Development of Newer Packing Materials for Protein Separations with Ultra Performance Liquid Chromatography

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INTRODUCTION

Ion exchange and size exclusion chromatography are commonly used for the analysis and purification of proteins. The complete analysis and characterization of these species is most efficient when orthogonal analytical techniques are applied to the samples. Each technique should be based on different physical properties of the protein and can include chromatographic methods such as size exclusion, ion exchange and reversed-phase chromatography. UltraPerformance Liquid Chromatography® (UPLC® Technology) has been used for protein characterization in several ways. We can now add IEX and SEC to the range of techniques available for protein analysis that benefit from UPLC Technology. Combining the low dwell volume of the ACQUITY UPLC® system and the higher pressure capabilities of newer ion exchange and size exclusion materials, improved resolution, sensitivity and speed can be achieved over conventional methods for bioseparations. In the following presentation the impurity detection of biomolecules, specifically the determination of charged variants and aggregates is demonstrated. The application of these techniques gives improved detection of impurity and often leads to faster analysis.

METHODS

Chromatographic Conditions

Size-Exclusion Chromatography

LC System: ACQUITY UPLC® System with TUV detector and 30cm Column Heater
Columns: ACQUITY UPLC BEH200 SEC, 1.7µm, 4.6 x 300 mm and 4.6 x 150 mm
Injection Volume: 20.0 µL
Flow Rate: 0.4 mL/min (unless otherwise noted)
Mobile Phase A: 25mM Sodium Phosphate, pH 6.8, 0.15M NaCl
Weak Needle Wash: Mobile Phase A
Strong Needle Wash: Mobile Phase A
Seal Wash: 90/10 H_2O/MeOH
Temperature: 30°C
Detection: UV @ 280 nm
Flow Cell: Stainless Steel (P/N 205000610)
Sample Diluent: 25mM Sodium Phosphate, pH 6.8, 0.15M NaCl (unless otherwise noted)

Ion-Exchange Chromatography

LC System: ACQUITY UPLC® System with TUV detector
Column: Protein-Pak™ Hi Res CM, 7µm, 4.6 x 100 mm
Protein-Pak™ Hi Res SP, 7µm, 4.6 x 100 mm
Protein-Pak™ Hi Res Q, 5µm, 4.6 x 100 mm

CM– Carboxymethyl Q– Quaternary Ammonium
SP—Sulfopropyl

Mobile Phase A: 20mM Tris (anion-exchange) or 20mM Phosphate Buffer (cation-exchange).
Mobile Phase B: 20mM Tris or 20mM Phosphate Buffer, 0.5M NaCl
Specific buffer pH in figure legends.
Weak Needle Wash: Buffer A
Strong Needle Wash: Buffer B
Seal Wash: 90/10 H_2O/MeOH
Temperature: 30°C
Detection: UV @ 280 nm
Flow Cell: Stainless Steel
Sample Diluent: Buffer A

Additional description in figure headings.

SIZE-EXCLUSION CHROMATOGRAPHY

A new column was developed for UPLC size-exclusion chromatography. This chromatographic techniques is often used for the analysis of protein aggregates. When combined with the low dispersion UPLC system, better resolution and higher sensitivity can be achieved.

Analyte	MW
1. β-Amylase (3mg/mL)	200,000
2. BSA (3mg/mL)	66,000
3. Carbonic Anhydrase (3mg/mL)	29,000
4. Ribonuclease A (3mg/mL)	13,700
5. Uracil (100 µg/mL)	112

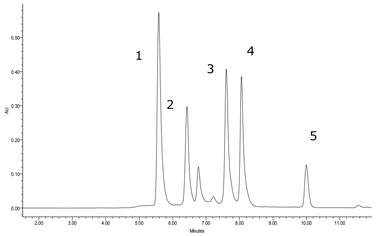

Figure 1. Protein calibration standard on ACQUITY UPLC BEH200 SEC, 1.7µm, 4.6 x 300mm column. These chromatographic data were used to construct the calibration curve as shown in Figure 2.

Analyte	MW
Blue Dextran	2,000,000
Thyroglobulin	669,000
β-Amylase	200,000
IgG,	150,000
Amyloglucosidase	97000
Conalbumin	75000
BSA	66,400
Ovalbumin	44,000
Carbonic Anhydrase	29,000
Myoglobin	17,000
Lysozyme	14,400
Ribonuclease A	13,700
Aprotinin	6,500
Uracil	112

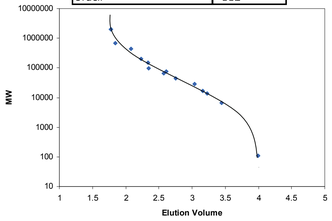

Figure 2. Protein calibration curve, ACQUITY UPLC BEH200, 1.7 µm SEC, 1.7 µM, 4.6 x 300mm. Recommended molecular weight range for ACQUITY UPLC BEH200, 1.7 µM SEC column is 10,000– 500,000.

Analyte	MW
1.Thyroglobulin, 3 mg/mL	669,000
2. IgG, 2 mg/mL	150,000
3. BSA, 5 mg/mL	66,400
4. Myoglobin, 2 mg/mL	17,000
5. Uracil, 0.1 mg/mL	112

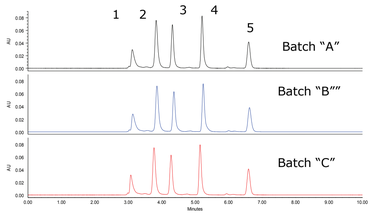

Figure 3. Batch to batch reproducibility, ACQUITY UPLC BEH200 SEC, 1.7 µm, 4.6 x 150mm column. Buffer: 100mM Sodium Phosphate, pH 6. 8. Flow rate: 0.3 mL/min.

