Stability Studies of a Novel Silica-Based, Diol-Bonded Size Exclusion Chromatography Analytical Column Specifically Designed for the Separation of Aggregates

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Highlights of This Study

- TSKgel UltraSW Aggregate, a novel silica-based, diol-bonded, 3 µm particle size, 30 nm pore size, size exclusion chromatography analytical column is robust and inherently stable.
- The durability of this inherently stable column can further be extended by proper maintenance of the column.
- This column lifetime study is currently ongoing with >900 injections.

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.
- Analytical size exclusion chromatography 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 lot-to-lot variations.

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

- The ideal SEC columns are expected to not have any secondary interaction, so that the proteins are separated based strictly on their hydrodynamic volume in solution.
- TSKgel SW columns are silica-based. A hydrophilic diol-type bonded phase shields the silica surface from interacting with protein samples.
- In a previous presentation (Fall ACS 2012, poster #54), it was shown that silica-based, diol-bonded SEC TSKgel G3000SW$_{XL}$, 5 µm, 25 nm columns are robust, with excellent reproducibility in retention time with a very low percent relative standard deviation (%RSD) of <1% (n=10) over 1,000 injections, irrespective of silica lot or bonding chemistry lot.
- The study showed the reliability and dependability of the TSKgel G3000SW$_{XL}$, 5 µm columns in the separation of proteins.
- A novel silica-based, diol-bonded, 3 µm, 30 nm SEC analytical column (TSKgel UltraSW Aggregate) was specifically designed for the separation of aggregates.
- Here we report a preliminary study on the column lifetime of a TSKgel UltraSW Aggregate column using protein standards.
    - Specification for TSKgel UltraSW Aggregate column passing QC:

      N(PABA) >35,000 and AF = 0.8 - 1.4

Objective

To study the column lifetime of a novel silica-based, diol-bonded, 3 µm, 30 nm SEC analytical column (TSKgel UltraSW Aggregate) specifically designed for the separation of aggregates.

Material and Methods

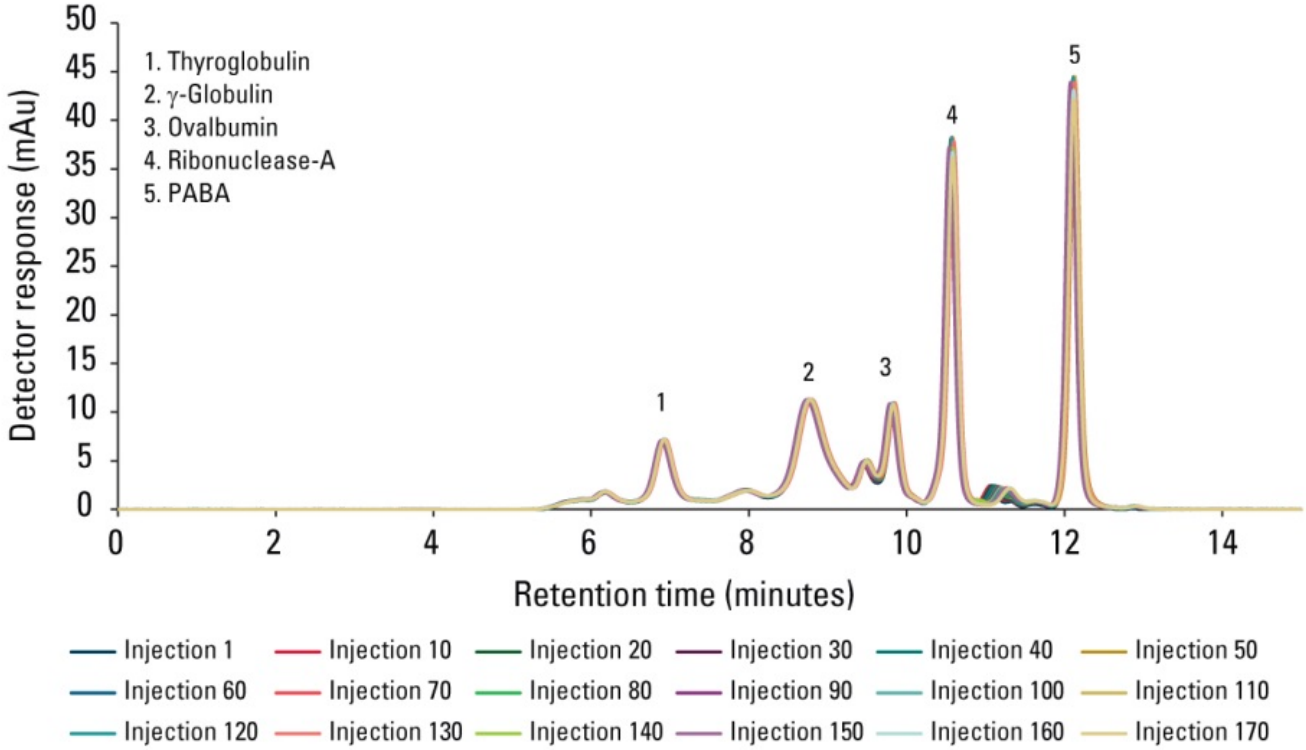
Column:	TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm
Instrument:	All analyses were carried out using an Agilent 1200 HPLC system run by Chemstation (ver B.04.02).
Mobile phase:	100 mmol/L KH_2PO_4/K_2HPO_4, pH 6.7, 100 mmol/L Na_2SO_4 + 0.05% NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	ambient
Injection vol.:	10 µL (approximately 0.594 mg of total protein content per injection)
Samples:	standard TSKgel SWXL test mixture: thyroglobulin, 0.5 g/L ɣ-globulin, 1 g/L ovalbumin, 1 g/L ribonuclease A , 1.5 g/L para-Aminobenzoic acid (PABA), 0.01 g/L

