Three novel prototype silica-based SEC columns designed 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 3 prototype SEC columns designed for the analysis of monoclonal antibodies

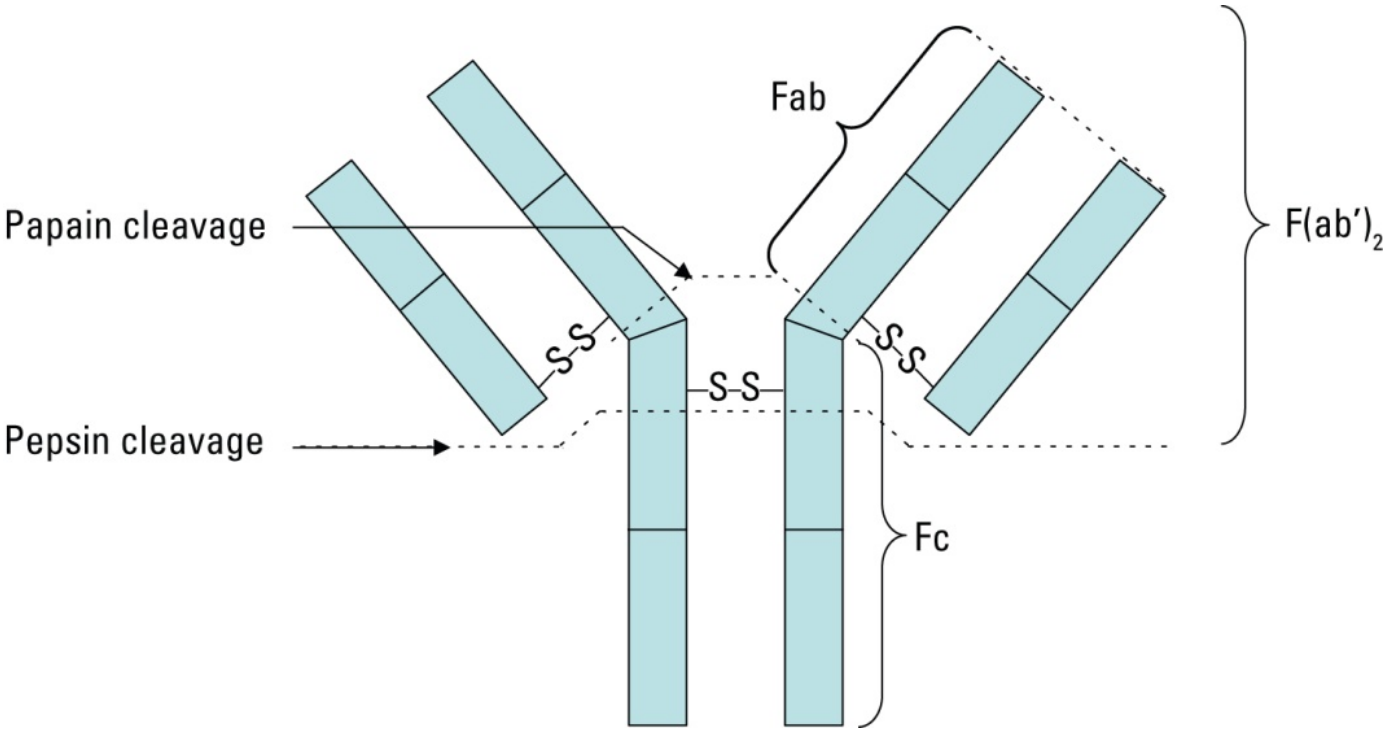
Introduction

- Monoclonal antibodies (mAbs) are widely used as biopharmaceuticals. 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 ~$4x10^6$ 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.
- We also report data which highlights the columns' reproducibility and lot-to-lot consistency while being used for mAb analysis.

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

Instrumentation

- 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 (0.5 g/L), γ-globulin (1 g/L), ovalbumin (1 g/L), ribonuclease A (1.5 g/L), PABA (0.01 g/L)
- Pullulan standards were obtained from Showa Denko (Tokyo, Japan).
- Monoclonal antibodies:
  - Kaketsuken (Kumamoto, Japan) *(figures 1-2)*
  - 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: protocol for 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

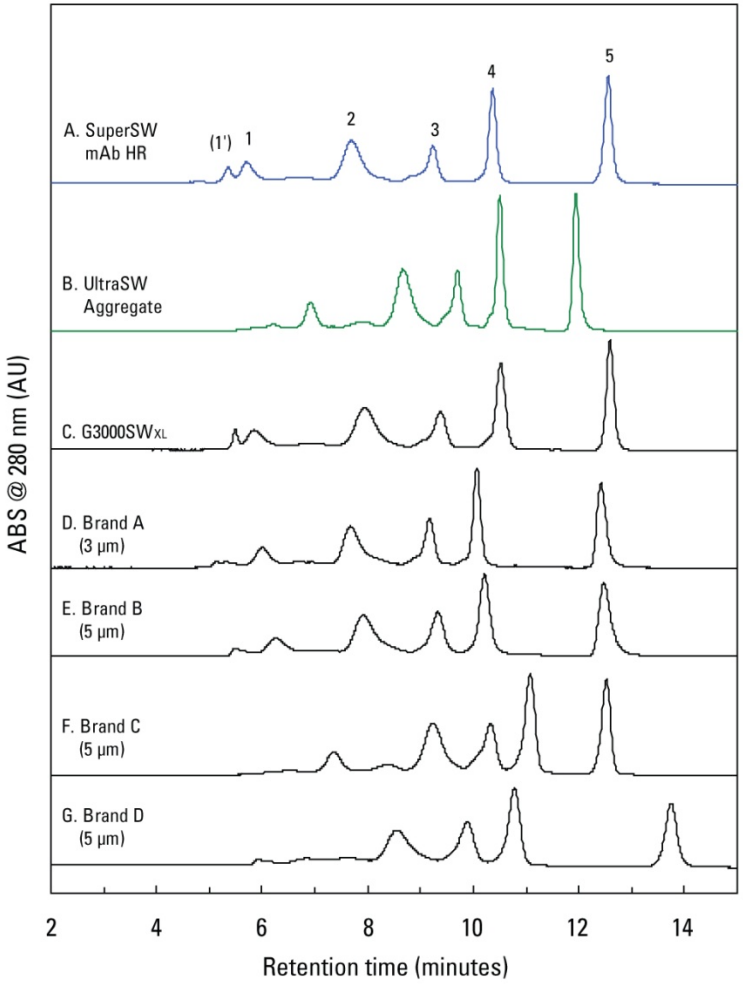
- **Mobile Phase:** 100 mmol/L potassium phosphate buffer, 100 mmol/L sodium sulfate, pH 6.7 + 0.05% $NaN_{3;}$ unless mentioned otherwise
- **Flow rate:** 1.0 mL/min (0.35 mL/min for 15 cm column)
- **Detection:** UV @ 280 nm
- **Temperature:** ambient/25 °C except during heat denaturation study
- **Injection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

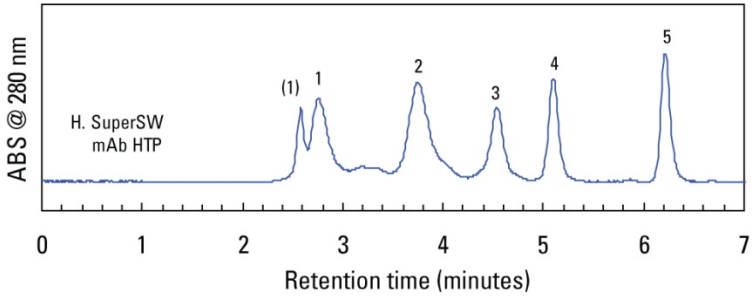
- High purity HPLC grade Sigma Aldrich chemicals were used in this study.
- High purity 18.2 m.Ohm-cm quality water was used to make buffer and samples.

