Effect of Larger Exclusion Limit on the Separation of HMW Species – a Study Using a 3 µm Size Exclusion Chromatography Column

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Introduction

- Immunogenicity Assessment for Therapeutic Protein Products for the existence of protein heterogeneity, other than the monomer of therapeutic interest, is very important for manufacturers and clinical investigators.
- FDA in their guidance recommends adopting a risk-based approach in evaluating these impurities to prevent any adverse immunological responses.
- To reach that goal, the separation of these species is important.
- The formation of these heterogenic species, including high molecular weight (HMW) species, need to be analyzed. These species can occur at all different stages of the manufacturing process, starting with the fermentation step.
- Conformational changes of proteins by solvent induced unfolding may produce these impurities as well, even if the protein is not completely denatured.
- Besides the inherent characteristics of the protein, there are many environmental factors such as chromatographic conditions, storage and handling, etc., that need to be monitored as well.

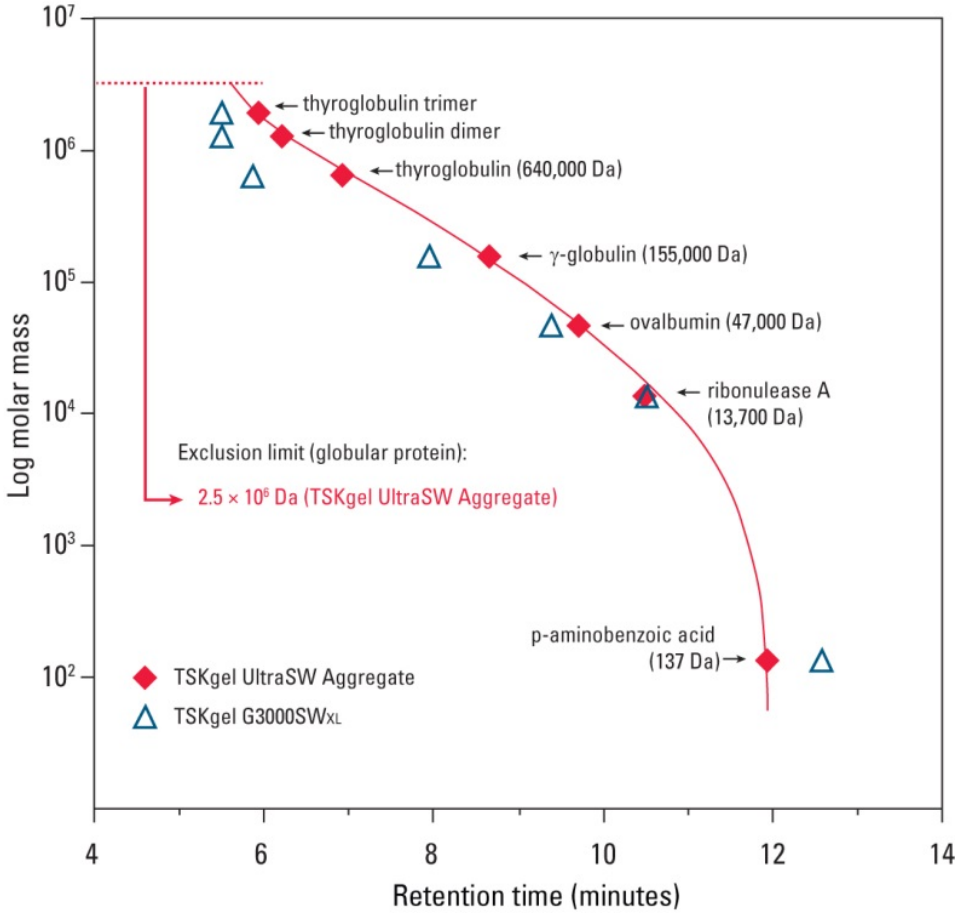
Introduction

- The following environmental factors common to the purification process may contribute to aggregate formation:
  - Cell culture process - up to 30% aggregation
  - Temperature
  - Storage
  - Shipping
  - Freeze and thaw cycles
  - Oxygen exposure
  - Light exposure
  - Physical stress
  - Protein concentration
  - pH
  - Shear forces
  - Ionic strength

