Optimising Core-Shell UHPLC Columns for Improving Protein and Peptide Separations

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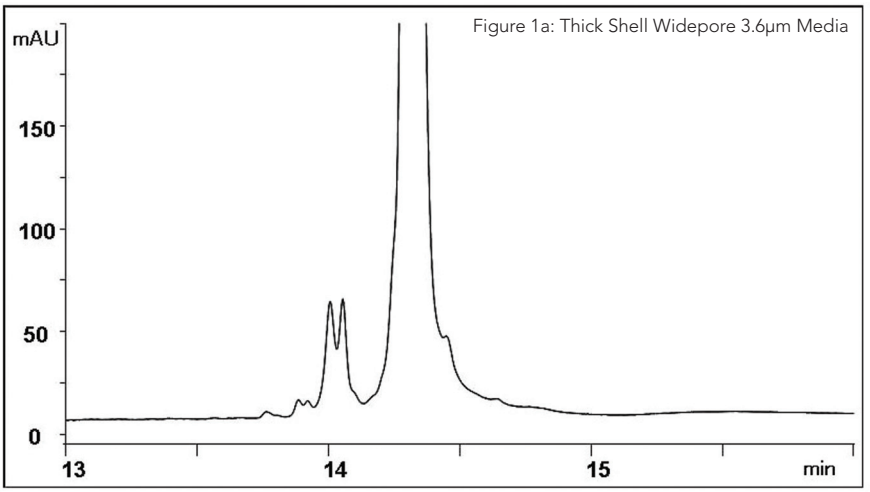
The introduction by several chromatography column manufacturers of sub-2 µm fully-porous as well as core-shell sub-3 µm UHPLC columns over the last several years have profoundly impacted the chromatography industry in the analysis of small molecules. These advances in silica particle technologies have for the most part focused on using increased efficiency as a means of reducing run times while maintaining resolution of existing separations.[1,2] The fundamental concept for all new silica media (sub-2 µm or core-shell) has been to reduce analyte diffusion leading to sharper peaks. By narrowing particle size distribution and optimising packing band broadening effects due to eddy diffusion (the so-called 'A' term of the Van Deemter curve) are minimised leading to an overall increase in performance independent of mobile phase velocity.

The more critical source of band broadening, mass transfer kinetics (the 'C' term of the Van Deemter curve) is closely linked to the diffusion of the analyte in and out of a porous media.[3,4] UHPLC columns (either sub-2µm fully-porous or core-shell) minimise the 'C' term by reducing analyte diffusion distance. Diffusion distance is reduced either through using a smaller fully-porous particle (sub 2 µm columns) or by manipulating the particle geometry such that a thin porous layer "shell" is grafted on a non-porous silica "core". By minimising both the "A" and "C" terms one can realise ultra high performance at very high mobile phase velocities, resulting in reduced run times and high sample throughput.

Continued interest in protein and peptide therapeutics has led to research efforts by many UHPLC column manufacturers for optimised reversed phase separation solutions for proteins and peptides. Unlike small molecule separations where reducing run time and increasing throughput are the principal goals for improved chromatography, for protein and peptide separations the principal focus is improving resolution of closely eluting analytes because any impurities are typically closely related post-translationally modified (PTM) versions of the protein of interest with minimal chemical differences. Furthermore, the parameters required for optimal separation of protein and peptides are often very different; proteins (especially above 30 kilodaltons) are usually fairly hydrophobic due to the large



