

## PEPTIDE ES-C18 UHPLC COLUMNS

# HALO<sup>®</sup>

BRINGING UHPLC PERFORMANCE  
*to the* SEPARATION of PEPTIDES

*Compatible with UHPLC and conventional HPLC equipment.*

# HALO

The name that defines a new direction in UHPLC separation of peptides.

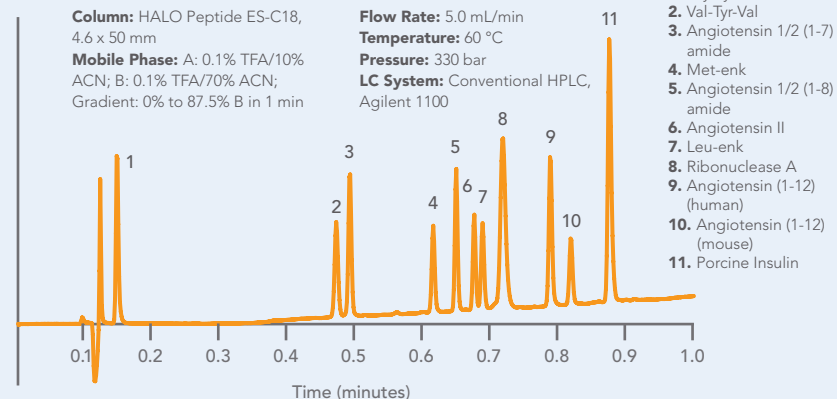
- ♦ Fast separations
- ♦ High peak capacity
- ♦ Rugged, reliable performance
- ♦ Use with either UHPLC or conventional HPLC equipment

“The fused-core silica column providing the reduced diffusional mass transfer path allows the use of shorter columns and higher flow rates to achieve remarkably fast high-resolution separations.”

Analytical Chemistry, August 2007

HALO Peptide ES-C18 columns are specifically designed for ultra-fast and ultra-high resolution separation of peptides (Figures 1 and 2). A specifically selected pore size and the use of extra stable (ES) bonding chemistry provides optimum separation of peptides up to 20 kDa. Most important, however, is the use of Fused-Core® particle technology to produce columns that bring UHPLC performance to the separation of peptides, using either UHPLC or conventional HPLC equipment (Figure 3).

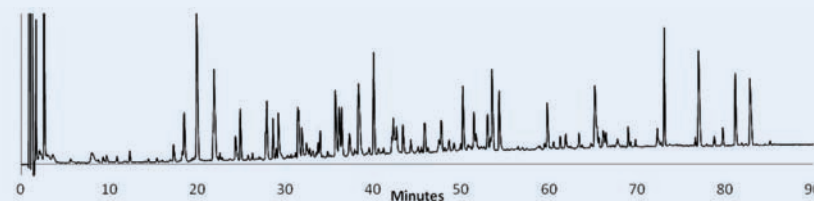
**FIGURE 1:** High Speed Separation Using HALO Peptide ES-C18



This chromatogram illustrates the high-speed separations of peptides that are possible with HALO Peptide ES-C18 columns. These 9 peptides and 2 proteins are separated with excellent resolution in less than 60 seconds.

