# Separation of Proteins with a Wide Variety of Hydrophobicity using a Reversed Phase 30 nm Pore Size Short Alkyl Chain Column

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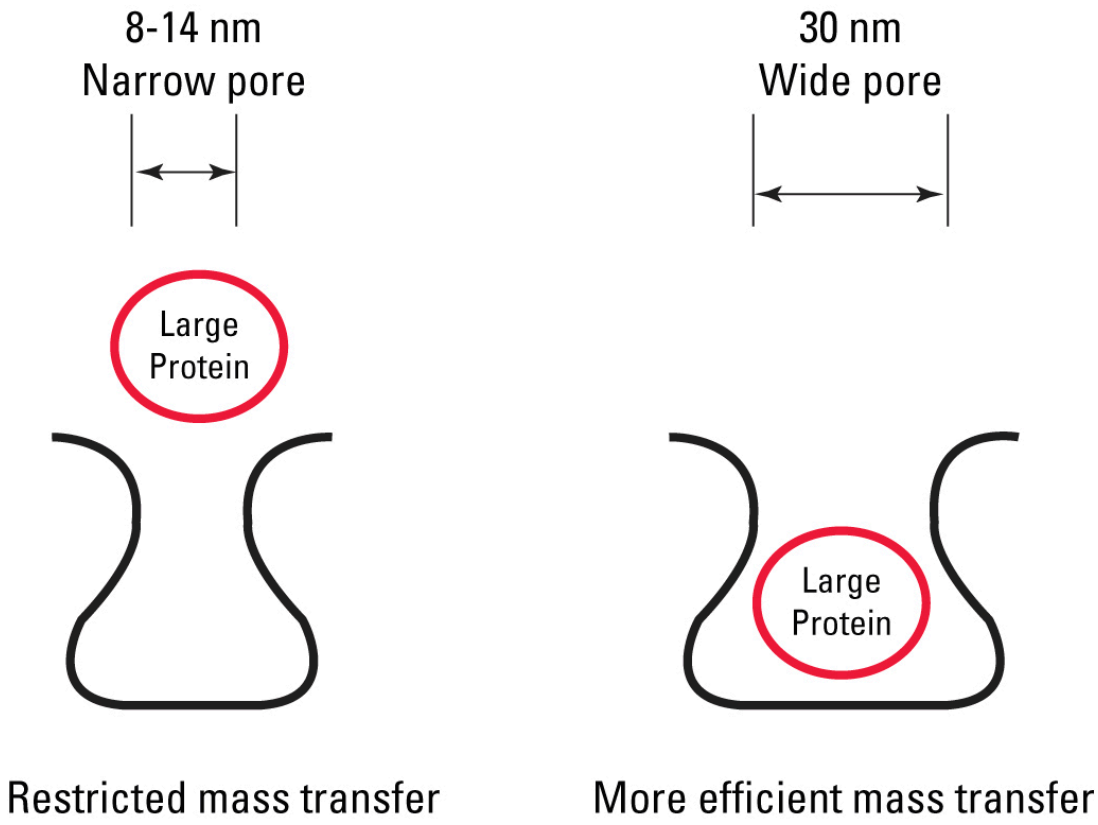
# Introduction

- Reversed phase chromatography is one of the most frequently used chromatographic modes for analytical separations, particularly for the analysis of small molecules.
- But conventional reversed phase HPLC packing materials with 8 -14 nm pore sizes are generally not suitable for the analysis of large intact proteins, due to less accessible pores.
- The packing of the TSKgel® Protein $C_4$-300 column is prepared by polymeric butyl (C4) groups and subsequent endcapping with trimethylsilyl (TMS) groups to 3 µm spherical silica gel with 30 nm pore size.
- Controlled bonding density of C4 short alkyl chain and the large pore size, allowing macromolecules better access to the interior of the pore and subsequently more efficient mass transfer, provides higher retention and higher peak capacities than reversed phase columns with 10 nm pore size.
- Moderate hydrophobicity of the TSKgel Protein $C_4$-300 column is suitable for protein separation with good recovery.
- Recently at Spring ACS 2013, studies were published on the TSKgel Protein $C_4$-300 column for the analysis of proteins and monoclonal antibody.
- Here we report some additional data about the separation of proteins and monoclonal antibodies using this column.

