Optimization of Reversed-Phase HPLC Separations of Biomolecules

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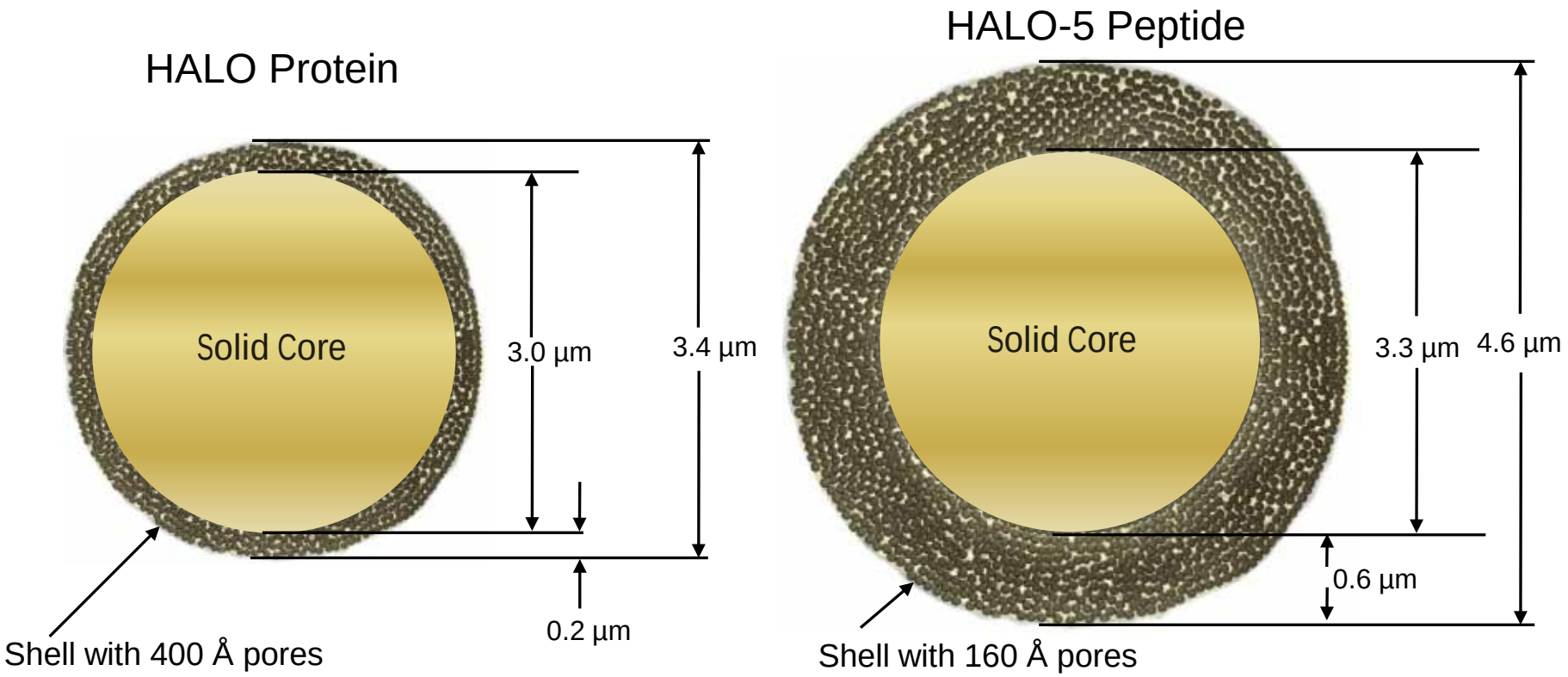
Abstract

Superficially porous particles were originally created with pore sizes and particle sizes suited for small molecule analytical separations. To improve reversed phase HPLC separations of biomolecules, known to have slower mass transfer kinetics and broader peak shapes, modification of the particles is required. To resolve these limitations, we introduce superficially porous silica particles with properties specifically designed for larger biomolecules. New particle designs with specially selected bonded phases for peptide and protein separations are discussed. Fast, high resolution separations of a variety of peptides and proteins demonstrate the advantage of the superficially porous particle design. The high temperature stability of the columns of these new particles and bonded phase characteristics are ideally suited for the conditions that are often used for analytical and small scale preparative biomolecule separations. Examples of high molecular weight protein separations highlight the advantages of using columns of superficially porous particles with 400 Å pores.

