Characterization of a New Wide Pore C4 Phase Silica Gel Reversed Phase Column Designed for Protein Separation

Justin Steve and Atis Chakrabarti, Ph.D.

Introduction

- Biopolymers are highly complex and large molecules.
- Characterizing biological macromolecules, such as proteins and peptides, is important to ensure consistent product quality.
- The importance of this is increasing along with the progress of the biopharmaceutical industry and the enthusiastic growth of proteomics research.
- Reversed phase chromatography (RPC) is one of the most frequently used chromatographic modes for analytical separations.
- RPC is often used for the analysis of small molecular weight compounds, but there are also various standard applications for the separation of biomolecules, such as proteins.
- Conventional reversed phase HPLC packing materials with 8-14 nm pore sizes are not generally suitable for the analysis of large intact proteins.
- This is because the analytes are not able to access the surface area within these pores.

Introduction

- A wide pore 30 nm, silica-based butyl (C4) column, the TSKgel® Protein C_4-300, is now available from Tosoh.
- The new column, with 3 µm spherical silica gel, has optimized ligand density and 30 nm pore size, useful for the separation of large biomolecules such as proteins.
- The packing is prepared by polymeric binding of butyl (C4) alkyl groups.
- The polymeric butyl group reduces the protein adsorption on the stationary phase compared to C18 stationary phase.
- Stationary phase is fully endcapped with trimethylsilyl (TMS) groups to prevent interaction with free silanol groups. This incurs higher stability of the phase and reduction of peak tailing.
- Optimized ligand density and alkyl length in the stationary phase result in lower adsorption of the protein.
- A particle size of 3 µm yields high theoretical plate counts.

Introduction

- The large pore size of this column allows macromolecules to enter the interior of the pore.
- The larger pore size also provides higher peak capacities than reversed phase columns with 10 nm pore size.
- Moderate hydrophobicity is suitable for protein separation with good recovery.
- The column is designed for the optimal recovery and resolution of proteins, such as recombinant proteins, antibody fragments or PEGylated proteins.

Introduction

- Analytical reversed phase chromatography columns are costly.
- A stable column yielding a high degree of reproducibility of retention time, peak symmetry, and column efficiency is therefore very important to the analyst.
- For many years now, column to column reproducibility remains the top factor, above price, to chromatographers when selecting an appropriate column.
- 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 to column reproducibility over price to chromatographers in the selection of a column.
- The survey also shows the same pattern over a number of years.
- Column to column reproducibility can be tested as a function of different peak parameters, such as peak retention time, capacity factor, peak area, asymmetry, efficiency, and resolution, etc.

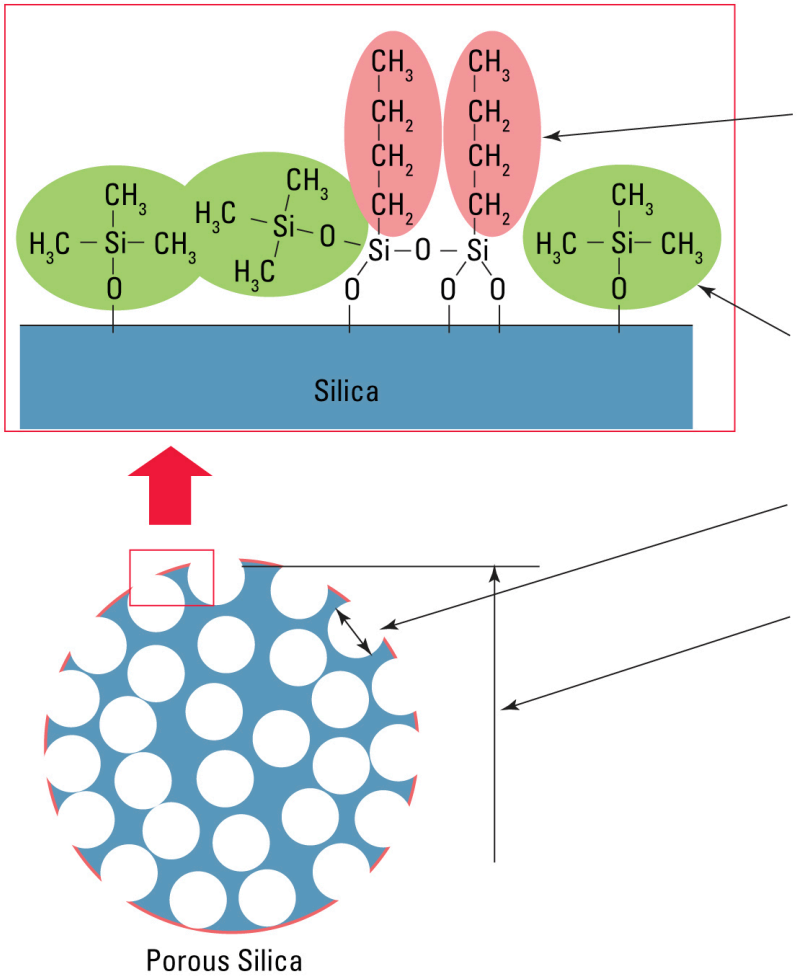
Introduction

- Retention time shift can sometimes be related to a loss of packing material or stationary phase.
- A loss of resolution, peak broadening, or significant tailing, factors that may affect quantitation, are symptoms of column failure.
- 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.
- Here we report the study on the reproducibility of the peak parameters during the analysis of standard proteins using TSKgel Protein C_4-300 columns.

