HALO®

BIOANALYSIS SOLUTIONS GUIDEBOOK


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GUIDEBOOK

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INTRODUCTION TO HALO® BIOCLASS SOLUTIONS FOR BIOANALYSIS

Pharmaceutical companies employ many orthogonal techniques and methodologies to better develop a complete picture of biopharmaceuticals which are inherently more complex than small molecules. The use of various separation technologies in the analysis of biological drugs is pervasive in new product development as well as release assays. While not all separations involve HPLC, most do and can benefit from the unique characteristics of superficially porous particle (SPP) columns.

Biomolecules are subject to multiple types of molecular transformations that can have an effect on their biological activity and structural confirmation. Adequate resolution of target biomolecules from their degradation products and impurities becomes paramount in protein and peptide analyses.

A clear advantage of SPP technology is in the ability to perform high efficiency separations in less time with reduced back pressures over fully porous particle columns. This is an important aspect when the desire is to completely characterize a protein, such as a peptide map which may contain 50 or more individual species. While fully porous particle columns could do an adequate job, the additional efficiencies gained from HALO® Fused-Core® columns provide more separation for more peak identification. An additional benefit is the ability to adapt a high efficiency assay that may be used in a characterization method to a fast, QC-like release assay where the separation of only a few critical peaks may be needed with the same column technology just with slightly modified conditions.

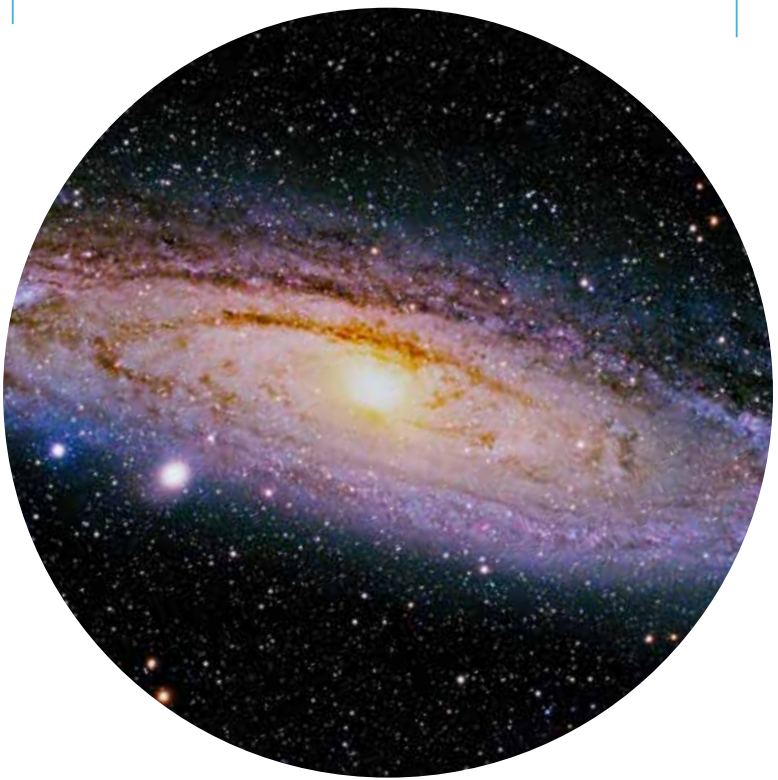
BIOANALYSIS

This guide will discuss improved reversed phase biomolecule separation solutions for monoclonal antibodies, as well as chromatography columns used to develop an understanding of the protein backbone via peptide mapping. Glycosylation analysis using HILIC will also be discussed. Examples will highlight areas in the workflow where HALO® Fused-Core® columns with appropriate pore sizes and phases offer unique advantages.

The bioanalysis landscape is expansive and is comprised from many elements. It may help to visualize this as a galaxy - filled with suns, planets and moons.

Proteins, which are the largest masses can be expressed as large (represented by a sun), mid-size (represented by Jupiter), and small proteins (represented by Saturn).

Peptide masses in the galaxy, would be represented by Earth followed by Glycan masses which relate to the moons in the solar system.



PROTEINS

PEPTIDES

GLYCANS

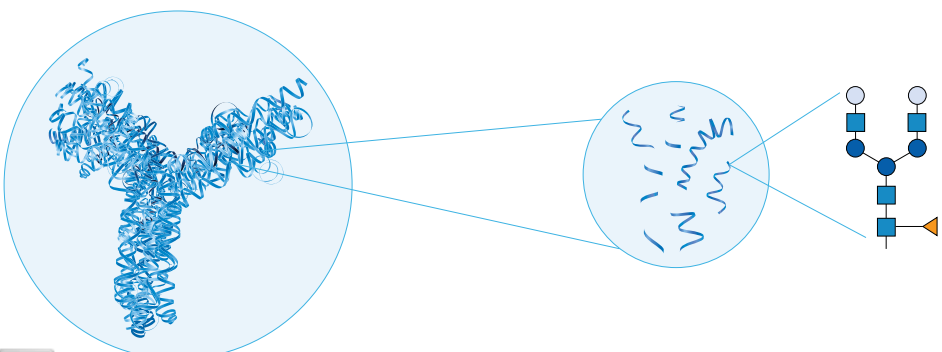
Very Large Protein
Ex: mAb
M.W.: >150 kDa

Mid-Size Protein
Ex: Erythropoietin
M.W.: 30.4 kDa

Small Protein
Ex: Myoglobin
M.W.: 17 kDa

Peptides
Ex: Beta-Endorphin
M.W.: 3.4 kDa

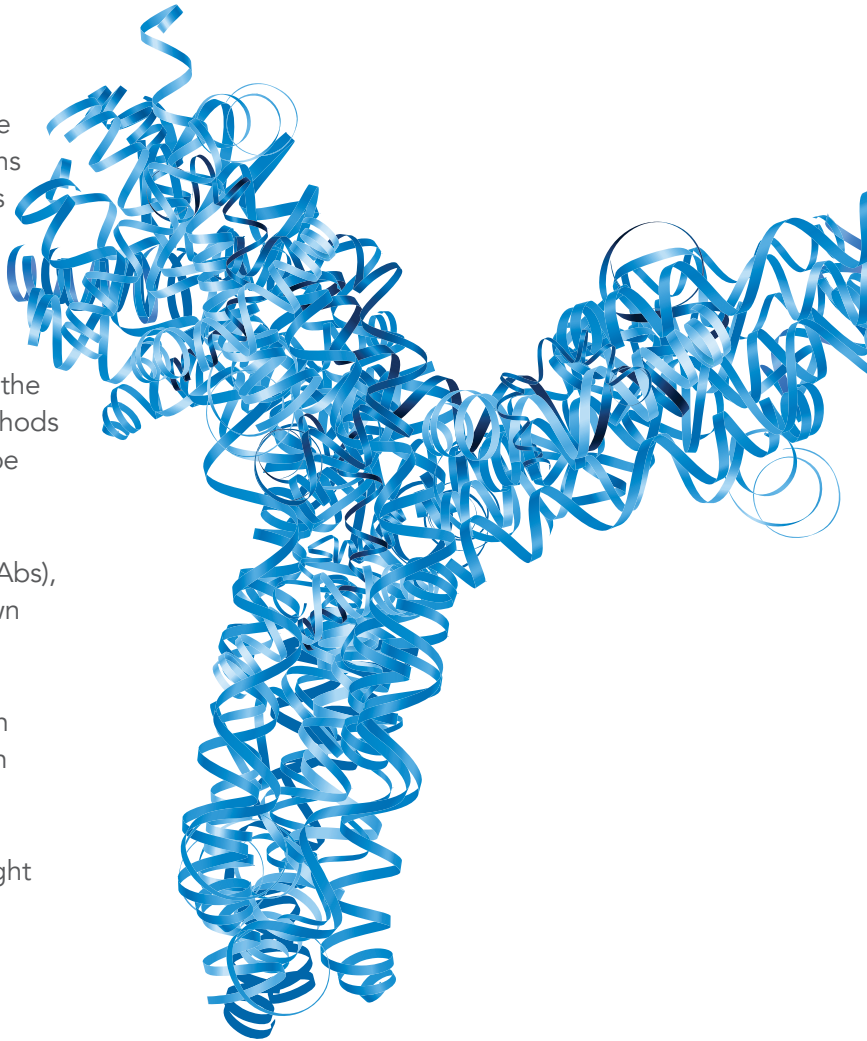
Glycans
Ex: Cellulose
M.W.: 162 Da



PROTEIN ANALYSIS

Proteomics can be broadly summarized as the qualitative and quantitative analysis of proteins that are located in a biological medium. Mass spectrometry, combined with separation techniques, such as HPLC, is imperative for a successful proteomic analysis. Two common proteomic methodologies target the analysis from both the peptide level (bottom-up) and the intact protein level (top-down), and both methods offer a multitude of challenges that need to be overcome.

For the analysis of monoclonal antibodies (mAbs), the techniques of middle-up and middle-down have recently been employed. Middle-up incorporates digestion of the mAb into large fragments/subunits (25 - 50 kDa) that are then analyzed by mass spectrometry. Middle-down starts with digestion of the mAb into large fragments, but then gas-phase dissociation is performed, which separates the heavy and light chains before MS/MS analysis. [1]



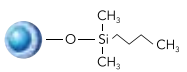
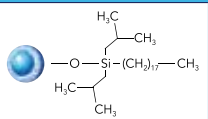
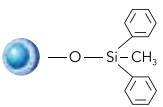
CHARACTERISTIC	PROPERTY MEASURED
Protein Backbone	Amino acid sequence Molecular Weight Amino acid composition Charge profile distribution
PTMs Glycosylation	Galactosylation Galactose-a-1,3 galatose Sialylation N-glycolylneurominic acid Core fucosylation High mannose structure Low abundance glycan species Aglycosylation
Protein Backbone Modifications	N-terminal variation C-terminal variation Deamidation Oxidation C-terminal amidation Glycation
Higher Order Structure	Protein Folding Disulfide connectivity Free cysteine Enthalpy of unfolding Tertiary structure Spectroscopic properties
Aggregation	Percent Monomer Aggregates Fragmentation Sub-visible particles Hydrodynamic radius
Formulation and Drug Product Properties	Protein extinction coefficient Protein concentration Solution properties Formulation Components Container Closure Components Process Impurities Leachables and Extractables
Stability Profile	Comparative stress stability Stress stability Long term stability studies
Host Derived Impurities	Host Cell Protein Host Cell DNA

For a protein to be targeted for a specific biotherapeutic function or application, such as a biosimilar or for mAb development, extensive analysis and characterization are required. The specified protein must be well characterized and a thorough understanding of the protein's structure, purity and impurities, biological activity, immunochemical and physicochemical properties, such as solubility, viscosity, and aggregation need to be established. Typical characteristics that need to be investigated are listed in the table.

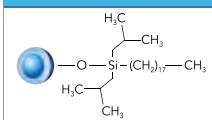
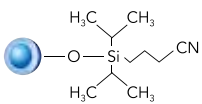
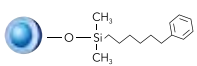
Table 1. Properties Measured for Physicochemical Characterization of Biological Drugs. Properties Listed in Red Utilize LC Separation Techniques.

HALO® BioClass from Advanced Materials Technology offers columns that fit the separation needs for the critical methods used in drug development and release. Whether intact mAb analysis, reduced and alkylated heavy and light chain fragment analysis, protein digests, or glycan analysis, HALO® BioClass has tailored particle solutions to meet the most challenging separation requirements.

