Separation of Proteins and Monoclonal Antibodies using New Wide Pore C4 Phase Silica Gel Reversed Phase Column with Moderate Hydrophobicity Designed for Protein Separation

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Introduction

- 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.
- 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.

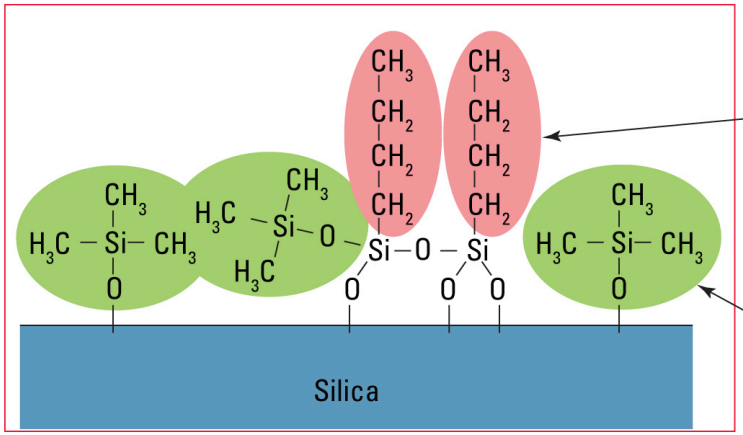
Introduction

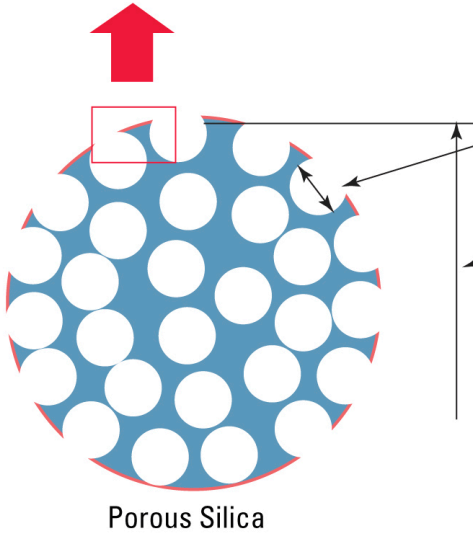
- The stationary phase is fully endcapped with trimethylsilyl (TMS) groups to prevent interaction with free silanol groups. This incurs higher stability of the phase and reduction of peak tailing.
- Optimized ligand density and alkyl length in the stationary phase result in lower adsorption of the protein.
- A particle size of 3 µm yields high theoretical plate counts.
- The large pore size of this column, 30 nm, 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.
- TSKgel Protein C_4-300 columns are designed for the optimal recovery and resolution of proteins, such as recombinant proteins, antibody fragments or PEGylated proteins.
- Here we report the separation of proteins and monoclonal antibodies using this column.

Specifications of the TSKgel Protein C_4-300 Column

Column	TSKgel Protein C_4-300
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%