TSKgel Protein C_4-300 Column – Product Attributes

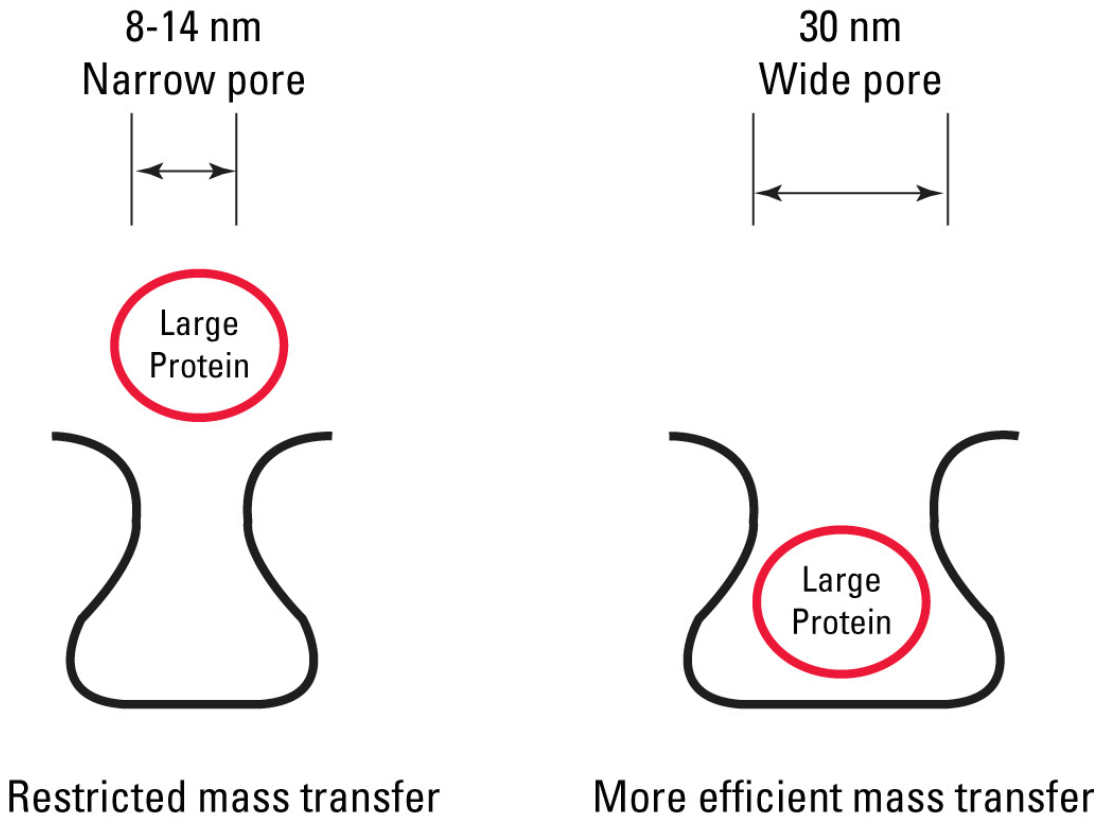
Pore size (silica):	30 nm
Particle size:	3 µm
Endcapped:	Yes (Trimethylsilyl)
pH stability:	1.5 - 7.5
Ligand:	C4 (butyl)
Specific surface area:	100 m^2/g
% carbon	3%

TSKgel Protein C_4-300 Column



- Polymeric butyl groups – shorter alkyl chain ligand with lower hydrophobicity results in less protein adsorption compared to C18. It also helps in high recovery.
- Full endcapping of residual silanol groups – leads to higher stability
- 30 nm pore size – accessible to proteins and hence higher resolution
- 3 µm particle size results in higher efficiency

TSKgel Protein C_4-300 Column



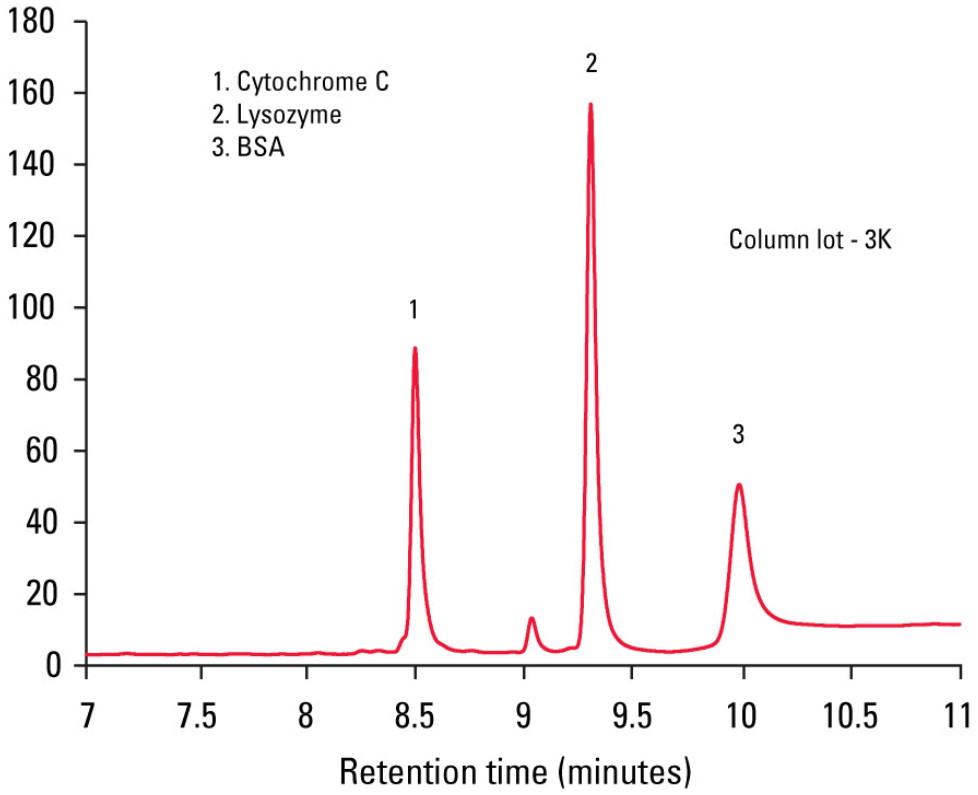
- The larger pore size of TSKgel Protein C_4-300 column helps in more efficient mass transfer during chromatographic analysis.

Materials and Methods

Columns:	TSKgel Protein C_4-300, 3 µm, 4.6 mm ID × 10 cm TSKgel Protein C_4-300, 3 µm, 2.0 mm ID × 10 cm
Instrumentation:	Agilent 1100 and an Agilent 1200 HPLC systems Agilent Chemstation (Rev B.04.02)
Mobile phase:	A: 90:10:0.05: water:acetonitrile:TFA (v/v/v) B: 20:80:0.05: water:acetonitrile:TFA (v/v/v)
Linear gradient:	0-100% B over 20 minutes
Flow rate:	1.0 mL/min (4.6 mm ID column) and 0.189 mL/min (2.0 mm ID column)
Detection:	UV @ 210 nm
Temperature:	40º C
Injection vol.:	20 µL
Samples:	1. cytochrome C (Sigma C2037-5G), 12 kDa, 3.43 mg/mL 2. lysozyme (Sigma L6876-25G), 14 kDa, 4.65 mg/mL 3. bovine serum albumin (Sigma A7906-100G) , 66 kDa, 3.99 mg/mL

- 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 Proteins using a TSKgel Protein C_4-300, 4.6 mm ID × 10 cm Column - Overlay of 5 Consecutive Injections



- A number of standard proteins with a wide variety of size and hydrophobicity could be well separated using this column.
- Excellent intra-day reproducibility was obtained from injection to injection.
- Further analyses are shown in Figure 2 and Table 1.

