Separation of BSA monomer from its dimer and aggregates using a 125 Å pore size diol coated 7.8 mm ID × 30 cm, 5 µm gel filtration column

Suhita Gayen-Betal[1], Atis Chakrabarti[2]

[1]Nemours Foundation, Philadelphia, PA
[2]Tosoh Bioscience LLC, King of Prussia, PA

Introduction

TSKgel SWXL columns:

- were first introduced in 1987
- represent the second generation of high performance Gel Filtration columns that have become synonymous with analyzing protein molecular weights in the ever emerging field of biotechnology
- contain 5 and 8 µm particles
- feature highly porous silica particles, the surface of which has been shielded from interacting with proteins by derivatization with ligands containing diol functional groups
- stand out from other silica- or polymer-based high performance size exclusion columns by virtue of their large pore volumes per unit column volume
- are commonly used in the quality control of monoclonal antibodies and other biopharmaceutical products

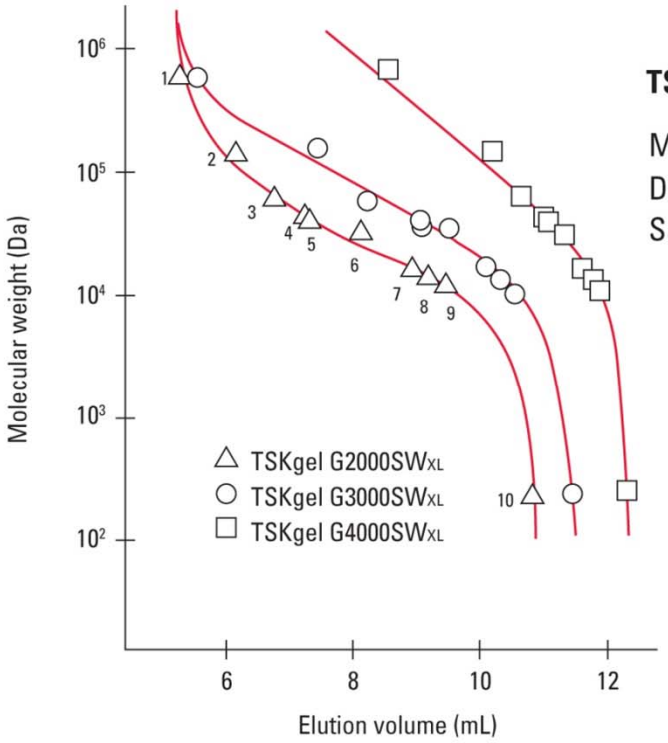
Introduction

- Bovine serum albumin (BSA) is a commonly used model protein in the pharmaceutical industry.
- BSA is widely popular because of its stability, inertness, and its low cost.
- Gel Filtration Chromatography (GFC) is commonly employed to assay its release from various dosage forms.
- GFC is popular among biochemists for the isolation of protein fractions or for the removal of aggregates in a final polishing step in the biotech industry.
- It is an excellent technique for investigating the formation of protein conjugates in solution, such as dimers and trimers.

Introduction

- The presence of aggregates affects properties of proteins and may provoke immune response.
- The presence of the impurities reduces the overall therapeutic effect or potency of the final product.
- There is increasing regulatory and industrial interest in identifying and separating protein aggregates.
- In this study we have used a 125 Å pore size diol coated 7.8 mm ID × 30 cm, 5 µm gel filtration column to separate BSA monomer from its dimer and aggregates.
- Forced degradation products of BSA were also analyzed.
- Native PAGE (polyacrylamide gel electrophoresis) was used for further characterization of the peaks.
- System suitability, loading capacity, limit of quantitation (LOQ), and limit of determination (LOD) are also reported.

Protein calibration curves for TSKgel SWXL columns



TSKgel SWXL Series, 7.8 mm ID × 30 cm

Mobile phase: 0.3 mol/L NaCl in 0.1 mol/L sodium phosphate buffer, pH 7.0

Detection: UV @ 220 nm

Samples:

1. thyroglobulin (660,000 Da)
2. IgG (156,000 Da)
3. bovine serum albumin (67,000 Da)
4. ovalbumin (43,000 Da)
5. peroxidase (40,200 Da)
6. β-lactoglobulin (35,000 Da)
7. myoglobin (16,900 Da)
8. ribonuclease A (13,700 Da)
9. cytochrome C (12,400 Da)
10. glycine tetramer (246 Da)

Properties and separation ranges for TSKgel SW packings

			Molecular weight of samples (Da)		
TSKgel column	**Particle size**	**Pore size**	**Globular proteins**	**Dextrans**	**Polyethylene glycols & oxides**
G2000SW	10 µm and 13 µm	125 Å	$5 \times 10^3 - 1.5 \times 10^5$	$1 \times 10^3 - 3 \times 10^4$	$5 \times 10^2 - 15 \times 10^3$
G3000SW	10 µm and 13 µm	250 Å	$1 \times 10^4 - 5 \times 10^5$	$2 \times 10^3 - 7 \times 10^4$	$1 \times 10^3 - 3.5 \times 10^4$
G4000SW	13 µm and 17 µm	450 Å	$2 \times 10^4 - 7 \times 10^6$	$4 \times 10^3 - 5 \times 10^5$	$2 \times 10^3 - 2.5 \times 10^5$
G2000SW$_{XL}$, BioAssist G2SW$_{XL}$, QC-PAK GFC 200	5 µm	125 Å	$5 \times 10^3 - 1.5 \times 10^5$	$1 \times 10^3 - 3 \times 10^4$	$5 \times 10^2 - 15 \times 10^3$
G3000SW$_{XL}$, BioAssist G3SW$_{XL}$, QC-PAK GFC 300	5 µm	250 Å	$1 \times 10^4 - 5 \times 10^5$	$2 \times 10^3 - 7 \times 10^4$	$1 \times 10^3 - 3.5 \times 10^4$
G4000SW$_{XL}$, BioAssist G4SW$_{XL}$	8 µm	450 Å	$2 \times 10^4 - 7 \times 10^6$	$4 \times 10^3 - 5 \times 10^5$	$2 \times 10^3 - 2.5 \times 10^5$
SuperSW2000	4 µm	125 Å	$5 \times 10^3 - 1.5 \times 10^5$	$1 \times 10^3 - 3 \times 10^4$	$5 \times 10^2 - 15 \times 10^3$
SuperSW3000	4 µm	250 Å	$1\ 10^4 - 5 \times 10^5$	$2 \times 10^3 - 7 \times 10^4$	$1 \times 10^3 - 3.5 \times 10^4$
BioAssist DS	15 µm	Excludes 2500 Da PEG			

Data generated using the following conditions:

Columns: TSKgel SuperSW columns in series, 4 µm, 4.6 mm ID × 30 cm × 2
TSKgel SW$_{XL}$ columns in series, 5 µm, 7.8 mm ID × 30 cm × 2
TSKgel SW columns in series, 10 µm, 7.5 mm ID × 60 cm × 2

Mobile Phase: globular proteins: 0.3 mol/L NaCl in 0.1 mol/L (0.05 mol/L for TSKgel SW$_{XL}$ columns) phosphate buffer, pH 7.0; dextrans and polyethylene glycols and oxides (PEOs): distilled water

This table summarizes the molecular weight ranges for TSKgel G2000SW$_{XL}$, G3000SW$_{XL}$ and G4000SW$_{XL}$ columns, in addition to other TSKgel SW columns, for globular proteins, polyethylene oxides and dextrans that can, at least partially, access the pores.

Objective

- To show the usefulness of the 125 Å pore size diol coated 7.8 mm ID × 30 cm, 5 µm gel filtration column in the separation of a protein monomer from its dimer and aggregates - Bovine Serum Albumin, a commonly used standard protein is used for this study as a reference

Material and methods: chromatographic conditions

- Column: TSKgel G2000SW$_{XL}$, 5 µm, 7.8 mm ID × 30 cm (S5018-03S)
- 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: 1.0 mL/min
- Detection: UV @ 280 nm
- Temperature: ambient
- Injection vol.: 20 µL (unless mentioned otherwise)
- 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)

  Bovine Serum Albumin (Sigma Aldrich A7906, Lot # 080M1251V; >98% purity, 10.2 mg/mL

Material and methods: chromatographic conditions

- Reduction of BSA: Protein was reduced by 10 mmol/L DTT (final concentration) for 30 minutes at 37 °C. QC buffer containing 10 mmol/L DTT (final concentration) was incubated for 30 minutes at 37 °C and used as a blank or control. FR = 0.2 mL/min
- Forced denaturation: forced denaturation to form aggregation was induced by heating the protein at 60 °C for 30 minutes and 70 minutes.
- PAGE: the presence of monomer, dimer and higher aggregates were identified by native PAGE; the details are provided in slide #16.

