Optimized Superficially Porous Particles for Peptide and Protein Analysis

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Abstract

For several years we have been designing and producing superficially porous (Fused-core®) particles for HPLC columns. The characteristics of these particles were specifically created to separate certain solutes optimally, usually based on molecular size. The original 2.7 µm superficially porous particles were created with an average pore size of 90 Å, which was suitable for small molecule analytical separations. This particle technology has been expanded to include wider pore sizes and larger particle sizes that are specifically designed for larger biomolecules. Novel particle designs with specially selected bonded phases for peptide and protein separations are described. This presentation includes fast separations of peptides and intact protein mixtures, as well as examples of very high resolution separations of larger proteins and associated variants and contaminants. Columns with bonded phases for these particles demonstrate high temperature stability, which is ideally suited for the conditions that are often used for analytical and small scale preparative biomolecular separations. Protein recovery and sample loading investigations are included. The optimized shell thickness of the new particles represents a compromise between a short diffusion path versus adequate retention and mass load tolerance. Examples of high molecular weight protein separations highlight the advantages of using columns of superficially porous particles with wider pores. Some comparisons with conventional totally porous particles are also shown.

Physical Characteristics of Fused-Core Particles

Fused-Core Particle	Particle Size (µm)	Pore Size (Å)	BET Surface Area (m^2/g)	Shell Thickness (µm)	% Porosity	Pore Volume (cm^3/g)	Separation Utility
Halo	2.7	90	135	0.5	75	0.26	Small molecules < 5000 MW
HALO-5	4.6	90	90	0.6	56	0.21	Small molecules < 5000 MW
Halo Peptide	2.7	160	80	0.5	75	0.29	Peptides < 15 kDa
HALO-5 Peptide	4.6	160	60	0.6	56	0.21	Peptides < 15 kDa
HALO Protein	3.4	400	15	0.2	31	0.11	Proteins < 400 kDa

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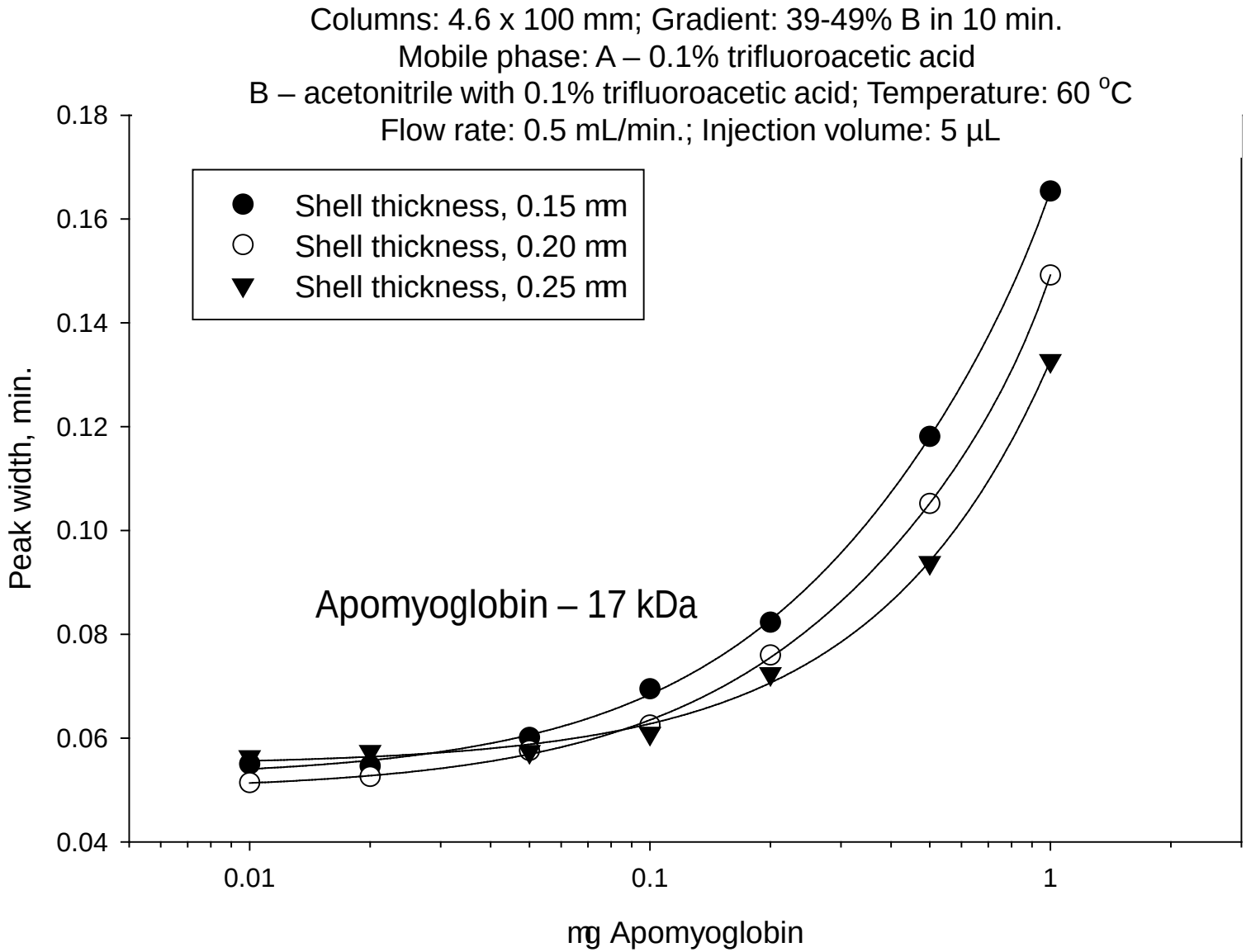
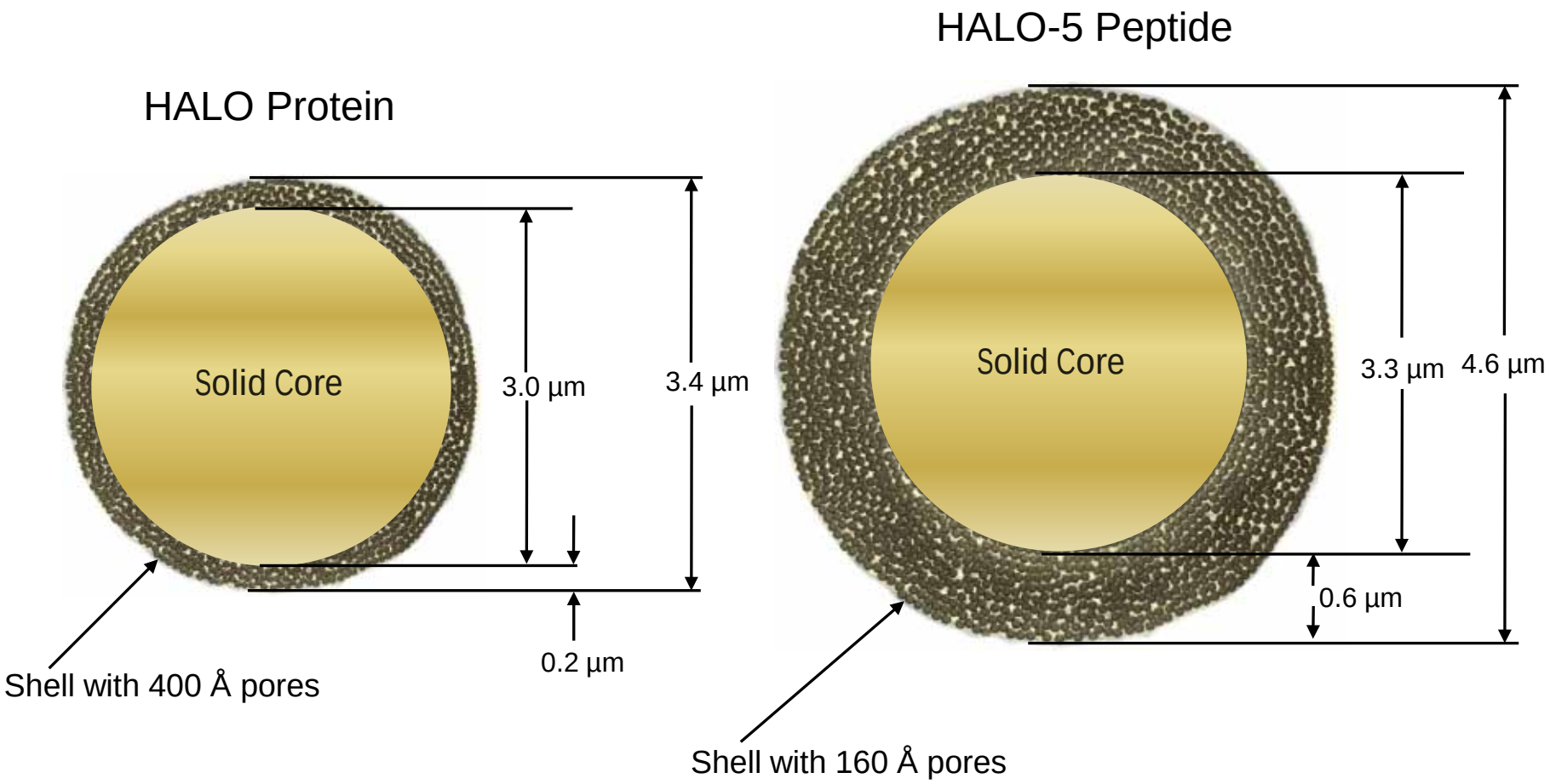
Sample Loading Study for HALO Protein C4