Material and Methods

In this study:

- Sample and mobile phase were not filtered through a 0.45 µm syringe filter.
- Purposefully no guard column was used during the study with the column (W00116-507W) to provide extra stress on the column.
- Guard columns were used for the analysis with the other columns (W00116-508W and W00134-504W) to monitor the effect of a guard column on increasing the column lifetime.
- The column was not cleaned in between consecutive injections until the QC specifications fell below the criteria mentioned previously.
- High purity HPLC grade Sigma Aldrich chemicals were used in this study. The samples prepared were not filtered through 0.45 µm or 0.22 µm filters as generally recommended to end users, another important precautionary step to prevent column fouling.
- High purity 18.2 m.Ohm-cm quality water was used to make buffer and samples, but the mobile phase made using this water was not filtered for the same reason as mentioned previously.

Figure 1: Analysis of Protein Standard Mixture using a TSKgel UltraSW Aggregate Column (W00116-507W) – an Overlay of 170 Consecutive Injections (n=10) – No Guard Column Used



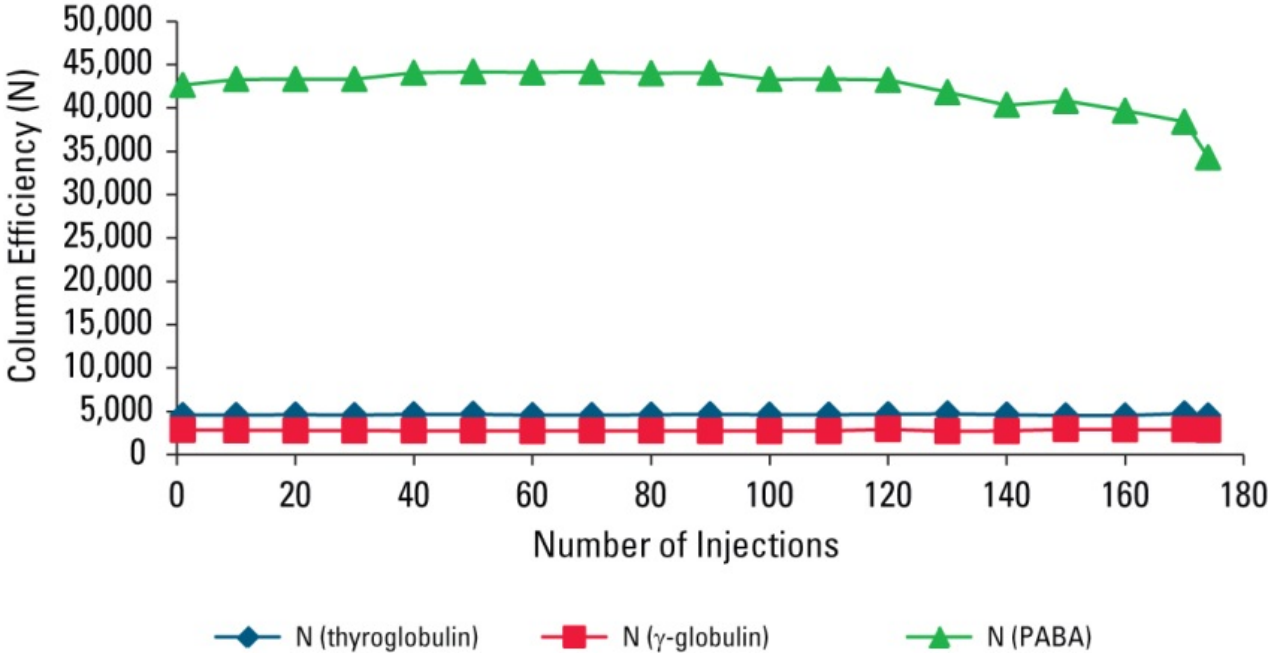
- The above figure clearly shows the overlap of all of the peaks.
- There was no shift in retention time and no change in the peak area, which are both common symptoms of secondary interaction. This shows the effectiveness of the diol-bonded coating used to prevent free silanol activity.

