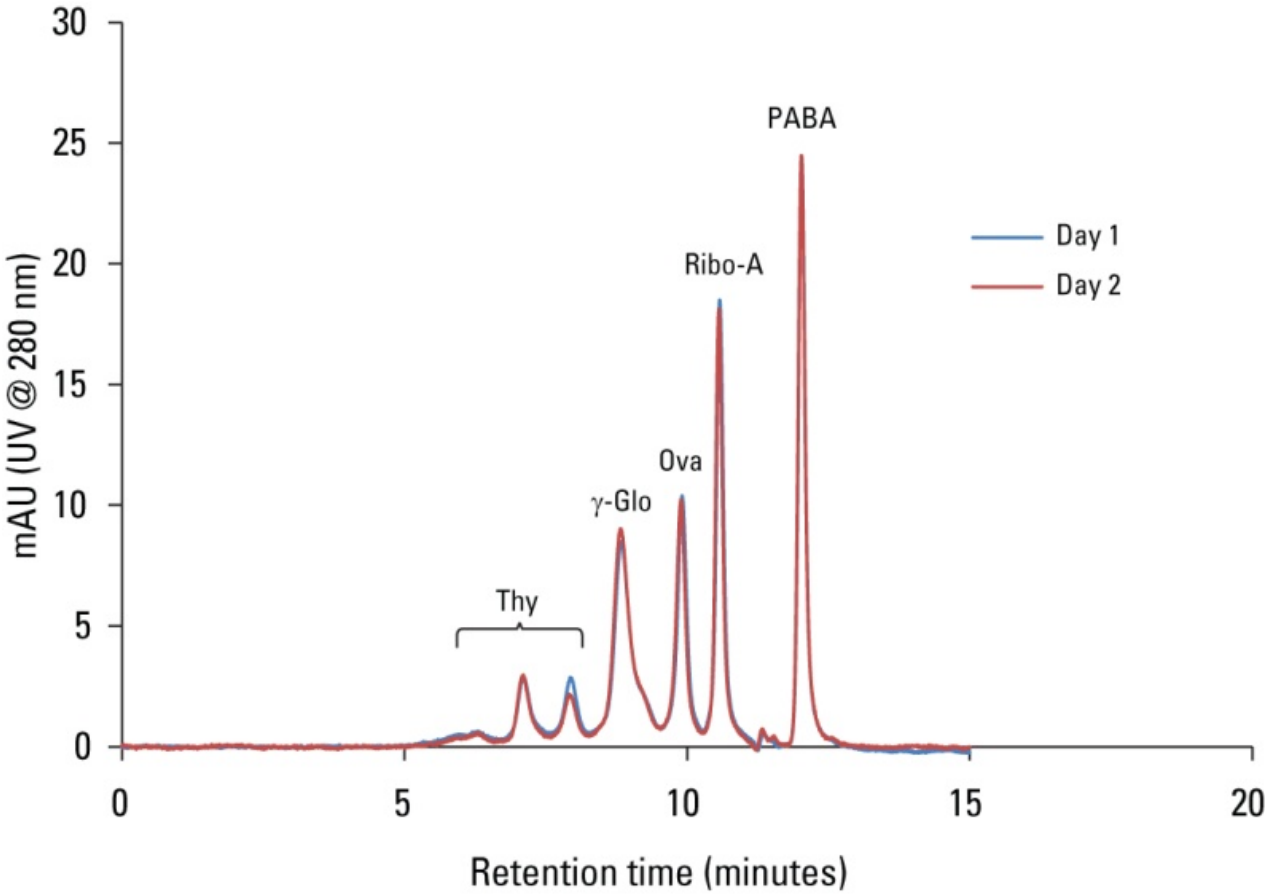
The TSKgel UltraSW Aggregate prototype column possesses a wider separation window for IgG oligomers and aggregates with high MW ($5 \times 10^5 \sim 5 \times 10^6$ Da) than other SEC columns.

Figure 10: Reproducibility (same day): Separation of protein standard mixture using TSKgel UltraSW Aggregate prototype column, 3 µm, 7.8 mm ID × 30 cm



Five consecutive injections yielded excellent reproducibility with low %RSD values in RT, As and N for all peaks.

