Stability Studies of a Silica-Based Diol-Bonded Size Exclusion Chromatography Column For Protein Separations

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Objective:

To study the column lifetime of TSKgel G3000SWXL: a silica-based, diol-bonded size exclusion chromatography column widely used for the separation of monoclonal antibodies.

Introduction

- Size exclusion chromatography (SEC) performed under aqueous conditions, also known as Gel Filtration Chromatography (GFC), is popular for isolation and quality control of monoclonal antibodies and other therapeutic proteins and peptides.
- Analytical size exclusion chromatography (SEC) columns are costly, so a stable column yielding a high degree of reproducibility of retention time, peak symmetry, and column efficiency over a large number of injections is very important to the analyst.
- Over many years now, column to column reproducibility and column lifetime consistently remain as the top two factors - above price - to chromatographers when selecting an appropriate column (as shown in the next slide).
- Development of a reliable analytical HPLC method requires these qualities to be independent of the lot of base silica as well as the bonding and packing procedures.

Introduction

Factors considered when selecting an HPLC column supplier			
Factor	**Respondents (Normalized %)**		
	2007	**2009**	**2011**
Column-to-column reproducibility	21	21	19
Column lifetime	16 **37%**	17 **39%**	15 **34%**
Price	12	14	13
Reputation of company	14	12	9.6
Column plate number	8.7	9.1	9.1
Technical assistance	5.7	5.8	6.7
Variety of phases available	4.5	4.0	6.4
Tailing factor	6.3	5.4	5.0

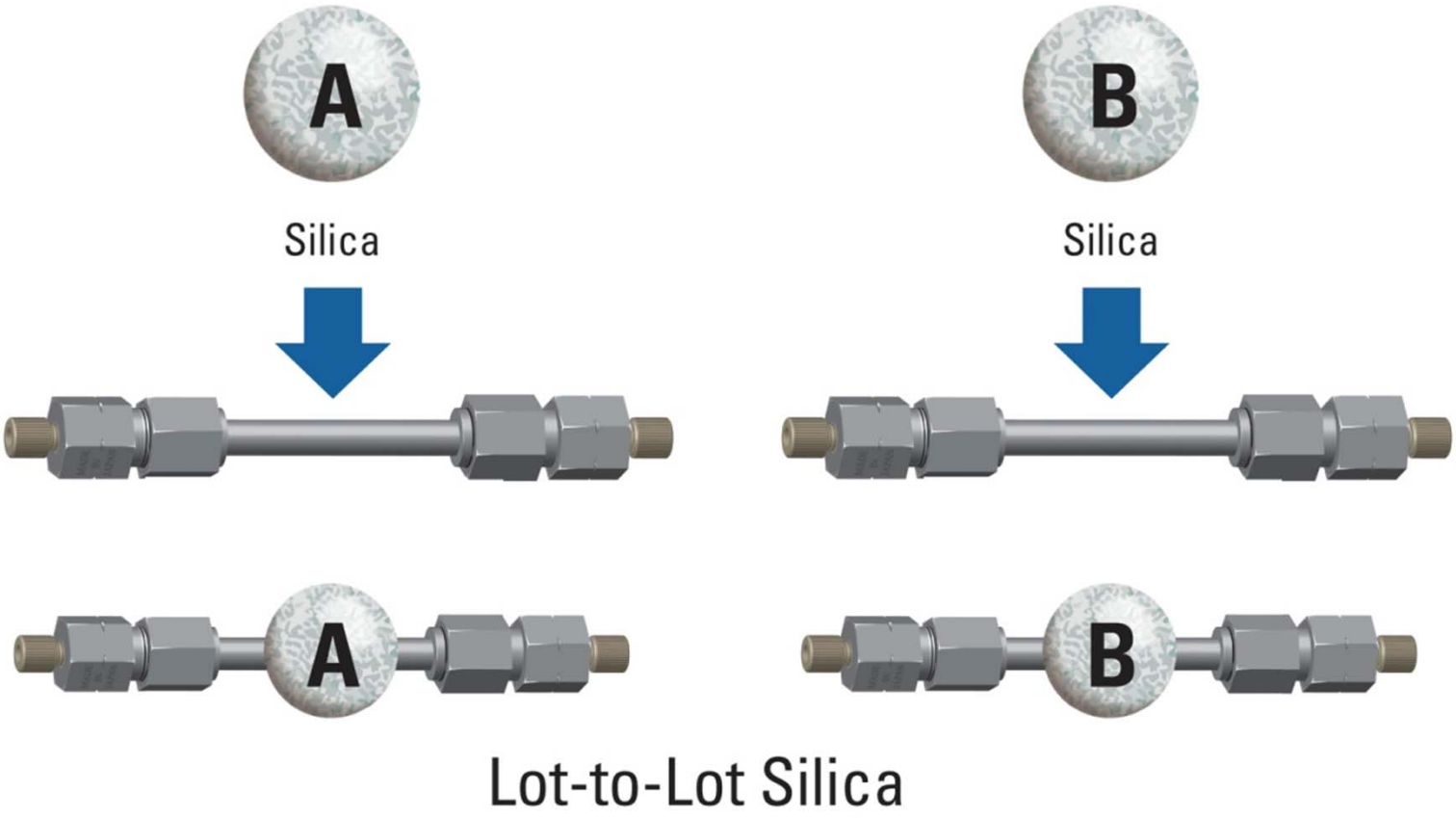
Ref: LCGC: Jan 1, 2012; Article: Current trends in HPLC column usage – By: Ron Majors

- The survey clearly shows the importance of column lifetime and reproducibility over price to chromatographers in the selection of a column.
- The survey also shows this same pattern over a number of years.