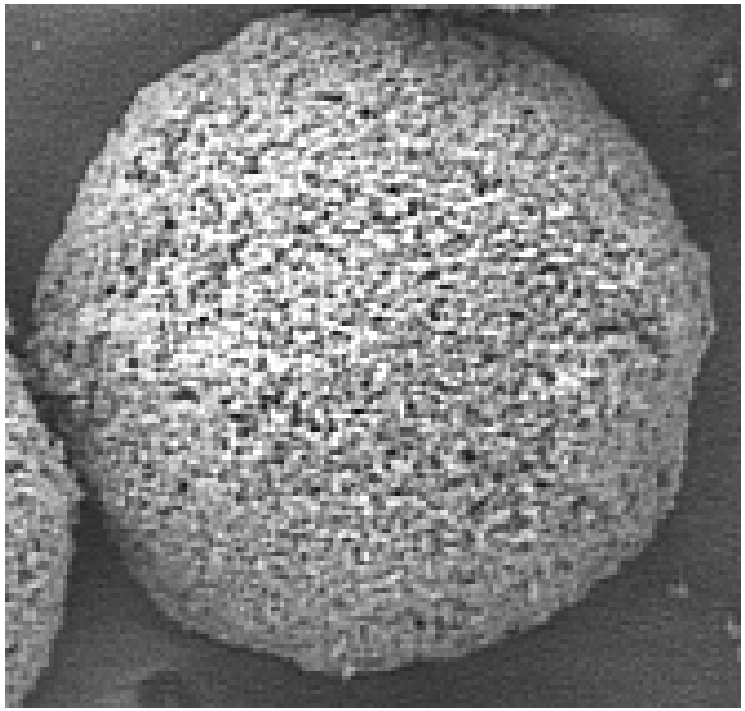


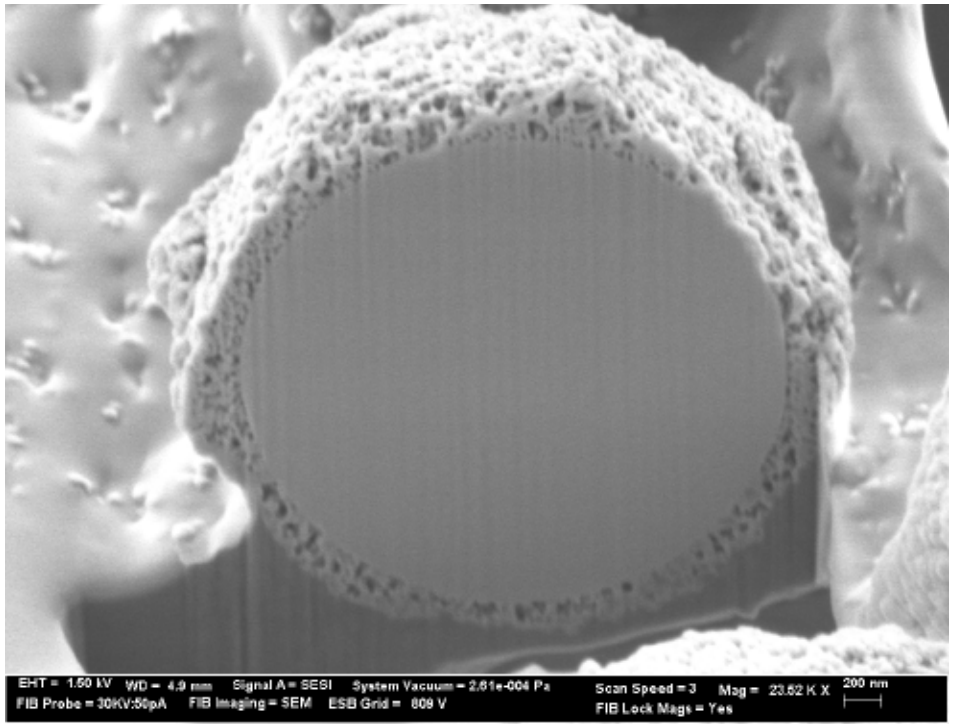
The particle with 0.20 µm shell thickness offers a compromise between sample loadability and retention, as well as an optimized diffusion path for large MW biomolecules.