Material and methods

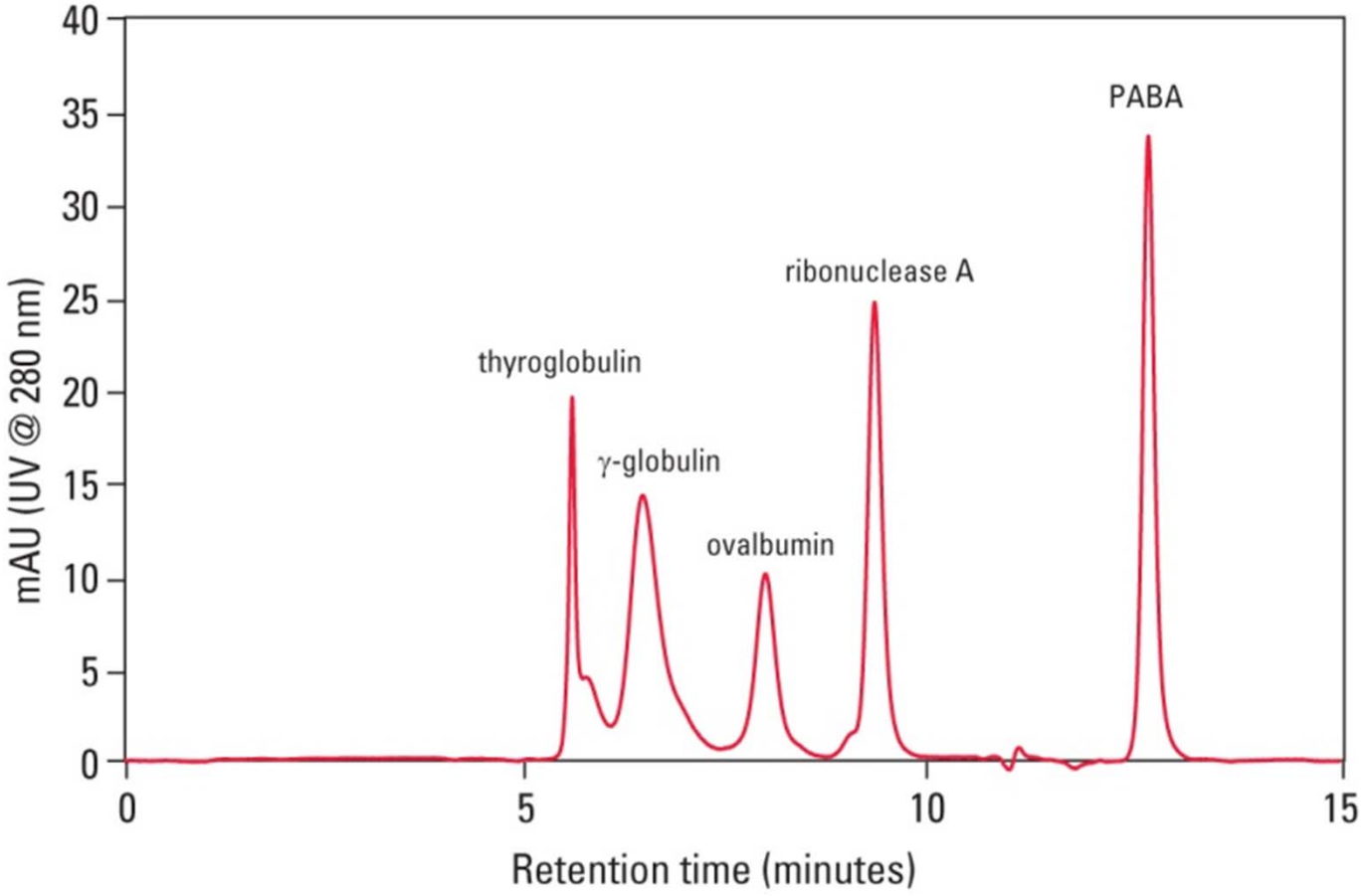
LOD and LOQ

- The limit of detection (LOD) is a parameter to measure the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantitated, under the stated experimental conditions.
- This is measured by a procedure for the validation of compendial methods as mentioned in USP under section 1225.
- The standard deviation of the baseline response (mAU at the wavelength selected for detection) using a blank sample is calculated.
- The standard deviation in mAU is multiplied by a factor of 2 to provide an estimate of the limit of detection (LOD).
- The LOD is subsequently validated by the analysis of the sample near that limit.
- For determination of limit of quantitation (LOQ), the LOD sample concentration is multiplied by a factor of 10.

High purity HPLC grade Sigma Aldrich chemicals were used in this study.

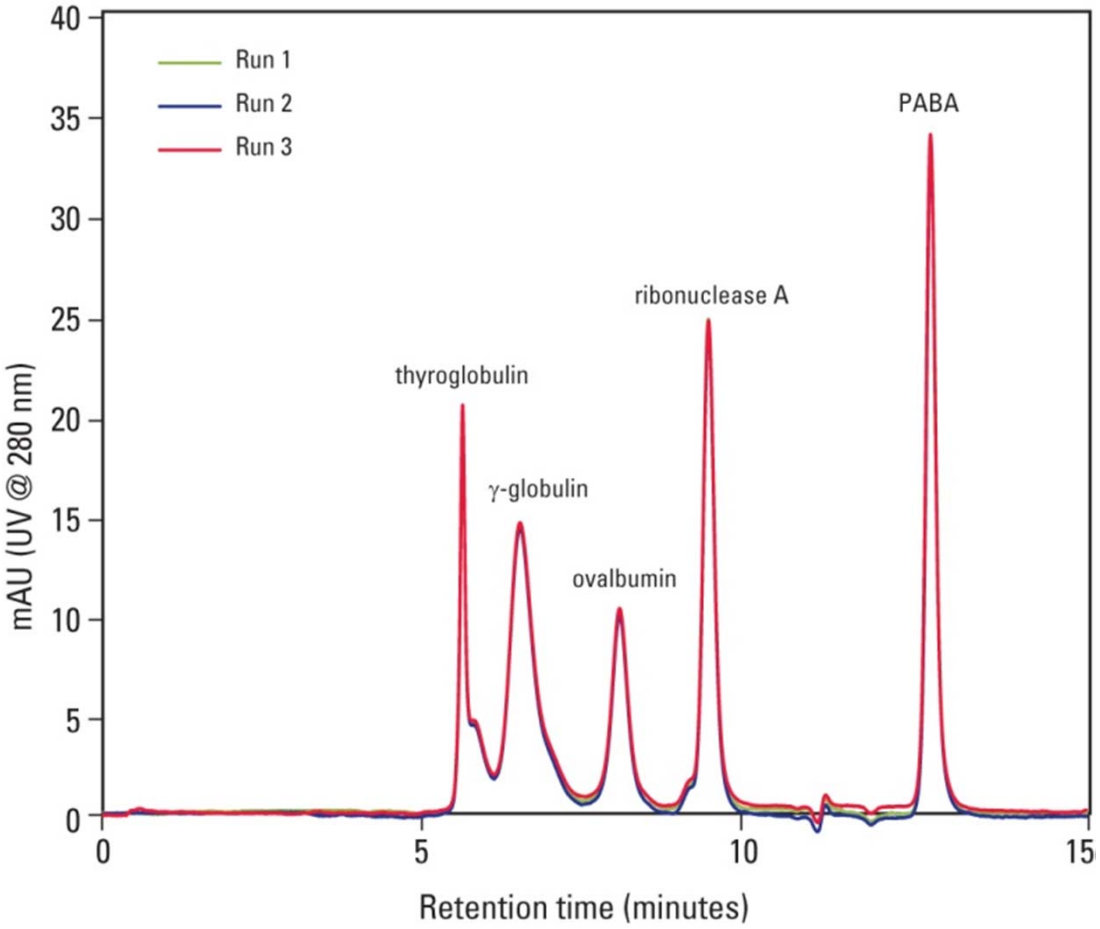
All the standards and samples were filtered through a 0.45 µm membrane before injecting into the column.