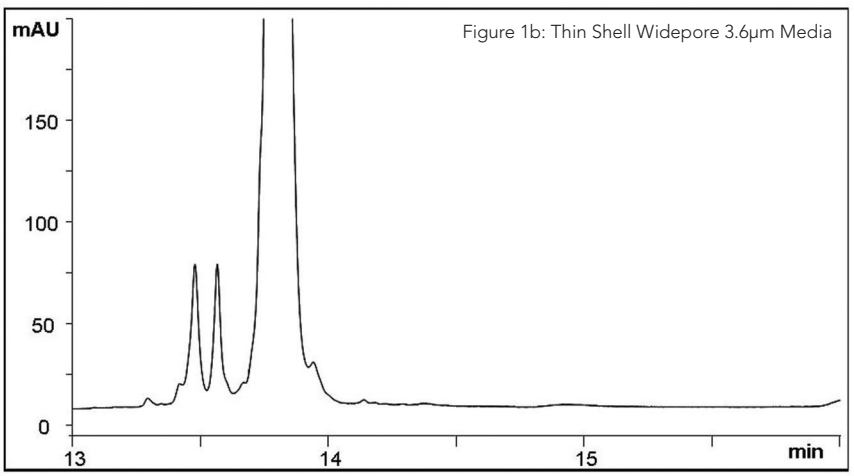


Figure 1: Effect of shell thickness on protein separations with thick shell (a) and thin shell (b) materials. Wide-pore core-shell HPLC columns with varying thickness of the porous shell were evaluated for the separation of degraded lysozyme. Note the increased resolution and narrower peak width for the thin-shell wide-pore media. The conditions used were: Core-Shell Wide-Pore 3.6 µm XB-C18 100 x 4.6 mm column with a flow rate of 1.5 mL/min and column temperature of 50°C. After a 3 minute hold at 3%B, a gradient from 3-65%B in 20 minutes was used.

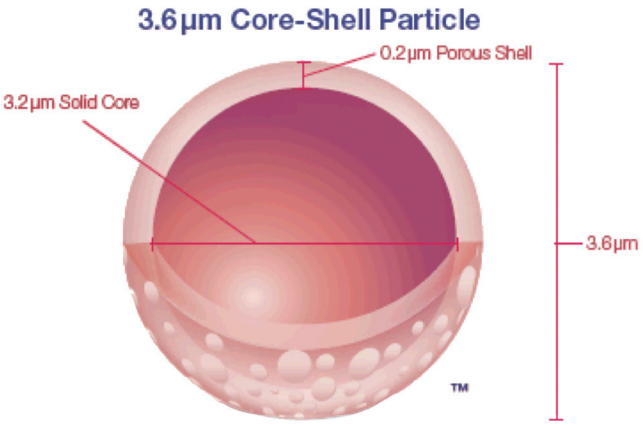


Figure 2: Graphic representation of an optimised protein core-shell silica particle. The Aeris widepore is a 3.6 µm core-shell particle with a 3.2 µm solid core surrounded by a 0.2 µm thin porous shell. Varying selectivities used for the particle include XB-C18, XB-C8, and C4 bonded phases.

stokes radius of protein excluding water and thus protein recovery is usually a major concern in reversed phase chromatography. For peptide separations recovery is less of a concern (due to peptides smaller size) and amino acid sequence plays the primary role in retention. Finally, diffusion and pore accessibility are factors that affect proteins and peptides separations differently; proteins larger than 5-10 kilodaltons molecular weight require the use a wide-pore (typically 300 Å) media to allow access of the media surface to the protein, peptides (being smaller than 5 kilodaltons) have minimal pore access issues with 100 Å media (unless the peptide has been modified with a PEG or other size increasing modification) and can take full advantage of the increased selectivity that the larger surface area of a standard pore-sized column provides.

In the development of core-shell columns for proteins and peptides several observations were made regarding the behaviour of proteins and peptides resulting in the production of two widely divergent particle geometries for protein or peptide separations.

Material and Methods

All chemicals and standard proteins were obtained from Sigma Chemical (St. Louis MO USA). Recombinant Human EGF and Alpha Interferon were purchased from R&D Systems (Minneapolis, MN, USA). Mouse and Human Ig-G were purchased from Dako (Carpenteria, CA, USA) Solvents were purchased from EMD (San Diego, CA, USA). Fully porous 300 Å C18 columns were purchased for various HPLC column vendors. Core-shell Kinetex, Aeris peptide, Aeris widepore as well as developmental beta-test columns were manufactured by Phenomenex (Torrance, CA, USA).

Myoglobin samples were partially degraded by incubation at room temperature for up to a week in dilute acid. Ribonuclease samples were reduced with 100 mM DTT in 50 mM NH_4HCO_3 pH 8.0 for 20 minutes at 45°C; reduced/non-reduced mixtures were generated by spiking different ratios of the native to the reduced sample prior to injection on HPLC. For generating peptide map samples, various proteins were digested with MS grade trypsin generally at a 1:50 E/S ratio (w/w) for 18 hours at 37°C in 0.1 M ammonium bicarbonate pH 8.0. The various protein and peptide digest samples were analysed on an Agilent 1200 HPLC system with autosampler, column oven, solvent degasser, and UV detector set at 214 nm. Data was collected using Chemstation software (Agilent, Santa Clara, CA, USA). Mobile phases used were 0.1% TFA in water (A) and 0.085%TFA in acetonitrile (B) for all chromatograms except 6a and b where mobile phase (A) comprised 0.1% Formic acid in water and mobile phase (B) comprised 0.085% formic acid in acetonitrile While it helps with peptide peak shape and resolution, TFA is especially important for large intact protein analysis in resolving closely related proteins and is still widely used by protein chemists despite its tendency to supress MS signals significantly. Different gradients, flow rates and column temperatures are listed with the corresponding chromatograms.