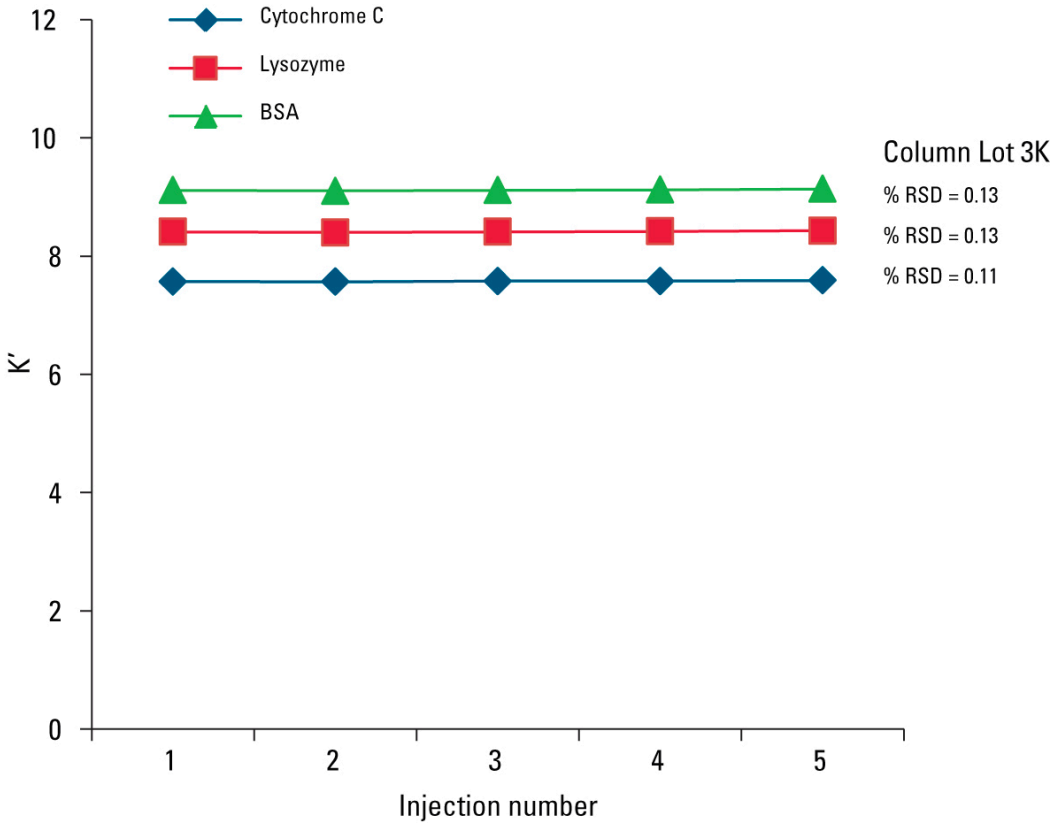
Table 1: Analysis of Proteins using a TSKgel Protein C4-300, 4.6 mm ID × 10 cm Column - Overlay of 5 Consecutive Injections

Column Lot 3K

Cytochrome C	Rt	k	As	N	Rs (cytochrome C/lysozyme)
Run 1	8.561	7.57	1.59	208126	11.00
Run 2	8.557	7.57	1.59	216030	10.91
Run 3	8.566	7.57	1.59	216476	10.94
Run 4	8.567	7.58	1.59	208452	11.00
Run 5	8.58	7.59	1.59	217198	10.97
Average	8.57	7.57	1.59	213256	10.96
STDEV	0.01	0.01	0.00	4555	0.04
%RSD	**0.10**	**0.11**	**0.00**	**2.14**	**0.36**

- Analysis of cytochrome C data (Fig 1) shows that 5 consecutive injections yielded a very low %RSD value for all the peak parameters, such as retention time, capacity factor, asymmetry, and efficiency within the same day.
- The resolution between cytochrome C and lysozyme also yielded a very low %RSD value.

Figure 2: Intra-day Reproducibility During the Analysis of Proteins using a TSKgel Protein C4-300, 4.6 mm ID × 10 cm Column



- Excellent reproducibility was obtained with a very low value of %RSD in capacity factors of the standard proteins.
- The degree of retention is very consistent.

Figure 3: TSKgel Protein C4-300, 4.6 mm ID × 10 cm Column - Lot-to-Lot Reproducibility in Capacity Factor

