Physical Stability of a Silica-Based Size Exclusion Column for Antibody Analysis

Atis Chakrabarti and Roy Eksteen

Tosoh Bioscience LLC, King of Prussia, PA 19406

Abstract

- There are many answers to the question of how long a column lasts. The most trivial reply is still the most appropriate - it all depends on what you do with the column. Particle structure, bonded phase composition, number of injections, mobile phase, pH, flow rate, sample type/mass/volume, temperature, pressure, storage, cleaning protocol, et cetera - all play a more or less important role in determining when to write the column's inevitable obituary.
- When it comes to gel filtration chromatography, particle stability and thus column stability is even more of an issue than in reversed phase chromatography for the simple reason that the pore volume of SEC columns is maximized to provide optimal mass resolution, and the higher the pore volume the more fragile the particle.
- From a user's perspective, it would be most desirable if no precautions would be required to obtain a long column lifetime. From a manufacturer's perspective, such a column would be impossible to make and even if it could be made, such a column would be too costly to sell.
- In this initial report of our ongoing study about the stability of the TSKgel G3000SW$_{XL}$ column for quality control of monoclonal antibodies, we evaluated three columns each of three bonding lots. Preliminary indications are that retention times and efficiency did not change over at least 1,000 injections of a 10µL mixture of protein standards into a 100µL injection loop. During the lifetime studies the analytical column was neither protected by a guard column nor by inline filters other than the ones that are a standard fixture in the Agilent 1100 HPLC system.

Introduction

- Column lifetime is a critical factor in all modes of liquid chromatography but particularly so for silica-based SEC columns as the benefit of increased particle porosity has to be balanced with the requirement to maintain physical integrity of the particles.
- While the physical stability of reversed phase HPLC columns is taken for granted by its practitioners, the same cannot be said for the physical stability of polar bonded phase columns.
- Although polar-bonded phase columns can display comparable efficiency and peak symmetry as RPLC columns, polar bonded phase columns are sometimes plagued by a sudden loss of performance; usually from the development of a void due to resettling of the packing at the top of the column.

Factors playing a more or less important role in determining column lifetime

- Particle structure
- Bonded phase composition
- Number of injections
- Sample type, sample mass, and sample volume
- Mobile phase conditions (pH, buffer, salt)
- Pressure
- Temperature
- Column cleaning
- Storage

In this study we report on variations in column efficiency, peak symmetry, and retention when making 1,000 injections of a 10uL standard mixture of globular proteins on five (5) TSKgel G3000SW$_{XL}$ columns operated under standard operating conditions.

Objective

This study was conducted to determine the long term physical stability of TSKgel G3000SW$_{XL}$ columns as a function of operating conditions.

In this initial study we report on experiments performed using a common mobile phase composition, a standard flow rate, and a small injection volume of globular protein standards.

Characteristics of TSKgel SW columns

- Particle size: 5µm
- Pore size: 250Å
- Bonded phase: diol-containing ligands
- Protein calibration range: 10,000 – 500,000Da

Material and methods: Columns

- TSKgel G3000SW$_{XL}$, 5µm, 7.8mm ID x 30cm
  - Bonding Lot 08R
    - **S1237**
    - **S1238**
    - **S1239**
  - Bonding Lot 09R
    - **S1261**
    - S1262
    - S1263
  - Bonding Lot 30P
    - **S6210**
    - S6211
    - S6212

Silica Lot A (Bonding Lots 08R and 09R)

Silica Lot B (Bonding Lot 30P)

In this poster we report on the stability of the highlighted columns prepared from three bonding lots, made from two silica lots.