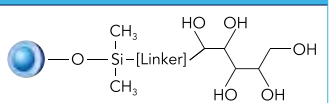
PROTEIN

C4	ES-C18	DIPHENYL
		

PEPTIDE

ES-C18	ES-CN	Phenyl-Hexyl
		

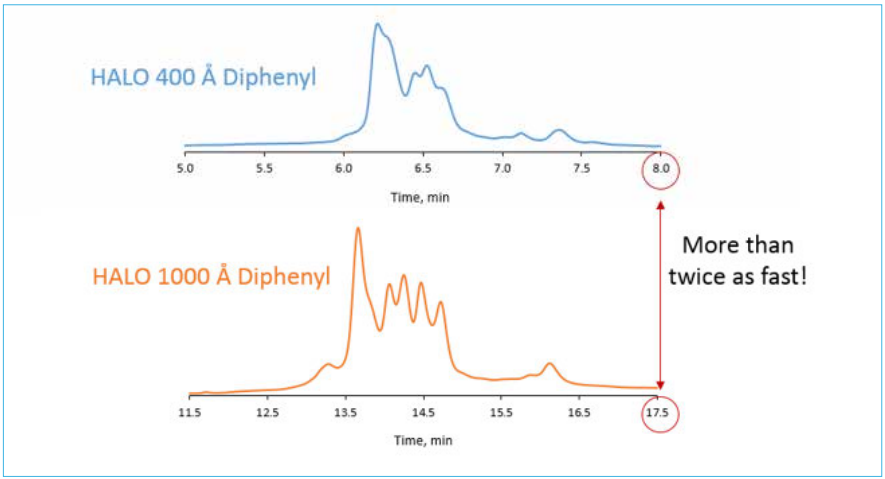
GLYCAN

Proprietary Poly-Hydroxy


Particle morphology plays an important part in efficient separations so optimizing surface area and volume is paramount. Shell thickness determines the diffusion path of the molecules, so it is imperative to have the correct size and particle distribution.

There are two particle designs for protein analysis: a 1000 Å 2.7 µm particle and a 400 Å 3.4 µm particle. The 1000 Å particle is used for the ultimate resolution of mAbs and other large proteins, while the 400 Å particle is designed for fast analysis of these compounds at lower back pressures.

Comparison of HALO 1000 Å vs. 400 Å Highlighting Speed and Resolution Solutions



TEST CONDITIONS:
Column: HALO 400 Å Diphenyl, 3.4 µm, 2.1 x 150 mm
Mobile Phase A: 88/10/2 Water/ Acetonitrile/ n-Propanol/ 0.1% DFA
Mobile Phase B: 70/20/10 n-Propanol/ Acetonitrile, Water/ 0.1% DFA
Gradient:

Time	%B
0.0	18
10.0	28

Flow rate: 0.4 mL/min
Temperature: 60 °C
Detector: UV 220 nm
Injection Volume: 2.0 µL

TEST CONDITIONS:
Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: 88/10/2 Water/ Acetonitrile/ n-Propanol/ 0.1% DFA
Mobile Phase B: 70/20/10 n-Propanol/ Acetonitrile, Water/ 0.1% DFA
Gradient:

Time	%B
0.0	18
20.0	28

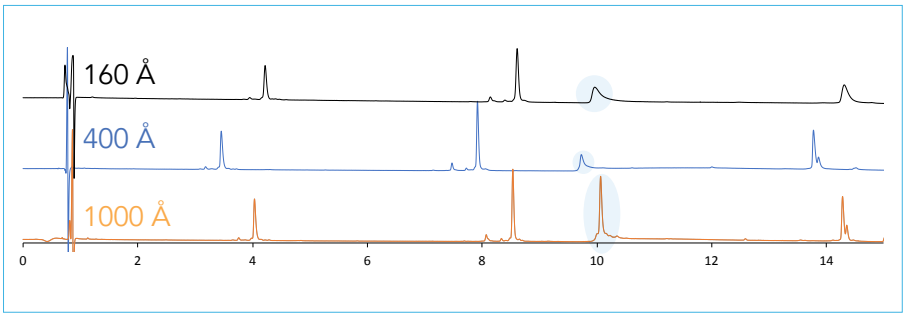
Flow rate: 0.2 mL/min
Temperature: 60 °C
Detector: UV 220 nm
Injection Volume: 2.0 µL

CONSIDERATION FOR PROTEIN ANALYSIS

Pore Size

Because biomolecules have a wide range of molecular weights, the pore size of the particle used in the separation is very important, and must fit the molecule size. High molecular weight biomolecules, such as proteins, require large pores to allow improved access to the stationary phase enabling increased resolution. If the pore size is too small, diffusion can be restricted, which limits the efficiency and load capacity. The figure shows the effect of pore size on the peak widths of proteins. Notice how much sharper both the SigmaMAb and enolase peaks become when analyzed with the 400 Å and 1000 Å pore HALO® particles, with the narrowest peak widths on the 1000 Å HALO.

Effect of Pore Size on Peak Width



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)

TEST CONDITIONS:
Column: HALO ES-C18, 2.1 x 150mm
Part Number: 92122-702 (160Å)
93412-702 (400Å)
92712-702 (1000Å)
Mobile Phase A: H_2O (0.1% TFA)
Mobile Phase B: 80/20 ACN/H_2O(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

To optimize the resolution of the separation during method development the appropriate conditions must be investigated and optimized. These include several parameters, including pH, modifiers, and temperature.

Optimal pH and Mobile Phase Modifiers

Important properties for good separation and peak shape include low pH and the need for a high level of volatility in the mobile phase which leads to effective desolvation in the MS. This is accomplished through mobile phase modifiers.

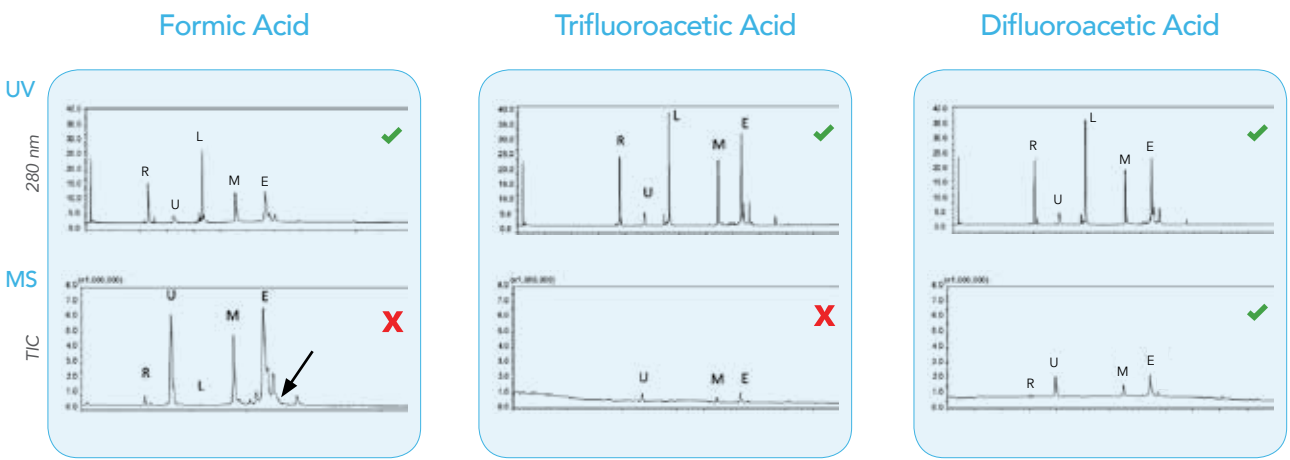
Formic, Trifluoroacetic, and Difluoroacetic Acids

Formic acid (FA) is a popular additive for increasing ionization efficiencies, however, for many large molecules, chromatography will suffer. Often, an increase in tailing, peak width, and poor recovery is observed because the pH is not low enough and FA has low buffering capacity which also often leads to unreproducible retention times.

Trifluoroacetic acid (TFA) is a popular additive when not using LC detectors which are not mass spectrometers, providing excellent peak shape and high recoveries. In MS however, TFA has limitations due to the ion pairing effect which can cause decreased ionization efficiencies due to partial charge masking of the analyte. Additionally, elimination of TFA from LC and MS instrument components needs attention to restore highest sensitivity operation. Flushing regimes usually consist of 50:50 ACN/H_20 with 1% acetic acid for 18-24 hours to remove traces of TFA.

Difluoroacetic acid (DFA), a less fluorinated ion pairing acidic mobile phase modifier provides MS sensitivity improvement relative to TFA [2], particularly with small to mid size molecules. DFA has the practical advantage of similar chromatographic benefits of TFA, with ready elimination from instrument components. The typical DFA wash regime is a 50/50 ACN/$H_2$0 flush for 10-15 minutes.

The figure shows a comparison of the peak shapes of five different proteins using both UV and MS detection. DFA offers the best compromise of symmetrical peak shape and good ionization efficiency.

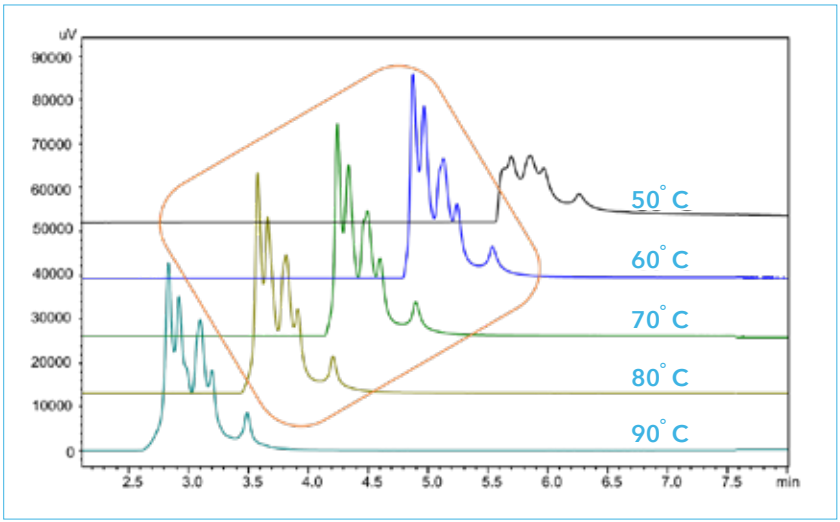


R – Ribonuclease A, U – Ubiquitin, L – Lysozyme, M – apo-Myoglobin, E – Enolase

Temperature

As part of any method development, it is recommended to perform temperature studies for determining the ideal separation conditions. Intact mass separations are optimal with proteins unfolded and denatured so the use of elevated temperature is often required for the best peak shape.

Increased Temperature Increases Speed and Resolution



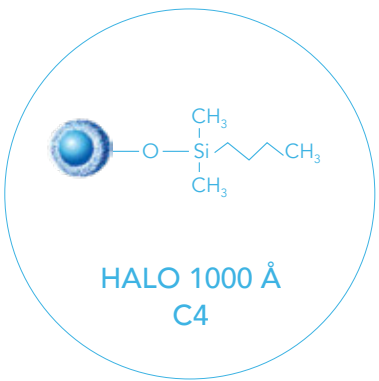
Denosumab with n-propanol in the mobile phase run at different temperatures on HALO 1000 Å C4

TEST CONDITIONS

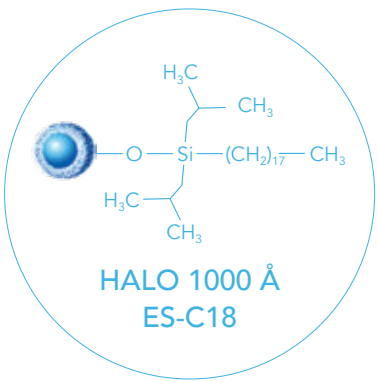
Column: HALO 1000 Å C4, 2.1 x 150 mm
Mobile Phase A: 88/10/2 water/ACN/nProp/0.1% TFA
Mobile Phase B: 70/20/10 nProp/ACN/H_2O/0.1% TFA
Gradient: 18–30% B in 8 min
Injection Volume: 0.4 mL/min

Why is it Necessary to Screen Multiple Phases?