HALO® Wide-Pore Fused-Core Particles

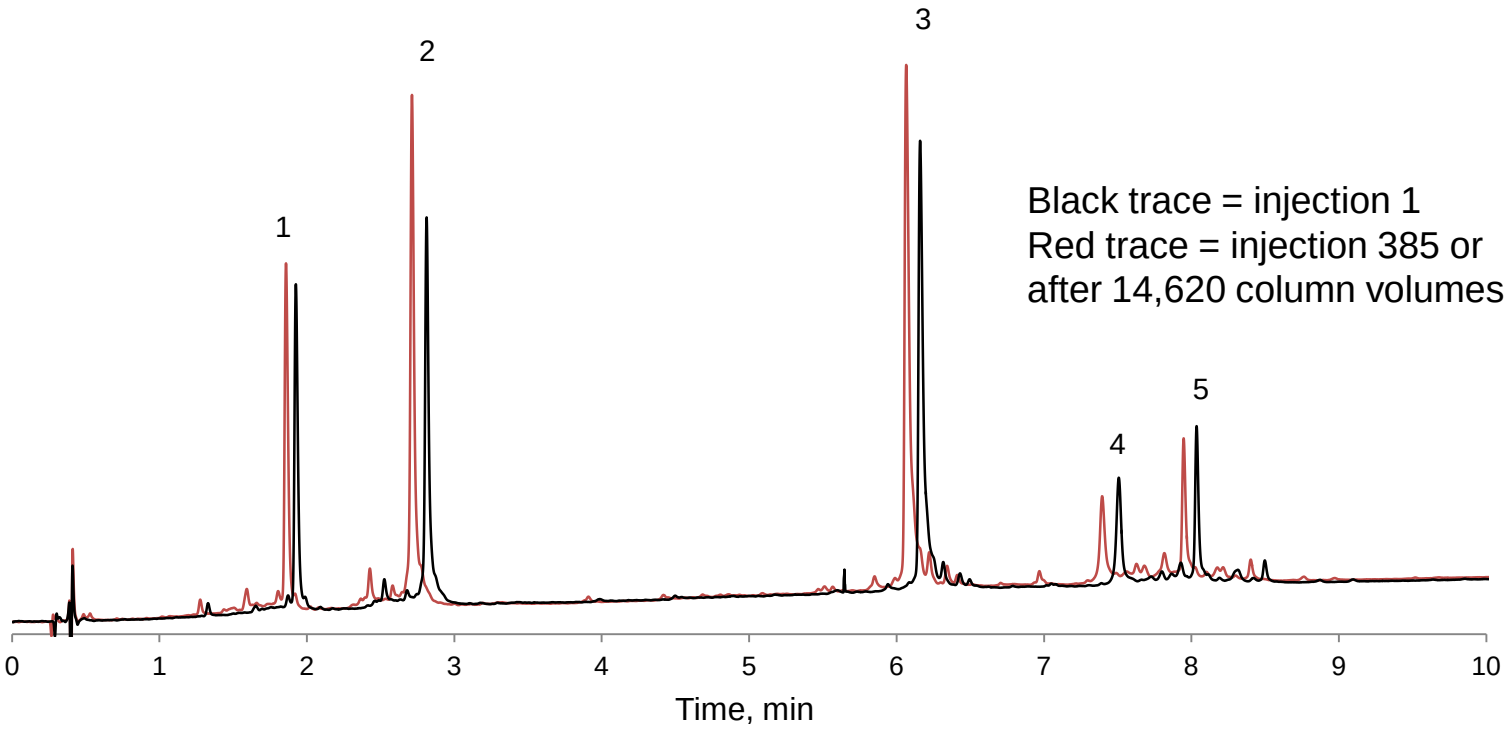


HALO Protein C4 Stability

Column: 2.1 x 100 mm
Instrument: Shimadzu Nexera
Injection Volume: 1.0 µL
Detection: 215 nm
Temperature: 90 ^{o}C

Flow rate: 0.5 mL/min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: acetonitrile/0.1% TFA
Gradient: 25-40% B in 10 min

Peak identities:
1. cytochrome *c* (12.4 kDa)
2. lysozyme (14.3 kDa)
3. apomyoglobin (17 kDa)
4. catalase (250 kDa total; tetramer of ~60 kDa each)
5. enolase (93 kDa total; dimer of 46.7 kDa each)



- The HALO Protein C4 bonded phase is stable up to 90 ^{o}C, showing very little loss of retention and no changes in peak shapes after almost 15,000 column volumes of mobile phase.

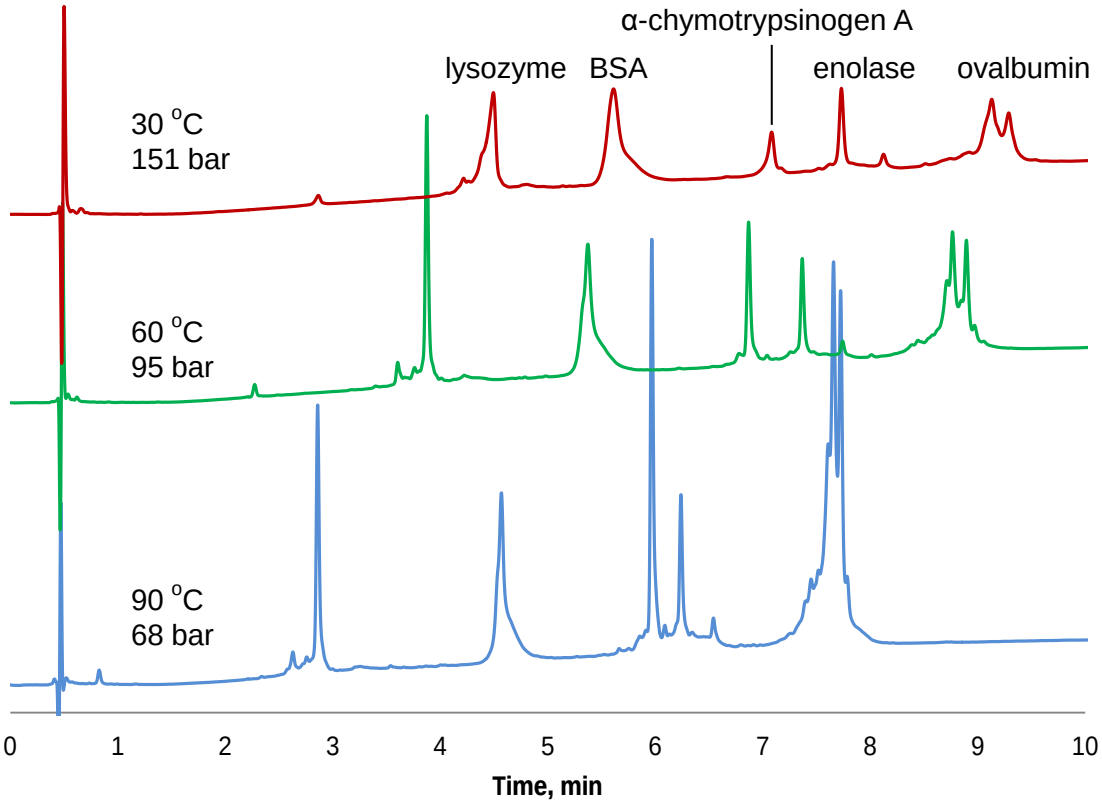
Protein Separations: Effect of Temperature

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 separation.