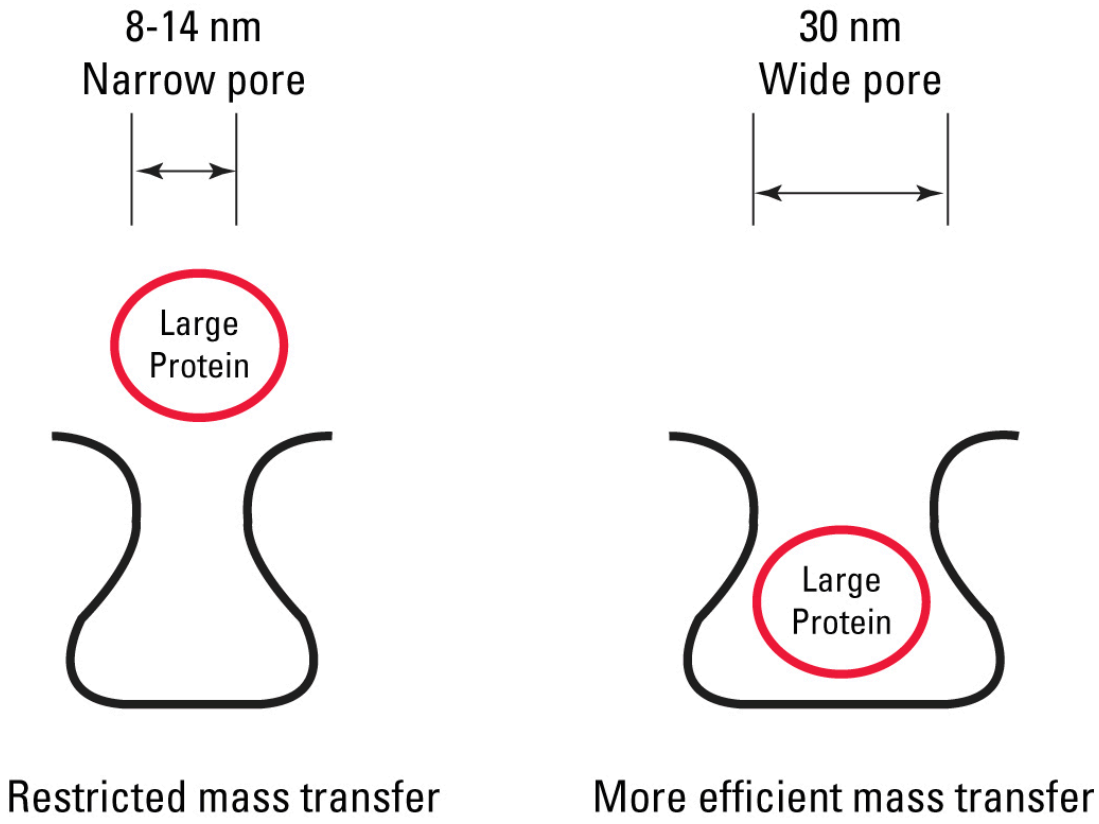
TSKgel Protein C_4-300 Column





- 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.
- The packing is prepared subsequent to endcapping with trimethylsilyl (TMS) groups
- Controlled bonding density of C4 short alkyl chain provides moderate hydrophobicity to the stationary phase, suitable for protein separation with high recovery.

TSKgel Protein C_4-300 Column



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

Specifications of the TSKgel UltraSW Aggregate SEC column

Column	TSKgel UltraSW Aggregate
Dimensions	7.8 mm ID × 30 cm
Particle size	3 µm
Pore size	30 nm
Features	Larger MW exclusion limit than TSKgel G3000SWXL, optimal for high MW samples
Applications	Separation of mAb aggregates (larger than trimer) with high resolution

- Base material: Silica gel
- Functional group: Diol
- This new SEC column is designed for mAb aggregate separation from its monomer.
- This column will be launched soon.

Materials and Methods

Columns:	TSKgel Protein C_4-300, 3 µm, 4.6 mm ID × 10 cm TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm
Instrumentation:	Agilent 1100 HPLC systems Agilent Chemstation (Rev B.04.02)

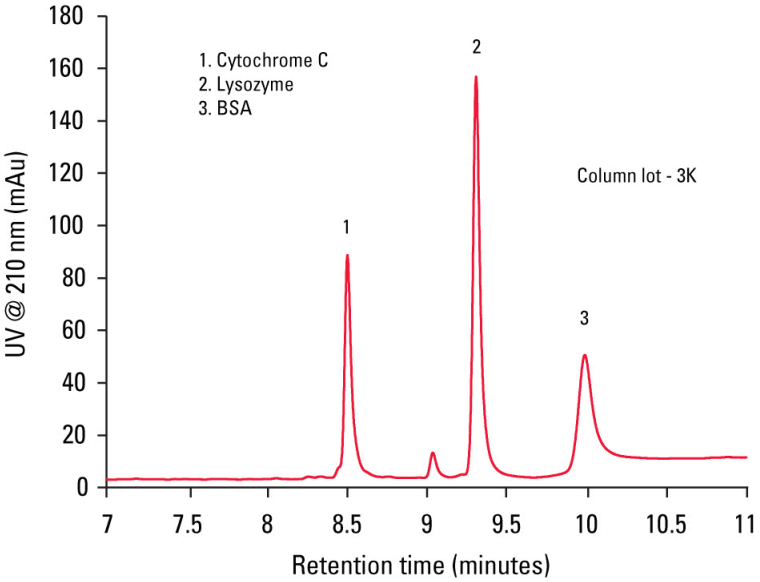
Chromatographic conditions: as mentioned in the respective chromatograms