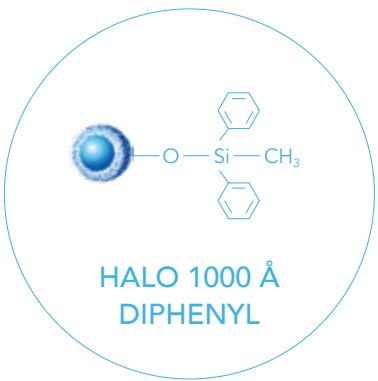
HALO® offers three different stationary phases for protein analysis: C4, ES-C18, and Diphenyl. When mAbs or biosimilars are being developed, there are often slight variations which benefit from selectivity differences. This is why it is recommended to screen multiple stationary phases to be assured of optimum separations. The phases offer selectivity options for the separations of highly similar mAbs.



HALO 1000 Å
C4

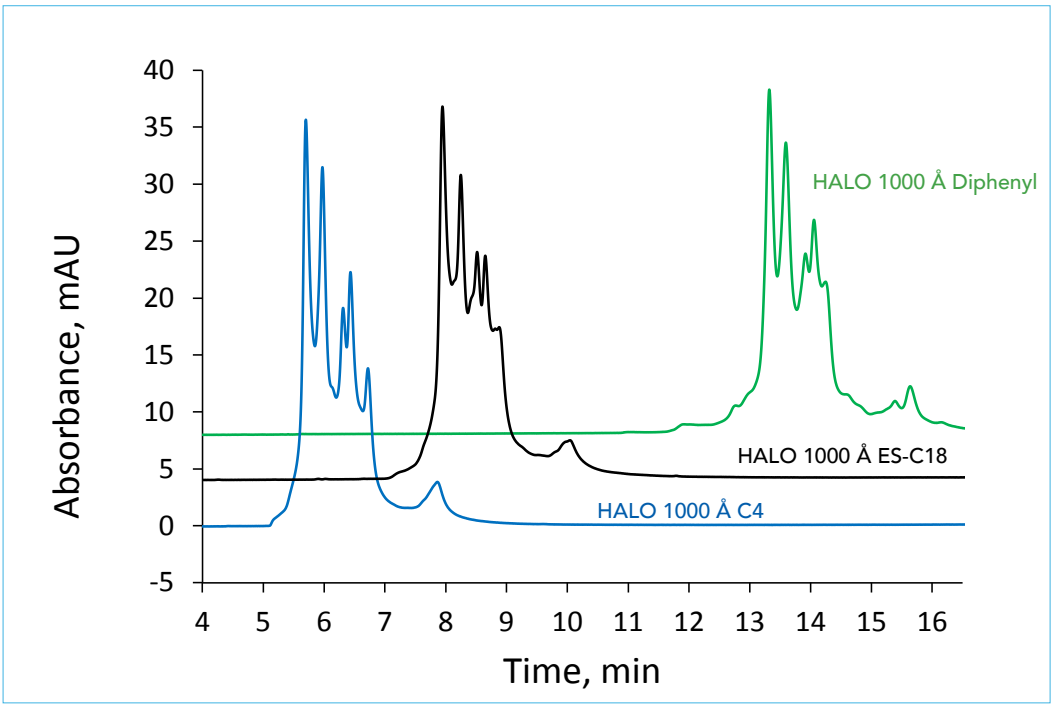


HALO 1000 Å
ES-C18



HALO 1000 Å
DIPHENYL

HALO 1000 Å Phase Comparison

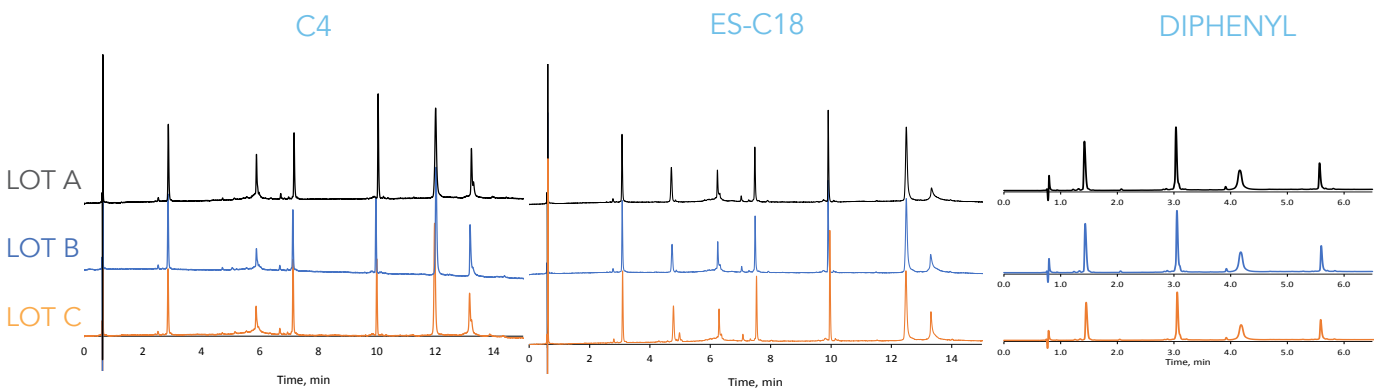


TEST CONDITIONS:
Column: HALO 1000 Å, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: 2:10:88 n-propanol/ Acetonitrile/ Water/ 0.1% DFA
Mobile Phase B: 70:20:10 n-propanol/ Acetonitrile/ Water/ 0.1% DFA
Gradient:

Time, min	%B
0.0	16
20.0	26

Flow rate: 0.2 mL/min
Temperature: 80 °C
Detector: UV 280 nm
Injection Volume: 2.0 µL

HALO® 400 Å LOT TO LOT REPRODUCIBILITY



TEST CONDITIONS:
Column: HALO 400 Å C4, 3.4 µm, 4.6 x 100 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.1% TFA
Gradient:

Time	%B
0.0	28.5
15.0	62.5

Flow rate: 1.5 mL/min
Temperature: 60 °C
Detector: UV 215 nm
Injection Volume: 5.0 µL

TEST CONDITIONS:
Column: HALO 400 Å ES-C18, 3.4 µm, 4.6 x 100 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.1% TFA
Gradient:

Time	%B
0.0	28.5
15.0	62.5

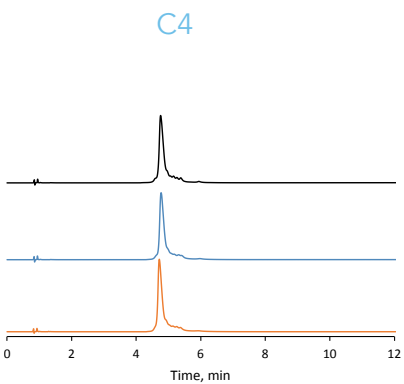
Flow rate: 1.5 mL/min
Temperature: 60 °C
Detector: UV 215 nm
Injection Volume: 5.0 µL

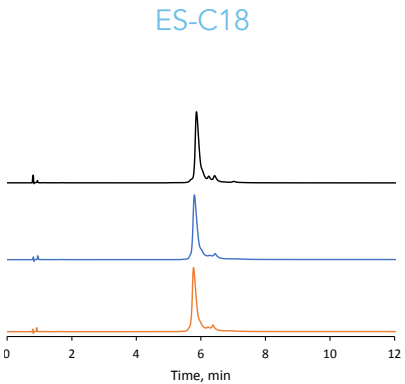
TEST CONDITIONS:
Column: HALO 400 Å Diphenyl, 3.4 µm, 2.1 x 150 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.1% TFA
Gradient:

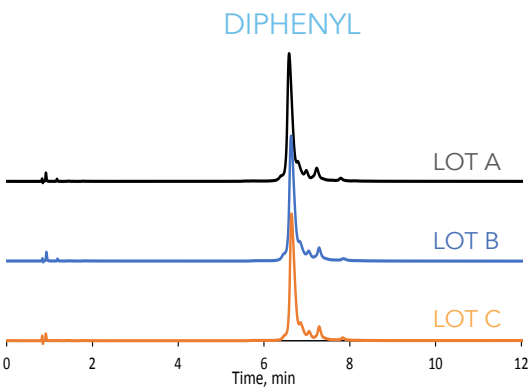
Time	%B
0.0	31
15.0	62.5

Flow rate: 0.4 mL/min
Temperature: 60 °C
Detector: UV 215 nm
Injection Volume: 2.0 µL

HALO® 1000 Å LOT TO LOT REPRODUCIBILITY







TEST CONDITIONS:
Column: HALO 1000 Å C4, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.085% TFA
Gradient:

Time	%B
0.0	32
12.0	42

Flow rate: 0.4 mL/min
Temperature: 80 °C
Detector: UV 280 nm
Injection Volume: 2.0 µL

TEST 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 Acetonitrile/ Water/ 0.085% TFA
Gradient:

Time	%B
0.0	40
12.0	47.5

Flow rate: 0.4 mL/min
Temperature: 80 °C
Detector: UV 280 nm
Injection Volume: 2.0 µL

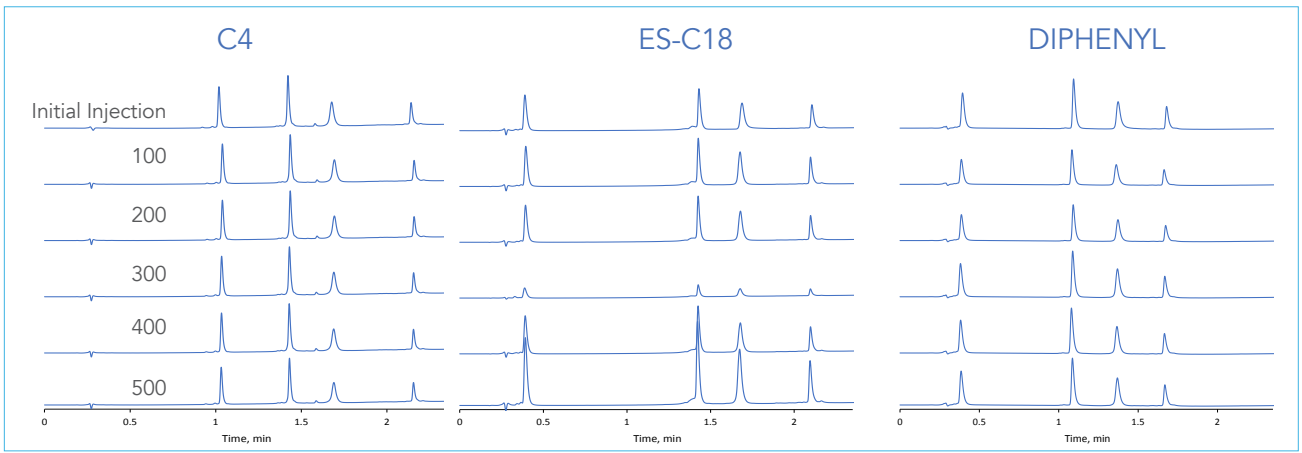
TEST CONDITIONS:
Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.085% TFA
Gradient:

Time	%B
0.0	40
12.0	47.5

Flow rate: 0.4 mL/min
Temperature: 80 °C
Detector: UV 280 nm
Injection Volume: 2.0 µL

HALO® 400 Å STABILITY

Column stability is paramount in HPLC, and a hallmark of all HALO® products. Advanced Materials Technology led the revolution in Fused-Core® particle technology with the development of the first commercially available sub-3 µm particle with the original HALO® 2.7 µm particle in 2006. Our manufacturing expertise has carried forward into being the wide pore leader, and this tradition continues in our BioClass products.



