Chromatography Solutions

Technical note #037

Avantor® ACE® Wide Pore HPLC Columns for the Separation and Purification of Proteins in Biopharmaceuticals

INTRODUCTION

The analysis of peptides and proteins is critical for ensuring the safety and efficacy of biopharmaceuticals. The comprehensive characterisation protein-based therapeutics, is a highly complex challenge and will typically involve a combination of complimentary analyses, targeting different critical quality attributes of the protein. Chromatography plays a crucial role in confirming product characterisation and the determination of related impurities. Figure 1 summarises the key chromatographic techniques that are typically employed. Determination of the target protein in its intact and aggregated forms, the assessment of charge variants, along with detailed analysis of various enzymatically cleaved fragments and characterisation of non-proteinaceous components (e.g. glycan distributions of glycoproteins) are commonplace.

Reversed-phase liquid chromatography (RPLC) is a powerful and widely applied tool for the separation and characterisation of both intact proteins and peptides. The technique shows excellent robustness, reproducibility and, crucially, is readily coupled to mass spectrometry. Additionally, RPLC is a high-resolution technique and is therefore ideally suited to the analysis of complex samples, such as those produced by enzymatic digestion in peptide mapping,[1] but is also valuable for intact protein analysis. In the latter case, the high resolving power can be used to resolve hydrophobic variants, including modified proteins that are similar in structure to the target protein, such as single amino acid post-translational modifications.

Whilst high resolution separations of peptides and complex protein digests can be readily achieved using columns packed with high-purity, high-performance silica-based particles with a pore size of approximately 80-100 Å (e.g. Avantor® ACE® Excel® C18), intact proteins require a modified approach. Proteins have considerably higher molecular weights and are therefore physically much larger. For example, a monoclonal antibody (mAb) has a molecular weight of ~150,000 Da compared to ~150-1,000 Da for typical small molecule drugs. Protein RPLC separations therefore require a stationary phase with a much larger pore size, typically of the order

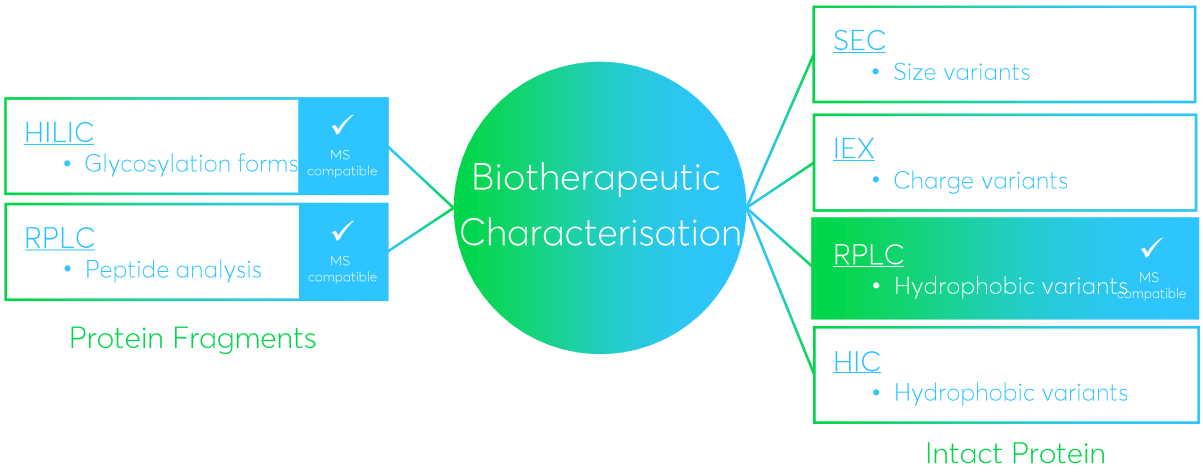

Figure 1: Summary of the key chromatographic techniques used for the analysis of critical quality attributes in protein biotherapeutics.

300-500 Å. Using a 100 Å phase for a protein separation results in poor accessibility of the analyte into the stationary phase pores, thereby restricting diffusion and increasing peak broadening and potentially tailing. The use of wider-pore materials was found to solve this issue by improving pore accessibility to large molecules.