HALO® Wide-Pore Fused-Core Particles



SEM of Images of HALO Protein Particles





FIB (Focused Ion Beam) sliced particle

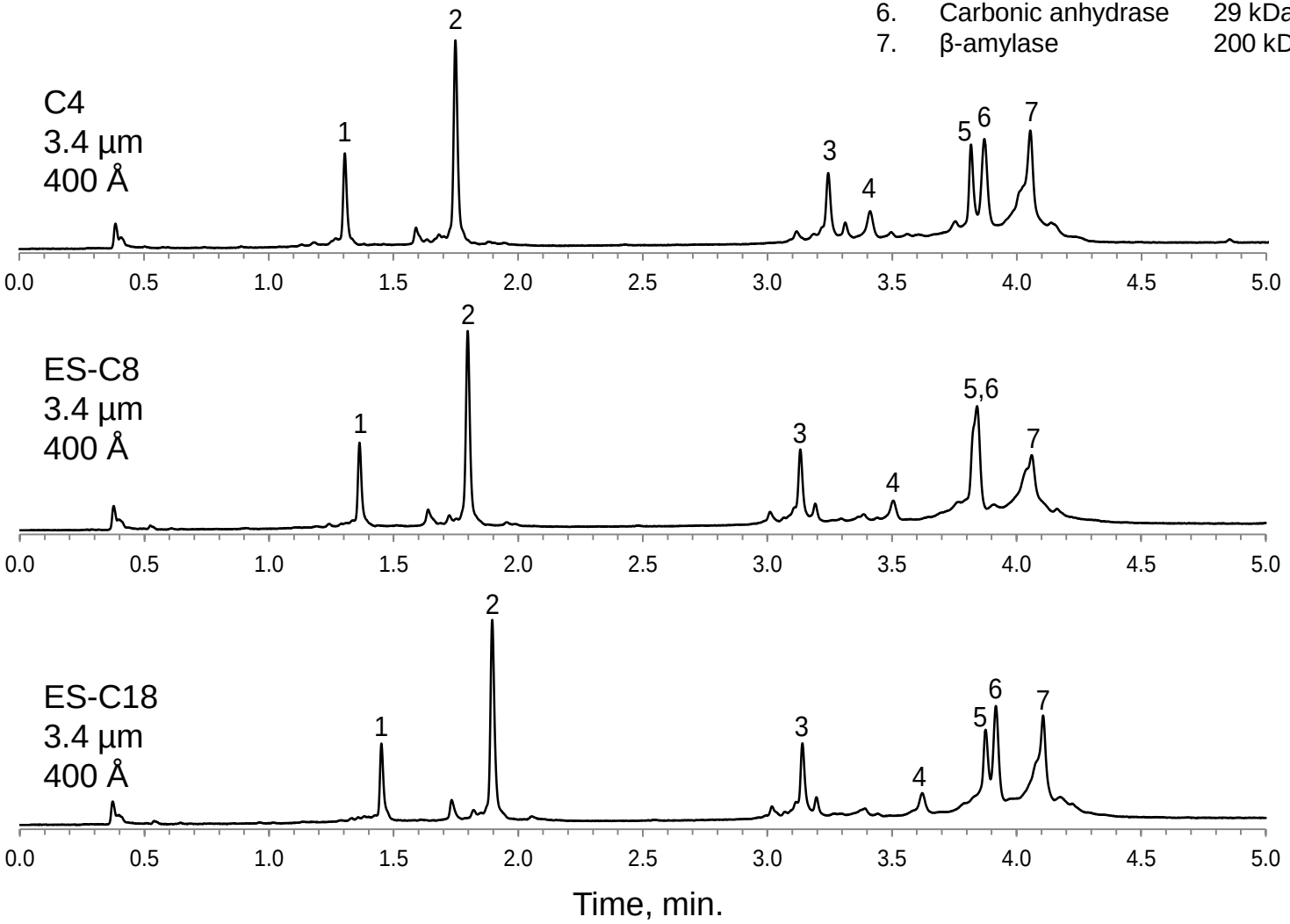
Comparison of Bonded Phases

Columns: 2.1 x 100 mm HALO Protein
Instrument: Shimadzu Nexera
Injection Volume: 1 µL
Detection: PDA @ 280 nm
Temperature: 60 °C

Flow rate: 0.5 mL/min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 35-66.3% B in 5 min.