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
4. ferritin (Sigma F4503-100), 450 kDa, 4.7 mg/mL
5. apoferritin (Sigma A-3660) 450 kDa, 5.0 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 C4-300, 4.6 mm ID × 10 cm Column - Overlay of 5 Consecutive Injections



Mobile phase:	A: H_2O/ACN/TFA = 90/10/0.05 (v/v/v)
	B: H_2O/ACN/TFA = 20/80/0.05 (v/v/v)
Linear gradient:	0-100% B over 20 minutes
Flow rate:	1.0 mL/min
Detection:	UV @ 210 nm
Temperature:	40° C
Injection vol.:	20 µL

- 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.

Table 1: System Suitability 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.

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

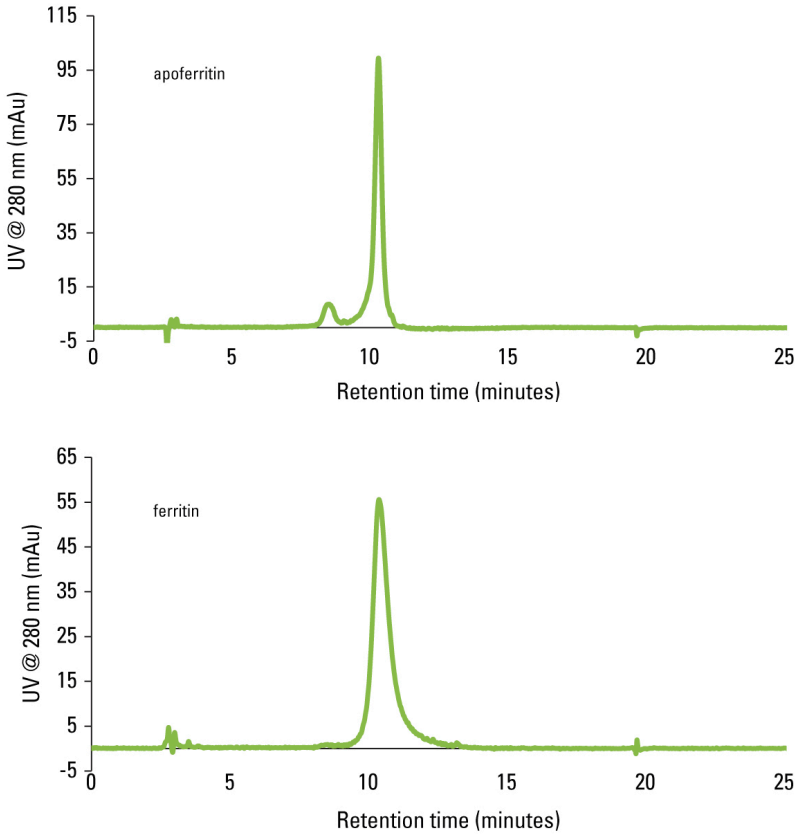
Lot	Lysozyme	Rt	k	Peak Area
1K	Run 1	9.301	8.31	535.10
	Run 2	9.301	8.31	537.67
	Run 3	9.299	8.31	538.89
2K	Run 4	9.421	8.43	549.73
	Run 5	9.422	8.43	544.73
	Run 6	9.424	8.43	546.58
3K	Run 7	9.400	8.41	541.88
	Run 8	9.389	8.40	543.55
	Run 9	9.401	8.41	542.98
	Average	9.37	8.38	542.35
	STDEV	0.06	0.06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