Avantor® ACE® wide pore columns have been specifically engineered to produce highly reproducible separations for a wide range of peptides, proteins and other high molecular weight biomolecules. Based on ultra-high purity silica, they provide exceptional chemical stability, peak shape, sensitivity and column lifetime, allowing highly efficient separations of proteins to be achieved. Both fully porous (FPP) and superficially porous or solid-core silica particles (SPP) are available, as summarised in Table 1. Multiple column chemistries are available to fully explore column selectivity during method development.

Table 1: Specifications of Avantor® ACE® fully porous and solid-core wide pore columns.

Phase	Particle type	USP listing	Functional group	End capped	Particle size (µm)	Pore size (Å)	Surface area (m^2/g)	Carbon load (%)	pH range
Fully porous phases									
C18-300	FPP	L1	Octadecyl	Yes	3, 5, 10	300	100	9.0	2-8
C8-300	FPP	L7	Octyl	Yes	3, 5, 10	300	100	5.0	2-8
C4-300	FPP	L26	Butyl	Yes	3, 5, 10	300	100	2.6	2-8
CN-300	FPP	L10	Cyano	Yes	3, 5, 10	300	100	2.8	2-8
PHENYL-300	FPP	L11	Phenyl	Yes	3, 5, 10	300	100	2.6	2-8
Solid core phases									
UltraCore BIO C18	SPP	L1	Octadecyl	Yes	3.5	300	16	1.0	1-8
UltraCore BIO C18	SPP	L1	Octadecyl	Yes	2.5	500	23	1.4	1-8
UltraCore BIO C4	SPP	L26	Butyl	Yes	3.5	300	16	0.4	2-9
UltraCore BIO C4	SPP	L26	Butyl	Yes	2.5	500	23	0.6	2-9
UltraCore BIO Phenyl2	SPP	L11	Diphenyl	Yes	3.5	300	16	0.7	2-9
UltraCore BIO Phenyl2	SPP	L11	Diphenyl	Yes	2.5	500	23	1.0	2-9

ANALYSIS OF INTACT PROTEINS BY RPLC USING AVANTOR® ACE® FULLY POROUS PARTICLES (FPP)

Intact protein separations are typically achieved using an acetonitrile/water gradient, with the addition of trifluoracetic acid (TFA) as an ion-pairing agent. Generally, separations are run at elevated temperatures up to 90 °C to improve analyte diffusivity and mass transfer, thereby improving peak shape, resolution and reducing protein adsorption.[2] Figure 2 demonstrates the high efficiency separation of a set of peptides and proteins with varying molecular weights using a broad scouting gradient run on a fully porous Avantor® ACE® 3 C4-300 column. Of particular note is the ability to separate the three insulin variants of human, bovine and porcine origin. These insulins have molecular weights of approximately 5,800 Da with only slight variation in their amino acid sequences. Despite this, the ACE 3 C4-300 provides sufficient chromatographic performance to separate these sample components.

0.1% TFA has historically proved popular for use as a mobile phase additive for RPLC protein analysis, however other non-ion-pairing additives such as 0.1% formic acid are gaining popularity, as they avoid potential issues with ion-suppression when coupled with mass spectrometry. Modern columns based on type B ultra-pure silica, such as Avantor® ACE® columns offer distinct advantages in terms of efficiency and peak shape for biomolecule analysis. The presence of metal ion impurities can result in undesirable secondary interactions with the target analytes and also increases the acidity of residual silanols at the silica surface. This can result in excessive peak tailing, low performance and reproducibility challenges. These effects are dramatically reduced for type B ultra-pure silica based columns and it is often possible to significantly reduce the amount of mobile phase additive (Figure 3). This is highly beneficial