Chromatographic conditions stability study

- TSKgel G3000SW_{XL}, 5µm, 7.8mm ID x 30cm
  - **Silica lot A**
    - Bonding lot 08R: columns **S1237, S1238, S1239**
    - Bonding lot 09R: Columns **S1261**, S1262, S1263
  - **Silica lot B**
    - Bonding lot 30P: columns **S6210**, S6211, S6212
- Mobile Phase: 0.1mol/L KH_2PO_4/Na_2HPO_4, pH 6.7, + 0.1mol/L Na_2SO_4 + 0.05% NaN_3
- Flow rate: 1.0mL/min
- Detection: UV@280nm
- Temperature: ambient
- Injection vol.: 10µL
- Samples
  - TSKgel SW_{XL} test mixture: thyroglobulin, γ-globulin, ovalbumin, ribonuclease A, p-ABA (see next slide for more details)
  - Monoclonal antibody: BI-MAb-2 (Boehringer-Ingelheim), 4.5g/L in glycine/sodium phosphate, pH 6.0

Chemicals

- Protein test mixture

Globular Proteins	Sigma PN	Conc. (g/L)
thyroglobulin, *bovine* (670kDa)	T1001	0.50
γ-Globulin, *bovine* (150kDa)	G5009	1.00
ovalbumin, *chicken*, (45kDa)	A5503	1.00
ribonuclease A, *bovine* (14.7kDa)	R4875	1.50
p-Amino benzoic acid (137g/mol)	A9878	0.01

Notes:

- Sample solvent: 100mmol/L phosphate buffer, pH 6.7, unless mentioned otherwise.
- The small molecular weight compound *p*-aminobenzoic acid is used to mark the mobile phase volume in the column, while the efficiency and peak symmetry of the pABA peak are indicators of the integrity of the packed bed.

- All chemicals and standards were obtained from Sigma-Aldrich and were of electrophoretic or analytical grade.
- High purity HPLC grade solvents were used for the preparation of stock standards, samples and mobile phases.

Column protection

- Although it is recommended practice to protect the column from potential sources of contamination no such precautions were made in this study.
  - Standards and mobile phases were not filtered through a 0.45µm syringe filter.
  - A frit filter was not used between injector and column.
  - TSKgel G3000SW$_{XL}$ columns were not protected by a guard column in any of these studies.
- Note: the PTFE Purge Valve Frit on the high pressure side of the Agilent 1100 HPLC system was replaced after about 3,000 injections (~ 45L mobile phase).

Dirty Frit New Frit

Results

- Tables
  - (I) N, AF and t_R at 1st , 500th and 1000th injection on S1261-09R
  - (II) %RSD values of N, AF and t_R during 1000 injections

- Figures
  1. Precision: column S1261-09R
  2. Precision: columns from bonding lot 08R
  3. Precision: columns from bonding lots 08R + 09R
  4. Precision: between silica lots A and B
  5. Sample load – protein standards
  6. Sample load – monoclonal antibody

Table I: Retention, efficiency and peak symmetry at the 1st, 500th and 1000th injection

	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 II: %RSD (n=100) over 1000 injections

	% RSD of Peak Parameter Values for each 10th injection during the 1000 injection cycle (n=100)				
	thyroglobulin	γ-globulin	ovalbumin	rib. A	p-ABA
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