Figure 11: Reproducibility (day to day): Analysis of protein standard mixture using TSKgel UltraSW Aggregate prototype column, 3 µm, 7.8 mm ID × 30 cm



Excellent day to day reproducibility was observed with low %RSD values in RT, As and N for all peaks.

Figure 12: Acid denaturation study of monoclonal antibody (BI-mAb-02) using a TSKgel UltraSW Aggregate prototype column, 3 µm, 7.8 mm ID × 30 cm

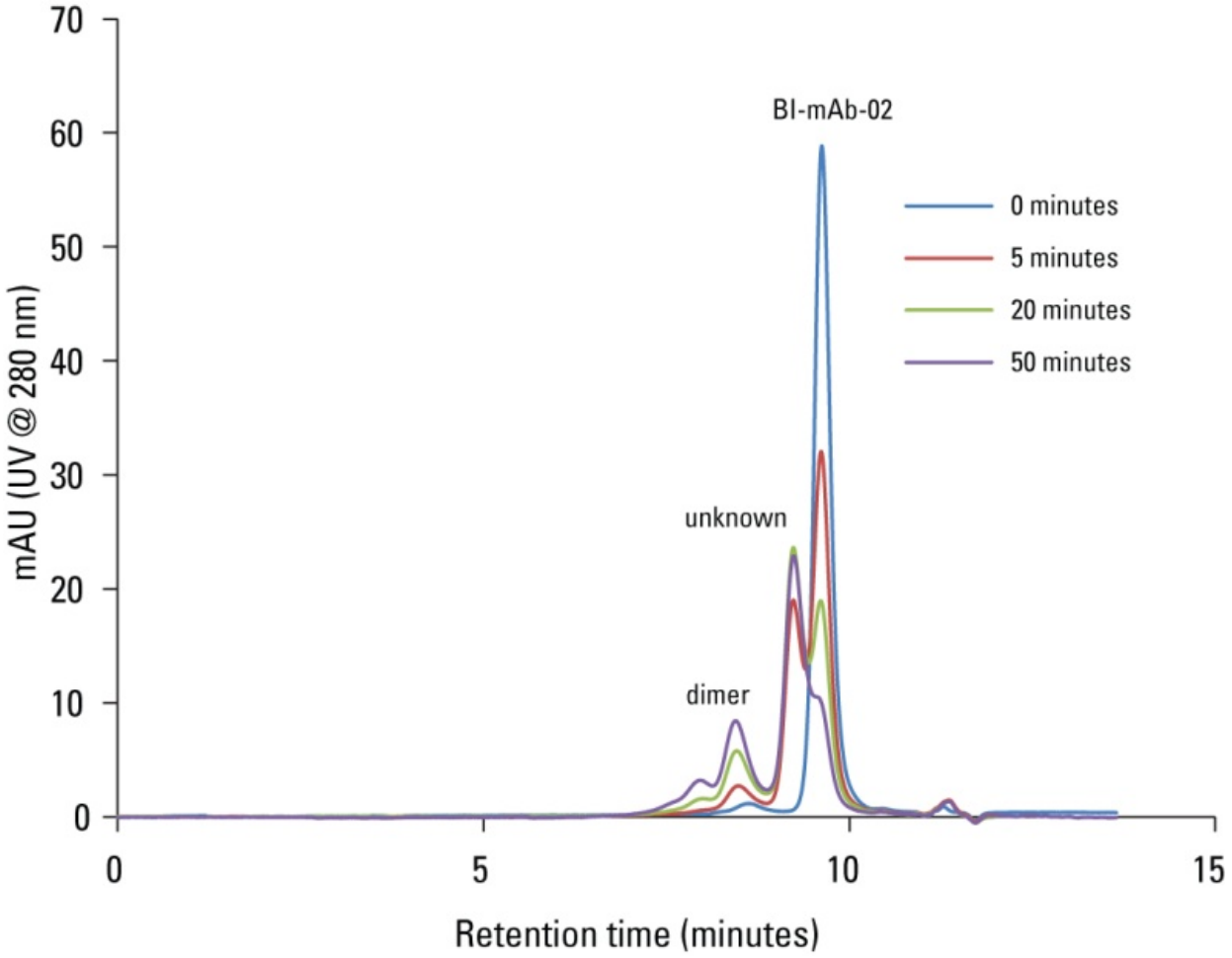


Figure 12: Results

- After reducing the pH of the BI-mAb-02 sample solution down to 4.7 by dilute phosphoric acid, aliquots were analyzed at 5, 20 and 50 minutes and the response was compared to that of the original sample solution. The blue trace shows the intact mAb and what is (presumably), its dimer eluting at 8.65 min.
- The degradation of the monoclonal antibody creates a larger MW entity (unknown) that elutes directly after the monomer and before the dimer. Continued decay increases both peaks, but more so for the dimer.
- Clearly the dimer increases in size while the peak height of the monomer decreases. Hints of higher order ‘multimers’ show between 7 and 8 minutes.

Figure 13: Heat denaturation study of monoclonal antibody (BI-mAb-02) using a TSKgel UltraSW Aggregate prototype column, 3 µm, 7.8 mm ID × 30 cm

