Selecting the Correct Pore Size of Your Analytical Columns for Better RPLC Separations of Biomolecules

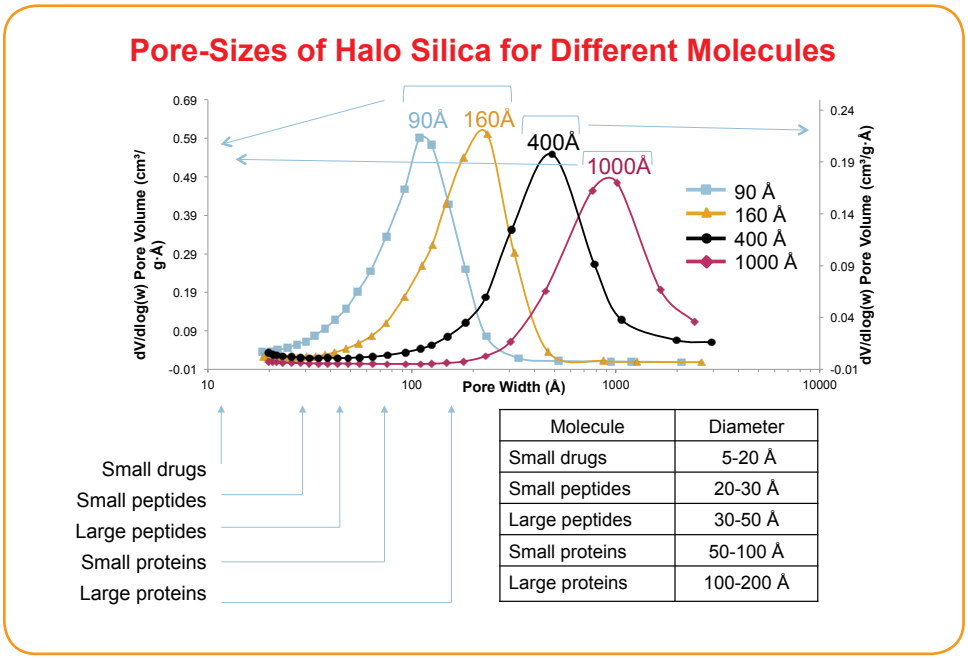
Molecular Weights of Biomolecules and Particle Pore Sizes

The physical size of an analyte in solution, and that size relative to the aperture of the particles' pores is what matters most for obtaining unrestricted diffusion into and out of the pores, where most of the bonded phase and surface area resides. The relatively thin porous layer, which superficially porous particles possess, also improves stationary phase access and makes diffusion less hindered compared to totally porous particles—even those that have diameters less than 2 microns.

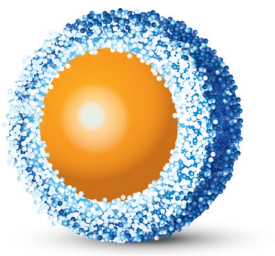
Some Key Points to Remember

- Biomolecules can have different shapes and sizes in solution for the same or similar MWs.
- Column particle pore sizes are typically characterized by column manufacturers using nitrogen adsorption, mercury porosimetry or other techniques, and are usually expressed in Ångstrom units (Å).
- Particles for HPLC and UHPLC columns usually have a broad pore size distribution range as shown in Figure 1 for HALO particles having different nominal average pore sizes.
- Consensus has not yet been reached on how large pore size should be relative to molecular size to avoid unnecessary loss of LC performance (broader and shorter peaks with less retention).
- Rule of thumb: Particle pore size should be a minimum of 8–10 fold larger than the molecular diameter for minimal size exclusion effects and for better access to stationary phase and less restricted diffusion for large analytes (Figure 1).

Figure 1 Pore Size Selection Based on Analyte Type and Size



Molecule	Diameter
Small drugs	5-20 Å
Small peptides	20-30 Å
Large peptides	30-50 Å
Small proteins	50-100 Å
Large proteins	100-200 Å



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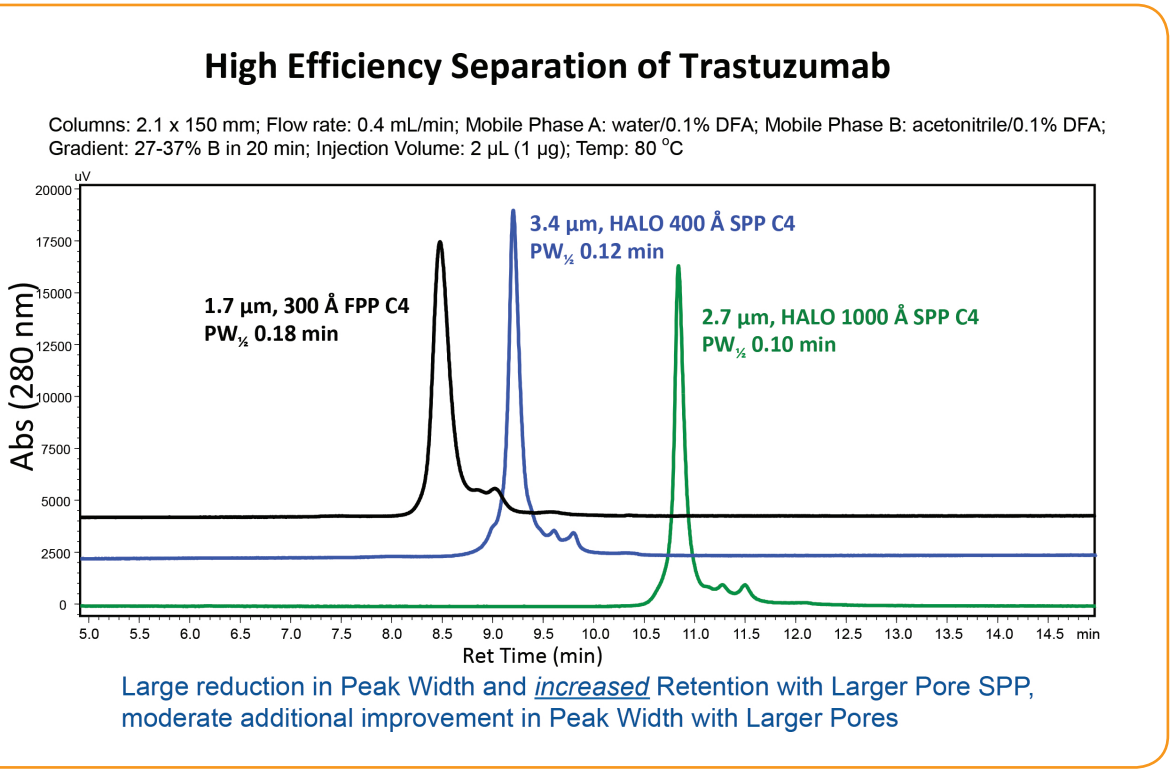
Because 300Å pore size columns have been the "go-to" size for peptides and proteins for many years, chromatographers assume that if they choose a 300Å pore size column, they will not observe any problems due to restricted pore access, slow diffusion, and size exclusion effects. However, for many proteins, especially those that are greater than ~50 kDa in size, narrower and taller peaks can be obtained using both 400Å and 1000Å pore size superficially porous columns.

The improvement in performance for the separation of a monoclonal antibody, with a MW of about 150 kDa is demonstrated in Figure 2. In this case, the larger pore size of a HALO 1000Å C4 column provided improved retention, sharper peaks and improved resolution of minor components compared to the 300 Ångstrom, 1.7 µm FPP column. In Figure 3, the improved resolving power of the HALO 1000Å C4, 2.7 µm SPP column is compared to that of a < 500Å SPP column and a 300Å, 1.7 µm FPP column for a mixture of IgG2 antibody structural variants. Clearly, more variants can be resolved using the HALO 1000Å C4 column because there is unrestricted access to the particle surface.

Note: FPP = fully porous particle and SPP = superficially porous particle

Figure 2 Comparison of 300Å, 400Å and 1000Å Columns for RPLC of 148 kDa Monoclonal Antibody



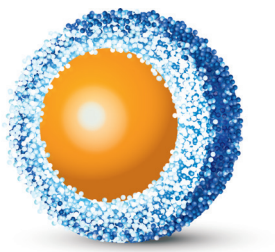
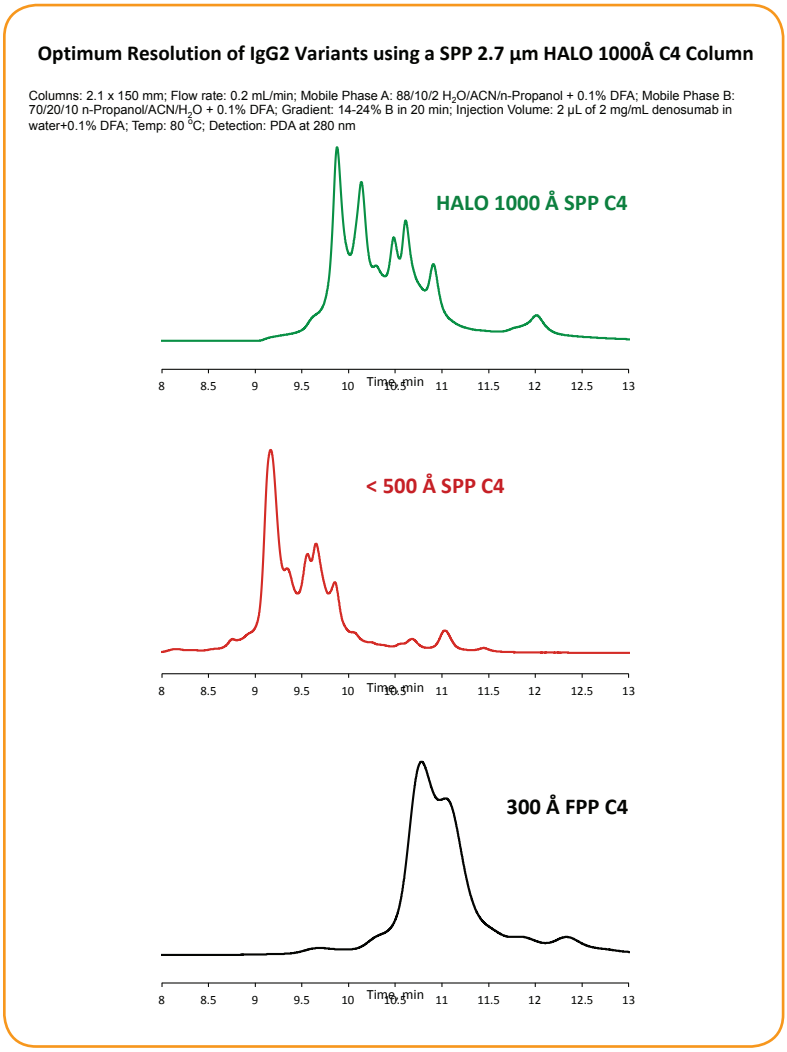


Figure 3 Optimum Resolution of IgG2 Variants using a SPP HALO 1000Å C4, 2.7 µm Column

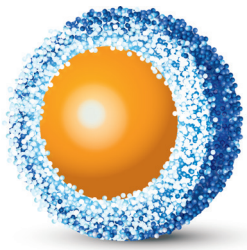


Columns: 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 H2O/ACN/n-Propanol + 0.1% DFA; Mobile Phase B: 70/20/10 n-Propanol/ACN/H2O + 0.1% DFA; Gradient: 14-24% B in 20 min; Injection Volume: 2 µL of 2 mg/mL denosumab in water+0.1% DFA; Temp: 80 oC; Detection: PDA at 280 nm

Conclusions

Larger pore size superficially porous particles provide improved chromatographic performance for larger biomolecules such as monoclonal antibodies. Increased retention, sharper peaks, and improved resolution of structural variants are some of the benefits provided by larger pores due to unrestricted access to the internal surface area of the particles and fast diffusion into and out of the thin porous shells.

For more information on the HALO 400Å and 1000Å columns and their usefulness for proteins, monoclonal antibodies and antibody fragments, please visit our web site.



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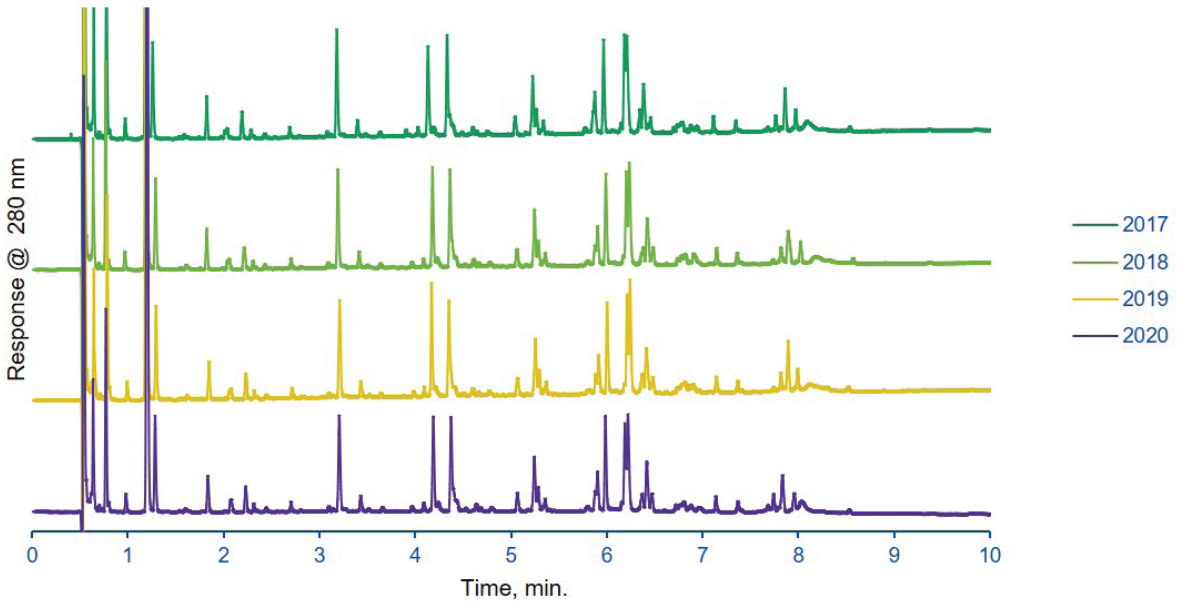
BIOPHARMACEUTICALS

Rapid Peptide Mapping of an Adalimumab (Humira®) Digest

Application Note 221-PE



TEST CONDITIONS:

Column: HALO 160 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Part Number: 92122-702
Mobile Phase: A: Water/0.1% DFA and B: ACN/ 0.1% DFA
Flow Rate: 600 µL/min
Pressure: 330 bar (4795 psi)
Temperature: 60 °C
Detection: 280 nm
Injection Volume: 3.0 µL
Sample Solvent: 90/10 mobile phase A/B
Response Time: 0.025 sec
Flow Cell: 1.0 µL
LC System: Shimadzu Nexera

The outstanding reproducibility and high throughput power of the HALO 160 Å ES-C18 column is demonstrated here with the separation of an adalimumab (immunosuppressive drug) tryptic digest achieved under 10 minutes (total analysis time of 15 min). The nearly identical gradient profiles highlight the reliability and reproducibility of four different column lots, over a four-year period (2017-2020).

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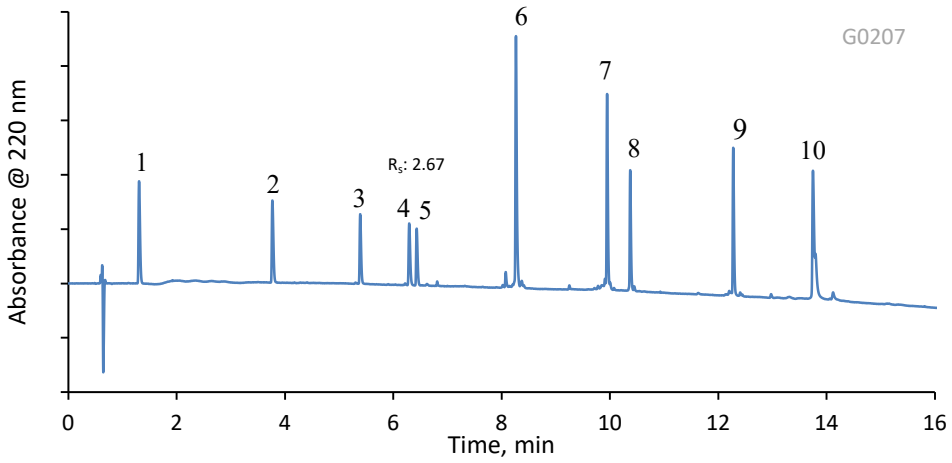
Application Note: 213-PR

Peptide and Protein Mix on HALO 400 Å ES-C18, 3.4 µm



PEAK IDENTITIES:

1. Gly-Tyr
2. Val-Tyr-Val
3. Methionine Enkephalin
4. Angiotensin II
5. Leucine Enkephalin
6. RNase A
7. Cytochrome C
8. Insulin
9. Alpha-lactalbumin
10. Enolase

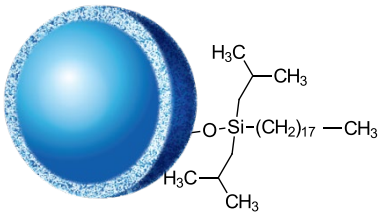
TEST CONDITIONS:

Column: HALO 400 Å ES-C18, 3.4 µm, 2.1 x 150 mm
Part Number: 93412-702
Mobile Phase A: Water + 0.1% DFA
Mobile Phase B: 80/20 Acetonitrile/Water + 0.1% DFA
Gradient:

Time	%B
0.0	0
15.0	60
16.0	60
16.1	0
20.0	0

Flow Rate: 0.5 mL/min
Initial Pressure: 165 bar
Temperature: 60 °C
Detection: UV 220 nm, PDA
Injection Volume: 1.5 µL
Sample Solvent: Water
Data Rate: 40 Hz
Response Time: 0.025 sec
Flow Cell: 1 µL
LC System: Shimadzu Nexera X2

STRUCTURE:



HALO 400 Å ES-C18, 3.4 µm

A mix of peptides and proteins was separated with excellent resolution and peak shape using the HALO 400 Å ES-C18. The steric protection of this phase makes it particularly ideal for the high temperature and low pH conditions often required for peptide and protein separations. Because of its smaller pore size compared to the 1000 Å ES-C18, the 400 Å ES-C18 easily separates mixtures of peptides and smaller proteins such as cytochrome C, alpha-lactalbumin, and enolase.