Table 1: %RSD of Peak Parameters During the 174 Consecutive Injections (n=10)

	Thyroglobulin (monomer)						γ-Globulin						PABA					
	Retention Time	Area	Height	As	Width	N	Retention Time	Area	Height	As	Width	N	Retention Time	Area	Height	As	Width	N
Average	6.92	108.48	6.48	1.04	0.24	4,598.53	8.77	284.16	10.42	1.33	0.39	2,797.58	12.12	416.99	43.43	1.07	0.14	42,213.05
Std. Dev	0.01	1.88	0.05	0.02	0.00	64.53	0.01	11.53	0.19	0.04	0.01	75.26	0.01	1.38	0.63	0.04	0.00	1,686.91
%RSD	0.07	1.73	0.70	1.85	0.66	1.40	0.10	4.06	1.81	2.71	1.35	2.69	0.09	0.33	1.45	4.14	2.01	4.00

- 174 consecutive injections yielded an excellent reproducibility with a very low %RSD value of all of the peak parameters.
- This also shows the inherent stability of the diol-bonded coating to prevent secondary interactions.

Figure 2: Column Efficiency Over 174 Consecutive Injections

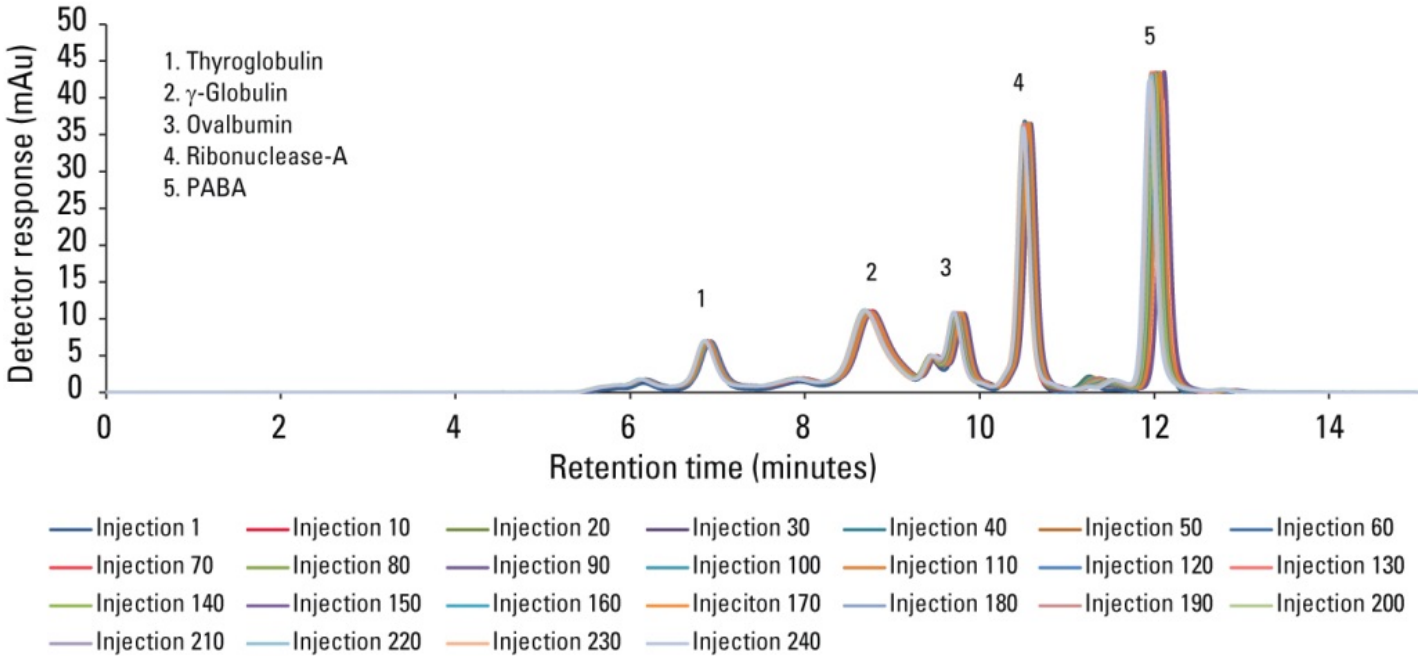


- This study shows the change in the column performance as a function of the number of consecutive injections as measured by thyroglobulin, γ-globulin and PABA standards.
- The consecutive runs of the unfiltered sample and mobile phase are expected to provide extra stress on the unprotected column, particularly with 3 µm particle size.
- A gradual decrease in column efficiency is observed beginning at injection 120 and the column performance fell below QC specifications after injection 170 [N(PABA) <35,000], suggesting the need to clean the column to prevent the complete failure of the column.

Figure 2: Column Efficiency Over 174 Consecutive Injections – Conclusions Continued

- Gradual loss of efficiency is a symptom of column failure – column failure of the QC criteria (after 170 consecutive injections) was intentional.
- This was to induce extra pressure on the column and to see whether the column could be fully regenerated.
- It is recommended that an end user must not wait until the column totally fails.
- A gradual increase in system pressure was also observed – another symptom of column failure – meaning the column is getting compromised with contaminants building up on the phase.
- The column was cleaned for 10 CV with 1.0 mol/L NaCl, followed by a water rinse and re-equilibration in mobile phase.
- Following the cleaning procedure, the column was re-tested with the Tosoh QC procedure.
- PABA verification following cleaning yielded N >42,000 plates. This shows that column performance is suitable for further runs.
- Based on these results, the column lifetime study was continued.

Figure 3: Overlay of Additional 240 Consecutive Injections of Protein Standard Mixture (n=10) Following Column Cleaning – 410 Injections in Total



As shown, the chromatograms are reproducible and all of the peak parameters of individual standards are also reproducible.

Table 2: Summary of Peak Parameters after Column Cleaning and an Additional 240 Consecutive Injections (410 Injections in Total)