**FIGURE 2:** High Resolution Separation Using HALO Peptide ES-C18

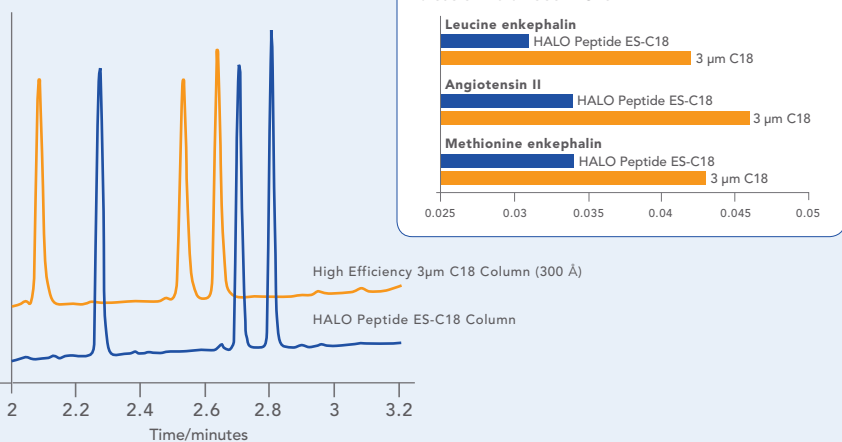
**Columns:** HALO Peptide ES-C18, two each, 2.1 x 100 mm, columns connected in series  
**Mobile Phase:** A: Water/0.1% TFA; B: 80% ACN/20% Water/0.1% TFA; Gradient: 5% to 65% B in 120 min  
**Flow Rate:** 0.5 mL/min  
**Temperature:** 45 °C  
**Pressure:** maximum 476 bar  
**LC System:** Agilent 1200  
**Sample:** Apotransferrin Tryptic Digest



This chromatogram illustrates how longer HALO Peptide ES-C18 columns can be used for high-resolution separations of peptides. The lower back pressure offered by Fused-Core particle technology allows HALO UHPLC columns to be used with either UHPLC or conventional HPLC equipment for circumstances where high peak capacity is demanded.

**FIGURE 3:** HALO Peptide ES-C18 UHPLC columns provide ultra-high resolution

**Columns:** 2.1 x 100 mm  
**Mobile Phase:** A: water/0.1% TFA  
 B: 70% ACN/30% water/0.1% TFA  
 Gradient: 9-65% B in 5 min  
**Flow Rate:** 1.0 mL/min  
**Temperature:** 60 °C



This comparison separation illustrates the ultra-high “UHPLC” resolution offered by HALO Peptide ES-C18 columns compared to other high efficiency columns.

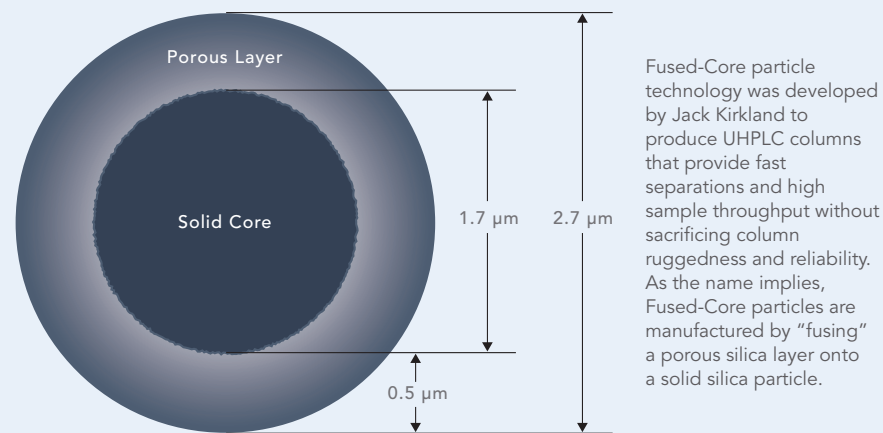
HALO UHPLC columns are not your typical HPLC or UHPLC columns. The particles packed into HALO columns are manufactured using Fused-Core particle technology that was developed to deliver ultra-fast and ultra-high resolution chromatographic separations while avoiding problems associated with UHPLC.

HALO columns, by virtue of their Fused-Core particles, generate significantly less back pressure compared to other UHPLC columns. This lower pressure puts less stress on equipment and facilitates more trouble free operation. It is this moderate back pressure of HALO columns that also permits them to be used with conventional HPLC equipment as well as UHPLC equipment. Furthermore, HALO columns utilize a column inlet frit with a porosity that is significantly larger than other UHPLC columns (2 µm versus 0.5 µm). This larger porosity column inlet frit reduces a problem that plagues UHPLC columns, inlet frit plugging. In fact, the porosity of the inlet frit on HALO columns is the same size as that typically used on columns packed with 5 µm particles. Imagine that, a UHPLC column with the ease of use and reliability of a column packed with 5 µm particles.