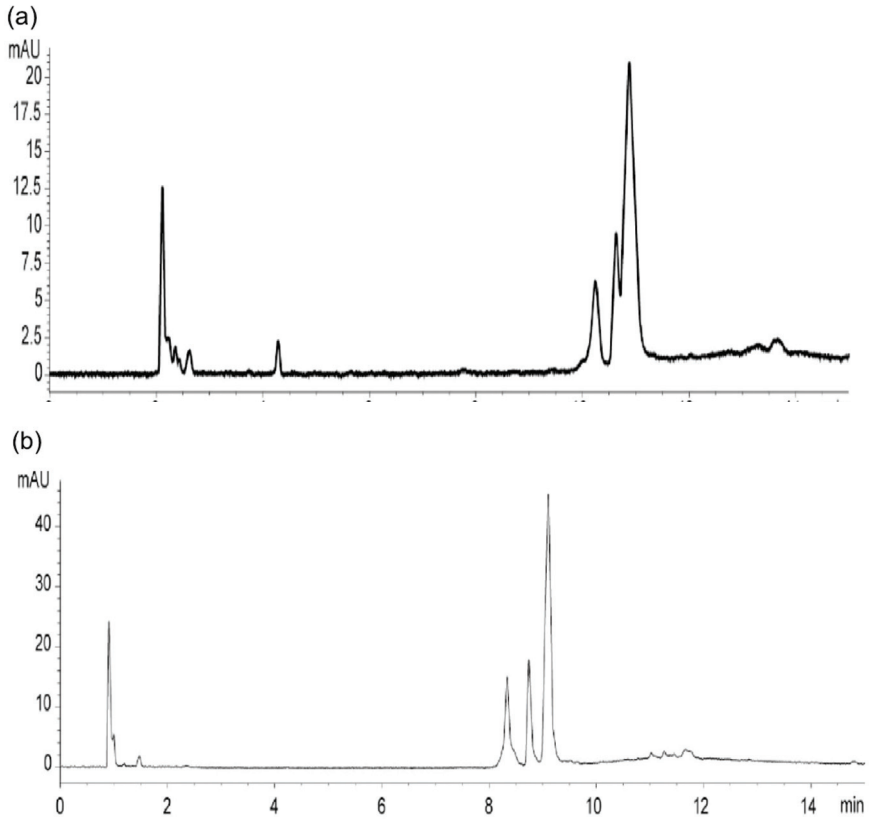


Figure 3: Comparative separation of Ig-G on a fully porous 5 µm 300 Å C18 column (a) and an Aeris widepore 3.6 µm XB-C18 column (b) . Note the increased resolution, narrow peak width, and increased recovery for the widepore core-shell column. The elevated column temperature 80°C is above the maximum of the fully porous column yet well below the temperature limit of the widepore core-shell column. Conditions used for both columns: 100 x 4.6 ID column at flow rate of 1 mL/min. The gradient was from 10%B to 40% B in 15 minutes.

Results and Discussion

Protein Core-Shell Column

In developing an optimised core-shell particle for protein separations several factors were considered including surface area, pore size, shell thickness, particle size and redendant ligand methods. Previous reports[5,6] have shown that diffusion rates of peptide and proteins are inversely proportional to the log of the molecular weight of a protein, thus as proteins increase in size the speed of diffusion through the porous layer becomes the limiting factor in minimising peak width of a protein in reversed-phase chromatography. Unlike fully-porous media where

backpressure considerations limit how much one can reduce the particle size, with core-shell media the thickness of the shell of the particle can be adjusted to improve protein separations. An example is shown in Figure 1 where porous shells of varying thickness were compared in their ability to resolve degraded proteins. Results verify that thin porous layers provide a good way of minimising band dispersion of protein peaks. However, optimising shell thickness is a balance between loading and performance; a thinner shell has a shorter diffusion path, but also lower surface area which limits loading and results in reduced analyte retention. A thicker shell allows for greater loading and retention but reduces performance. Optimising for analytical separations where loading is typically below 20 µg (for a 4.6 mm ID column) led to the development of a particle with a 3.2 µm solid core surrounded by a 0.2 µm porous shell for a total particle size of 3.6 µm (Figure 2).