Introduction

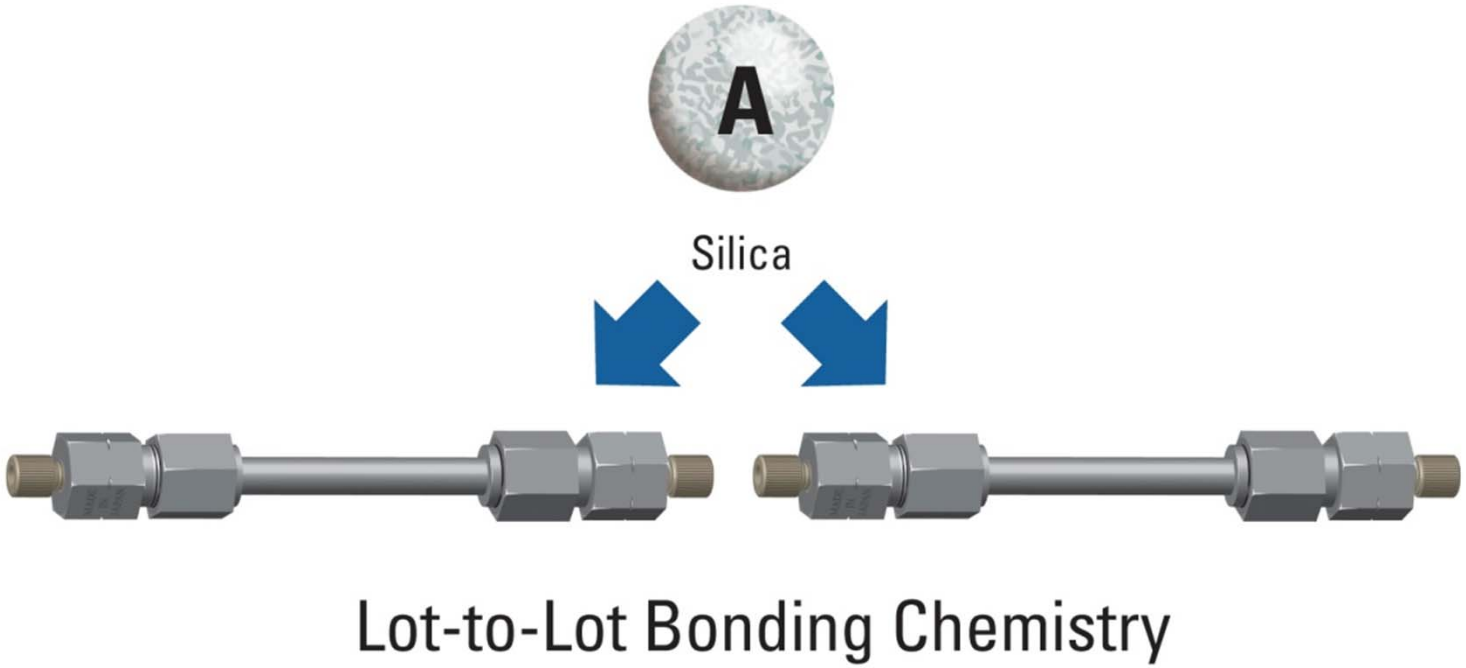
- A loss of resolution, peak broadening, or significant tailing, factors that may affect quantitation, are symptoms of column failure.
- One of the most important warning signs that a column may be on the verge of failing can be predicted by a gradual increase in backpressure.
- Retention time shift can sometimes be related to a loss of packing material or stationary phase.
- The other factors chromatographers consider before declaring the column dead are: failure of an established method specification, failure to pass QC test using a standard protein mixture, failure to pass system suitability requirements, a high % RSD (relative standard deviation) value over a number of consecutive injections.
- Although the use of a guard column to protect the analytical column is highly recommended and ought to be part of a standard operating procedure, in practice not all users do so.

Introduction



Two different sources of silica can be a factor in lot-to-lot reproducibility.

Introduction



Bonding chemistry developed on the same silica at different times can also be a factor in lot-to-lot reproducibility.

Introduction

- In this presentation we report the results of a column lifetime study of a silica-based TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm SEC column: a column type that is widely used for the separation of monoclonal antibodies.
- Altogether 9 columns were used to study the effect of reproducibility arising from both silica and bonding chemistry, as explained below.

  - Lot 09R – 3 columns (S1261, S1262, S1263)
  - Lot 08R – 3 columns (S1237, S1238, S1239)

  Silica Lot A

  - Lot 30P – 3 columns (S6210, S6211, S6212)

  Silica Lot B

- Specification for TSKgel G3000SWXL column passing QC:

  N (PABA) >20,000 and AF = (0.7 – 1.6)

Material and methods

Chromatographic Conditions

Instrument:	all analyses were carried out using an Agilent 1200 HPLC system run by Chemstation (ver B.04.02).
Mobile phase:	100 mmol/L KH_2PO_4/K_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.:	10 µL
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)
	Monoclonal antibody: BI-mAb-2 from Boehringer-Ingelheim; concentration: 4.5 g/L in glycine/Na phosphate, pH 6.0
	Bovine Serum Albumin (Sigma Aldrich A7906, Lot # 080M1251V; >98% purity, 10.2 mg/mL
	Human sera (Sigma S7023-50mL), neat - no dilution

Material and methods

In this study:

- sample and mobile phase were not filtered through 0.45 micron syringe filter
- no guard column was used to provide extra stress on the column
- In one case the mobile phase was recycled to provide additional stress to the column, basically to encourage column failure
- column was not cleaned throughout the experiment
- 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

Figure 1: Analysis of protein standard mixture using a TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm column (S6212-30P)