“The unusually high efficiency for columns of these particles is believed to be a feature of the very narrow particle size distribution and the higher particle density.”

American Laboratory, April 2007

**FIGURE 4:** HALO Fused-Core particle

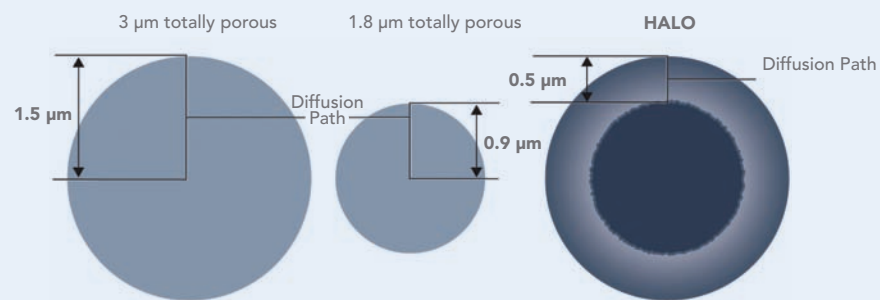


Fused-Core particle technology was developed by Jack Kirkland to produce UHPLC columns that provide fast separations and high sample throughput without sacrificing column ruggedness and reliability. As the name implies, Fused-Core particles are manufactured by “fusing” a porous silica layer onto a solid silica particle.

### HALO particles are designed for hyper-fast separations at modest column back pressure

The ability of HALO to generate hyper-fast separations comes not only from their small particle size (2.7 µm) but also from the unique Fused-Core particle technology that creates a 0.5 µm porous shell fused to a solid core particle (Figure 4). As mobile phase flow rate is increased to speed up a separation, the slow mass transfer of solute molecules inside the particles limits resolving power. Fused-Core particle technology

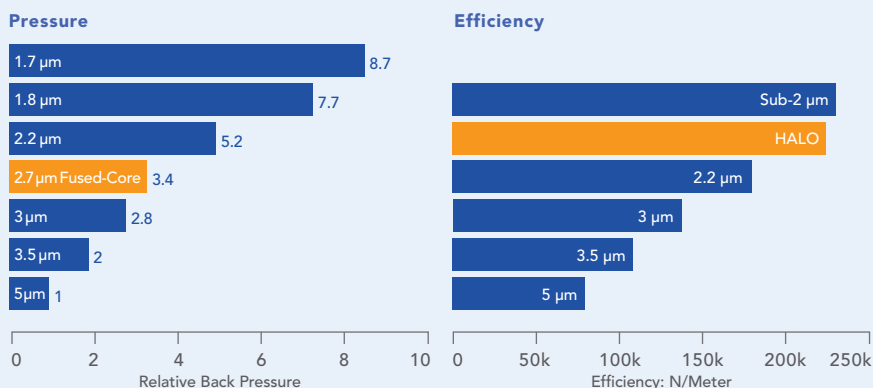
**FIGURE 5:** The shorter diffusion path of HALO reduces axial dispersion



The shorter diffusion path of HALO particles reduces axial dispersion of solutes and minimizes peak broadening. Because of the shorter diffusion path, the performance advantages of HALO become even more apparent when separating peptides and operating at faster mobile phase flow rates.

addresses this limitation by providing an incredibly small path (0.5  $\mu\text{m}$ ) for diffusion of solutes into and out of the stationary phase, thereby reducing the time solute molecules spend inside the particles and minimizing a major barrier to fast chromatographic separations (Figure 5).

**FIGURE 6:** HALO UHPLC columns deliver UHPLC performance at conventional HPLC pressure.



HALO UHPLC columns deliver over 90% more separating power (theoretical plates) than columns of the same length packed with 3.5  $\mu\text{m}$  particles and almost three times the separating power of columns packed with 5  $\mu\text{m}$  particles. And, unlike UHPLC columns packed with conventional sub-2  $\mu\text{m}$  particles, HALO columns generate only modest back pressure, thereby, permitting their use with both UHPLC and conventional HPLC equipment.