Fast Protein Separation

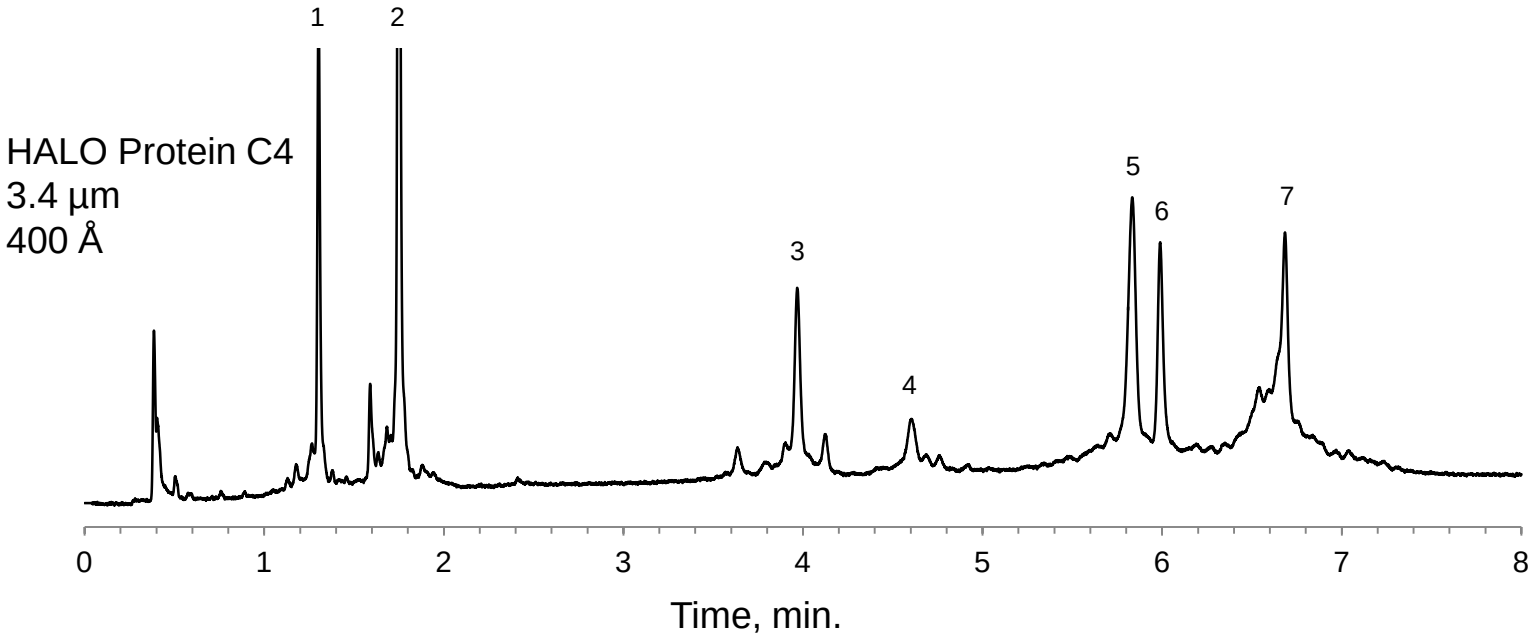
Column: 2.1 x 100 mm
Instrument: Shimadzu Nexera
Injection Volume: 1 µL
Detection: PDA @ 280 nm
Temperature: 60 °C
Flow rate: 0.5 mL/min
Pressure: 109 bar

Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient:

Time	Mobile Phase Composition
0 min.	35% B
0-2 min.	35-47.5% B
2-8 min.	47.5-60% B

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. Carbonic anhydrase, 29 kDa
6. Enolase, 46.7 kDa
7. β-amylase, 200 kDa (4 x 50 kDa)



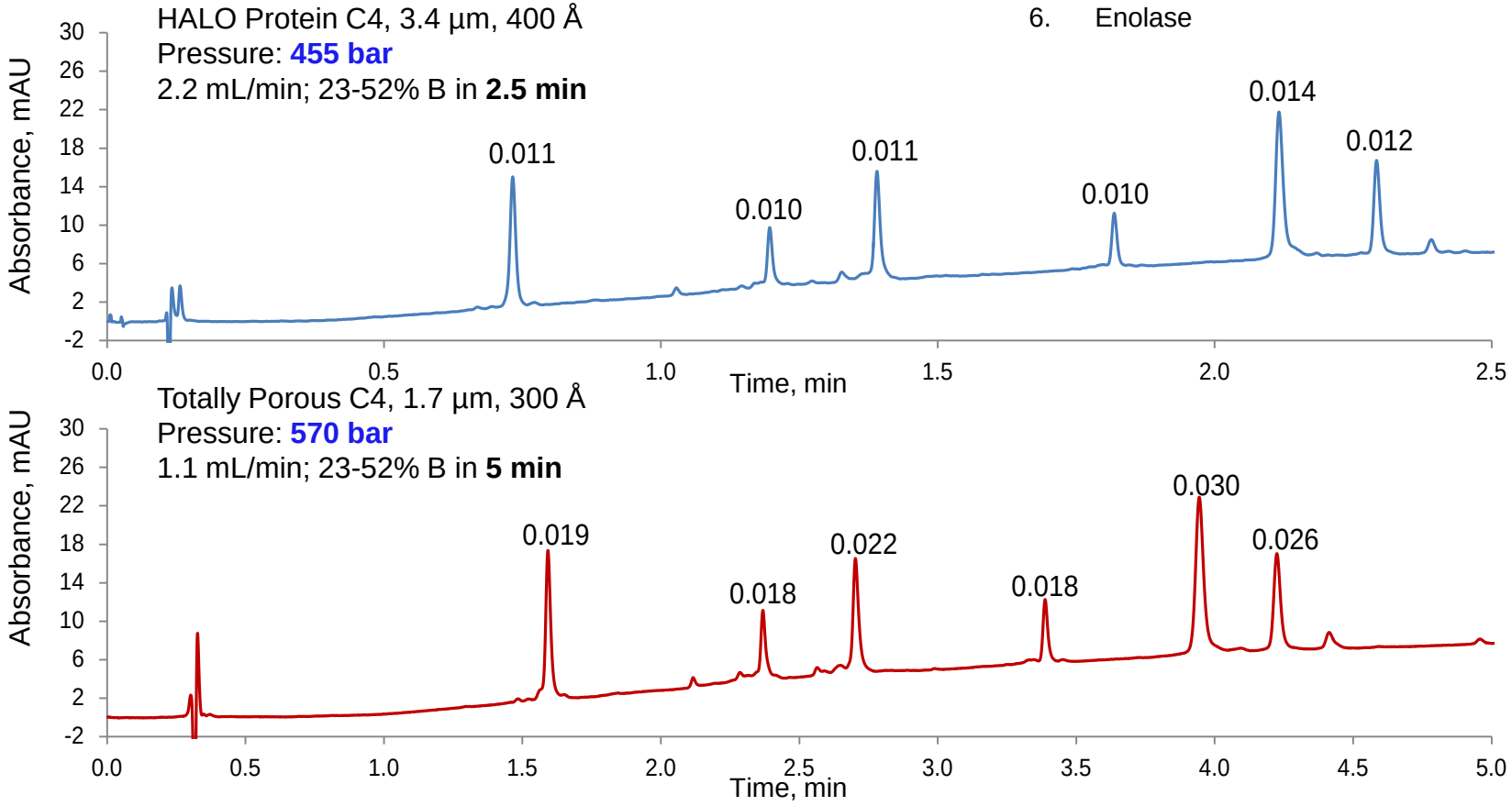
- A very wide range of molecular weights of small to large proteins is separated in less than 7 minutes using HALO Protein C4.