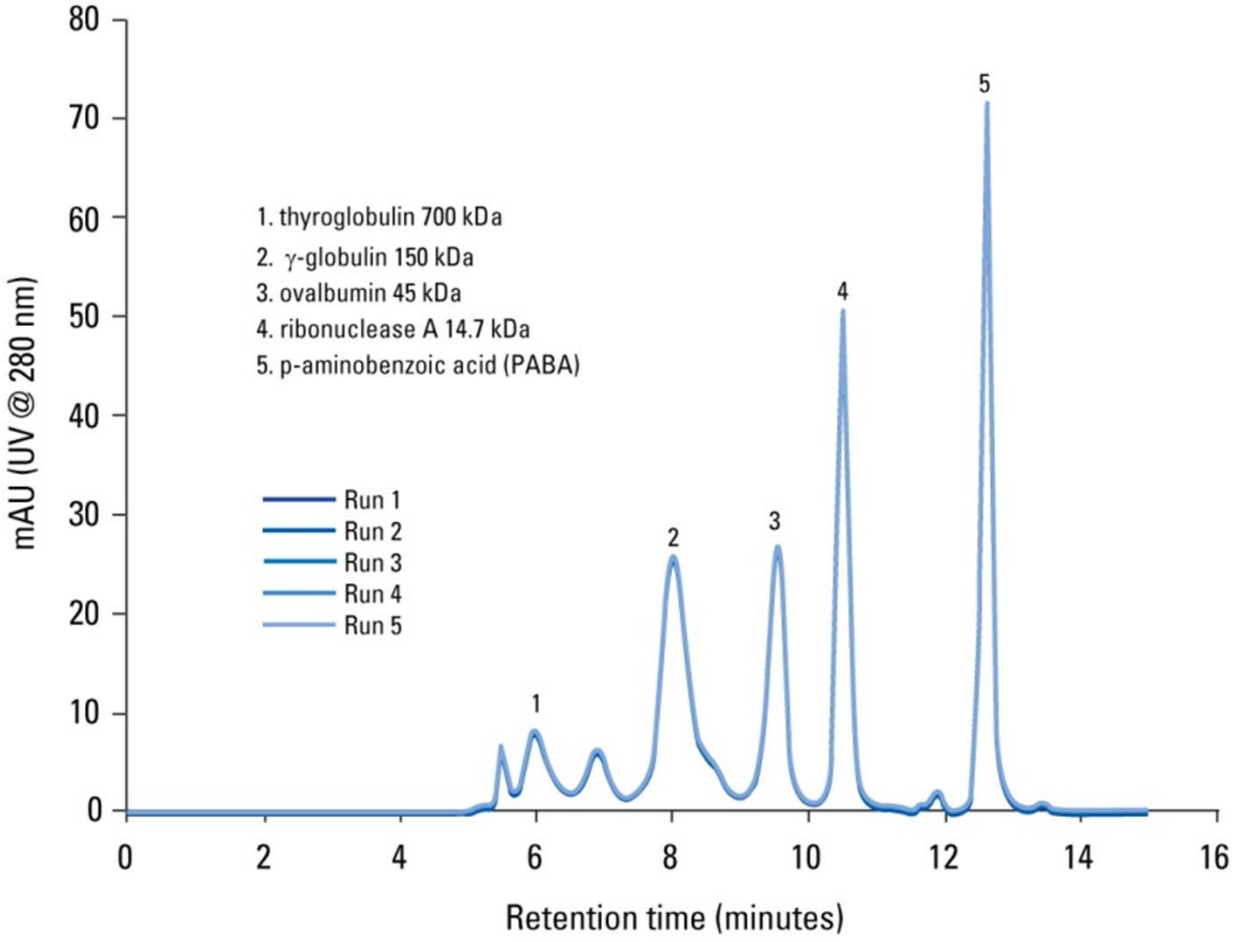


Figure 1: Analysis of protein standard mixture: conclusions

- 5 consecutive runs yielded an excellent reproducibility: very low % RSD value of all the peak parameters.
- Similarly, all 9 columns from different silica and bonding lots yielded excellent reproducibility in 5 consecutive runs: N was always ~32,000 and other peak parameters also remained consistent
- The following table shows the change of retention time of protein peaks as a function of silica and bonding chemistry

Table 1: Change of retention time of protein peaks as a function of silica and bonding chemistry

Retention times								
Column #		Thy			γ-Glo	Ova	Ribo-A	PABA
		Peak 1	Peak 2	Peak 3				
S1237-08R		5.547	5.993	6.971	8.084	9.546	10.596	12.638
S1238-08R		5.571	6.072	6.994	8.110	9.636	10.620	12.740
S1239-08R		5.546	5.996	6.977	8.083	9.601	10.579	12.672
S1261-09R		5.472	5.922	6.895	8.000	9.528	10.506	12.639
S1262-09R		5.476	5.989	6.914	8.024	9.544	10.513	12.613
S1263-09R		5.469	5.993	6.924	8.038	9.552	10.518	12.577
S6210-30P		5.544	5.969	6.939	8.050	9.588	10.577	12.725
S6211-30P		5.499	5.993	6.924	8.039	9.572	10.539	12.613
		5.493	5.981	6.904	8.018	9.549	10.517	12.622
	Average	5.513	5.990	6.938	8.050	9.568	10.552	12.649
	STDEV	0.039	0.039	0.035	0.036	0.034	0.042	0.054
	%RSD	0.707	0.645	0.502	0.446	0.358	0.398	0.427

Figure 2: Injection-to-injection reproducibility: within column

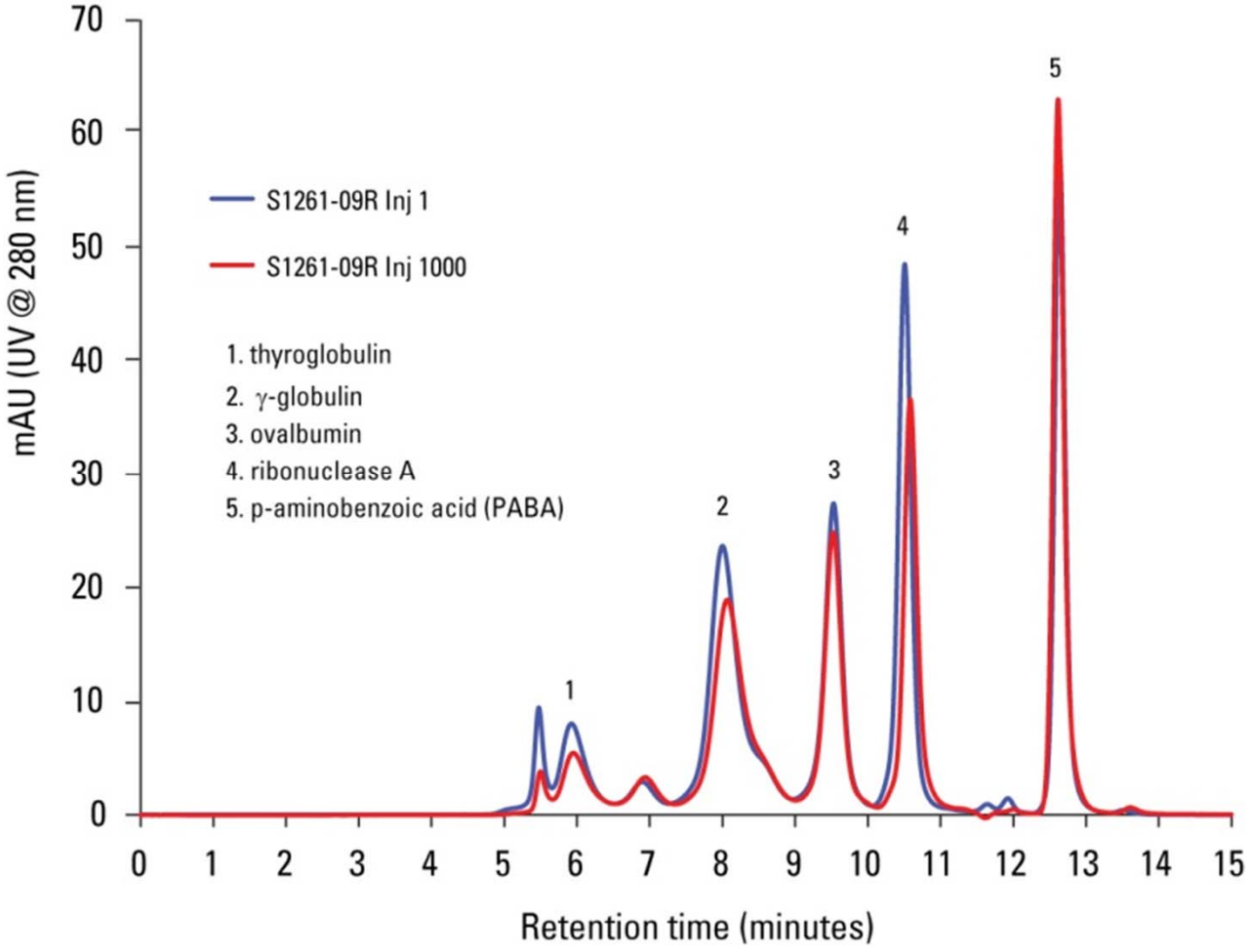


Figure 2: Injection-to-injection reproducibility: conclusions

- This study shows that the column remained stable and precise over 1000 injections. More analytical data is shown in the following tables.
- The difference in protein peak heights and areas between injection 1 and 1000 is due to the fact that protein concentrations were different; PABA concentration remained the same.
- Proteins were individually weighed and added to the fresh aliquot from the same stock of 10× PABA stock followed by final volume make-up

Table 2: Retention time, efficiency, and peak symmetry at the 1st, 500th and 1000th injections