A standard protein for demonstrating separation of large proteins is immunoglobin Ig-G which is often difficult to separate using existing fully-porous 300Å reversed phase columns. Often elevated temperature and isopropanol in the organic mobile phase are used to overcome peak tailing due the hydrophobicity of this 150 kilodalton protein. Figure 3 shows a comparison between the widepore core-shell 3.6 µm XB-C18 column and a fully porous 5 µm 300 Å C18 column. In this separation (which does not use isopropanol in the organic mobile phase) the wide-pore core-shell column achieves better resolution and recovery over existing fully porous 300 Å media, which illustrates the influence of the reduced diffusion distance that a core-shell material provides over fully porous columns. Another observation of note is the greatly reduced retention of the wide-pore core-shell column over fully porous media. The low hydrophobicity of the thin shell can be an advantage in the separation of large hydrophobic proteins where recovery is a major issue, but limits its utility when working with small polar peptides. In addition, this low hydrophobicity requires one to potentially lower the initial organic concentration of a gradient as the core-shell column may potentially not bind a protein in a tight band if loaded at an elevated organic concentration.

Peptide Core-Shell Column

As previously mentioned, separation requirements for peptides and peptide map applications are significantly different to protein separations. While reduced hydrophobicity may be an advantage for improving protein recovery, for peptides and peptide mapping applications reduced retention can lower resolution of polar peptides. Further, since diffusion is significantly faster for peptides versus proteins, diffusion advantages of thin porous shell are outweighed by increased separation, retention, and capacity that a thicker shell provides. The resultant 3.6 µm peptide core-shell particle scales to other core-shell particles with a 2.6 µm solid core surrounded by a 0.5 µm porous shell (Figure 4). When compared against fully porous sub-2 µm particles, similar peak counts and peak widths are observed for the 3.6 µm core-shell peptide media at a backpressure approximately a quarter of the sub-2 µm column (125 bar vs. 465 bar) which makes the peptide column amenable to operation on a standard 400 bar limited HPLC (Figure 5). Use of a scaled core to shell ratio allows for method portability to smaller (Aeris PEPTIDE

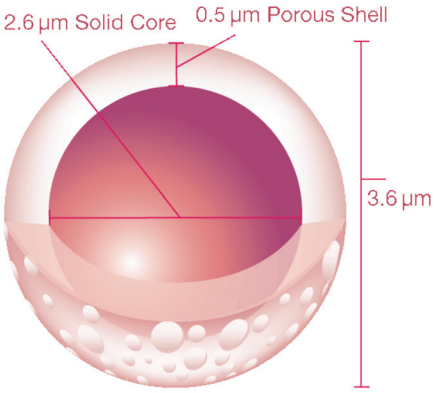


Figure 4: Graphic representation of an optimised peptide core-shell silica particle. The Aeris PEPTIDE 3.6 µm XB-C18 is a 3.6 µm core-shell particle with a 2.6 µm solid core surrounded by a 0.5 µm thin porous shell. The core to shell ratio is similar to an Aeris PEPTIDE 1.7 µm XB-C18 which allows for some method portability between HPLC and UHPLC instrumentation.

1.7 µm XB-C18) core-shell particle UHPLC columns if 1000 bar system capability is available. However the development of a 3.6 µm core-shell column allows for use of longer