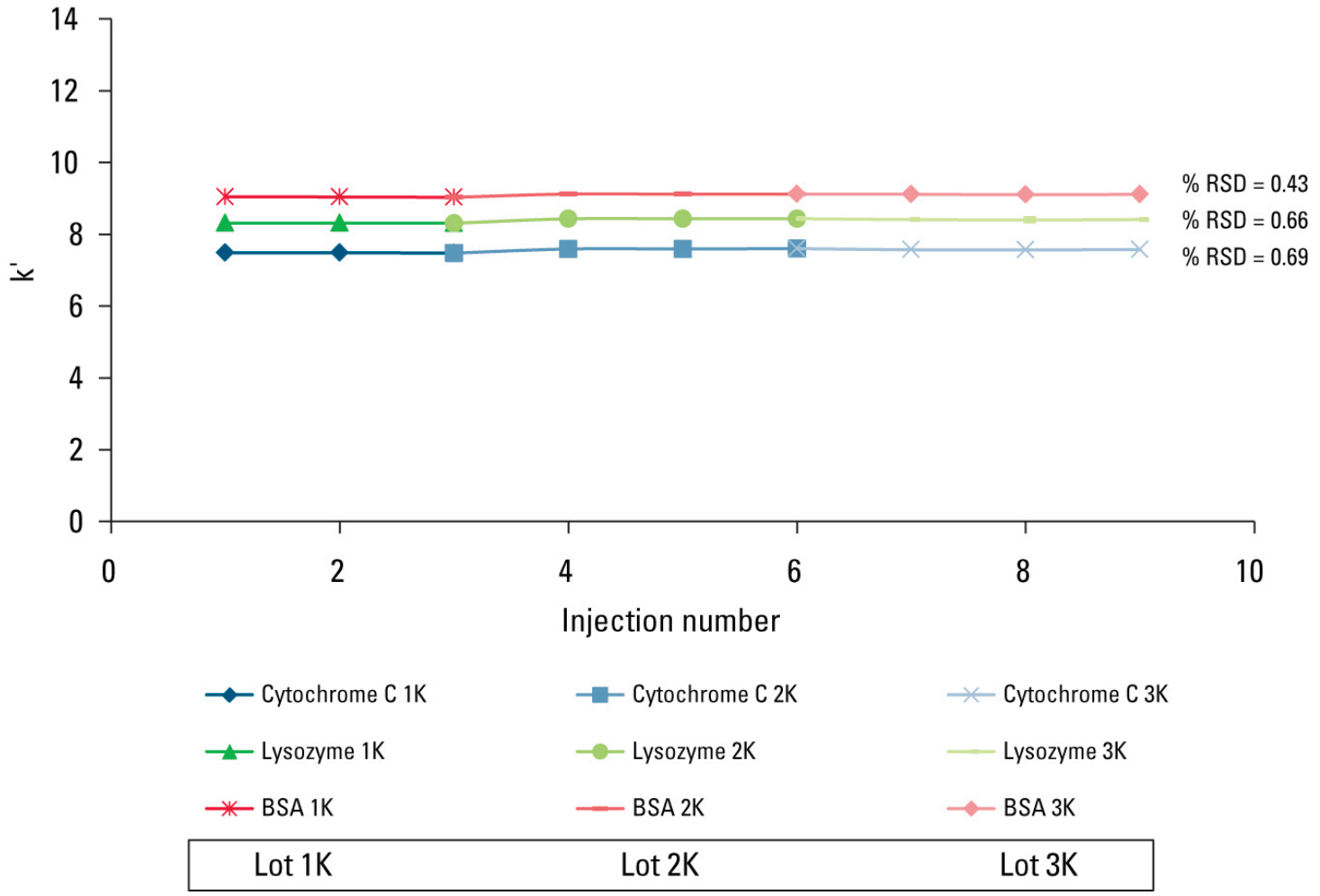


Table 2: TSKgel Protein C_4-300, 4.6 mm ID × 10 cm Column - Lot-to-Lot Reproducibility in Peak Retention Time, Capacity Factor, and Peak Area

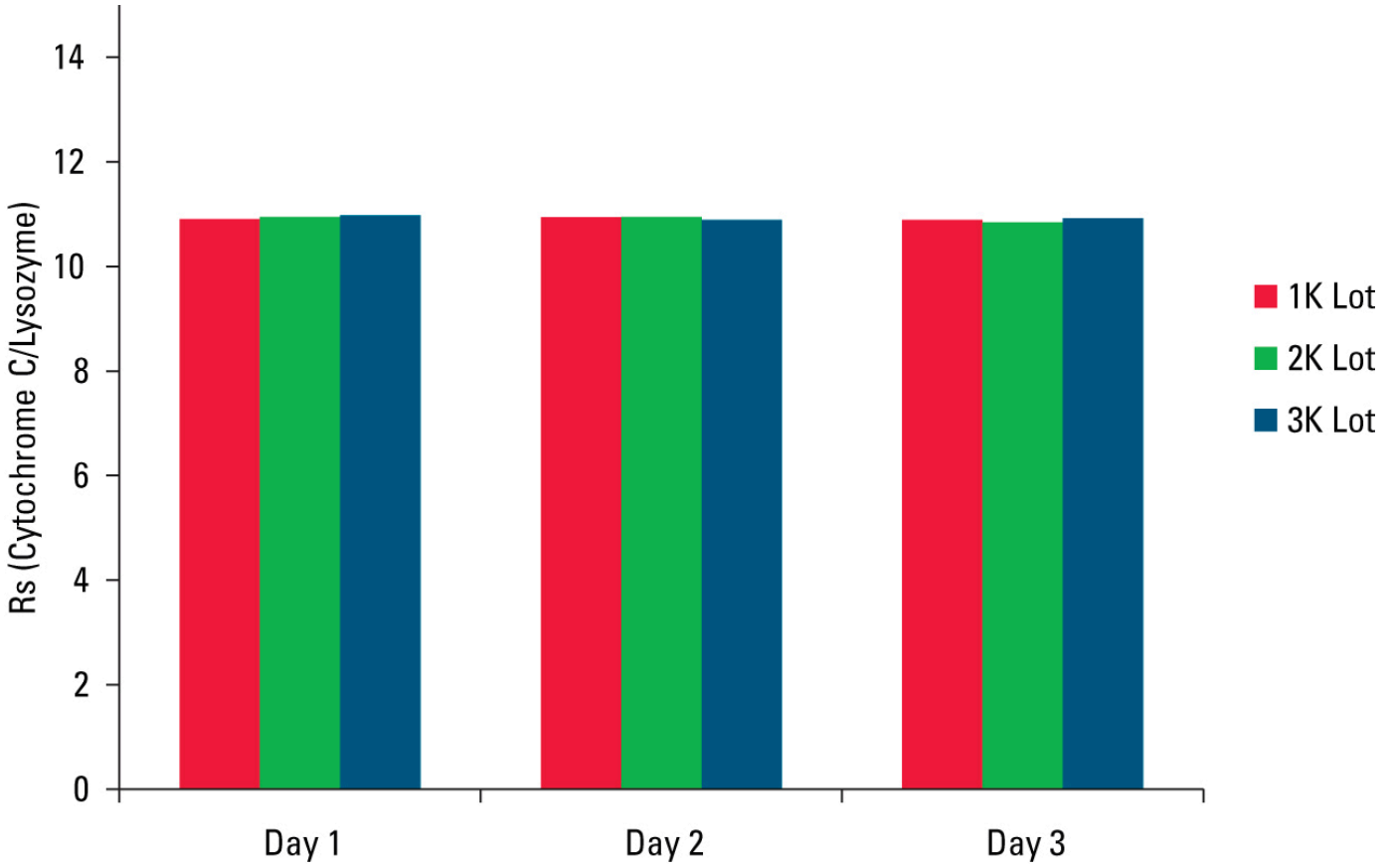
Lot	Lysozyme	Rt	k	Peak Area
1K	Run 1	9.301	8.31	535.10
	Run 2	9.301	8.31	537.67
	Run 3	9.299	8.31	538.89
2K	Run 4	9.421	8.43	549.73
	Run 5	9.422	8.43	544.73
	Run 6	9.424	8.43	546.58
3K	Run 7	9.400	8.41	541.88
	Run 8	9.389	8.40	543.55
	Run 9	9.401	8.41	542.98
	Average	9.37	8.38	542.35
	STDEV	0.06	0.06	4.56
	%RSD	**0.60**	**0.67**	**0.84**

An analysis of lysozyme data shows that excellent lot-to-lot reproducibility was obtained with a very low value of %RSD in retention time, capacity factor and peak area.