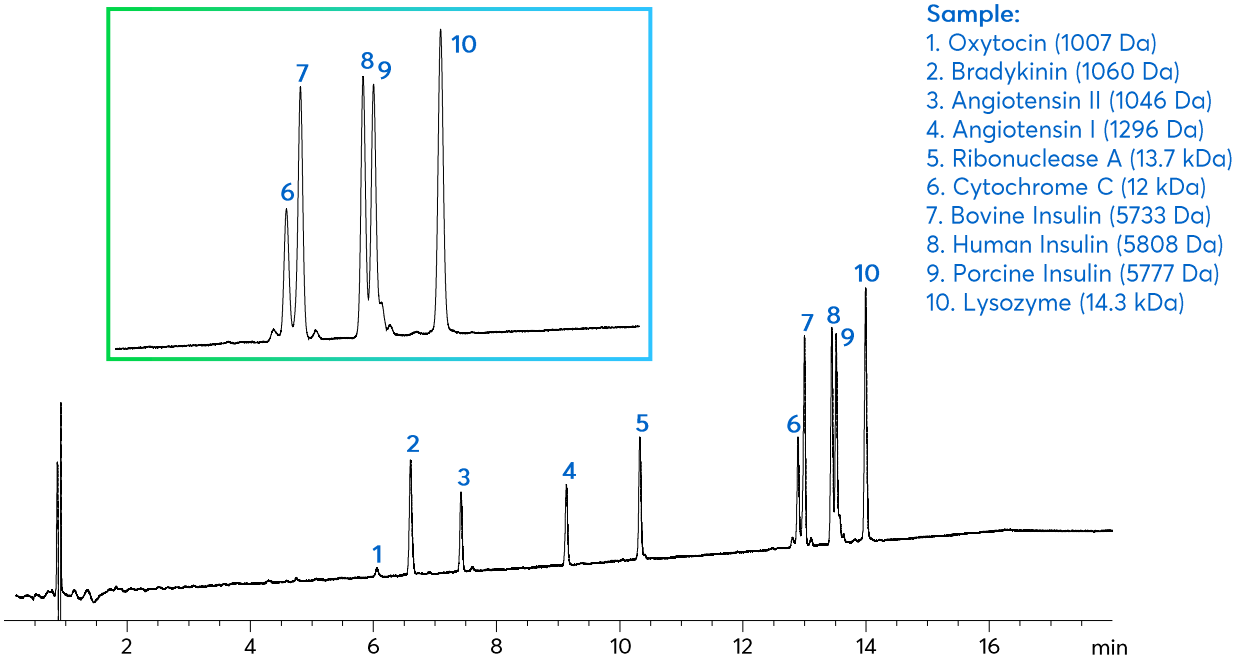

Figure 2: Reversed-phase separation of a range of peptides and proteins with varying molecular weights on an Avantor® ACE® 3 C4-300 (150 x 2.1 mm). Mobile phase A: 0.1% TFA in H_2O, B: 0.1% TFA in MeCN/H_2O 80:20 v/v; Gradient: 10 to 50% B in 15 minutes; Flow rate: 0.5 mL/min; Temperature: 60 °C; Detection: UV (220 nm).

for both UV (reduced background adsorption and better sensitivity) and MS detection (reduced ion suppression and increased sensitivity).

The separation of closely related protein variants is a common theme in protein characterisation. In this situation, shallower gradient profiles are often employed to provide enhanced resolution of closely related proteins. Figure 4 shows how this approach can be used for the analysis of milk proteins. A much shallower acetonitrile/water gradient was employed on an Avantor® ACE® 5 C18-300 column to elute a series of