HALO UHPLC columns deliver over 90% more separating power (theoretical plates) than a column of the same length packed with 3.5  $\mu\text{m}$  particles and almost three times the plates of a column packed with 5  $\mu\text{m}$  particles (Figure 6).

And, because of Fused-Core particle technology, HALO columns maintain their resolving power at high flow rates. This means that shorter columns and higher flow rates can be used to achieve remarkably fast high resolution separations (Figure 1).

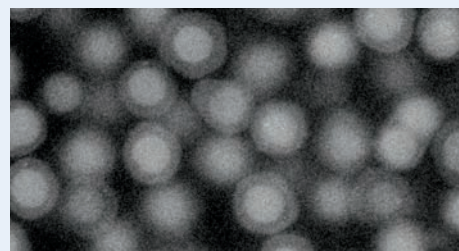
### HALO UHPLC columns are designed to be super-rugged

Packing HPLC columns can be as much art as it is science. There are many variables that have to be optimized in order to pack a column well for even non-high throughput applications. But, the demands placed upon columns used in high speed applications, i.e., high flow rate and high pressure, make it especially difficult to pack a column that will hold up for a satisfactory period of time. HALO particles facilitate the packing process in two ways. First, the unique Fused-Core particle technology produces particles that have extremely narrow size distribution. Second, these particles are significantly more dense than conventional totally porous particles, allowing them to be more easily packed into stable and efficient columns. This

combination of extremely narrow particle size distribution and very dense particles allows the production of columns that are incredibly rugged and reliable, as well as very reproducible from column to column.

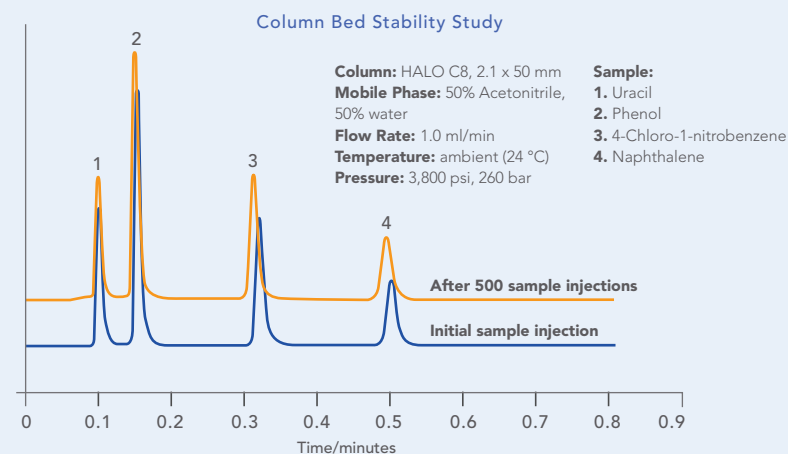
Also of importance, the extremely narrow particle size distribution permits the use of 2  $\mu\text{m}$  porosity inlet frits on the HALO columns. This is the same inlet frit porosity typically found on columns packed with 5  $\mu\text{m}$  particles. The result is a column capable of delivering incredibly high sample throughput, much higher than 3  $\mu\text{m}$  packed columns, but with the ease of use and durability of a column packed with 5  $\mu\text{m}$  particles (Figure 8). Who says you can't have both high speed and ruggedness? HALO delivers both.

**FIGURE 7:** Scanning electron microscope (SEM) photograph of HALO particles



This SEM photograph of HALO particles illustrates two important attributes of this unique column packing. First, the incredibly narrow particle size distribution is apparent. Second, this SEM photo shows some of the HALO particles "sliced in half" so that the solid core and the porous outer layer, the "halo" of the particles, is evident.

**FIGURE 8:** Stability testing of a HALO UHPLC column



A HALO column was run under high flow conditions to test bed stability. After 500 sample injections and over 40,000 column volumes, there was no evidence of any change to the packing bed.