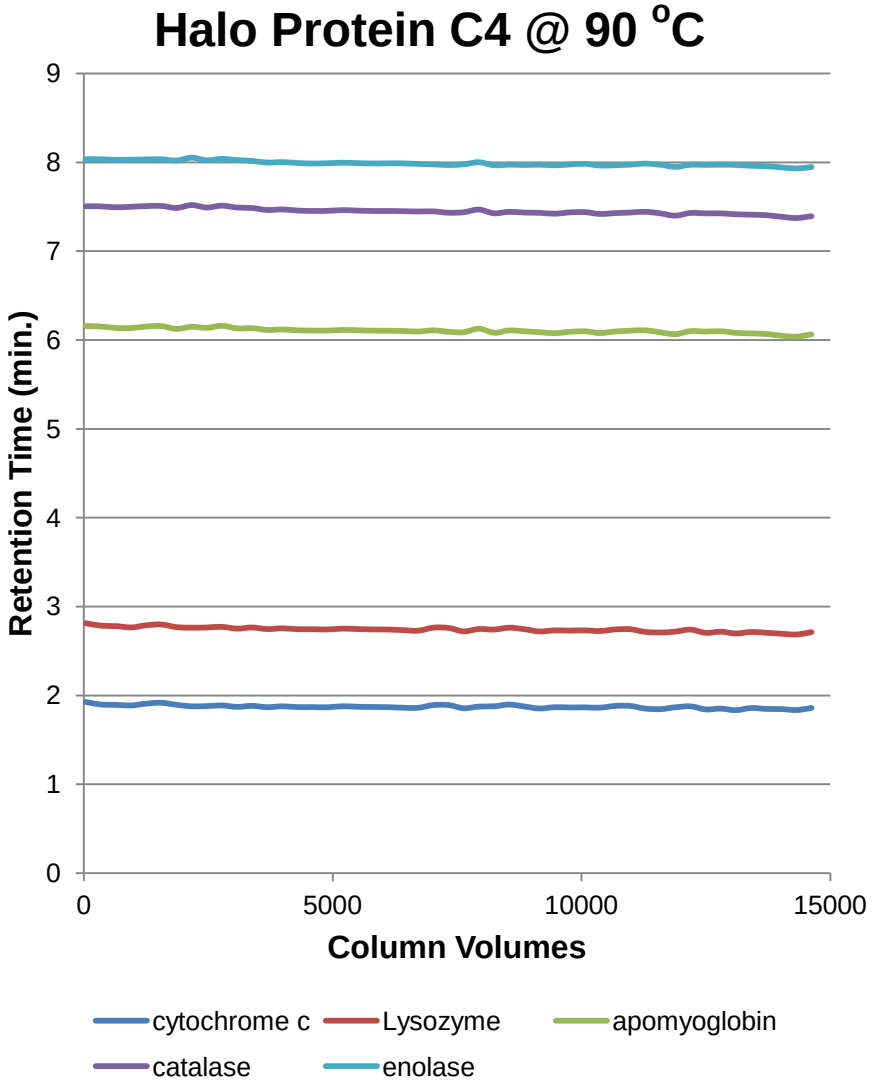
Sample: In order

1.	Cytochrome *c*	12.4 kDa
2.	Lysozyme	14.3 kDa
3.	α-chymotrypsin	25 kDa
4.	Catalase	250 kDa (4 x 60 kDa)
5.	Enolase	46.7 kDa
6.	Carbonic anhydrase	29 kDa
7.	β-amylase	200 kDa (4 x 50 kDa)



Column Stability Study

Column: 2.1 x 100 mm; Mobile phase gradient: 25-40% acetonitrile/0.1% aqueous trifluoroacetic acid in 10 min;
Temperature: 90 °C; Flow rate: 0.5 mL/min; Detection: 215 nm



- The HALO Protein C4 bonded phase is stable up to 90 °C, showing very little loss of retention.

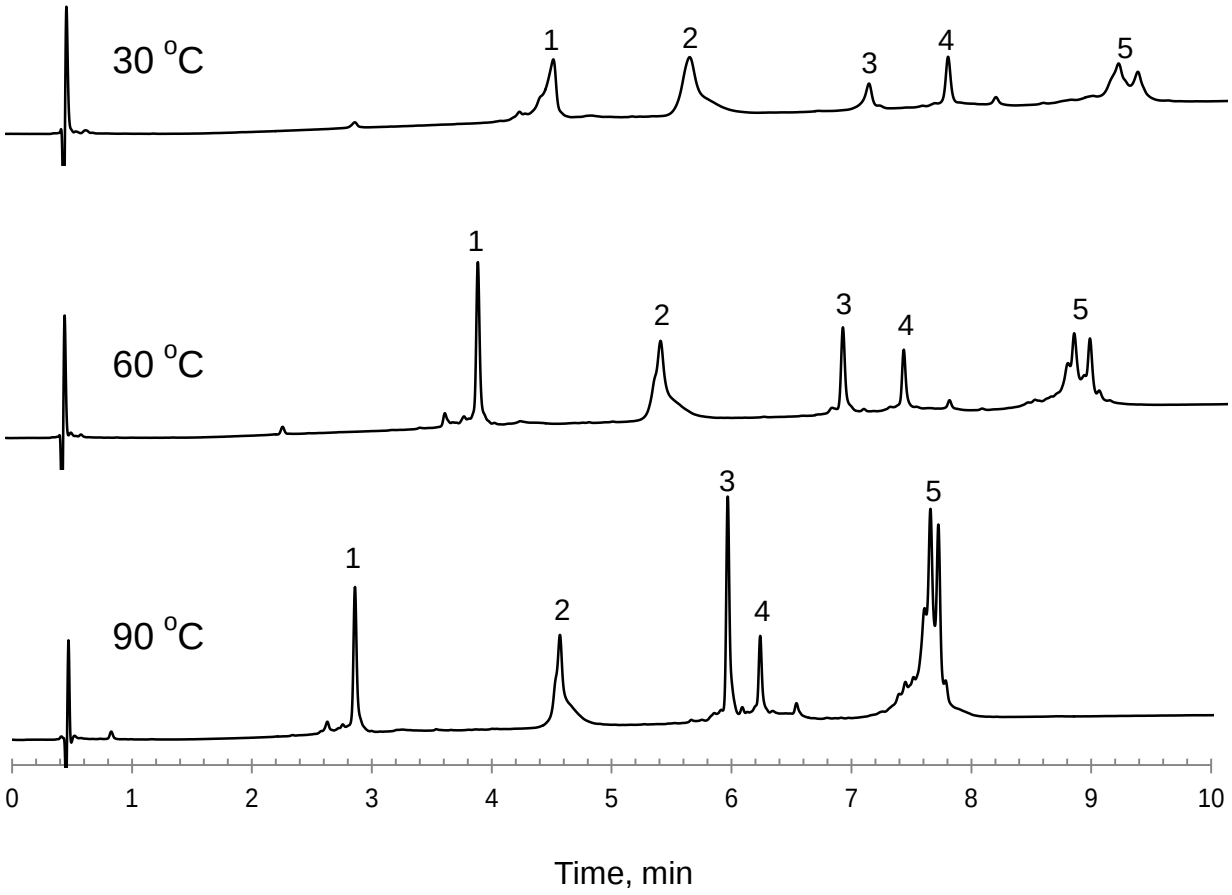
Protein Separations: Effect of Temperature