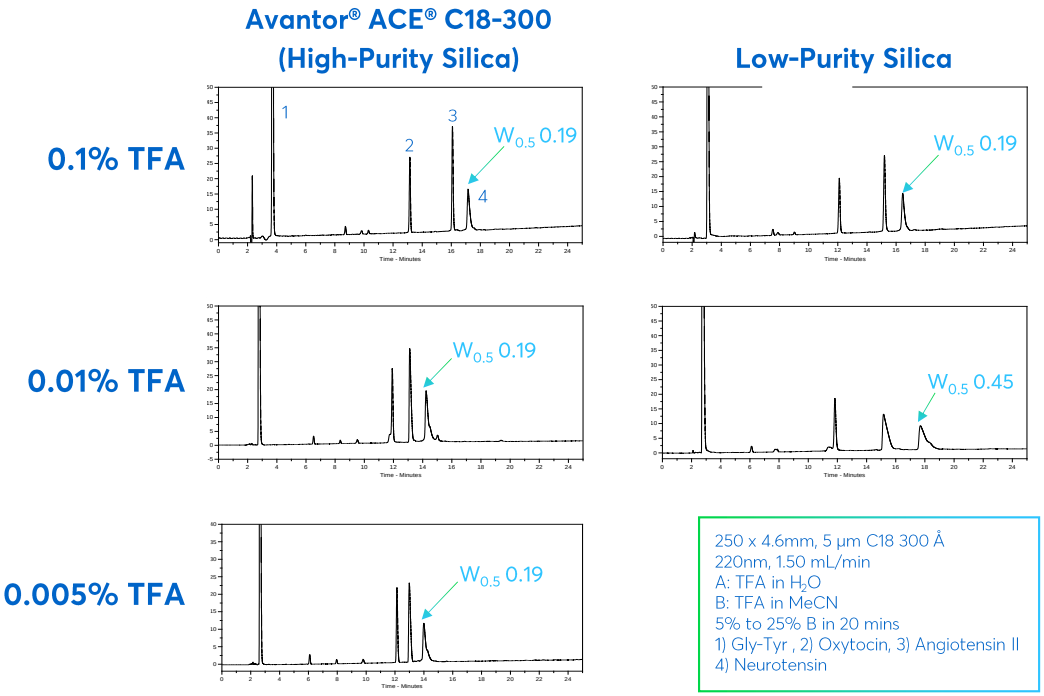

Figure 3: The effect of lowering mobile phase modifier (TFA) concentration with low- and high-purity silica base columns.

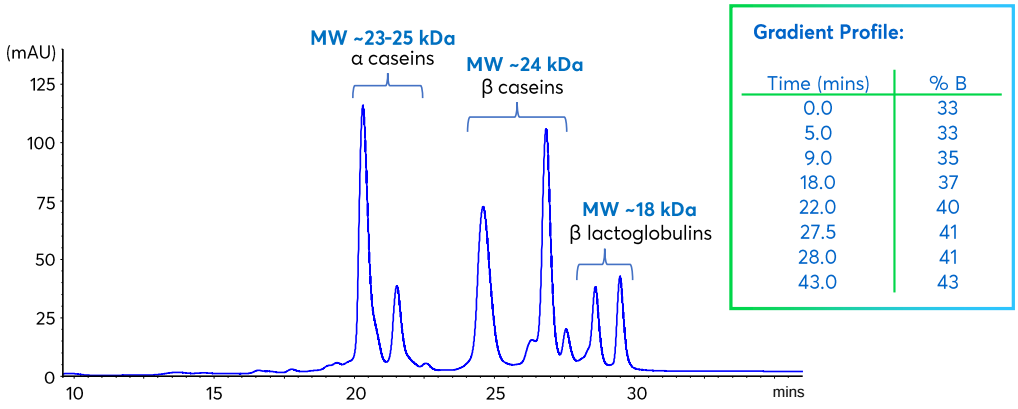

Figure 4: Separation of milk proteins using the Avantor® ACE® 5 C18-300. Column dimension: 150 x 2.1 mm; Mobile phase A: 0.01% TFA in H_2O, B: 0.01% TFA in MeCN; Gradient: as shown above; Flow rate: 0.2 mL/min; Temperature: 45 °C; Detection: UV 214 nm. Reproduced with permission of The Chemical Analysis Facility, University of Reading, UK.

casein and lactoglobulin proteins between 20 and 30 minutes. The high efficiency and excellent peak shape of the ACE column allows for the successful separation of casein variants which differ in their structure by as little as one amino acid substitution. In this example, the concentration of TFA in the mobile phase has been reduced by a factor of 10 to just 0.01%, which would dramatically improve sensitivity if MS detection was employed. This is possible because of the high inertness of the ACE silica surface, which allows excellent peak shape to be obtained for proteins, even at low concentrations of TFA.

INTACT PROTEIN ANALYSIS USING AVANTOR® ACE® ULTRACORE SOLID-CORE COLUMNS

One of the key performance limitations of RPLC analysis of proteins is that the large analyte molecular weights give rise to slow molecular diffusion, resulting in broadening of chromatographic peaks. RPLC separation of intact proteins tend to be run at elevated temperature to counteract this. An alternative resolution could be to use non-porous stationary phase particles to reduce mass transfer, however, non-porous particles suffer from poor loadability.[3] More recently, solid-core (or superficially porous, SPP) particles were introduced to the market, so called as they are comprised of a solid, non-porous core, surrounded by a porous outer shell (Figure 5).