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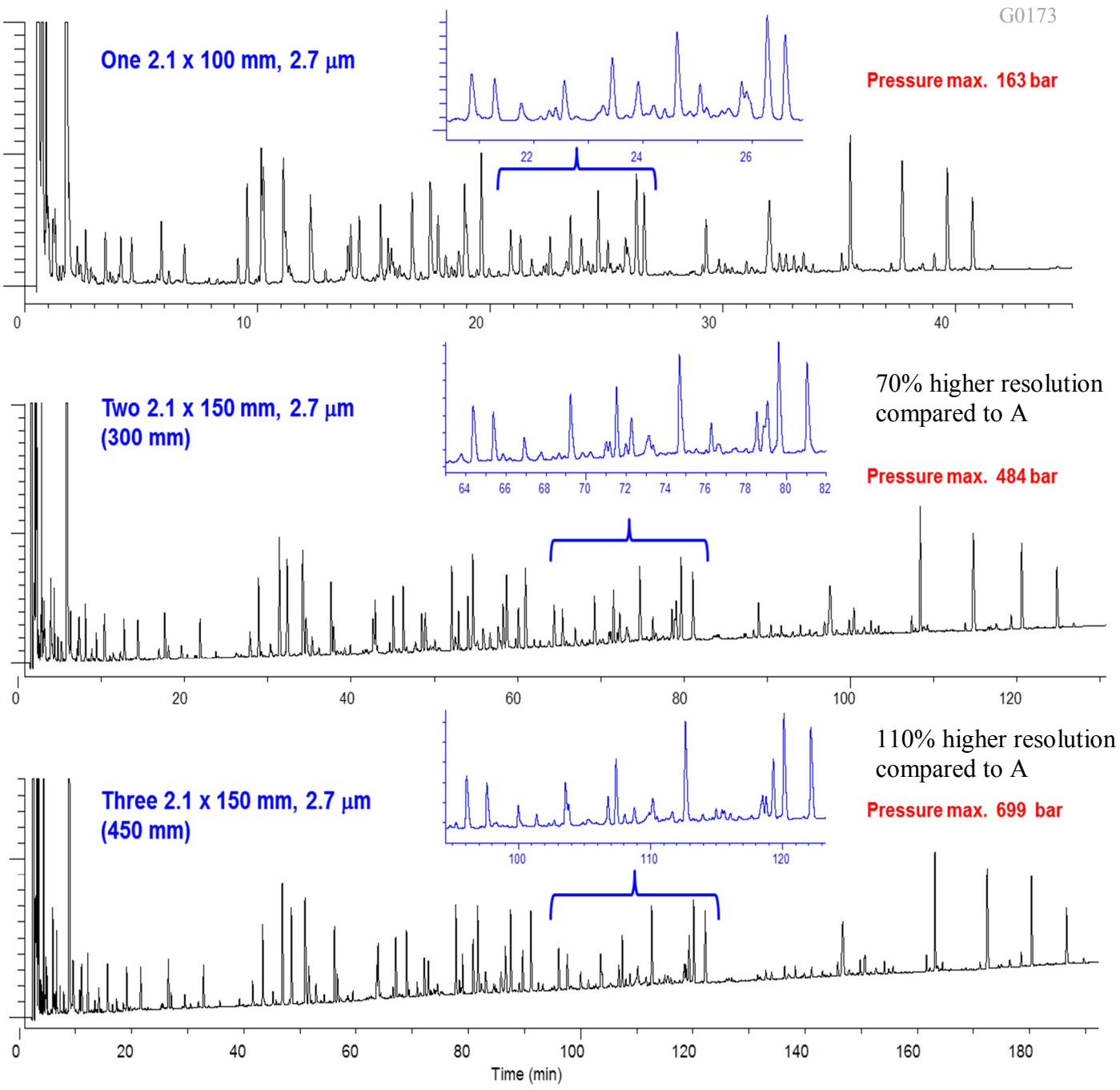
FOR MORE INFORMATION OR TO PLACE AN ORDER, CONTACT:

Application Note: 179-PE

Analysis of Apotransferrin Tryptic Digest on HALO® Peptide Columns



FOR MORE INFORMATION OR TO PLACE AN ORDER, CONTACT:

Application Note: 179-PE

TEST CONDITIONS:

Column: HALO 160 Å ES-C18, 2.7 µm
Part Number: 2.1 x 100 mm 92122-602
Part Number: 2.1 x 150 mm 92122-702

Mobile Phase A: water with 0.1% TFA
Mobile Phase B: 80/20 acetonitrile/ water with 0.1% TFA

Gradient A:

Time	%B
0.0	5
60	60

Gradient B:

Time	%B
0.0	5
180	60

Gradient C:

Time	%B
0.0	5
270	60

Flow Rate: 0.4 mL/min

Temperature: 60°C

Detection: UV 215 nm, PDA

Injection Volume: 10 µL

Sample Solvent: water

Data Rate: 40 Hz

Response Time: 0.05 sec

Flow Cell: 1 µL

LC System: Shimadzu Nexera X2

The chromatograms on the preceding page show a comparison of an apotransferrin tryptic digest sample analyzed on three different lengths of HALO® Peptide ES-C18 columns: a single 2.1 x 100 mm, two 2.1 x 150 mm columns in series, and three 2.1 x 150 mm columns in series. The insets show examples of the improved performance obtained using longer column lengths along with longer gradient times for demanding samples. Resolution increases of approximately 70% and 110% are achieved by increasing column length by 3-fold and 4.5-fold respectively. Gradient times of 60, 180 and 270 minutes were used for the top, middle and bottom chromatograms, respectively.

Lower pressures afforded by both 2.7 and 5 µm HALO® Peptide particles allow two or more columns to be used in series for additional resolution and peak capacity for challenging peptide mapping analyses. HALO® Peptide ES-C18 is also available in 2 µm particle sizes in 2.1 and 3 mm IDs up to 150 mm length for additional options in run time and peak capacity.

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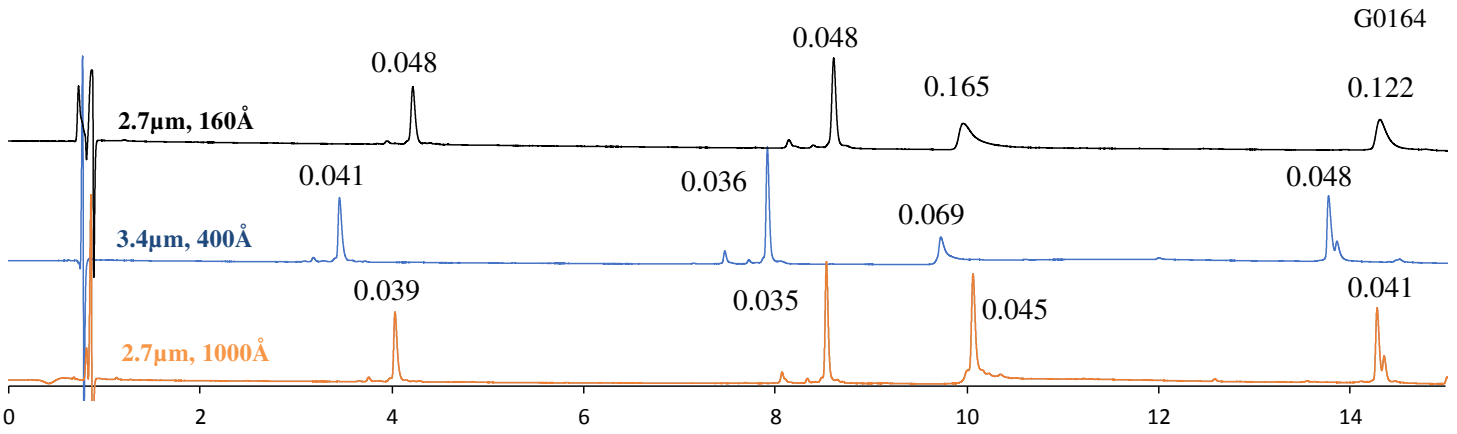
Application Note: 170-PR

Effect of HALO ES-C18 Pore Size on Protein Peak Shape and Width



(Superpositioned numbers are peak widths at half height in minutes)

TEST CONDITIONS:

Column: HALO ES-C18, 2.1 x 150mm
Part Number: 92122-702 (160Å)
93412-702 (400Å)
92712-702 (1000Å)
Mobile Phase A: Water (0.1% TFA)
Mobile Phase B: 80/20 Acetonitrile/ Water (0.085% TFA)
Gradient: 27–60% B in 15 minutes
Flow Rate: 0.4 mL/min
Temperature: 60°C
Detection: UV 280 nm, PDA
Injection Volume: 4 µL
Sample Solvent: Water (0.1% TFA)
Data Rate: 40 Hz
Response Time: 0.025 sec
Flow Cell: 1 µL
LC System: Shimadzu Nexera X2

PEAK IDENTITIES:

1. Ribonuclease A (13.8 kDa)
2. Lysozyme (14.4 kDa)
3. SILu™ Lite SigmaMAb Antibody (~150 kDa)
4. Enolase (46.7 kDa)

Pore size can play an important part in your HPLC separations. A range of proteins and a monoclonal antibody are separated on HALO ES-C18 160Å, 400Å, and 1000Å columns. Peak widths decrease as the column packing's pore size becomes larger, especially for the monoclonal antibody. The 160Å pore size is recommended for molecules in the range of 100 Da to 15kDa. The 400Å pore size is recommended for molecules between 2kDa to 500 kDa. The 1000Å pore size is used for molecules over 50 kDa.

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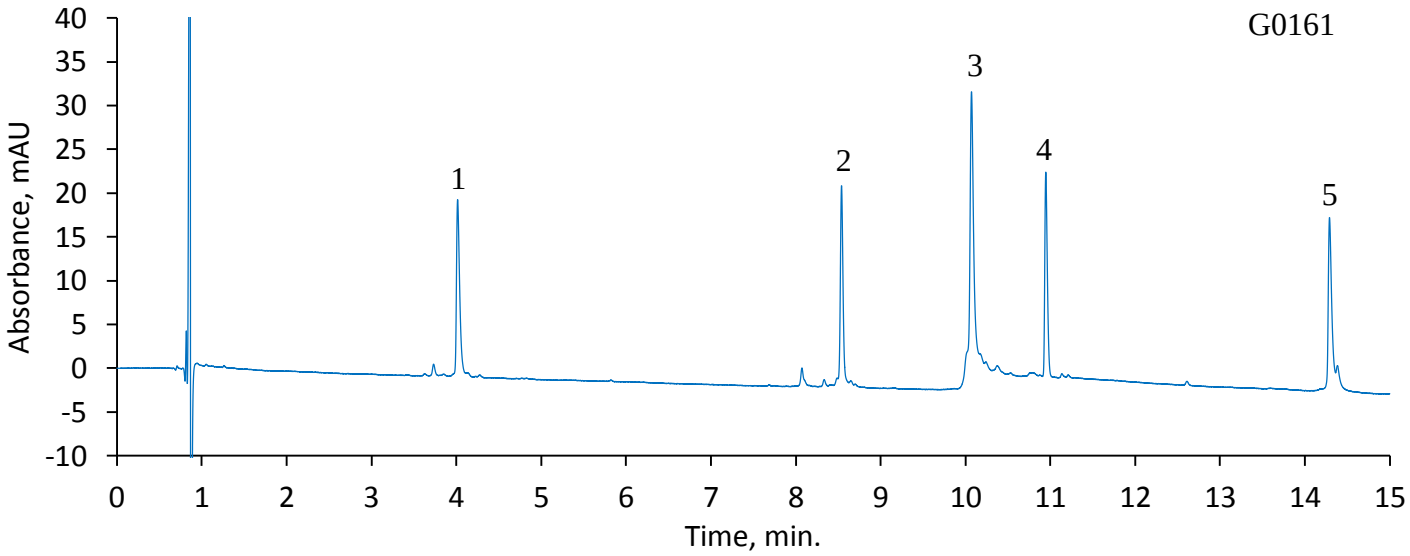
Application Note: 167-PR

Protein Separation on HALO 1000Å ES-C18, 2.7 µm



TEST CONDITIONS:

Column: HALO 1000Å ES-C18, 2.7µm, 2.1 x 150 mm
Part Number: 92712-702
A= Water, 0.1% TFA
B= 80/20 ACN/ Water, 0.085% TFA
Gradient:

Time (min.)	%B
0.00	27
15.00	60

Flow Rate: 0.4 mL/min.
Pressure: 268 bar
Temperature: 60 °C
Injection Volume: 2 µL
Sample Solvent: Water/0.1% TFA
Detection: UV 280 nm, PDA
Data Rate: 12.5 Hz
Response Time: 0.05 sec.
Flow Cell: 1 µL
LC System: Shimadzu Nexera X2

PEAK IDENTITIES:

1. Ribonuclease A 13.7 kDa
2. Lysozyme 14.3 kDa
3. SigmaMAb ~150 kDa
4. α-Lactalbumin 14.2 kDa
5. Enolase 46 kDa monomer

This mix of proteins with a wide range of molecular weights is separated with high efficiency on a HALO 1000Å ES-C18 column. With improved access to the particle surface, the 1000Å pore size enables large biomolecule analysis with excellent peak shape and high resolution.

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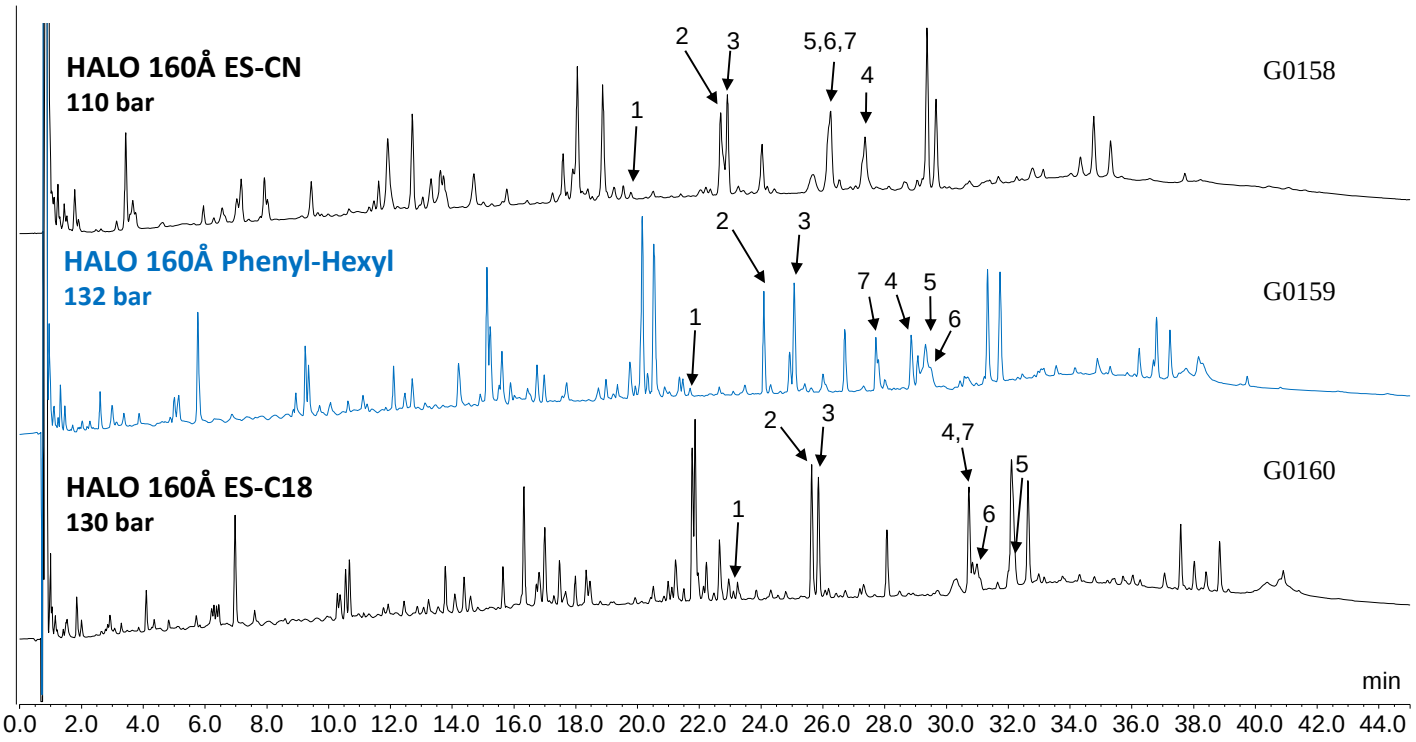
FOR MORE INFORMATION OR TO PLACE AN ORDER, CONTACT:

HALO | Fused-Core® Particle Technology

Application Note: 166-PE

Enhanced Selectivity with HALO 160Å Phenyl-Hexyl for a Tryptic Digest using LC-MS



TEST CONDITIONS:

Columns: HALO 160Å ES-CN, 2.7 µm, 2.1 x 100 mm
Part Number: 92122-604
HALO 160Å Phenyl-Hexyl, 2.7 µm, 2.1 x 100 mm
Part Number: 92112-606
HALO 160Å ES-C18, 2.7 µm, 2.1 x 100 mm
Part Number: 92122-602

Mobile Phase:
A = water + 10 mM difluoroacetic acid (DFA)
B = ACN + 10 mM difluoroacetic acid
Flow Rate: 0.3 mL/min
Gradient: 2–50%B in 60 min
Temperature: 60 °C
Detection: UV 220 nm, VWD
Injection Volume: 5 µL of 0.2 mg/mL digest
Sample Solvent: 50 mM Tris-HCl/1.5 M Guanidine-HCl with 0.25% formic acid
Response Time: 0.15 sec
Data Rate: 10 Hz
LC System: Shimadzu Nexera
Flow Cell: 2.5 µL semi-micro

PEAK IDENTITIES (using one-letter amino acid abbreviations):

1. FTISADTSKNTAYLQMNSLR (754 m/z)
2. LScAASGFNIKDTYIHWVR (747 m/z)
3. GFYPSDIAVEWESNGQPENNYK (849 m/z)
4. LLIYSASFLYSGVPSR (592 m/z)
5. SGTASVVcLLNNFYPR (899 m/z)
6. ScDKTHTcPPcPAPELLGGPSVFLFPPKPK (834 m/z)
7. VVSVLTVLHQDWLNGKEYK (1115 m/z)

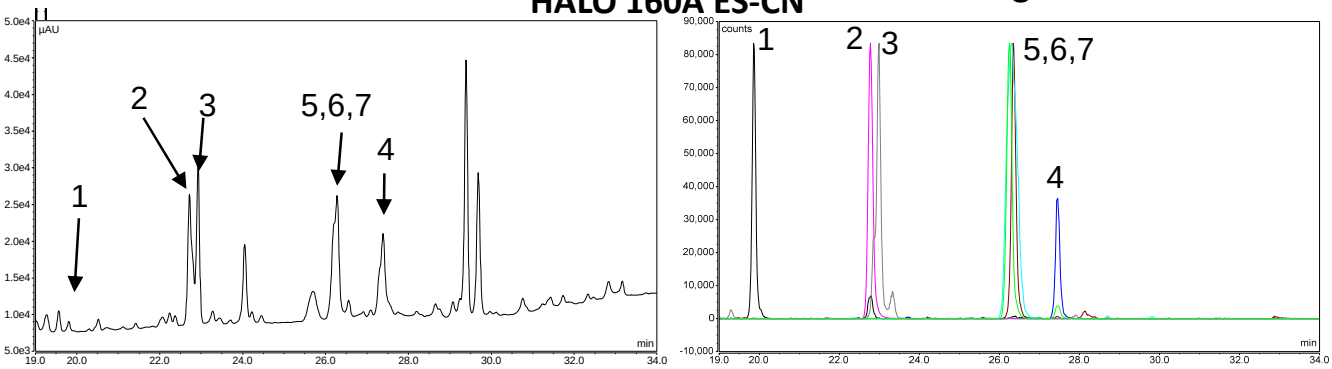
The HALO 160Å Phenyl-Hexyl column provided improved resolution between tryptic digest fragments 2 and 3 compared to the 160Å ES-CN column and the 160Å ES-C18 column. Peptide identification was accomplished by using MS-MS fragmentation spectra.