Recent LCGC survey showed that chromatographers consider column to column reproducibility as most important criteria while choosing a column. (Ref: LCGC Jan 1, 2012)

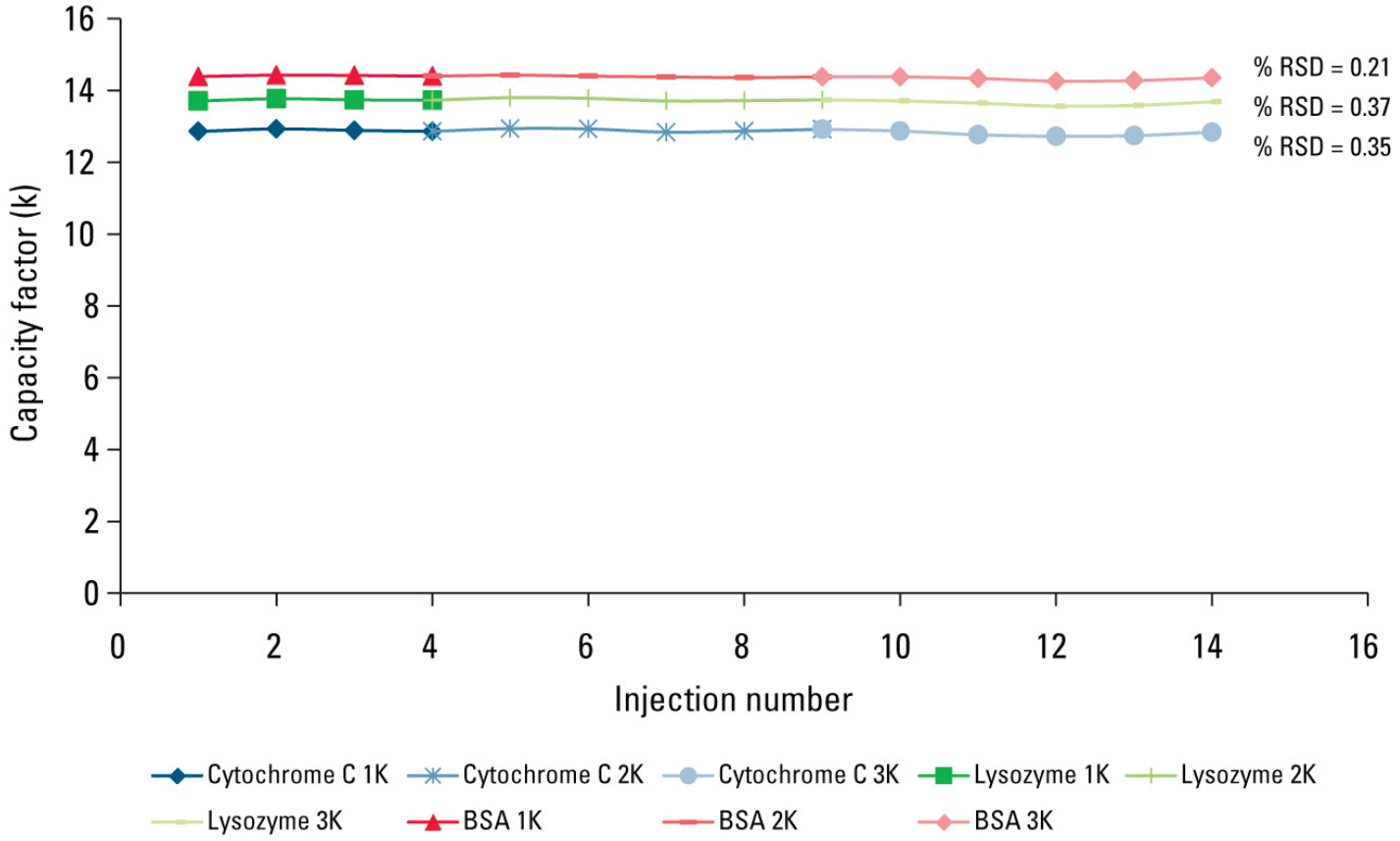
This result shows that the TSKgel Protein C_4-300 column is very dependable for the analysis of proteins.

Figure 4: TSKgel Protein C_4-300, 4.6 mm ID × 10 cm Column - Lot-to-Lot Reproducibility in Resolution Between Cytochrome C and Lysozyme