## HALO UHPLC columns do not require ultra-high pressure

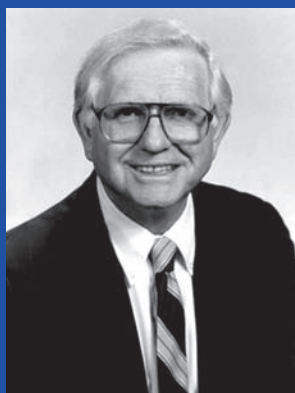
Fused-Core particle technology produces hyper-fast columns that can be used with both UHPLC and conventional HPLC equipment. Figure 6 provides a comparison of system back pressure and efficiency for the HALO column versus other HPLC and UHPLC columns. Columns packed with stationary phases smaller than 2  $\mu\text{m}$  often require pressures in excess of what is achievable with typical HPLC instrumentation. A very real bonus that comes with using a HALO column is that expensive ultra-high pressure instrumentation does not have to be purchased and new laboratory protocols do not have to be developed. HALO columns can turn almost any HPLC system into a high speed workhorse for your lab.

## The science behind HALO

The well known van Deemter equation identifies the three main sources of band broadening.

$$H = A + B/\mu + C\mu$$

The value of the A term, eddy diffusion, reflects the multiple flow paths through a column. Packing particle size, particle size distribution, and the uniformity of the packed bed all determine the value of A. Because of the high density and extremely narrow size distribution of Fused-Core particles, HPLC columns can be packed with well ordered beds that have A term values significantly smaller than what is typically seen with columns packed with totally porous particles. This is one of the



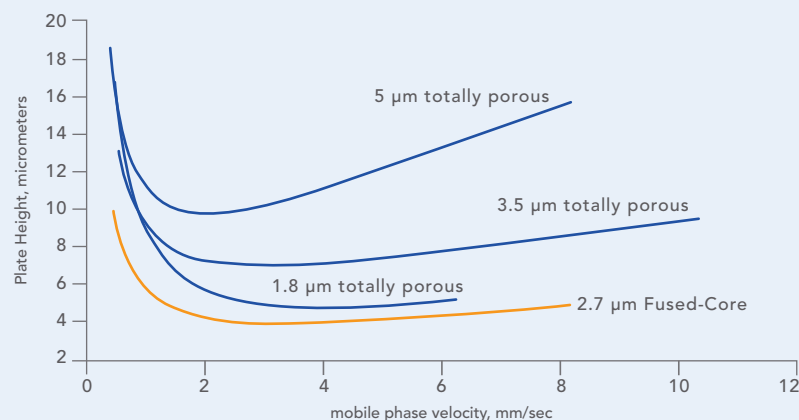
### FUSED-CORE TECHNOLOGY

Fused-Core technology was developed by Jack Kirkland. Dr. Kirkland is widely regarded as one of the “founders” of HPLC and is well recognized for his research and contribution to the understanding of chromatography. He’s authored over 150 major research publications and 8 textbooks. Dr. Kirkland holds over 30 patents and has received several prestigious awards within the field of chromatography.

reasons that HALO columns deliver column plate numbers that are much higher than what would normally be expected from their particle size.

The C term of the van Deemter equation, the coefficient of mass transfer, reflects the time it takes for an analyte to diffuse in and out of the stationary phase. The C term is directly related to mobile phase velocity because higher velocity interferes with the equilibrium between the analyte, mobile phase and stationary phase. The longer the path an analyte has to travel within the pores of the stationary phase support particles, the more detrimental the effect of mobile phase velocity will be on column efficiency (Figure 9).

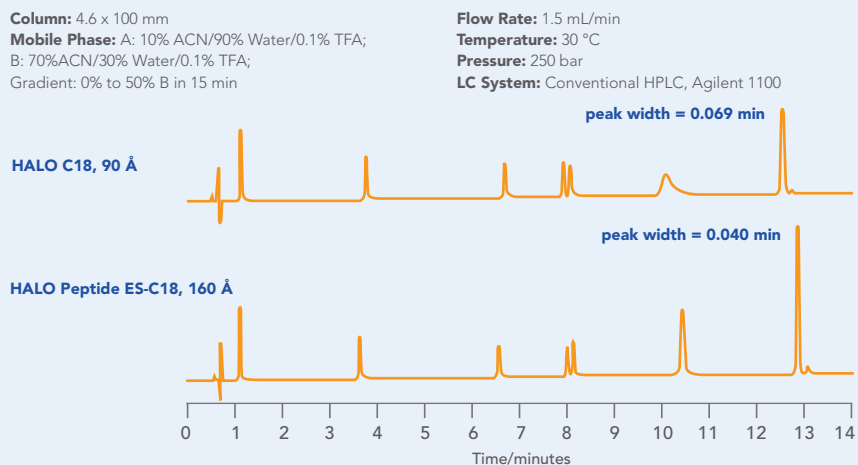
FIGURE 9: Comparison of van Deemter plots