Column: 2.1 x 100 mm HALO Protein C4
Instrument: Agilent 1200 SL
Injection Volume: 2 µL
Detection: 215 nm
Temperature: as indicated

Mobile Phase A: water/0.1% TFA
Mobile Phase B: acetonitrile/0.1% TFA
Gradient: 28-58% B in 10 min.
Flow rate: 0.45 mL/min

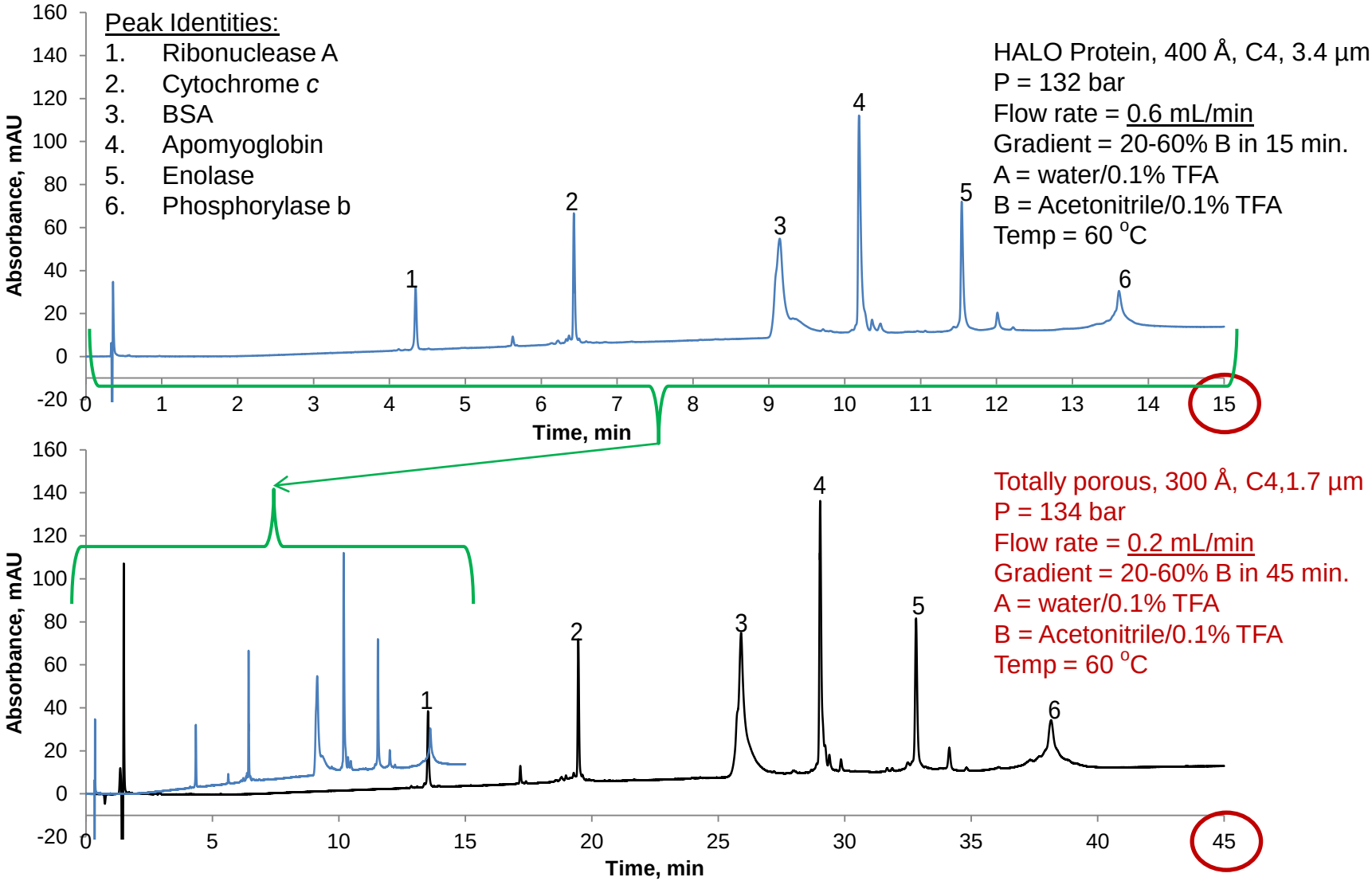
Peak Identities (in order):

1.	Lysozyme	14.3 kDa
2.	BSA	66.4 kDa
3.	α-Chymotrypsinogen A	25.0 kDa
4.	Enolase	46.7 kDa
5.	Ovalbumin	44.0 kDa



- Protein peak shape and recovery improve with increased temperature of analysis.

Protein Separations: Fused-Core compared to Totally Porous



- Separation is 3 times faster at the same back pressure on the HALO Protein column compared to the same sample run on a sub-2-µm totally porous particle column

Protein Recovery Studies

Protein	Recovery
Cytochrome *c*	100 (5.8 SD)
Catalase	92 (18 SD)

- Proteins were fraction collected from a 4.6 x 100 mm HALO Protein C4 column run at 60 ^{o}C under gradient conditions with water/ACN/0.1% TFA mobile phase. Blanks were obtained by replacing the column with a union
- Lyophilized proteins were reconstituted using 3 M Urea/1% Triton X-100/0.25% acetic acid
- Protein recoveries were measured using QuantiPro™ BCA Assay Kit for 0.5-30 µg/mL protein (Sigma-Aldrich, St. Louis, MO)
- Samples were incubated at 37 ^{o}C for 100 min.
- Each sample was run in duplicate
- Absorbance values were measured at 562 nm
- HALO Protein C4 shows good recovery of proteins