TEST CONDITIONS:
Column: HALO 400 Å C4, 3.4 µm, 2.1 x 50 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: Acetonitrile/ 0.1% TFA
Gradient:

Time, min	%B
0.0	21
2.35	50

Flow rate: 0.4 mL/min
Back Pressure: 47 bar
Temperature: 80 °C
Detector: UV 220 nm
Injection Volume: 0.5 µL

TEST CONDITIONS:
Column: HALO 400 Å ES-C18, 3.4 µm, 2.1 x 50 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: Acetonitrile/ 0.1% TFA
Gradient:

Time, min	%B
0.0	25
2.35	55

Flow rate: 0.4 mL/min
Back Pressure: 57 bar
Temperature: 80 °C
Detector: UV 220 nm
Injection Volume: 1.0 µL

TEST CONDITIONS:
Column: HALO 400 Å Diphenyl, 3.4 µm, 2.1 x 50 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: Acetonitrile/ 0.1% TFA
Gradient:

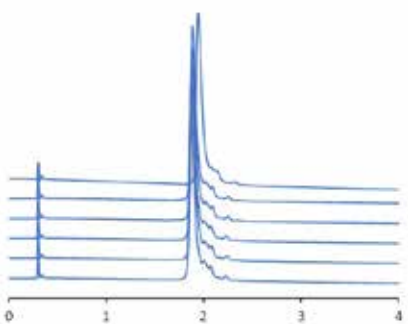
Time, min	%B
0.0	25
2.35	55

Flow rate: 0.4 mL/min
Back Pressure: 55 bar
Temperature: 80 °C
Detector: UV 220 nm
Injection Volume: 0.5 µL

HALO® 1000 Å STABILITY

Note the stability seen throughout 1000 Å products for the analysis of trastuzumab. The retention times for trastuzumab show stability for over 500 injections.

C4

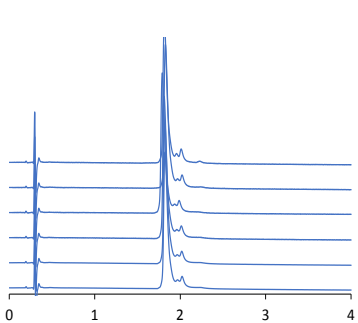


TEST CONDITIONS:
Column: HALO 1000 Å C4, 2.7 µm, 2.1 x 50 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: Acetonitrile/ 0.1% TFA
Gradient:

Time	%B
0.0	32
5.0	40

Flow rate: 0.4 mL/min
Back Pressure: 86 bar
Temperature: 80 °C
Detector: UV 280 nm
Injection Volume: 2.0 µL

ES-C18

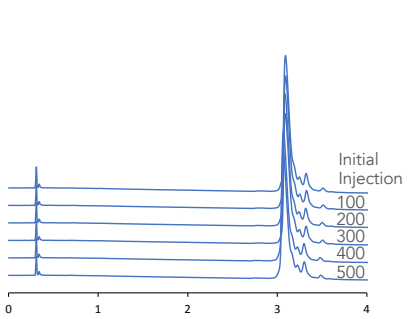


TEST CONDITIONS:
Column: HALO 1000 Å ES-C18, 2.7 µm, 2.1 x 50 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: Acetonitrile/ 0.1% TFA
Gradient:

Time	%B
0.0	32
4.0	38

Flow rate: 0.4 mL/min
Temperature: 80 °C
Detector: UV 280 nm
Injection Volume: 2.0 µL

DIPHENYL



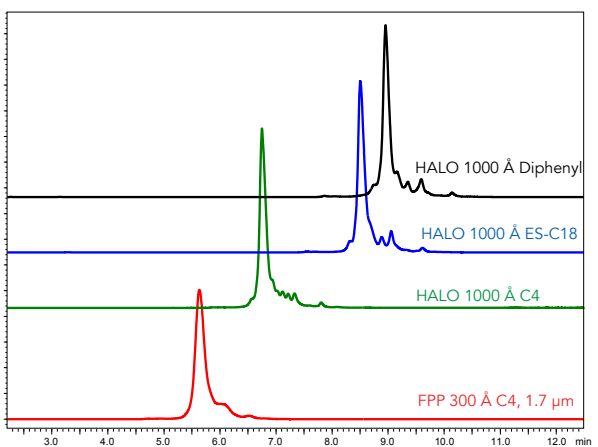
TEST CONDITIONS:
Column: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 50 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: ACN/ 0.1% TFA
Gradient:

Time	%B
0.0	32
4.0	38

Flow rate: 0.4 mL/min
Temperature: 80 °C
Detector: UV 280 nm
Injection Volume: 2.0 µL

HALO® PORE SIZE ADVANTAGE VS COMPETITION

High resolution analysis of large proteins using very wide pore silica based superficially porous particles (SPPs) has been well established. Fused-Core® particles provide better peak widths and resolution of these biomolecules in comparison to fully porous particles, due to accessibility. With a 1000 Å pore size, large, bulky protein structures have unrestricted access to the bonded phase. In the case of biotherapeutics, mAbs are large molecules and as a general guideline, the pore size should be ~ 10 x larger than the analyte of interest in order to avoid restricted diffusion. Note the improved resolution of the mAb (trastuzumab) on all phases of a 1000 Å pore on a HALO® particle versus a 300 Å pore on a fully porous particle.

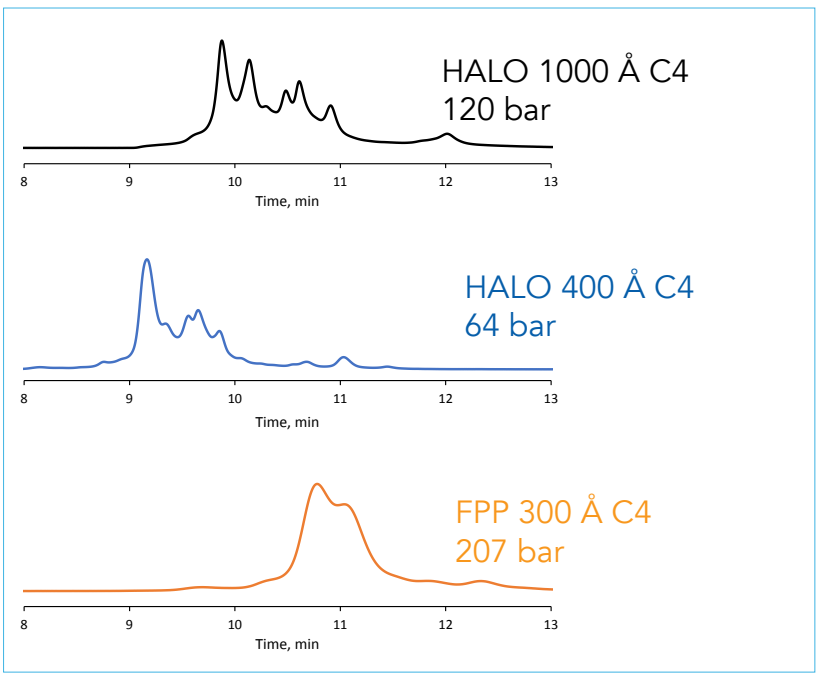


Comparison of HALO 1000 Å Bonded Phases Against a 300 Å FPP Column. Note the advantage in resolution for the 1000 Å pore size.

TEST CONDITIONS:
Columns: 2.1 x 150 mm
Flow rate: 0.4 mL/min
Mobile Phase A: H_2O/0.1% TFA
Mobile Phase B: ACN/0.1% TFA
Gradient: 32-40 %B in 16 min
Instrument: Shimadzu Nexera
Injection Volume: 2 µL
Detection: 280 nm
Temp: 80 °C

This point can be further illustrated by improved resolution over FPP in the comparison of IgG2 variants below.

Demonstration of Resolution Differences Impacted by Pore Size

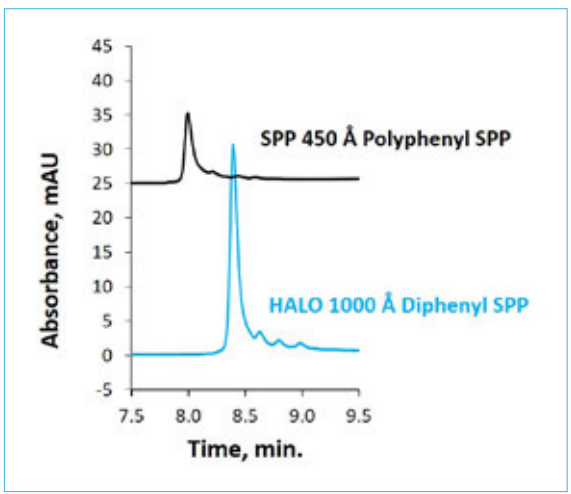


TEST CONDITIONS:
Column: C4, 2.1 x 150 mm
Mobile Phase A: 88/10/2 Water/ Acetonitrile/ n-Propanol/ 0.1% DFA
Mobile Phase B: 70/20/10 n-Propanol/ Acetonitrile/ Water/ 0.1% DFA
Gradient:

Time	%B
0.0	14
20.0	24

Flow rate: 0.2 mL/min
Temperature: 80 °C
Detector: UV 280 nm
Injection Volume: 2.0 µL

The total pore access is evident in this separation, and this superior performance can also be demonstrated when comparing the other phases of HALO® Protein to both FPP and SPP columns. Lower operating temperatures can be advantageous for temperature sensitive analyses, while still meeting the demands for high recovery. The HALO® Diphenyl phase can be operated at 40 °C without showing peak area loss. As seen in the example below, the HALO 1000 Å Diphenyl shows improved resolution, retention, and peak area compared to a competitor column of 450 Å SPPs with Polyphenyl phase. The increased retention clearly demonstrates the benefit of unrestricted large pore access to the bonded phase as well as high performance at lower operating temperatures.



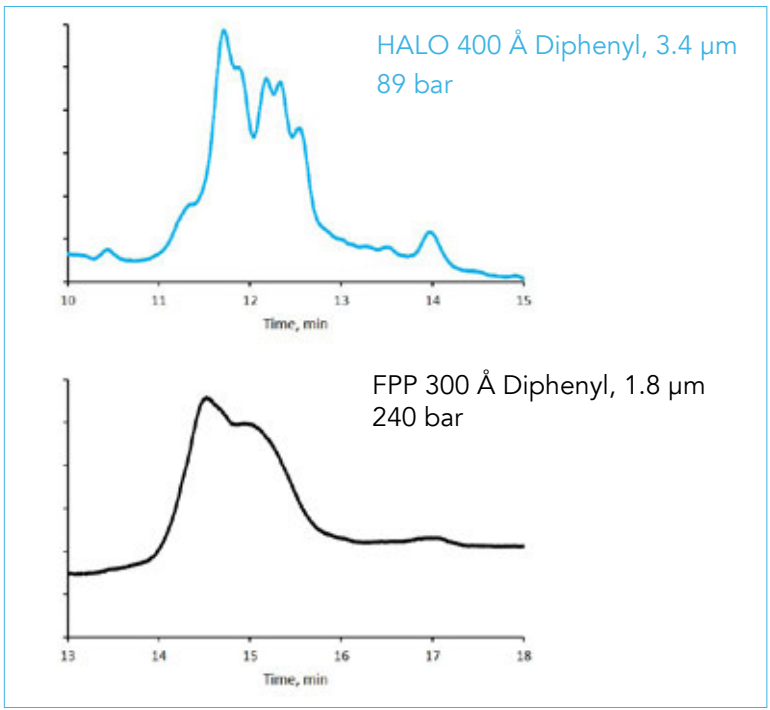
Improved Resolution with HALO® Diphenyl vs Competitor Polyphenyl SPP

TEST CONDITIONS:
Column: 2.1 x 150 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: Acetonitrile/ 0.1% TFA
Gradient:

Time	%B
0.0	30
15.0	45

Flow rate: 0.4 mL/min
Temperature: 40 °C
Detector: UV 280 nm
Injection Volume: 2.0 µL

Benefits of the HALO 400 Å Diphenyl when comparing to a 1.8 µm FPP 300 Å Diphenyl column are also realized. This higher resolution separation is clearly indicative of the accessibility afforded by the wider pore size.