Protein Separations: 3.4µm Fused-Core vs. 1.7µm Totally Porous

Column: 2.1 x 100 mm
Instrument: Agilent 1200 SL
Injection Volume: 1 µL
Detection: 215 nm
Temperature: 60 °C

Flow rate: as indicated
Mobile Phase A: water/0.1% TFA
Mobile Phase B: acetonitrile/0.1% TFA
Gradient: as indicated

Peak Identities:
1. Ribonuclease A
2. Cytochrome *c*
3. Lysozyme
4. α-Lactalbumin
5. Catalase
6. Enolase



Given the low back pressure of the HALO Protein column, the flow rate can be doubled while the gradient time is cut in half. This yields a separation in half of the time as the one run on the sub-2-µm totally porous column. Numbers above peaks are widths in minutes.

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

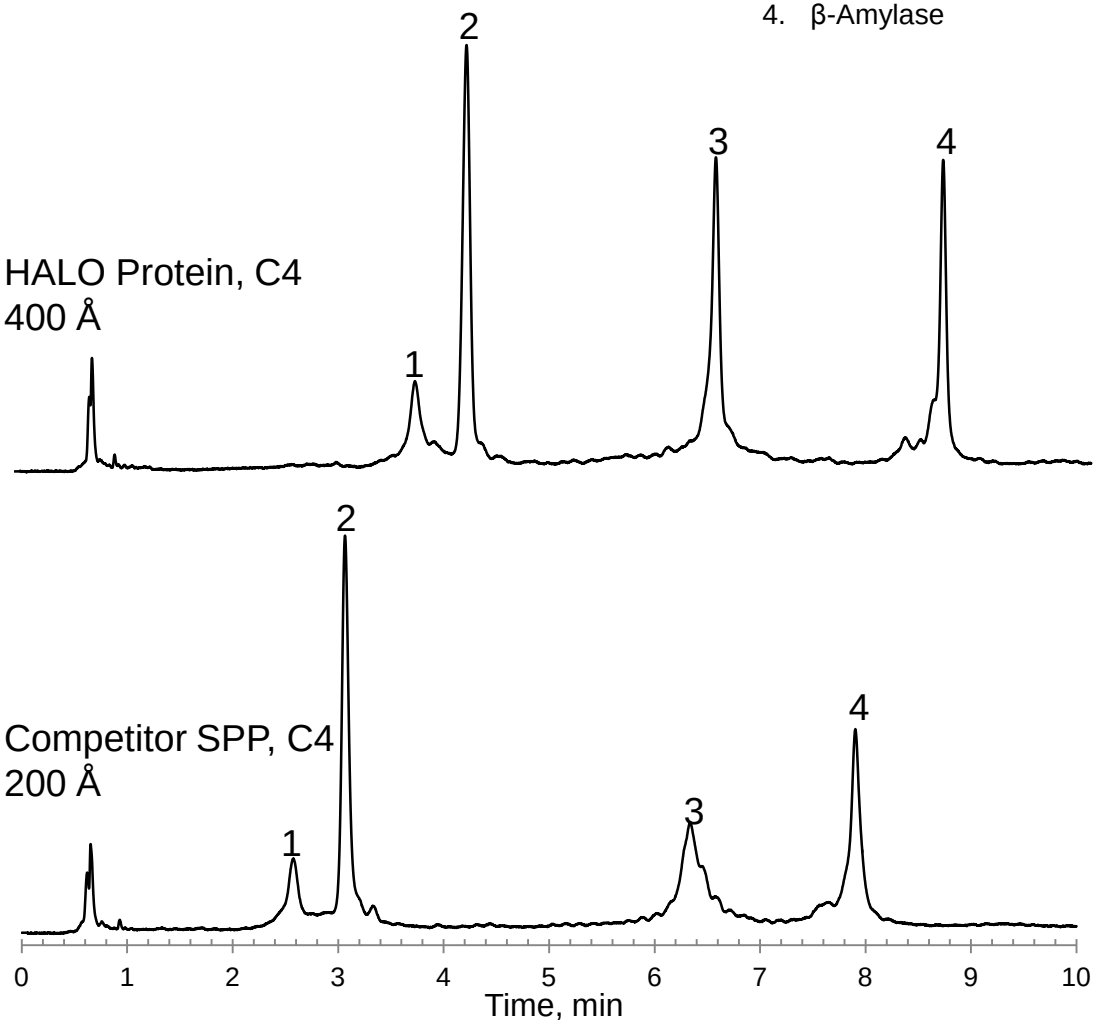
Protein Separations: Effect of Pore Size

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:

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]