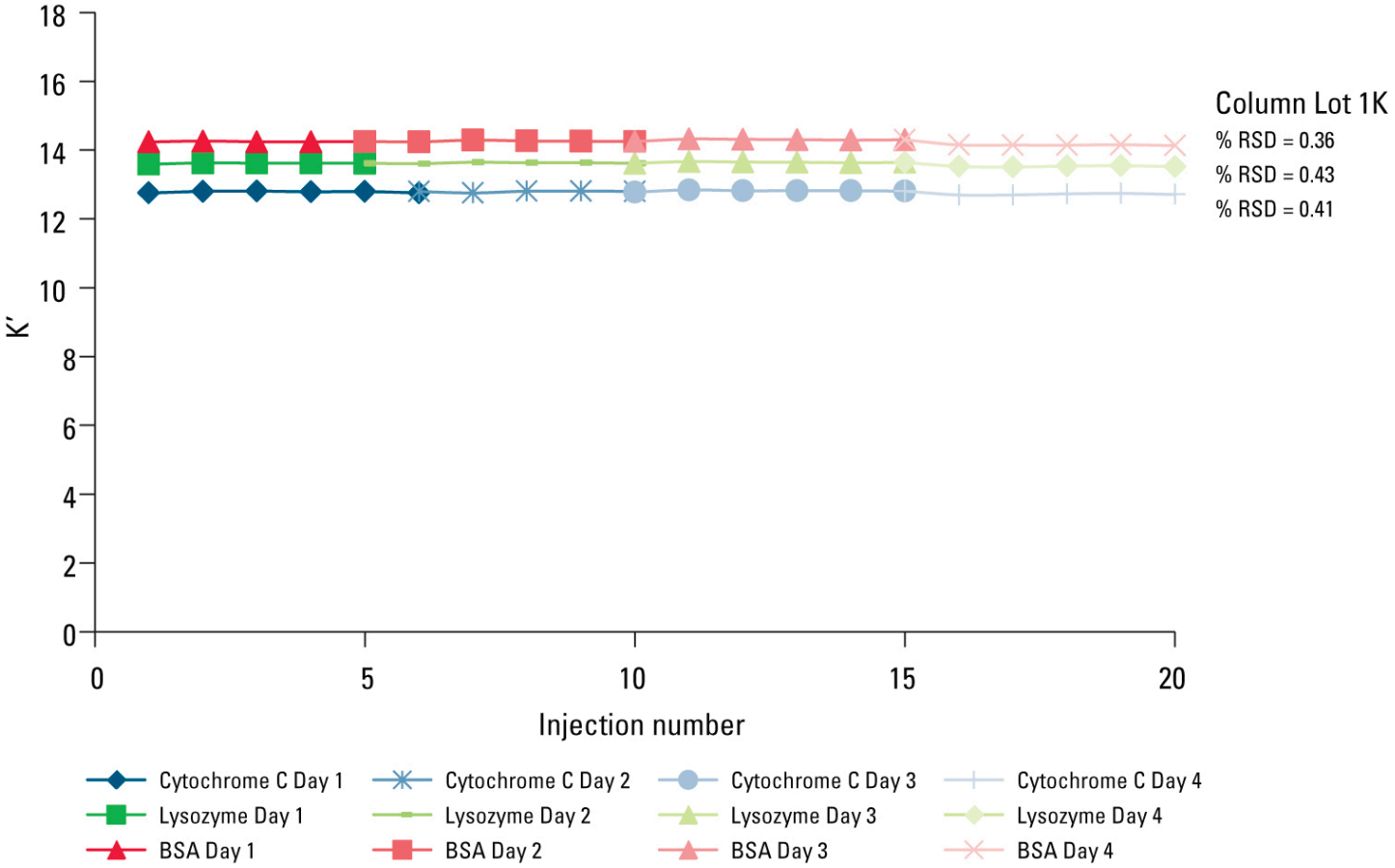
- Resolution remained constant with low %RSD when 3 different lots (1K, 2K and 3K) were tested.

Figure 5: Lot-to-Lot Reproducibility in Capacity Factor of the Proteins Analyzed using a TSKgel Protein C_4-300, 2.0 mm ID × 10 cm Column



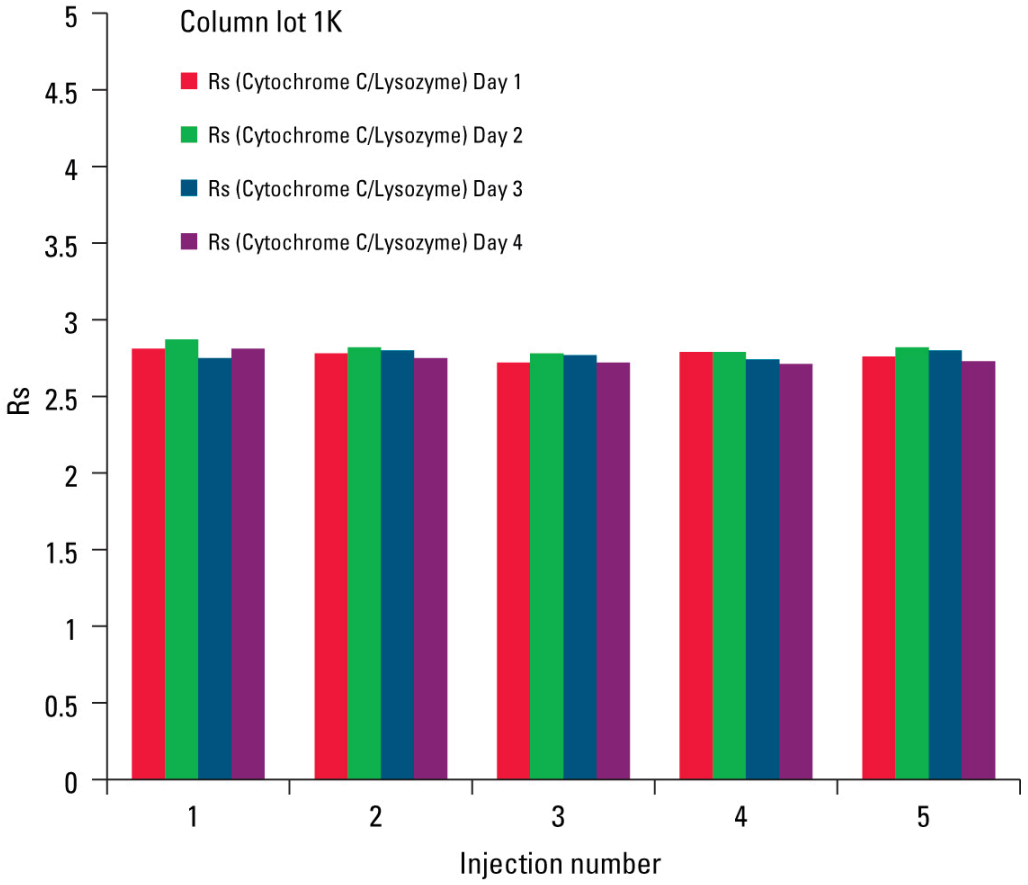
- The results show consistency in k' from day to day across multiple silica lots 1K, 2K and 3K with a very low %RSD.