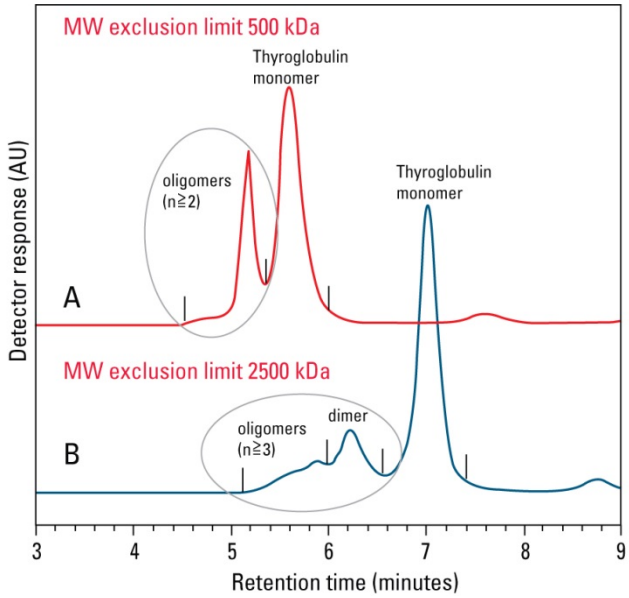
Introduction

- In size exclusion chromatography, the upper limit of molecular weight (or size) beyond which molecules will elute together at the same retention volume is called the exclusion volume.
- The exclusion limit defines the molecular weight (MW) at the upper end of the column 'working' range and is where molecules are too large to be trapped in the stationary phase.
- Traditionally GFC columns with a dimension of 7.8 mm ID x 30 cm L with 500 kDa MW exclusion limit are widely used for analytical purposes in quality control to monitor the stability of the protein.
- 500 kDa exclusion limit is very close to the molecular weight of the trimer of a monoclonal antibody, which is 450 kDa.
- Therefore, the trimer and higher order aggregates may elute in the excluded or void volume and cannot be separated as a function of their size or hydrodynamic radii.
- A column with a higher exclusion limit of 2,500 kDa will be more helpful in preventing the HMW species from going into the void and avoid being quantified.
- A GFC column with a higher exclusion limit of 2,500 kDa and a smaller particle size (3 µm) compared to traditional GFC columns should be useful for its higher resolution of multimers and aggregates of large proteins, including thyroglobulin, IgGs and their derivatives.
- In this presentation we demonstrate the performance of a TSKgel® UltraSW Aggregate SEC column with a larger exclusion limit and a 3 µm particle size for the separation of HMW species using both conventional HPLC and UHPLC systems. See the calibration curve in the next figure.

Calibration Curve of TSKgel UltraSW Aggregate Column



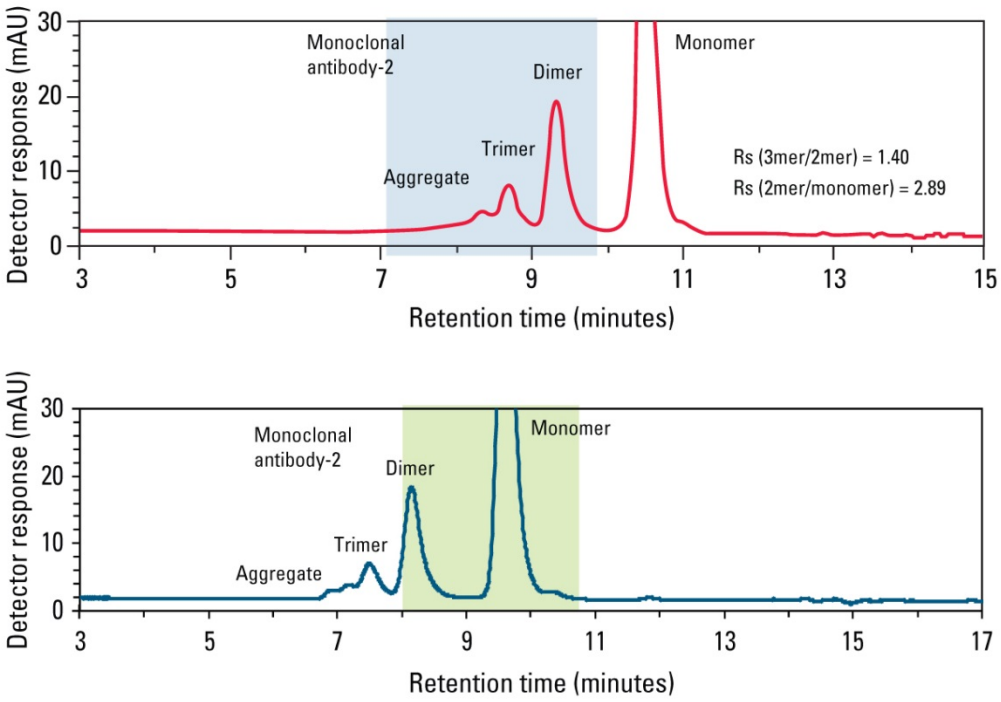
Larger Molecular Exclusion Limit Allows Better HMW Separation of Monoclonal Antibodies