	γ-globulin			p-aminobenzoic acid		
	Inj. 1	Inj. 500	Inj. 1000	Inj. 1	Inj. 500	Inj. 1000
t_R	8.065	8.027	8.065	12.635	12.647	12.616
N	2021	2002	1847	32,483	33,187	32,381
AF	1.47	1.47	1.49	1.19	1.21	1.28

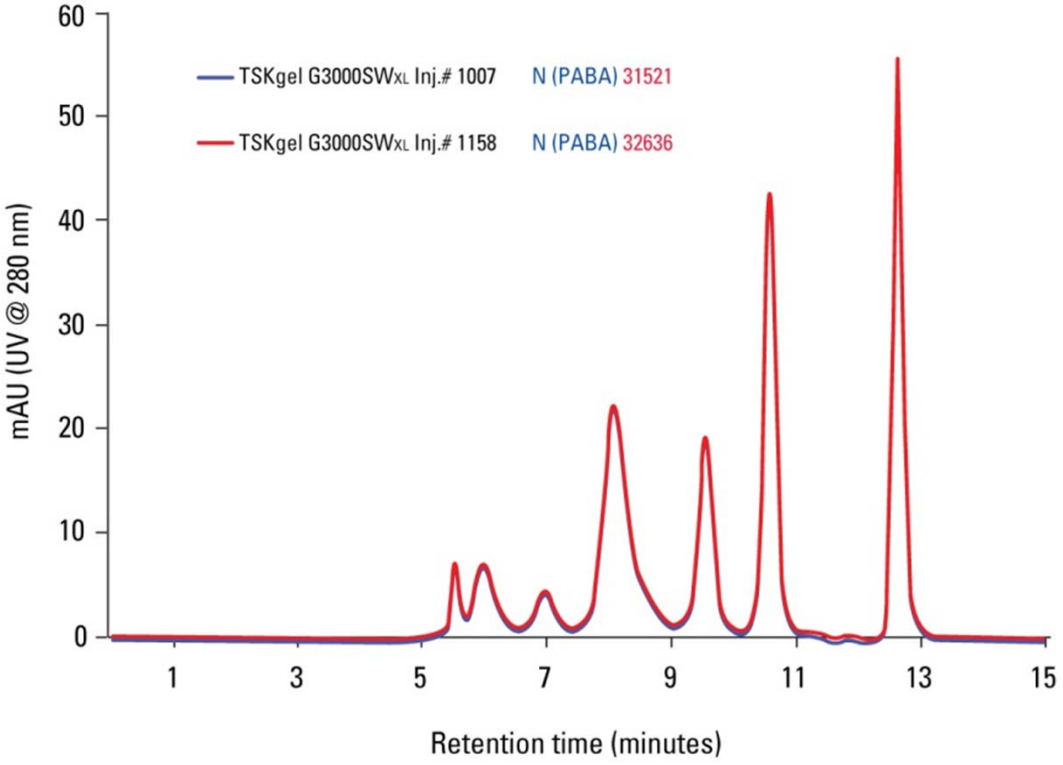
Table 3: % RSD of peak parameters for each 10th injection during the 1000 injection cycle (n = 100)

	thyroglobulin	γ-globulin	ovalbumin	rib. A	PABA
t_R	0.16	0.21	1.05	0.20	0.28
N	5.48	5.27	2.40	2.87	1.70
AF	4.32	1.38	2.24	2.56	2.66

The results shown in tables 2 and 3 show that 3 important chromatographic peak parameters: 1) retention time (t_R), 2) theoretical plate count (N), and 3) asymmetry factor (AF) remained consistent over 1000 runs.

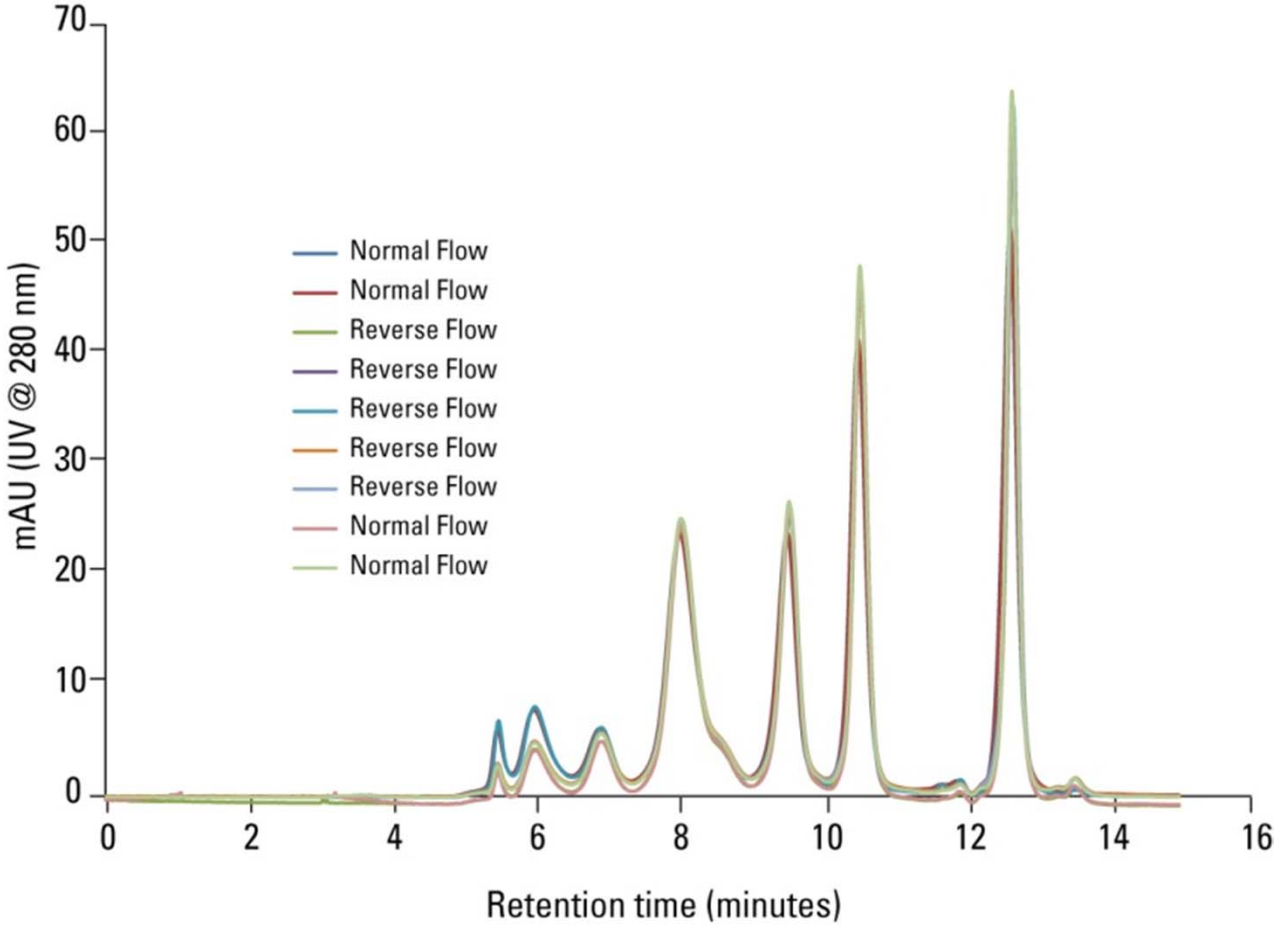
Figure 3: Column integrity test of a TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm column (S1239-08R)

- This column was already used for **1007** injections of protein standard mixture with **N (PABA) = 31,521**.
- Then, 100 uL QC buffer was injected each time (instead of 10 µL).
- Injections were repeated **150 times every 1 minute to provide pressure on the column bed.**
- This is **equivalent to 1500 injections of 10 µL** buffer.
- This is also equivalent to a total of 2507 injections of 10uL injection volume each time.