Injection-to-injection reproducibility: within column

S1261-09R Inj 1
S1261-09R Inj 1000

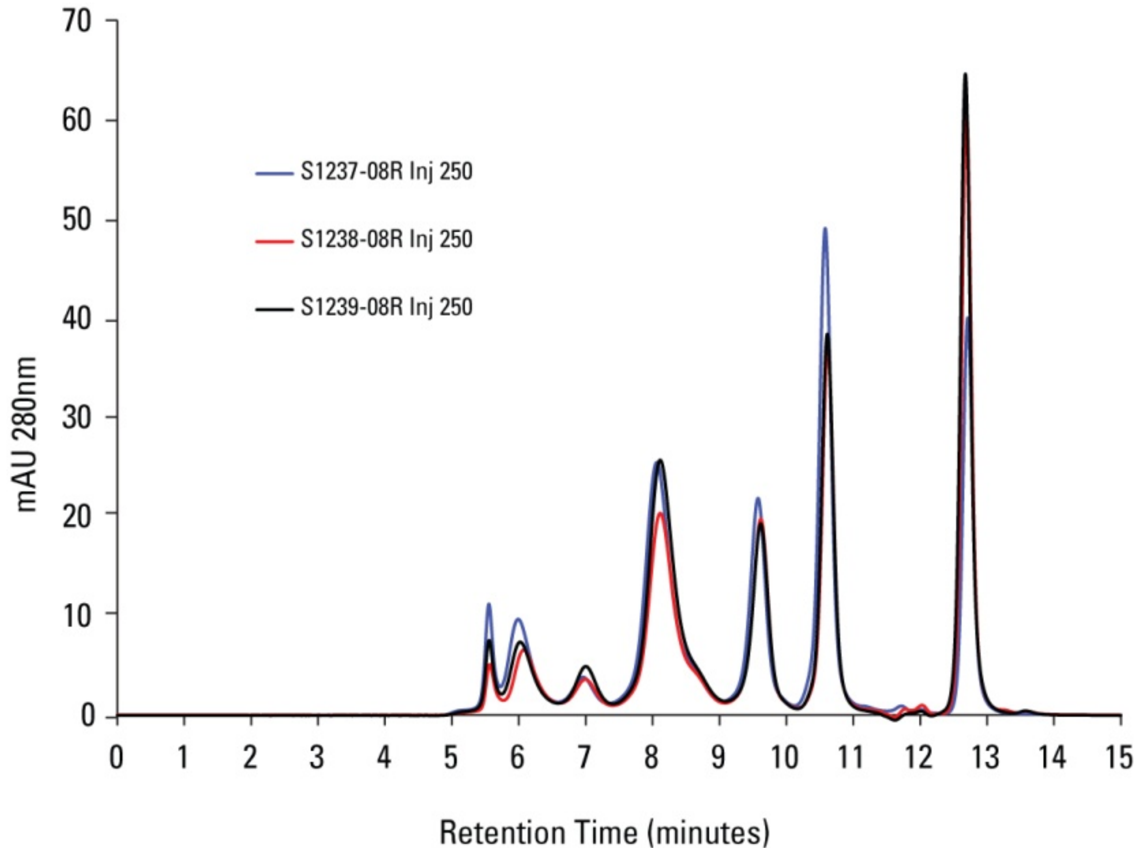
1. thyroglobulin
2. γ-globulin
3. ovalbumin
4. ribonuclease A
5. p-Aminobenzoic acid (pABA)

mAU 280nm

Retention Time (minutes)

High degree of reproducibility from injection to injection is illustrated by superimposed chromatograms of the 1st and 1000th injection on column S1261-08R.

Column-to-column reproducibility within bonding lot



High degree of reproducibility within a bonding lot is illustrated by superimposed chromatograms of the 250th injection on three columns from bonding lot 08R.