Columns:	A: TSKgel SuperSW mAb HR, 500 kDa exclusion limit 7.8 mm ID × 30 cm B: TSKgel UltraSW Aggregate, 2,500 kDa exclusion limit 7.8 mm ID × 30 cm
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 g/L

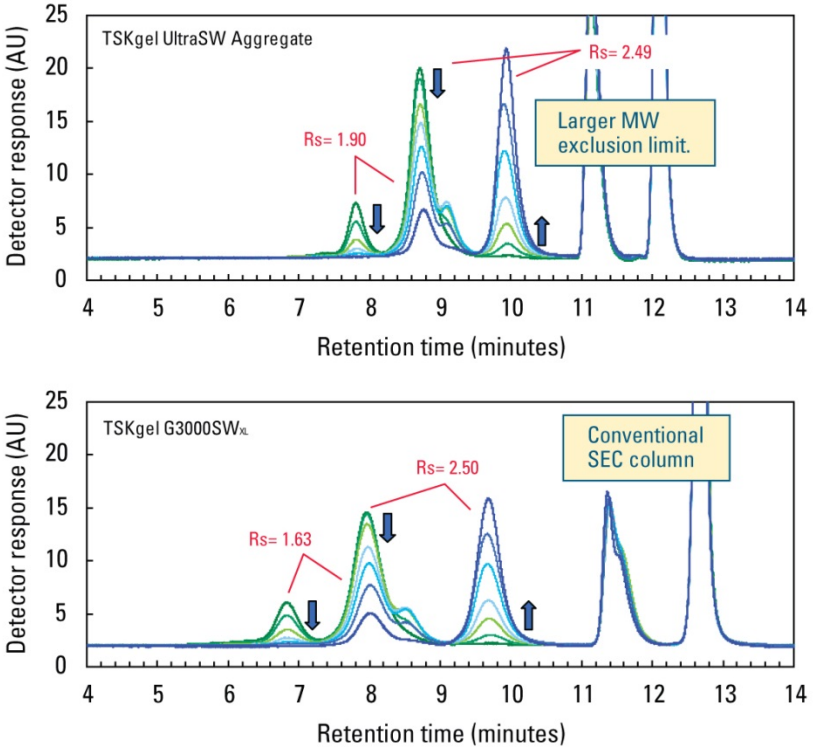
- Larger molecular weight exclusion limit of 2,500 kDa (B) compared to 500 kDa (A) results in the better separation of HMW impurities
- In the analysis of proteins, particularly in the case of mAbs and ADCs, a large exclusion limit of 2,500 kDa is expected to help in eluting the higher order aggregates in the inclusion volume so they will be separated as a function of their size or hydrodynamic radii

Aggregate Analysis of Therapeutic Antibody



Column:	TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm
Mobile phase:	0.2 mol/L phosphate buffer, pH 6.7 + 0.05% NaN_3
Flow rate:	0.8 mL/min
Detection:	UV @ 280 nm
Temperature:	25 °C
Sample:	monoclonal antibody-2 (mouse-human chimeric IgG, Erbitux), 10 µL