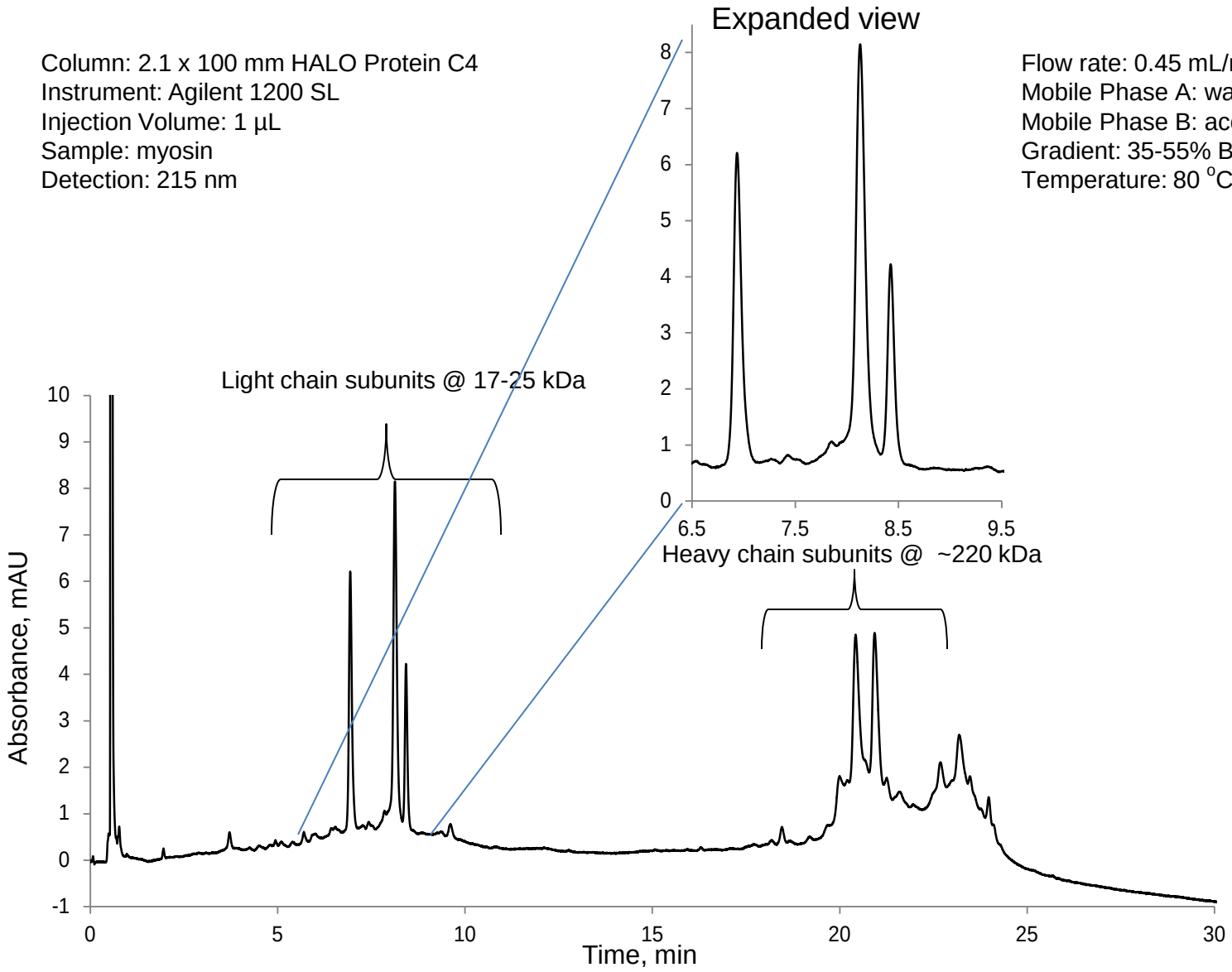


- The 400 Å pores of HALO Protein enable sharp peaks for high MW biomolecules.

Large Protein Separation using 3.4 µm Fused-Core

Column: 2.1 x 100 mm HALO Protein C4
Instrument: Agilent 1200 SL
Injection Volume: 1 µL
Sample: myosin
Detection: 215 nm

Flow rate: 0.45 mL/min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: acetonitrile/0.09% TFA
Gradient: 35-55% B in 30 min
Temperature: 80 °C



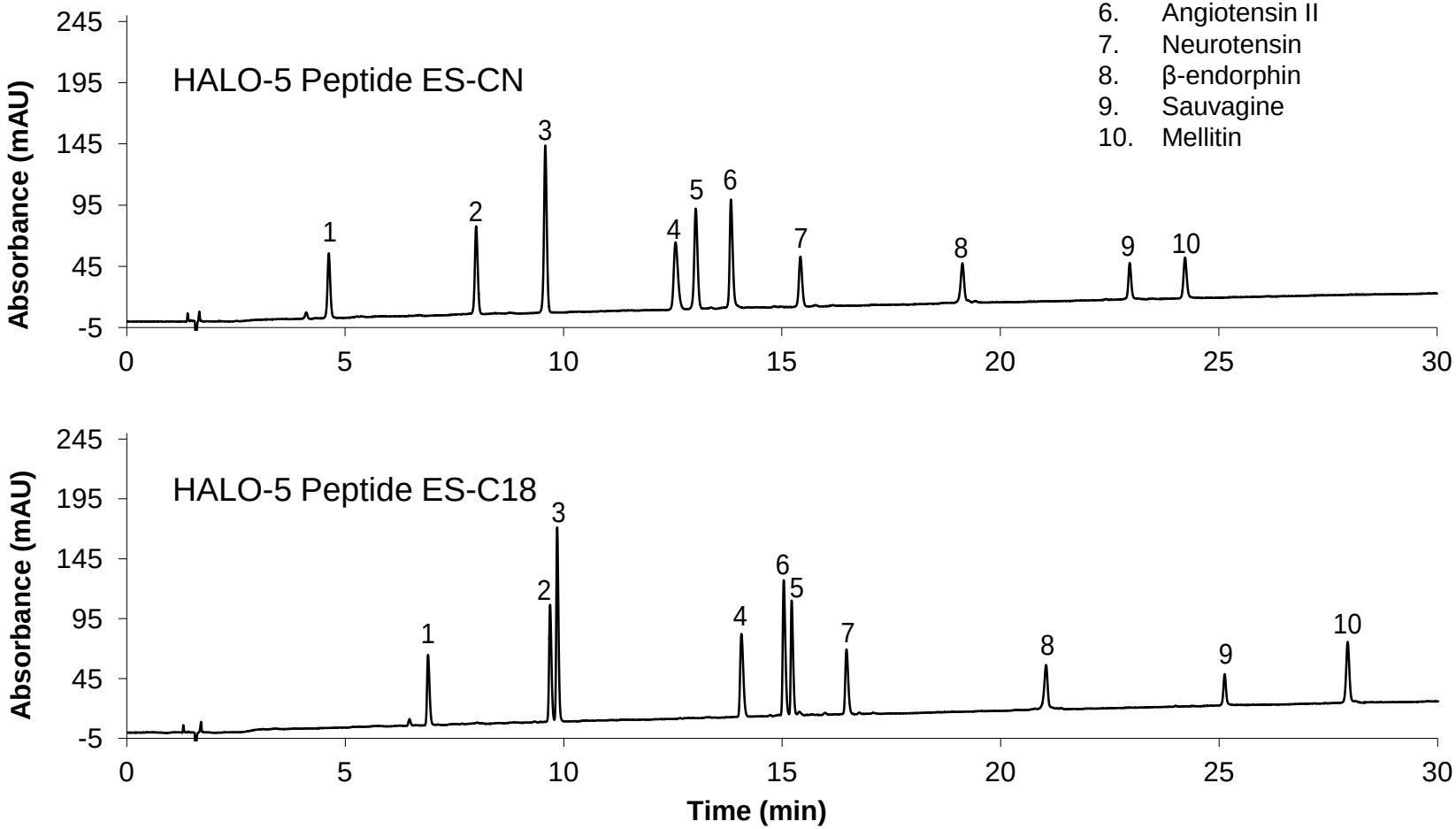
High resolution is obtained for the separation of this denatured contractile protein (purified rabbit skeletal muscle whole myosin (Cytoskeleton, Inc.) using HALO Protein C4.