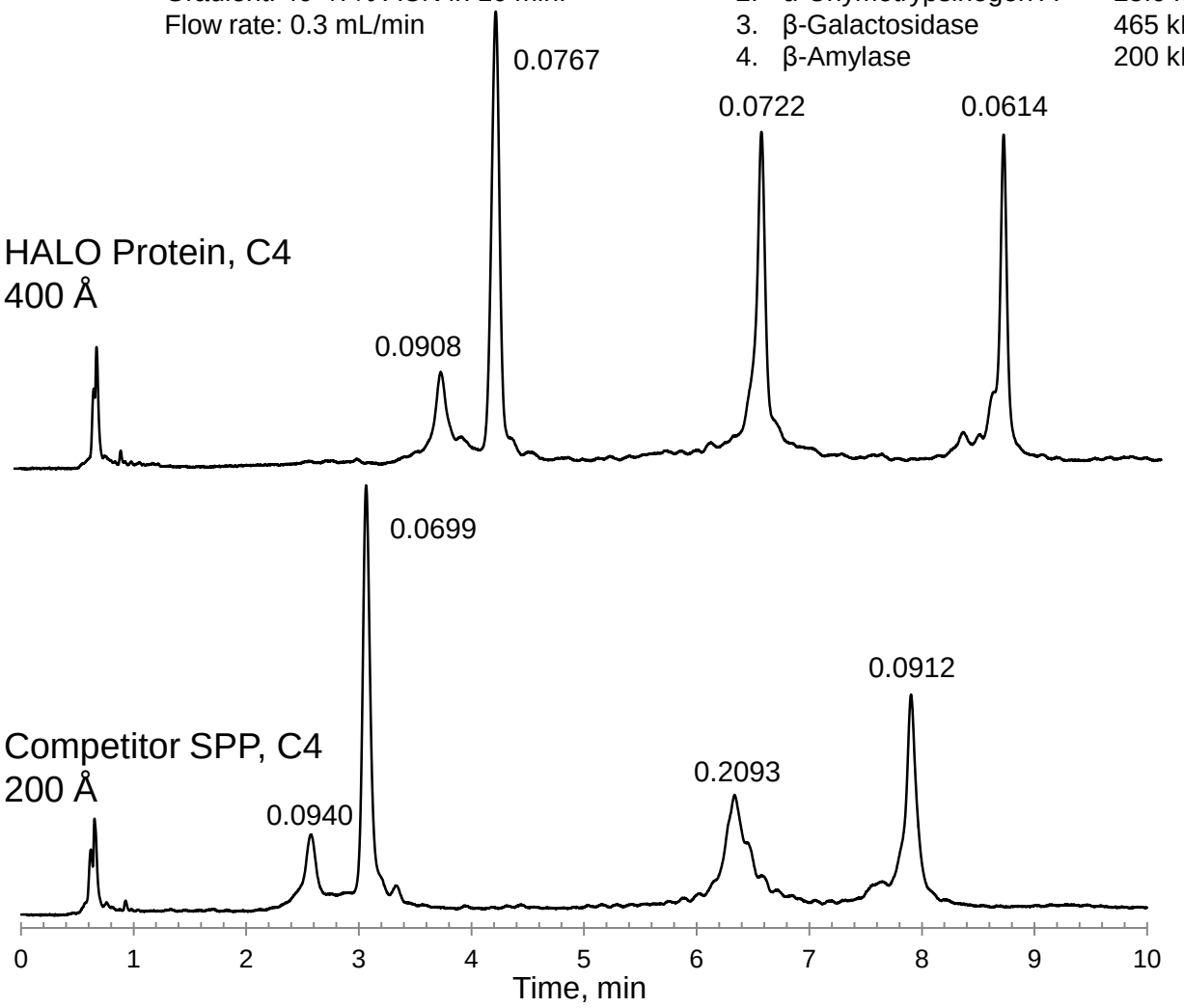
Protein Separations: Effect of Pore Size

Columns: 2.1 x 100 mm
Instrument: Shimadzu Nexera
Injection Volume: 1 µL
Detection: 280 nm
Temperature: 60 °C

Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 40-47% ACN in 10 min.
Flow rate: 0.3 mL/min

Peak Identities (in order):

1.	Catalase	250 kDa [~60 kDa subunit]
2.	α-Chymotrypsinogen A	25.0 kDa
3.	β-Galactosidase	465 kDa [116 kDa subunit]
4.	β-Amylase	200 kDa [~50 kDa subunit]

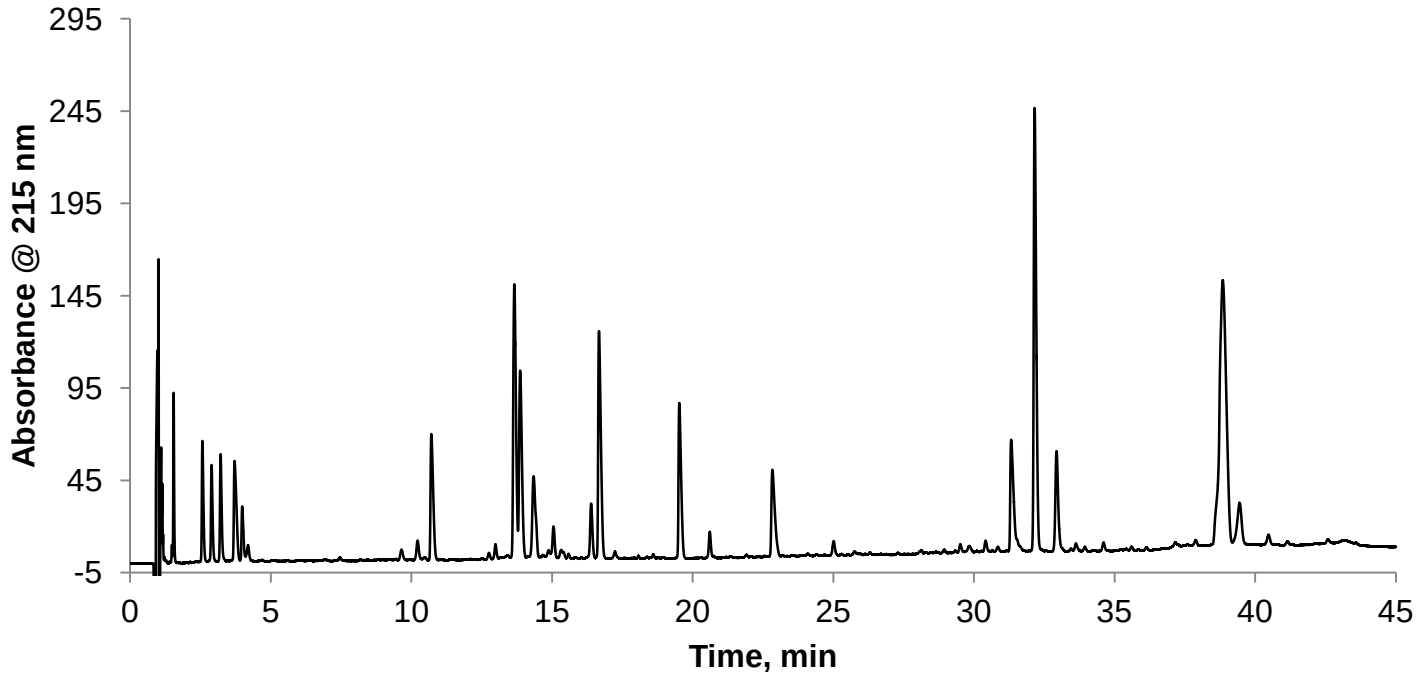


- Peak widths in minutes provided above each peak.
- The 400 Å pores of HALO Protein enable sharp peaks for high MW biomolecules.