Separation of protein standard mixture using TSKgel G2000SWXL, 5 μm, 7.8 mm ID × 30 cm column (S5018-03S)



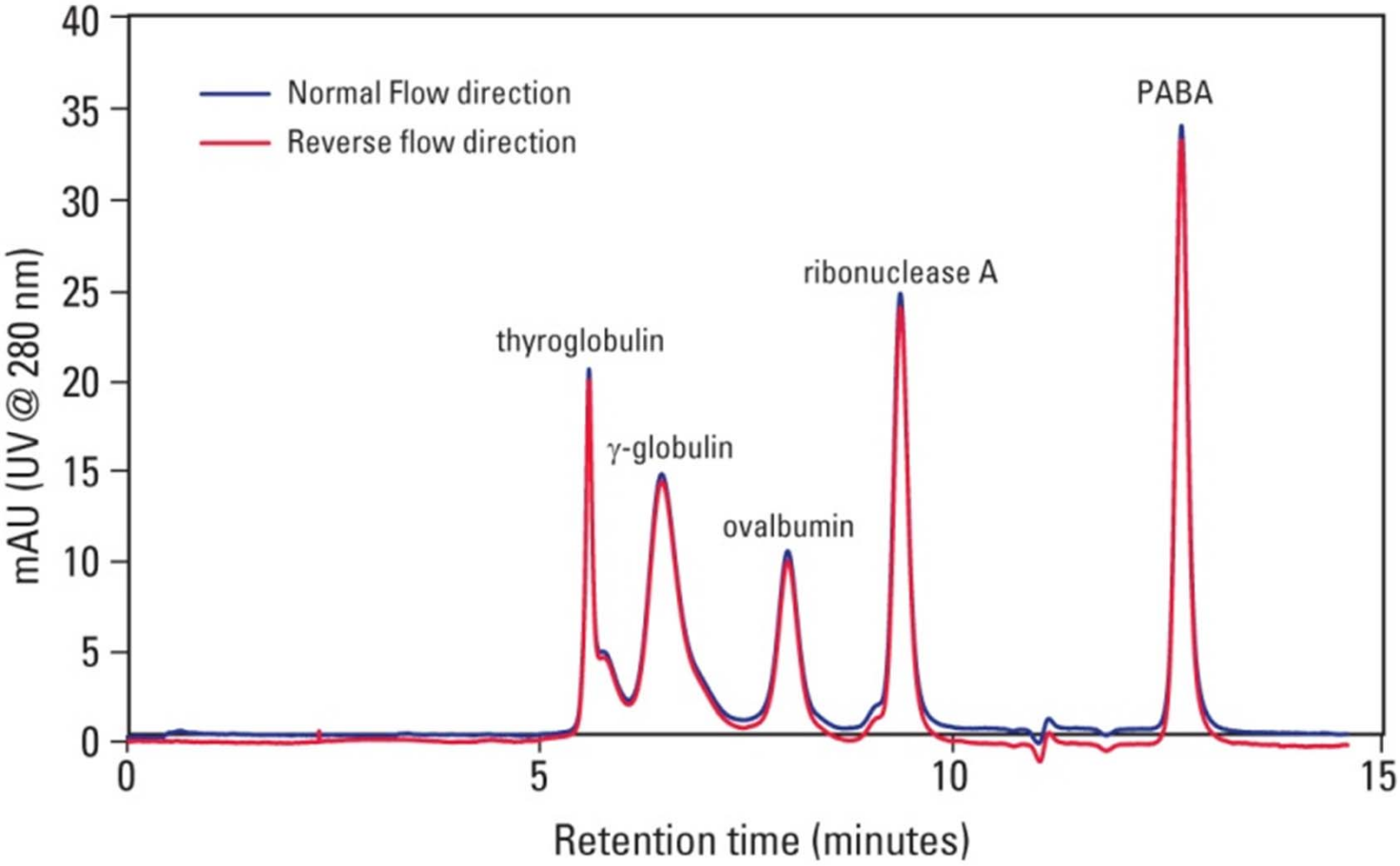
- N = 33,944 (PABA), which is well above the failure cut off limit of >20,000
- As = 1.18, which is well within the failure cut off limit range of 0.7-1.6

System suitability study using protein standard mixture using TSKgel G2000SWXL, 5 μm, 7.8 mm ID × 30 cm column (S5018-03S)



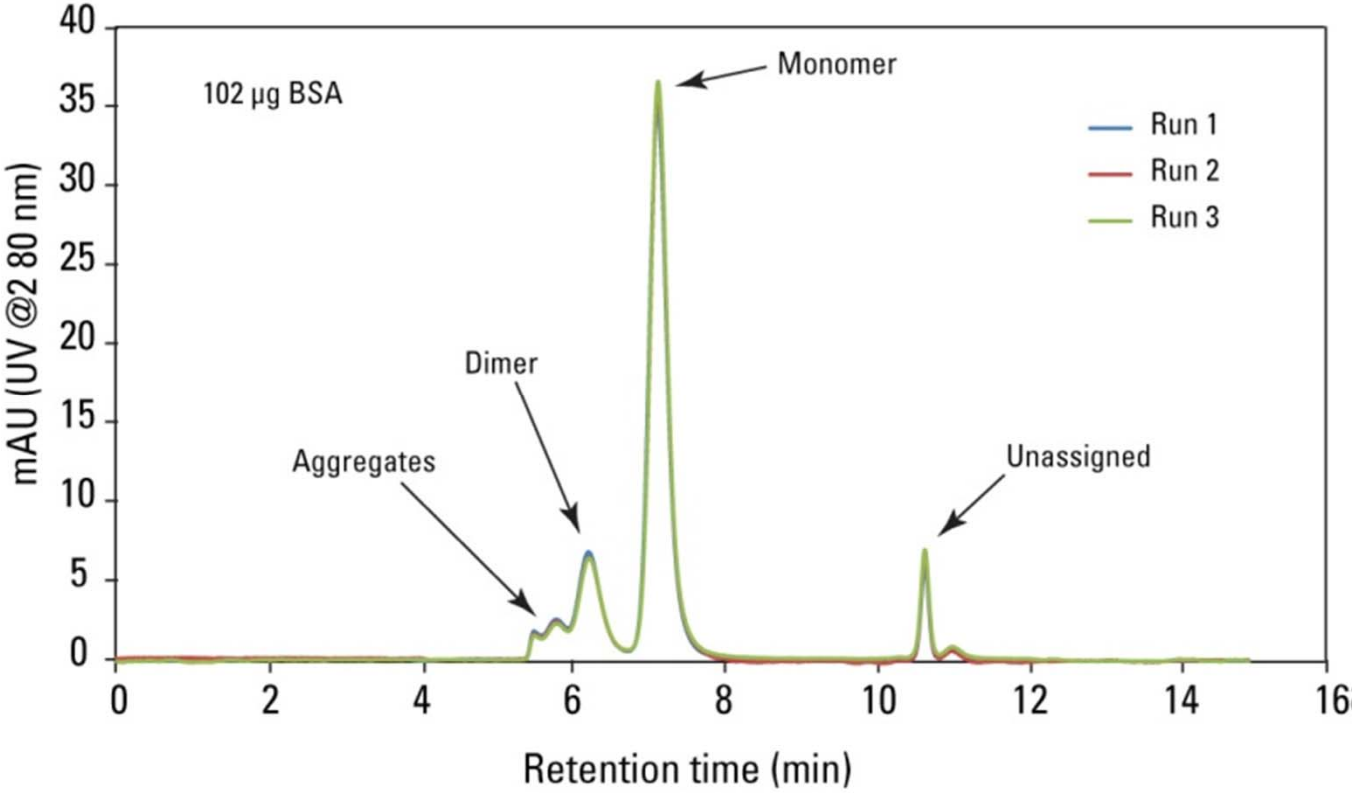
3 consecutive runs yielded excellent reproducibility with all the proteins.

Column packing conditions



No shift in retention time was observed when the column was run in reverse flow – suggesting stable packing with no void.