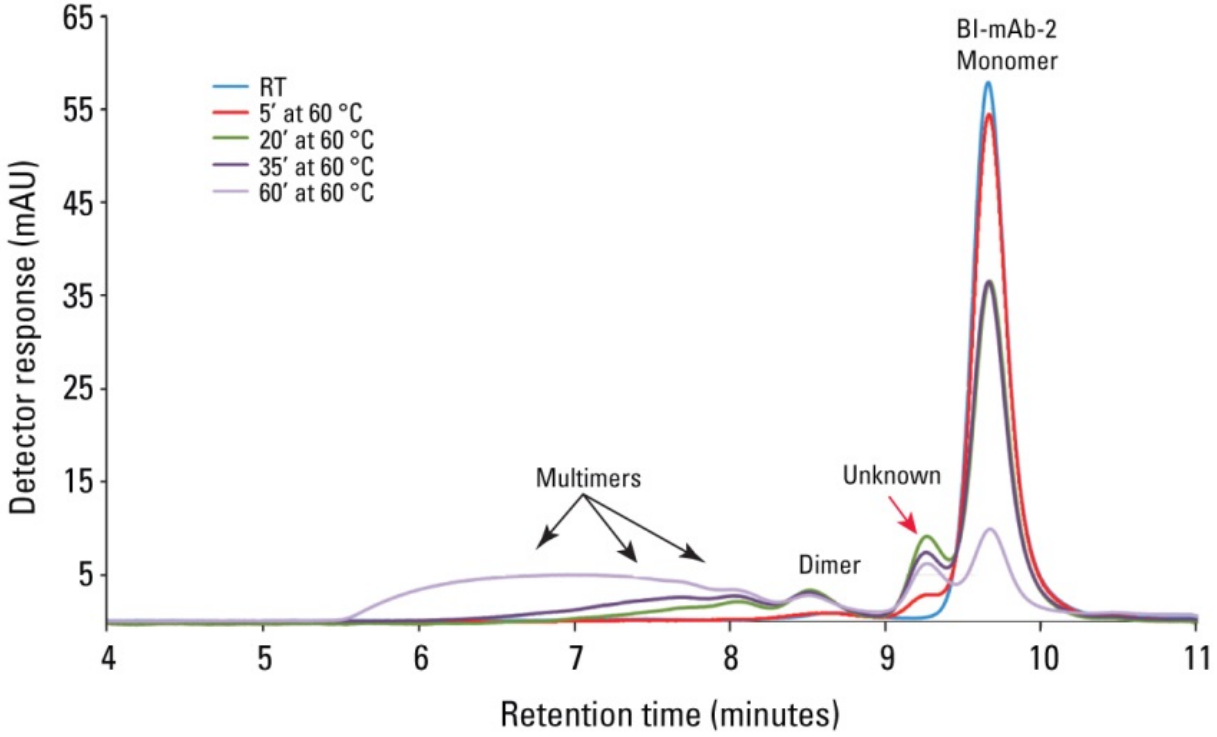
Analytical Chromatography in QC: Analysis of Monomer, Dimer and Fragments



- The TSKgel UltraSW Aggregate column, packed with 3 µm particles and 30 nm pores, has a larger molecular weight exclusion limit of 2,500 kDa compared to the conventional TSKgel G3000SW$_{XL}$ column packed with 5 µm particles, 25 nm pores and 500 kDa exclusion limit.
- The TSKgel UltraSW Aggregate column yields higher resolution between the monomer and dimer of IgG.

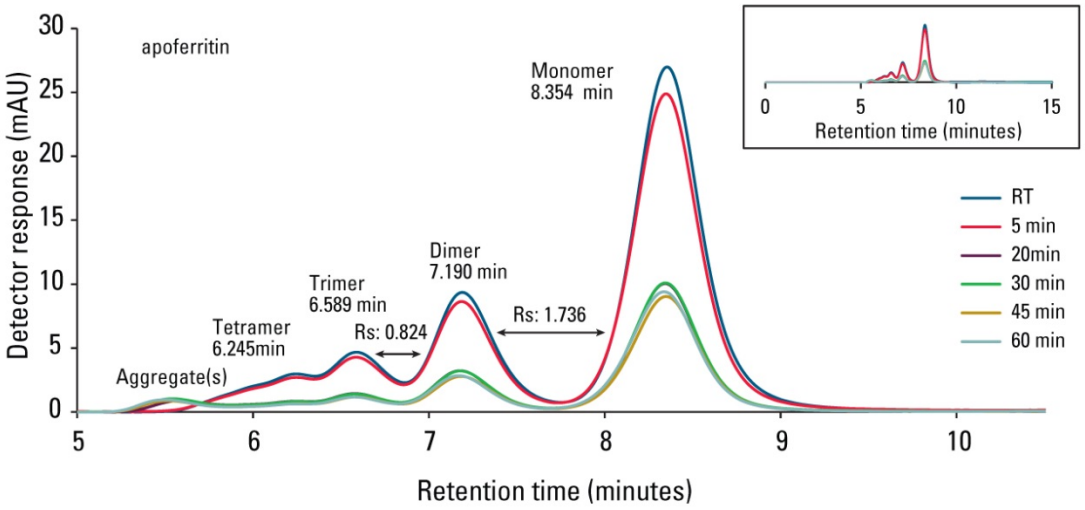
Column	Undigested IgG				IgG digested with papain for 1,440 min	
	ET (min) (Monomer)	TP (Dimer)	TP (Monomer)	Rs (d/m)	TP (Fragments)	Rs (m/f)
TSKgel UltraSW Aggregate, 7.8 mm ID × 30 cm	8.710	5,563	4,279	1.90	7,807	2.49
TSKgel G3000SW$_{XL}$, 7.8 mm ID × 30 cm	7.963	1,912	1,781	1.63	3,883	2.50