Reproducibility between bonding lots

S1237-08R Inj 1000
S1261-09R Inj 1000

mAU 280nm

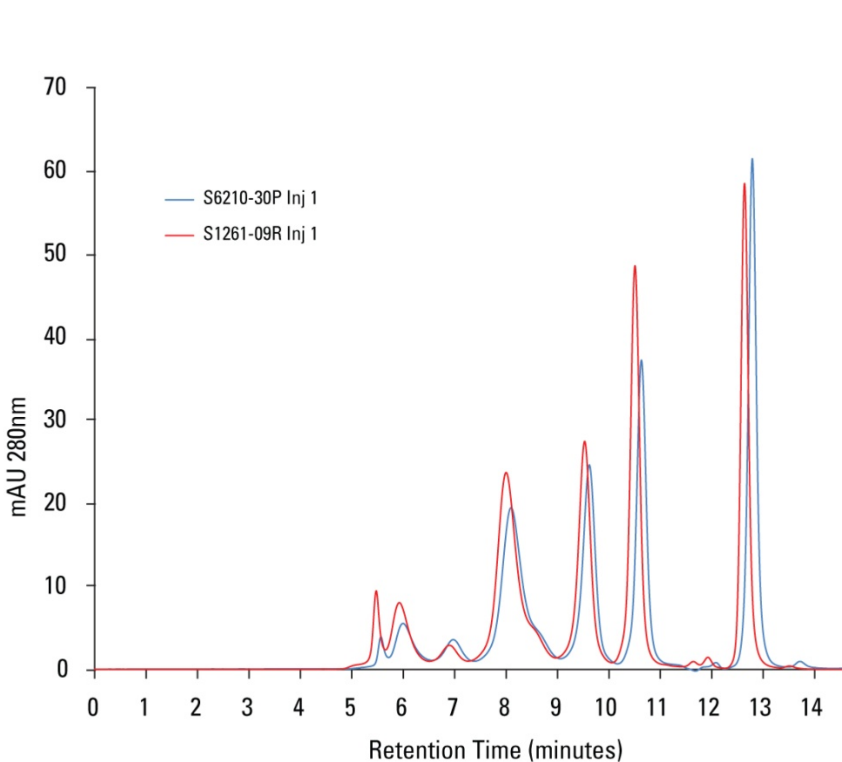
70
60
50
40
30
20
10
0

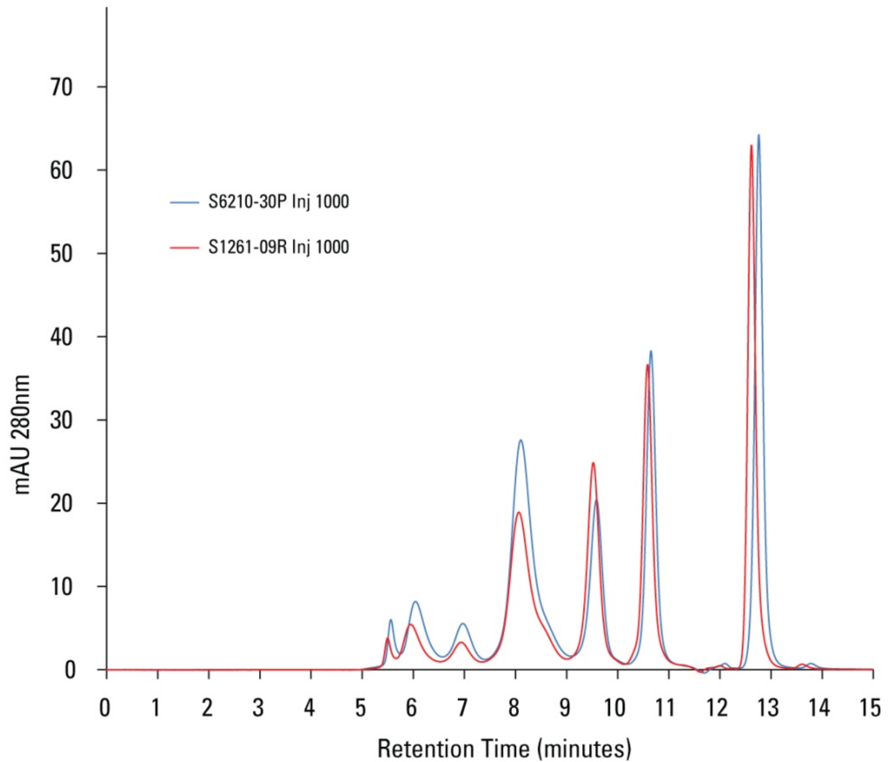
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

Retention time (minutes)

High degree of reproducibility between bonding lots is illustrated by superimposed chromatograms of the 1000th injection on one column each of bonding lots 08R and 09R.