Van Deemter plots are a convenient way to compare the efficiency of HPLC columns. In this comparison we see that HALO columns are more efficient than columns packed with totally porous particles and that they can be run at higher mobile phase linear velocity and still maintain their resolving power.

The path a solute has to travel within the pores of a stationary phase support particle can be reduced by using smaller size particles and this is typically the strategy that is used by column manufacturers when making UHPLC columns. Smaller particles have shorter diffusion path lengths and, therefore, are less affected by increases in mobile phase velocity. As the molecular size of the solute increases, its diffusion rate slows, making shorter diffusion path lengths even more important. HALO particles, by virtue of their 0.5  $\mu\text{m}$  porous shell, have reduced the diffusional mass transfer path by one third compared to 3  $\mu\text{m}$  particles. This is why HALO Fused-Core particles are so well suited for the separation of peptides. In fact, the research project that led to the development of Fused-Core particles was motivated by the desire to produce a better stationary phase support for fast, high resolution separation of peptides.

**FIGURE 10:** Effect of Pore Restriction on Chromatographic Performance



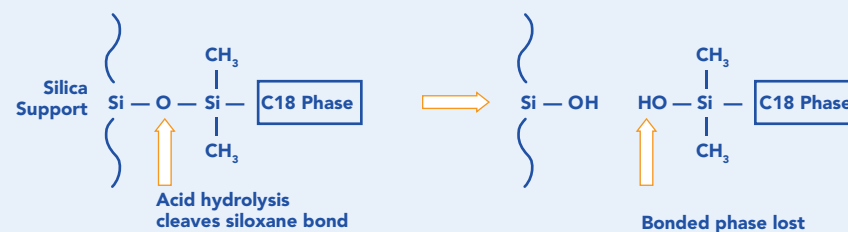
The short diffusion path of Fused-Core particles provides great advantages in speed and resolution, especially for larger molecules that have slower diffusion rates. However, if the pore size of the Fused-Core particle is so small that it restricts the diffusion of larger molecules, such as some peptides, then chromatographic performance will be compromised. In this comparison of the separation of a peptide mixture on columns packed with Fused-Core particles with two different pore sizes, the larger pore size (160 Å) column generated narrower peaks and higher peak capacity.

### HALO Peptide ES-C18 silica support particles are designed specifically for peptide separations

Reversed-phase retention requires that molecules partition into and out of the bonded-phase of the column packing material. Most of the bonded-phase is within the interior of the stationary phase support particle, not on the particle surface. This is true for both totally porous particles as well as the porous layer of the Fused-Core particles. To permit fast, high resolution separation of peptides, the pores must be large enough to permit efficient diffusion of peptide molecules in and out of the pores where they can fully interact with the bonded-phase. If the pore size is too small for the peptides being separated, diffusion will be restricted and the peptides will elute from the column with broad peaks, thus compromising resolution. The advantage offered by the short diffusion path of Fused-Core particles is eliminated if a peptide molecule's diffusion is restricted by the pore size (Figure 10). On the other hand, if the pore size is too large, there will be less surface area and bonded-phase volume for peptides to interact with, thus negatively affecting retention time, resolution, and sample capacity without any corresponding benefit in column efficiency. The 160 Angstrom pore size of the Fused-Core particles used in HALO Peptide ES-C18 columns was specifically selected to provide an optimum pore size for separating peptides.