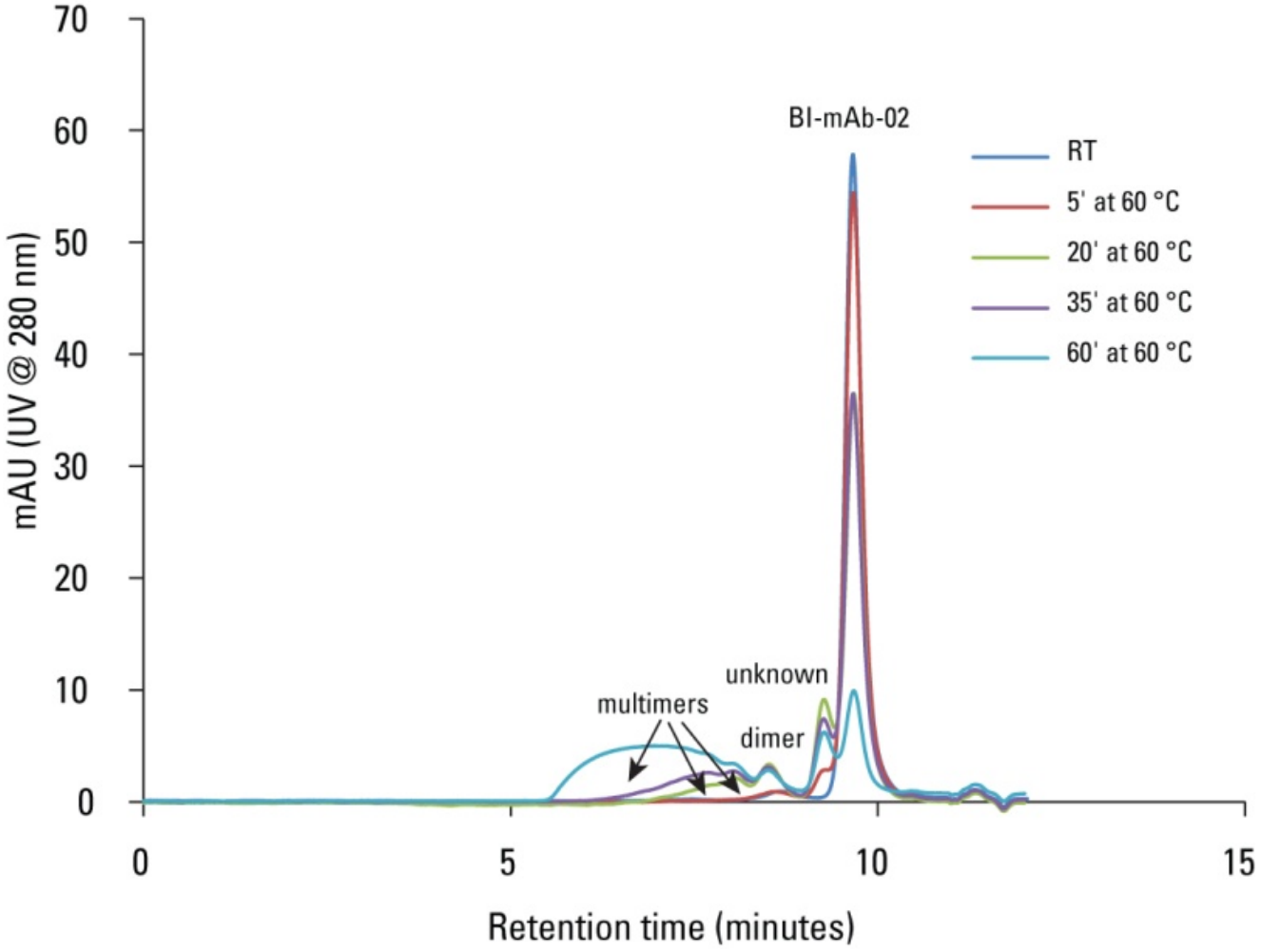