# Specifications of the TSKgel Protein C4-300 Column

| Column | TSKgel Protein C4-300 |
|---|---|
| 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 C4-300 Column




**The larger pore size of the TSKgel Protein C4-300 column helps in more efficient mass transfer during chromatographic analysis.**

# Specifications of the TSKgel UltraSW Aggregate SEC Column used for Orthogonal Separation

| 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 G3000SW$_{XL}$, 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.

# Material and Methods

## Columns

- TSKgel Protein C4-300, 4.6 mm ID x 10 cm, 3 µm particle
- TSKgel TMS-250, 4.6 mm ID x 7.5 cm, 10 µm particle
- TSKgel UltraSW Aggregate, 7.8 mm ID × 30 cm; 3 µm

## Instrumentation

- Agilent 1100 HPLC and an Agilent 1200 system with Chemstation (Rev B.04.02)

## Samples

- cytochrome C (2.1 mg/mL, Sigma C2037-5G)
- lysozyme (2.1 mg/mL, Sigma L6876-25G)
- bovine serum albumin (2.0 mg/mL, Sigma A7906-100G)
- α-Chymotrypsinogen (2.0 mg/mL, Sigma C-4879)
- ferritin (4.6 mg/mL, Sigma F4503-100MG)
- apoferritin (5.0 mg/mL, A-3660)
- mAb 02 (4.6 mg/mL) – A gift from Tosoh Bioscience, GmbH
- rHGH (5.0 mg/mL)

# Chromatographic Conditions

Mobile phase:

- A: $H_2O$ + 0.1% TFA (unless noted otherwise)
- B: ACN + 0.1% TFA (unless noted otherwise)

Linear gradient: 30-50% B over 10 minutes (unless noted otherwise)

Flow rate: 1.0 mL/min (0.5 mL/min for TSKgel TMS-250)

Detection: UV @ 280 nm (unless noted otherwise)

Temperature: 40 °C (unless noted otherwise)

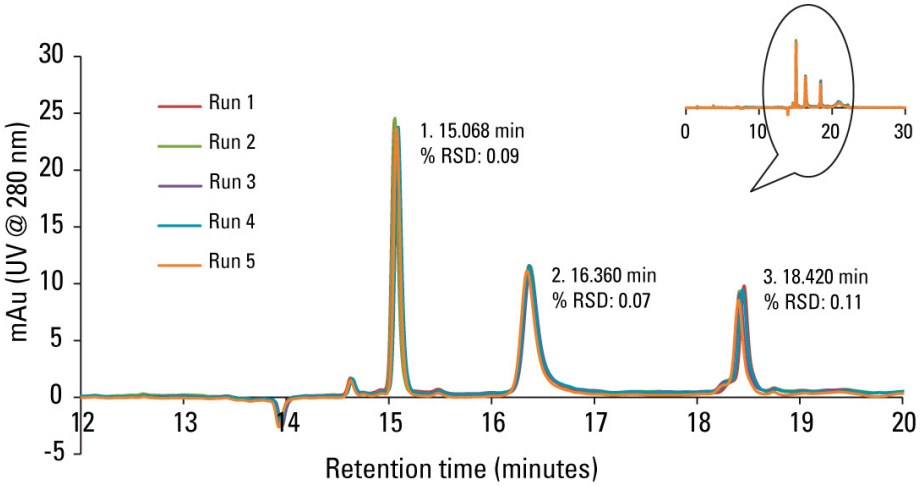
Injection vol.: 10 µL (unless noted otherwise)

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.

Individual chromatographic conditions may vary from experiment to experiment – please refer to the respective chromatograms.