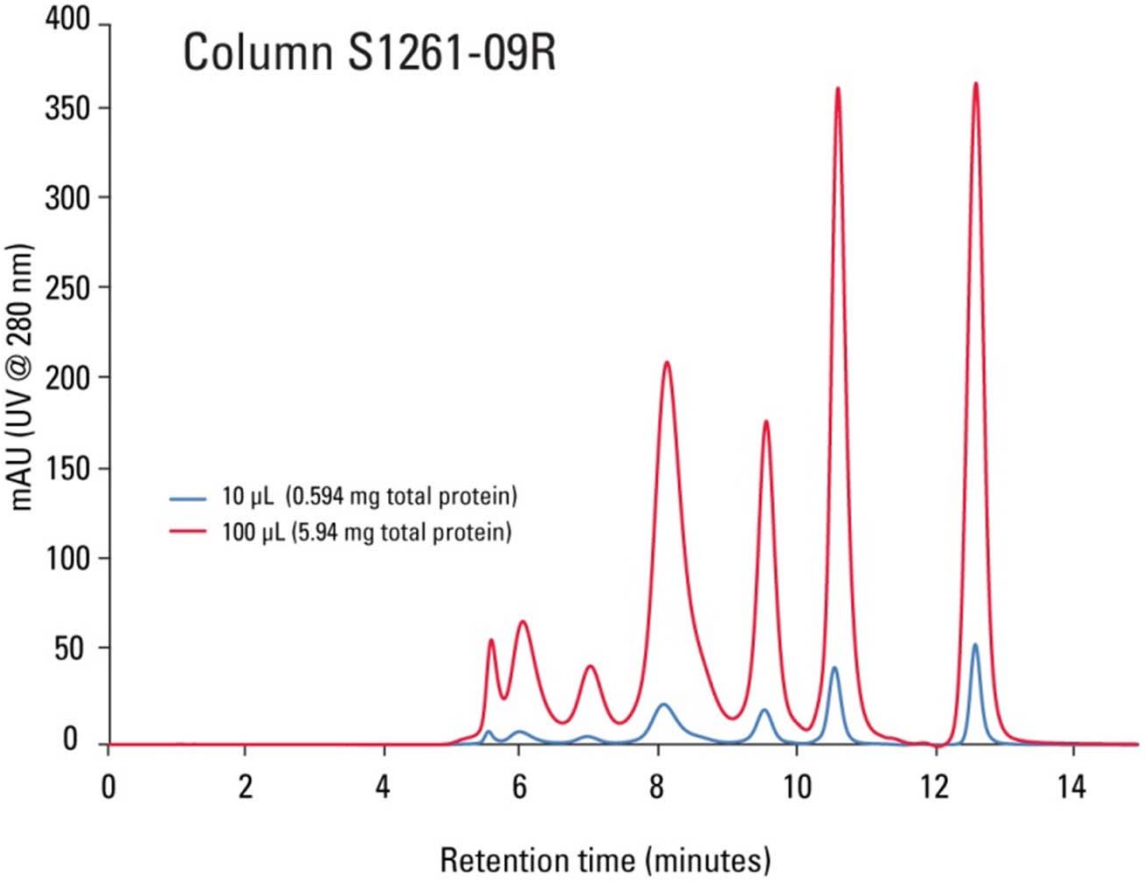
- The results show superb reproducibility in RT, AF and N.
- Result could be reproduced with another column.

Figure 4: Analysis of protein standard mixture using a TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm column (S1262-09R)



- No change in RT, AF or N observed over 200 injections in reverse flow on this column.
- The result shows that the column has an excellent packing integrity.

Figure 5: Loading capacity of protein standard

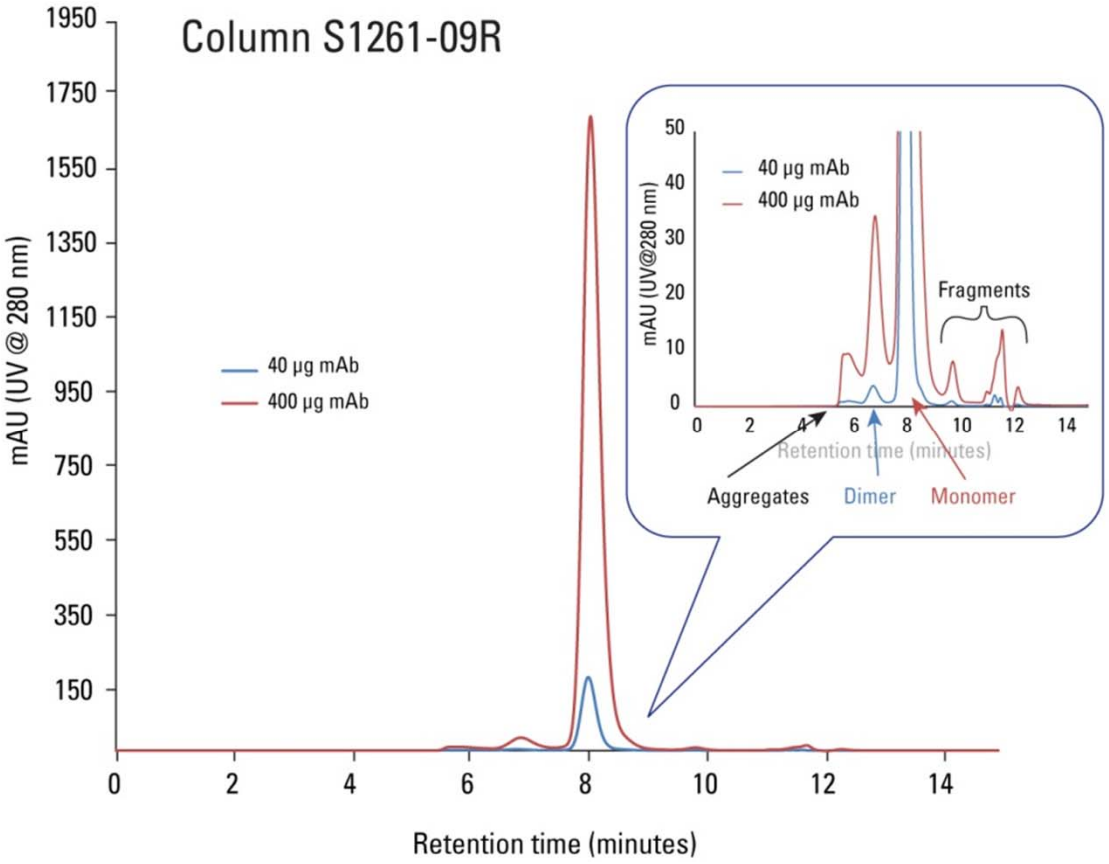


	γ-globulin	
	10 µL Inj.	100 µL Inj.
t_R	8.084	8.132
N	2007	1688
AF	1.42	1.45

Figure 5: Loading capacity of protein standard: conclusions

- 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.
- A 10 fold increase in total protein content did not affect the retention time, symmetry of the peak, or efficiency of the column.