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

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

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

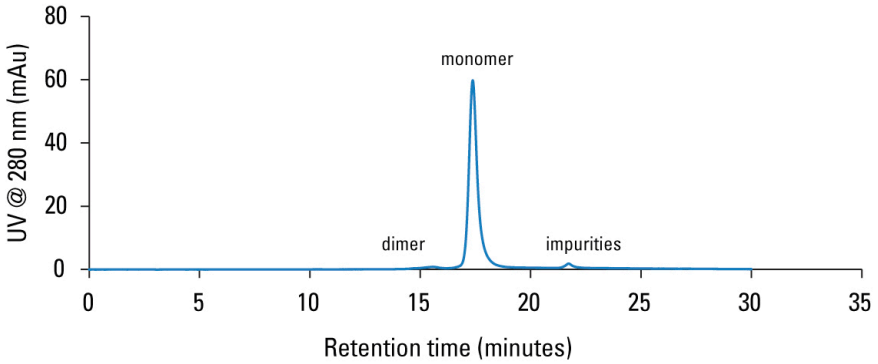
Figure 2: Analysis of Large Metalloproteins using a TSKgel Protein C4-300, 4.6 mm ID × 10 cm Column



Mobile phase:	A: H_2O + 0.05% TFA
	B: CH_3CN + 0.05% TFA
Gradient:	25-80% B in 20 minutes
Flow rate:	0.5 mL/min
Detection:	UV @ 280 nm
Temperature:	40 °C
Injection vol.:	10 µL
Samples:	ferritin, 450 kDa, 4.7 mg/mL
	apoferritin, 450 kDa, 5.0 mg/mL

- A large metalloprotein and corresponding apo-protein could be analyzed using this column.
- Chromatograms shown above are an overlay of 3 consecutive injections.
- Excellent intra-day reproducibility was obtained from injection to injection.

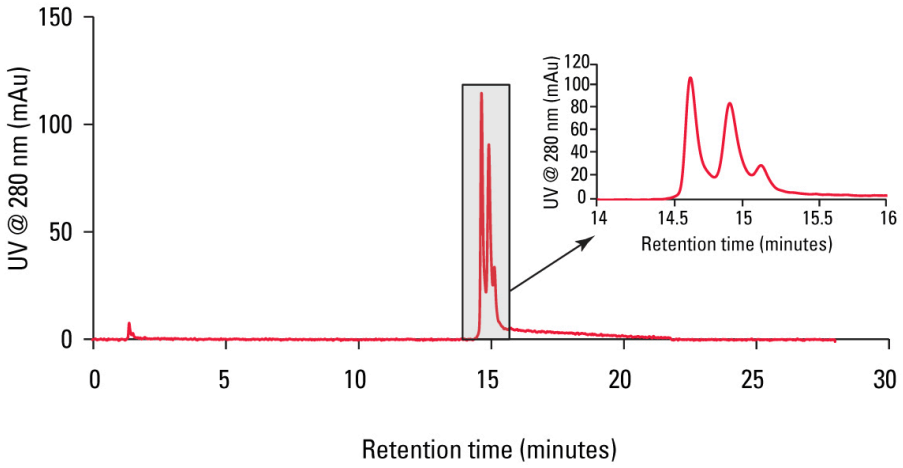
Figure 3: Analysis of Monoclonal Antibody using a TSKgel UltraSW Aggregate, 7.8 mm ID × 30 cm Column



Column:	TSKgel UltraSW Aggregate, 3 µm, 7.8 mm ID × 30 cm
Mobile phase:	0.1 mol/L phosphate buffer, 0.1 mol/L Na_2SO_4, 0.05 NaN_3
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	ambient
Injection vol.:	10 µL

- The separation of this same antibody is carried out using a TSKgel Protein C4-300, 4.6 mm ID × 10 cm column to show more heterogeneity as shown in the next slide.