10 Peptides: Selectivity changes with different stationary phases

Columns: 4.6 x 150 mm
Instrument: Agilent 1100
Injection Volume: 10 µL
Detection: 215 nm
Temperature: 40 °C

Flow rate: 1.0 mL/min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: ACN/0.1% TFA
Gradient: 5-50% ACN in 30 min

Peak Identities:
1. Asp-Phe
2. Angiotensin (1-7) amide
3. Tyr-Tyr-Tyr
4. Bradykinin
5. Leu-Enk
6. Angiotensin II
7. Neurotensin
8. β-endorphin
9. Sauvagine
10. Mellitin

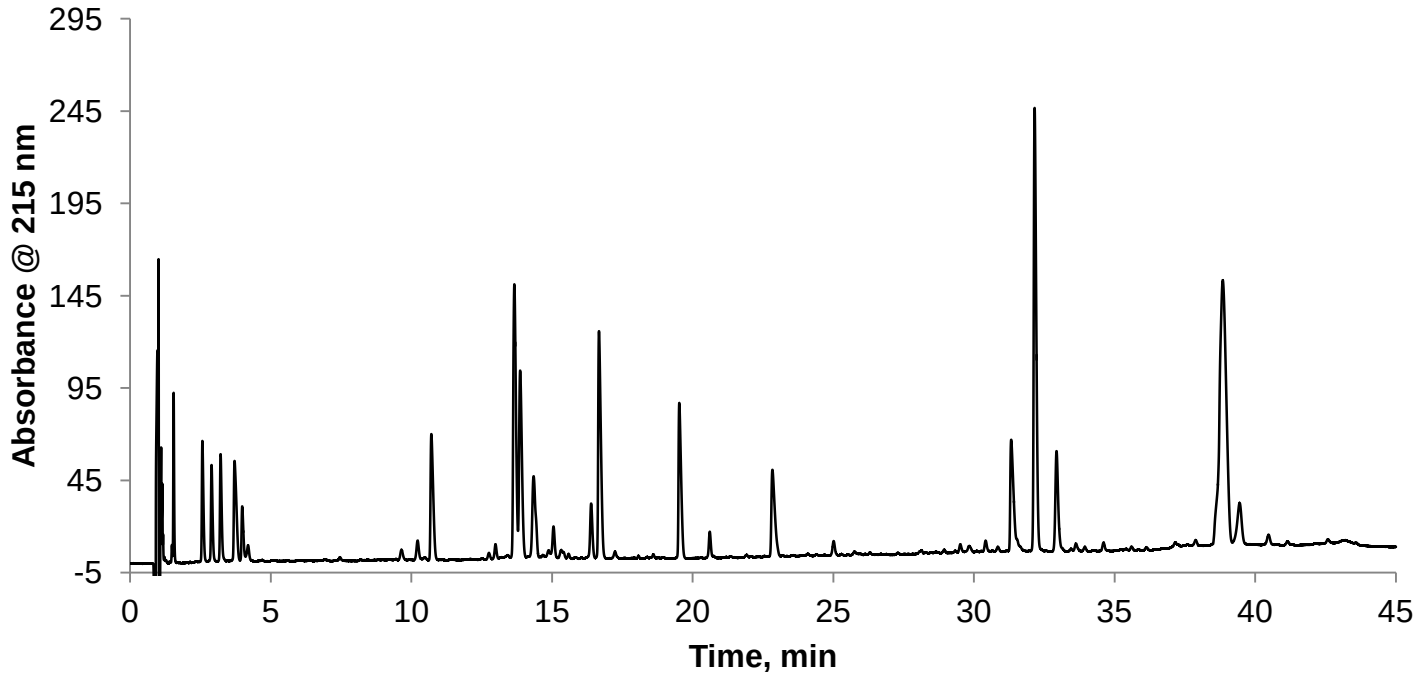


The selectivity between peak pairs 2 & 3 and 5 & 6 is different on the ES-CN phase compared to the ES-C18 phase.

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]



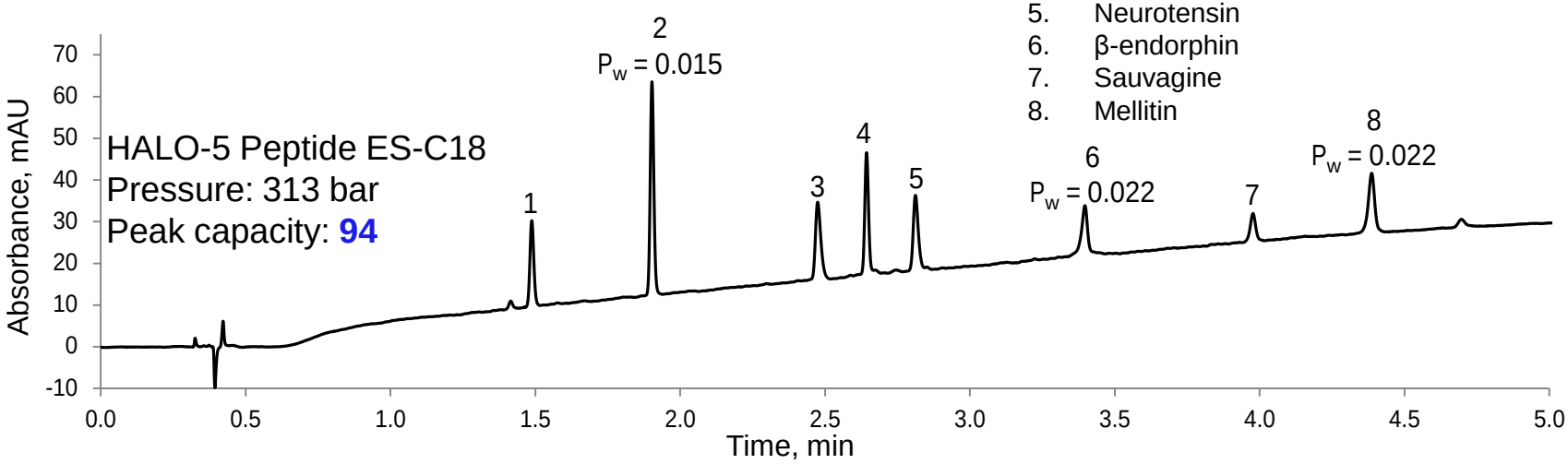
The extremely low back pressure of the HALO-5 Peptide ES-C18 column enables fast, efficient proteomic separations with a low potential for plugging.