Table 1: Specification 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 - 500,000 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





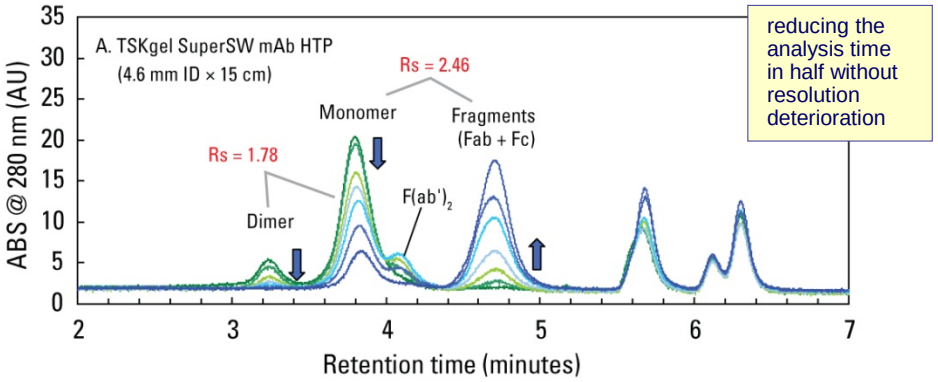
Column dimension: A-E: 7.8 mm ID × 30 cm
F&G: 8.0 mm ID × 30 cm
H: 4.6 mm ID × 15 cm
Mobile phase: 200 mmol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate: A-G: 1.0 mL/min H: 0.35 mL/min
Detection: UV @ 280 nm
Temperature: 25 °C
Injection vol.: A-G: 10 µL H: 3.5 µL
Samples: 1 thyroglobulin (MW 640,000) (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)

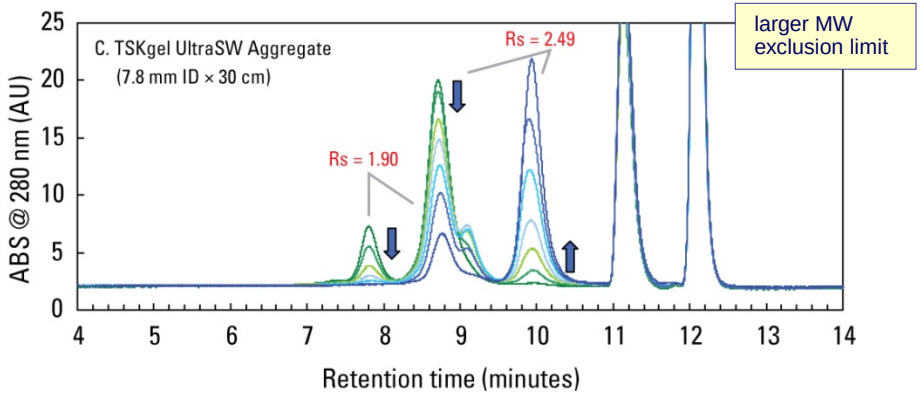
Prototype TSKgel SEC columns show their superior performance over other columns.

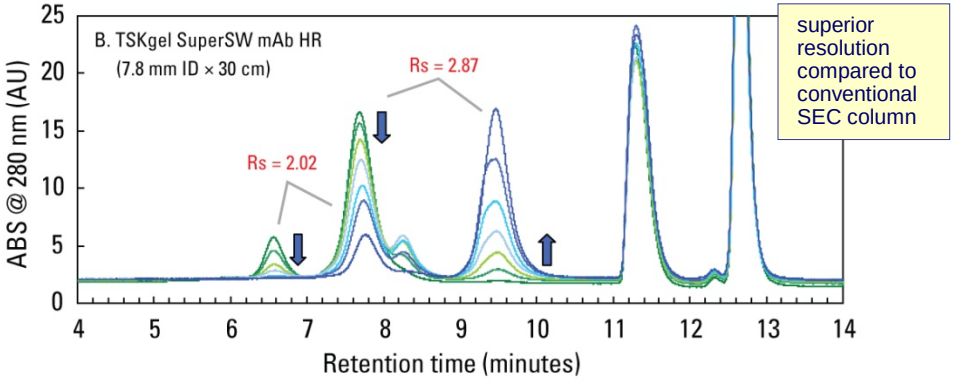
Summary of column performance

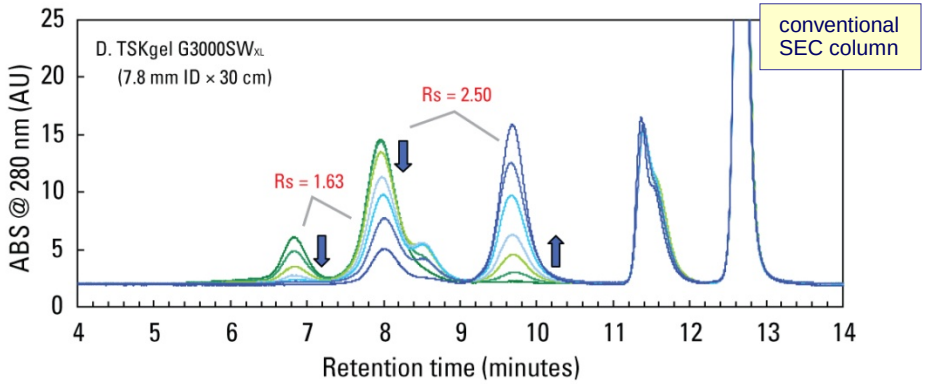
- The TSKgel SuperSW mAb HTP prototype column exhibited equal separation between IgG monomer and dimer in half the analysis time compared to the conventional SEC column, TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm.
- The TSKgel SuperSW mAb HR prototype column exhibited superior resolving power for IgG 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 2: Separation of IgG monomer, dimer, and fragments by novel SEC columns – TSKgel SuperSW mAb HTP, SuperSW mAb HR, UltraSW Aggregate









Mobile phase:	200 mmol/L phosphate buffer + 0.05% NaN_3, pH 6.7
Flow rate:	A 0.35 mL/min; B-D 1.0 mL/min
Injection volume:	A 5 µL; B-D 10 µL
Temperature:	25°C,
Detection:	UV @ 280 nm
Samples:	10 g/L IgG digested with papain for 0-24 hr

Columns:
A. TSKgel SuperSW mAb HTP, 4.6 mm ID × 15 cm*
B. TSKgel SuperSW mAb HR, 7.8 mm ID × 30 cm*
C. TSKgel UltraSW Aggregate, 7.8 mm ID × 30 cm*
D. TSKgel G3000SWXL, 7.8 mm ID × 30 cm