System suitability study using BSA and TSKgel G2000SWXL, 5 μm, 7.8 mm ID × 30 cm column (S5018-03S)

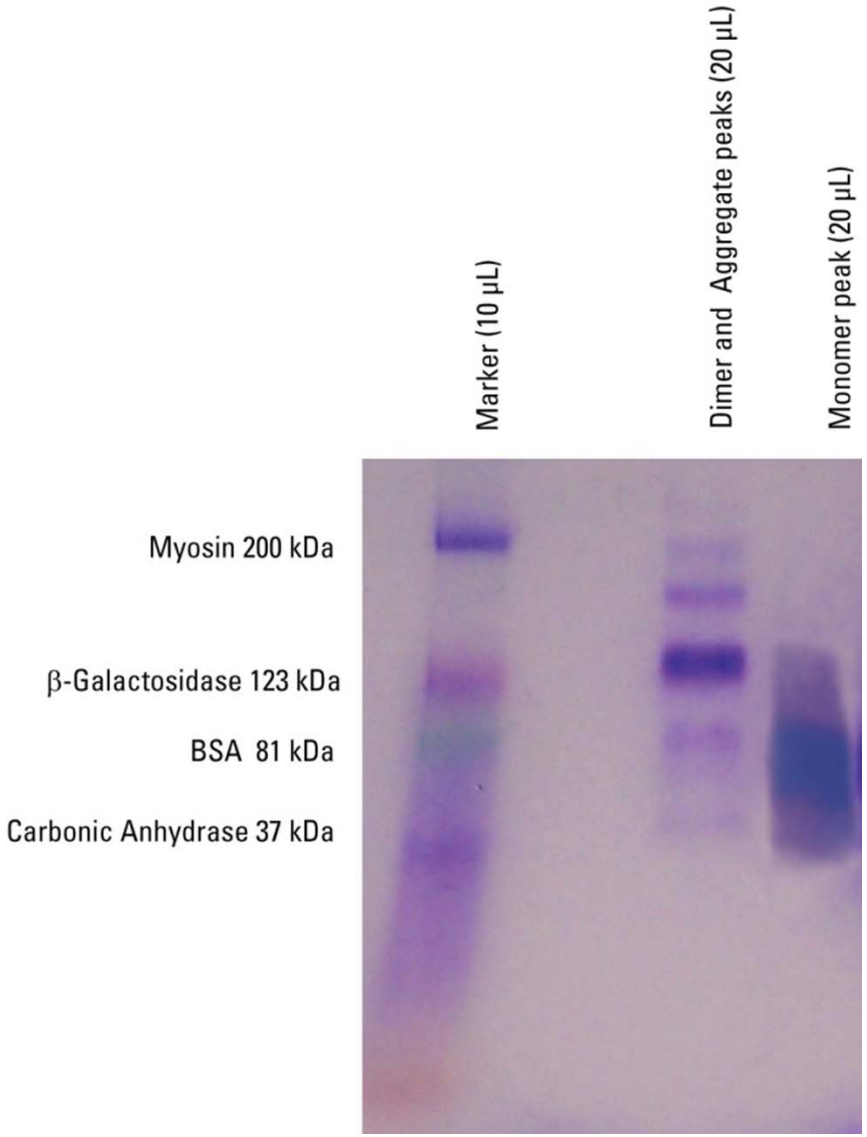


- 3 consecutive runs yielded excellent reproducibility with BSA also.
- The monomer peak was clearly resolved from the dimer peak.
- No splitting of the monomer peak was noticed.
- Monomer peak, dimer and aggregate molecular weights were further identified by native PAGE, against Bio-Rad MW marker as shown in the next slide.

Material and methods

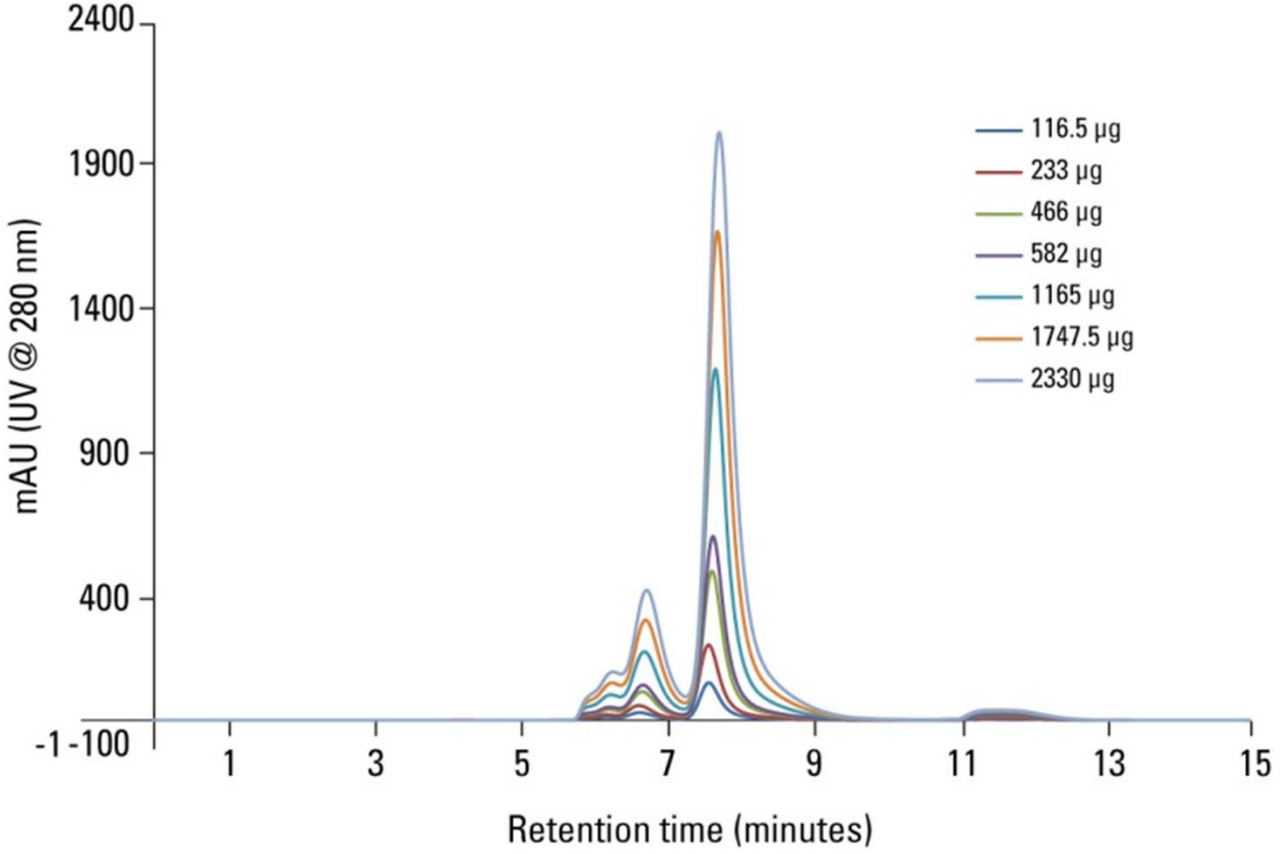
- Native PAGE is a convenient way to observe aggregation.
- For native PAGE gels, sodium dodecyl sulfate (SDS) is not used.
- Under this condition the proteins retain their native conformation and structure.
- The mobility of the protein is solely governed by the ratio of electric charge to hydrodynamic friction.
- Native PAGE neither denatures nor dissociates proteins.
- Hence the BSA monomer peak and pool of the peaks of dimer and aggregates were identified using native PAGE.