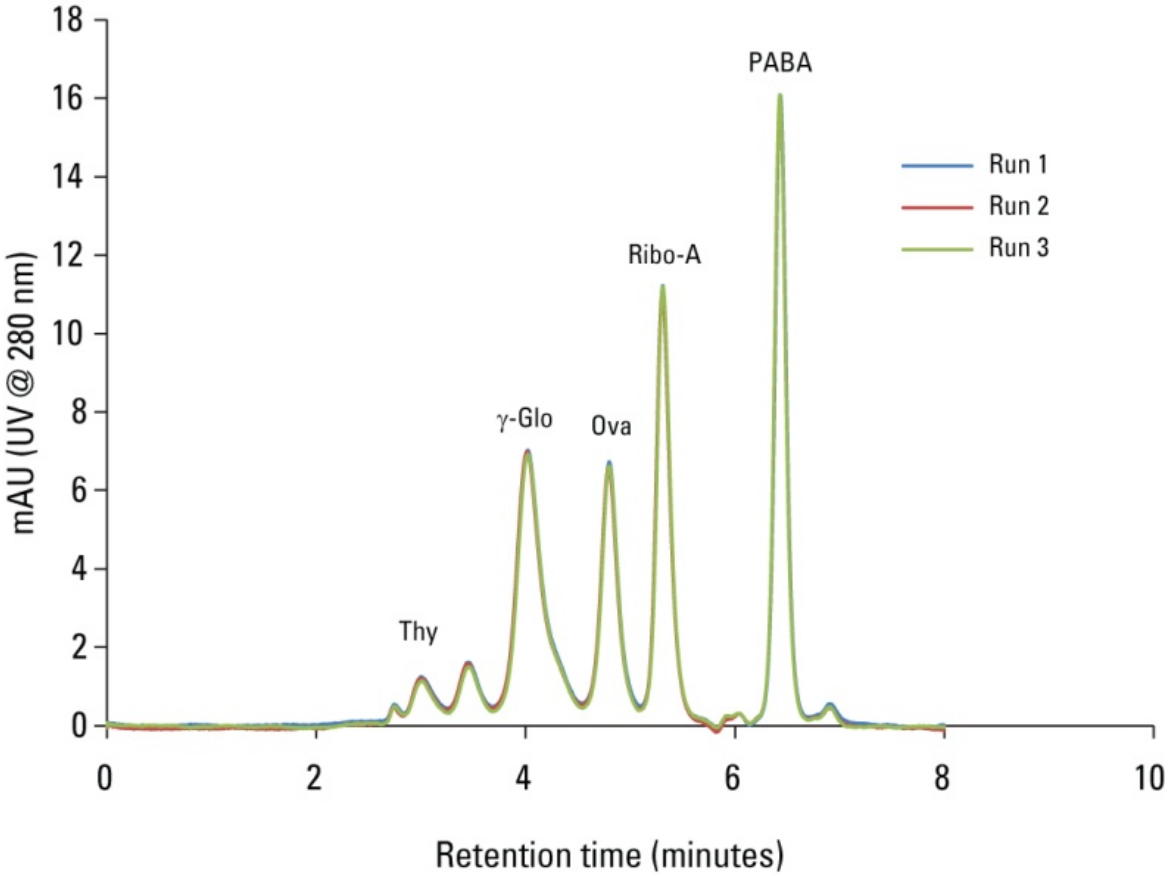