The Fused-Core particles used in HALO Peptide ES-C18 UHPLC columns are made from extremely pure (>99.99%) silica with no detectable amounts of metal contamination to interfere with the separation of peptides. These unique silica particles also have an extremely homogeneous surface chemistry with minimum acidic silanols to interfere with separations. They benefit from extremely high mechanical stability and are able to withstand very high pressure and flow velocity.

**FIGURE 11:** Acid Hydrolysis of the Siloxane Bond



Acid hydrolysis of the siloxane bond and the resulting loss of bonded phase is one of the major causes of column failure when using acidic mobile phase, such as the typical mobile phase used for peptide separations. Elevated column temperature will accelerate the loss of bonded phase.

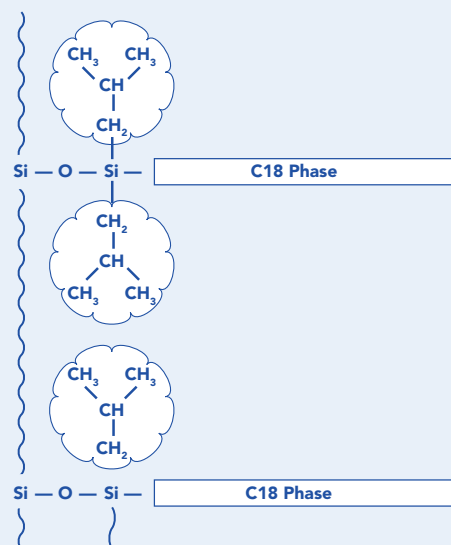
### Extra Stable (ES) bonded phase provides rugged, reliable performance

Typical reversed phase columns are known to be vulnerable to loss of bonded phase due to acid hydrolysis when operating under low pH mobile phase conditions. (Figure 11) This loss in bonded phase results in constantly changing retention times and degradation of resolution as the column is used. The lower the pH of the mobile phase and the higher the column temperature the faster bonded phase will be lost from the stationary phase support.

Commonly used mobile phase conditions for separating peptides include water/acetonitrile gradients with trifluoroacetic acid (TFA) added to both the water and acetonitrile components of the mobile phase. Typically, the mobile phase pH will be 2 or less and elevated column temperatures are commonly used to improve reproducibility and resolution. Unfortunately, these conditions hasten the loss of bonded phase and shorten column lifetime.

HALO Peptide ES-C18 columns avoid the problem of bonded phase loss through the use of sterically protecting bonding technology that inhibits acid hydrolysis of siloxane bonds, even under extremes of high column temperature and low mobile phase pH. This Extra Stable bonding is achieved through the use of bulky side chains on the alkylsilanes that provide steric protection of the siloxane bond (Figure 12).

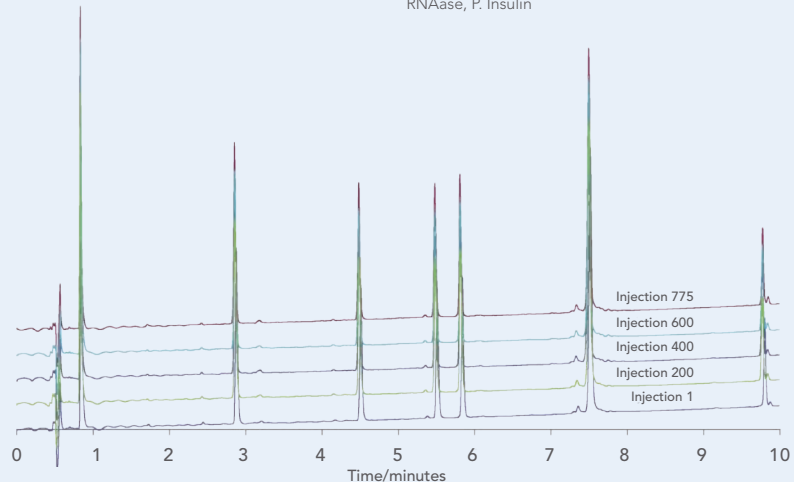
**FIGURE 12:** Extra Stable (ES) Bonded Phase



Specially designed organosilanes with bulky side groups are used for the bonding chemistry of HALO Peptide ES-C18 column packing material. The bulky side groups sterically protect the siloxane bond from acid hydrolysis and yield a stationary phase that is especially stable to the conditions typically used for the separation of peptides.