Tryptic Digest using HALO-5 Peptide ES-C18

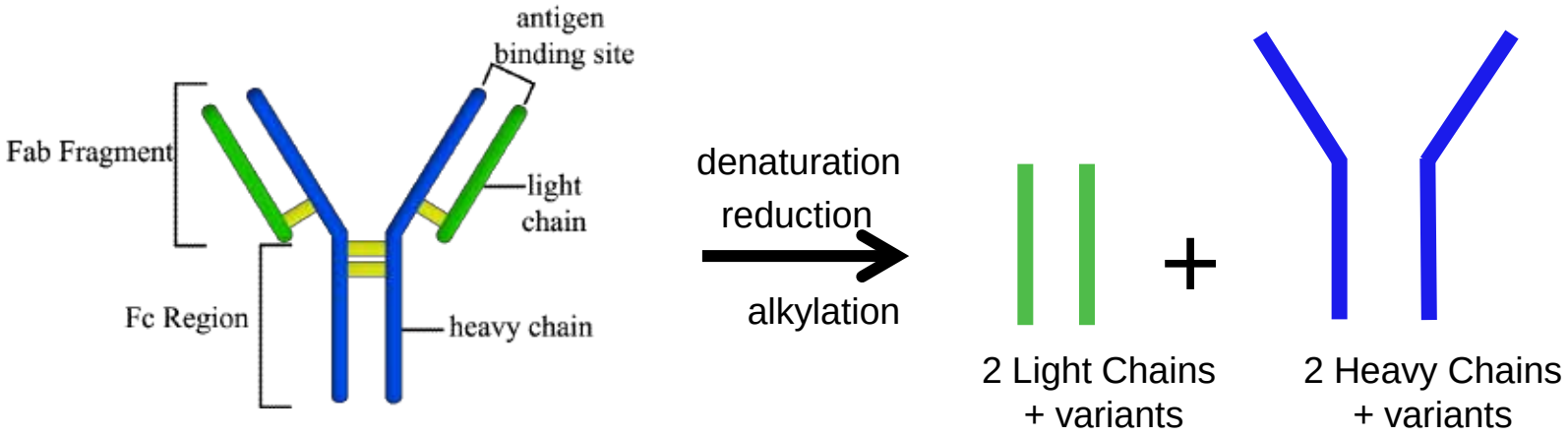
Column: 4.6 x 100 mm, HALO-5 Peptide ES-C18
Instrument: Agilent 1100
Injection Volume: 10 µL
Detection: 215 nm
Temperature: 45 °C
Pressure: 54 bar initial

Mobile Phase A: water/0.1% TFA
Mobile Phase B: ACN/0.1% TFA
Gradient: 5-40% B in 45 min.
Flow rate: 1.0 mL/min
Sample: Apomyoglobin Tryptic Digest [2 mg/mL]



The extremely low back pressure of the HALO-5 Peptide ES-C18 column enables fast, efficient proteomic separations with a low potential for plugging.

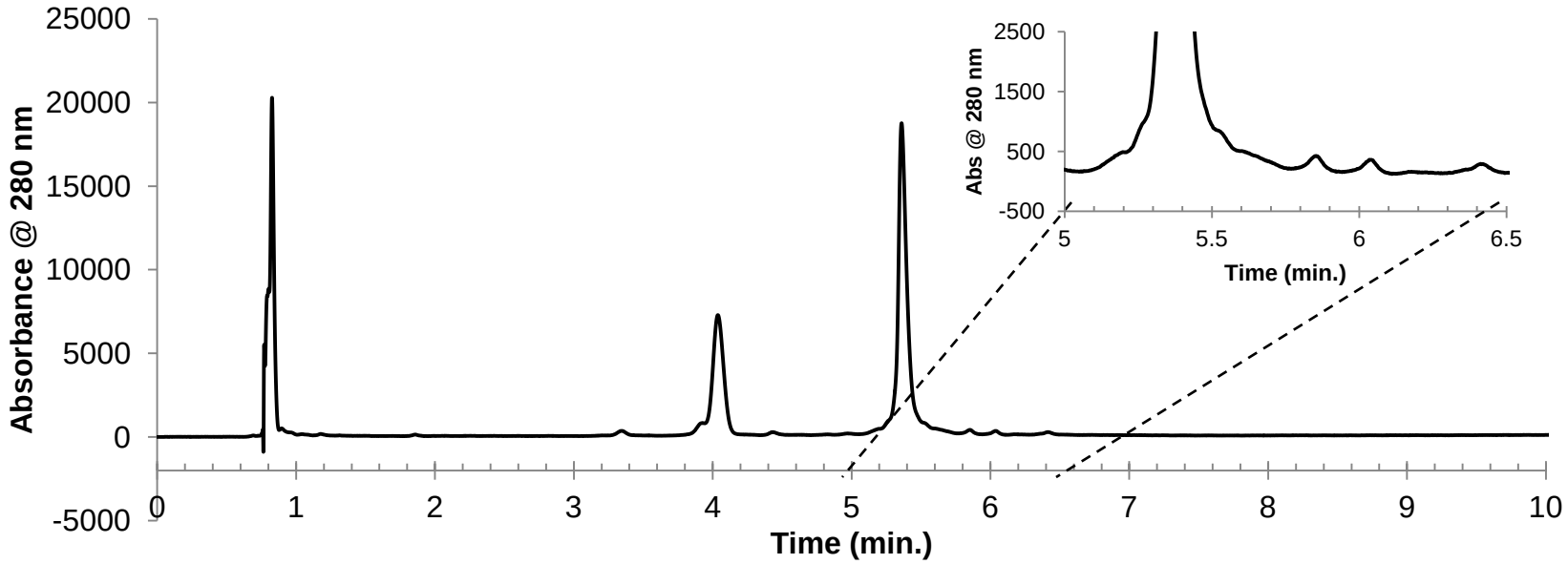
Separation of Reduced IgG1 using TFA Mobile Phase