Figure 13: Results

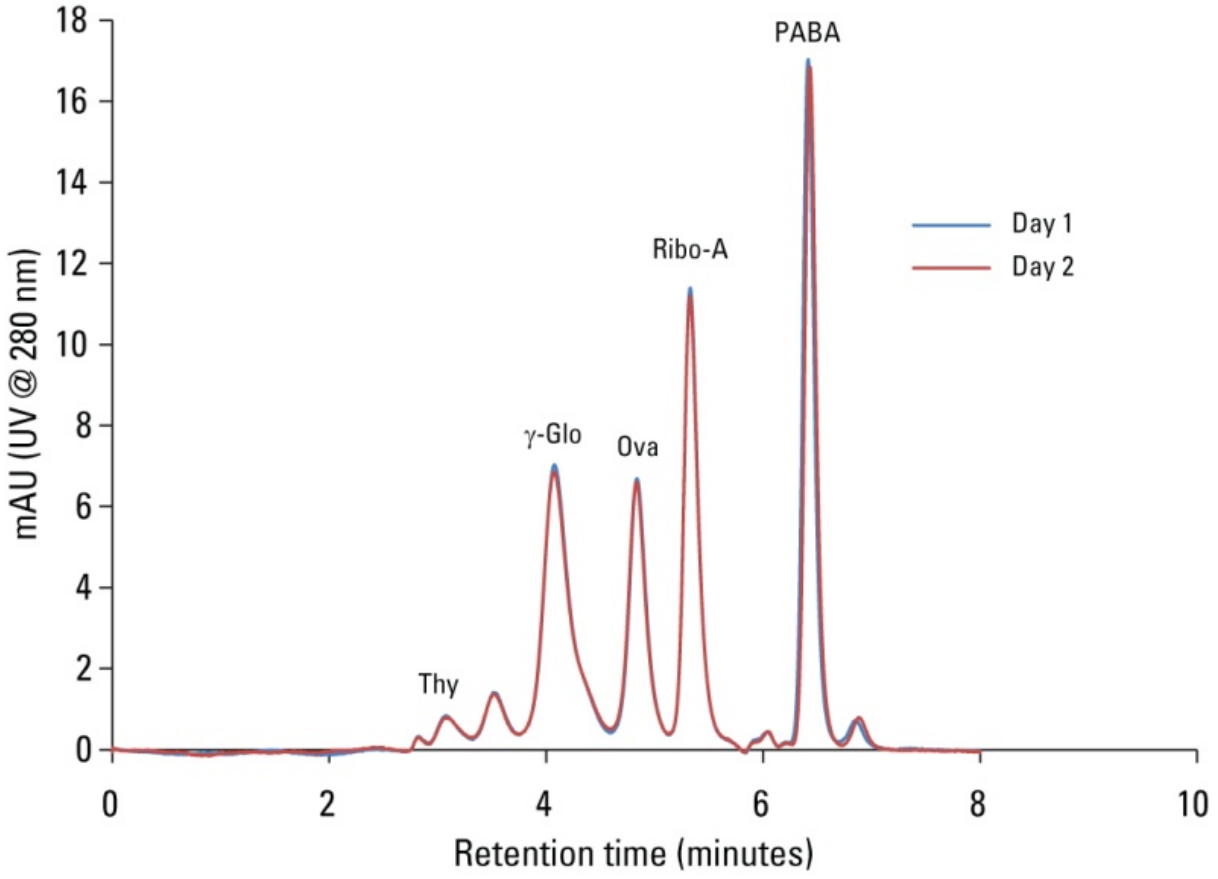
- Since the mAb degradation occurred very fast at pH 4.7, degradation at pH 5.5 and a temperature of 60 °C was also monitored.
- 50 µL of antibody (pH 6.0) was mixed with 50 µL of 0.1 mol/L phosphate buffer, pH 4.65; final pH was 5.5; 20 µL was injected.
- In addition to the ‘unknown’ aggregate, what is presumably the dimer peak at 8.5 minutes and several higher order aggregate peaks is now seen.
- Heating for one hour at 60 °C results in almost complete breakdown of the monoclonal antibody and the formation of very large aggregates that extend to the exclusion volume of the column.