The HALO 160Å Phenyl-Hexyl column also provided improved resolution between tryptic digest fragments 4 and 7 compared to the 160Å ES-C18 column. The extracted ion current chromatogram (EIC) and the mass spectrum, corresponding to each peptide fragment, are shown. The use of difluoroacetic acid (DFA) in the mobile phase facilitates symmetrical peak shape and good retention, while enabling good ionization efficiency and sensitivity.

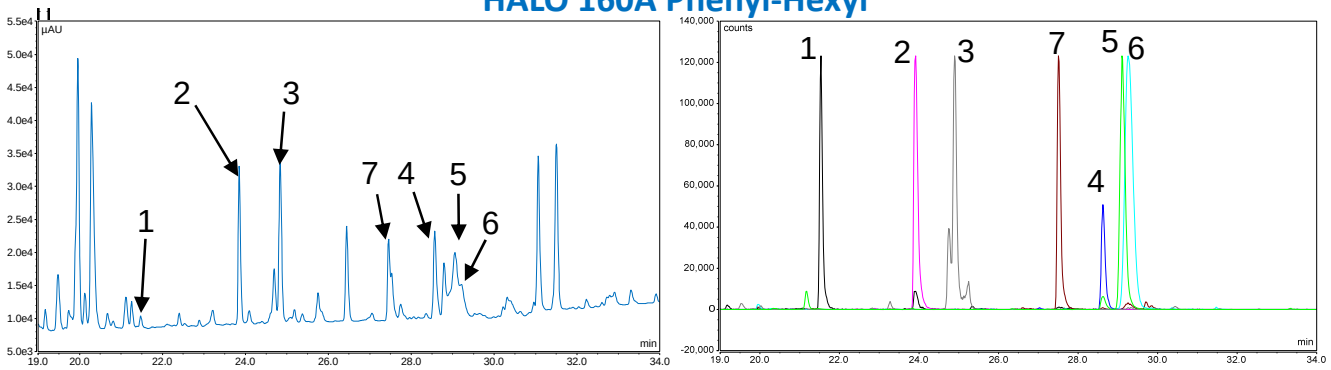
UV 220 nm traces

HALO 160Å ES-CN

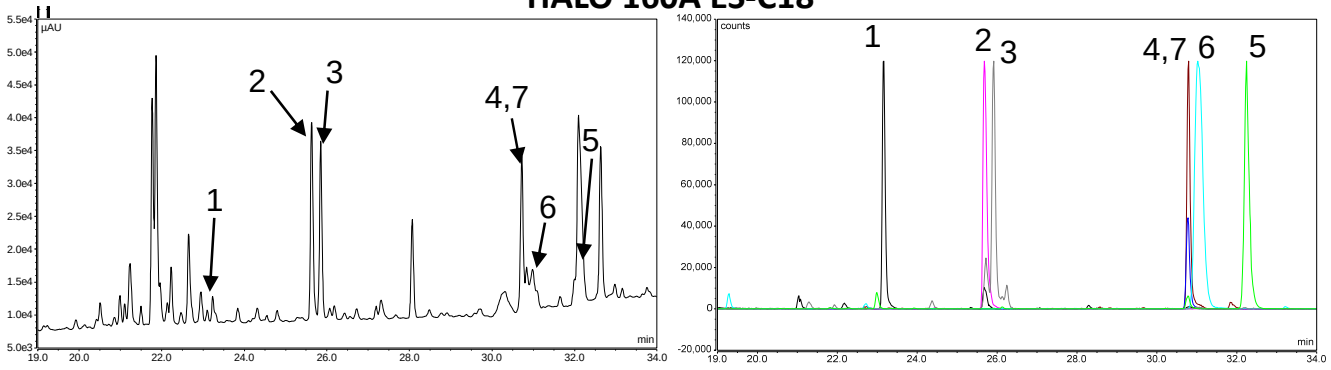
EIC signals normalized



HALO 160Å Phenyl-Hexyl



HALO 160Å ES-C18



MS CONDITIONS:

MS System: Thermo Fisher Orbitrap VelosPro ETD
ESI: +3.5 kV
Scan range: 50-2000 m/z
Scan rate: 2 pps
Capillary: 225 ˚C

Sheath gas: 35
Auxiliary gas: 10
Scan Time: 2 µscans/200 ms max inject time

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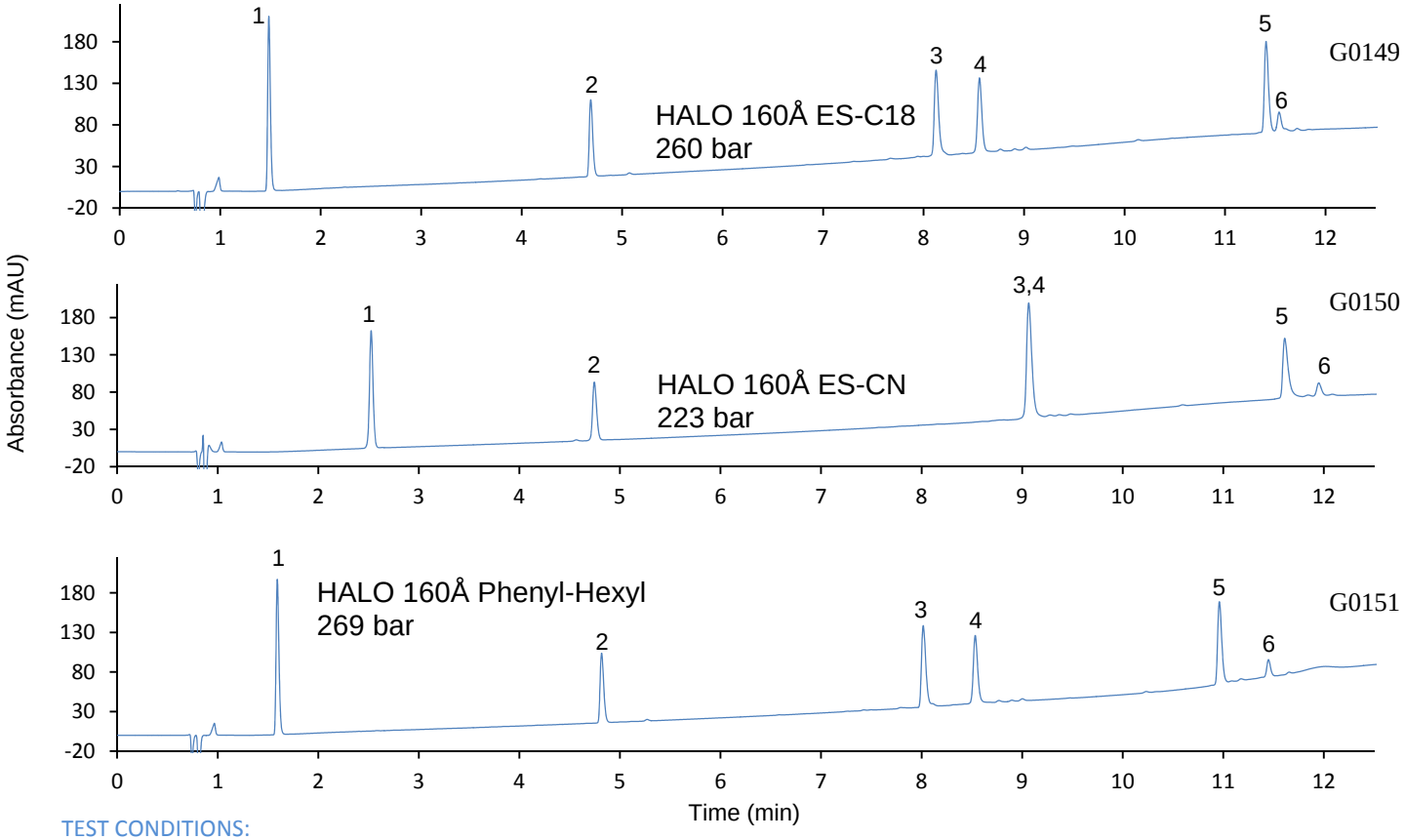
Application Note: 159-PE

Enhanced Selectivity for the Separation of Peptides Comparing HALO 160Å with Three Different Bonded Phases



TEST CONDITIONS:

Columns: HALO 160Å ES-C18, 2.7 µm, 2.1 x 150mm
Part Number: 92122-702
HALO 160Å ES-CN, 2.7 µm, 2.1 x 150mm
Part Number: 92122-704
HALO 160Å Phenyl-Hexyl, 2.7 µm, 2.1 x 150mm
Part Number: 92112-706

Mobile Phase:
A = 0.1% formic acid in water + 10mM ammonium formate
B = 50/50 n-propanol/water + 0.1% formic acid + 10mM ammonium formate (pH: 3.45)
Flow Rate: 0.4 mL/min
Gradient: 10-60%B in 15 min
Temperature: 60 °C
Detection: UV 220 nm, PDA
Injection Volume: 2 µL
Sample Solvent: water, 0.1% TFA
Response Time: 0.24 sec
Data Rate: 12.5 Hz
LC System: Shimadzu Nexera
Flow Cell: 1 µL

PEAK IDENTITIES:

1. Tyr-Tyr-Tyr
2. Angiotensin II
3. Angiotensin 1-12
4. Melittin
5. Sauvagine
6. β-Endorphin

The initial separation using a HALO 160Å ES-C18 column showed inadequate resolution of peaks 5 and 6. The same separation was attempted on a 160Å ES-CN column which provided improved resolution of peaks 5 and 6, but resulted in coelution of peaks 3 and 4. The HALO 160Å Phenyl-Hexyl column delivered excellent resolution between both peak pairs.

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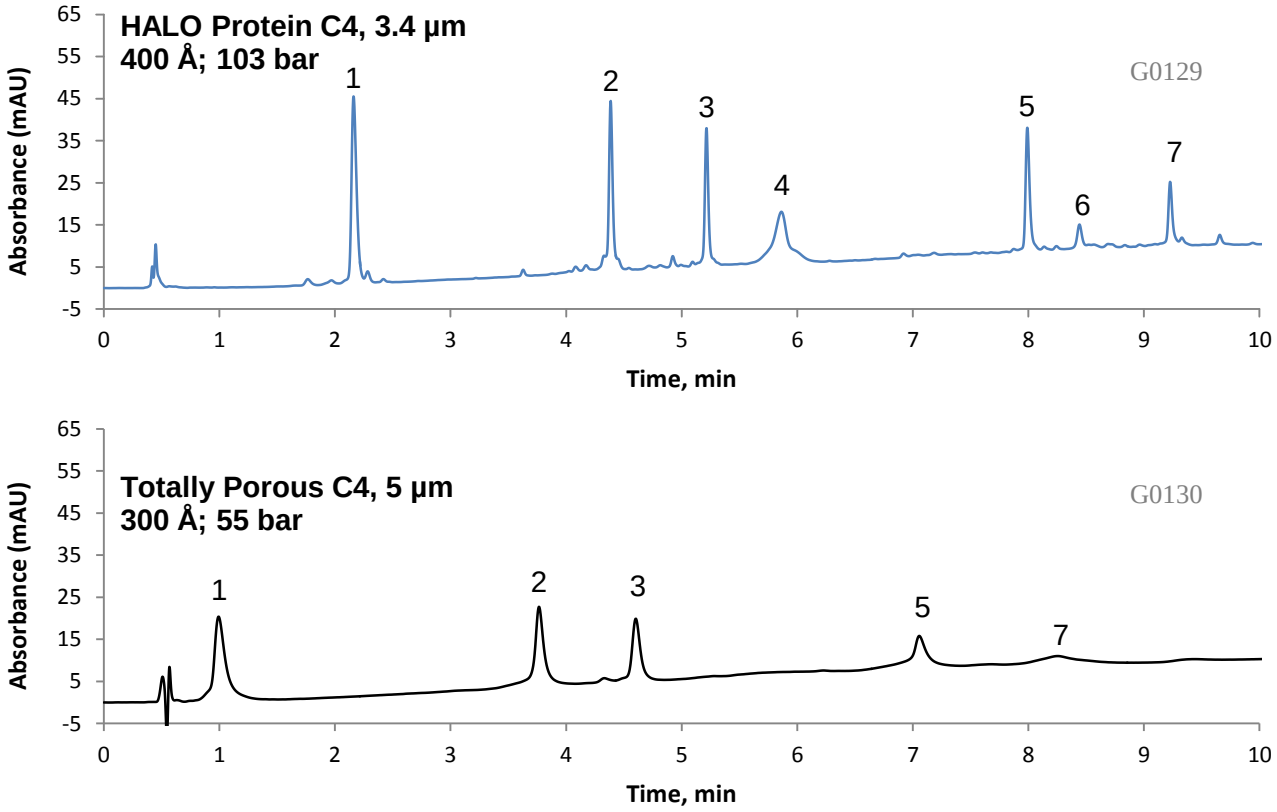
Application Note: 141-PR

Improved Protein Separations with HALO Protein C4 Compared to Totally Porous C4



TEST CONDITIONS:

Columns:
HALO Protein C4, 2.1 x100 mm, 3.4 µm
Part Number: 93412-614
Totally Porous C4, 2.1 x100 mm, 5 µm
Mobile Phase:
A = water/0.1% TFA
B = acetonitrile/0.1% TFA
Flow Rate: 0.5 mL/min.
Gradient: 25% B to 52% B in 10 minutes
Starting pressure: As indicated on chart
Temperature: 60°C
Injection Volume: 1 µL
Sample Solvent: mobile phase A
Detection: UV 215 nm, PDA
Data Rate: 5 Hz
Response Time: 1 sec.
Flow Cell: 2 µL micro cell
LC System: Agilent 1200 SL

PEAK IDENTITIES:

1.	Ribonuclease A	13.7 kDa
2.	Cytochrome *c*	12.4 kDa
3.	Lysozyme	14.3 kDa
4.	Holotransferrin	77 kDa
5.	Apomyoglobin	17 kDa
6.	Catalase	tetramer of ~ 60 kDa each
7.	Enolase	46.7 kDa

Sharper, taller peaks are observed using the HALO Protein C4 column compared to a conventional totally porous C4 column. Additionally, the HALO Protein C4 column provides improved recoveries for holotransferrin, apomyoglobin, catalase, and enolase.

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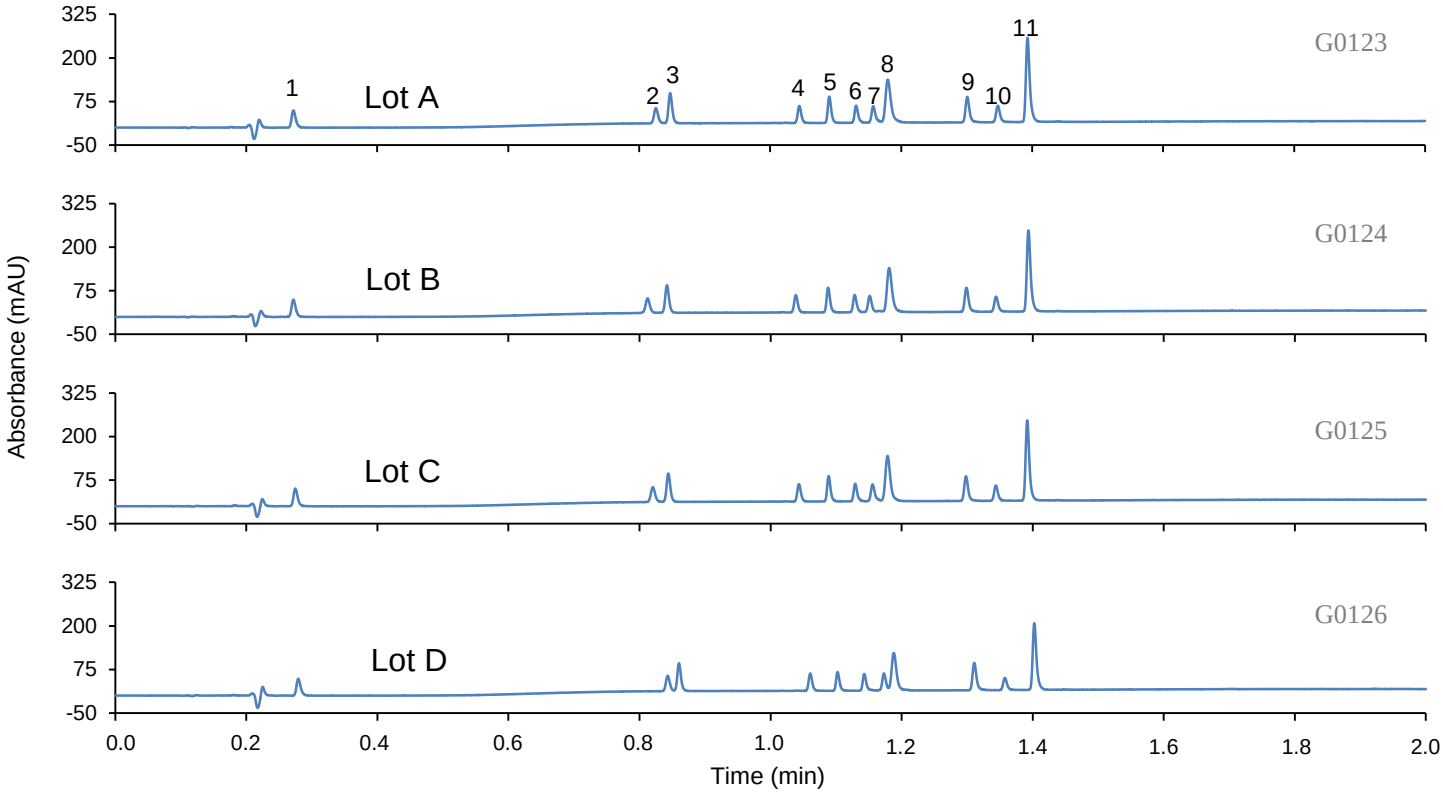
Application Note: 138-PE

HALO 2 Peptide ES-C18 Lot Reproducibility



TEST CONDITIONS:

Columns:
3.0 x 50 mm, HALO 2 Peptide ES-C18, 2 µm
Part Number: 91123-402

Mobile Phase:
A = 0.1% Trifluoroacetic acid in water
B = 0.1% Trifluoroacetic acid in 80/20 acetonitrile/water
Flow Rate: 1.1 mL/min.
Gradient: Hold at 12.5% B for 0.1 min;
12.5% B to 93% B from 0.1 - 2.0 min.
Initial pressure: 278 bar
Temperature: 60 °C
Detection: UV 215 nm, PDA
Injection Volume: 0.5 µL
Sample Solvent: mobile phase A
Response Time: 0.025 sec.
Data Rate: 200 Hz
LC System: Shimadzu Nexera X2
Flow Cell: 1 µL

PEAK IDENTITIES:	MW (g/mol)	%RSD (retention times)
1. Gly-Tyr	238	1.21
2. Val-Tyr-Val	380	1.59
3. Angiotensin 1/2 (1-7) amide	898	0.95
4. Met-enkephalin	574	0.92
5. Angiotensin 1/2 (1-8) amide	1045	0.60
6. Angiotensin II	1046	0.61
7. Leu-enkephalin	556	0.82
8. Ribonuclease A	13,700	0.35
9. Angiotensin (1-12) (mouse)	1573	0.46
10. Bovine Insulin	5733	0.49
11. Angiotensin (1-12) (human)	1509	0.36

The lot-to-lot reproducibility of HALO 2 Peptide ES-C18 is maintained by tightly controlled manufacturing practices and quality assurance testing. This ensures the reliability of the product over its lifetime.