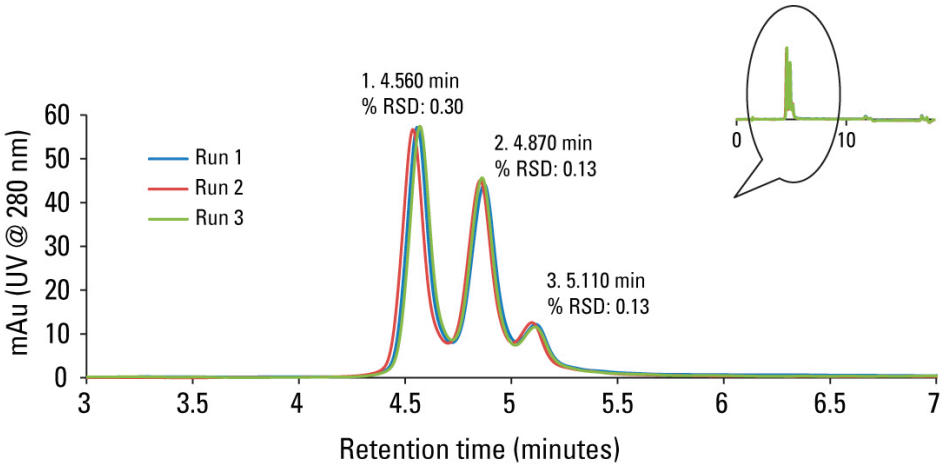
# Figure 1: Analysis of Proteins using a TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 10 cm Column – Overlay of 5 Consecutive Injections




| | |
|---|---|
| Mobile phase: | A: $H_2O$ + 0.05% TFA |
| | B: ACN + 0.05% TFA |
| Gradient: | 5-50% B over 20 min |
| Flow rate: | 1.0 mL/min |
| Detection: | UV @ 280 nm |
| Temperature: | 40 °C |
| Injection vol.: | 10 µL |
| Samples: | standard protein mixture |
| | 1. lysozyme |
| | 2. BSA |
| | 3. chymotrypsinogen |

- **A number of proteins with a wide variety of size and hydrophobicity were well separated.**
- **5 consecutive injections illustrate extremely high reproducibility of results.**
- **% RSD was very low (< 0.2) which conforms to the system suitability.**

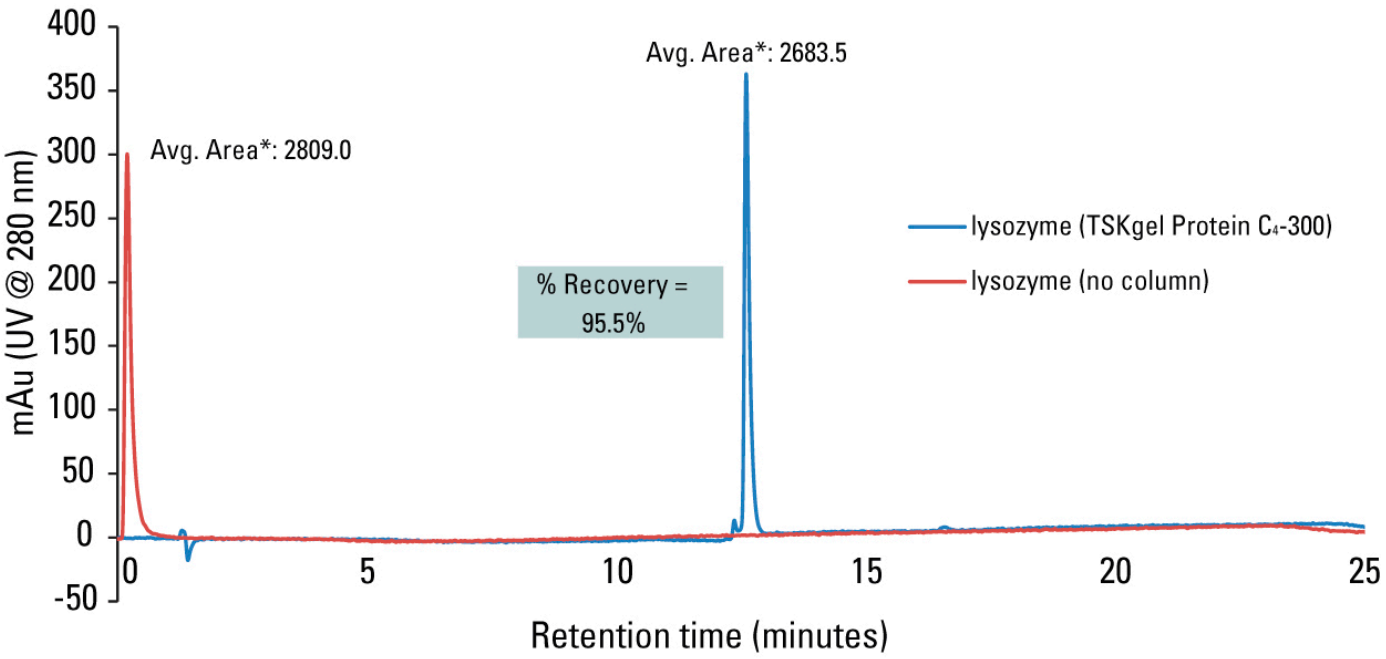
# Figure 2: Analysis of a Monoclonal Antibody using a TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 10 cm Column