Figure 14: Analysis of protein standard mixture using a TSKgel SuperSW mAb HTP prototype column, 4 µm, 4.6 mm ID × 15cm



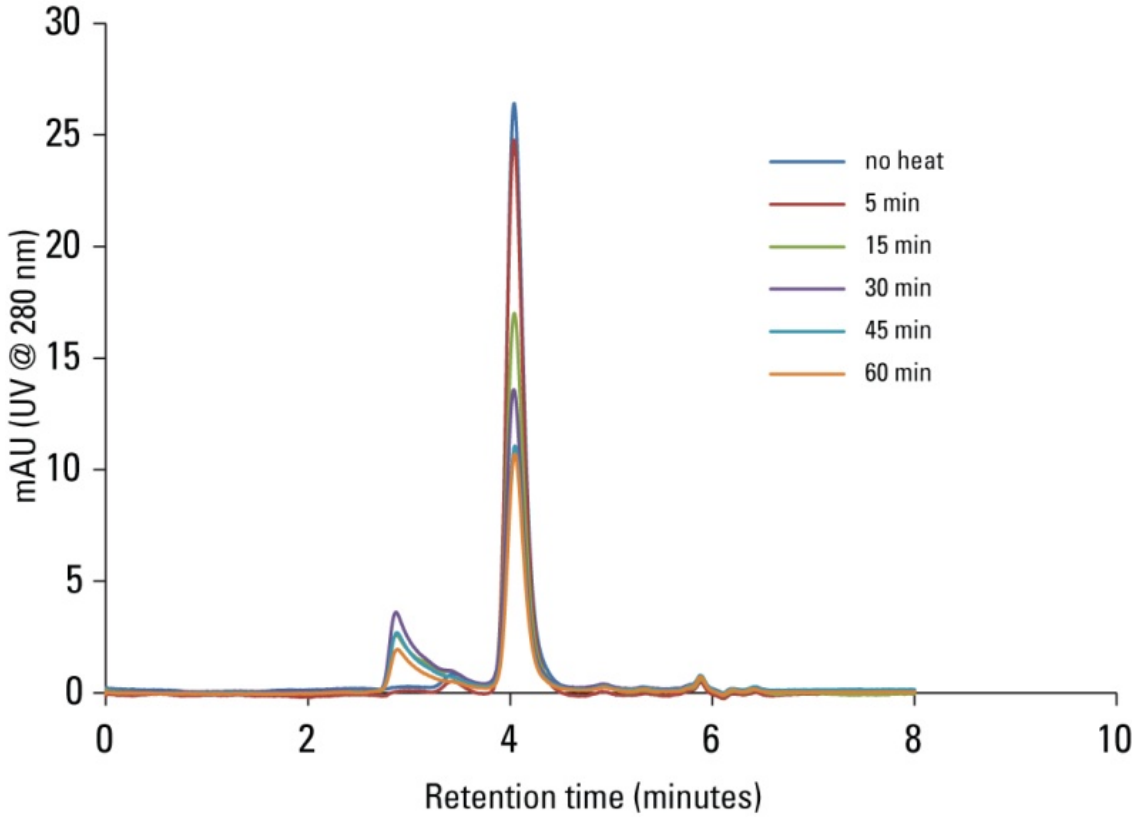
Three consecutive runs yielded excellent reproducibility with low %RSD values in RT, As and N for all peaks.

Figure 15: Reproducibility (day to day): Analysis of protein standard mixture using a TSKgel SuperSW mAb HTP prototype column, 4 µm, 4.6 mm ID × 15 cm