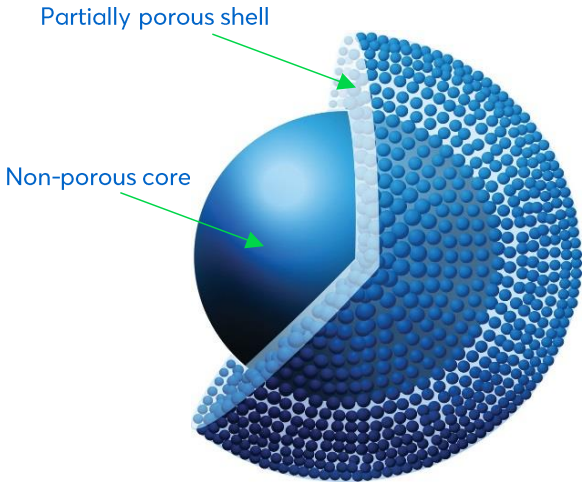

Figure 5: Schematic representation of a solid-core particle.

This particle morphology provides several benefits over traditional porous particles for the analysis of therapeutic proteins. Firstly, the monodispersed nature of solid-core particles results in more uniformly, "better" packed columns, reducing intra-column flow path variation (eddy-diffusion, van Deemter A-term).[4] Additionally, longitudinal dispersion is reduced because of a reduction in column dead volume provided by the non-porous core (B-term). Finally, detrimental mass transfer effects are reduced as the analyte diffusion path length is reduced by the thin, porous shell compared to fully porous particles (C-term). This final effect provides a particular benefit to the analysis of larger molecular weight analytes, such as proteins, due to their inherently slower diffusion rates. This improved mass transfer is also exaggerated at higher mobile phase flow rates. The improved kinetic performance for solid-core particles therefore means that they have great potential to provide higher throughput and efficiency for intact protein separations, compared to fully porous particles.

Avantor® ACE® UltraCore BIO columns have been developed to offer multiple solutions for the high-efficiency separation of large biomolecules, such as proteins (Table 1). The range features solid-core particles in two different particle size/pore size combinations to ensure that the optimum pore size can be determined for any separation. These wide-pore columns are suitable for the analysis of proteins and peptides with molecular weights over 5 kDa. Three different stationary phases are available to help fine tune the selectivity of protein separations. The C4 phase provides less hydrophobicity than a C18 phase, whilst the Phenyl2 phase can provide additional pi-pi interactions with the aromatic amino acids phenylalanine, tryptophan and tyrosine, and can therefore provide alternative selectivity and retention to the C4 and C18 phases.[5]

Figure 6 demonstrates the use of a wide-pore 500 Å Avantor® ACE® UltraCore BIO column to provide a high efficiency RPLC separation of a mixture of proteins and peptides. High performance and resolution are obtained for this broad range of analytes, which ranged in mass from 5.8 to 45 kDa, demonstrating the broad applicability of wide-pore UltraCore BIO phases for the analysis of intact proteins.