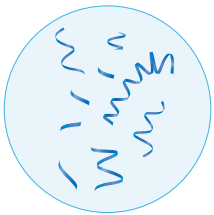


TEST CONDITIONS:
Column: Diphenyl, 2.1 x 150 mm
Mobile Phase A: 88/10/2 Water/ Acetonitrile/ n-Propanol/ 0.1% DFA
Mobile Phase B: 70/20/10 n-Propanol/ Acetonitrile/ Water/ 0.1% DFA
Gradient:

Time	%B
0.0	18
20.0	28

Flow rate: 0.2 mL/min
Temperature: 60 °C
Detector: UV 220 nm
Injection Volume: 2.0 µL

PEPTIDE ANALYSIS



Bottom up proteomics is essentially the domain of peptide analysis. HPLC is the most widely used method of peptide analysis, predominantly due to its high resolution and effective separations. Many techniques have been employed in the chromatographic separation of peptides, including those based on size (GPC), ion exchange (IEC), and on hydrophobicity (reversed-phase and interaction chromatography). RP-HPLC however is well suited to be coupled to ESI MS ion sources and will be the focus of this guide.

For peptide analysis, Advanced Materials Technology offers three particle sizes from which to choose: 2 µm columns which offer the highest efficiency and are desirable for UHPLC systems, 2.7 µm columns for a good compromise between efficiency and back pressure, and 5 µm columns where longer columns can be utilized. Due to lower back pressure (larger particle size), they are amenable to being connected in series for even greater peak capacity when needed.

The HALO 160 Å peptide columns and phases are specifically designed to offer selectivity, stability, and reproducibility to address complex peptide separations. The columns are excellent options for both fast and high resolution UHPLC separations, have high peak capacity and are extremely stable at high temperatures and low pH. For peptide characterization, the 160 Å pore size is ideal for small protein fragments and peptides.

PEPTIDE MAPPING TECHNIQUES

Peptide Mapping

Peptide mapping is widely used and needed for both characterization and release of biological molecules. To completely characterize the protein a digestion is done. Typically, a protein is digested using one or more of three most commonly used enzymes:

- **Trypsin**
- **Chymotrypsin**
- **Lys-C**

Trypsin cleaves peptide chains at the carboxyl side of the amino acids lysine or arginine. Using this enzyme will give about 90% coverage of the protein sequence.

Chymotrypsin is site specific and will only cleave the carboxyl side of large hydrophobic or aromatic amino acids (phenylalanine, tyrosine, and tryptophan), but it will not cleave if the amino acid is followed by proline. Using both trypsin and chymotrypsin results in about 95% coverage of the protein sequence.

Lys-C cleaves proteins on the C-terminal side of lysine residues. With these three enzymes, nearly 100% coverage of the protein sequence can be expected.

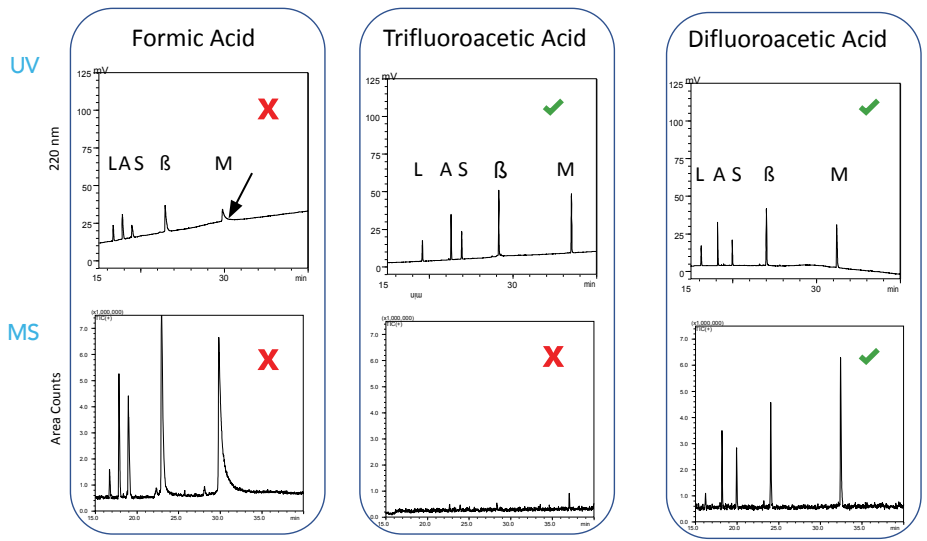
The peptides generated via digestion are separated using reversed phase chromatography, and interrogated by either HRAM-MS or both HRAM and UV.

CONSIDERATIONS FOR PEPTIDE ANALYSIS

Mobile Phase Modifiers

Five peptides were screened using the same HALO 160 Å ES-C18 column using both UV (220 nm) and MS (ESI) detection. Formic acid (FA), TFA, and DFA were compared for retention, resolution, sensitivity and baseline stability.

- **FA** tailing is excessive because ionic interactions are not adequately suppressed by lower pH and higher ionic strength, but it has highest MS sensitivity;
- **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;
- **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. For LC-MS of peptides, 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 DFA is a good option for a balanced result between good peak shape and good ionization efficiency.



L = leucine enkephalin, A = angiotensin I, S = substance P, ß = ß-endorphin, M = melittin

TEST CONDITIONS:
Column: HALO 160 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: Water/ as specified
Mobile Phase B: Acetonitrile/ as specified

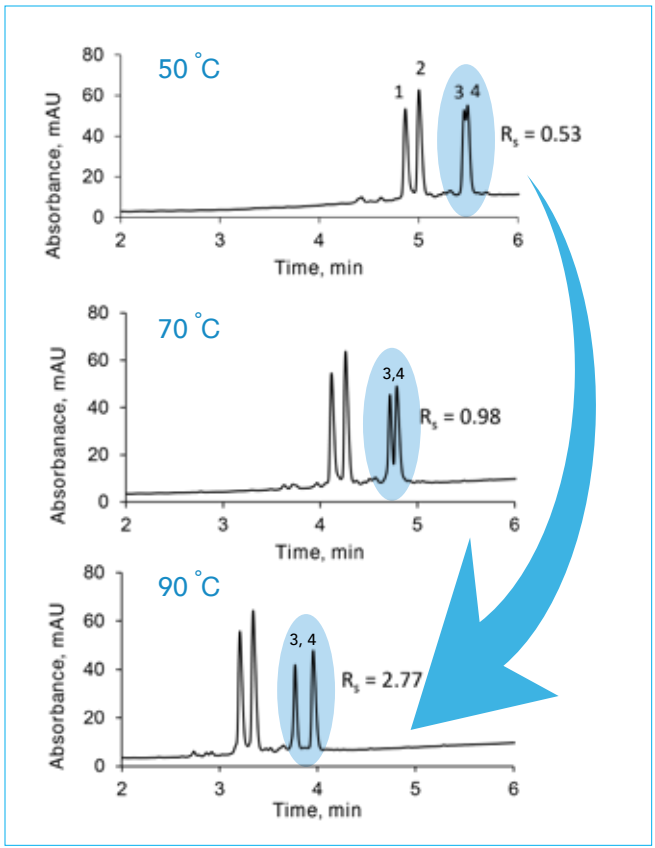
Gradient:	Time, min	%B
	0.0	2
	40.0	47

Flow rate: 0.3 mL/min
Temperature: 60 °C
Detector: UV 220 nm
Injection Volume: 0.5 µL

Temperature

Understanding temperature in relation to the peptides being studied is important. In this example notice how increasing temperature improves the resolution of amyloid β peptides and fragments. These compounds are important in the study of Alzheimer's disease (AD). The dramatic effect of higher column temperatures on these amyloid peptides is shown in the figure. As column temperature is increased, both mobile phase viscosity and peak retention decrease, and peaks widths become narrower. In addition, a temperature-dependent shift in selectivity occurs for peptides 3 and 4, which is beneficial in this case.

Increasing column temperature may not always improve resolution, but it is an easy parameter to manipulate.

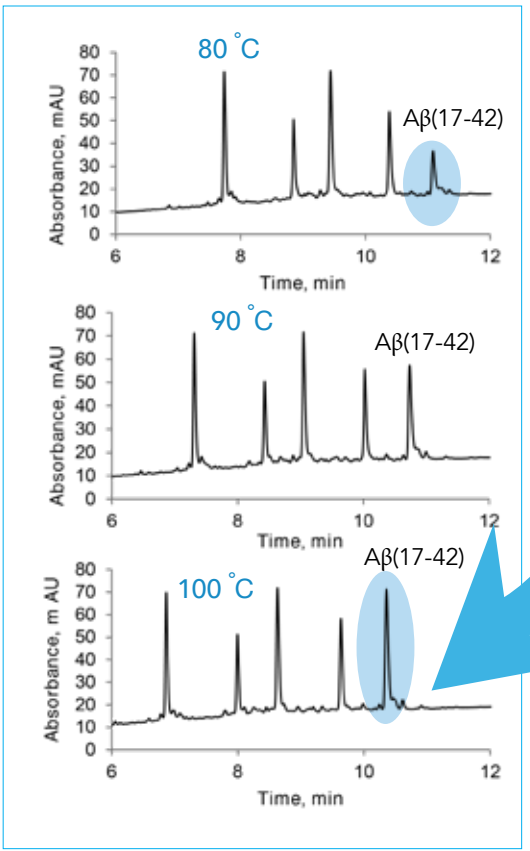


Temperature Effect on Recovery

Recovery for the Aβ(17-42) fragment increases substantially with higher column temperature as shown in the figure. Note when the column temperature is increased to 100 °C, the Aβ(17-42) fragment peak area experiences a 206% increase over the 80 °C trace.

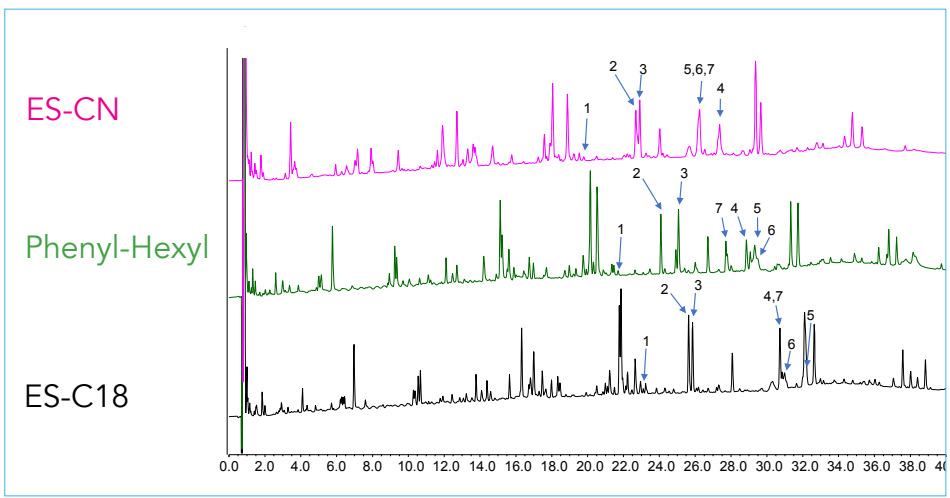
To take advantage of higher column temperatures, it is important to use columns that can perform reproducibly using the combination of high temperature and low pH mobile phases.

HALO® Peptide columns utilize steric-protection silane technology, which prevents the bonded phase from being removed by hydrolysis under the high temperature and acidic pH conditions typically used for peptide and tryptic digest separations. The HALO 160 Å ES-C18 is an unendcapped stationary phase in order to maintain its retention for very long lifetimes.



Why is it Necessary to Screen Multiple Phases?