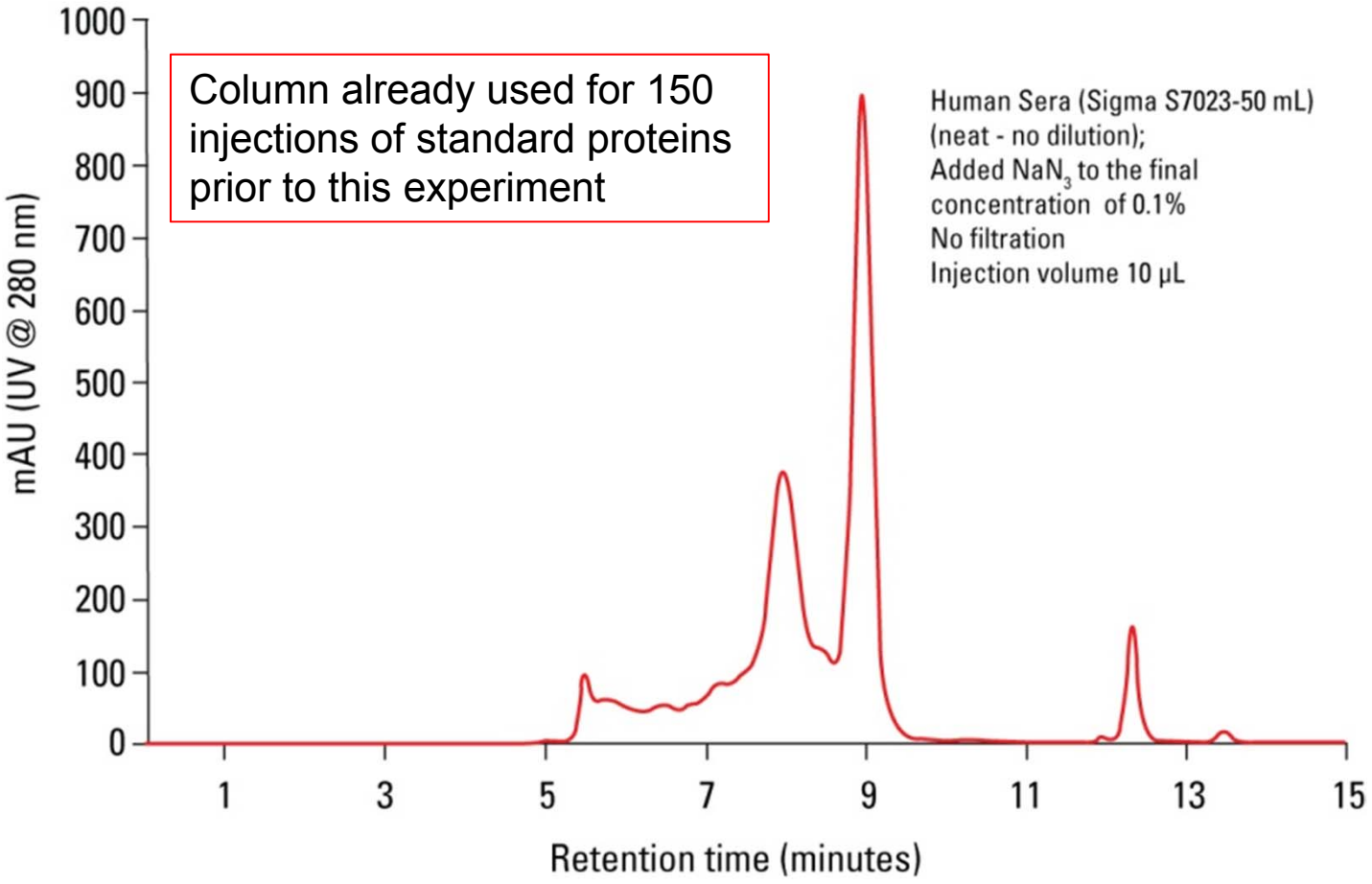
Figure 6: Loading capacity of monoclonal antibody upon a TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm column



	Monoclonal antibody	
	40 µg Inj.	400 µg Inj.
t_R	8.023	8.061
N	4500	3508
AF	1.22	1.35

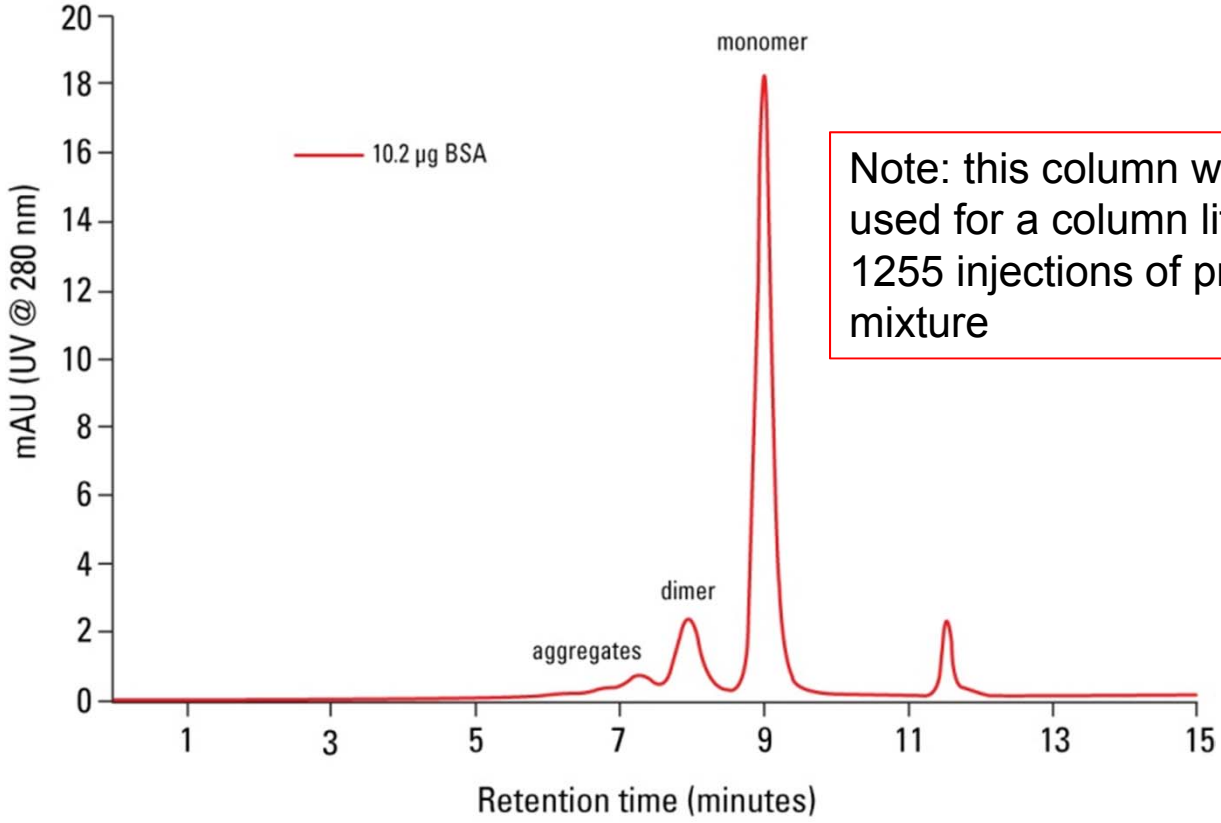
Peak shape and efficiency were not affected when injecting 400 µg of a monoclonal antibody preparation.