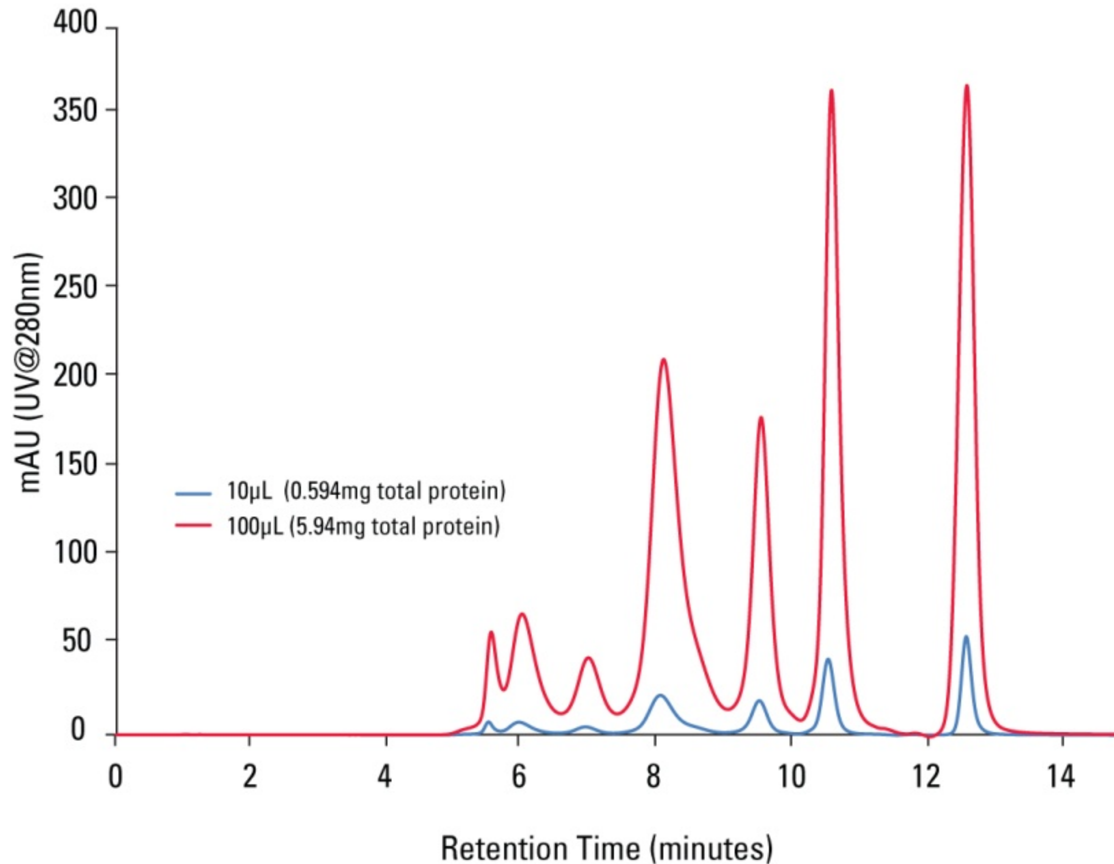
Reproducibility between silica lots





High degree of reproducibility between silica lots is illustrated by superimposed chromatograms of the 1st and 1000th injection on one column each of silica lots A and B.

