Chromatography Solutions

Technical note #028

Analysis of intact proteins using Avantor® ACE® UltraCore BIO columns

INTRODUCTION

In recent years, interest in the chromatographic analysis of proteins has increased markedly, largely driven by the emergence and successes of protein-based therapeutics. Due to the inherent complexity of proteins compared to small molecules, a range of analytical approaches are typically employed for their characterisation in accordance with the requirements from regulatory bodies. This Knowledge Note discusses some of the key concepts for the analysis of proteins in their intact forms by reversed-phase liquid chromatography (RPLC) and how the Avantor® ACE® UltraCore BIO range of columns has been developed to meet the challenges involved in such separations.

ANALYSIS OF INTACT PROTEINS BY RPLC

The comprehensive characterisation of proteins, including protein-based therapeutics, is a highly complex challenge and will typically involve carrying out a combination of complimentary analyses targeting different critical quality attributes of the protein, with the ultimate aim of ensuring drug product safety and efficacy (Figure 1). For protein-based drugs, determinations 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. The use of RPLC for the analysis of peptides and proteins is well established and is widely used. 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 this 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. Additionally, RPLC shows excellent robustness and reproducibility and, crucially, is readily coupled to mass spectrometry for use in structural characterisation. The application of RPLC to intact protein analysis is the focus for this article.

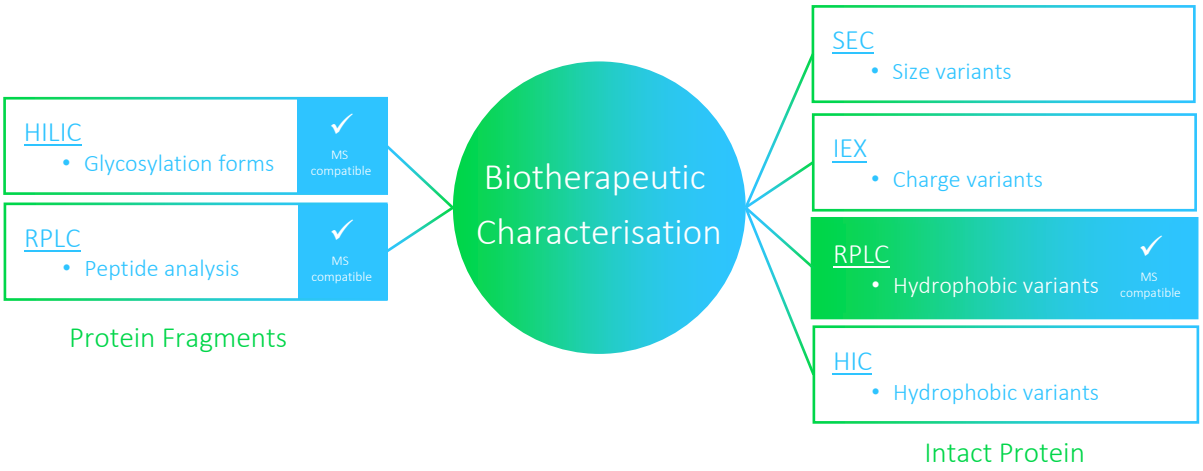


Figure 1: Summary of the chromatographic modes used for the analysis of critical quality attributes in protein biotherapeutics.

Small molecule reversed-phase separations are typically carried out using stationary phase particles with pore sizes of 80-100 Å. However, 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. One of the fundamental aspects of protein RPLC separations is that proteins require a stationary phase with much larger pore sizes, typically of the order 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 resulting in peak broadening and potentially tailing. The use of wider-pore materials was found to solve this issue by improving pore accessibility to large molecules. Figure 2 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 phases for the analysis of intact proteins. Wide-pore stationary phases are typically bonded with a hydrophobic ligand, such as C18, with shorter chain ligands such as C8 and C4 also proving useful for larger/more hydrophobic proteins. Stationary phases bonded with alternative ligands (e.g. phenyl) are also available to provide options for alternative selectivity.

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]. It is important to note that not all columns are compatible with the use of high temperatures, potentially being prone to acid hydrolysis of the stationary phase at elevated temperature. Optimisation of the separation is typically achieved by adjusting gradient range and slope, or by utilising columns with different stationary phase chemistries. Mobile phases often include an ion-pairing agent to reduce secondary interactions with the stationary phase surface and improve peak shape and efficiency. 0.1% TFA has historically proved popular, 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. For modern columns based on type B ultra-pure silica, it is often possible to reduce the amount of additive to 0.05% or less, as secondary interactions are much less problematic [3].