| | |
|---|---|
| Mobile phase: | A: $H_2O$ + 0.1% TFA |
| | B: ACN + 0.1% TFA |
| Gradient: | 30-50% B over 10 min |
| Flow rate: | 1.0 mL/min |
| Detection: | UV @ 280 nm |
| Temperature: | 70 °C |
| Injection vol.: | 10 µL |
| Sample: | mAb 02 |

- **Determination of protein purity and heterogeneity derived is of critical importance in the biopharmaceutical industry.**
- **Separation of protein hydrophobic variants by RPC requires high resolution and selectivity to fully identify the heterogeneous profile of the protein.**
- **3 hydrophobic variants were easily identified from analysis of a mAb using the TSKgel Protein C4-300 column.**
- **3 consecutive injections yielded low % RSD for k' for all peaks (% RSD < 0.5)**

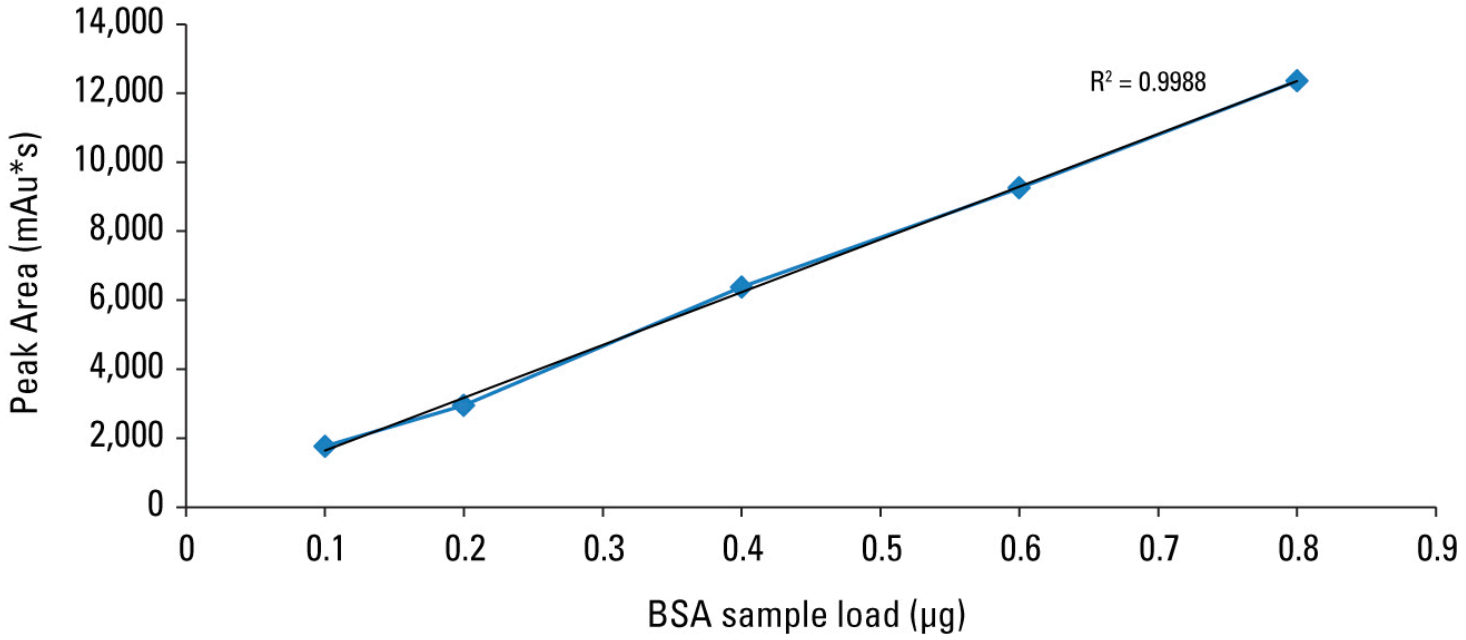
# Figure 3: Recovery of Proteins using a TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 10 cm Column