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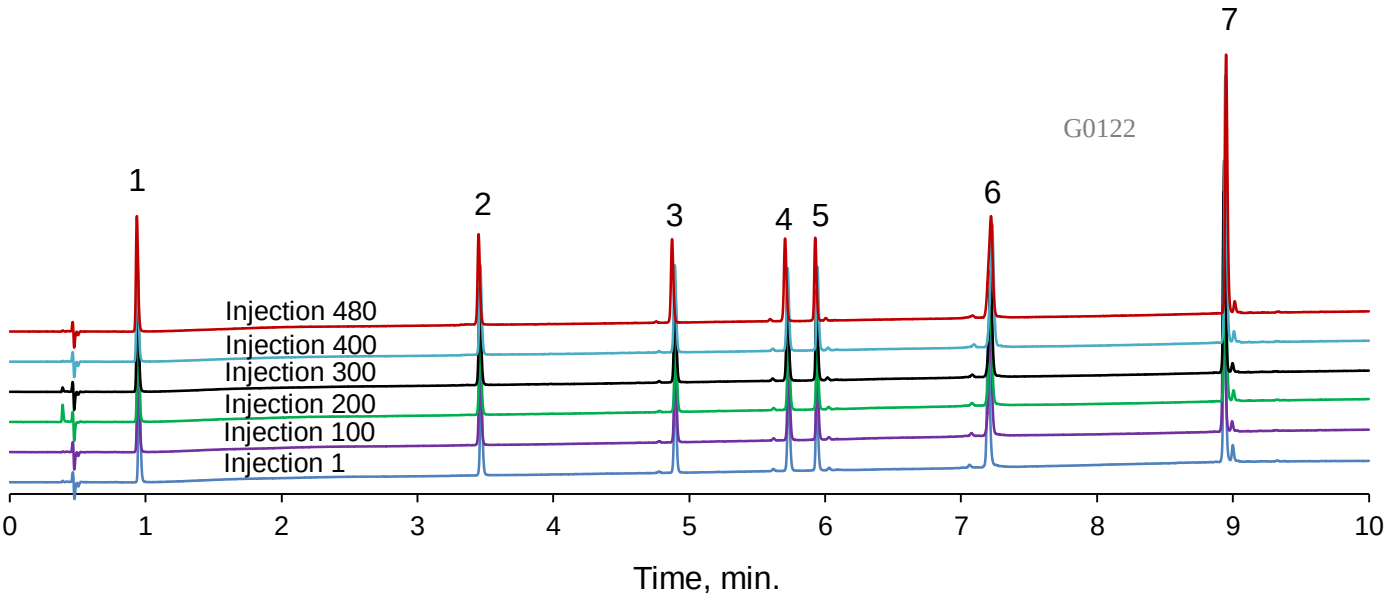
Application Note: 137-PE

High Temperature/Low pH Stability with HALO 2 Peptide ES-C18



TEST CONDITIONS:

Column:
2.1 x 100 mm, HALO 2 Peptide ES-C18, 2 µm
Part Number: 91122-602

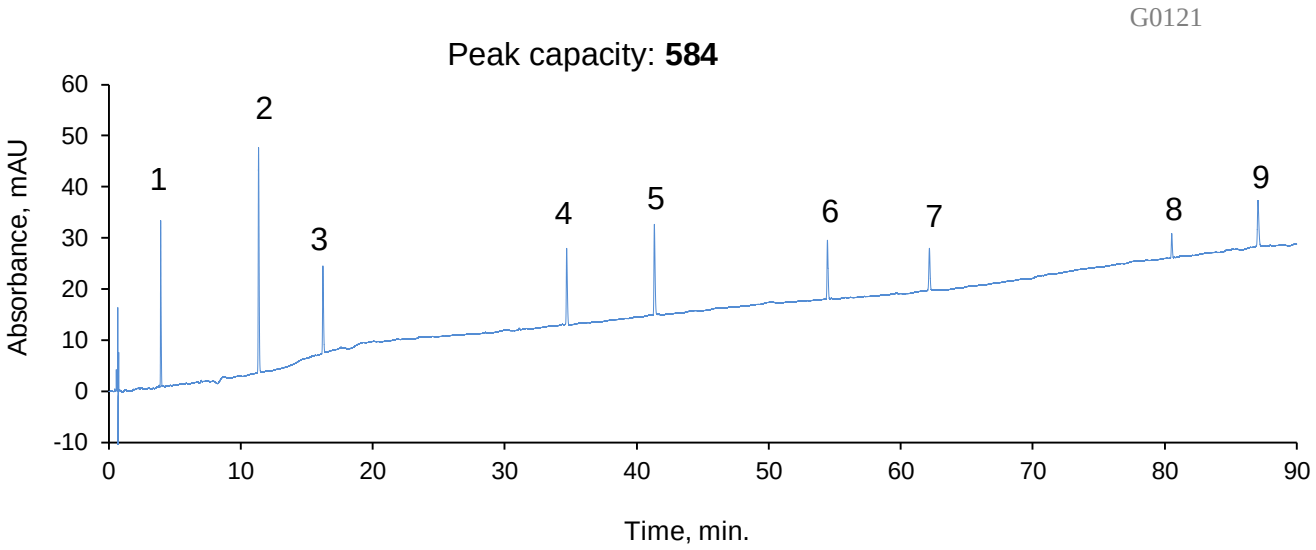
Mobile Phase:
A = 0.1% Trifluoroacetic acid in water
B = 0.1% Trifluoroacetic acid in 80/20 acetonitrile/water
Flow Rate: 0.5 mL/min.
Gradient: 6% B to 54% B in 10 min
Initial pressure: 395 bar
Maximum pressure: 417 bar
Temperature: 60 °C
Detection: UV 215 nm, PDA
Injection Volume: 0.5 µL
Sample Solvent: mobile phase A
Response Time: 0.025 sec.
Data Rate: 40 Hz
LC System: Shimadzu Nexera X2
Flow Cell: 1 µL

PEAK IDENTITIES	MW (g/mol)
1. Gly-Tyr	238
2. Val-Tyr-Val	380
3. Met-enkephalin	574
4. Angiotensin II	1046
5. Leu-enkephalin	556
6. Ribonuclease A	13,700
7. Bovine Insulin	5733

The sterically-protected C18 phase on the HALO 2 Peptide column enables high temperature stability with low pH mobile phases. The replicate injections were stopped at injection 480 (15,500 column volumes). The column is expected to have a lifetime of ~ 1000 injections, depending on the type of sample and conditions used.

Application Note: 136-PE

Very High Peak Capacity with HALO 2 Peptide ES-C18



TEST CONDITIONS:

Column:
2.1 x 150 mm, HALO 2 Peptide ES-C18, 2 µm
Part Number: 91122-702

Mobile Phase:
A= 0.1% Trifluoroacetic acid in water
B= 0.1% Trifluoroacetic acid in 80/20 acetonitrile/water
Flow Rate: 0.5 mL/min.
Gradient: 5% B to 50% B in 90 minutes
Maximum pressure: 577 bar
Temperature: 60 °C
Detection: UV 215 nm, PDA
Injection Volume: 0.5 µL
Sample Solvent: mobile phase A
Response Time: 0.025 sec.
Data Rate: 40 Hz
LC System: Shimadzu Nexera X2
Flow Cell: 1 µL

PEAK IDENTITIES	MW (g/mol)
1. Asp-Phe	280
2. Tyr-Tyr-Tyr	508
3. Angiotensin (1-7) amide	898
4. Angiotensin II	1046
5. Angiotensin (1-12) human	1509
6. Neurotensin	1673
7. ß-endorphin	3465
8. Sauvagine	4599
9. Mellitin	2847

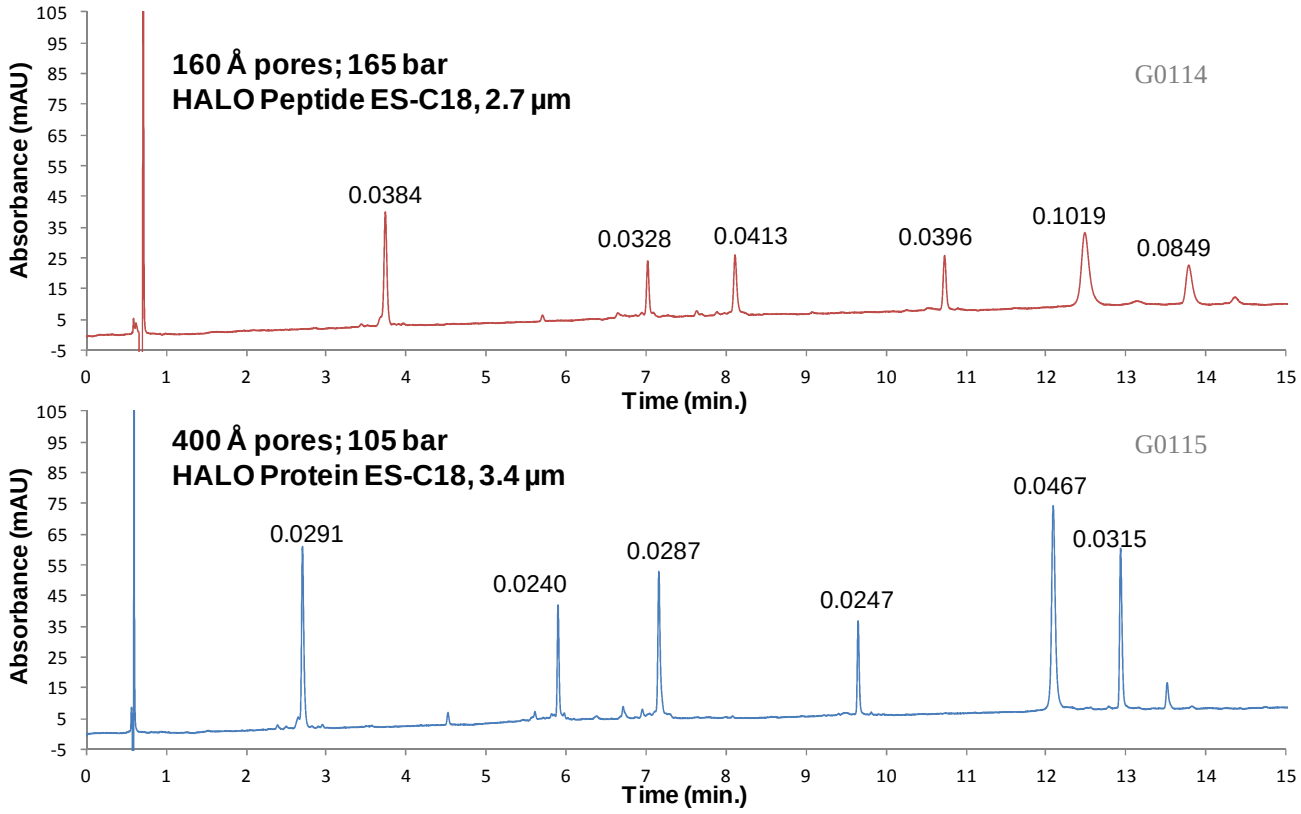
With a HALO 2 Peptide ES-C18 column, very high peak capacity values can be obtained within 90 minutes. The sharp, narrow peaks facilitate separations of complex, challenging samples, such as tryptic digests.

Peak capacity: $n_{pc} = \frac{(t_f - t_i)}{W_{4\sigma}}$

where t_i is the time for initial measurable peak in the gradient, t_f is the time for final peak and $W_{4\sigma}$ is the average four-sigma width in time for the peaks in the chromatogram

Application Note: 130-PR

Effect of Silica Pore Size on Protein Separations



TEST CONDITIONS:

Columns:
4.6 x100 mm, HALO Peptide ES-C18, 2.7 µm
Part Number: 92124-602
4.6 x 100 mm, HALO Protein ES-C18, 3.4 µm
Part Number: 93414-602
Mobile Phase:
A= 0.1% Trifluoroacetic acid in water
B= 0.1% Trifluoroacetic acid in acetonitrile
Flow Rate: 1.5 mL/min.
Gradient: 23% B to 50% B in 15 minutes
Starting pressure: As indicated on chart
Temperature: 60℃
Detection: UV 215 nm, VWD
Injection Volume: 5 µL
Sample Solvent: mobile phase A
Response Time: 0.12 sec.
Data Rate: 14 Hz
LC System: Agilent 1100 Quaternary
Flow Cell: 5 µL semi-micro

PEAK IDENTITIES:

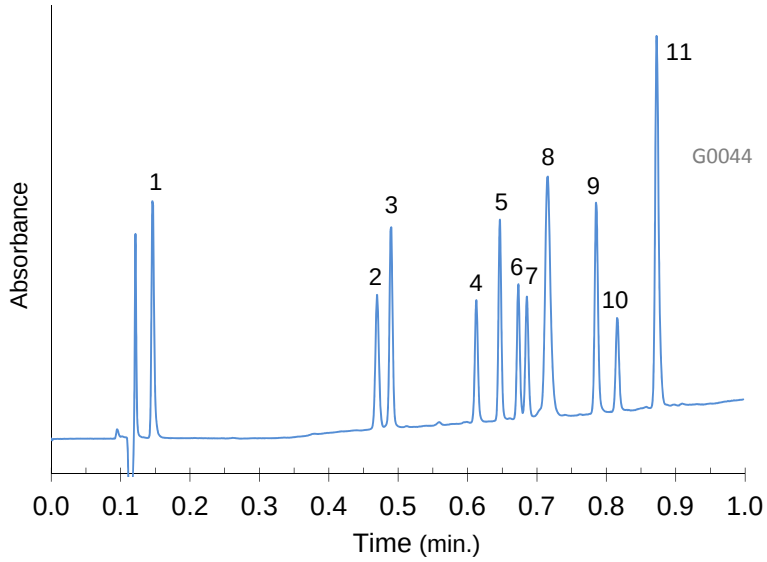
1.	Ribonuclease A	13.7 kDa
2.	Cytochrome *c*	12.4 kDa
3.	Lysozyme	14.3 kDa
4.	α-Lactalbumin	14.2 kDa
5.	Catalase	tetramer of ~ 60 kDa each
6.	Enolase	46.7 kDa

Sharper, taller peaks are observed using the HALO 400 Å Protein ES-C18 column because the larger pore size allows unrestricted diffusion for these biomolecules into and out of the porous shell. The half height peak widths above each protein peak are significantly smaller with the HALO Protein column despite the larger particle size of the packing material, emphasizing the importance of larger pores when separating proteins.

HALO® | Fused-Core® Particle Technology

Application Note: 62-PT

Separation of Peptides and Small Proteins on HALO Peptide ES-C18



PEAK IDENTITIES:

1. Gly-Tyr
2. Val-Tyr-Val
3. Angiotensin (1-7) amide
4. Met-Enk
5. Angiotensin (1-8) amide
6. Angiotensin II
7. Leu-Enk
8. Ribonuclease A
9. Angiotensin (1-12) (human)
10. Angiotensin (1-12) (mouse)
11. Porcine insulin

TEST CONDITIONS:

Column: 4.6 x 50 mm, HALO Peptide ES-C18
Part Number: 92124-402
Mobile Phase:
A= 90%(0.1% TFA in water)/10% acetonitrile
B= 30% (0.1% TFA in water)/70% acetonitrile
Gradient: 0% B to 87% B in 1 minute
Flow Rate: 5.0 mL/min.
Pressure: 330 Bar
Temperature: 60°C
Detection: UV 220 nm, VWD
Injection Volume: 1.0 µL
Sample Solvent: Mobile Phase A
Response Time: < 0.12 sec.
Flow Cell: 5 µL semi-micro
LC System: Quaternary Agilent 1100
Gradient dwell volume is 0.88 mL

DISCUSSION:

This separation shows the utility of the HALO Fused-Core Peptide ES-C18 stationary phase for the separation of peptides by HPLC. An average pore size of about 160 Angstroms enhances the mass transfer of peptides and small proteins of up to a molecular weight of approximately 15 kD, depending on the molecular configuration. Also, the stationary phase is a sterically protected C18 bonded silane to increase resistance to low pH mobile phases and elevated temperatures (up to 100 °C.) that are commonly used in the separation of many biological materials.

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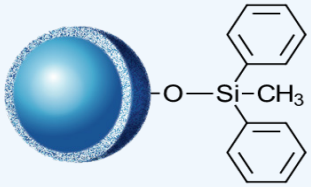
FOR MORE INFORMATION OR TO PLACE AN ORDER, CONTACT:

The HALO® BioClass Diphenyl Phase: Discussion and Best Uses

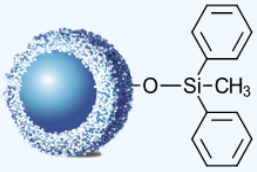
Advanced Materials Technology offers two diphenyl bondings on the well-known superficially porous particle. The new HALO 400 Å Diphenyl, 3.4 µm column and 1000 Å Diphenyl, 2.7 µm columns are primarily used for protein and monoclonal antibody analysis due to its larger pore size. The columns provide narrow peak shapes and better sample recoveries for large biomolecules that range from 2kDa and higher when compared to smaller pore sizes and fully porous particles. The new 3.4 µm superficially porous particle consists of a 3 µm core and a 0.2 µm shell with 400 Å pores while the 2.7 µm superficially porous particle consists of a 1.7 µm core and a 0.5 µm shell with 1000 Å pores. Figure 1 below shows a comparison of the two particles.

Figure 1. HALO® BioClass Diphenyl Options

HALO 400 Å Diphenyl, 3.4 µm Particle

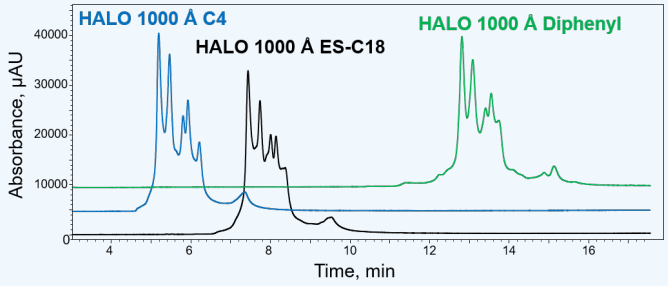


HALO 1000 Å Diphenyl, 2.7 µm Particle



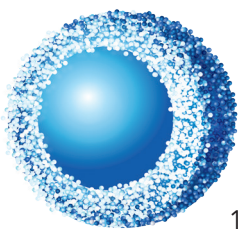
The diphenyl phase offers a unique selectivity to help separate complex samples such as IgG1 and IgG2 monoclonal antibodies. Figure 2 shows a comparison resolving denosumab isoforms (mAb used to help treat bone cancer) on three different 1000 Å bonded phases. The diphenyl phase is retained the longest. While there are minor differences for this IgG2 in this particular comparison, since biopharmaceutical production involves designing custom mAbs with particular characteristics, screening multiple bonded phases could reveal important differences. This is especially true when looking at protein variants.