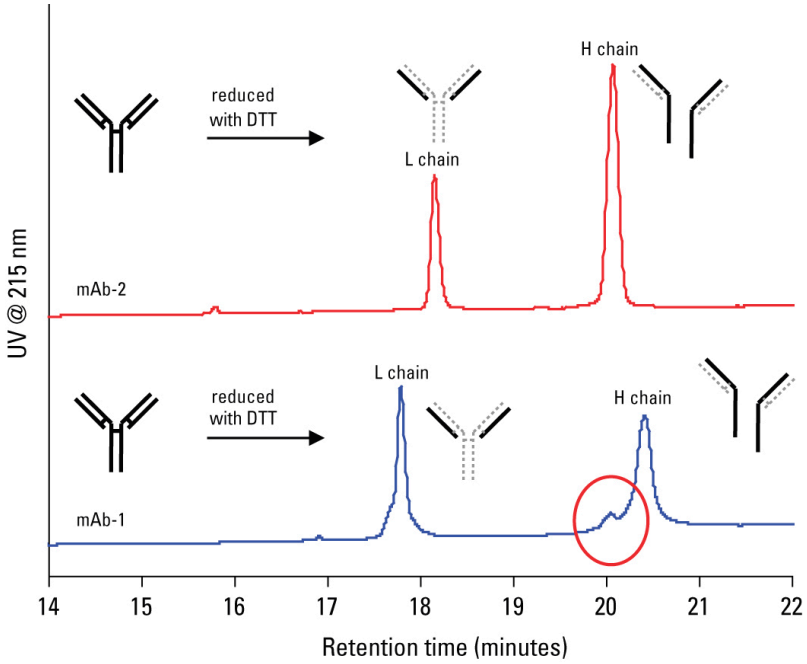
Figure 4: Analysis of Monoclonal Antibody using a TSKgel Protein C4-300, 4.6 mm ID × 10 cm Column



Column:	TSKgel Protein C_4-300, 3 µm, 4.6 mm ID × 10 cm
Mobile phase:	A: H_2O + 0.5% TFA
	B: CH_3CN + 0.5% TFA
Gradient:	5-50% B in 20 min
Flow rate:	1.0 mL/min
Detection:	UV @ 280 nm
Temperature:	70 °C
Injection vol.:	10 µL

- The monoclonal antibody could be analyzed using this column.
- Inset in the chromatogram shows a number of hydrophobic variants which could be separated.
- Excellent intra-day reproducibility was obtained from injection to injection during this analysis.

Figure 5: Separation of mAbs (Reduced Form by Dithiothreitol) using a TSKgel Protein C4-300, 4.6 mm ID × 10 cm column



Column:	TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 10 cm
Mobile phase:	A: H_2O/ACN/TFA = 90/10/0.05 (v/v/v) B: H_2O/ACN/TFA = 20/80/0.05 (v/v/v)
Gradient:	0% B (0 min) - 100% B (45 min)
Flow rate:	1.0 mL/min
Detection:	UV @ 215 nm
Temperature:	50 °C
Injection vol.:	100 µL
Samples:	mAb-1 (mouse monoclonal) mAb-2 (mouse monoclonal) each was reduced with dithiothreitol

- mAb-1 and -2 were reduced with dithiothreitol (DTT) to dissociate into heavy chain and light chain, and then separated with TSKgel Protein C4-300.
- There were small differences in hydrophobicity between mAb-1 and -2.
- A hydrophilic variant of H chain was observed in mAb-1.

Table 3: Recovery of Protein During the Analysis of Proteins using a TSKgel Protein C_4-300, 4.6 mm ID × 10 cm Column

Protein	Recovery
lysozyme	96%
ferritin	92%

A preliminary study (data not shown here) yielded excellent recovery (>90%) during the analysis of the proteins using a TSKgel Protein C_4-300, 4.6 mm ID × 10 cm column.

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 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 column yielded an excellent recovery (>90%) of proteins.
- 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 large proteins.