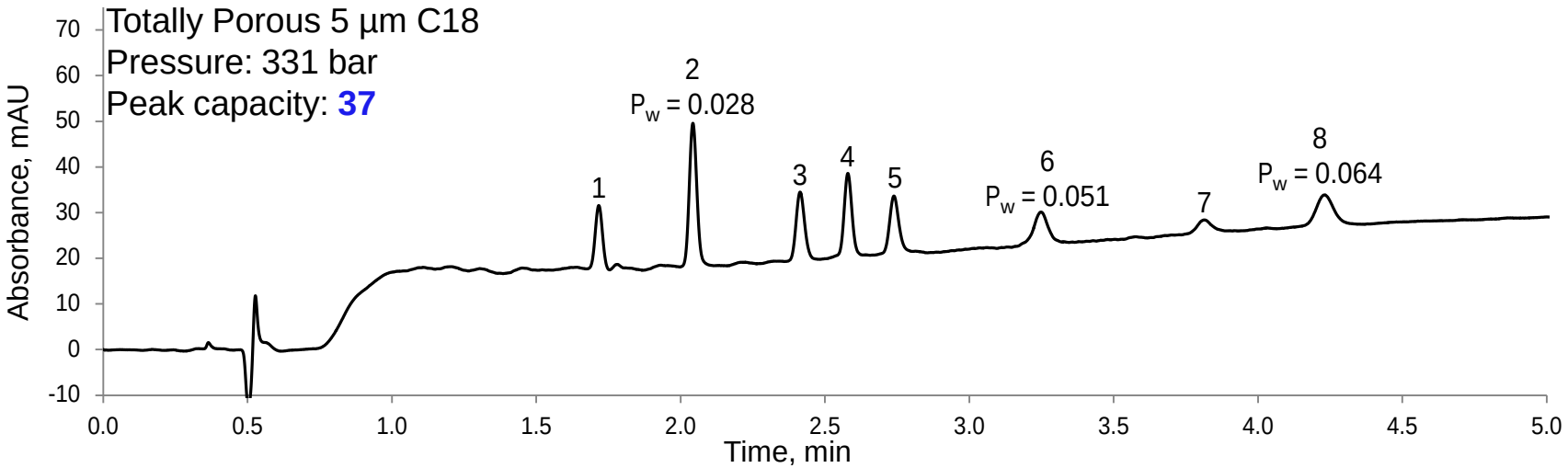
Peptide Separations: Fused-Core compared to Totally Porous

Columns: 4.6 x 150 mm
Instrument: Agilent 1100
Injection Volume: 5 µL
Detection: 215 nm
Temperature: 40 °C

Flow rate: 4.0 mL/min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: acetonitrile/0.1% TFA
Gradient: 5-60% B in 5 min

Peak Identities (in order):
1. Asp-Phe
2. Tyr-Tyr-Tyr
3. Bradykinin
4. Angiotensin II
5. Neurotensin
6. β-endorphin
7. Sauvagine
8. Mellitin





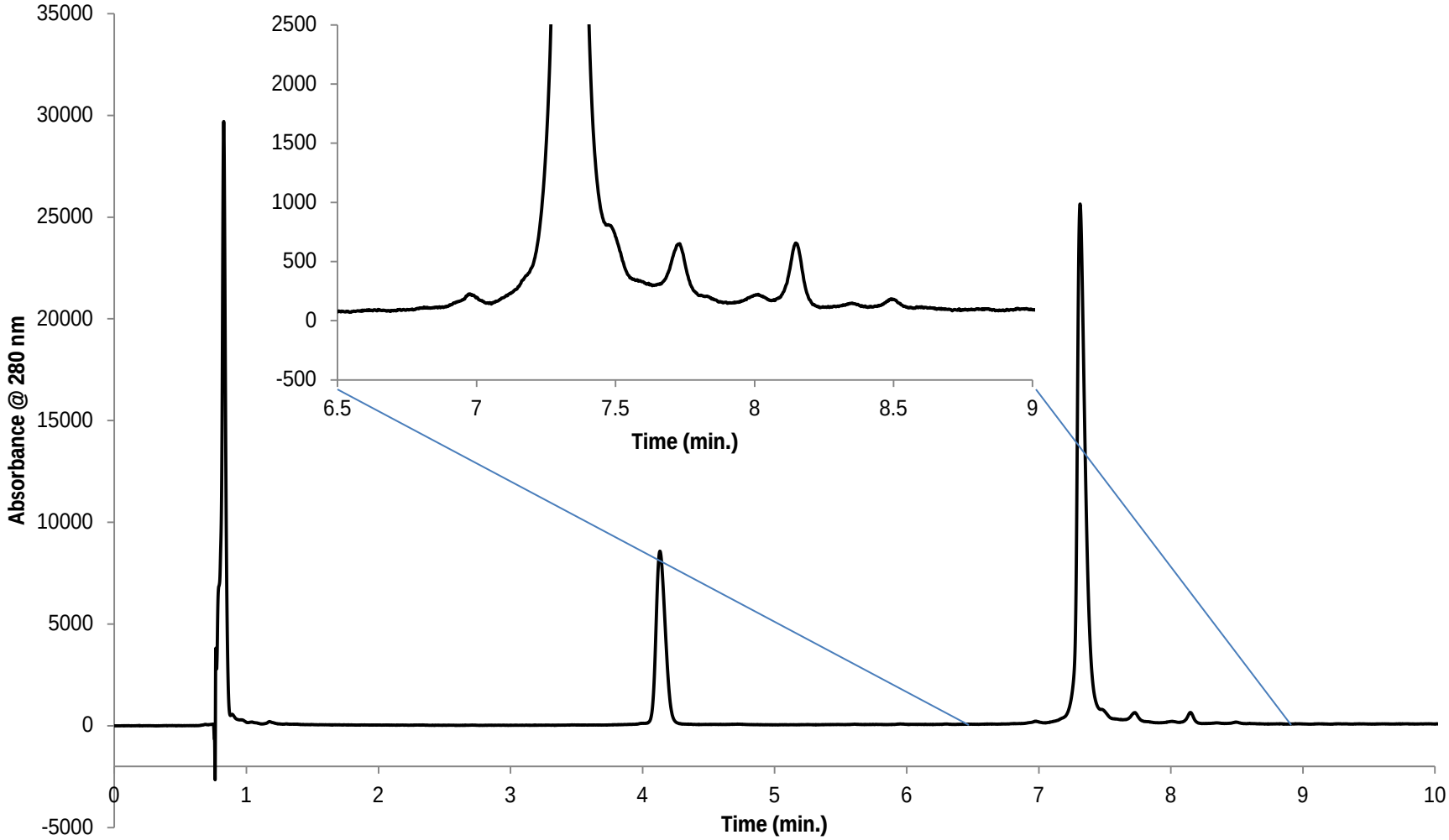
Broad peaks are exhibited by totally porous particles at high flows due to long diffusion paths compared to the thin shells of SPP. The peak capacity is 2.5 times greater with the SPP column compared to the totally porous particle column. P_w is peak width in minutes.