Figure 2. IgG2 Comparison On HALO 1000 Å C4, ES-C18, And Diphenyl



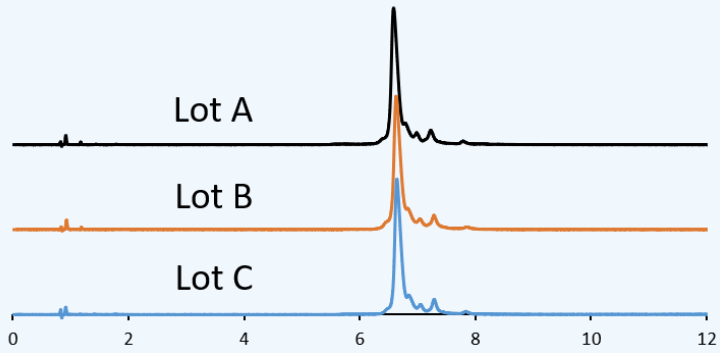
TEST CONDITIONS

Columns: HALO 1000 Å C4, 2.7 µm, 2.1 x 150 mm
HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 150 mm
HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
Flow Rate: 0.2 mL/min
Temperature: 80°C
Injection Volume: 2 µL of 2 mg/mL denosumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 280 nm
Mobile Phase A: 2:10:88 n-propanol/ACN/H2O + 0.1% difluoroacetic acid (DFA)
Mobile Phase B: 70:20:10 n-propanol/ACN/H2O + 0.1% DFA
Gradient: 16-26% B in 20 min



Both 400 and 1000 Å Diphenyl phases show excellent lot to lot reproducibility in order to maintain reliable and repeatable results for the user. Tight manufacturing processes used by Advanced Materials Technology ensure that the highest quality column performance is achieved. For example, Figure 3 shows trastuzumab (mAb used to treat breast cancer) on three different lots of HALO 1000 Å Diphenyl. Resolution of minor components are repeatable along with the retention time of the IgG1 monoclonal antibody.

Figure 3. Lot-To-Lot Reproducibility Of HALO 1000 Å Diphenyl

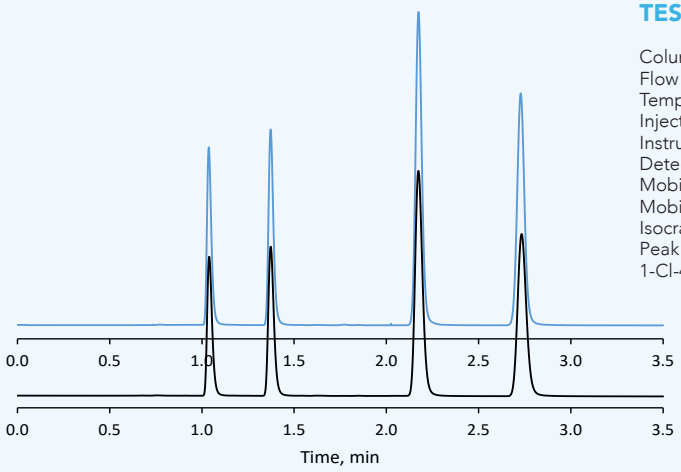


TEST CONDITIONS

Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
Flow Rate: 0.4 mL/min
Temperature: 80 °C
Injection Volume: 2 µL of 2 mg/mL trastuzumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 280 nm
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/ Water/0.085% TFA
Gradient: 40-47.5% B in 12 min

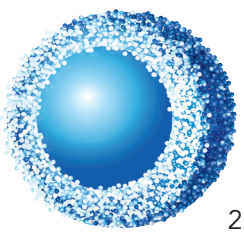
Both 400 Å and 1000 Å Diphenyl columns are also very stable allowing for long column lifetimes. Figure 4 shows the results of a high-pressure stability test using neutral compounds on a HALO 400 Å Diphenyl column. The column has experienced 10,000 column volumes at 600 bar and maintained its peak shape, retention, and held a consistent back pressure.

Figure 4. HALO 400 Å Diphenyl Stability



TEST CONDITIONS

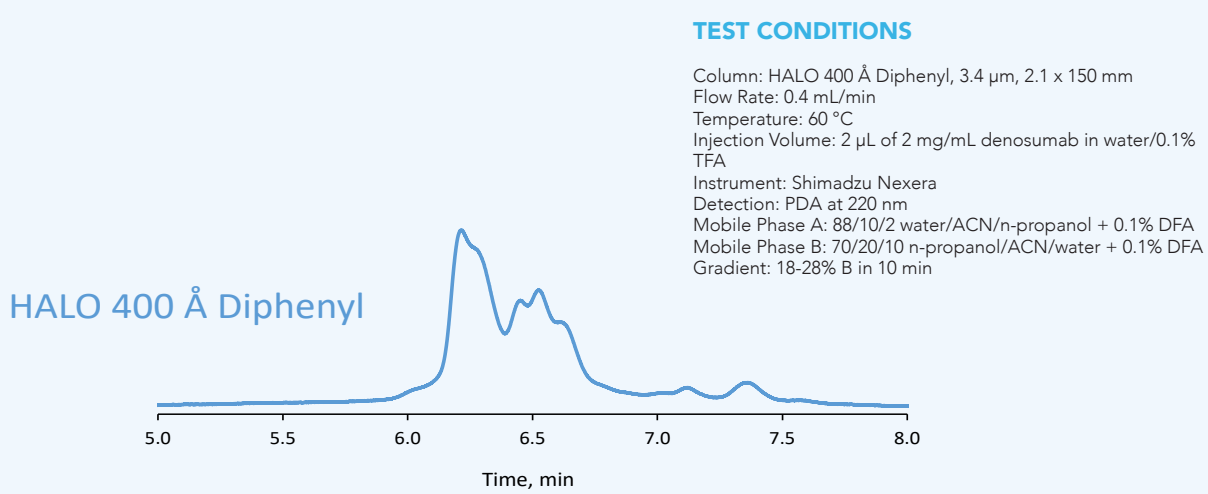
Column: HALO 400 Å Diphenyl, 3.4 µm, 2.1 x 150 mm
Flow Rate: 0.3 mL/min
Temperature: 30 °C
Injection Volume: 0.5 µL
Instrument: Shimadzu Nexera
Detection: PDA at 254 nm
Mobile Phase A: water
Mobile Phase B: ACN
Isocratic: 75/25 A/B
Peak Identities: uracil, phenol, propiophenone, 1-Cl-4-nitrobenzene (in elution order)



HALO 400 Å DIPHENYL: BEST USES

For analysts whose goal is high throughput and/or less emphasis on ultimate resolution yet looking for the critical quality attributes of their biopharmaceutical in the shortest amount of time, the larger particle size and thinner shell of the HALO 400 Å Diphenyl may be used to their advantage while developing release assay methods. The HALO 400 Å Diphenyl with 3.4 µm particle size will have lower overall back pressures when compared to the 1000 Å, 2.7 µm particle or a sub-2 µm fully porous particle. Figure 5 shows a fast analysis of denosumab in under 8 minutes on the HALO 400 Å Diphenyl column. Shorter run times allow reduced mobile phase consumption and higher throughput.

Figure 5. Fast Separation Of Denosumab On HALO 400 Å Diphenyl



If resolution is more important than speed, then the method may be adjusted accordingly. The 400 Å Diphenyl phase shows excellent resolution for monoclonal antibodies when compared to similar columns on the market. For example, denosumab was analyzed again on a HALO 400 Å Diphenyl column compared to a 300 Å fully porous diphenyl column. The HALO 400 Å column outperformed the 300 Å fully porous diphenyl column by providing much better resolution at 2.5x lower back pressure along with a shorter analysis time. See Figure 6.

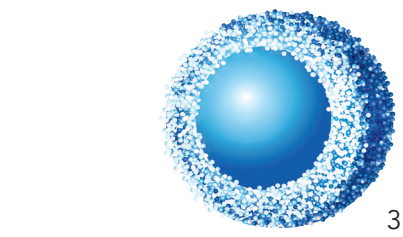
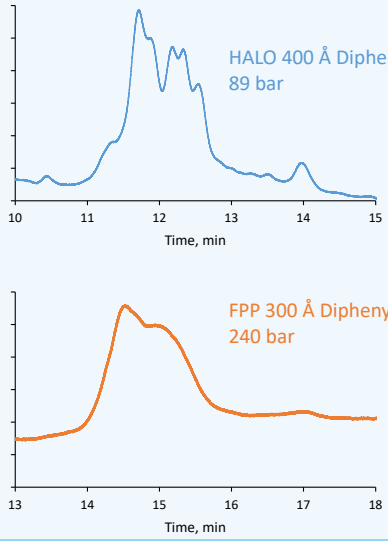


Figure 6. Increased Resolution And Lower Back Pressure with HALO 400 Å Diphenyl Compared To An FPP 300 Å Diphenyl



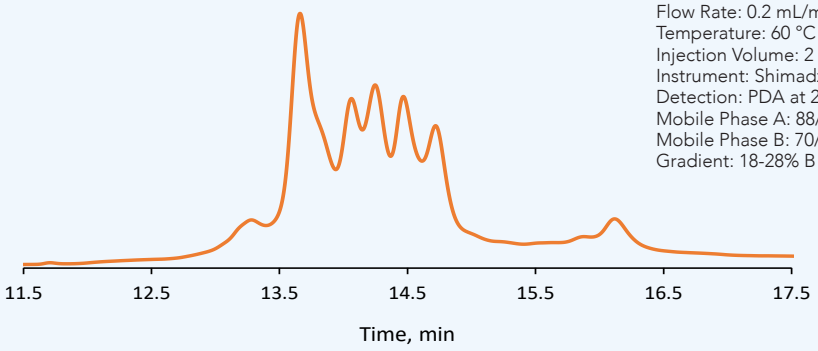
TEST CONDITIONS

Columns: HALO 400 Å Diphenyl, 3.4 µm, 2.1 x 150 mm
FPP 300 Å Diphenyl, 1.8 µm, 2.1 x 150 mm
Mobile Phase A: 88/10/2 water/ACN/n-propanol + 0.1% DFA
Mobile Phase B: 70/20/10 n-propanol/ACN/water + 0.1% DFA
Gradient: 18-28% B in 20 min
Flow Rate: 0.2 mL/min
Temperature: 60 °C
Injection Volume: 2 µL of 2 mg/mL denosumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 220 nm

HALO 1000 Å DIPHENYL: BEST USES

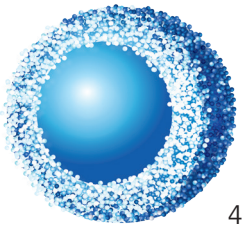
For the ultimate resolution of monoclonal antibodies or other large proteins, HALO 1000 Å Diphenyl phase is recommended. The large pores allow unrestricted access of mAbs to the bonded phase, while the higher surface area/thicker shell enables high resolution separations of various mAb isoforms. Figure 7 shows a high resolution separation of denosumab.

Figure 7. High Resolution Separation Of Denosumab On HALO 1000 Å Diphenyl Column



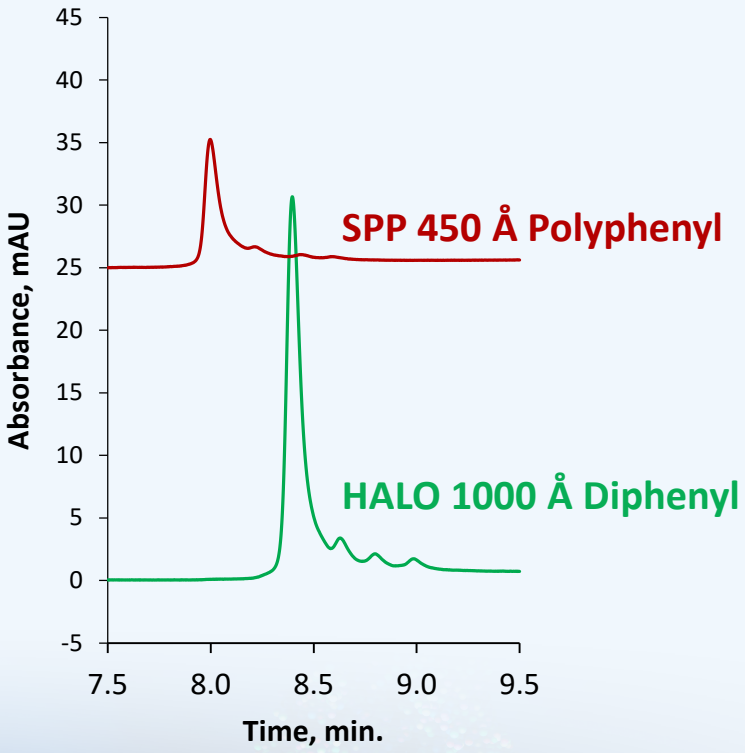
TEST CONDITIONS

Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
Flow Rate: 0.2 mL/min
Temperature: 60 °C
Injection Volume: 2 µL of 2 mg/mL denosumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 220 nm
Mobile Phase A: 88/10/2 water/ACN/n-propanol + 0.1% DFA
Mobile Phase B: 70/20/10 n-propanol/ACN/water + 0.1% DFA
Gradient: 18-28% B in 20 min



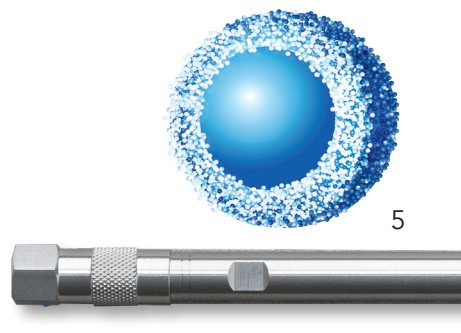
Both HALO 400 Å and 1000 Å Diphenyl columns are stable up to 90 °C for high temperature separations. It is generally accepted that sample recovery of proteins and mAbs improves as temperature increases, therefore temperatures of 60 °C or greater are often used to maximize sample recovery. However, temperature related artifacts have been observed when operating at these higher temperatures. Because of this, a temperature gradient is recommended and operating at lower temperature may prove desirable. A comparison of trastuzumab (Figure 8) at 40 °C on both a HALO 1000 Å Diphenyl column and a 450 Å SPP Polyphenyl column shows impressive protein recovery with the HALO® column. The HALO® Diphenyl column also demonstrates improved resolution, retention, and peak area compared to the competitor SPP column. This increased retention and resolution clearly demonstrate the benefit of unrestricted large pore access to the bonded phase and that high recoveries are possible with lower operating temperatures.

Figure 8. Comparison of HALO 1000 Å Diphenyl to Competitor Polyphenyl at 40 °C



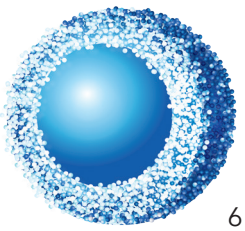
TEST CONDITIONS

Columns: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
SPP 450 Å PolyPhenyl, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: water/0.1% TFA
Mobile Phase B: ACN/0.1% TFA
Gradient: 30-45% B in 15 min
Flow Rate: 0.4 mL/min
Temperature: 40 °C
Injection Volume: 2 µL of 2 mg/mL trastuzumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: PDA at 280 nm



CONCLUSIONS

For biopharmaceutical separation scientists, the HALO 400 Å and 1000 Å Diphenyl columns are two beneficial additions to the protein chemist's separation toolbox for mAb release assays and characterization methods. The HALO® Diphenyl offers a unique selectivity compared to C4 and C18 and demonstrates excellent stability with good sample recovery while outperforming the competitors to deliver quality and performance – every time.



Mobile Phase Additive Selection for LC-MS

INTRODUCTION

Acids have always been preferred additives for use in HPLC methods because silica bonded phases typically exhibit highest performance under acidic conditions. Volatile acids and buffers are required for mass spectrometric detection. Selected properties of common acids and additives are shown in Table 1.

Table 1. Mobile Phase Additives for HPLC (UV and MS Detection)

Acidic Additive	pK_a	pH (aq. sol.)	BP (°C)	Ion-pairing
Phosphoric	2.1 (pK_1)	2	158	no
Formic	3.8	3	100	no
Acetic	4.8	4	118	no
3,3,3-Trifluoropropanoic	3.2	3	145	weak
Difluoroacetic	1.3	2	134	strong
Trifluoroacetic	0.3	<2	74	very strong
Formate/Formic acid	n. a.	3-5 (buffer)	volatile	no
Acetate/Acetic acid	n. a.	4-6 (buffer)	volatile	no

Table properties are approximate and were obtained from several sources. The pH values for acid solutions are estimates that will vary with concentration. Volatile organic reagents should always be used in a well-ventilated environment.

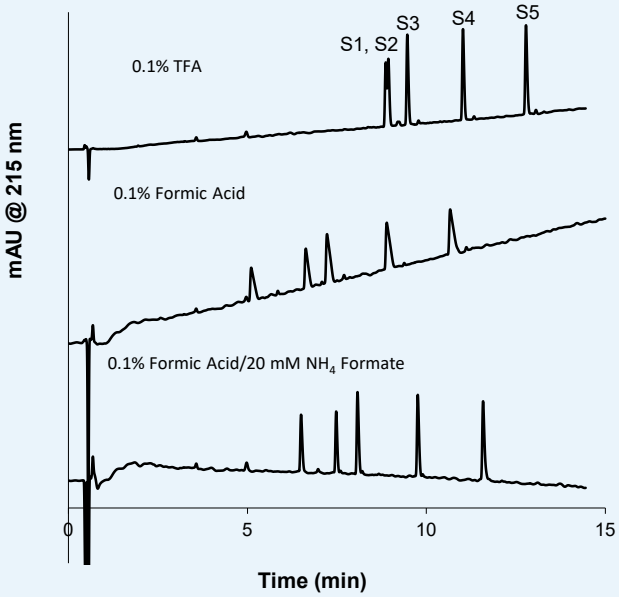
More information is readily available in modern texts (Practical HPLC Method Development by L.R. Snyder, J.J. Kirkland and J.L. Glajch, 2nd ed., John Wiley & Sons. Hoboken, NJ, 1997.) and also in A Guide to HPLC and LC-MS Buffer Selection (prepared by John Dolan) from http://mac-mod.com. Also refer to an oral presentation given by Barry Boyes at HPLC 2016 for more details.

DISCUSSION AND EXAMPLES

Figure 1 shows a comparison of low MW peptide standards separated on a HALO® 160 Å ES-C18, 2.7 µm column with three different mobile phase additives from Table 1 at similar concentration levels. HALO® ES-C18 is a sterically protected bonded phase that is stable at the low pH conditions that are typically used for peptide analysis. Note that the very low pH condition fully suppresses silanol interaction to create very sharp peaks; and causes maximum retention because TFA is a strong ion-pair reagent that interacts with peptides to make them seem more hydrophobic to the stationary phase. Since selectivity for peaks 1 and 2 is lost, more screening is needed. Changing to formic acid raises pH to 2.7 and brings possible silanol ionization into the picture. Peptides are well separated, but rather broad because of competing ionic and RP interactions at the higher pH, and the ionic strength is too low to suppress ionic effects. The ammonium formate/ formic acid gradient shows the most promise of the three conditions.

Although the threat of mixed ionic and RP retention is even greater at pH 3.3, the formate increases ionic strength and keeps peaks as sharp as TFA does at much lower pH. Although this study was done with low wavelength UV detection, note that all mobile phase additives are volatile. It has been reported by some analysts that combinations of TFA and FA are useful to offset weaknesses in both acids. Alternatively, in some cases, dilute TFA (0.03%) is as effective as 0.1% FA/0.05% TFA, yielding conditions that are compatible with MS detection.