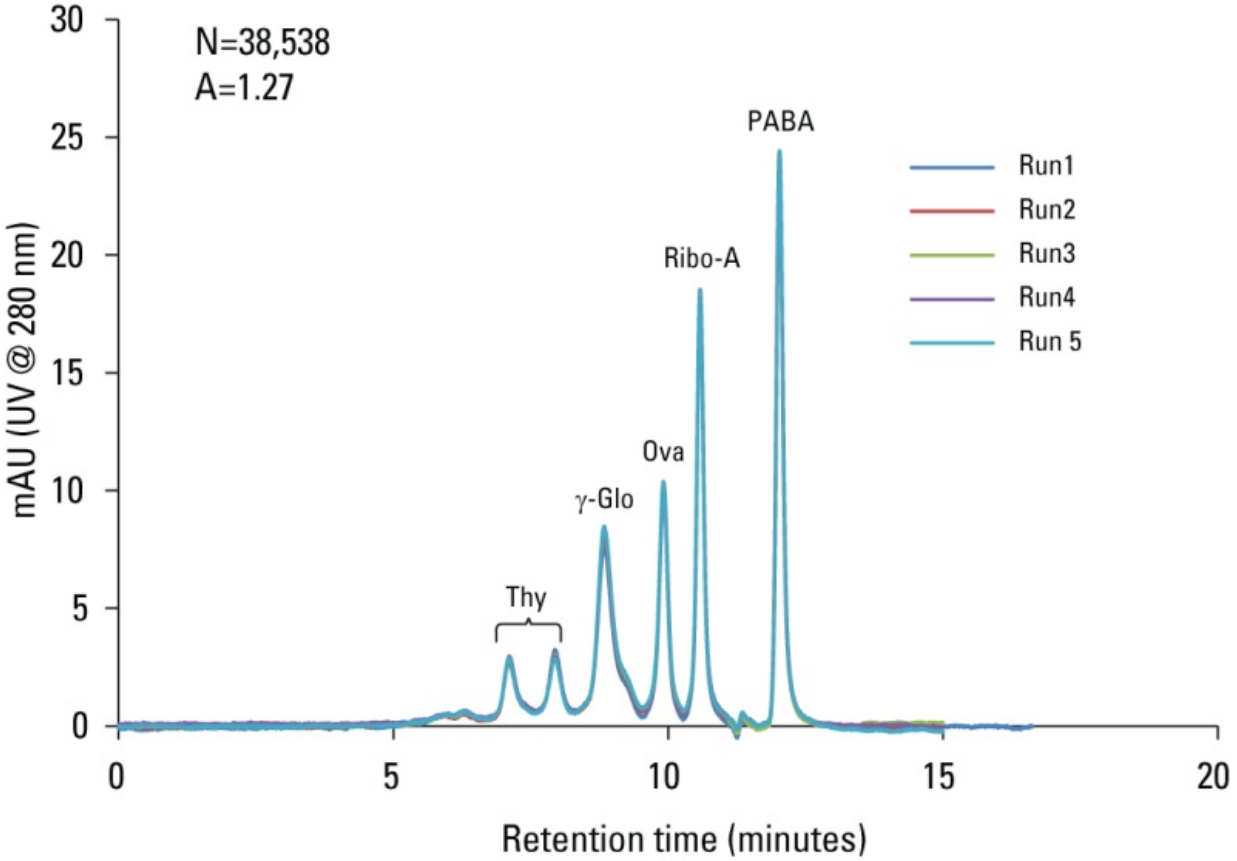
*prototype columns

Table 2: Summary of the 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

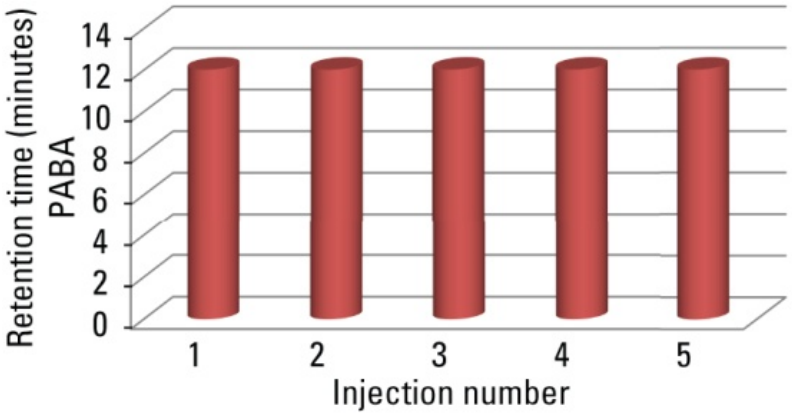
- **The TSKgel SuperSW mAb HTP prototype column reduced the overall analysis time in half compared to a conventional TSKgel G3000SW$_{XL}$ SEC column without any compromise in resolution between monomer/dimer in undigested IgG or monomer/fragments.**
- **The TSKgel SuperSW mAb HR prototype column exhibited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

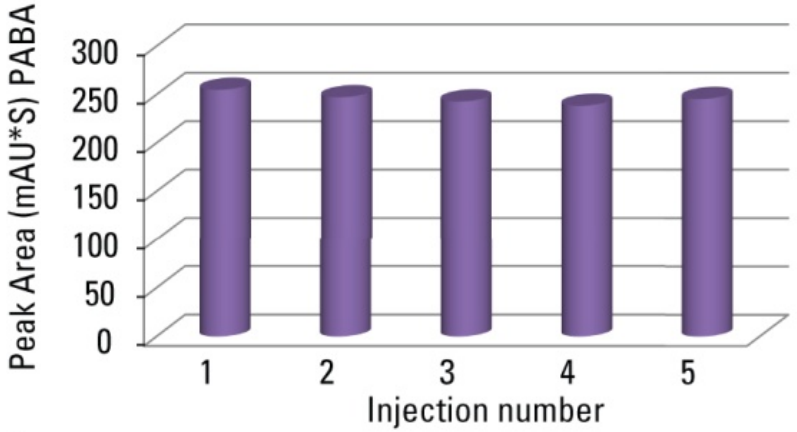
Figure 3: Intra-day repeatability - separation of protein standard mixture using a prototype TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column (Y1002S)

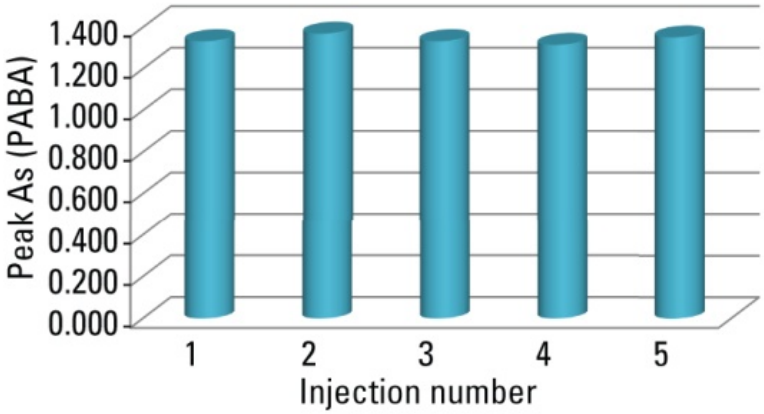


Excellent intra-day reproducibility was obtained using a TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column (Y0002T)

Figure 4: Intra-day repeatability - 5 consecutive injections - separation of protein standard mixture using a prototype TSKgel UltraSW Aggregate, 3 μm, 7.8 mm ID × 30 cm column (Y1002S)







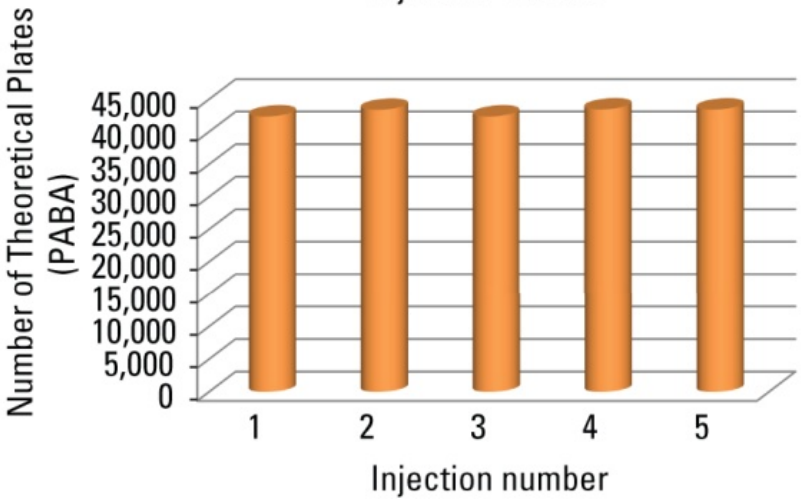
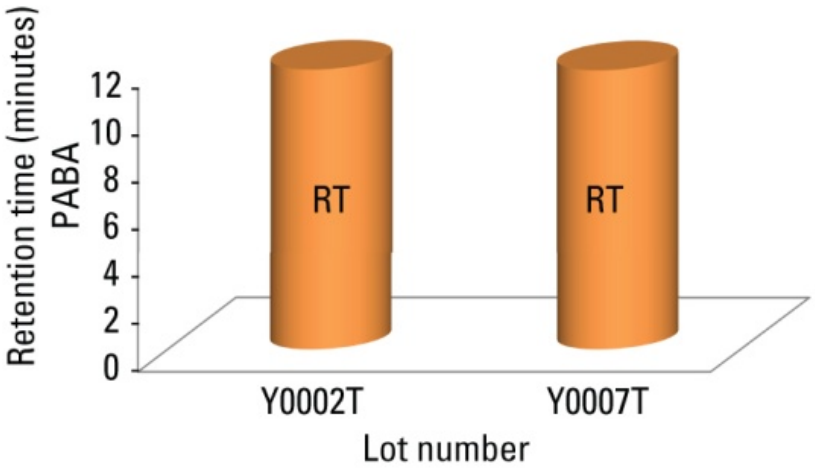
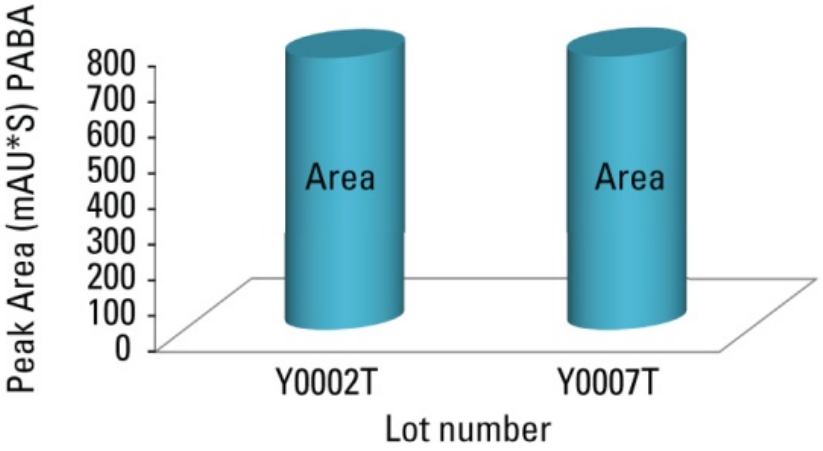
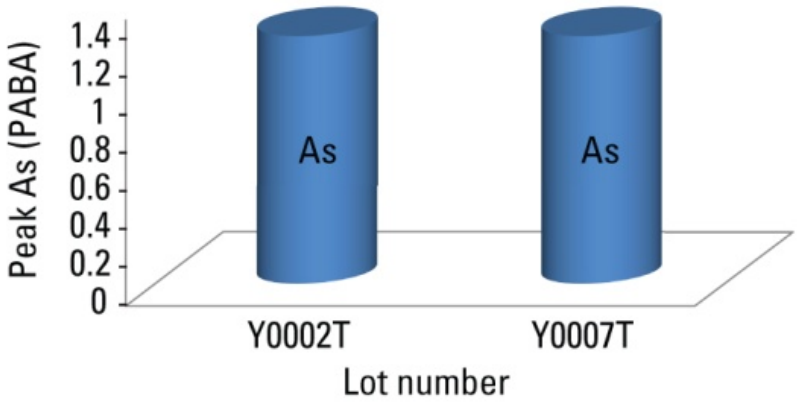