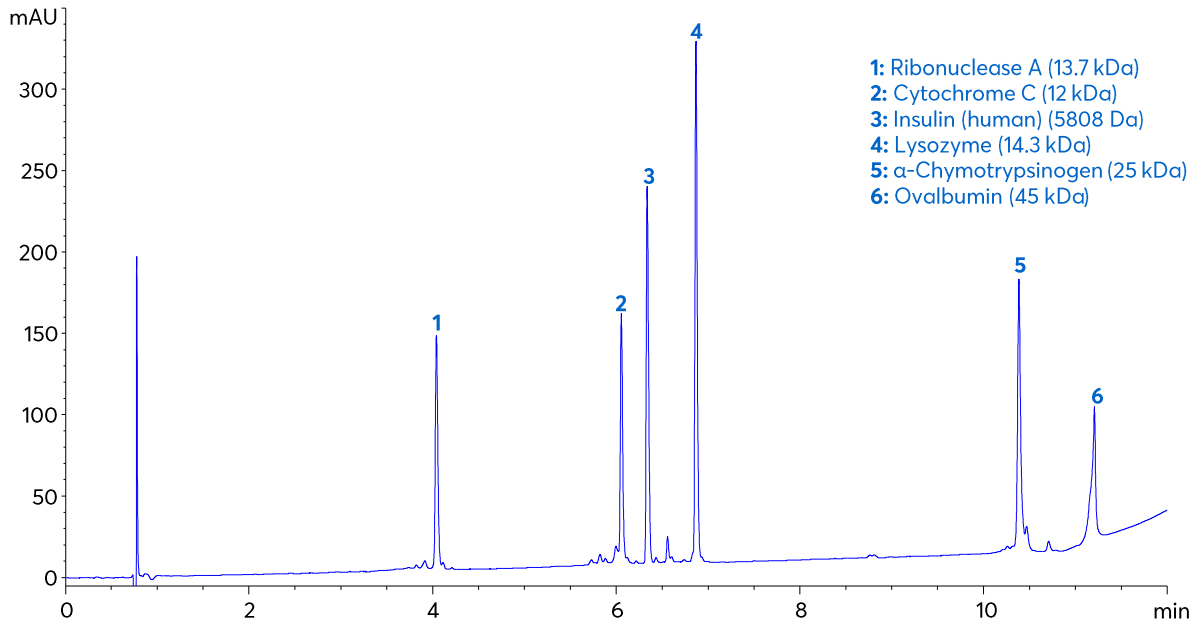

Figure 6: Separation of a range of peptides and proteins on an Avantor® ACE® UltraCore BIO C4-500 column. Column dimensions: 100 x 3.0 mm; Mobile phases: A: 0.1% TFA in H_2O, B: 0.1% TFA in MeCN/H_2O 90:10 v/v; Gradient: 20 to 50% B in 10 minutes, then 50 to 100% B in 2 minutes; Flow rate: 0.6 mL/min; Injection volume: 5 µL; Temperature: 60 °C; Detection: UV, 220 nm.

The high molecular weight of mAbs means that achieving high-resolution, high-throughput analyses of higher molecular weight proteins such as mAbs can be challenging, due to slow molecular diffusion and hindered pore access. The particle morphology of Avantor® ACE® UltraCore BIO means that highly efficient separations can be achieved for these analytes. Figure 7 demonstrates how the 500 Å C4 phase can achieve a similarly high-resolution separation of a mAb. In this case the NIST mAb analysed has a much greater molecular mass of 150 kDa. Using 0.1% TFA as the modifier, along with an elevated temperature of 80 °C, a rapid, high-resolution separation is readily achieved, with sample impurities clearly discernible from the main API peak.

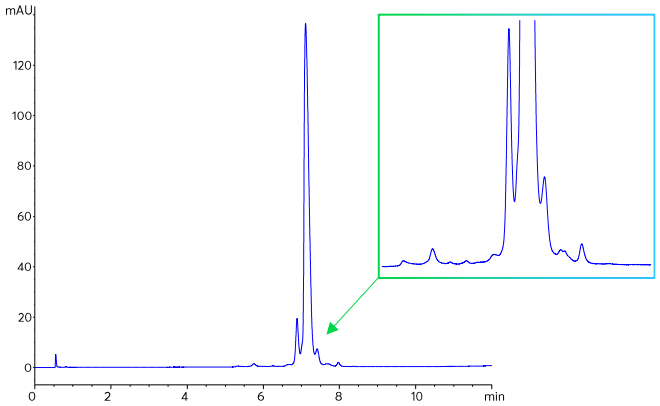

Figure 7: Analysis of NIST mAb on an Avantor® ACE® UltraCore BIO C4-500 column. Column dimensions: 100 x 3.0 mm; Mobile phases: A: 0.1% TFA in H_2O, B: 0.1% TFA in MeCN/H_2O 90:10 v/v; Gradient: 36 to 45% B in 10 minutes, then 45 to 80% B in 2 minutes, hold at 80% B for 2 minutes; Flow rate: 0.8 mL/min; Injection volume: 1 µL; Temperature: 80 °C; Detection: UV, 280 nm.

CONCLUSION

The analysis and characterisation of intact protein biotherapeutics is a complex analytical challenge requiring application of a range of analytical techniques. The high The high resolving power of reversed-phase liquid chromatography and compatibility with mass spectrometry makes it a valuable tool for the separation of intact proteins and their closely related variants. The

successful analysis of such compounds requires the use of a wide pore (300-500 A) stationary phase, operated at elevated temperatures, low pH and with relatively shallow gradients. Avantor® ACE® wide pore columns offer a comprehensive range of options for the high resolution analysis of intact proteins. Both fully porous and solid-core particles are available, bonded with a variety of complementary stationary phase chemistries, enabling the optimisation of stationary phase selectivity during method development. The use of ultrapure and highly inert base silica means that lower concentrations of mobile phase modifiers can be used without compromising analyte peak shape, which is potentially highly beneficial for LC-MS applications.

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