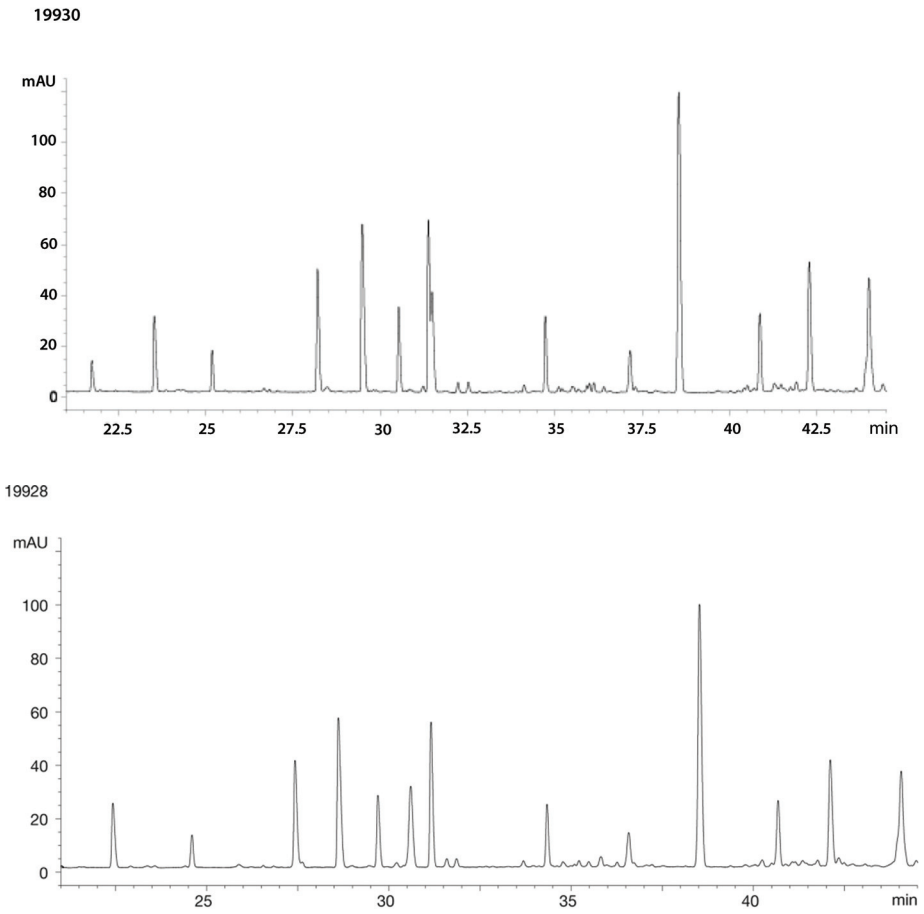


Figure 5: Comparative separation of an Ig-G peptide between fully-porous sub-2 µm (a) C18 and 3.6 µm peptide core-shell (b) columns. Note the similar peak width and peak count between the two columns. The most notable differences between the peptide maps are the column backpressures where the core-shell column has a backpressure of 125 bar and the fully-porous sub-2 µm column has a backpressure of 465 bar. The low backpressure of the Aeris PEPTIDE 3.6 µm XB-C18 column makes it amenable to using longer column lengths to improve resolution. Columns used were 150 x 2.1 mm for both the core-shell and the fully-porous column. Flow rate was 0.3 mL/min and column temperature was 40°C. The gradient used was held isocratically at 3% organic for 5 minutes followed by a 3% to 45%B organic gradient.

columns for improved resolution of complex peptide map separations. An example separation is shown in Figure 6 where 150 mm and 250 mm length core-shell peptide columns were compared for a peptide map separation of BSA. One can note the improved resolution of several peptides using the 250 mm length column while still maintaining HPLC compatible backpressures (200 bar). Because time is not typically the major constraint in peptide map applications, one could conceivably couple columns to get additional resolution of critical components (though not shown in this paper).

Conclusions

Historically separation of peptide and proteins have often been coupled together because they both consist of oligomers of amino acids. Many assume incorrectly that features that make up a good separation device for a small peptide would apply equally to a large protein 10 to 100 fold larger in size. The two characteristic differences between large proteins and small peptides that influence chromatography are diffusion and tertiary structure.

For proteins the 3-dimensional folding of a protein plays an increasingly important role in chromatographic behaviour as domains of a protein displace water leading to a general increase in hydrophobicity as a protein gets larger. This increase in hydrophobicity necessitates a less retentive stationary phase for better protein recovery while still attempting to retain some degree of selectivity over small differences in a protein's chemical and physical structure. The second discriminating feature of protein separation versus peptides is the dramatically slower rate of diffusion observed as proteins increase in size. This slower rate of diffusion suggests the use of a very thin porous layer in the development of core-shell columns specifically designed for the separation of proteins. Data showing the impact on performance by varying the thickness of the Aeris widepore column confirm previous data on protein diffusion and highlights the value of 'tuning' the porous layer for optimising large protein separations. Resolution increases in the separation of immunoglobin Ig-G and other large proteins adds credibility to designing core-shell particles specifically for protein separations.