Heat Denaturation Study of Monoclonal Antibody (BI-mAb-02)



Forced heat denaturation of a mAb results in an almost complete breakdown of the monoclonal antibody and the formation of very large aggregates that extend to the exclusion volume of the TSKgel UltraSW Aggregate column of 2,500 kDa.

Separation of the Metalloprotein Apoferritin

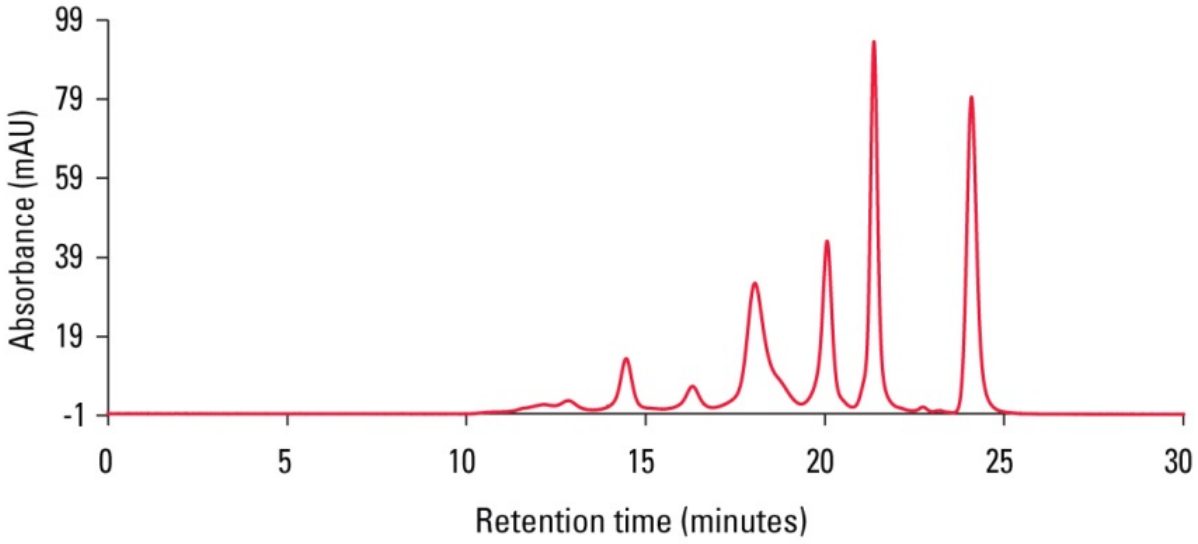


Protein	Molecular weight (kDa)			
	Monomer	Dimer	Trimer	Tetramer
ferritin and apoferritin	450	900	1350	1800

- The analysis of a heat denatured, large hydrophobic metalloprotein, apoferritin, analyzed using a TSKgel UltraSW Aggregate column yielded high resolution between the monomer (450 kDa) and dimer (900 kDa).
- The trimer, tetramer and higher order aggregates of apoferritin were well separated.
- Tetramer of apoferitin is equivalent to 13 mer of mAb.
- Larger exclusion limit yielded better resolution of the higher order aggregates.