Figure 1. Improving Retention and Peak Shape Using Ammonium Formate. Comparison of TFA, Formic Acid (FA), and FA/Ammonium Formate for Peptide Separations.



PEAK IDENTITIES

1. S1 RGAGGLGLGK-Am
2. S2 Ac-RGGGGLGLGK-Am
3. S3 Ac-RGAGGLGLGK-Am
4. S4 Ac-RGVGGLGLGK-Am
5. S5 Ac-RGVVGLGLGK-Am

	TFA 0.1%	Formic Acid 0.1%	Ammonium Formate	AF/FA
Concentration	0.013 M	0.026 M	0.020 M	20 mM AF 26 mM FA
pH	1.8	2.7	c. 7	3.3
Ionic strength	26 mM	4.4 mM	40 mM	c. 44 mM

TEST CONDITIONS

Column: HALO 160 Å ES-C18, 2.7 µm, 4.6 x 100 mm
Mobile Phase A: Water/acid modifier
Mobile Phase B: ACN/0.1% TFA or Formic Acid
Flow rate: 2.0 mL/min
Gradient: 1.5% to 26% B in 15 min
Temperature: 30 °C
Detection: UV @ 215 nm
Injection: 8 µL (800 ng) of synthetic peptides S1-S5

McCalley, D. V., Effect of buffer on peak shape of peptides in reversed-phase high performance liquid chromatography. J Chromatogr A 2004, 1038 (1-2), 77-84. Schuster, S. A.; Boyes, B. E.; Wagner, B. M.; Kirkland, J. J., Fast high performance liquid chromatography separations for proteomic applications using Fused-Core® silica particles. J Chromatogr A 2012, 1228, 232-241.

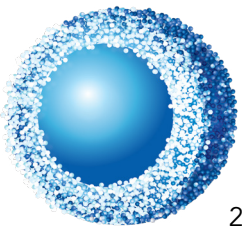
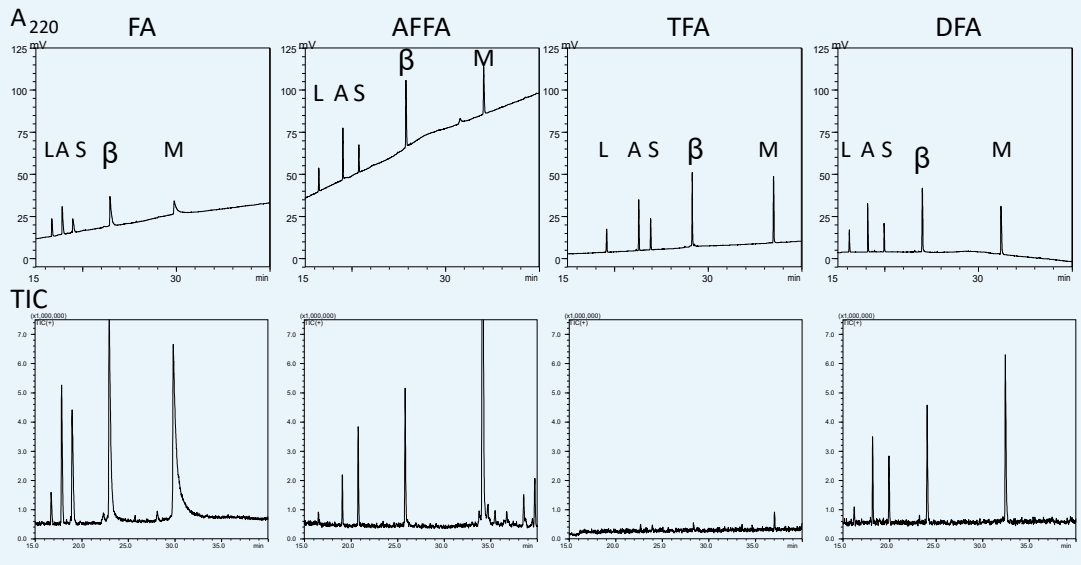


Figure 2 shows five natural peptides screened using the same HALO® Peptide ES-C18 column and the same three mobile phase additives with a stronger gradient and higher temperature using both UV (220 nm) and MS (ESI) detection. TFA, formic acid (FA) and ammonium formate/formic acid (AFFA) buffer were compared for retention, resolution, sensitivity and baseline stability to a fourth additive from Table 1, Difluoroacetic acid (DFA), that is growing in popularity for MS detection. Tailing is excessive with FA because ionic interactions are not adequately suppressed by lower pH and higher ionic strength, but it has highest MS sensitivity; AFFA yields excellent results and next highest MS sensitivity, but some baseline drift with UV is seen at 220 nm; TFA shows high performance and excellent baseline stability with UV, but the MS signal is completely suppressed due presumably to the formation of strong ion-pairs between peptides and TFA anion [1]; DFA offers an excellent combination of performance and baseline stability with both UV and MS. With DFA, pH is very low like TFA to suppress all silanol ionization and improve peak shape, while the ion-pairing is strong enough to maximize retention and resolution, but not so strong as to completely suppress the MS-ESI signal. This latter MS advantage may be related to a much higher boiling point (lower volatility) for DFA. With lower volatility, DFA also should not evaporate as quickly as TFA and change chromatographic conditions during use.

Figure 2. Comparison of UV and MS Intensities Using Peptide Synthetic Mixture (10 mM Acid; 50 pmol of a 5 peptide mix)



PEAK IDENTITIES

Peptide	Abbrev.	MW (g/mol)
[Leu5]-enkephalin	L	555.6
angiotensin I, human acetate hydrate	A	1297
substance P acetate salt hydrate	S	1348
Melittin, honey bee venom	M	2847
beta-endorphin, human	β	3465

TEST CONDITIONS

10 mM Acid; 50 pmol of a 5 peptide mix
Column: HALO 160 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Flow Rate: 0.3 mL/min
Mobile Phase Gradient: 2-47% ACN in 40 min
Temperature: 60 °C
Detection: UV @ 220 nm 300-1800 m/z, 4kV, 0.33s

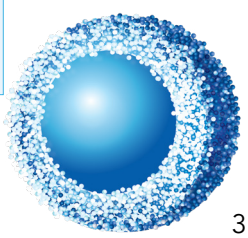
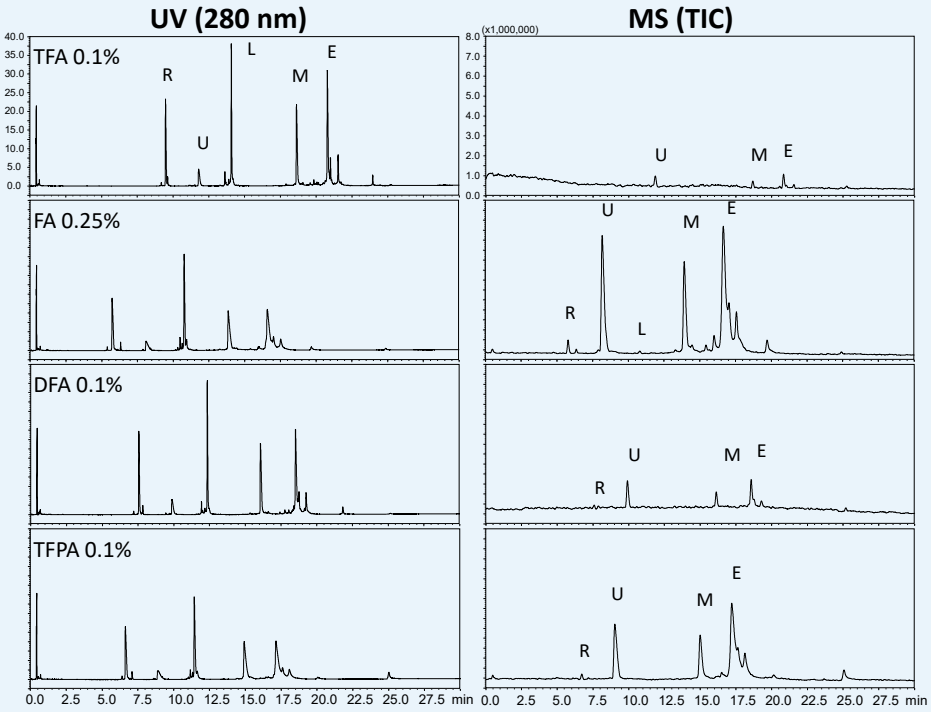


Figure 3 compares mobile phase additives from Table 1 for separating small proteins (under 50 kDa) using UV (this time at 280 nm) and MS (ESI) detection. In this case, pore-size has been eliminated as a performance factor by choosing the HALO® 400 Å C4 Protein column where the average pores are more than 10 times as large as the largest sample molecule to allow free diffusion within the particle. Similar to peptides, TFA shows highest retention and performance, but suffers from signal suppression in MS-ESI; higher boiling fluorinated acids DFA and 3,3,3-trifluoropropanoic acid (TFPA) can often improve MS sensitivity over TFA. The highest overall performance for this sample is shown with FA and TFPA.

Figure 3. Comparison of the Effect of TFA, FA, DFA, and TFPA on UV and MS Signals



PEAK IDENTITIES

R – Ribonuclease A:	13.7 kDa
U – Ubiquitin:	10.7 kDa
L – Lysozyme:	14.4 kDa
M – apo-Myoglobin:	17 kDa
E – Enolase:	46 kDa

25 pmol each protein

TEST CONDITIONS

Column: HALO 400 Å C4, 3.4 µm, 2.1 x 100 mm
Gradient: 15-55% AcN in 30 min
Flow Rate: 0.35 mL/min
Temperature: 50 °C
Detection: Nexera LC system
UV @ 280 nm
MS-2020 Single Quad
400 – 2000 m/z 3 pps, 3.8 kV ESI

Figure 4 compares three popular acid additives for a monoclonal antibody protein separation with the HALO® 1000 Å C4 Protein column using UV (280 nm) detection. The ability for this very large (150 kDa) protein to enter 1000 Å particle pores should be relatively unhindered. As seen for many proteins and peptides, FA does not completely suppress interaction with acidic silanols and shows peak broadening; however, both DFA and TFA operate at much lower pH and introduce extra retention from ion-pairing. If MS detection were needed, DFA would be preferred for higher MS sensitivity due to lower volatility and weaker ion-pair formation.

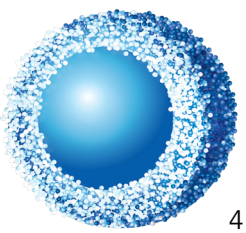
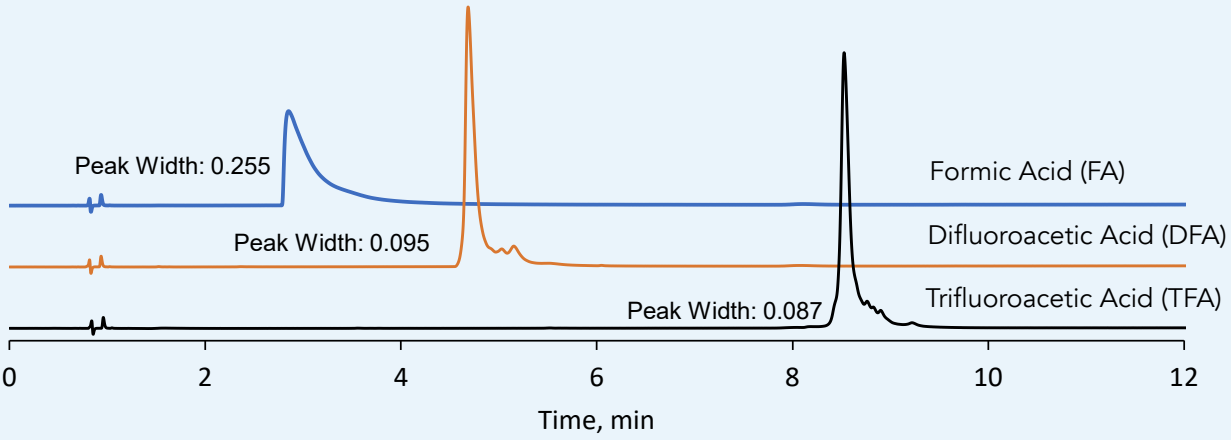


Figure 4. Mobile Phases for Improved mAb LC
Effect of FA, DFA, and TFA on Retention and Peak Shape



TEST CONDITIONS
Column: HALO 1000 Å C4, 2.7 µm, 2.1 x 150 mm
Gradient: 35-47.5% AcN/0.1% acid as indicated in 12 min
Flow Rate: 0.4 mL/min
Temperature: 80 °C
Sample: 2 µL of trastuzumab - 2 µg/µL (30/70 ACN/H2O)

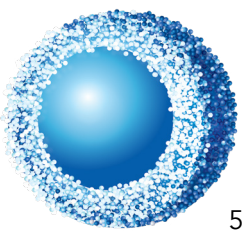
PEAK IDENTITY
trastuzumab

CONCLUSIONS

For LC-MS of peptides and proteins, a compromise must be made between sharp peak shape and adequate ionization efficiency. While TFA gives sharp peaks and good retention, the ionization efficiency is poor. With FA, the ionization efficiency is high, but the peak shapes are poor. The use of either FA/AF or DFA are good options for a balanced result between good peak shape and good ionization efficiency.

REFERENCE

[1] U.A. Mirza, B.T. Chait, Effects of anions on the positive ion electrospray ionization mass spectra of peptides and proteins, Anal. Chem., 66 (1994) 2898-2904.



Analyses of Large Proteins, Antibodies, and Modified Proteins Using A New Series of Advanced Large Pore HPLC Materials

Introduction

The list of FDA-registered and commercialized therapeutic proteins continues to expand rapidly, including more than 400 biotherapeutics, such as enzymes, monoclonal antibodies (mAbs), antibody-drug conjugates (ADCs), and hybrid antigen binding structures. Various human and veterinary diseases, such as cancers, autoimmune diseases, neurodegenerative diseases, and many others can be treated using these new molecules, employing a more effective targeted approach [1].

Protein biopharmaceuticals have higher molecular weights (MWs), are large in aqueous solution, with shapes that can be very complex having tertiary and quaternary structural elements. The polypeptide chains fold in specific ways, with highly hydrated charged amino acid side chains oriented towards the surface containing domain structures that are stabilized by specific and cumulative ion pairs and hydrophobic interactions. Many of these proteins are composed of more than one polypeptide chain, associated by both covalent and non-covalent bonds. In many cases, the proteins are based on molecules from the human or animal immune systems, and often their MWs usually exceed 100 kilodaltons (kDa). For example, the MWs of antibodies, including therapeutic mAbs, range between 140 and 150 kDa (Figure 1). The volumes of these molecules in solution are very large, compared to small molecule pharmaceuticals, peptides, or even small to medium MW polypeptides and proteins.

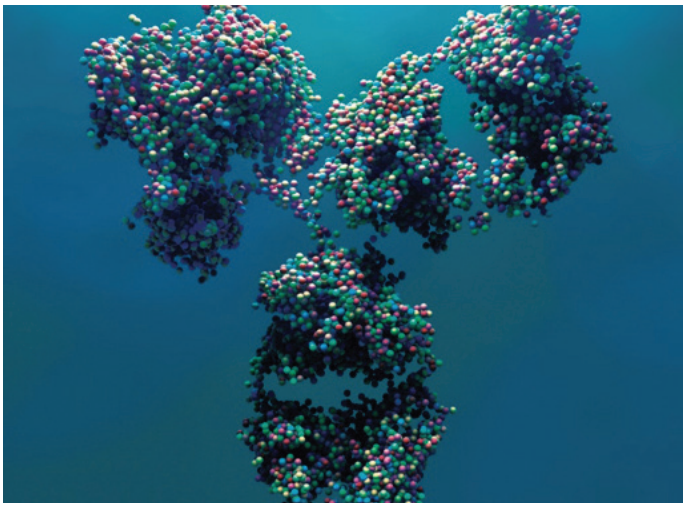


Figure 1. Representative Therapeutic Monoclonal Antibody Structure

In addition to their high MW and hydrated volumes, large biotherapeutic proteins have additional structural complexities due to inherent or induced heterogeneity. For example, many biotherapeutics are glycoproteins, often with a mixture of neutral and acidic glycan structures at one or more specific sites of the protein structures. As mentioned above, proteins may be composed of multiple protein chains, and some are covalently linked by specific interchain or intrachain disulfide linkages, or may have a mixture of fully bridged disulfide and free sulfhydryl groups. Moreover, the polypeptide chains themselves may have heterogeneous lengths, with terminus variations of one or two amino acids (ragged ends). In many therapeutic proteins these various sources of heterogeneity may coexist to a varying degree, as the biological and production process variables that lead to these heterogeneities are not all under complete control during the preparation of a useful therapeutic biopharmaceutical.

Characterization of Biopharmaceutical Proteins

The complexity of these biopharmaceuticals, and the regulatory requirements for thorough characterization of novel entities, or of biosimilars, to ensure identity, purity and quality, are substantial compared to those of small molecule drugs. Among the important analytical techniques that are applied for characterization of biotherapeutics such as mAbs, are various modes of liquid chromatography (LC) for separations of intact mAbs, their fragments, their enzymatic digests to yield smaller peptide fragments, and their associated glycans. A key analytical approach for protein characterization remains reversed-phase HPLC (RPLC), due to the unique and informative capabilities of this method.

Reversed-Phase HPLC as a Preferred Method for Protein Analysis

For several decades, RPLC has been the dominant method for pharmaceutical and small molecule analyses, and it is employed at every stage of drug discovery and development, manufacturing and quality assurance processes. Since the early 1990s, a central role for RPLC for the analysis of proteins has emerged—initially with protein fragments (chemical and enzymatic digests), protein subunits, and, more recently, for intact protein analyses.

This growing importance and application of RPLC for protein analyses coincides with the development of increasingly useful and appropriately designed chromatographic materials. Methods for their use include the analysis of protein enzymatic digests, intact polypeptides and intact proteins, and the various derivatives of all of the above.

The increased role of RPLC has also been accelerated by the development of improved LC instrumentation (hardware and software), and the advancements in high speed detectors, most notably, high speed and high resolution mass spectrometers (MS). Most advanced MS platforms are capable of being efficiently interfaced with HPLC and UHPLC instruments via electrospray ionization (ESI). Unlike some HPLC modes, RPLC is easily accommodated by MS detection, and RPLC-MS can be used at scaled flow rates ranging from nanoliter/min to mL/min accommodating nano (< 300 µm ID), capillary (300 µm –1 mm ID) and analytical (1 – 4.6 mm ID) columns.

The relative simplicity of RPLC and the widespread availability of automated, computer-controlled instrumentation and qualified software have made the technique indispensable for protein identification and quantitation. It was shown in the 1980s that RPLC of even moderate size proteins (ca. 15 – 20 kDa), because of their molecular size, required larger-pore-size column packing materials [2], and careful selection of mobile phases and analysis conditions that maintain protein solubility and enhance recovery. Most analytical RPLC protein separations use acidic mobile phase additives (e.g., formic or trifluoroacetic acid), elevated column temperatures (40 – 90 °C), and an organic modifier-water gradient using acetonitrile or acetonitrile mixed with a short chain aliphatic alcohol (propanol, butanol or isopropanol) [3]. An example RPLC separation of a mixture of intact proteins, separated using a typical acetonitrile/ water gradient, with UV absorbance detection at 215 nm, is shown in Figure 2.