Different stationary phase functionalities should be examined since each phase offers different selectivity. HALO Peptide is available in ES-C18, ES-CN, and Phenyl-Hexyl stationary phases. Most reversed phase HPLC phases interact with analytes via weak dispersive interactions, developing from an overlap of the electron clouds between the analyte and bonded phase. The phases that comprise unmodified alkyl chains (C18) interact solely via this mechanism. However, the other phases, including cyano and phenyl, can have other types of interactions. This often can manifest in different elution patterns, and peak order reversal, compared to alkyl-only phases providing selectivity manipulation. See figure below for an example of the selectivity differences that were observed with the three different HALO® phases when used to separate a trastuzumab tryptic digest.



PEAK IDENTITIES:

1. FTISADTSKNTAYLQMNSLR
2. LScAASGFNIKDTYIHWVR
3. GFYPSDIAVEWESNGQPENNYK
4. LLIYSASFLYSGVPSR
5. SGTASVVcLLNNFYPR
6. THTcPPcPAPELLGGPSVFLFPPKPK
7. VVSVLTVLHQDWLNGKEYK

TEST CONDITIONS:

Column: HALO 160 Å, 2.7 µm, 2.1 x 100 mm
Mobile Phase A: Water/ 0.1% DFA
Mobile Phase B: Acetonitrile/ 0.1% DFA

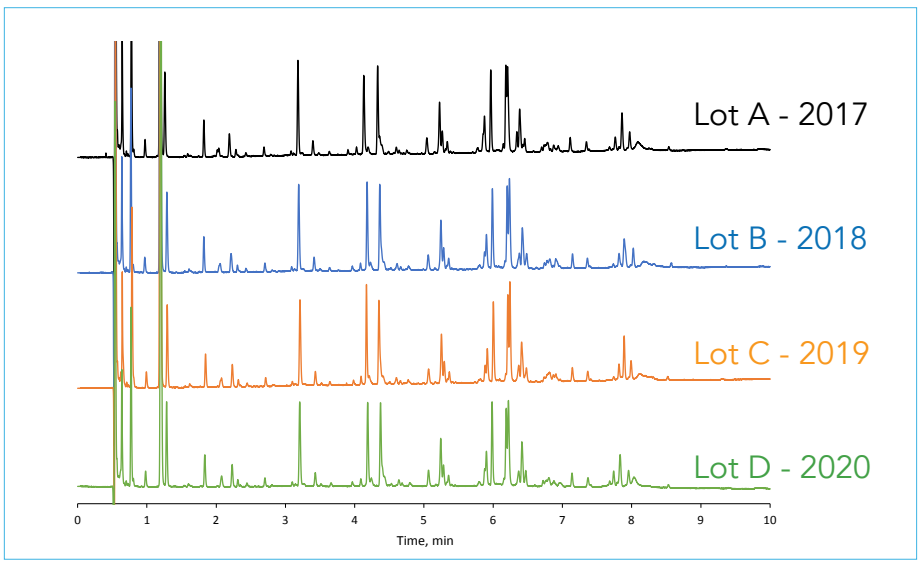
Gradient:	Time, min	%B
	0.0	2
	60.0	50

Flow rate: 0.3 mL/min
Temperature: 60 °C
Detector: UV 215 nm
Injection Volume: 5.0 µL

HALO® PEPTIDE LOT TO LOT REPRODUCIBILITY

The following examples demonstrate the excellent lot-to-lot reproducibility that is a hallmark of the HALO® peptide columns. An adalimumab digest was run on different HALO 160 Å ES-C18, 2.7 µm lots from 2017 through 2020.

High Retention and Resolution Reproducibility Among Multi-Year Lots Provides Reliable Performance Throughout Method Lifetimes

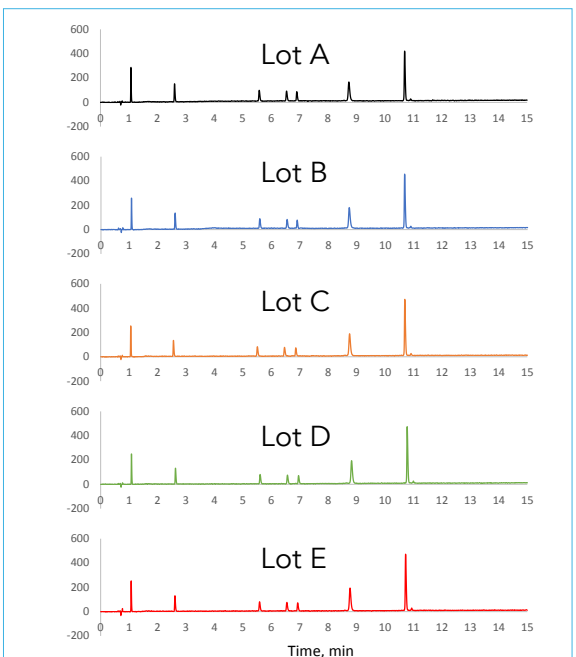


TEST CONDITIONS:
Column: HALO 160 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: Water/ 0.1% DFA
Mobile Phase B: Acetonitrile/ 0.1% DFA
Gradient:

Time, min	%B
0.0	45
10.5	100

Flow rate: 0.6 mL/min
Temperature: 60 °C
Detector: UV 220 nm
Injection Volume: 3.0 µL

Lot to lot reproducibility of the HALO 160 Å Phenyl-Hexyl, 2.7 µm using a mix of peptides and small proteins. As was seen with the ES-C18 stationary phase, there is excellent reproducibility demonstrated across these five different lots.



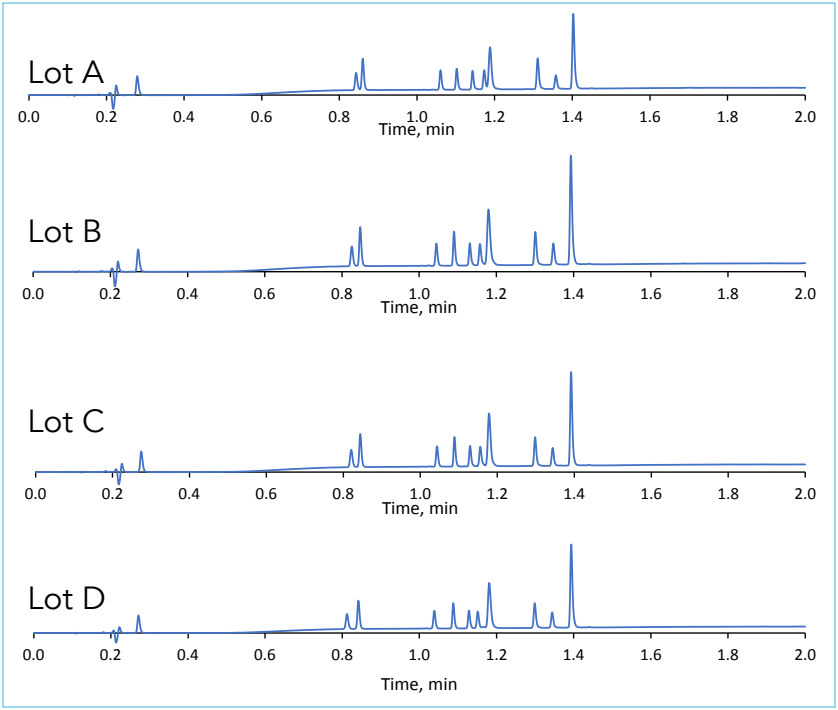
Multi-Lot Data for HALO® Peptide Phenyl-Hexyl Phase

TEST CONDITIONS:
Column: HALO 160 Å Phenyl-Hexyl, 2.7 µm, 4.6 x 100 mm
Mobile Phase A: 10/90 Acetonitrile/Water/ 0.1% TFA
Mobile Phase B: 70/30 Acetonitrile/ Water/ 0.1% TFA
Gradient:

Time, min	%B
0.0	0
15.0	50

Flow rate: 1.5 mL/min
Temperature: 30 °C
Detector: UV 220 nm
Injection Volume: 5.0 µL
Sample: Gly-Tyr, Val-Tyl, Methionine Enkephalin, Leucine Enkephalin, Bovine RNase A, and Bovine Insulin

Lot to lot reproducibility is demonstrated for the HALO 160 Å ES-C18, 2 µm. The lot to lot reproducibility of HALO products is maintained by tightly controlled manufacturing practices and quality assurance testing. This ensures the reliability of the product over its lifetime.



2 µm Multi-Lot Data for HALO® Peptide ES-C18 Phase

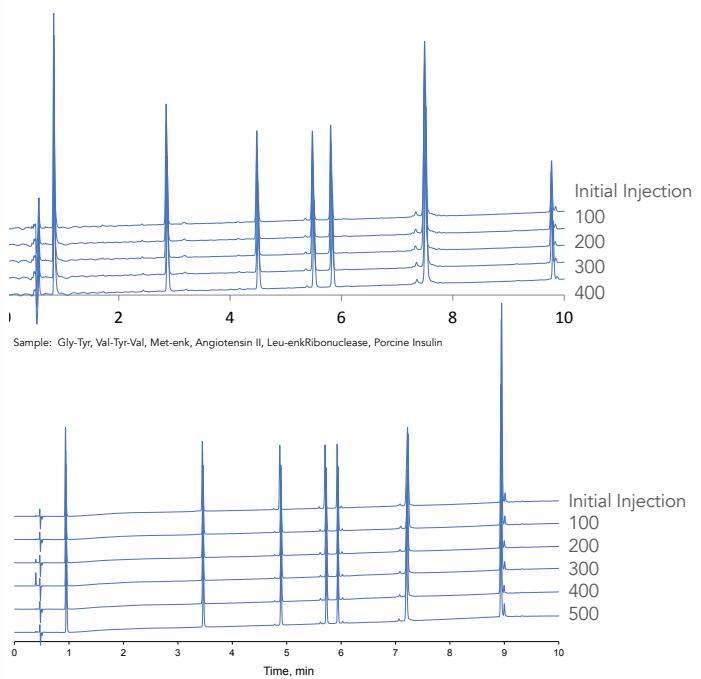
TEST CONDITIONS:
Column Dimension: HALO 160 Å ES-C18, 2µm 3.0 x 50 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.1% TFA
Gradient:

Time	%B
0.0	12.5
0.1	12.5
2.0	93

Flow rate: 1.1 mL/min
Temperature: 60 °C
Detector: UV 215 nm
Injection Volumn: 0.5 µL

HALO® PEPTIDE STABILITY

HALO® Peptide columns are designed for rugged and reliable operation at low pH and elevated temperatures. The figure shows examples averaging over 400 injections using different HALO 160 Å columns with the peak shape and back pressure maintained.