* % Recovery was calculated using average peak area from two consecutive injections with or without the column

- **Excellent protein recovery (95.5%) was observed using the TSKgel Protein C4-300 column.**
- **Figure 4 also illustrates the independence of sample load on protein recovery for the TSKgel Protein C4-300 column.**

# Figure 4: Effect of Protein Load on the Recovery of Proteins using a TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 10 cm Column

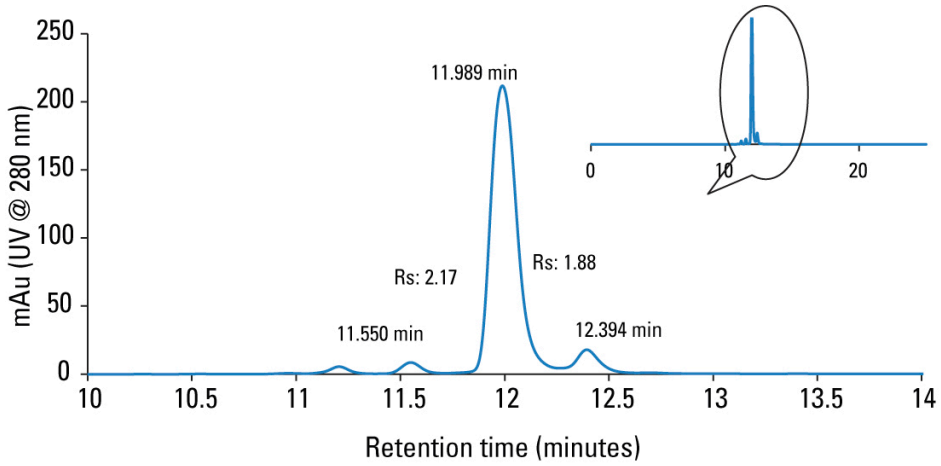



- **BSA protein recovery performed under same chromatographic conditions as figure 3.**
- **Linear response observed as a function of increasing protein load was within the experimental range studied on the TSKgel Protein C4-300 column.**
- **Linear regression yielded a coefficient of regression value of ~ 0.999 up to a protein load of 0.8 µg.**
- **High protein recoveries were obtained at all concentrations of BSA loaded onto the TSKgel Protein C4-300 column.**

# Analysis of r-Human Growth Hormone (rHGH) Protein using the TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 10 cm Column and Fluorescence as Detection

- Fluorescence detectors offer some of the highest sensitivities among currently available HPLC detectors.
- Detection of fluorescent analytes is 10-1,000 times more sensitive than that of strongly absorbing UV species using a traditional UV detector.
- Natural fluorescence is a property intrinsic to most proteins due to the presence of conjugated pi-electron systems found primarily in tryptophan residues located in the primary structure of the protein. Tyrosine and phenylalanine also exhibit fluorescence, but with a far lesser quantum yield in comparison to tryptophan.
- Tryptophan strongly absorbs energy at 280 nm and emits energy in the form of fluorescence at 340 nm.
- Tyrosine and phenyl alanine are the two other intrinsic fluorophores.
- The natural fluorescence found in proteins, combined with the fluorescence transparency of common reversed phase mobile phases, makes RPC-FLD a highly sensitive and specific method of analysis for a wide array of proteins.

# Figure 5: Analysis of r-Human Growth Hormone (rHGH) Protein using the TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 10 cm Column