Figure 6: Day-to-Day Reproducibility in Capacity Factor of the Proteins Analyzed using a TSKgel Protein C_4-300, 2.0 mm ID × 10 cm Column



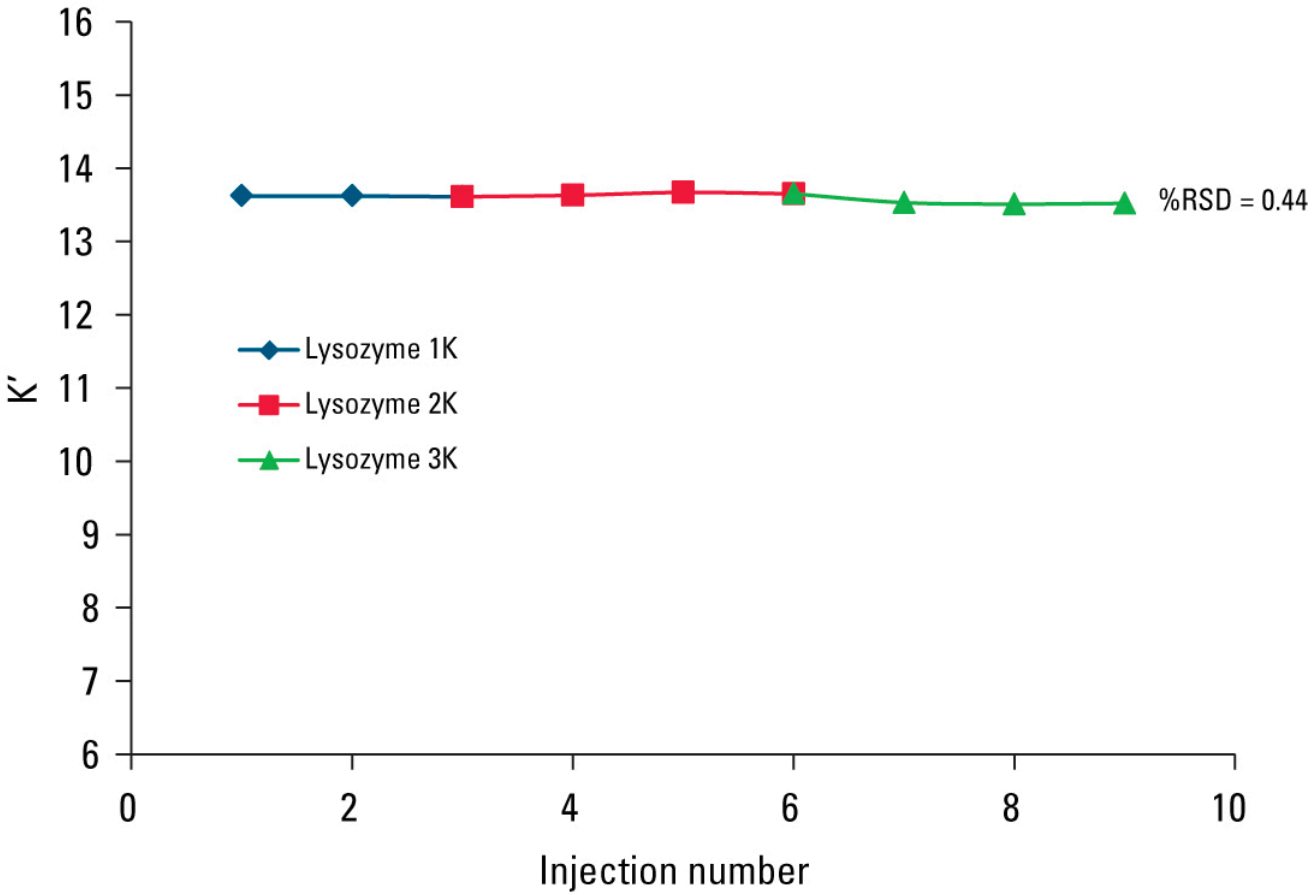
- The results show consistency in k' from day to day.
- Similarly, low %RSD values could be reproduced with lot 2K and 3K.

Figure 7: Day-to-Day Reproducibility in Peak Resolution During Protein Analysis using a TSKgel Protein C4-300, 2.0 mm ID × 10 cm Column



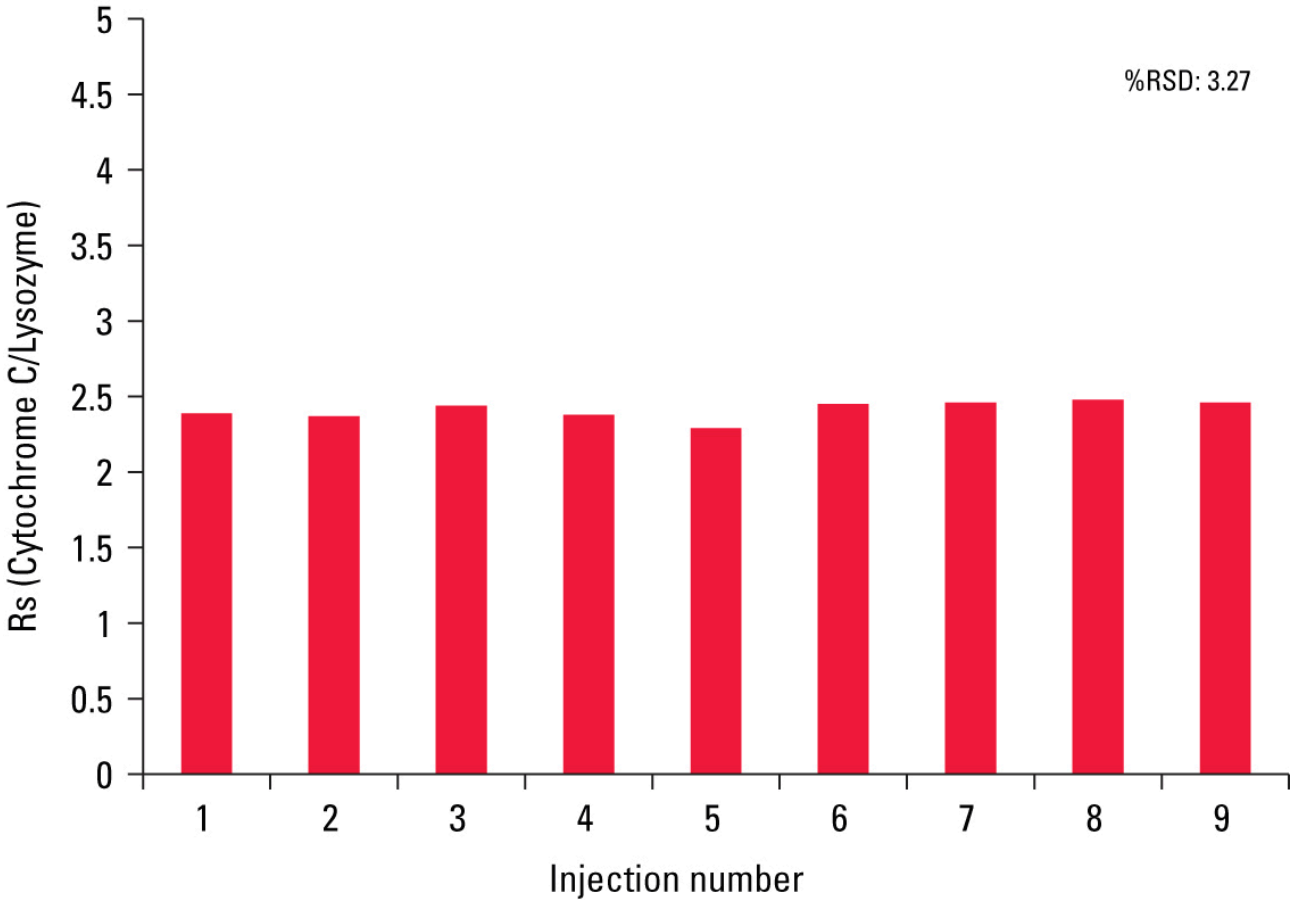
- Analysis was carried out on 4 different days.
- On each day, 5 injections were made.