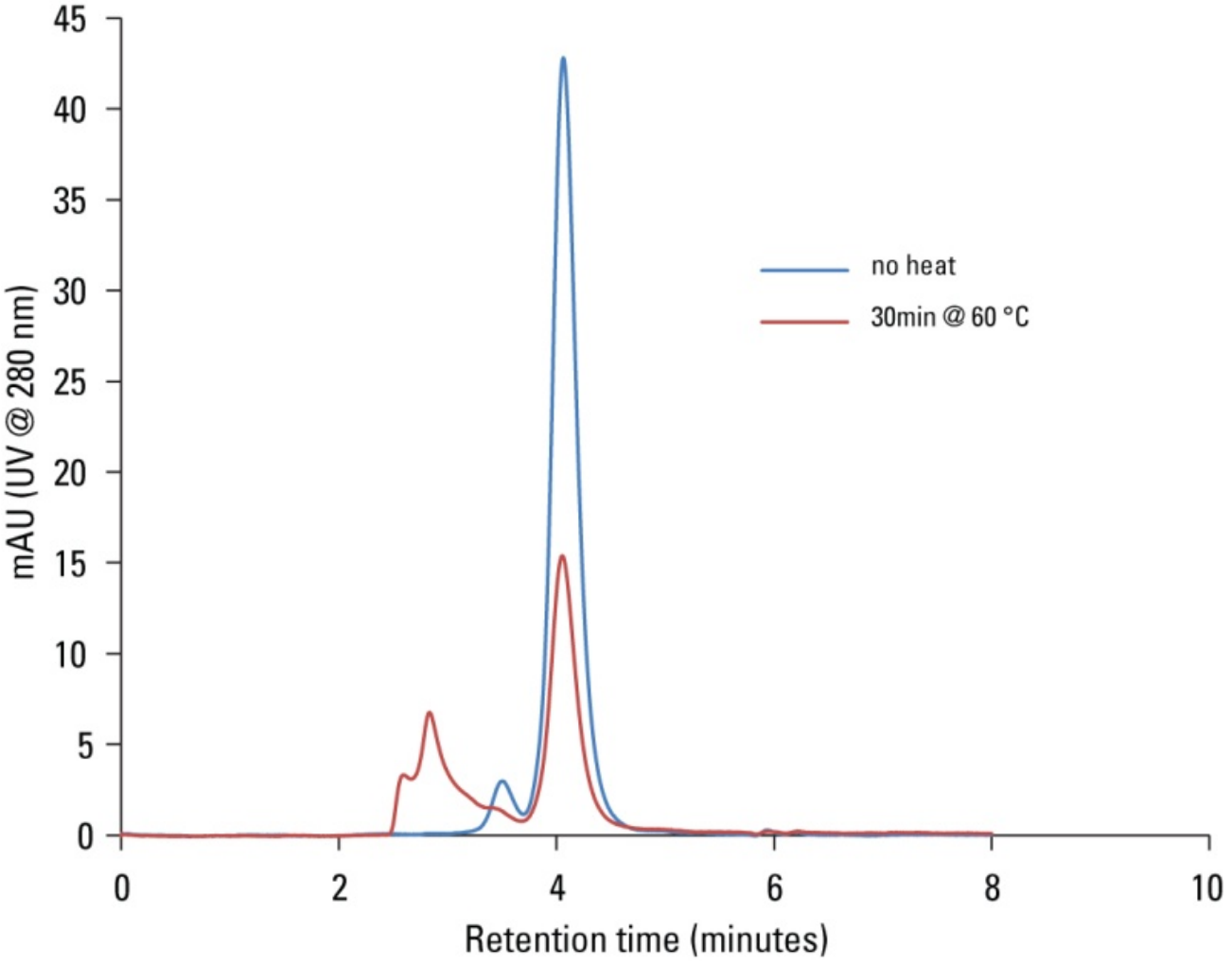
Day to day reproducibility yielded low %RSD values in RT, As and N for all peaks.

Figure 16: Heat denaturation study of monoclonal antibody (BI-mAb-02) using a TSKgel SuperSW mAb HTP prototype column, 4 µm, 4.6 mm ID × 15 cm



- **The column could be used to monitor the denaturation of the antibody as a function of time.**
- **Fragments and aggregates could be separated from the monomer peak to the baseline.**

Figure 17: Analysis of heat denatured monoclonal antibody (human IgG) using a TSKgel SuperSW mAb HTP prototype column, 4 µm, 4.6 mm ID × 15 cm



Conclusions

- The following three novel prototype SEC columns have been developed:
  - TSKgel SuperSW mAb HTP exhibited equal separation between IgG monomer and dimer in half the analysis time compared to the conventional SEC column, TSKgel G3000SWXL, 5 µm particle, 7.8 mm ID × 30 cm
  - TSKgel SuperSW mAb HR exhibited superior resolving power for IgG monomer and dimer compared to other SEC columns.
  - TSKgel UltraSW Aggregate, which possesses a larger MW exclusion limit, exhibited superior resolving power for oligomers and aggregates of large proteins, including thyroglobulin and IgG.
- The performance of these columns was demonstrated by the separation of IgG fragments generated by papain digestion and separation of IgG aggregates.