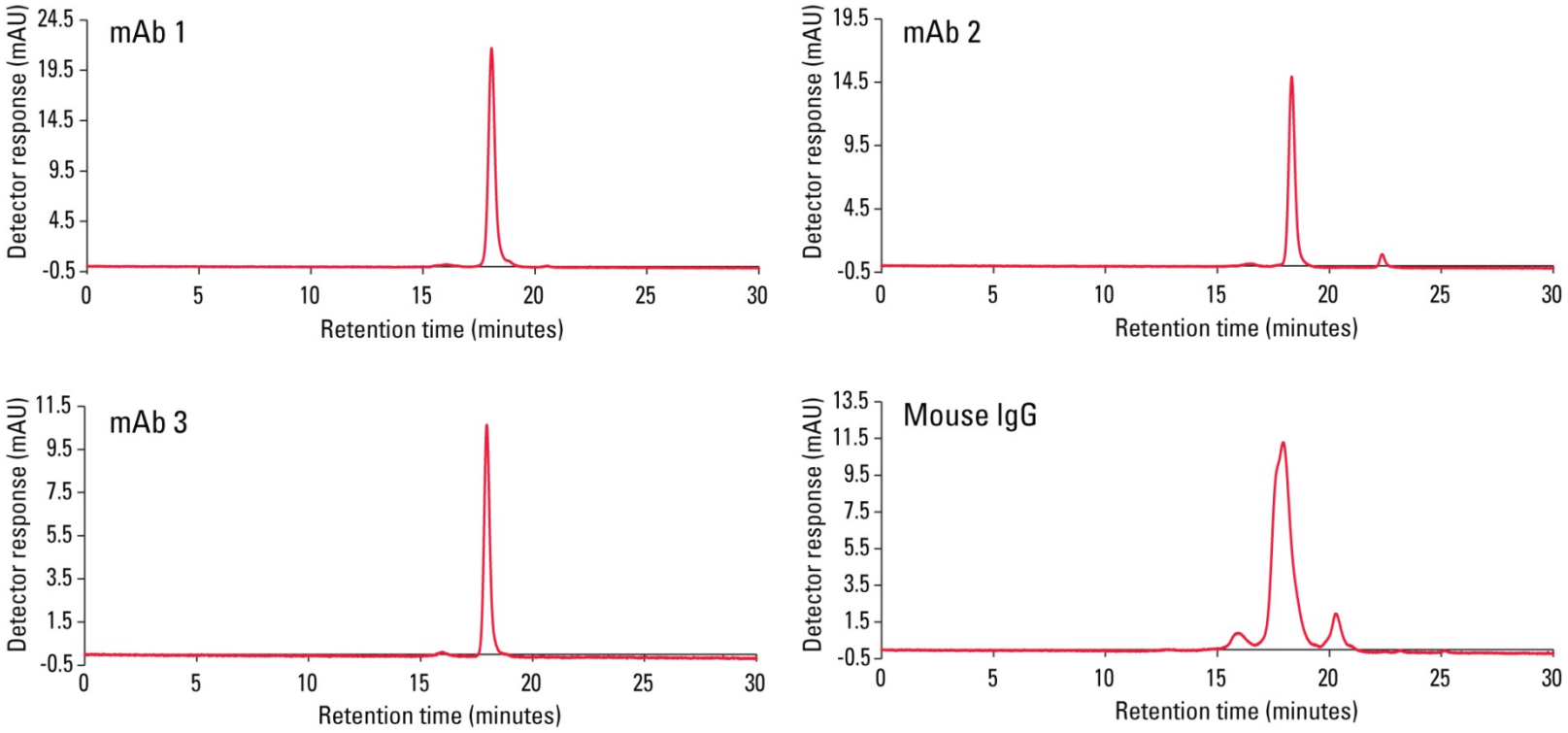
Ref: Tosoh AN 53

Analysis of Standard Proteins



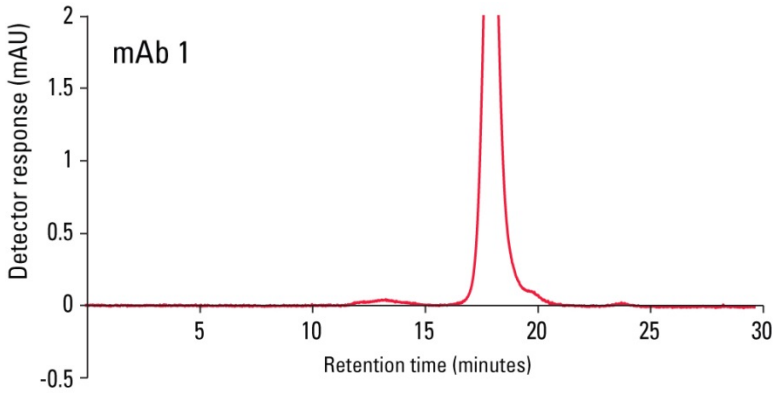
Column:	TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm
Instrument:	Thermo Scientific Ultimate 3000 UHPLC System with DAD and quaternary pump; Chromeleon 7
Flow Rate:	0.5 mL/min
Pressure:	7.5 MPa
Detection:	UV @ 280 nm
Temperature:	25 °C
Injection vol.:	20 µL
Samples:	standard TSKgel SW_{XL} test mixture: 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)