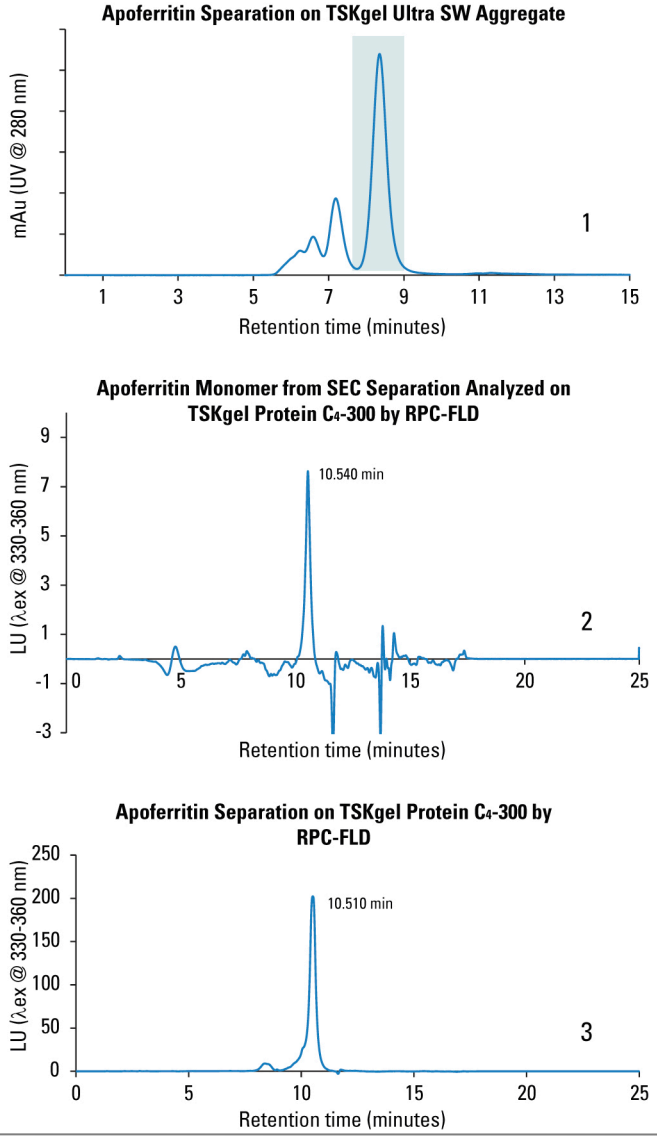

| | |
|---|---|
| Mobile phase: | A: $H_2O$ + 0.05% TFA<br>B: ACN + 0.05% TFA |
| Gradient: | 10-80% B over 15 min |
| Flow rate: | 1.0 mL/min |
| Detection: | FLD: Ex: 280 nm;<br>Em: 330-360 nm |
| Temperature: | 40 °C |
| Injection vol.: | 10 µL |
| Sample: | rHGH (5 mg/mL) |

- **Analysis of r-Human Growth Hormone (rHGH) using the TSKgel Protein C4-300 column and fluorescence as detection were carried out.**
- **The analysis yielded high resolution between the main peak and the peaks on either side (11.55 min and 12.394 min).**

# Orthogonal Separation of a Large Hydrophobic Protein using SEC and RPC

- Size exclusion chromatography (SEC) is commonly used in proteomics for the determination of the distribution of protein monomers, dimers, and higher order aggregates.
- While SEC can separate analytes of different hydrodynamic radii, differences in hydrophobicity go unnoticed and can co-elute during a SEC separation.
- Reversed phase chromatography (RPC) offers an orthogonal separation mode to SEC to further characterize analyte composition.
- Fraction collection from SEC usually results in highly dilute samples which may require additional preparation steps for accurate quantitation using UV/Vis detection with RPC.
- SEC-RPC-FLD results in extremely high sensitivity, eliminating additional sample preparation procedures prior to RPC analysis.

# Figure 6: Orthogonal Separation of a Large Hydrophobic Protein using SEC and RPC




- Panel 1: Separation of apoferritin (450 kDa) using the TSKgel UltraSW Aggregate SEC column (Shaded region depicts collected fraction used for analysis by RPC in panel 2)
- Panel 2: Analysis of apoferritin monomer (collected from SEC separation in panel 1) on TSKgel Protein C4-300 column using FLD detection
- Panel 3: Separation of apoferritin on TSKgel Protein C4-300 using FLD detection

The orthogonal analysis clearly shows that the monomer of the apoferritin could be separated completely from heterogenic impurities.