Figure 5: Lot-to-lot variation - separation of protein standard mixture using a prototype TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column







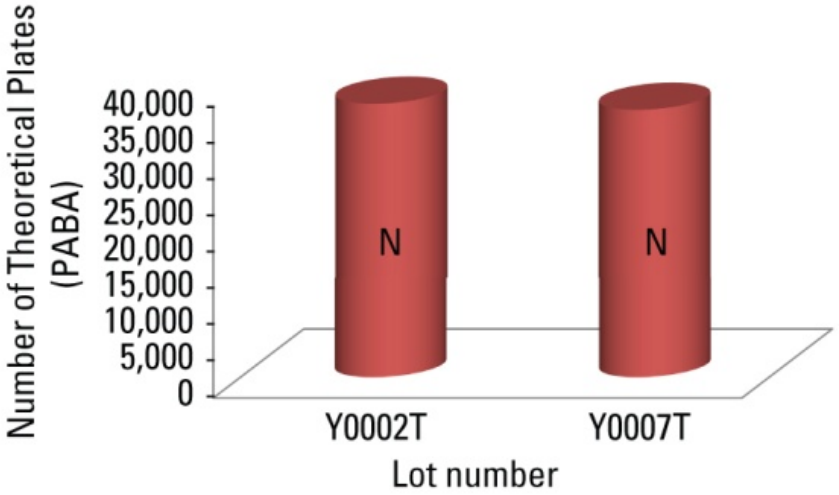
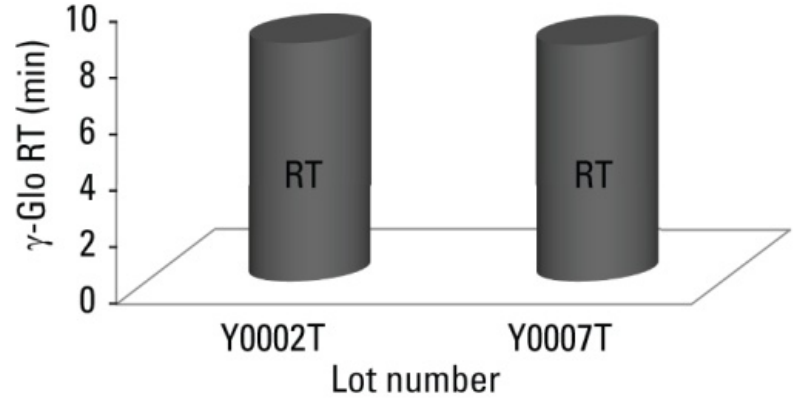
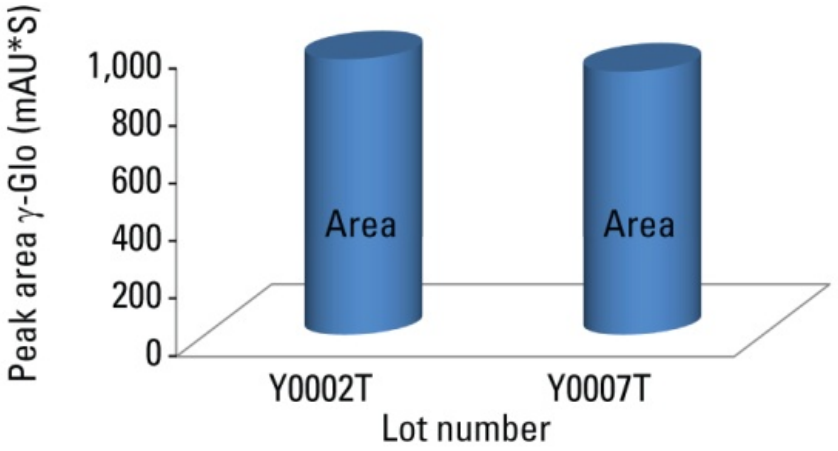
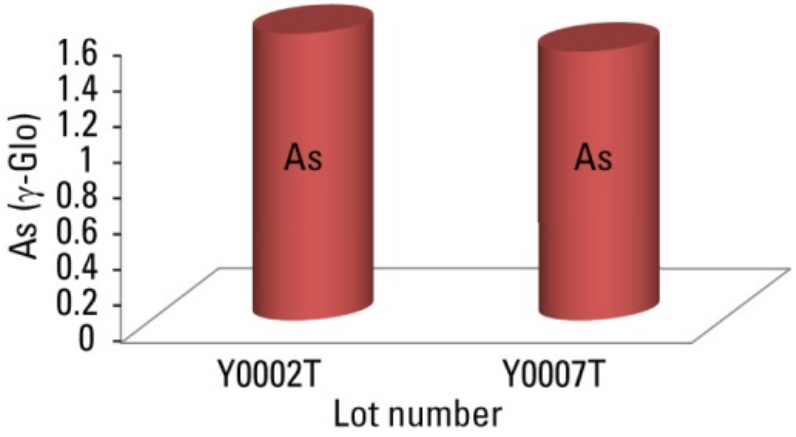


Figure 6: Lot-to-lot variation - separation of protein standard mixture using a prototype TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column







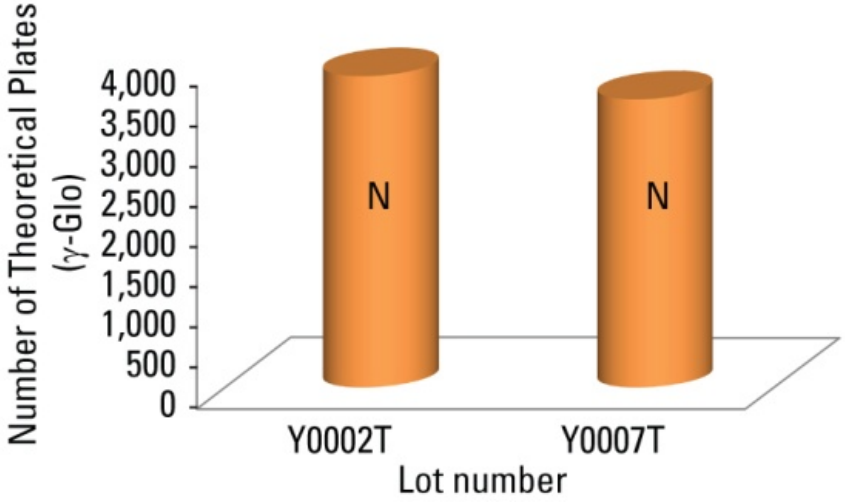


Figure 7: Intra-day repeatability - 3 consecutive injections - separation of monoclonal antibody (BI-mAb-01) using a prototype TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column (Y0007T)