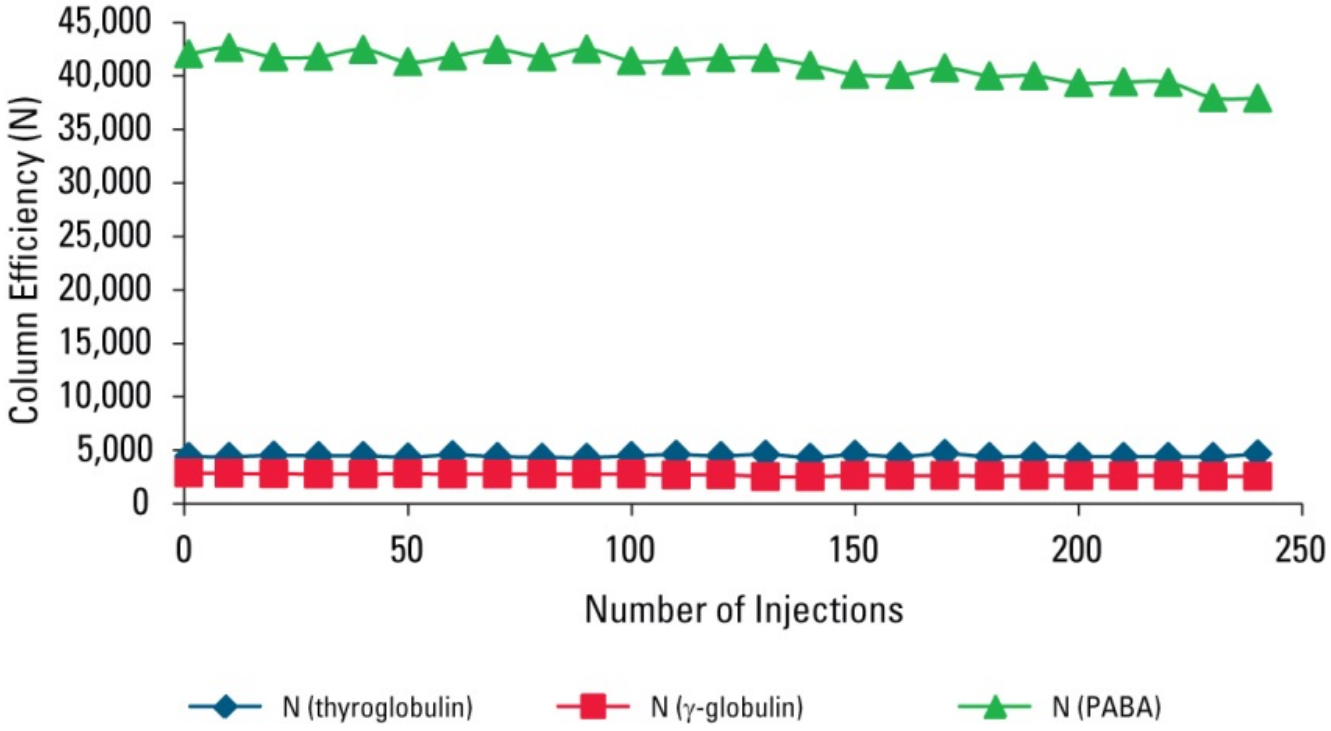
	Thyroglobulin (monomer)						γ-Globulin						PABA					
	Retention Time	Area	Height	As	Width	N	Retention Time	Area	Height	As	Width	N	Retention Time	Area	Height	As	Width	N
Average	6.87	106.34	6.30	1.06	0.24	4,475.84	8.71	273.42	10.06	1.35	0.39	2,704.44	12.01	418.78	42.93	1.14	0.14	40,901.96
Std. Dev	0.02	3.85	0.12	0.04	0.00	98.95	0.03	13.81	0.26	0.05	0.01	104.64	0.05	4.09	0.38	0.06	0.00	1,332.44
%RSD	0.32	3.62	1.83	3.41	1.20	2.21	0.33	5.05	2.62	3.95	2.18	3.87	0.38	0.98	0.88	5.16	1.40	3.26

The low %RSD of the peak parameters for thyroglobulin, γ-globulin and PABA clearly shows that the column is performing very well and is still useful for protein separations.

Summary of Peak Parameters after Column Cleaning and an Additional 240 Consecutive Injections (410 Injections in Total) – Conclusions Continued

- Column performance remained stable for an additional 240 consecutive injections, i.e. 410 injections to this point. The column still passed the QC criteria.
- After 410 injections in total, the column backpressure was high, and to avoid destruction of the column from over pressure, it was taken out of the system for cleaning before it reached the maximum pressure limit for the column.
- The following figure shows the trend of the QC analysis during this analysis.

Figure 4: Analysis of Protein Standard Mixture using a TSKgel UltraSW Aggregate Column (W00116-507W) for an Additional Set of 250 Consecutive Injections Following One Cleaning