HALO® Peptide Column Ruggedness Demonstrated Over Multiple Phases and Particle Sizes

TEST CONDITIONS:
Column: HALO 160 Å ES-C18, 2 µm, 2.1 x 100 mm
Mobile Phase A: Water/ 0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.1% TFA
Gradient:

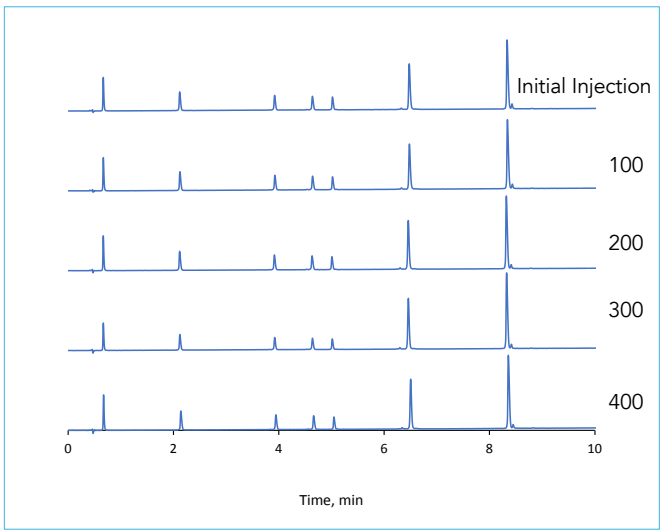
Time, min	%B
0.0	6
10.0	54

Flow rate: 0.5 mL/min
Temperature: 60 °C
Detector: UV 215 nm
Injection Volume: 0.5 µL

TEST CONDITIONS:
Column: HALO 160 Å, ES-C18, 2.7 µm, 2.1 x 100 mm
Mobile Phase A: Water/ 0.1% TFA
Mobile Phase B: 70/30 Acetonitrile/ Water/ 0.1% TFA
Gradient:

Time, min	%B
0.0	9
10.0	55

Flow rate: 0.5 mL/min
Temperature: 60 °C
Detector: UV 220 nm
Injection Volume: 5 µL



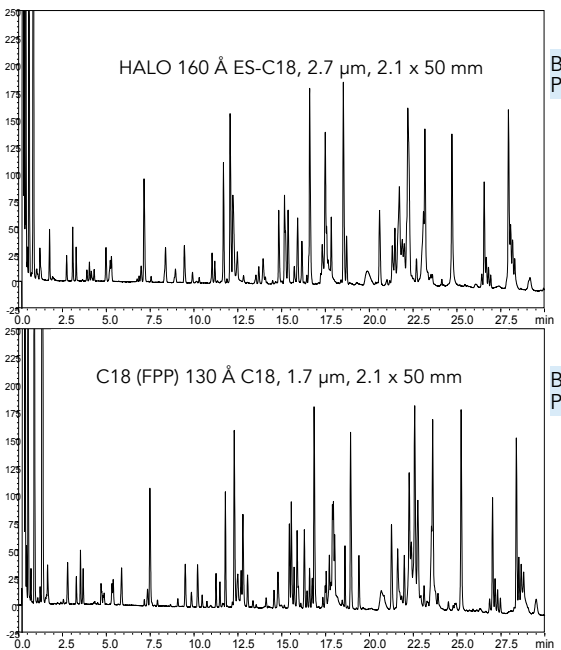
TEST CONDITIONS:
Column: HALO 160 Å Phenyl-Hexyl 2.7 µm, 2.1 x 100 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: 70/30 Acetonitrile/ Water/ 0.1% TFA
Gradient:

Time	%B
0.0	9
10.0	95

Flow rate: 0.5 mL/min
Temperature: 60 °C
Detector: UV 220 nm
Injection Volume: 2.0 µL
Sample: Gly-Tyr, Val-Tyr-Val, Methionine Enkephalin, Leucine Enkephalin, Bovine RNase A, and Bovine Insulin

COMPETITIVE ADVANTAGE WITH HALO®

While many separations can be done with multiple types of modern columns, one of the major advantages of HALO® columns is their ability to generate high efficiency separations in a reasonable time. The figure below shows the comparison of a trastuzumab tryptic digest run on a HALO 160 Å ES-C18, 2.7 µm column compared to a 1.7 µm FPP C18 column.



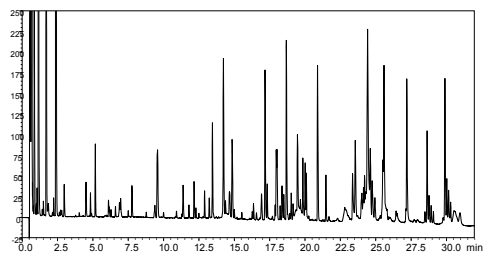
Comparison of Fused-Core® Technology to sub 2 µm FPP. Note the 50% reduction in back pressure

TEST CONDITIONS:
Column: 2.1 x 50 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.08% TFA
Gradient:

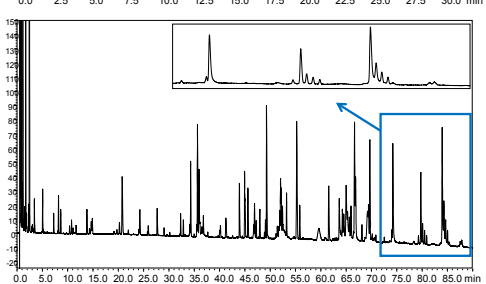
Time	%B
0.0	3
30.0	45

Flow rate: 0.6 mL/min
Temperature: 50 °C
Detector: UV 210 nm
Injection Volume: 10.0 µL

While slightly more peaks are observed on the FPP column, it is at a cost of twice the back pressure of the HALO 160 Å ES-C18 column. Since by design the back pressure is low with the HALO® Fused-Core® columns, a 150 mm length column can be used to increase the efficiency of the separation. The back pressure is now similar to the FPP column, and more peaks are observed (156 vs. 140). Ultimate performance is achieved when the gradient time is extended to 90 minutes, yielding 189 peaks.



Back Pressure: 385 bar
Peaks: 156



Back Pressure: 385 bar
Peaks: 189

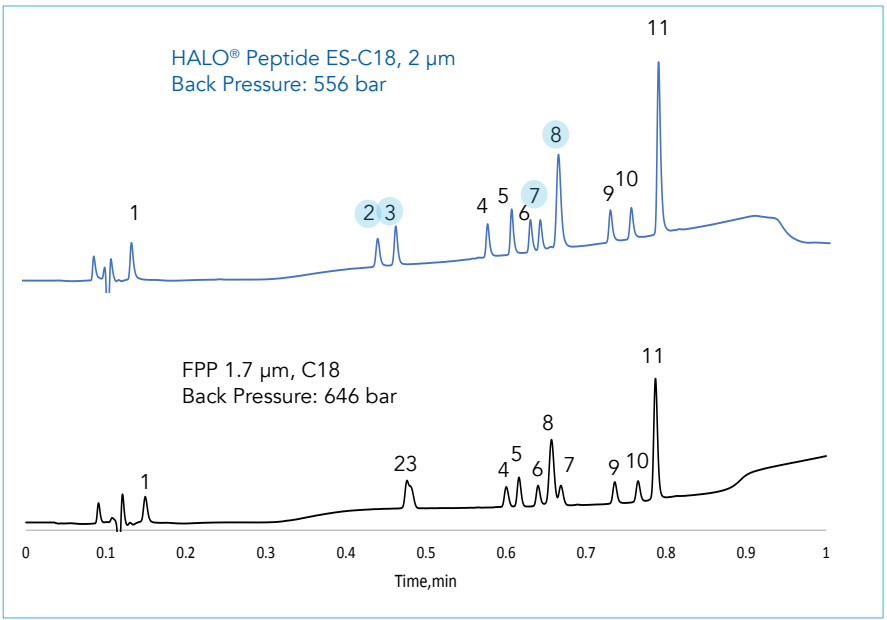
THIS RESULTS IN 35% MORE PEAKS WITH THE HALO® COLUMN!

TEST CONDITIONS:
Column: HALO 160 Å ES-C18, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: Water/0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.08% TFA
Gradient:

Time	%B
0.0	3
30 or 90	45

Flow rate: 0.6 mL/min
Temperature: 50 °C
Detector: UV 210 nm
Injection Volume: 10.0 µL

In the next example, a HALO 160 Å ES-C18, 2 µm column is compared to a FPP 1.7 µm column. Improved resolution is observed on the HALO® column with about 90 bar lower back pressure.



PEAK IDENTIFIES

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

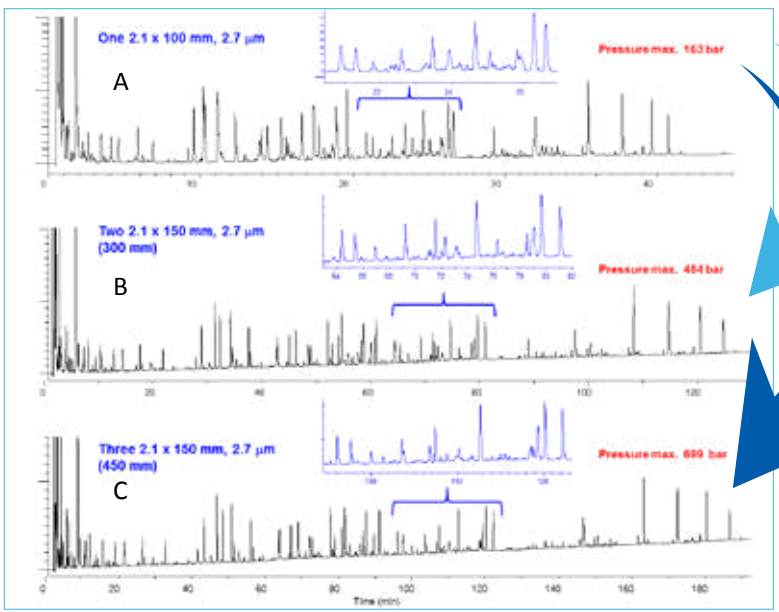
TEST CONDITIONS:

Columns: HALO 160 Å, ES-C18, 2 µm, 3.0 x 50 mm
FPP 1.7 µm, C18, 3.0 x 50 mm
Mobile Phase A: Water/ 0.1% TFA
Mobile Phase B: 80/20 Acetonitrile/ Water/ 0.1% TFA

Gradient:	Time, min	%B
	0.0	12.5
	0.1	12.5
	1.0	63.0

Flow rate: 2.2 mL/min
Temperature: 60 °C
Detector: UV 215 nm
Injection Volume: 0.5 µL

To demonstrate higher performance at lower pressure offered by SPP columns, an example of columns run in series is shown below. Figure B demonstrates the gains in resolution that can be attained by coupling two 2.1 x 150 mm HALO 160 Å ES-C18 columns for a **70% increase in resolution** compared to using a single 2.1 x 100 mm column. Figure C takes the example one step further and couples three 2.1 x 150 mm HALO 160 Å ES-C18 columns for a **110% gain in resolution** compared to when one 2.1 x 100 mm HALO 160 Å ES-C18 column is used.

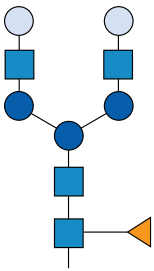


70% HIGHER RESOLUTION COMPARED TO A

110% HIGHER RESOLUTION COMPARED TO A

TEST CONDITIONS:
Columns: HALO 160 Å ES-C18, 2.7 µm, lengths as indicated
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/ Water/0.1% TFA
Gradient: 5-60% B in 60, 180, or 270 min
Flow Rate: 0.4 mL/min
Temperature: 60 °C
Injection Volume: 10 µL of 1 mg/mL apotransferrin tryptic digest
Instrument: Shimadzu Nexera
Detection: PDA at 215 nm

GLYCAN ANALYSIS



Glycans are simple and complex carbohydrates (sugars) that influence protein structure/ folding and transport.

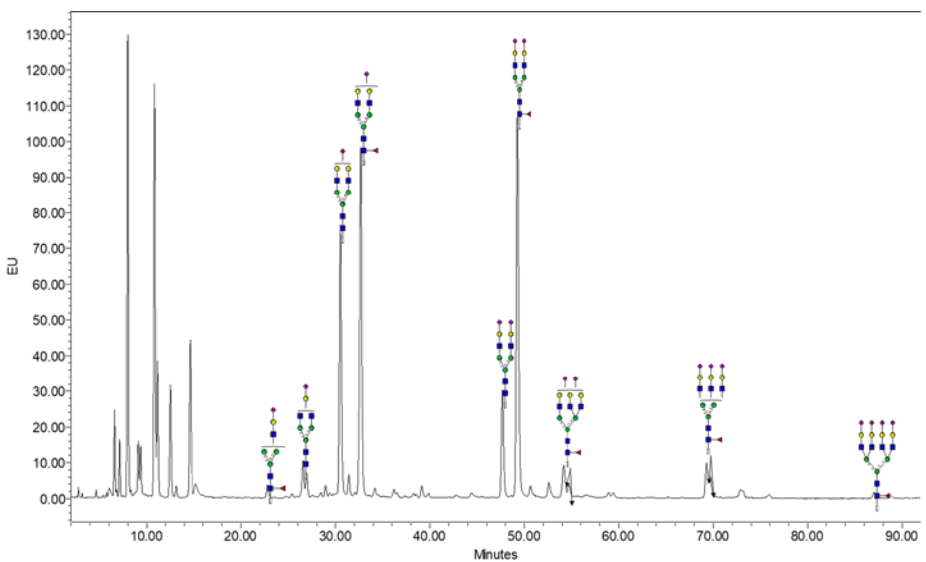
TECHNIQUES TO CHARACTERIZE GLYCANS

Glycosylation is one of the most important modifications of the protein structure. Typically, glycans are attached by N-linkage at Asn-X-Ser/Thr sequons at one or more sites in the protein. The N-glycans can affect the overall properties of the glycoprotein. [3]

Given the importance of glycosylation to overall performance of a glycoprotein, there is a need to completely characterize and then monitor these glycans for both research and manufacturing purposes.