Column: 2.1 x 100 mm HALO Protein C4
Instrument: Shimadzu Nexera
Injection Volume: 1 µL
Detection: 280 nm
Temperature: 80 °C

Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 33-40% B in 10 min.
Flow rate: 0.25 mL/min

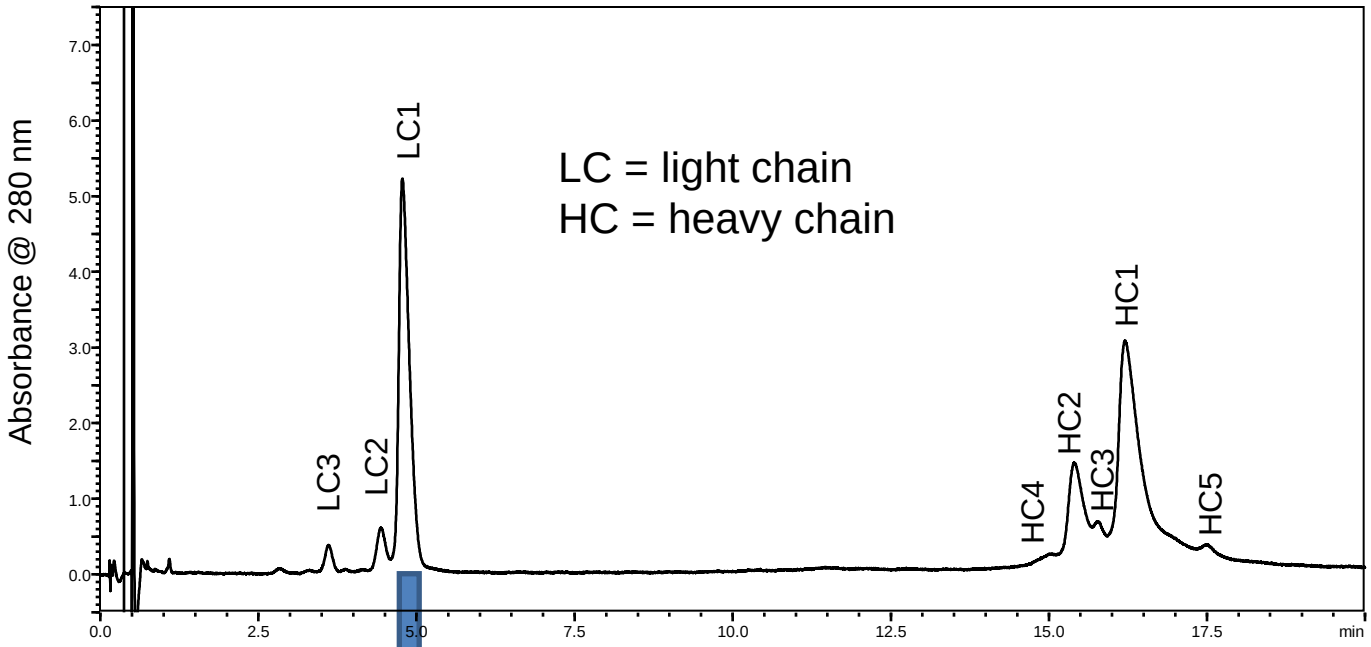
Sample: 0.5 mg/mL IgG1 treated with 100 mM DTT in 8 M Guanidine HCl at 50 °C for 35 min. Sample reduced only.

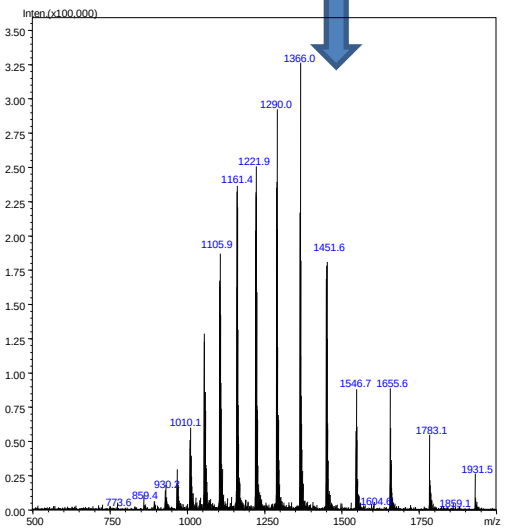


Antibody structure: Afaneh, C, Aull, MJ, Kapur, S, *Modern Immunosuppression Regimens in Kidney Transplantation*, 2012.

High Resolution Analysis of mAb IgG1 Light and Heavy Chains with LC/MS

2.1 mm ID x 100 mm HALO Protein C4; 0.4 mL/min.; A: 0.5 % formic acid with 20 mM Ammonium Formate
B: 45% AcN/45% IPA/ 0.5 % formic acid with 20 mM Ammonium Formate; Gradient: 29-32% B in 20 min.; 80 ^{o}C
Detection: 280 nm Abs; Shimadzu LCMS-2020, ESI +4.5 kV, 2 pps, 500-2000 m/z





ID	Mass (Da)
LC1	23,204
LC2	23,192
LC3	23,203
HC1	50,539
HC2	50,424
HC3	50,668
HC4	50,680
HC5	28,862

Masses deconvoluted using MagTran

Conclusions

- Fused-core particles with 400 Å pores are effective for efficiently separating proteins without restricted diffusion
- Protein separations can be run approximately 3 times faster on columns of Fused-core particles compared to columns of sub-2-µm particles at the same back pressure
- Fused-core particles have performance advantages over totally porous particles for separating peptides and proteins
- Columns of 400 Å particles are both efficient and stable up to 90 ^{o}C
- With the low back pressure afforded by 5-µm 160 Å Fused-core particles, columns of these particles are less prone to overpressurizing due to plugging and longer columns can be run for high resolution separations of proteomic samples
- With the correct choice of mobile phase, high resolution LC-MS data can be obtained for mAb separations using 400 Å Fused-core particles

Acknowledgment

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