Identification of protein peaks by native PAGE



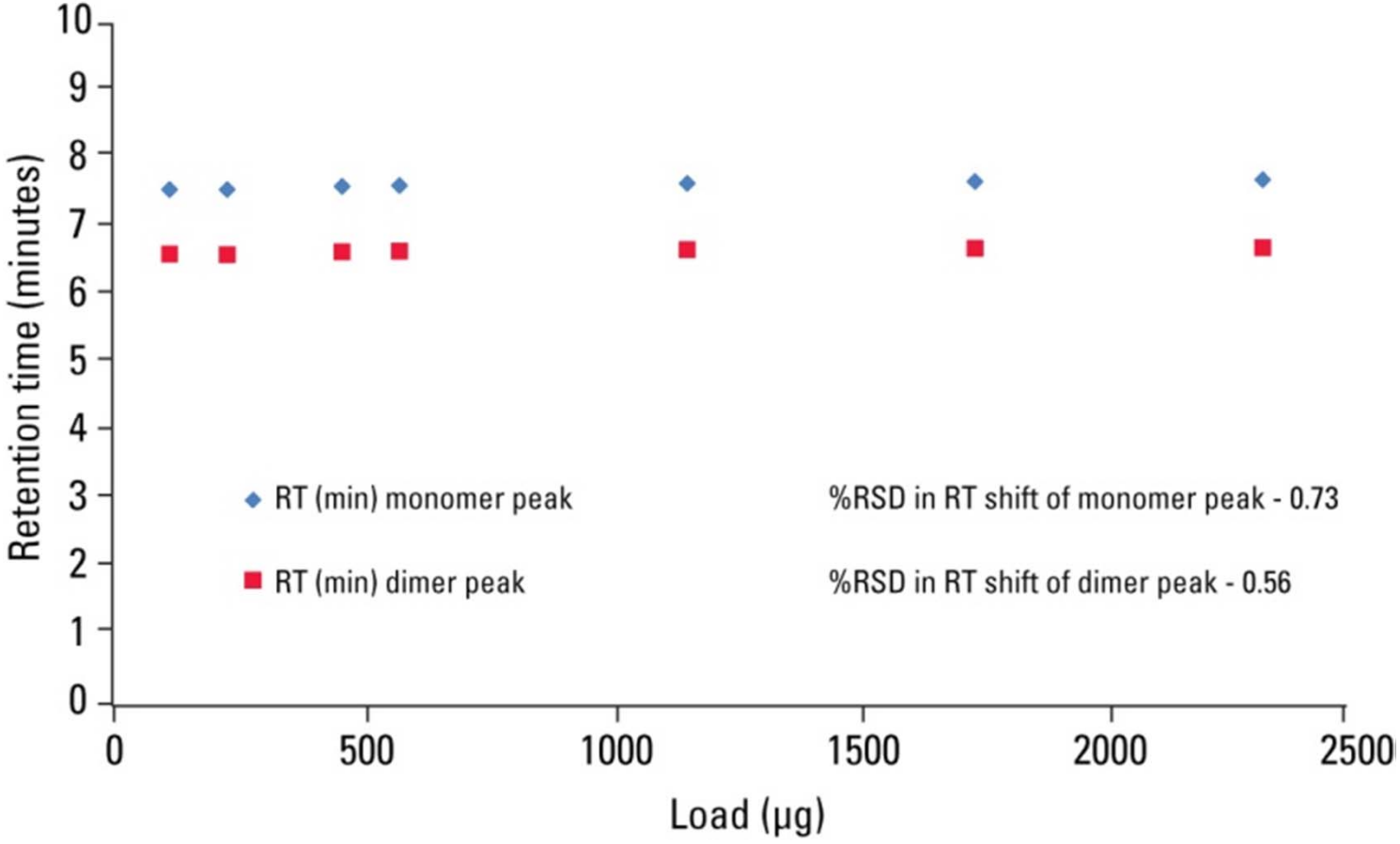
- Monomer peak and peaks containing dimer and aggregates were concentrated 25 fold by Amicon® Ultra centrifugal filters (Milipore Catalog UFC505024).
- 4-15% Tris-HCl pre-cast gradient gel (Bio-Rad Catalog # 161-1104).
- Tris-Glycine running buffer: 25 mmol/L Tris, 192 mmol/L glycine; prepared from 10 × stock (Bio-Rad Catalog # 161-0771).
- Sample buffer: 62.5 mmol/L Tris-HCl, pH 6.8, 25% (v/v), glycerol, 0.01% (w/v), bromophenol blue (Catalog # 161-0738).
- Sample was prepared by mixing equal volume of sample with native buffer (no heating).
- Gel running at 200 V for 1 hour at room temperature.
- Gel was fixed by 50% methanol and 10% glacial acetic acid, 1 hour at room temperature
- Gel was stained using 0.1% Cooumassie Brilliant Blue (R-250) (Sigma Catalog # 27816), 50% methanol and 10% glacial acetic acid, incubated at room temperature for 20 minutes.
- The gel was destained using 40% methanol and 10% glacial acetic acid in water.
- Bio-Rad Kaleidoscope pre-stained standards (Catalog # 161-0324) were used as molecular weight markers.

Loading capacity of TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



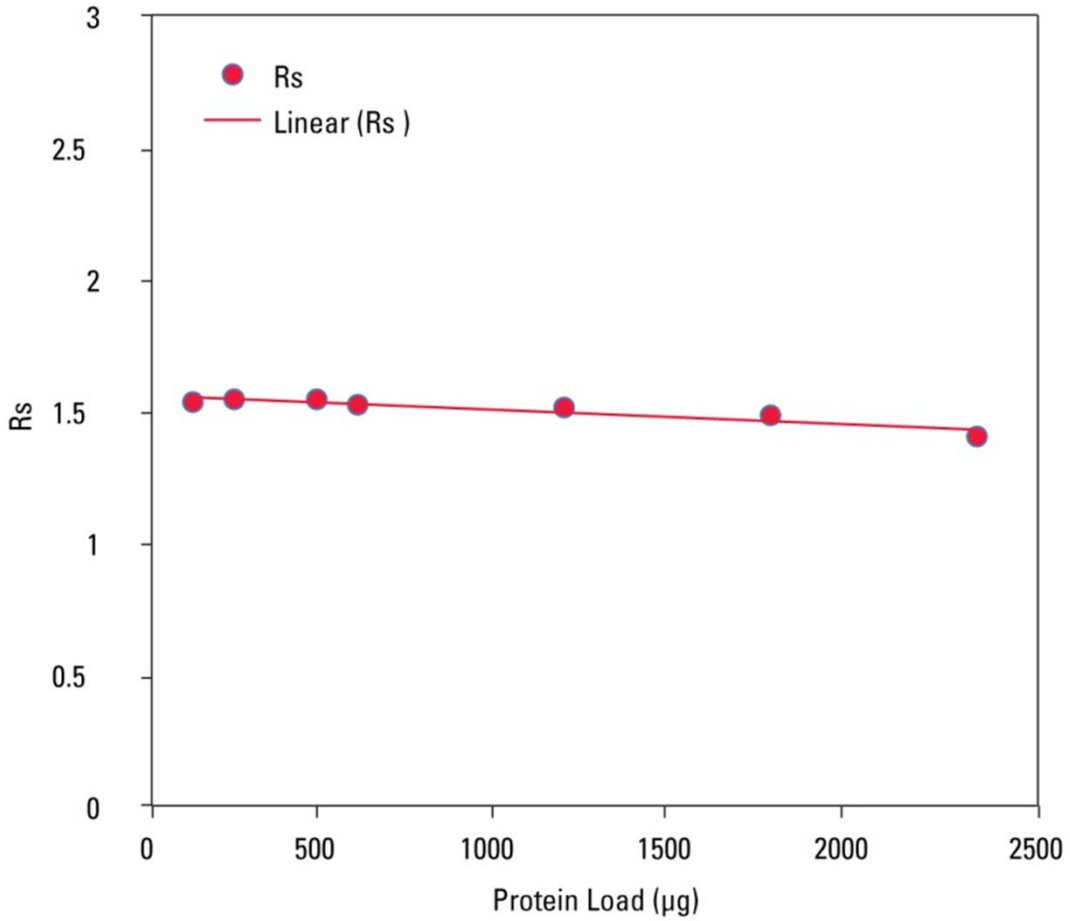
- The loading capacity of a SEC column with defined dimensions depends on the sample.
- The loading capacity can be increased by increasing the column length or diameter.
- Increasing column length also increases resolution and retention time, leading to additional separation time and mobile phase.
- This study shows that using a TSKgel G2000SWXL, 5µm, 7.8 mm ID × 30 cm analytical column even with a high load of Bovine Serum Albumin yielded a well resolved peak without any splitting.
- The monomer-dimer peak could be baseline resolved.

Effect of protein load on the retention time in the analysis of BSA using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