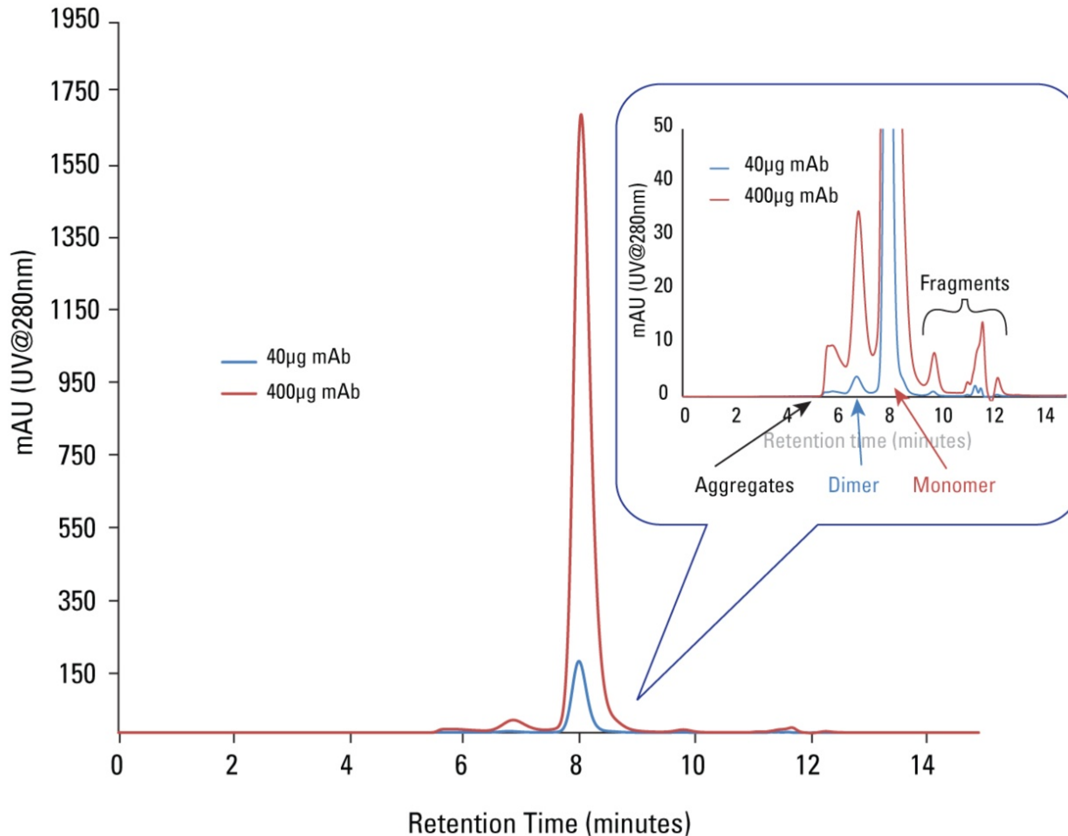
Loading capacity of protein standard



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

Efficiency of γ-globulin declined slightly, while peak shape and retention time were not affected when injecting 100µL sample containing 6mg protein.

Loading capacity of monoclonal antibody



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

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

Preliminary conclusions 1

- Based on five TSKgel G3000SW$_{XL}$ columns tested, each column was stable for at least 1,000,10µL injections of a protein standard mixture in 0.1mol/L, pH 6.7 phosphate buffer containing 0.1mol/L sodium sulfate and 0.05% sodium azide, operated at 1mL/min at room temperature.
- Efficiency (N) and peak symmetry (AF) values showed minimal variation from the first to the last (1000th) injection indicating that the integrity of the packed bed was maintained throughout the experiment.
- Retention times of globular protein standards did not change while pumping 15L mobile phase through the column, indicating that the integrity of the diol-bonded phase was maintained throughout the experiment.
- Plate numbers, peak symmetry values and retention times for each protein studied showed minimal variation for TSKgel G3000SW$_{XL}$ columns prepared from:
  - The same bonding lot – 3 columns tested.
  - Different bonding lots – one column each of 3 lots tested.
  - Different silica lots – one column each of two lots tested.

Preliminary conclusions 2

- Efficiency, peak symmetry and retention times did not change when injecting a 100µL of a standard protein mixture containing 6mg total protein.
- Chromatographic parameters were also not affected when injecting 0.4mg of a monoclonal antibody preparation.

Future studies

- When performing column stability studies one first needs to understand the effect of common chromatographic conditions on the integrity of the packed bed before one can study how ‘real’ samples influence column lifetime.
- In the next phase of this study we plan to investigate the role of injection volume and the role played by the nature of the injection solvent on column stability.
- Another topic of interest is the role of mobile phase additives, such as organic modifiers and detergents.