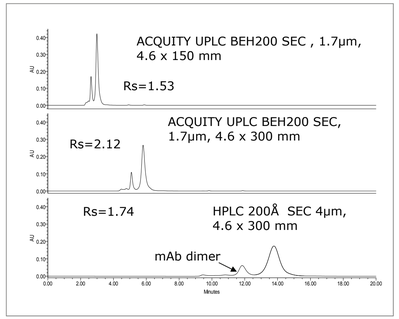

Figure 3. Effect of particle size on size-exclusion chromatography. With smaller particle SEC columns better resolution can be obtained. If additional resolution is not required the same packing material can be used in a shorter column for reduced run time. As expected the flow rate for optimal resolution is higher with smaller particle columns.
Top: ACQUITY BEH200 SEC 1.7µm, 4.6 x 150 mm: 3000 psi, 0.4mL/min.
Middle: ACQUITY BEH200 SEC 1.7µm, 4.6 x 300 mm: 6000 psi, 0.4 mL/min.
Bottom: HPLC 250Å 4 µm, 4.6 x 300 mm: 953 psi, 0.2mL/min.

ION-EXCHANGE CHROMATOGRAPHY

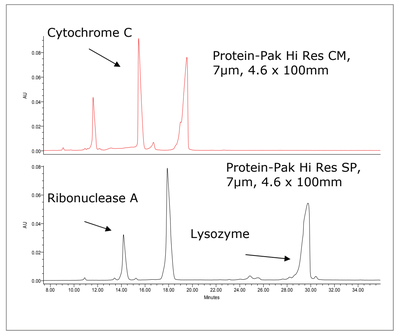

Figure 4. Protein separation on cation-exchange columns. Separation on strong (SP) and weak (CM) cation-exchange columns shows selectivity differences between two column chemistries. Conditions: Buffer A: 20mM Sodium Phosphate, pH 6.6; Buffer B: A with 0.5M NaCl, 0-60%B in 34 min.

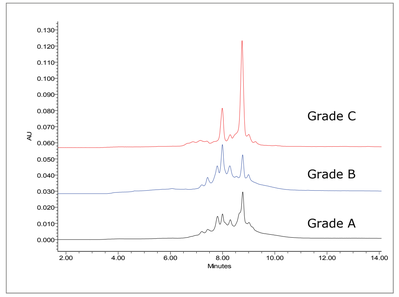

Figure 5. Analysis of a charge variants of chicken ovalbumin on Protein-Pak Hi Res Q 5µm, 4.6 x 100mm column. Three grades of purified ovalbumin contained different relative amounts of variant structures as shown in the pattern of charge variants. Separation was achieved in under 10 min. Conditions: A: 20mM Tris, pH8.5; B: A with 0.5 M NaCl, 0-100%B in 13 min.

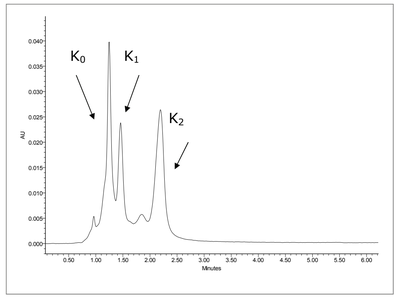

Figure 6. Separation of C- terminal lysine variants of a chimeric monoclonal antibody. In addition to the single residue truncation, additional charged variants are recognizable in the chromatogram. Protein-Pak Hi Res CM 7µm. Conditions: Protein-Pak Hi Res CM 7µm, 4.6 x 100mm; A: 20mM Sodium Phosphate, pH 7; B: A with 0.7M NaCl, pH 7; 0– 10% B in 25 min.

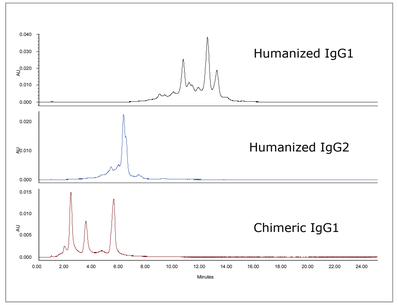

Figure 7. Ion-exchange analysis of monoclonal antibodies. A single column and method were used for the analysis of multiple antibody preparations. Each antibody preparation showed a unique pattern of charged variants Conditions: Protein-Pak Hi Res CM 7µm, 4.6 x 100mm; A: 20mM Sodium Phosphate, pH 7; B: A with 0.7M NaCl, pH 7; 0– 10% B in 25 min.

CONCLUSIONS

- **New packing materials for protein separations enable the benefits of UPLC Technology to be realized for both ion-exchange and size-exclusion chromatography.**
- **Size-Exclusion Chromatography**
  - ACQUITY UPLC BEH200 SEC, 1.7µm separates proteins over the molecular weight range of 10,000 to 500,000.
  - The SEC method can be adapted to varying requirements of run time and resolution.
  - Monoclonal antibody monomers and dimers are resolved on this column.
- **Ion-Exchange Chromatography**
  - A selection of ion-exchange columns, including strong and weak cation-exchangers and a strong anion-exchanger, has been developed for use with the ACQUITY UPLC system.
  - These columns can be used for the analysis of charge variants in monoclonal antibodies.
- **This work extends UPLC technology to a wider range of bioseparations.**

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