Figure 7: Analysis of human sera using a TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm column (1262-09R)



The column maintained its efficiency up to injection #300.

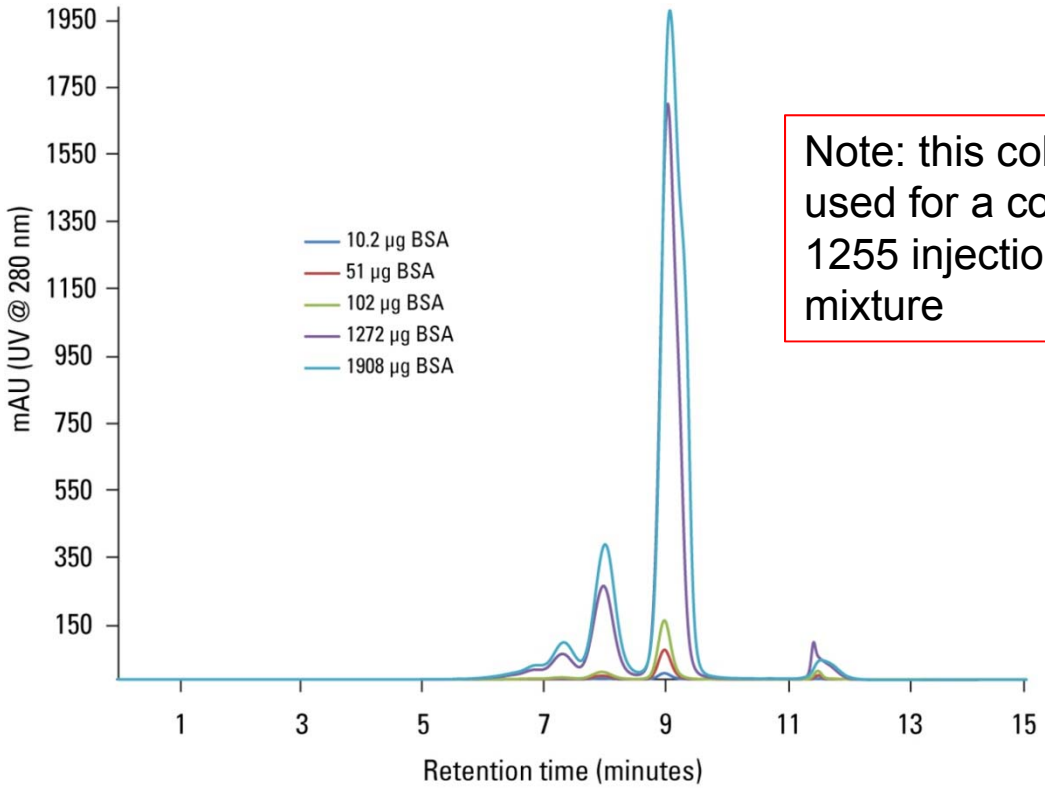
Figure 8: Analysis of BSA using a TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm column (1262-09R)



Note: this column was previously used for a column lifetime study for 1255 injections of protein standard mixture

- The TSKgel G3000SWXL column was very stable even after 1255 injections.
- In addition, baseline separation of the monomer from the dimer and aggregates was achieved.

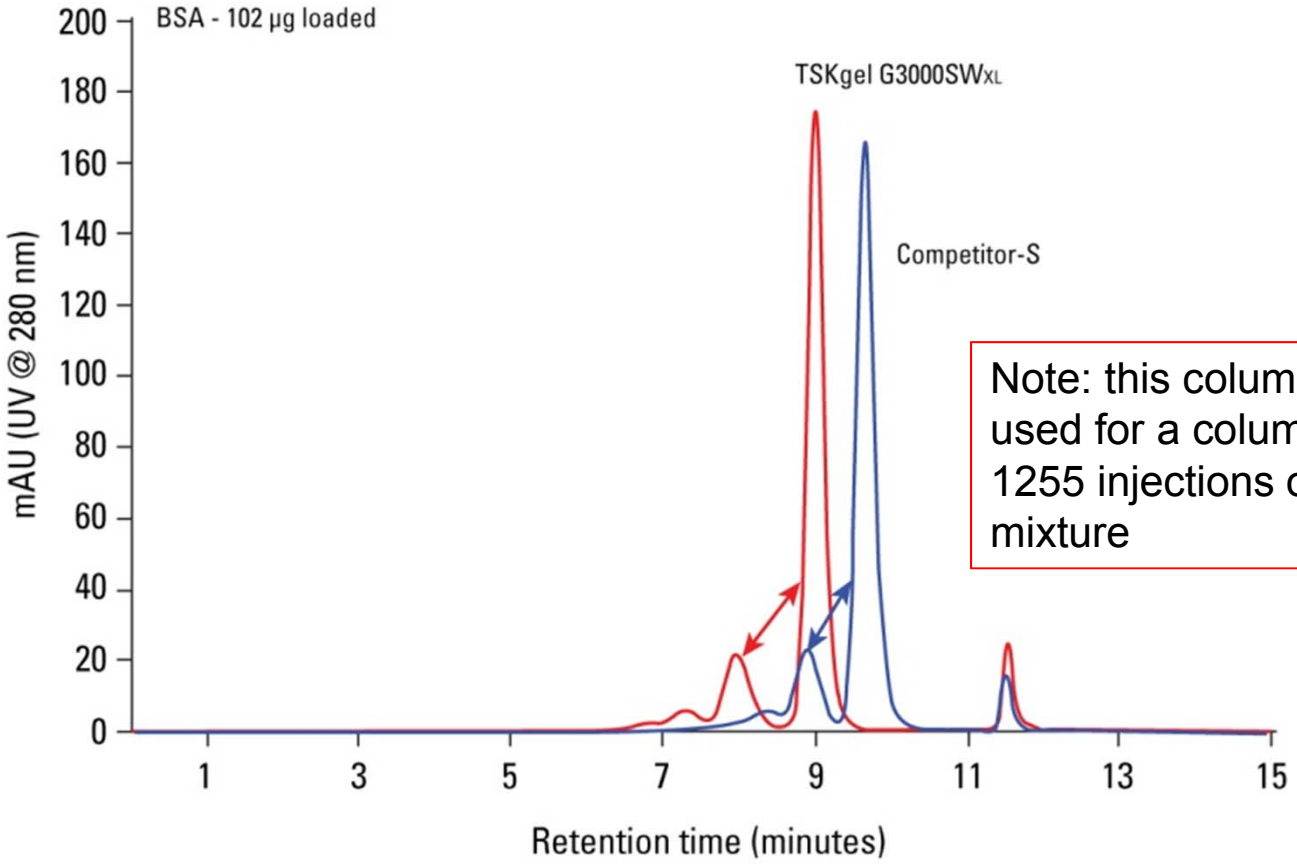
Figure 9: Analysis of BSA using a TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm column (1262-09R)