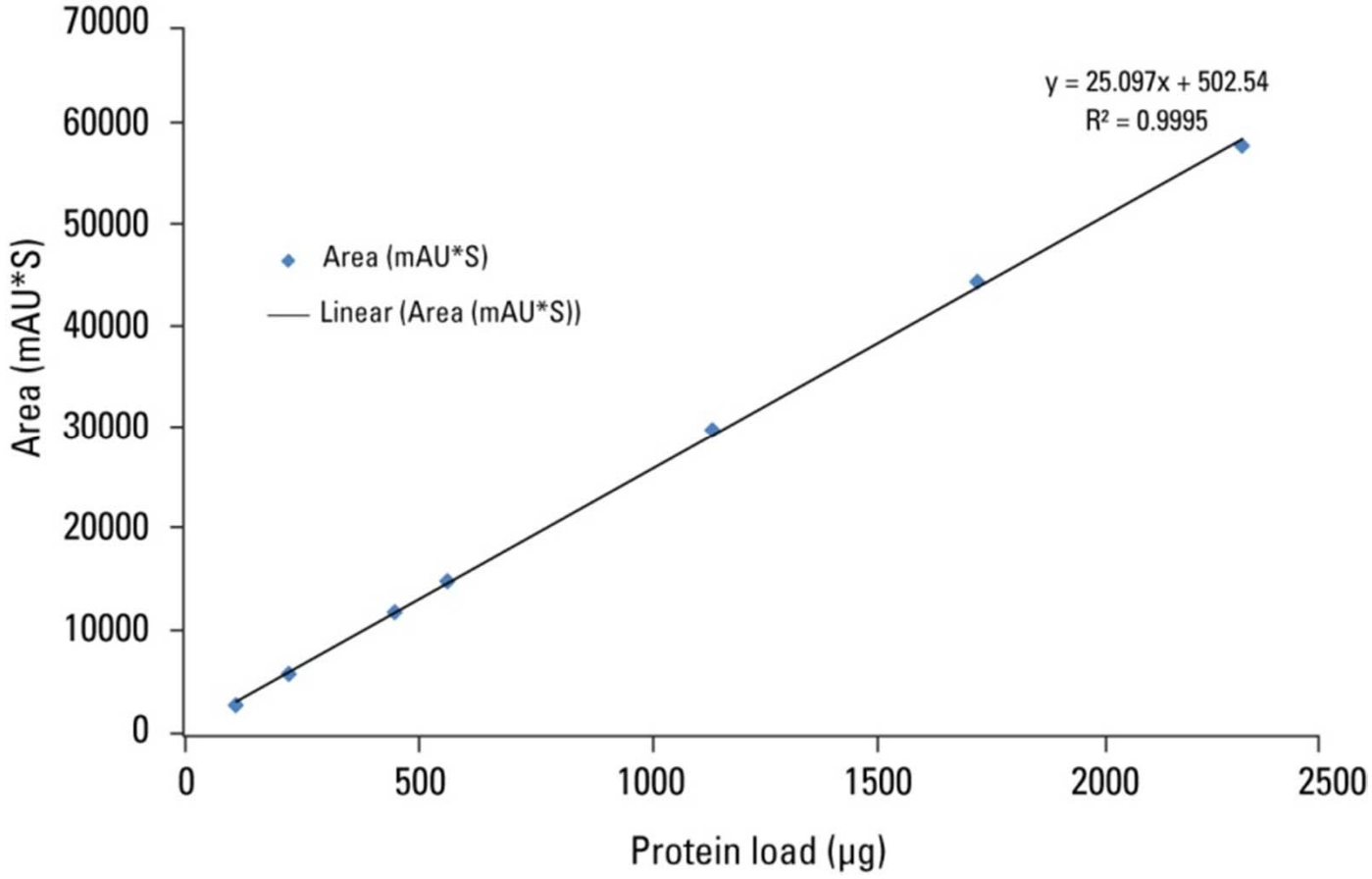
Retention times of both monomer and dimer peaks were consistent with very low %RSD over the range of protein load studied.

Effect of protein load on the resolution between dimer and monomer in the analysis of BSA using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



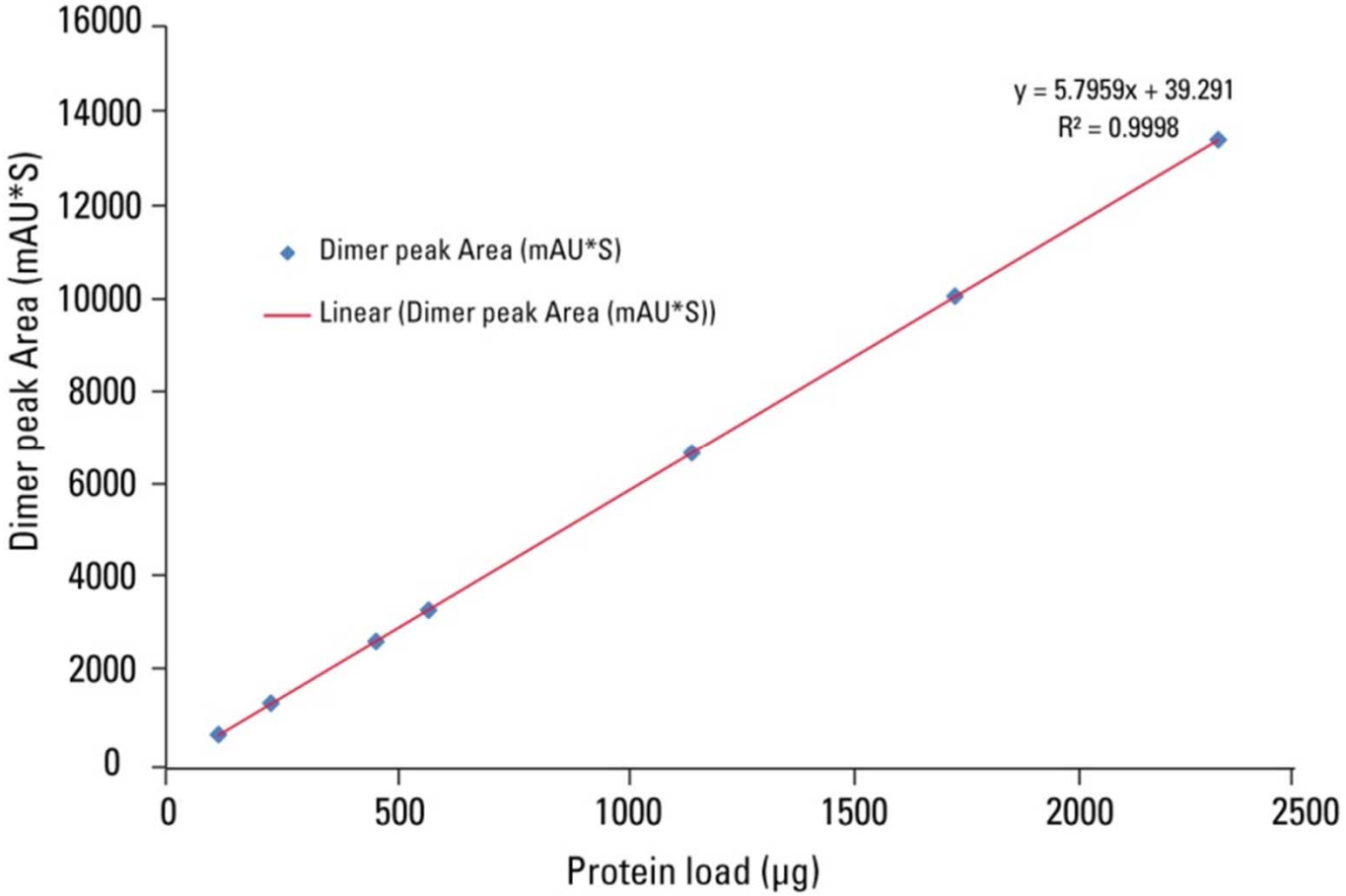
- The %RSD of resolution was <2 up to a load of 1.747 mg.
- The value increased to 3.3 with the load of 2.33 mg.

Effect of protein load on the monomer peak area in the analysis of BSA using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



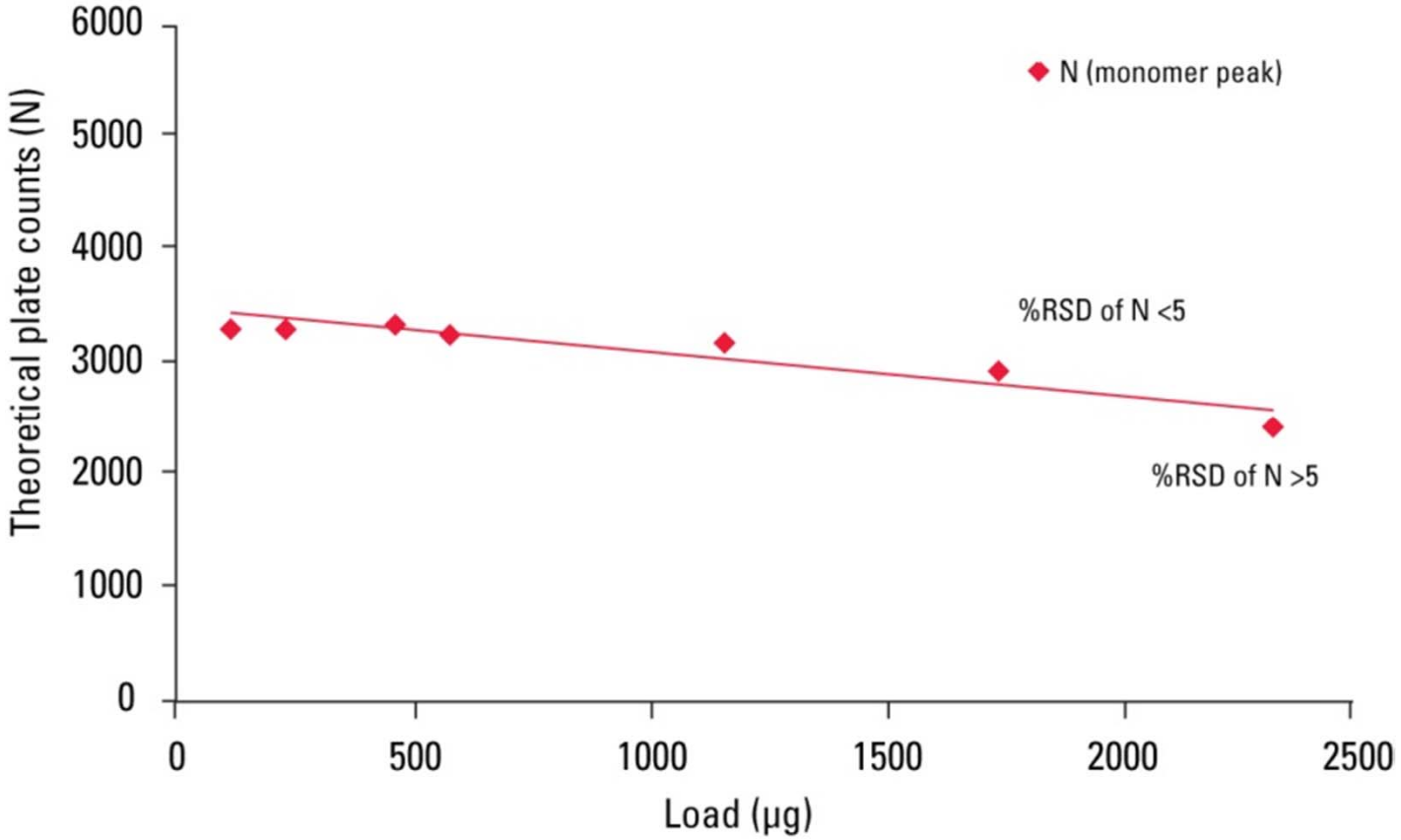
Excellent linearity in the monomer peak area was obtained over the experimental range of the protein load.