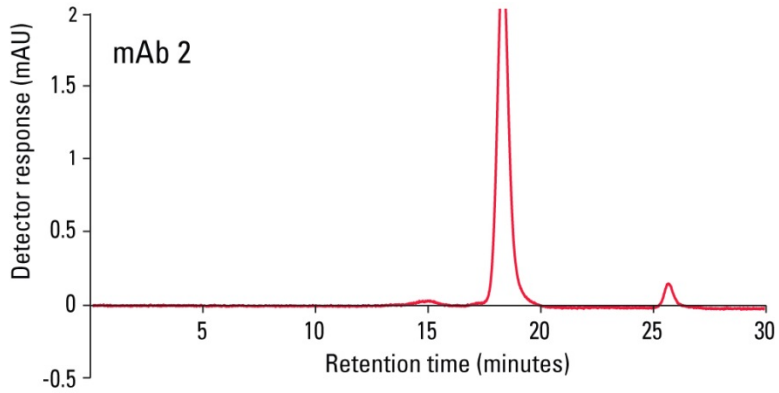
Analysis of Monoclonal Antibodies and Mouse IgG

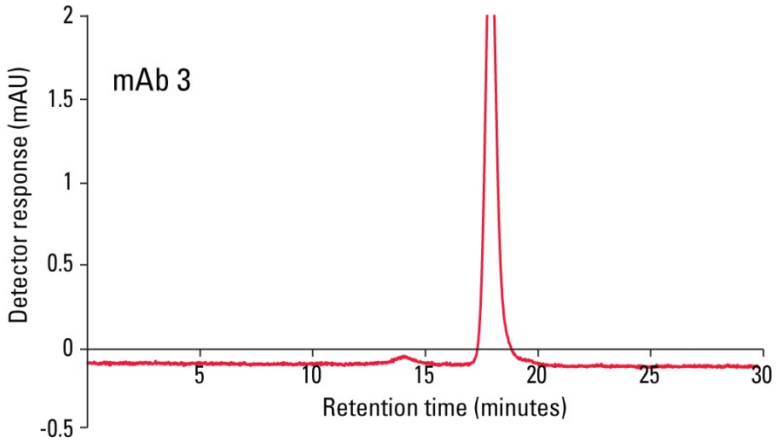


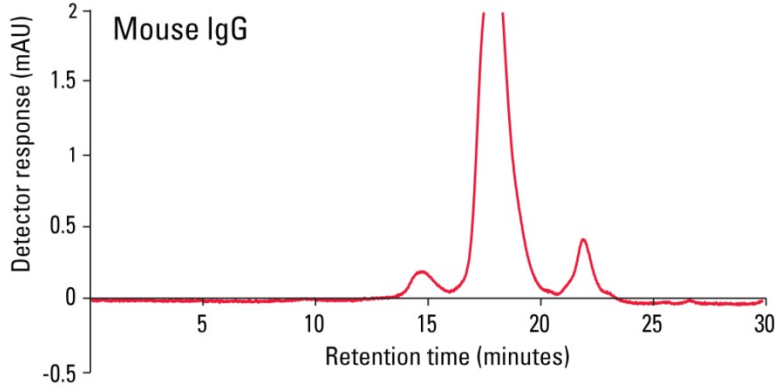
The results show that the degree of heterogeneity varies for different types of mAbs and mouse IgG which could be analyzed using the 3 µm, 30 nm TSKgel UltraSW Aggregate column with higher exclusion limit.

Analysis of Monoclonal Antibodies and Mouse IgG: Zoomed-In Figures









Conclusions

- The effect of larger exclusion limit on the separation of HMW species is useful in separating higher order aggregates.
- This study demonstrates the effectiveness of the TSKgel UltraSW Aggregate, 3 µm particle size, 30 nm pore size SEC column for protein stability studies.