For peptide separations, diffusion and 3-dimensional structure play a much smaller role in chromatographic behaviour compared to actual chemical differences in amino acid sequence and any post-translational modifications. In the case of peptide map applications one is typically faced with separating peptides across a large range of sizes, hydrophobicities and polar functionalities. Maximising surface interactions with the stationary phase thru increased surface area and densely bonded ligands are key in resolving closely related peptides. In addition, most peptide maps have one or several polar peptides in the mixture that require the use of a hydrophobic phase to both retain and resolve such peptides. The core-shell Aeris PEPTIDE 3.6 µm XB-C18 phase was developed to provide an alternate 'tuned' solution for peptide and peptide mapping applications. The use of a scaled porous layer on a large solid core resulted in a phase with comparable peak width of sub-2 µm phases at a fraction of the column backpressure. This lower backpressure provides options for modulating column length based on analysis time and resolution requirements.

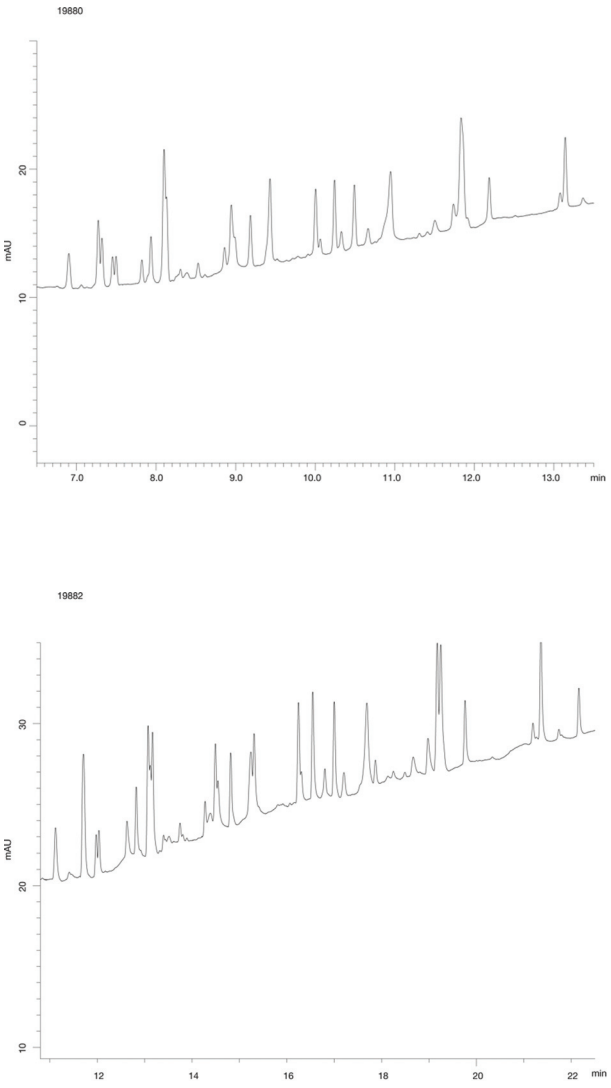


Figure 6: Influence of column length on peptide resolution. Tryptic digests of BSA were compared between 150 x 4.6 mm (a) and 250 x 4.6 mm (b) length columns of Aeris PEPTIDE 3.6 µm XB-C18. Note the increase resolution of peak clusters early (8 min vs. 13 min) and later (12 min vs. 19 min) in the map when the longer 250 x 4.6 mm column is used. As the larger particle Aeris PEPTIDE was developed on a large particle (3.6 µm) backpressures are low enough (140 bar and 200 bar respectively) for longer columns to be used to increase resolution of closely eluting peptides. Conditions used were a flow rate of 1.2 mL/min, a column temperature of 40°C, and an initial 3 minute hold at 3%B followed by a gradient from 3% to 65%B in 30 minutes.

While the debate continues regarding the optimal solution for reversed phase small molecule separations (core-shell versus fully-porous media), for protein and peptide separations core-shell media provide some unique advantages over fully porous media. As it is possible to decouple column backpressures from analyte diffusion (and thus performance) one can design silica particles for the specific separation requirements that a biomolecule analysis necessitates.

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