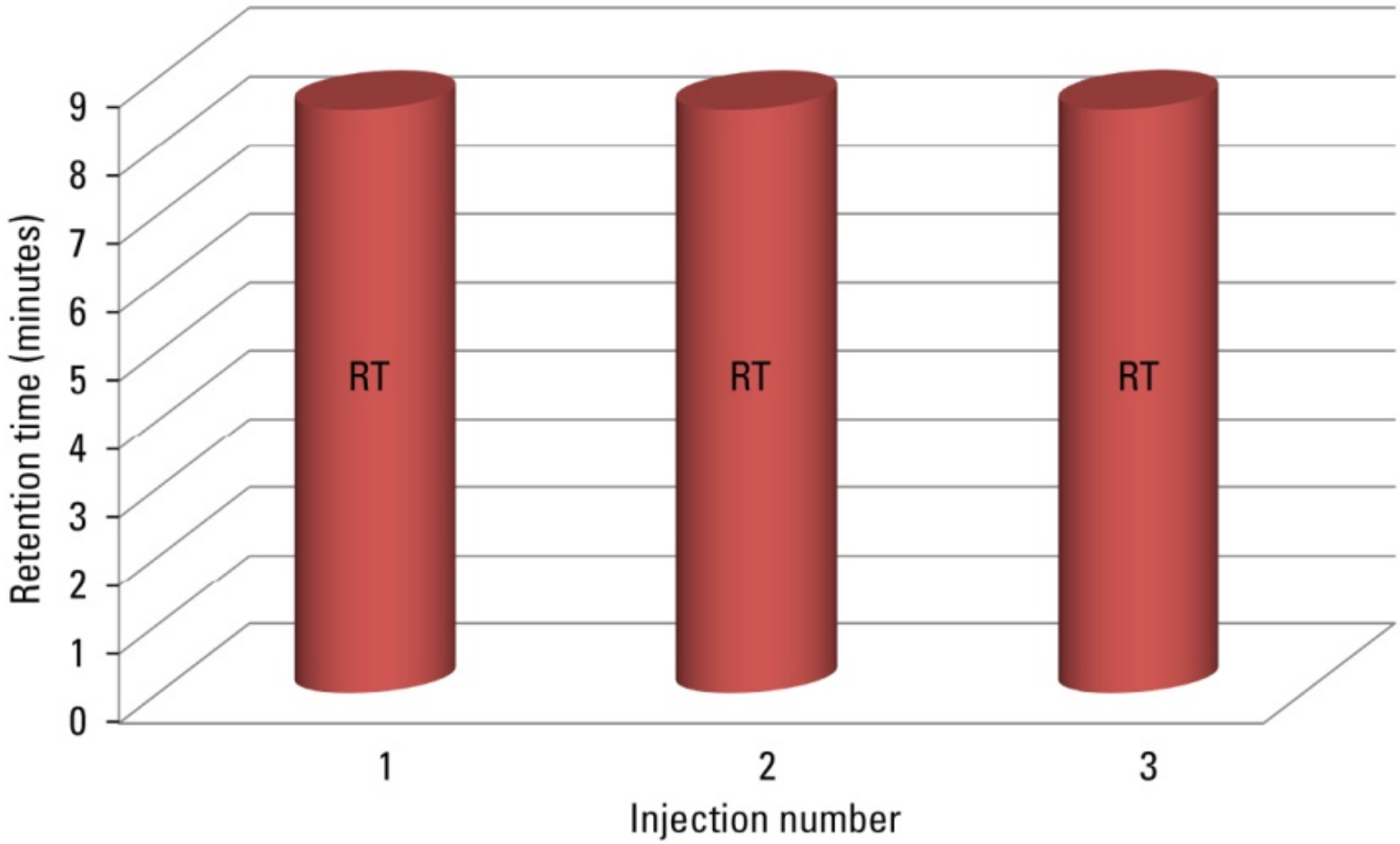


Figure 8: Intra-day repeatability - 3 consecutive injections - separation of monoclonal antibody (BI-mAb-01) using a prototype TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column (Y0007T)

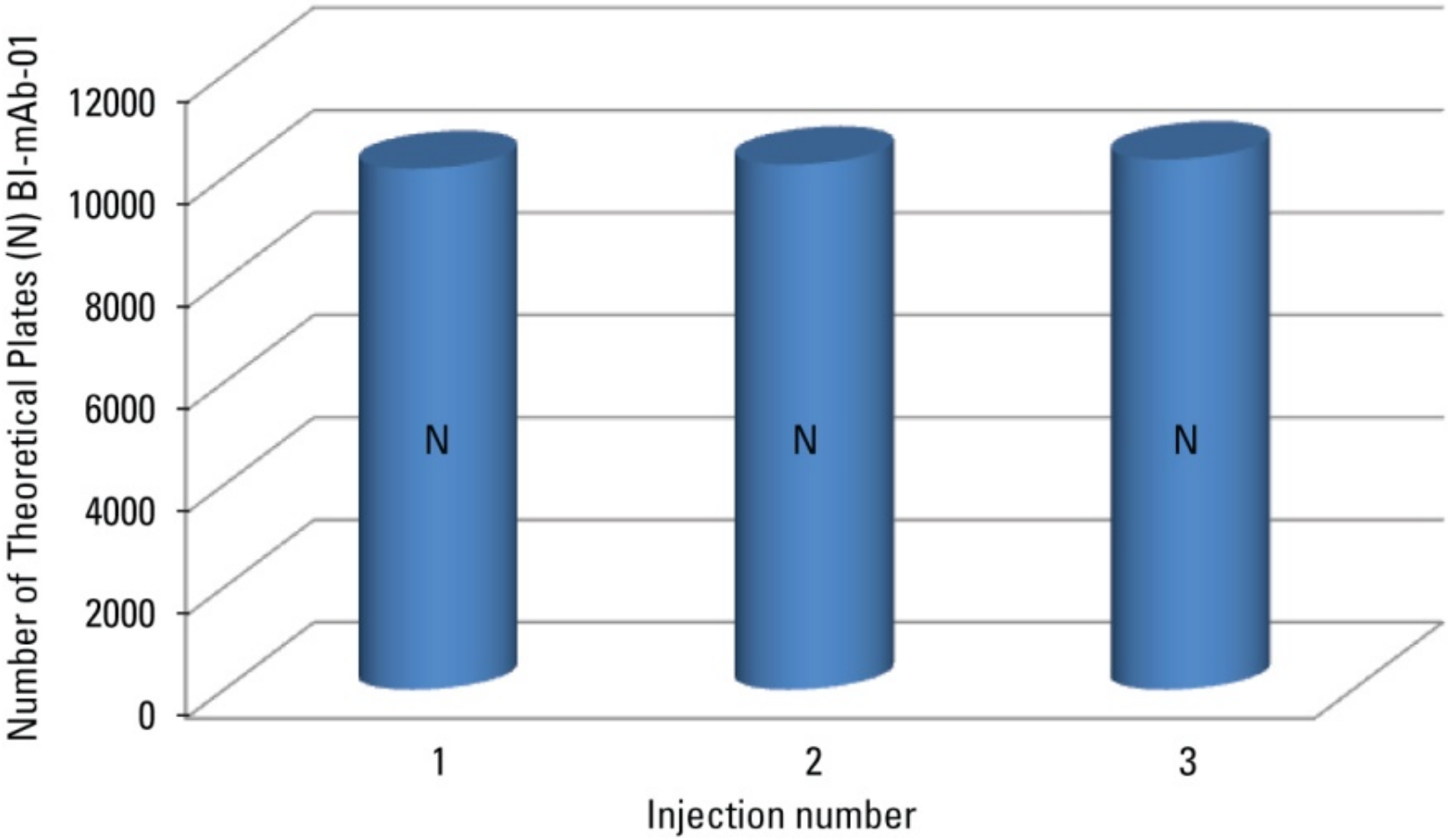


Figure 9: Inter-day repeatability - separation of monoclonal antibody (BI-mAb-01) using a prototype TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column (Y0007T) – retention time