Figure 8: Lot-to-Lot Reproducibility in Capacity Factor of Lysozyme Analyzed using a TSKgel Protein C_4-300, 2.0 mm ID × 10 cm Column on 3 Different Days



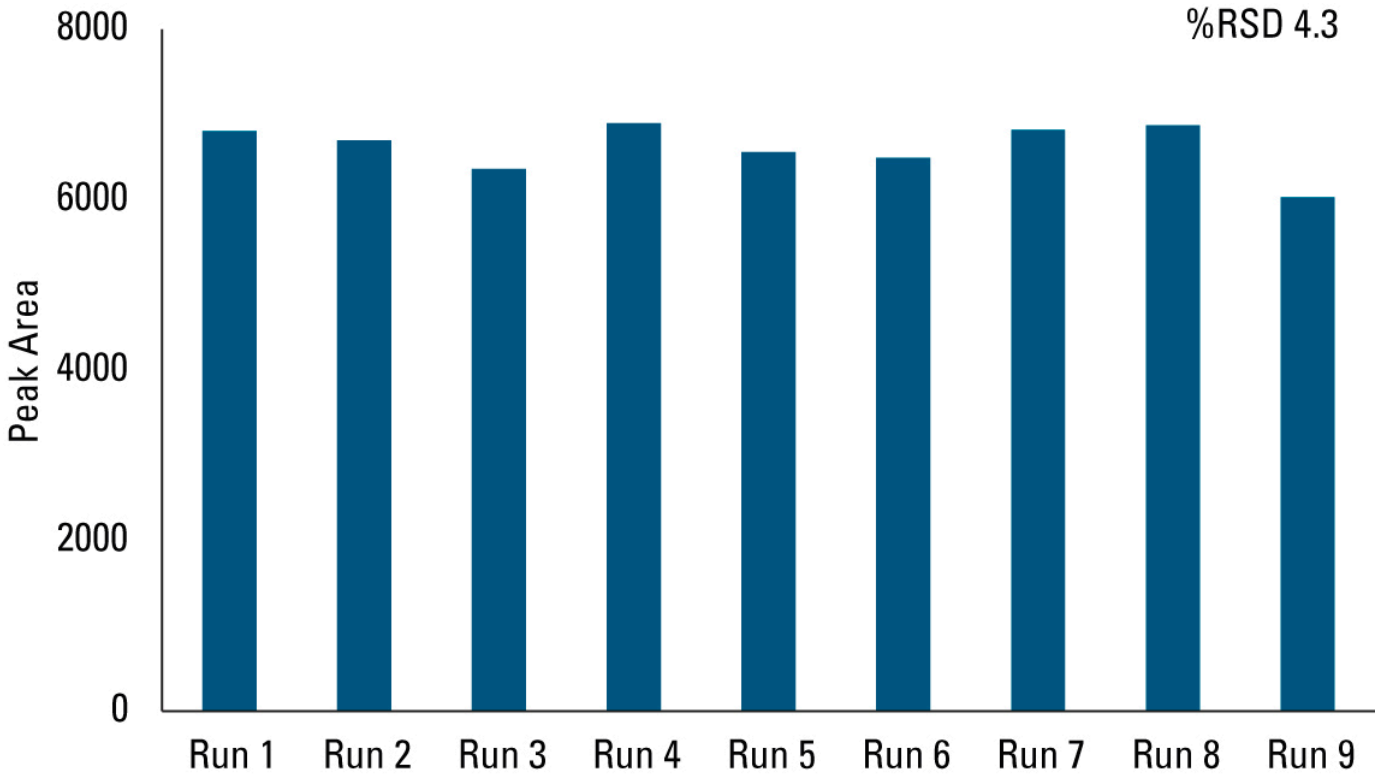
- Excellent reproducibility was obtained using 3 base silica gel lots.

Figure 9: Lot-to-Lot Reproducibility of Resolution Between Cytochrome C and Lysozyme Analyzed on 3 Different Days using a TSKgel Protein C_4-300, 2.0 mm ID × 10 cm Column



- All 3 base silica gel lots demonstrated excellent resolution

Figure 10: Lot-to-Lot Reproducibility of Peak Areas of Lysozyme Analyzed on 3 Different Days using a TSKgel Protein C_4-300, 2.0 mm ID × 10 cm Column



- All 3 base silica gel lots demonstrated excellent consistency.

Conclusions

- A number of proteins with a variety of sizes and hydrophobicity could be well separated using the TSKgel Protein C_4-300 column.
- Excellent intra-day reproducibility was obtained from injection to injection.
- Excellent day-to-day reproducibility was obtained from injection to injection.
- Excellent lot-to-lot reproducibility was obtained.
- The study shows that the columns are independent of the lot of base silica, as well as the bonding and packing procedures.
- The TSKgel Protein C_4-300 column, which has a large pore size of 30 nm, is suitable for highly efficient, reversed phase separations of proteins.