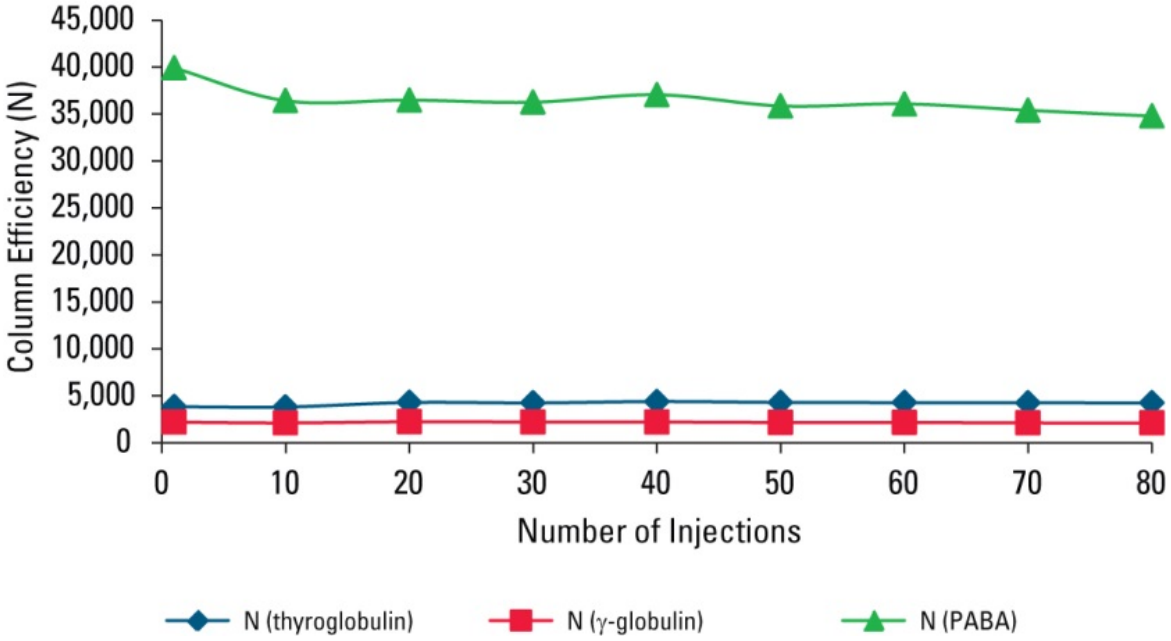
- This study shows that the column performance is perfect.
- After 410 injections, the column needed to be cleaned due to high backpressure.

Analysis of Protein Standard Mixture with an Additional Set of 250 Consecutive Injections Following One Cleaning – Conclusions Continued

- Column was cleaned for 10 CV with 1.0 mol/L NaCl, followed by a water rinse and re-equilibration in mobile phase.
- Pressure remained high (>90 bar) after cleaning.
- Column was then cleaned overnight with 20% ACN, followed by a water rinse and re-equilibration in mobile phase.
- Pressure remained high (>90 bar) after this cleaning.
- Column was then cleaned overnight with 0.1 mol/L sodium phosphate, pH 2.53, followed by a water rinse and re-equilibration in mobile phase.
- Pressure remained high (>90 bar) after this cleaning.
- Contamination (blockage) of frit at the inlet was observed and frit was rinsed in Milli-Q® water.
- The column was retested with PABA after cleaning the inlet frit: N(PABA) yielded >39,000 plates.
- Based on these results, the column lifetime study was continued.

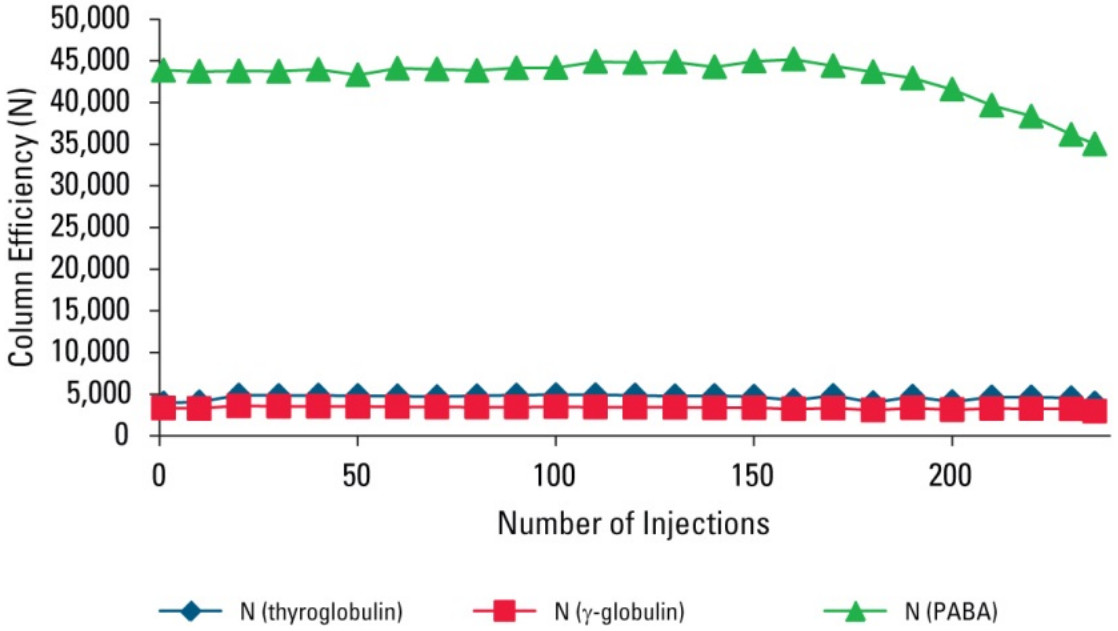
Figure 5: Column Efficiency vs. Number of Protein Standard Injections