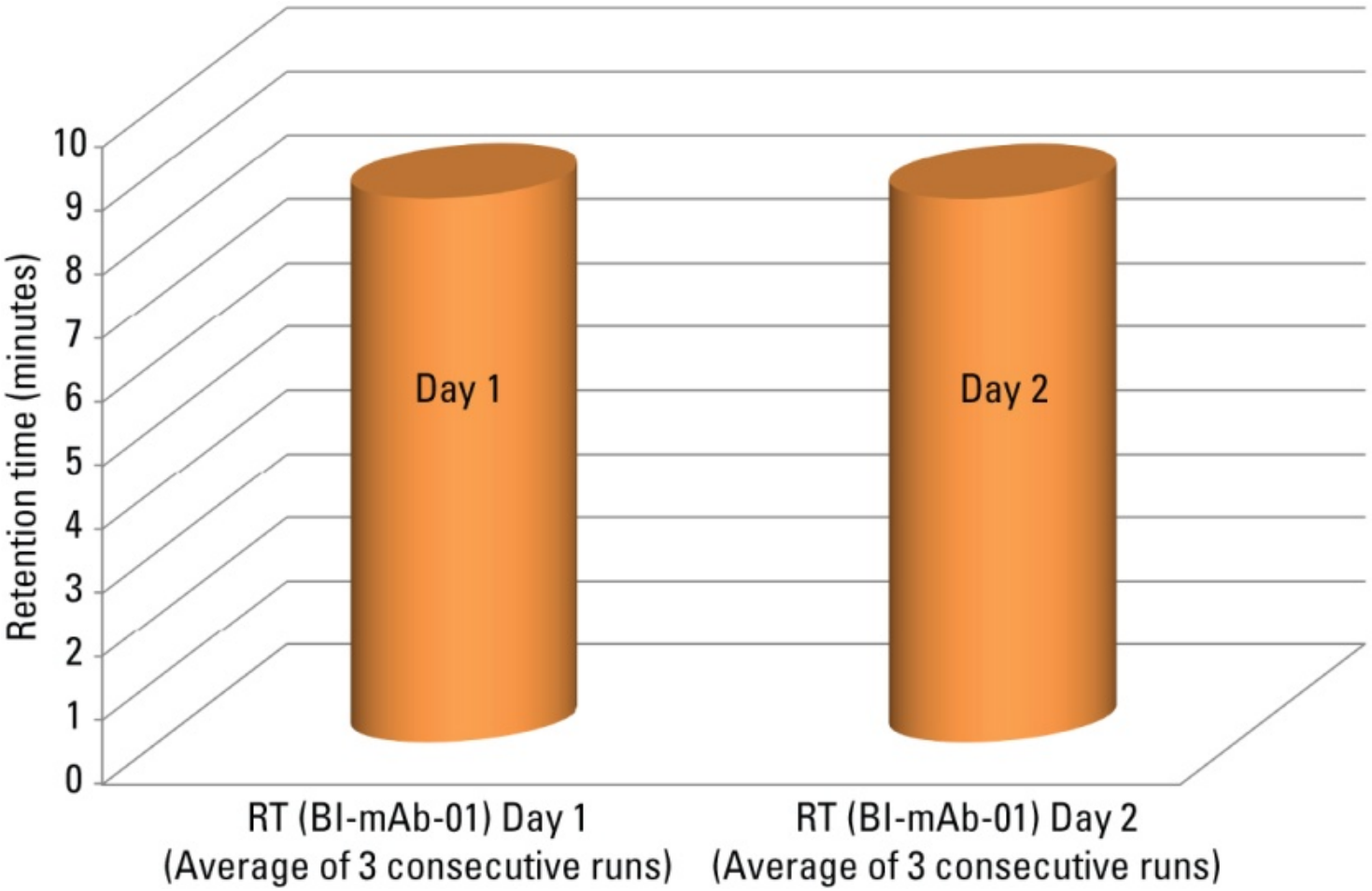
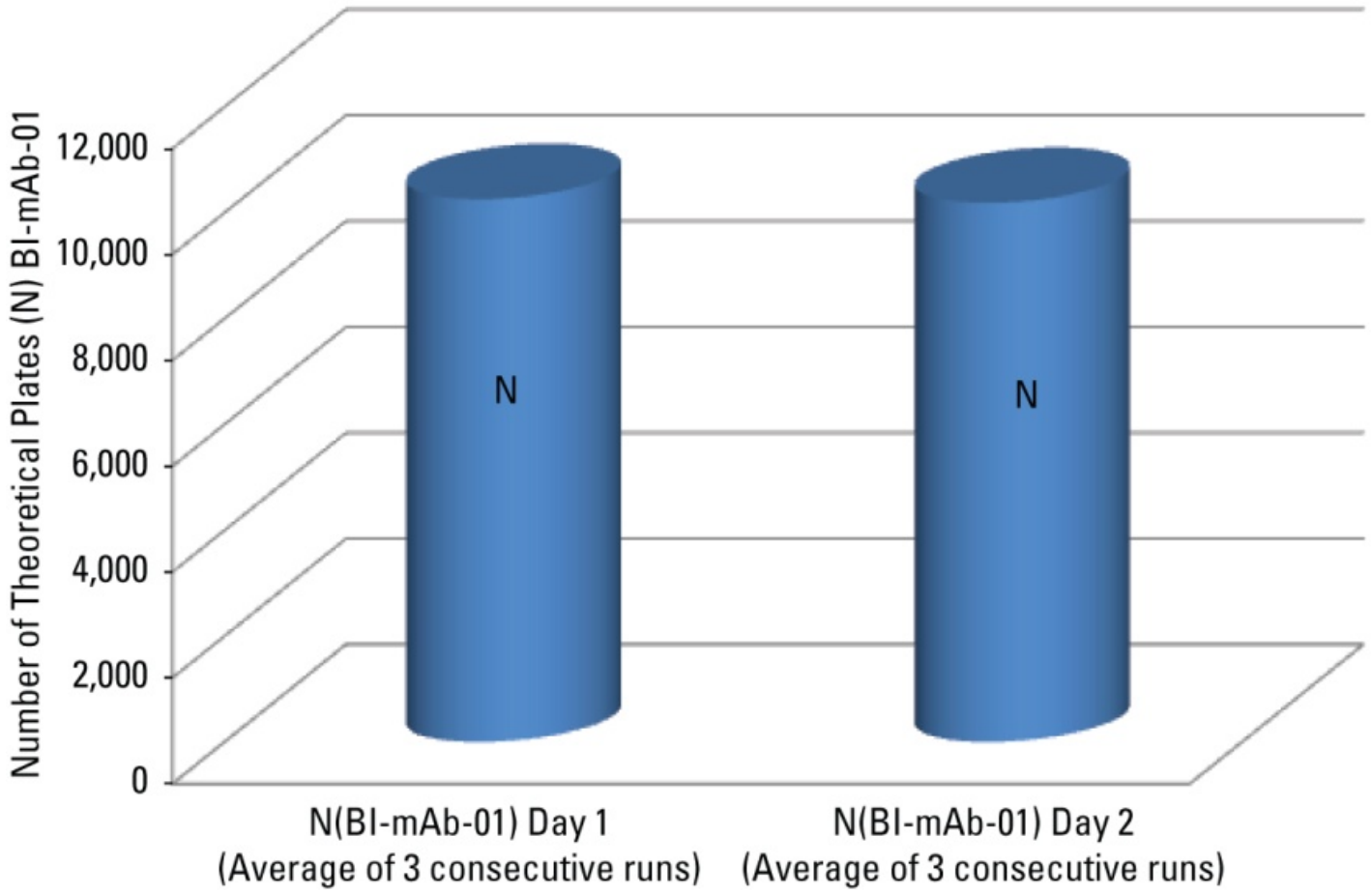


Figure 10: Inter-day repeatability - separation of monoclonal antibody (BI-mAb-01) using a prototype TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm column (Y0007T)



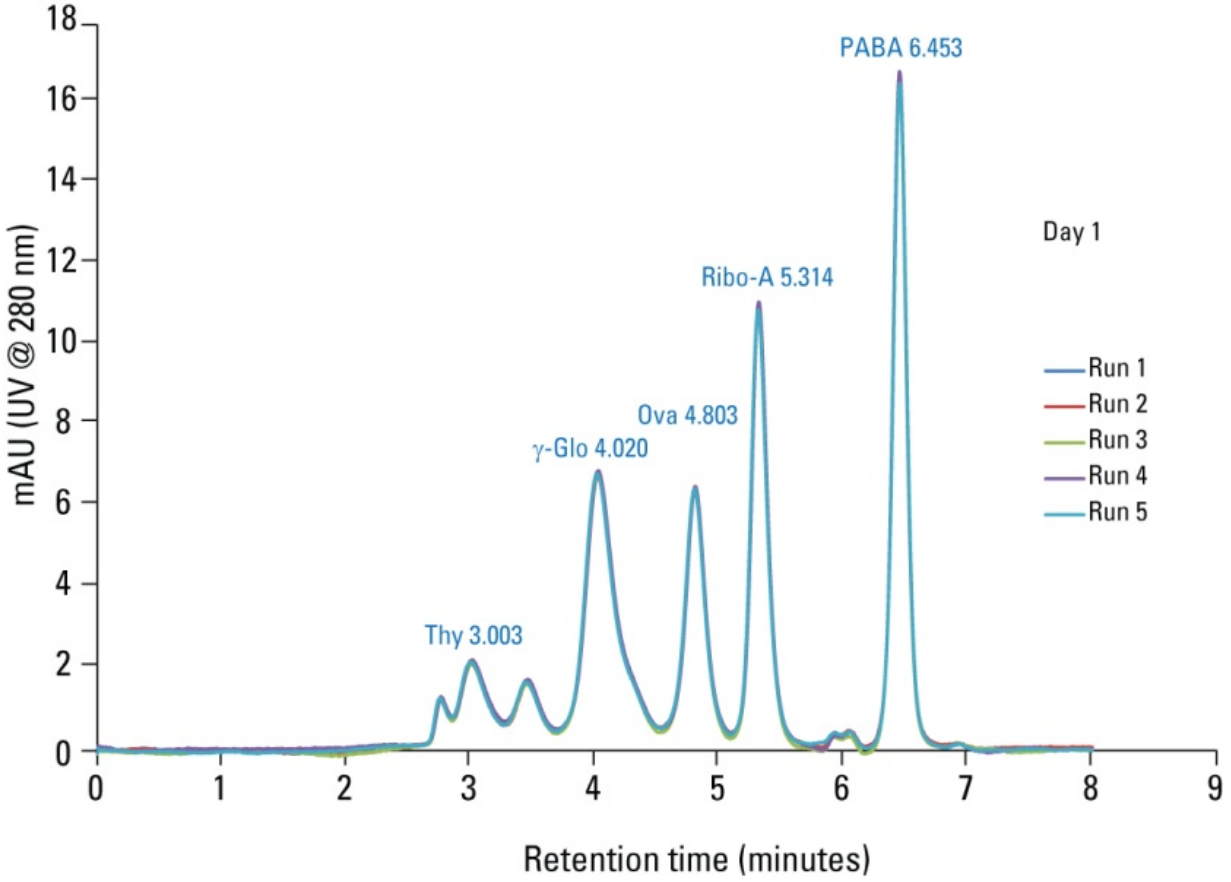
- **Excellent day-to-day reproducibility was observed.**
- **Excellent day-to-day reproducibility was also observed using the protein standard mixture.**