Glycan analysis consists of several steps. First, the protein is deglycosylated, which means the glycans are cleaved from the protein backbone. Then the released glycans are labeled with a UV or fluorescent small molecule tag. Several different tags have been investigated with one of the most sensitive being procainamide. The labeled glycans are separated using the HALO® Glycan column using HILIC mode. Mass spectrometry is used to identify the glycans during development while final quantitation is performed using fluorescence or UV detection with fluorescence being more sensitive.

HALO® Glycan column separating > 70 glycan species with a peak capacity of ~200



TEST CONDITIONS:
Column: HALO Glycan, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: 0.1% Formic Acid/ACN/Ammonium
Gradient: 90 min
Flow rate: 0.6 mL/min
Temperature: 60 °C

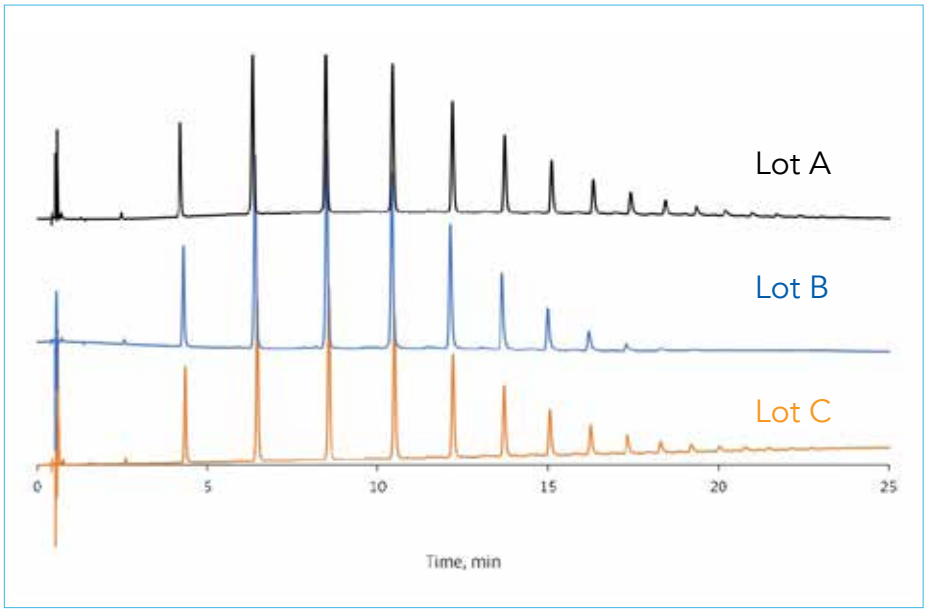
CONSIDERATIONS FOR GLYCAN ANALYSIS

Within biotherapeutic drug development the three main areas for separations are process support, characterization, and final release/QC. Process support can be rapid and low resolution, but more efficient separations can be developed and then "detuned" using the same column and mobile phase, thus not requiring a complete new qualification or validation. Characterization methods can be designed to be very highly efficient, focusing on the highest resolution, usually with longer times. This is often acceptable since these full characterizations are only done on a limited basis. Similar to the process work, high efficiency methods can be "detuned" for final release/QC and stability to be faster when the highest resolution is secondary to time.

For instance, one could develop the high resolution methods using long SPP columns (150 mm or longer) with 2.7 µm particles. These methods will be used for the characterization of the biotherapeutic drug which can then be modified by shortening the column length and/or increasing flow rate for process support, QC work and release assays. The advantage of this approach is that the same column is used for multiple purposes and translation from one method to another is easy. For glycan analysis, a universal method approach is ideal so that all of the possible glycans from the biotherapeutic can be fully characterized during development and then a fast method can be used for its QC/release assays.

HALO® GLYCAN LOT TO LOT REPRODUCIBILITY

The example below shows the lot to lot reproducibility of three different HALO® Glycan lots using a dextran ladder.



TEST CONDITIONS:
Column: HALO 90 Å, Glycan, 2.7 µm, 2.1 x 150 mm
Mobile Phase A: 50 mM Ammonium Formate, pH: 4.5
Mobile Phase B: Acetonitrile
Gradient:

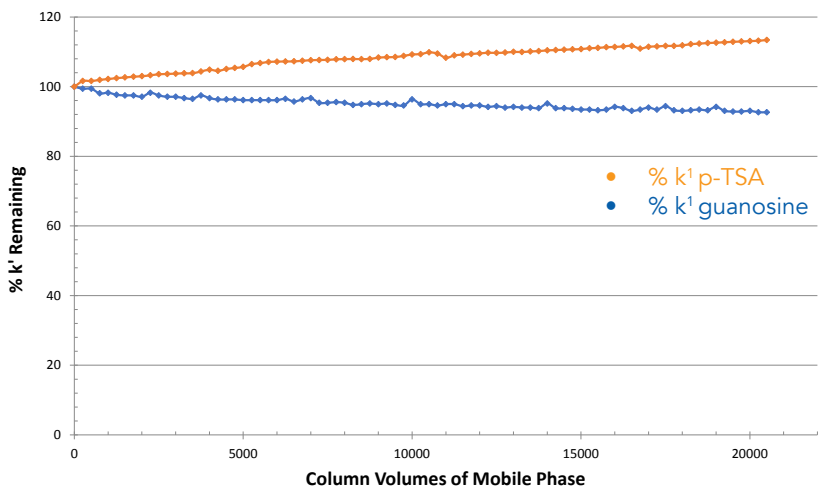
Time, min	%B
0.0	20
25	45

Flow rate: 0.6 mL/min
Temperature: 60 °C
Detector: UV 300 nm
Injection Volume: 3.0 µL

HALO® GLYCAN STABILITY

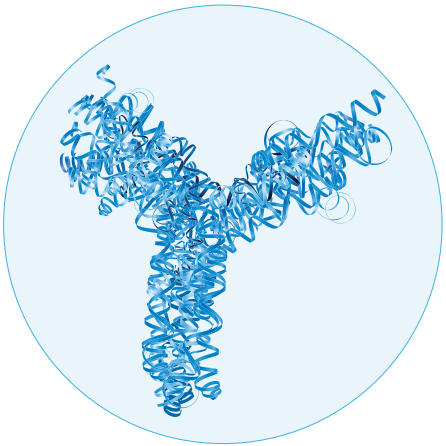
The HALO® Glycan column has been stability tested using acidic mobile phase and elevated temperature. Very little change in retention is observed after more than 20,000 column volumes.

Column Stability in Acidic Mobile Phase at 60 °C



TEST CONDITIONS:
Column: HALO 90 Å Glycan, 2.7 µm, 4.6 x 50 mm
Mobile Phase A: Water/0.05% TFA
Mobile Phase B: Acetonitrile/0.05% TFA
Isocratic: 90% B
Flow rate: 1.0 mL/min
Temperature: 60 °C
Detector: UV 254 nm
Injection Volume: 1.0 µL

CHOOSING THE PROPER COLUMN



FOR PROTEIN ANALYSIS

With the different options available from HALO® for protein analysis, what column should you select?

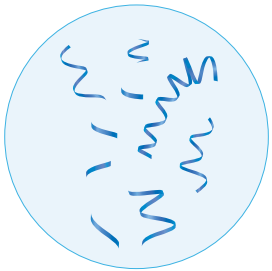
First ask, What is needed?

- Is it Speed? → Select 400 Å HALO® Protein
- Is it Resolution? → Select 1000 Å HALO® Protein
- Both speed and resolution? → Select 1000 Å HALO® Protein

Then ask, Is it for characterization or release assay?

- If characterization → Select 1000 Å HALO® Protein
- If release assay → Select 400 Å HALO® Protein or 1000 Å HALO® Protein

FOR PEPTIDE ANALYSIS

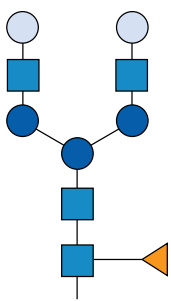


Choose the 160 Å pore size and the appropriate particle size:

- For UHPLC and maximum efficiency → Select 2 µm HALO® Peptide
- For UHPLC and a good compromise of efficiency and back pressure → Select 2.7 µm HALO® Peptide
- For HPLC → Select 2.7 or 5 µm HALO® Peptide

FOR GLYCAN ANALYSIS

Select the HALO® Glycan column length based on instrumentation and needed resolution.



REFERENCES

[1] J. Am. Soc. Mass Spectrom. (2019) 30:1149–1157.
[2] Talanta (2014)127: 219–224.
[3] Essentials of Glycobiology [Internet]. P. Stanley, N. Taniguchi, M. Aebi. 3rd edition. Chap. 9. 2017

Additional applications and technical information can be found at **fused-core.com**

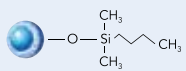
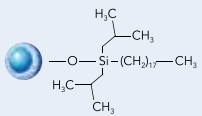
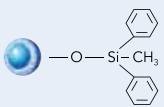
- **Method Conversion Guidebook** - Offers conversion tips for superficially porous particles from fully porous particles methods.

- **Guidebook on Reversed Phase Chemistries and Utilizing Selectivity** - Offers tips on choosing the proper stationary phase and manipulating selectivity for improved HPLC separations.

- **Contact your local HALO® distributor for more details.**

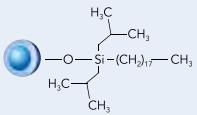
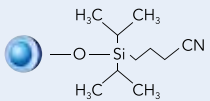
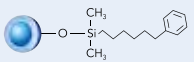
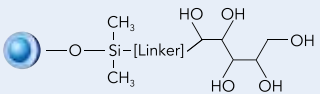
PRODUCT SPECIFICATIONS

PROTEIN

Bonded Phase	C4	ES-C18	DIPHENYL
Structure			
USP Designation	L26	L1	L11
Particle Size(s) (µm)	2.7 3.4	2.7 3.4	2.7 3.4
Pore Size (Å)	1000 400	1000 400	1000 400
Carbon Load (%)	0.6 0.4	1.4 1.0	1.0 0.7
Surface Area (m^2/g)	22 15	22 15	22 15
Low pH/T Limit	2/90 °C	1/90 °C	2/90 °C
High pH/T Limit	9/40 °C	8/40 °C	9/40 °C
Endcapped	Yes	Yes	Yes

PEPTIDE

GLYCAN

Bonded Phase	ES-C18	ES-CN	PHENYL-HEXYL	Proprietary Poly-Hydroxy
Structure				
USP Designation	L1	L10	L11	L95
Particle Size(s) (µm)	2 2.7 5	2.7 5	2.7	2.7
Pore Size (Å)	160	160	160	90
Carbon Load (%)	4.0 4.6 4.0	2.2 1.5	4.7	3.2
Surface Area (m^2/g)	65 90 60	90 60	90	135
Low pH/T Limit	1/90 °C	1/90 °C	2/90 °C	2/65 °C
High pH/T Limit	8/40 °C	8/40 °C	9/40 °C	9/40 °C
Endcapped	No	Yes	Yes	No

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