**FIGURE 13:** Stability testing of HALO Peptide ES-C18 column

**Column:** HALO Peptide ES-C18, 2.1 x 100 mm  
**Mobile Phase:** A: 0.1% TFA; B: 0.1% TFA/70% ACN;  
 Gradient: 9-55% B in 10 min  
**Flow Rate:** 0.5 mL/min  
**Temperature:** 60 °C  
**Sample:** Gly-Tyr, Val-Tyr-Val, Met-enk, Angio II, Leu-enk, RNAase, P, Insulin



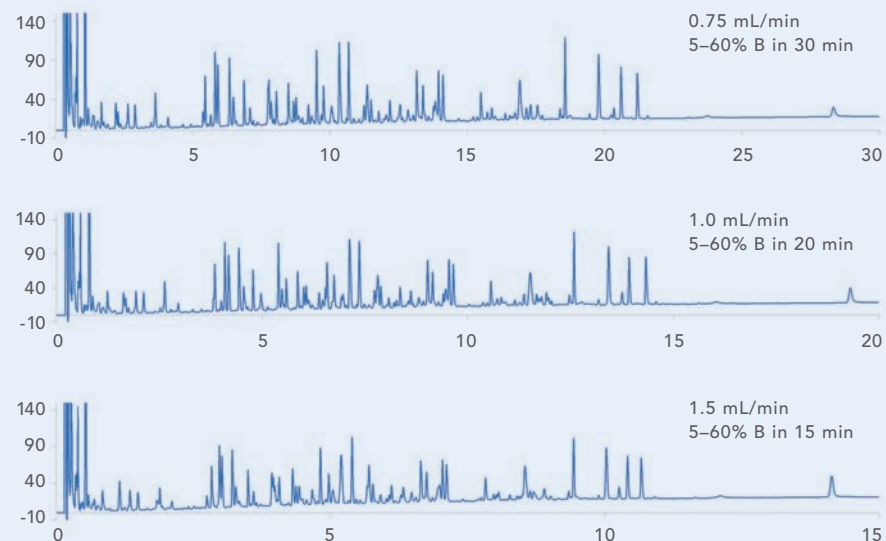
After 775 chromatographic separations of peptides, the HALO Peptide ES-C18 column continues to give highly reproducible results with no evidence of loss of bonded phase, packing bed settling, inlet frit plugging, or any other issues that would render the column unusable.

Extra Stable (ES) bonding technology combined with Fused-Core particle technology yields UHPLC columns that are rugged and reliable, even under adverse conditions that quickly destroy ordinary columns (Figure 13).

Ultra-pure silica, specifically selected 160 Angstrom pore size, and Extra Stable bonded phase yields a column packing material particularly well suited for the separation of peptides. Combine these characteristics with the inherent speed and efficiency offered by Fused-Core particle technology and you have UHPLC columns that provide higher resolution and faster separation of peptides than what has been possible with ordinary columns (Figure 14).

**FIGURE 14:** High speed separation of a tryptic digest

**Column:** HALO Peptide ES-C18, 2.1 x 100 mm  
**Mobile Phase:** A: water/0.1% TFA; B: 80% ACN/0.1% TFA;  
 gradient as indicated  
**Detection:** 215 nm  
**Injection Volume:** 15 µL  
**Sample:** apotransferrin tryptic digest  
**Temperature:** 60 °C



The short diffusion path of Fused-Core particles permits the use of high mobile phase flow velocities for fast separations, without sacrificing resolution. These chromatograms illustrate how increasing the flow rate can reduce separation time, while maintaining resolution. To maintain the same gradient slope, gradient time is scaled by the inverse of the change in flow rate.

“This fast HPLC technology is comparable with ultra high-pressure liquid chromatography (UHPLC) in terms of chromatographic performance but demands neither expensive ultra-high-pressure instrumentation nor new laboratory protocols.”

Analytical Chemistry, August 2007