Effect of protein load on the dimer peak area in the analysis of BSA using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



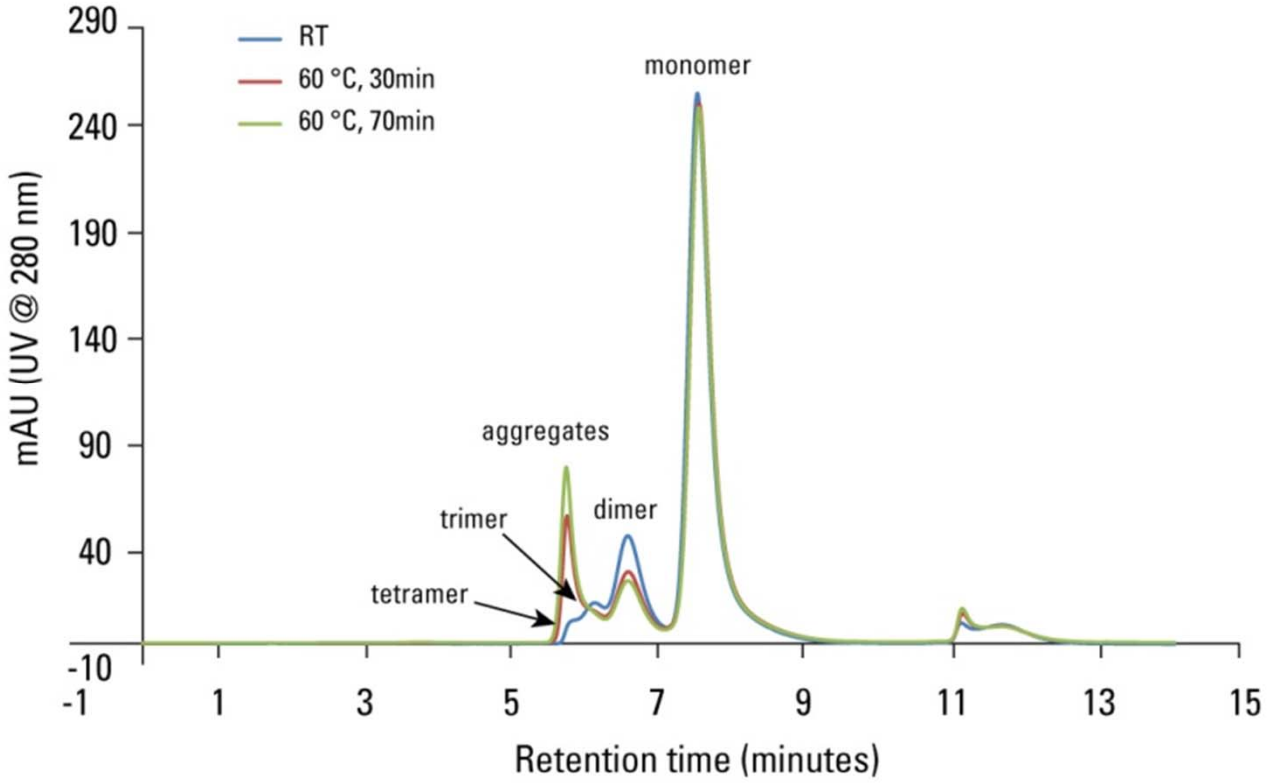
Excellent linearity in the dimer peak area was obtained over the experimental range of the protein load.

Effect of protein load on the efficiency of the column in the analysis of BSA using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



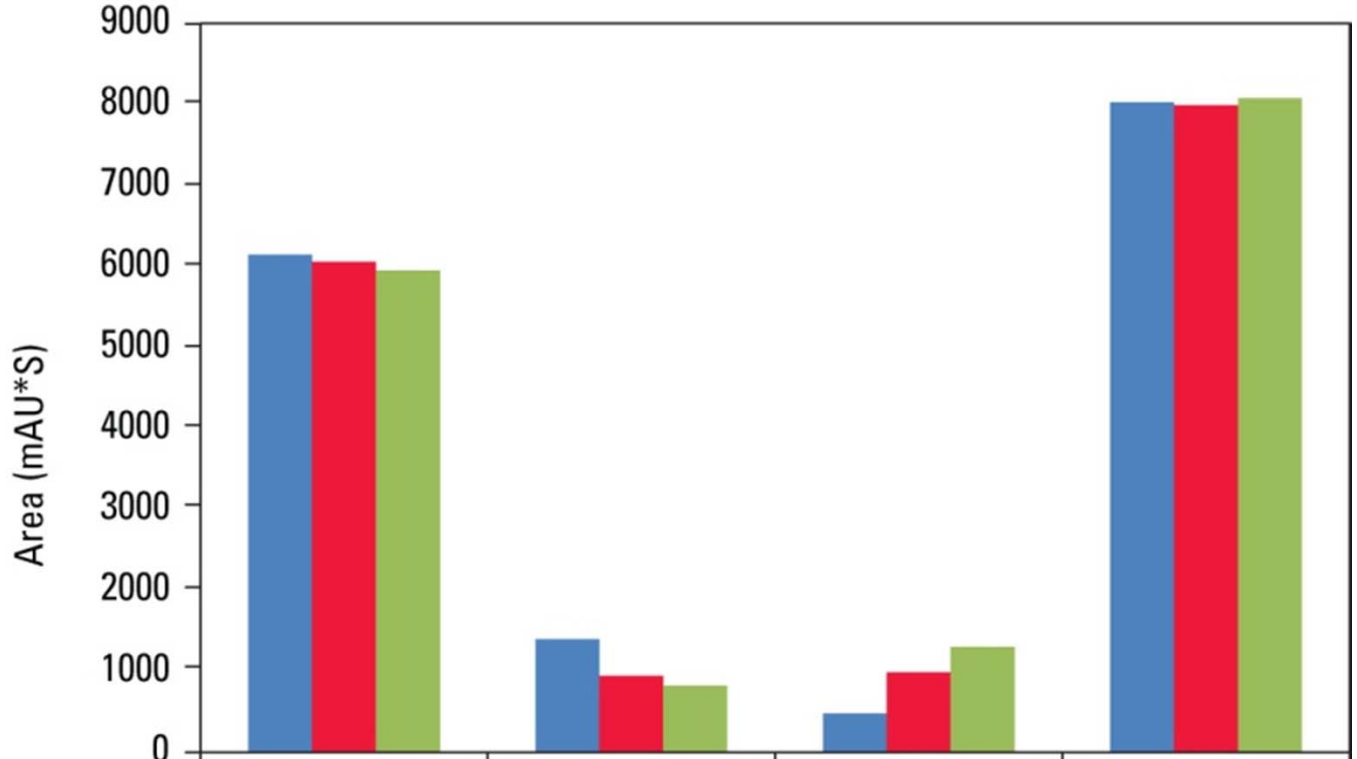
- The theoretical plate count was 3,311 for a load of 116.5 µg of BSA.
- This analytical column maintained high efficiency over a broad range of protein load.