Highlights

TSKgel UltraSW Aggregate, 3 μm, 7.8 mm ID × 30 cm column:

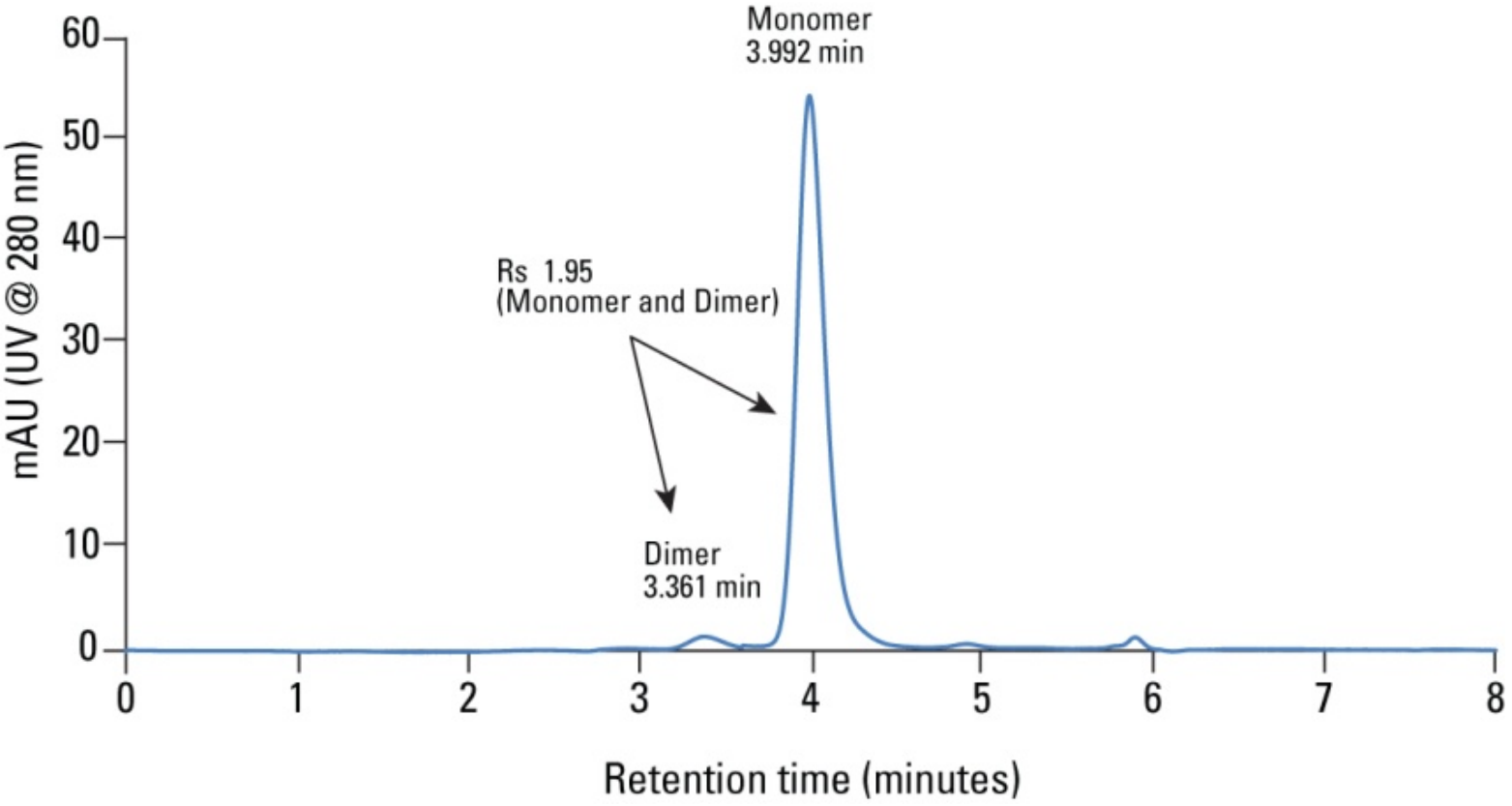
- High column efficiency (PABA) could be achieved.
- Excellent reproducibility in both intra-day and inter-day analysis was achieved in the analysis of mAb and proteins.
- No significant difference was found from lot-to-lot in the analysis of mAb and proteins.
- Rs between the peaks was consistent.

Figure 11: Analysis of protein standard mixture using a prototype TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm column (0216S4)



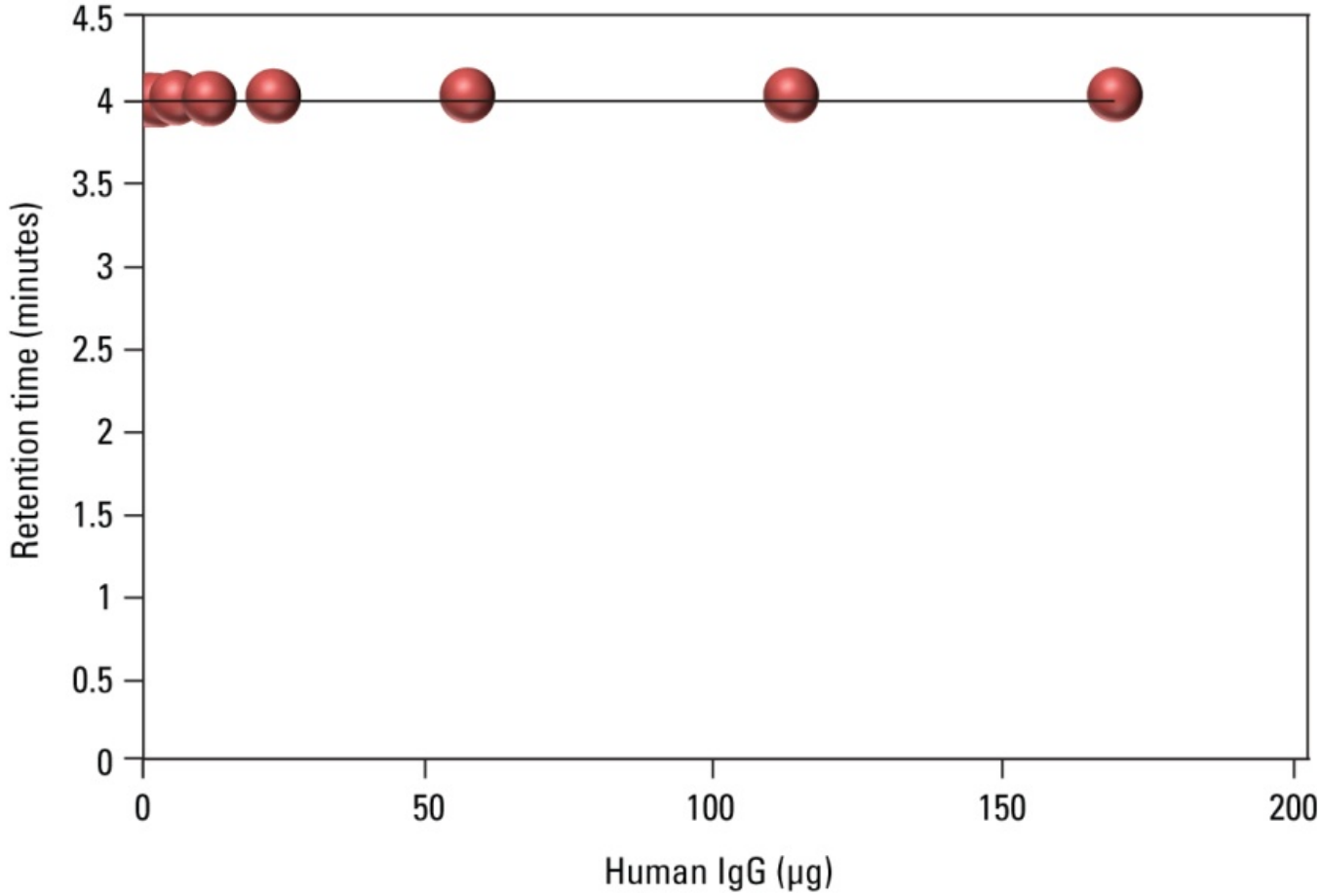
- **Five consecutive runs yielded excellent reproducibility with low %RSD values in RT, As. and N for all peaks.**
- **Excellent day-to-day reproducibility was also obtained (Ref: Poster presented at Fall ACS 2012).**
- **Excellent column-to-column reproducibility was obtained as well. (Ref: Poster presented at Fall ACS 2012)**

Figure 12: Analysis of human IgG using a prototype TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm column (0216S4)