- The column was used for an additional set of 80 injections – a total of 490 injections - with a very low %RSD for the peak parameters as shown in the next table.
- The column could not be used after 490 consecutive injections with two cleanings in between as mentioned previously.



- So the next study was performed with a TSKgel UltraSW Aggregate column from a different lot (W00200-508W) with the use of a guard column prior to the inlet of the column to protect the column from getting fouled.

Figure 6: Analysis of a Protein Standard Mixture using a TSKgel UltraSW Aggregate Column (W00200-508W)

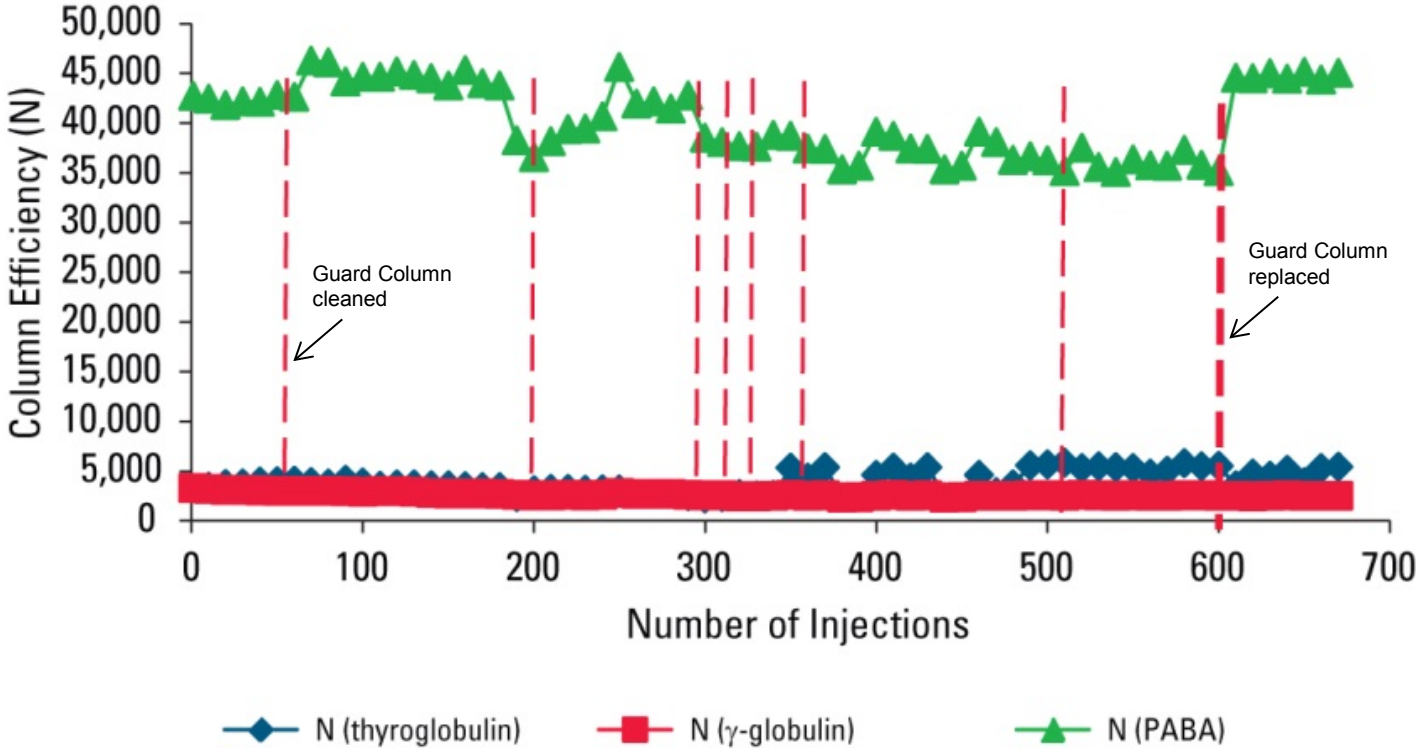


- A gradual decrease in column efficiency is observed beginning at injection 170.
- The column maintained performance up to injection 236 [N(PABA)] >35,000.
- A gradual increase in system pressure was also observed.
- With intermittent cleaning of the guard column or replacing the guard column, the column lifetime of the TSKgel UltraSW Aggregate (W00200-508W) column could be extended to 656 consecutive injections.
- %RSD was low for all of the peak parameters as shown in the following table.

Table 3: Analysis of Protein Standard Mixture using a TSKgel UltraSW Aggregate Column (W00200-508W)

	Thyroglobulin (monomer)						γ-Globulin						PABA					
	Retention Time	Area	Height	As	Width	N	Retention Time	Area	Height	As	Width	N	Retention Time	Area	Height	As	Width	N
Average	7.36	123.62	6.75	1.07	0.26	4,407.33	9.28	404.86	13.34	1.36	0.43	2,512.12	12.99	463.47	37.78	0.77	0.16	36,723.95
Std. Dev	0.01	12.39	0.22	0.06	0.01	222.56	0.01	13.95	0.28	0.05	0.01	100.06	0.02	13.40	0.33	0.02	0.00	454.06
%RSD	0.12	10.03	3.20	5.91	2.69	5.05	0.14	3.45	2.11	3.76	1.86	3.98	0.15	2.89	0.87	3.19	0.59	1.24

Figure 7: Column Efficiency vs. Number of Protein Standard Injections on TSKgel UltraSW Aggregate (W00134-504W) Column



- This study shows the use of a TSKgel UltraSW Aggregate column from a different lot (W00134-504W) with the use of a guard column prior to the inlet of the column to protect the column from getting fouled.