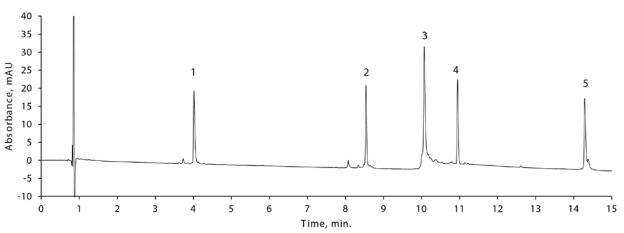


Figure 2. Gradient RPLC Separation of a Mixture of Intact Proteins

TESTING CONDITIONS:

Column: HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Mobile phase A: Water (0.1% TFA)
Mobile phase B: 80/20 ACN/Water/0.085% TFA
Gradient: 27–60 %B in 15 min
Flow rate: 0.4 mL/min
Temperature: 60 °C
Injection volume: 2 µL
Instrument: Shimadzu Nexera XR
Detection: UV at 280 nm
Sample: (1) ribonuclease A; (2) lysozyme; (3) SigmaMAb; (4) α-lactalbumin; (5) enolase

Until very recently, researchers have used column technology for RPLC of intact mAbs, ADCs and their resulting fragments which has been fundamentally unchanged since the mid-1990s. Typically, such packing materials have consisted of 200 – 300 Å pore size, silica-based, fully porous particle (FPP) bonded phases. However, new, very wide, 1000 Å pore size superficially porous packings for RPLC of very large molecules have been developed to improve the resolution for, and the information gained from, higher resolution chromatographic separations of protein mixtures.

HALO® 1000 Å Protein Column Series

The HALO 1000 Å Protein columns are innovative products that are built upon the success and performance of the family of small particle diameter

COLUMN NAME	PORE SIZE (Å)	PARTICLE SIZE(S) (um)	SURFACE AREA (m^2/g)	STATIONARY PHASES	TARGET ANALYTES
HALO Glycan	90	2.7	135	Proprietary	Glycans, glycopeptides, glycoproteins < 20 kDa
HALO Peptide	160	2,2.7,5	65, 90, 60	ES-C18, ES-CN, Phenyl-Hexyl	Peptides and polypeptides 100 Da < MW < 15 kDa
HALO Protein	400	3.4	15	C4, ES-C18	Peptides, polypeptides, and proteins 2 kDa < MW < 500 kDa
HALO Protein	1000	2.7	22	C4, ES-C18, Diphenyl	Large proteins, mAbs, mAb fragments, and ADCs > 50 kDa

Table 1. HALO Fused-Core Column Family: Pore Size Designated for Target Analytes

Fused-Core® superficially porous particle (SPP) HALO columns (Table 1), a particle type pioneered by Advanced Materials Technology. The objective in the design of the various HALO columns has always been to develop particles whose pore size had been chosen carefully to balance retention and pore access for the targeted analyte sizes.

The performance improvement afforded by HALO 1000 Å, 2.7 µm particles (Figure 3) for RPLC analyses of large biotherapeutics, compared to commercially available, smaller pore size columns, is attributable to these main advantages:

- Larger pore size allows completely unrestricted access of biomolecules to the interior domain of particles, and movement within the volume element adjacent to the particle surface and associated bonded phase.

- Superficially porous layer provides a shorter diffusion path (even vs. fully porous sub-2-µm particles) for larger biomolecules having much lower diffusion coefficients, while maintaining sufficient surface area for necessary loading capacity, resulting in reduced peak broadening and high resolution of minor components (impurities and structural variants).

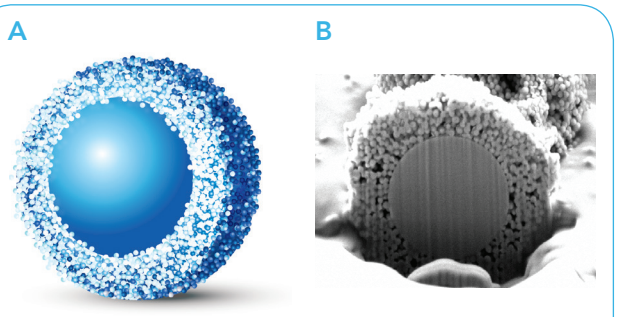


Figure 3. (A) Schematic drawing of a 1000 Å superficially porous 2.7 µm particle; (B) Focused-ion-beam-sliced SEM image of a 1000 Å SPP showing the 1.7 µm core with 0.5µm shell

Unrestricted Pore Access

Unrestricted pore access by large biomolecules to the large 1000 Å pores produces narrow peaks, which allows high resolution separations of protein and mAb variants, in addition to improved sample load tolerance before band broadening. For existing columns with smaller pore sizes, molecular exclusion and restricted diffusion of large molecules occurs and produces broader peaks, poorer loading behavior, and less resolution. While the minimum pore size required to fully accommodate very large biomolecules is determined by many factors, consideration of the effects of diffusion into an open cylindrical channel suggests [4, 5] that particle pore size should be on the order of 10 times the effective hydrodynamic diameter of an analyte for optimal chromatographic performance.

The HALO 1000 Å pore size distribution permits free access for mAbs and larger proteins to the stationary phase available within the porous shell structure.

Short Diffusion Path

Another contributing factor to the superior performance of the HALO 1000 Å particle is its porous shell morphology, which it shares with the other Fused-Core® product series (see Table 1 and Brief History Section). The unique particle design of SPPs includes a solid silica core surrounded by a porous shell of sub-micron thickness. This thin shell does not require that the slowly-diffusing biomolecules traverse the entire radius of the particle as it does for FPPs. This reduced diffusion path confers improved mass transfer and sharper peaks, and permits faster separations. For a review on SPPs, see Hayes, et al. [6], and for descriptions of superficially porous particles and their advantages for larger protein separations, see Kirkland, et al. [7].

Sample Loading

The very wide pores and short diffusion distances not only provide narrower peaks and improved resolution for large biotherapeutic molecules, but they also enable greater sample load capacity and tolerance. What this means is that a larger amount (mass) of sample can be injected, with less peak broadening, so that minor impurities such as subtle variants of mAbs and ADCs can be detected and quantified.

The ability of the HALO 1000 Å Protein SPP columns to tolerate increasing load for protein separations have been investigated using various preparations. The effects of sample load for a highly purified IgG1 mAb peak width are shown in Figure 4 both for a HALO 1000 Å C4 column and a 300 Å FPP C4 column (2.1 x 150 mm sizes) using trastuzumab as a model. These results show that, for all load levels, the HALO 1000 Å C4 column afforded smaller peak widths than those for the 300 Å FPP C4 column.

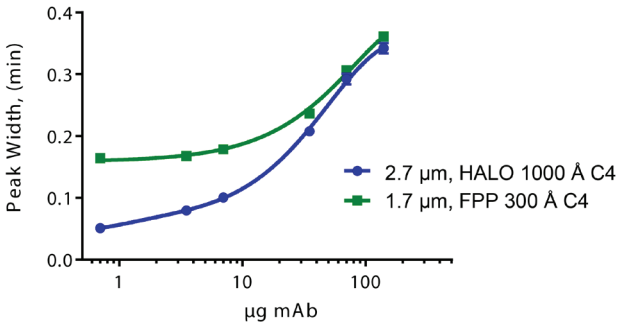


Figure 4. Effect of Sample Mass on Peak Width

TESTING CONDITIONS:

Column: 2.1 x 150 mm
Mobile phase A: Water (0.1% DFA)
Mobile phase B: ACN (0.1% DFA)
Gradient: 27–37% B in 10 min
Flow rate: 0.5 mL/min
Temperature: 80 °C
Sample: trastuzumab
Injection volume: 0.1, 0.5, 1, 5, 10, and 20 µL of 7 mg/mL mAb in Water
Instrument: Shimadzu Nexera
Detection: UV at 280 nm with 350 nm reference wavelength

Note: Peak widths measured at 50% height

These results are counterintuitive, when one considers the actual surface areas of these two silica column packing materials. The surface area (nitrogen adsorption, surface BET analysis) of the 300 Å FPPs is about 90 m2/g compared to about 20 m2/g for the 1000 Å SPPs. The surface area of the latter is about 4.5-fold smaller, yet the sample loading capacity (inversely correlated with peak width) is much better for the 1000 Å SPPs at lower sample loads, and comparable to the FPPs at high loads. These findings suggest that in the example of trastuzumab, it has much greater access to the bonded phase surface of the 1000 Å SPPs, compared to the 300 Å FPPs, which have higher absolute surface area.

The HALO® 1000 Å Protein Column Bonded Phase Series

The traditional bonded phase for RPLC separations of proteins has been the short chain alkyl bonded phase, typically based on a form of butyl-silane (C4). This material works well, and has repeatedly shown high performance separations of proteins using default gradient RP conditions. To enhance the utility of 1000 Å wide pore bonded phases for protein development, Advanced Materials Technology has recently introduced specially-selected versions of the octyldecyl-silane (ES-C18) and diphenyl-silane (Diphenyl) surface modified bonded phases (surface structures shown in Figure 5).

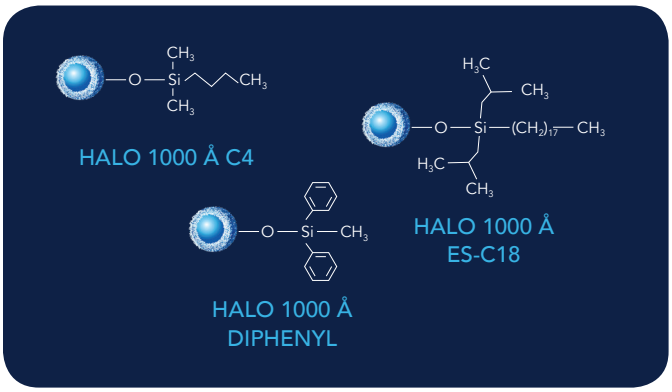


Figure 5. HALO 1000 Å Bonded Phase Structures

The three bonded phase RPLC materials selected for the HALO 1000 Å Protein series was derived from the comparison of many materials, with specific reference to stability for applications of low pH and elevated temperature that are often required to obtain high recovery of proteins in RPLC separations. All three materials exhibit acceptable column lifetimes when operated under such conditions, and an example of the HALO 1000 Å Diphenyl stability, when challenged with highly aggressive low pH (<2), elevated temperature conditions (90°C) is shown in Figure 6.

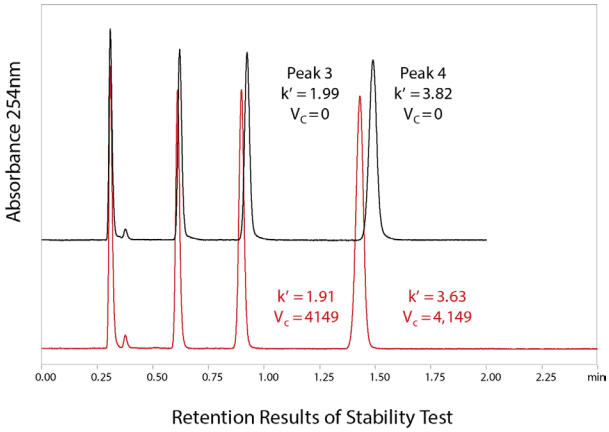


Figure 6. High Temperature and Low pH Stability

TESTING CONDITIONS:

Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 50 mm
Mobile phase A: Water
Mobile phase B: ACN
Isocratic: 55/45 A/B
Flow rate: 0.4 mL/min
Temperature: 25 °C
Injection volume: 0.2 µL
Instrument: Shimadzu Nexera
Detection: UV at 254 nm
Sample: (1) uracil; (2) hexanophenone; (3) octanophenone; (4) decanophenone

Black trace shows the initial results and red trace shows the column performance after 4000 column volumes.

Examples of HALO® 1000 Å Protein Column RPLC Performance

HALO® 1000 Å SPP C4 vs. 300 Å FPP C4

RPLC separations of intact denosumab (IgG2 type) are compared for HALO 1000 Å SPP and 300 Å FPP columns in Figure 7. For the HALO 1000 Å C4 column separation, the denosumab peak widths are not only narrower compared to that for the 300 Å C4 column, but there is also much better resolution of the IgG2 disulfide bridge isoform variants. The narrower peak widths using HALO 1000 Å have also been observed and previously described using IgG1 type mAbs [8].

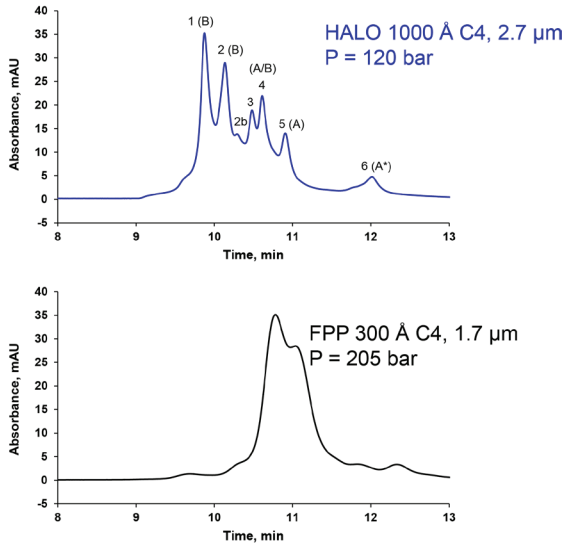


Figure 7. Intact Denosumab Separation Using 1000 Å SPPs and 300 Å FPPs

TESTING CONDITIONS:

Columns: 2.1 x 150 mm
Flow rate: 0.2 mL/min
Mobile Phase A: 88/10/2 H2O/ACN/n-Propanol + 0.1% DFA
Mobile Phase B: 70/20/10 n-Propanol/ACN/H2O + 0.1% DFA
Gradient: 14–24% B in 20 min
Injection Volume: 2 µL of 2 mg/mL denosumab in Water + 0.1% DFA
Temperature: 80 °C
Detection: PDA at 280 nm

These observations, along with the sample loading results shown in Figure 4 demonstrate that large molecules have much greater pore access for bonded phase interactions using the HALO 1000 Å SPP column, compared to the 300 Å FPP column.

RPLC analyses of four additional mAbs were carried out using both columns. The peak widths of these four mAbs (in addition to trastuzumab) were, on average, 69% narrower using the HALO 1000 Å C4 column compared to those obtained using the 300 Å FPP C4 column (Figure 8).

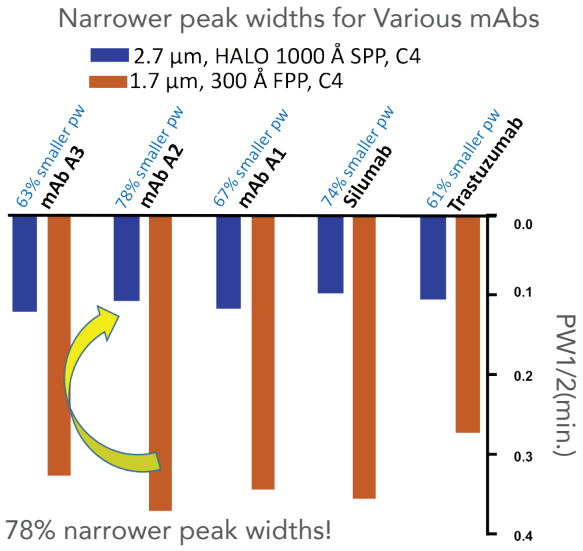


Figure 8. Narrower Peak Widths for Various mAbs. This bar graph compares the average peak widths for five different mAbs using a HALO 1000 Å C4 column and a 300 Å FPP C4 column.

TESTING CONDITIONS:

Columns: 2.1 x 150 mm
Mobile phase A: Water (0.1% DFA)
Mobile phase B: ACN (0.1% DFA)
Gradient: 27–37% B in 20 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Injection volume: 2 µL (1 µg each in 0.1% TFA)
Instrument: Shimadzu Nexera
Detection: UV at 280 nm with 350 nm reference wavelength
Samples: As Indicated

Note: Peak widths measured at 50% height

HALO® 1000 Å C4 SPP vs. 1500 Å Polymeric RP FPP

RPLC separations of intact trastuzumab were also compared using the HALO 1000 Å, 2.7 µm C4 column and a 1500 Å, 4 µm, polymeric FPP column (Figure 9). The trastuzumab peak was about 24% narrower using the 1000 Å C4 column, compared to the larger pore size polymeric column. The trastuzumab peak may be sharper due to the combination of smaller particle size and the thin shell of the 1000 Å SPP column (2.7 µm with 0.5 µm shell) compared to the 4 µm particle size of the 1500 Å fully porous polymeric particles. Resolution of the minor trastuzumab variants was much better using the 1000 Å C4 SPP column, which is expected, as generally the case with silica particles, to exhibit high pressure and mechanical shock resilience, in comparison to polymeric particles.

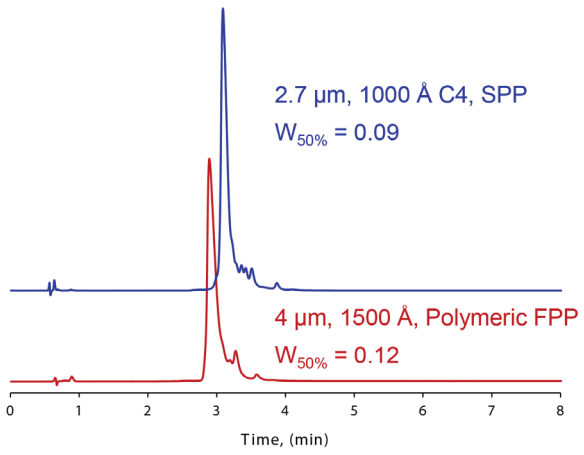


Figure 9. Intact Trastuzumab RPLC Separations Using 1000 Å C4 SPP and 1500 Å FPP Columns

TESTING CONDITIONS:

Columns: 1000 Å SPP and 1500 Å FPP columns 2.1 x 100mm
Mobile phase A: Water (0.1% TFA)
Mobile phase B: 80/20 ACN/Water (0.085% TFA)
Gradient: 40–47.5% B in 8 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Sample: trastuzumab
Injection volume: 2 µL of 2 mg/mL in Water
Instrument: Shimadzu Nexera
Detection: UV at 280 nm with 350 nm reference wavelength

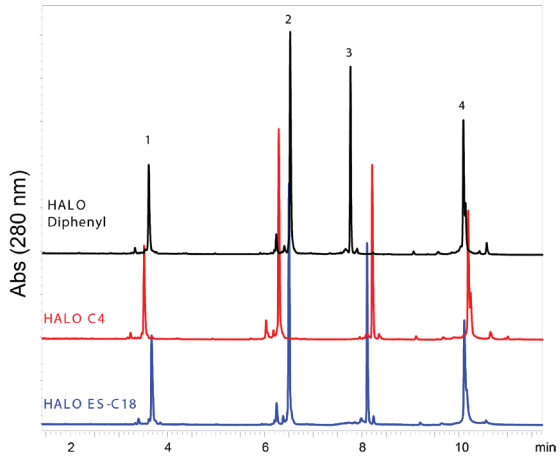


Figure 10. Comparison of Three HALO 1000 Å Bonded Phases Using a Protein Mix

TESTING CONDITIONS:

Columns: HALO 1000 Å, phase as indicated, 2.1 x 150 mm, 2.7 µm
Mobile phase A: Water/0.1% TFA
Mobile phase B: ACN/0.1% TFA
Gradient: 20–60 %B in 15 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Injection volume: 2 µL
Instrument: Shimadzu Nexera
Detection: UV at 280 nm
Sample: (1) Ribonuclease A; (2) lysozyme; (3) α-lactalbumin; (4) enolase

Use of HALO® 1000 Å Protein Bonded Phase Options for Protein Separations

The HALO 1000 Å Protein series is the only commercially available RPLC material that is a very wide pore SPP particle, and has available three highly stable and reproducible bonded-phases specific for resolving protein mixtures. The separation of four standard proteins is shown in Figure 10, using typical gradient RPLC conditions. Although the Diphenyl bonded phase is a weak RPLC retention material for small molecules, these results show that this bonded phase is comparable in retention to C4 and C18 for protein mixtures (for example comparing retention of peaks 1 and 4 for each column). In addition, this figure also shows that the three bonded phases exhibit subtle selectivity differences, appreciated when examining the relative elution positions for peaks 2 and 3, across the different bonded phase surfaces. Each bonded phase exhibits a unique pattern of separation for this mixture. Such selectivity differences, although appearing modest, become highly relevant when employing gradient elution conditions that are less aggressive, for example as is employed for protein variant analysis for a highly purified protein.