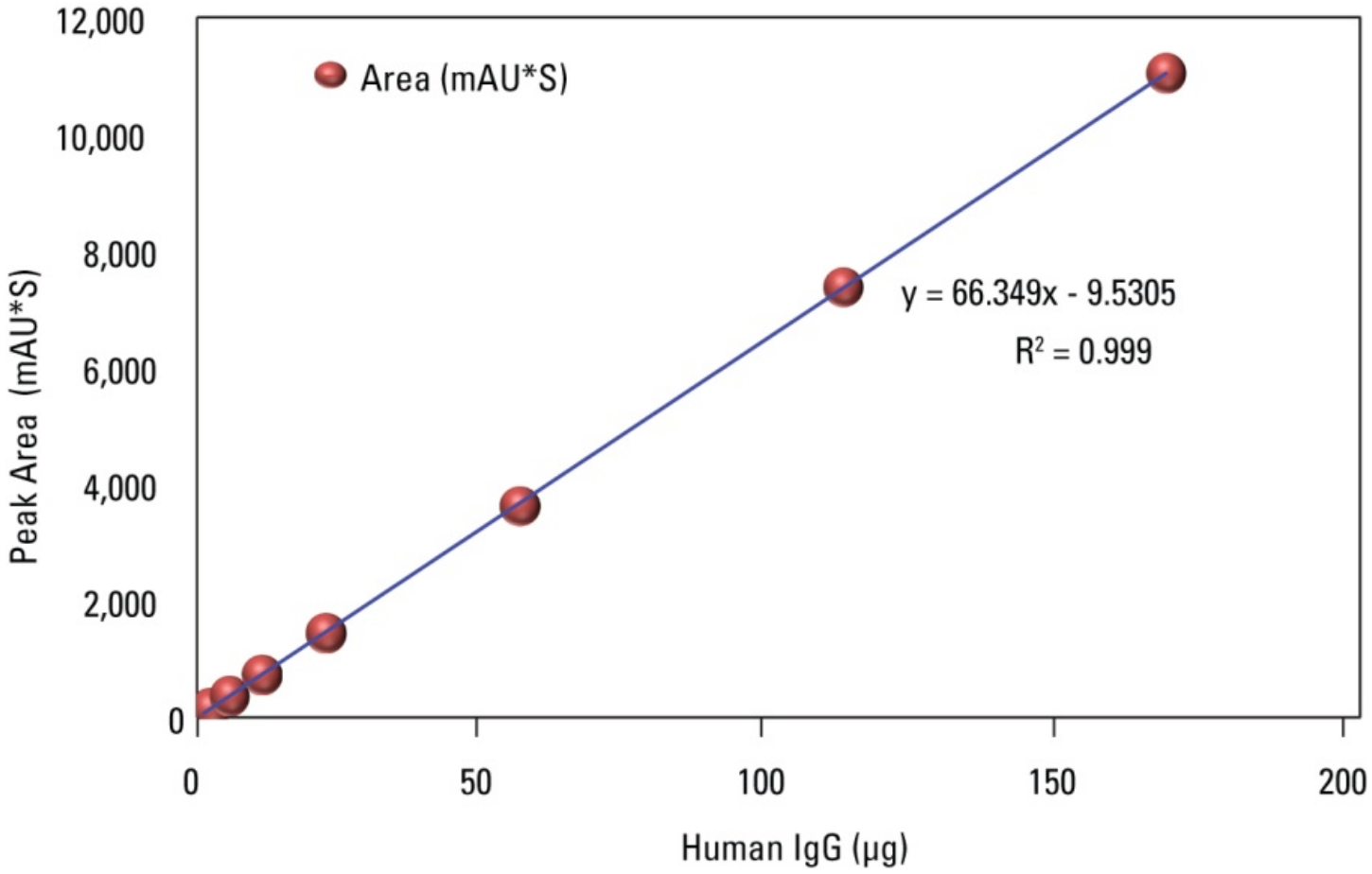
- **Monomer and dimer peak of Human IgG could be baseline resolved with a Rs of ~2.**
- **Fast separation of monoclonal antibody could be achieved.**

Figure 13: Analysis of monoclonal antibody (human IgG) using a prototype TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm column (0216S4)



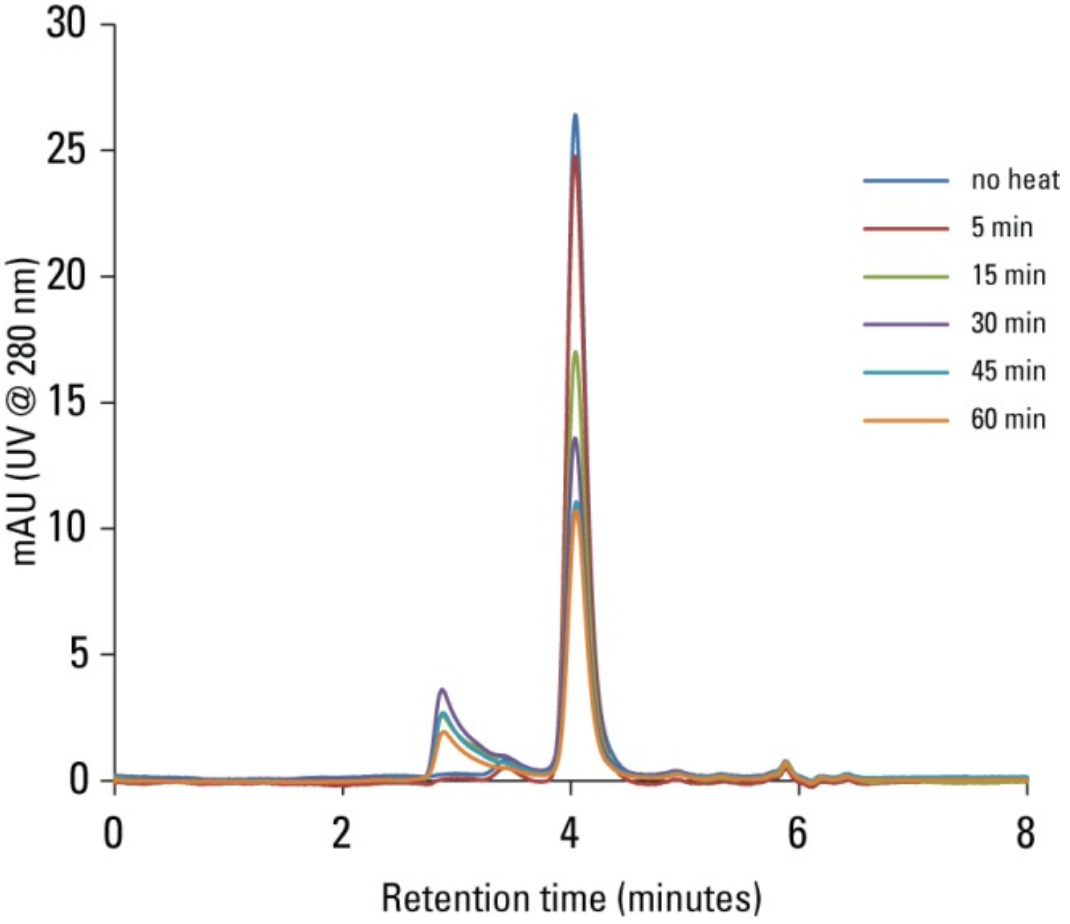
There was no significant shift in retention time of the monomer peak of human IgG over the range of loading used in this study.

Figure 14: Analysis of human IgG using a prototype TSKgel SuperSW mAb HTP, 4 µm, 4.6 mm ID × 15 cm column (0216S4)



Monomer peak area of human IgG yielded excellent linearity over the experimental range of loading amount.

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



- **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.**

Conclusions

- The following three novel prototype SEC columns have been developed:
  1. TSKgel SuperSW mAb HTP exhibited equal separation between IgG monomer and dimer in half the analysis time compared to the conventional SEC column, TSKgel G3000SW$_{XL}$, 5 µm particle, 7.8 mm ID × 30 cm.
  2. TSKgel SuperSW mAb HR exhibited superior resolving power for IgG monomer and dimer compared to other SEC columns.
  3. 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 superb performance of these columns was demonstrated by the separation of IgG fragments generated by papain digestion and separation of IgG aggregates.

Conclusions continued

- TSKgel UltraSW Aggregate has a wider separation window and higher resolving power for oligomers and aggregates with high molecular weight compared to a conventional column.
- TSKgel UltraSW Aggregate, 7.8 mm ID × 30 cm, 3 µm column can successfully be used for the separation of the dimer from the monomer peak of IgG with high resolution.
- TSKgel SuperSW mAb HTP, 4.6 mm ID × 15 cm, 4 µm column can be used for fast separation of dimer, monomer, and fragments with same resolving power and in half the analysis time compared to a convention column.
- The efficient separation of aggregates induced by heat denaturation could be achieved using these columns.
- No significant difference was noticed in the analysis of proteins or monoclonal antibodies as a function of time within a day, day-to-day or lot-to-lot.
- This study shows that these 3 novel SEC columns are suitable for the analysis and purification of antibodies.