- This study shows that the column is stable even after more than 600 injections.

Column Efficiency vs. Number of Protein Standard Injections on TSKgel UltraSW Aggregate (W00134-504W) Column – Conclusions Continued

- The original guard column (W00011) lasted 600 injections with intermittent cleanings of 1 mol/L NaCl throughout, as shown by the dashed lines.
- At 600 injections, the guard column was unrecoverable and was replaced with W00026.
- At each cleaning point, the column efficiency of the analyte column (without guard) was evaluated.
- The analytical column yielded N(PABA) >43,000 plates, illustrating the effectiveness of the guard column.
- The following table shows the peak parameters obtained for the standards used during these runs.

Table 4: Analysis of Protein Standard Mixture using a TSKgel UltraSW Aggregate Column (W00134-504W)

	Thyroglobulin (monomer)						γ-Globulin						PABA					
	Retention Time	Area	Height	As	Width	N	Retention Time	Area	Height	As	Width	N	Retention Time	Area	Height	As	Width	N
Average	7.42	69.22	3.01	1.21	0.29	3,715.10	9.30	506.92	16.65	1.32	0.43	2,654.17	12.95	941.74	84.70	1.33	0.15	39,815.52
Std. Dev	0.05	62.10	2.60	0.10	0.01	229.36	0.06	34.11	1.10	0.03	0.01	152.08	0.08	44.11	3.85	0.05	0.00	1,359.59
%RSD	0.66	89.72	86.44	8.42	3.12	6.17	0.61	6.73	6.60	2.55	2.30	5.73	0.63	4.68	4.55	3.47	1.73	3.41

Conclusions

- TSKgel UltraSW Aggregate, 3 µm column is robust and inherently stable.
- The column shows a very long lifetime.
- 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 lots.
- The study shows the reliability and dependability of the column in the separation of proteins.
- Since columns were studied with and without guard columns and filtration of the sample, mobile phase, etc., lifetime of the column can further be improved by using these features to protect the column.
- TSKgel UltraSW Aggregate, 3 µm column can be successfully used for the separation of aggregates.
- This column lifetime study is currently ongoing with >900 injections.

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.

www.tosohtalk.com