Analysis of heat denatured BSA (forced degradation) using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



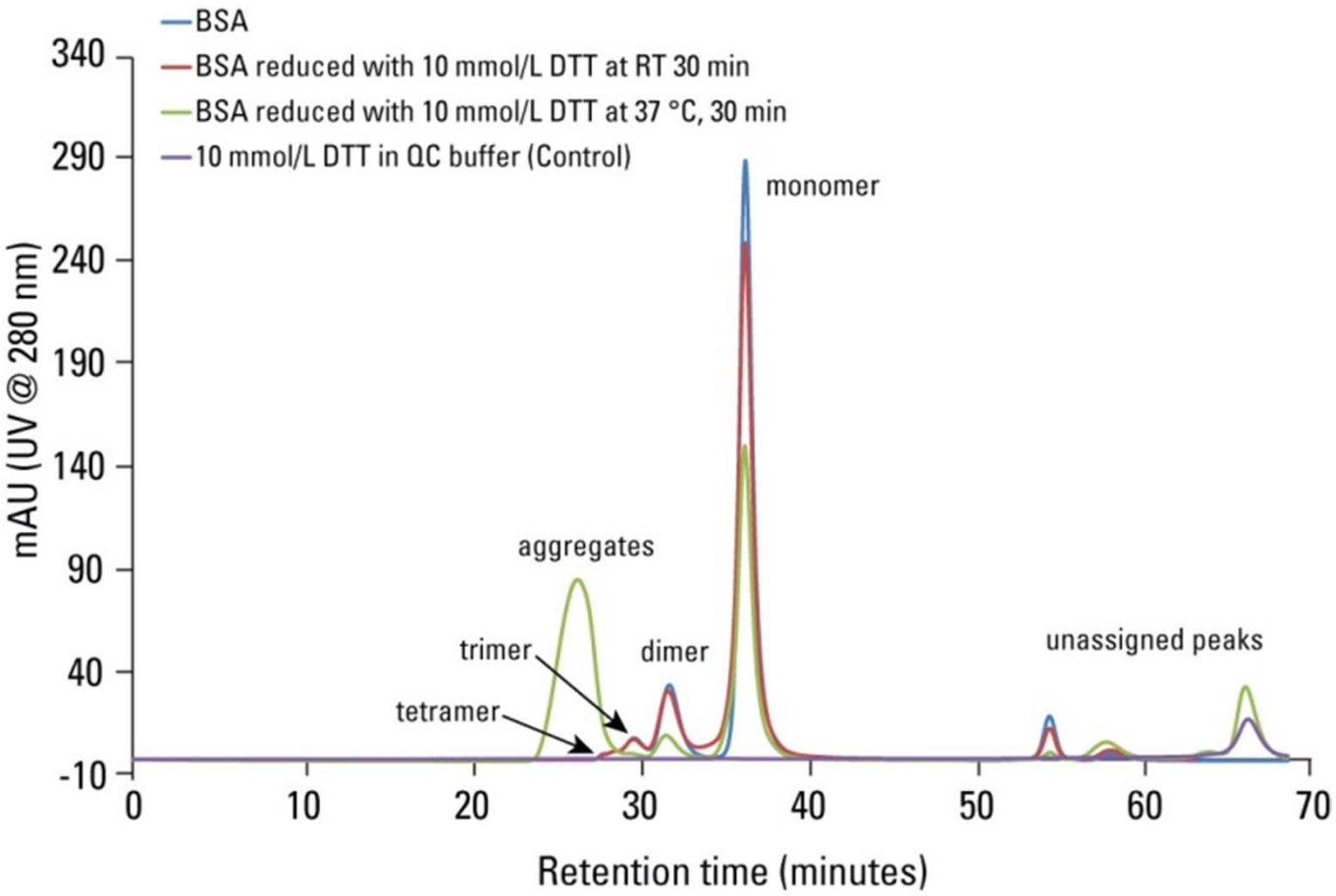
- The intensity of the dimer and monomer peaks are decreased while that of the aggregate is increased upon heating the protein sample at 60 °C.
- Similar forced denaturation of the proteins by acid denaturation can be separated using this column (data not shown here).

Analysis of heat denatured BSA (forced degradation) using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column: peak area distribution



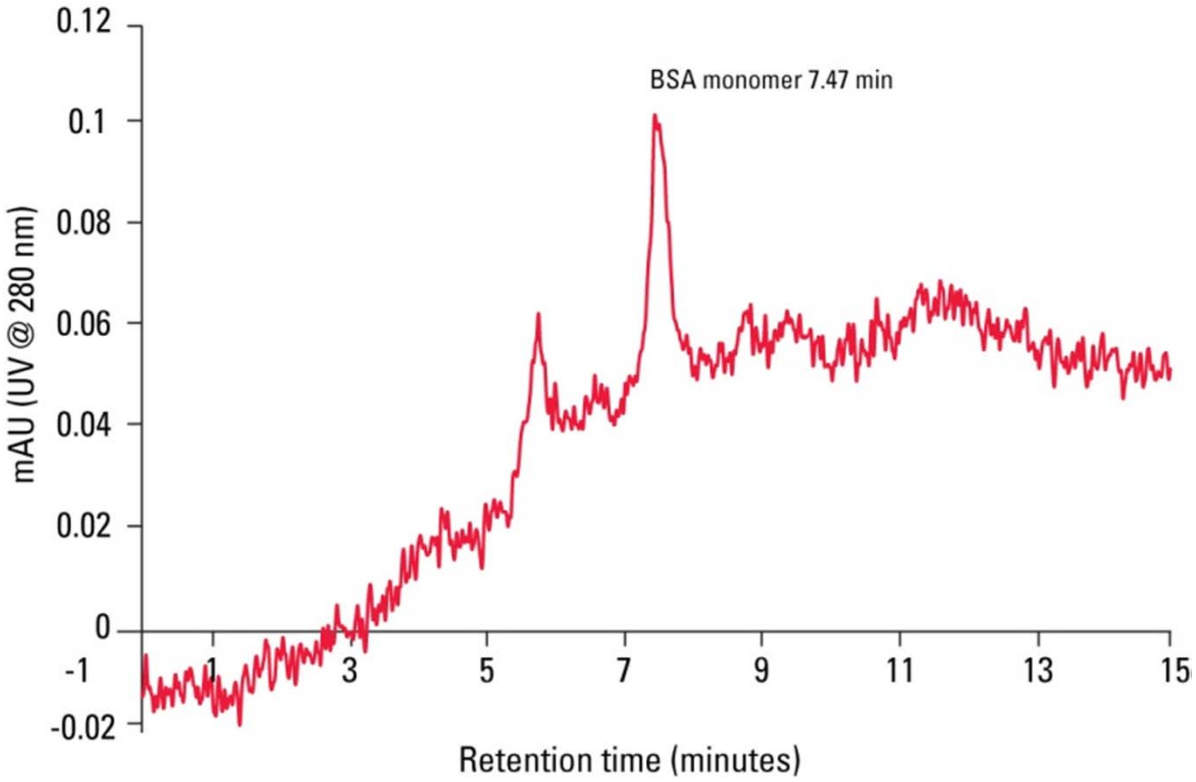
	Monomer peak Area (mAU*S)	Dimer peak Area (mAU*S)	Trimer peak Area (mAU*S)	Total peak Area (mAU*S)
RT	6147	1398	479	8024
60 °C, 30 min	6055	946	989	7990
60 °C, 70 min	5952	822	1301	8075

Analysis of reduced BSA using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



The increased amount of aggregate upon reduction of BSA could be successfully separated to the baseline from the dimer and monomer peak.

Determination of LOD and LOQ of BSA using a TSKgel G2000SWXL, 5 µm, 7.8 mm ID × 30 cm column



- The stock sample was diluted by QC buffer to reach the limit of detection.
- The LOD of BSA (monomer) was determined to be 46 ng.
- Therefore LOQ of BSA (monomer) was determined to be 460 ng.
- The LOD of BSA (for both monomer and dimer) was determined to be 92 ng.
- Therefore LOQ of BSA (monomer) was determined to be 920 ng.

Conclusions

- A TSKgel G2000SWXL column could be used for the separation of a BSA monomer from its dimer and aggregates.
- BSA did not yield any split peak even with a higher load.
- Protein standard mixture and BSA runs yielded excellent reproducibility.
- Efficiency of the column was measured and consistently yielded very high value.
- The column could be used for the forced degradation study.
- The TSKgel G2000SWXL, 5 μm, 7.8 mm ID × 30 cm column (S5018-03S) is suitable for the separation of monomer from dimer and aggregates of BSA and other proteins separable by this column.