Reduced IgG2-B in TFA mAb Separation

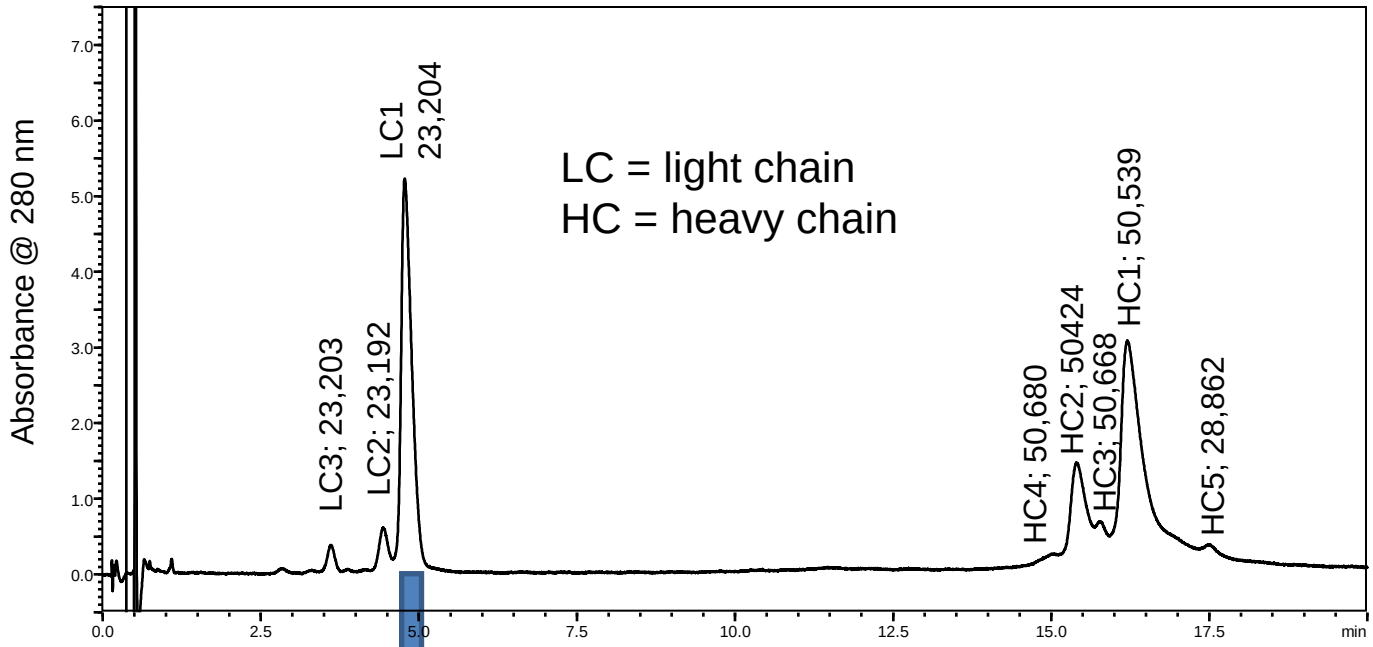
Column: 2.1 x 100 mm HALO Protein C4
Instrument: Shimadzu Nexera
Injection Volume: 1 µL
Detection: 280 nm

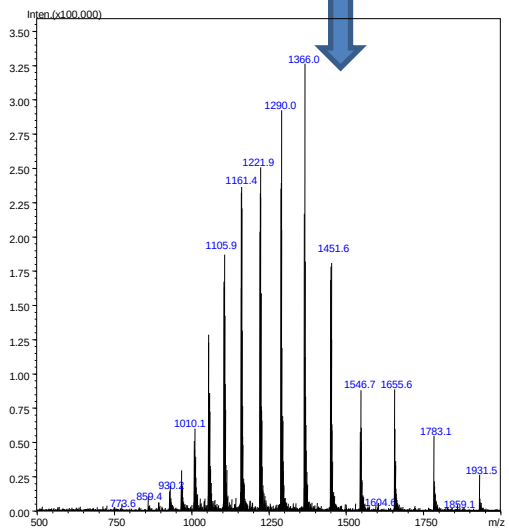
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 33-40% B in 10 min.
Flow rate: 0.25 mL/min
Temperature: 80 °C

Sample: 0.5 mg/mL IgG2-B treated with 100 mM DTT in 8 M Guanidine HCl at 50 °C for 35 min.



High Resolution LC/MS Analysis of IgG1 mAb Polypeptide Chains Using HALO Protein C4





Sample Preparation

IgGs were reduced and alkylated by sequential treatment with 10 mM DTT, 15 mM iodoacetamide, then quenched with an additional 10 mM DTT, all in 6 M guanidine HCl/20 mM Tris-HCl buffer at pH 7.8. Reduced and alkylated IgG solutions were buffer exchanged into 0.1% TFA using VivaSpin (Sartorius Stedim Biotech, Goettigen, Germany) centrifugal concentrators with 5 kDa cut-off HY polymeric membranes. The reduced and alkylated IgGs were adjusted to 2 mg/mL protein in 0.1% TFA and stored at -25 ^{o}C until use.

Column: 2.1 mm ID x 100 mm HALO Protein C4
Flow rate: 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.
Temperature: 80 ^{o}C
Detection: 280 nm
MS Conditions: Shimadzu LCMS-2020, ESI +4.5 kV, 2 pps, 500-2000 m/z

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 2 times faster and with lower backpressure on columns of Fused-core particles compared to columns of sub-2-um particles
- Fused-core particles have performance advantages over totally porous particles for separating peptides and proteins
- Columns of Fused-core particles with 400 Å pores are both efficient and stable up to 90 ^{o}C
- With the low back pressure afforded by 5-µm 160 Å Fused-core particles and the use of 2-µm frits, columns of these particles are less prone 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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