In Figure 11, a high resolution separation of the biotherapeutic mAb trastuzumab is shown. In this example, the materials of interest are the small peaks that elute after the main, correctly formed and fully disulfide bridged intact mAb structure. Following this main peak, a collection of smaller peaks is variably resolved by each of the specific bonded phases. The selectivity differences between these columns show up as a complex collection peaks for the many small protein variant structures. Subsequent studies have revealed that these later eluting peaks are subtle structural variants of trastuzumab, predominantly variants with a single reduced disulfide bridge, leading to pairs of free thiol variants. Similar disulfide bridge variants have been shown in previous studies of other IgG1 and IgG2 mAbs [10,11].

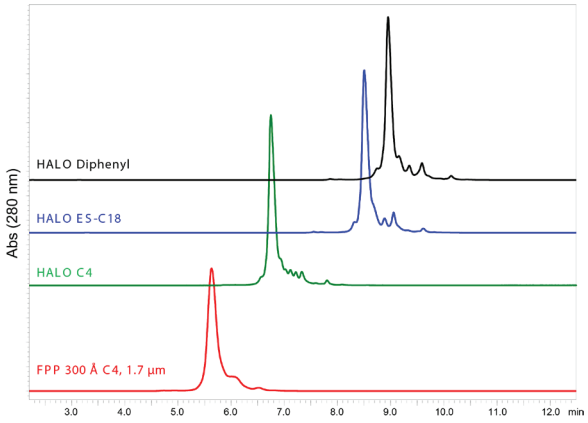


Figure 11. Comparison of Three HALO 1000 Å 2.7 µm Bonded Phases to a 300 Å 1.7 µm FPP C4 Column Using Trastuzumab

TESTING CONDITIONS:

Columns: as indicated, 2.1 x 150 mm
Mobile phase A: Water/0.1% TFA
Mobile phase B: ACN/0.1% TFA
Gradient: 32–40 %B in 16 min
Flow rate: 0.4 mL/min
Temperature: 80 °C
Injection volume: 2 µL
Instrument: Shimadzu Nexera
Detection: UV at 280 nm

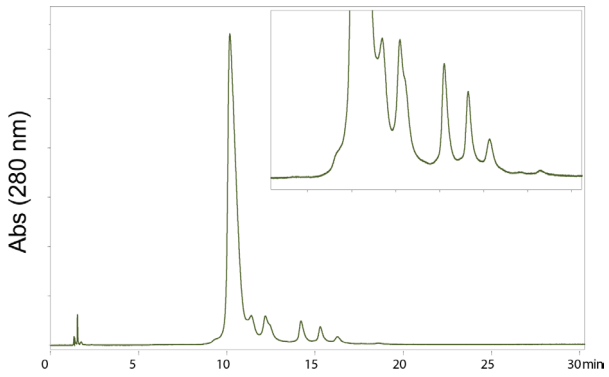


Figure 12. Example of Optimized Trastuzumab Method Development Using a HALO 1000 Å Diphenyl Column

TESTING CONDITIONS:

Mobile phase A: Water (0.1% DFA)
Mobile phase B: 50/50 ACN/n-propanol/0.1% DFA
Gradient: 29–33 %B in 29 min
Flow rate: 0.25 mL/min
Temperature: 60 °C
Injection volume: 2 µL of 2mg/mL trastuzumab in water/0.1% TFA
Instrument: Shimadzu Nexera
Detection: UV at 280 nm

The subtle selectivity difference in the HALO 1000 Å Protein bonded phases can be employed with gradient selectivity optimization and mobile phase modification as independent variables. As an example, Figure 12 shows the high resolution results that can be obtained with bonded phase, temperature and mobile phase optimization to yield a highly informative separation of the main peak and at least 7 variant protein structures present in this biotherapeutic mAb. The separation using this highly efficient HALO 1000 Å Protein Diphenyl column is completed in less than 30 minutes, while being compatible with both UV detection, as well as online high resolution MS detection.

List of Abbreviations:

ADC: antibody-drug conjugate **BET:** Brunauer, Emmett and Teller (method for calculating surface area) **DFA:** difluoroacetic acid **FPP:** fully porous particle **IgG:** immunoglobulin **kDa:** kilodaltons **mAb:** monoclonal antibody **SEM:** scanning electron microscope **SPP:** superficially porous particle **TFA:** trifluoroacetic acid

Conclusions

As pharmaceutical companies have shifted their development focus to large-molecule biotherapeutics, the ability to separate intact monoclonal antibodies and antibody-drug conjugates for characterization purposes has become extremely important. To enable this characterization work, new HPLC particle technology has been required. The large 1000 Å pore size of the superficially porous particles used for RPLC described herein enables full access to the bonded phase surface for these larger biomolecules. This improved access to the bonded surface produces narrower peak widths and enhanced resolution of minor mAb variants, and can lead to increased retention under most analysis conditions. Together with new mass spectrometric instrumentation and software, wide-pore superficially porous particle HPLC columns will greatly aid in the advancement of large-molecule biopharmaceutical characterization and development. The recent expansion of the very large pore superficially porous particle family to include several bonded phases (C4, C18, Diphenyl) permits very high resolution separations of lower abundance protein variants, permitting structure analysis and quantification of these variants.

Authors

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A Brief History of Superficially Porous Particles for HPLC and UHPLC

- Superficially porous particles (SPPs) were originally developed during the 1960s, but enjoyed a new renaissance in the mid-2000s with the commercialization of HALO® Fused-Core® technology by Advanced Materials Technology. This revolutionary HALO 90 Å, 2.7 µm SPP column, first introduced in 2006, rivals the speed, resolution and performance of sub-2-µm columns, which had been introduced in 2003-2004, for small molecule analyses. This new SPP technology has rapidly changed the HPLC materials landscape, and has become very popular in the last ten years.

- These particles consist of a non-porous silica core, surrounded by a porous shell (Figure 3A). The interest in and popularity of these particles is due to the very high column efficiencies at modest back pressures [9]. This superficially porous particle morphology has been so successful that it has been adopted and commercialized by a number of other column manufacturers.

- It is well known that selection of the correct pore size is important to allow unhindered analyte access to the silica surface. Pore access is not a consideration for all but the largest pharmaceuticals (e.g., macrocyclic antibiotics such as tylosin, etc.). However, for peptides, proteins and larger biopharmaceuticals, larger pore size is a critical factor in achieving high efficiencies and narrow peak widths.

- Building upon the success of the 90 Å HALO particles, 160 Å pore size HALO Peptide particles, designed for fast and high resolution separations of peptides and small polypeptides, were commercialized in 2011 [12]. HALO Peptide particles are bonded with sterically-protected silanes to promote excellent stability at the low pH, high temperature conditions often used for peptide mapping and other peptide analyses.

- Subsequently, in 2013 a new 400 Å, 3.4 µm HALO particle was introduced with C4 and sterically-protected C18 bonded phases to provide high efficiency gradient separations of larger polypeptides and proteins.

- The HALO 1000 Å, 2.7 µm particle is the newest addition to the HALO BioClass series of columns, and was designed to deliver superior performance for monoclonal antibodies, their fragments, and antibody-drug conjugates.

- Advanced Materials Technology is the innovator in this area of superficially porous packing materials, being first to offer very wide pore SPP materials, and is the only company that offers the choice of 400 Å and 1000 Å pore particle materials.

- The HALO 1000 Å Protein series has been extended to include the C4, ES-C18 and Diphenyl bonded phases, allowing manipulation of separation selectivity, while assuring highly stable and reproducible columns for high resolution separations.

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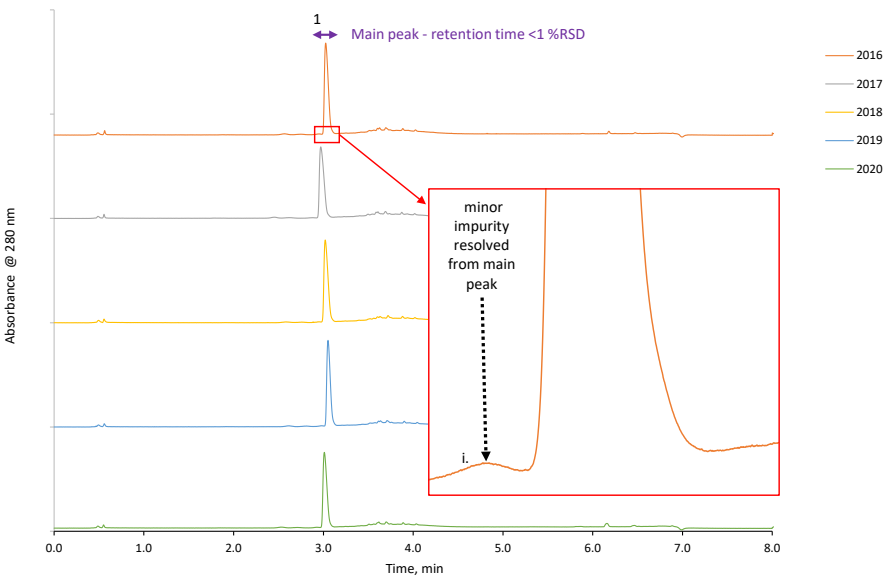
BIOPHARMACEUTICALS

Peptide Analysis of Bee Venom Assay for Antimicrobial Properties Using HALO® Peptide

251



PEAK IDENTITIES:

1. Melittin
i. Impurity of a honey bee venom standard

TEST CONDITIONS:

Column: HALO 160 Å C18, 2.7 µm, 2.1x150mm
Part Number: 92122-702
Mobile Phase: **A:** Water/0.1% TFA
B: ACN/0.1% TFA

Gradient:

Time	%B
0.0	40
2.0	40
6.0	100
6.1	100
6.2	40
7.0	40

Flow Rate: 0.6 mL/min
Pressure: 408 bar
Temperature: 60 °C
Detection: 280 nm
Injection Volume: 1 µL
Sample Solvent: Water/ 0.1% TFA
Data Rate: 100 Hz
Response Time: 0.025 sec
Flow Cell: 1 µL
LC System: Shimadzu Nexera

Antimicrobial peptides in animal venom (vAMPs) are natural antibiotics of emerging interest. As resistance over conventional antibiotics has become an area of concern, vAMPs are key alternative early drug discovery candidates. An assay of melittin from honey bee venom completed in <10 min (total analysis time) was demonstrated on five different manufactured HALO® 160 Å ES-C18 lots (2016, 2017, 2018, 2019, and 2020) illustrating the separation profile reproducibility over a five-year period.

The main active vAMP component in honey bee venom was resolved from minor related impurity peaks (unidentified) with a retention time reproducibility of <1% RSD. Furthermore, a closely related low abundant impurity peak could be separated. Critical aspects are achieved with HALO® column technology to develop reliable assays to support biomedical, and drug development research of vAMPs' physiological role in human diseases, as well as microbial and parasitic infections.

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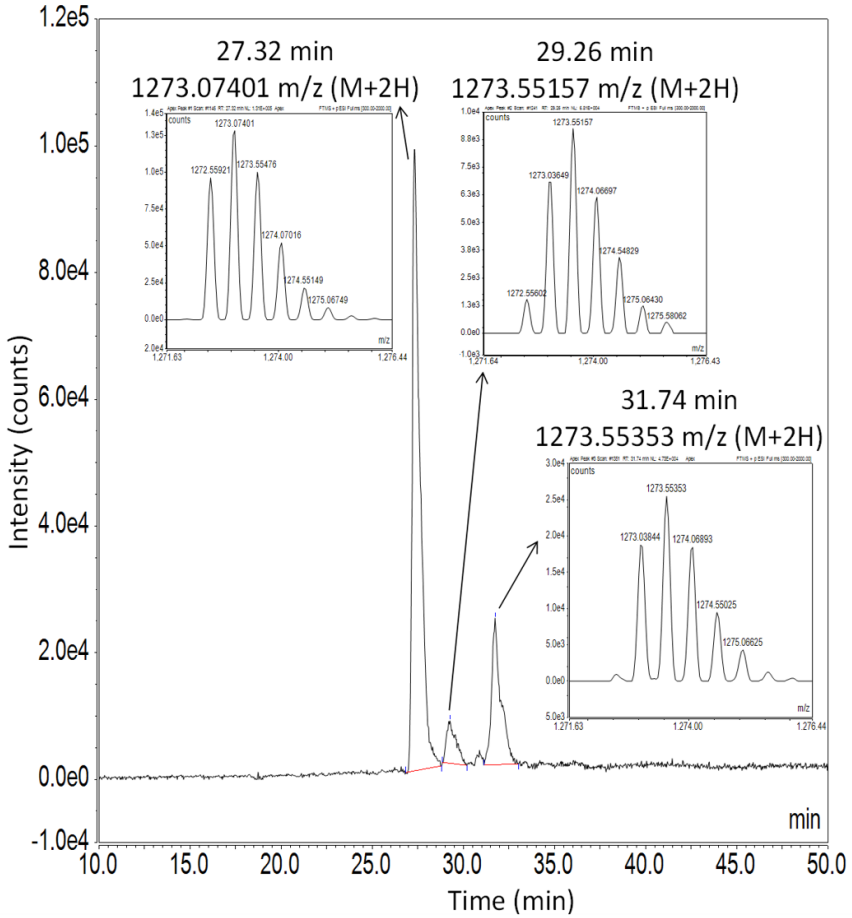
Capillary scale HILIC Separation of Deamidation Products of Trastuzumab

263-PE



PEAK IDENTITIES

Peptide fragments of GFYPSDIAVEWESNGQPENNYK

1. m/z= 1273.07401
2. m/z= 1273.55157
3. m/z= 1273.55353

The capillary HALO® Penta-HILIC column facilitated coupling of microflow LC conditions of 12 µL/min and a higher organic HILIC gradient separation. The column's high resolution capabilities resolved similar charged species required for examining peptide deamidation and isomerization products of Asn, Asp, and isoAsp forms of a peptide fragment of a trastuzumab tryptic digest.

TEST CONDITIONS:

Column: HALO 90 Å Penta-HILIC, 2.7 µm 0.5 x 150mm
Part Number: 98215-705
Mobile Phase A: 50 mM ammonium formate in water
Mobile Phase B: Acetonitrile/0.1% Formic acid
Gradient:

Time	%B
0.0	80
4.0	80
64.0	48

Flow Rate: 12 µL/min
Pressure: 123 bar
Temperature: 60 °C
Detection: ESI+
Injection Volume: 1 µL
Sample Solvent: 50 mM Tris-HCl /1.5M Guanidine-HCl, 0.5% formic acid
LC System: Thermo Ultimate 3000
MS System: Thermo Orbitrap Velos

MS CONDITIONS:

Spray Voltage (kV): 3.8
Capillary temperature: 300 °C
Sheath gas: 40
Aux gas: 10
RF lens: 50

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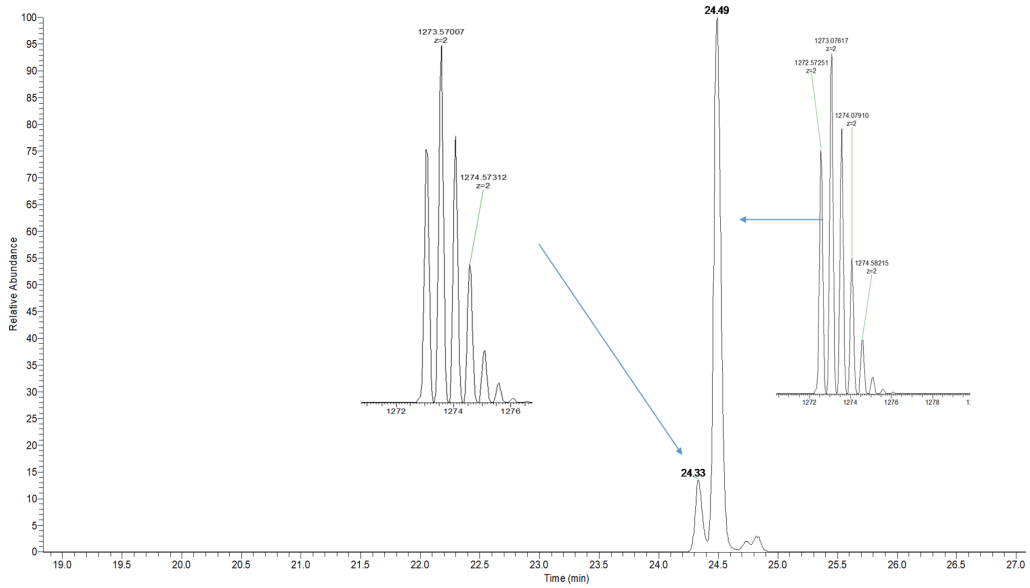
Separation of Deamidation Products of the NIST mAb on HALO® ES-C18

264-PE



PEAK IDENTITIES

Peptide fragments of GFYPSDIAVEW-ESNGQPENNYK

1. m/z= 1273.57007
2. m/z= 1273.07617

Deamidation is a reaction in which an amide functional group in the side chain of the amino acids asparagine or glutamine is removed or converted to another functional group. Deamidation products are of increasing importance in proteomics because they can alter a protein's structure, or possibly its function and stability, resulting in degradation. This is especially of interest in monoclonal antibody (mAb) development as well. The HALO® ES-C18 has the high resolution nessessary to separate the deamidation products of the NIST mAb.

TEST CONDITIONS:

Column: HALO 160 Å ES-C18, 2.7 µm 2.1 x 100mm
Part Number: 95814-902
Mobile Phase A: Water/0.1% Formic acid
Mobile Phase B: Acetonitrile/0.1% Formic acid
Gradient:

Time	%B
0.0	2.0
45.0	40
45.5	80
48.0	80
48.5	2.0
55.0	End

Flow Rate: 0.3 mL/min
Pressure: 124 bar
Temperature: 60 °C
Detection: ESI+
Injection Volume: 5 µL
Sample Solvent: 50 mM Tris-HCl /1.5M Guanidine-HCl, 0.5% formic acid
LC System: Shimadzu Nexera X2
MS System: Orbitrap Velos Pro

MS CONDITIONS:

Spray Voltage (kV): 4.0
Capillary temperature: 300 °C
Sheath gas: 40
Aux gas: 10
RF lens: 50

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