Note: this column was previously used for a column lifetime study for 1255 injections of protein standard mixture

- Even with 1.9 mg load of BSA, the dimer and monomer peak was separated to the baseline.
- The monomer peak did not split with the higher load.

Figure 10: Analysis of BSA using a TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm column (1262-09R) and Competitor-S, 5 µm, 7.8 mm ID × 30 cm column



Note: this column was previously used for a column lifetime study for 1255 injections of protein standard mixture

Compared to a competitive column, the TSKgel G3000SWXL column has much higher resolution of monomer and dimer peaks.

Figure 11: Backpressure of TSKgel G3000SWXL, 5 µm, 7.8 mm ID × 30 cm column

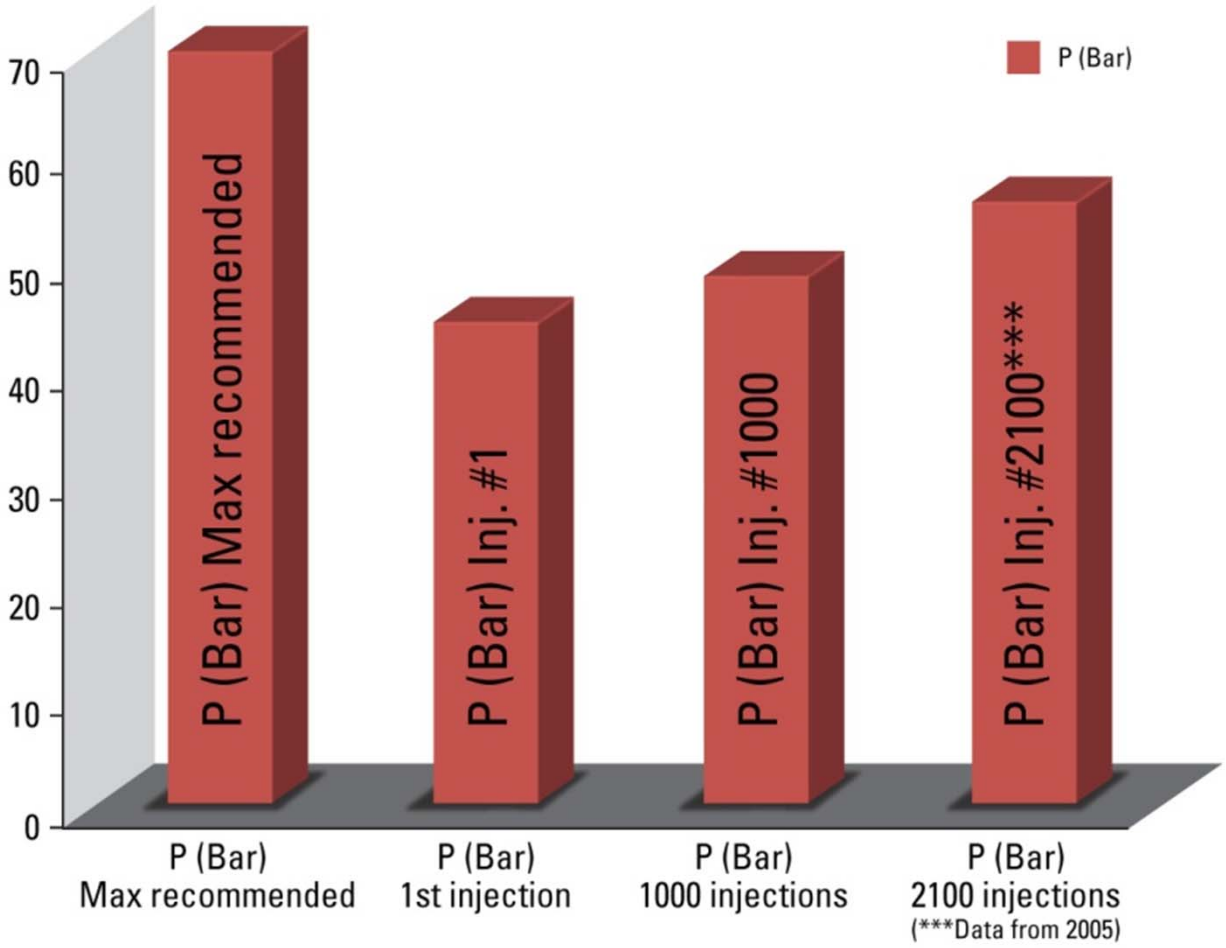


Figure 11: Backpressure: conclusions

- In the study in 2005 (marked by *** in the chart), the mobile phase was recycled to encourage column failure.
- Still the column maintained consistency in RT, AF and N over ~2900 injections of protein standard mixture, after that the % RSD was greater than 5%.
- The columns never reached or exceeded pressure limit in this study.
- This study shows that the column maintained its consistency in terms of packing and bonding chemistry over the years.

Conclusions

- Silica-based diol-bonded size exclusion chromatography TSKgel G3000SW$_{XL}$ columns are robust.
- Results show excellent reproducibility in retention time with a very low percent relative standard deviation (% RSD) of <1% (n=10) within the same lot and between the lots.
- The column had a very long lifetime under the experimental conditions of this study.
- Even with the monoclonal antibody and human sera samples the columns had a long lifetime.
- There was no change in the column matrix consistency over the years from 2005 until the present time.
- Lot-to-lot consistency is excellent irrespective of silica lot or bonding chemistry lot.
- The study shows the reliability and dependability of the column in the separation of proteins.
- Since columns were studied without guard column and filtration of the sample, mobile phase, etc., lifetime of the column can further be improved by using these features to further protect the column.

Further reading

Please refer to the TosohTalk blog on the Tosoh Bioscience LLC website: “How long does a column last?” – a discussion on how to take care of columns to increase their lifetime.