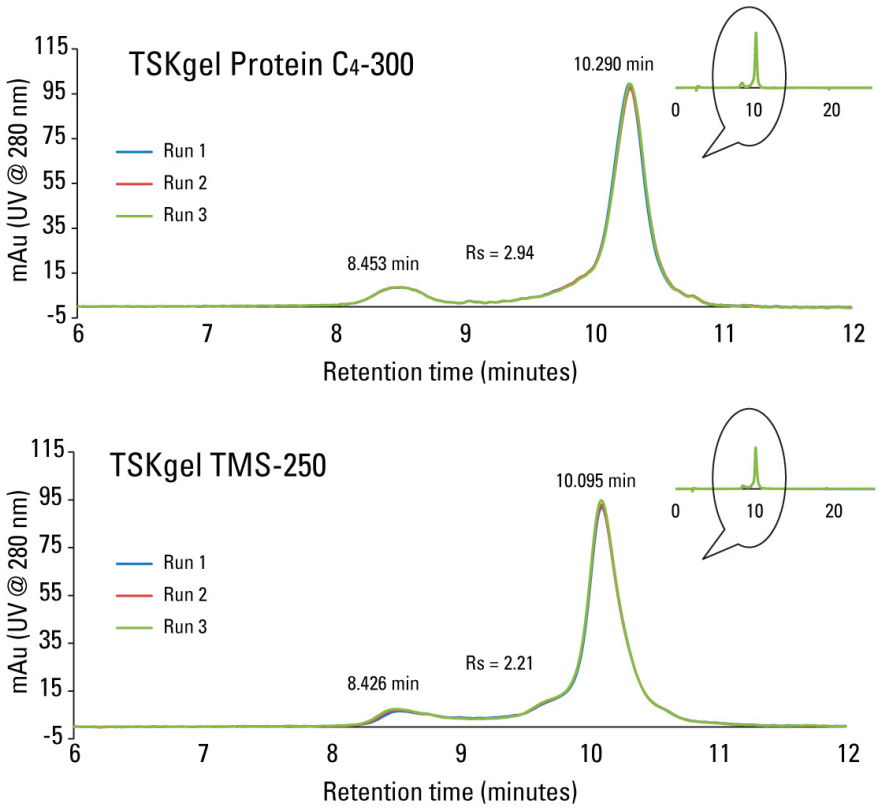
# TSKgel Protein C4-300 vs. TSKgel TMS-250

**Column Parameters:**

| TSKgel Protein C4-300 | | TSKgel TMS-250 | |
|---|---|---|---|
| Pore size: | 30 nm | Pore size: | 25 nm |
| Particle size: | 3 µm | Particle size: | 10 µm |
| Endcapped: | Yes (Trimethylsilyl) | Endcapped: | Yes |
| pH stability: | 1.5 - 7.5 | pH stability: | 2.0 - 7.5 |
| % carbon | 3% | % carbon | 5% |
| Ligand: | C4 (butyl) | Functional group: | C1 (monomeric bonding chemistry) |
| Specific surface area: | 100 $m^2/g$ | | |

- **Both columns offer a large pore size for high mass transfer within the stationary phase.**
- **The 3 µm particle size of the TSKgel Protein C4-300 columns yields higher resolution compared to the TSKgel TMS-250.**

# Figure 7: Separation of Apoferritin using 30 nm TSKgel Protein C4-300, 3 µm, 4.6 mm ID × 10 cm Column and 25 nm TSKgel TMS-250, 10 µm, 4.6 mm ID × 7.5 cm Column




| | |
|---|---|
| Mobile phase: | A: $H_2O$ + 0.05% TFA |
| | B: ACN + 0.05% TFA |
| Gradient: | 25-80% B over 15 min |
| Flow rate: | 0.5 mL/min |
| Detection: | UV @ 280 nm |
| Temperature: | 40 °C |
| Injection vol.: | 10 µL |
| Sample: | apoferritin (5 mg/mL) |

- **The TSKgel Protein C4-300 column yields higher resolution compared to the TSKgel TMS-250.**
- **Both columns yielded consistency over 3 consecutive injections.**

# Conclusions

- A number of proteins with a variety of sizes and hydrophobicity were well separated using the TSKgel Protein $C_4$-300 column.
- Excellent reproducibility was obtained from injection to injection.
- The column yielded an excellent recovery (> 90%) of proteins.
- The TSKgel Protein $C_4$-300 column could be used for the orthogonal separation 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.