ADVANTAGES OF 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 bands. Increased

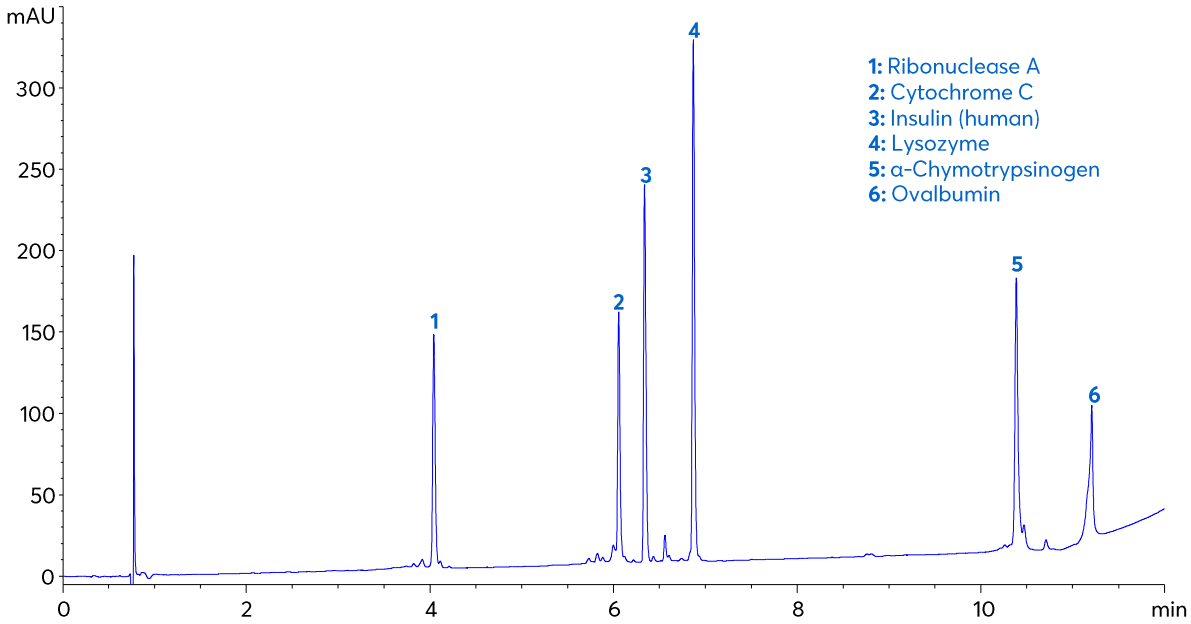


Figure 2: Separation of a range of peptides and proteins on an Avantor® ACE® UltraCore BIO C4-500 column. Column dimensions: 100 x 3.0 mm; Mobile phases: A: 0.1% TFA in H_2O, B: 0.1% TFA in MeCN/H_2O 90:10 v/v; Gradient: 20 to 50% B in 10 minutes, then 50 to 100% B in 2 minutes; Flow rate: 0.6 mL/min;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

molecular diffusion at higher temperature is the reason for protein RPLC separations typically being run at elevated temperatures, to partially aid this issue. An alternative resolution could be to use non-porous stationary phase particles to reduce mass transfer, however, non-porous particles suffer from poor loadability [4]. More recently, solid-core 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 3).

This novel particle morphology offers several key 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) [5]. 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. This 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.

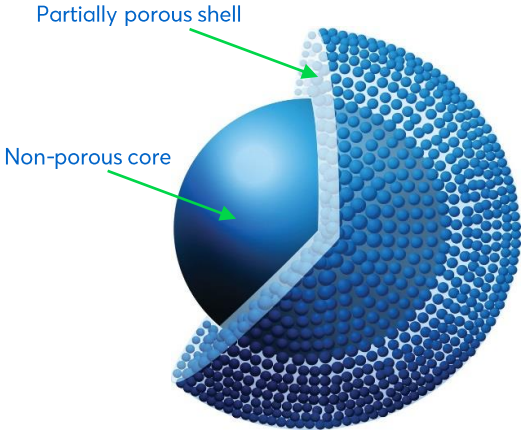


Figure 3: Schematic representation of a solid-core particle.

AVANTOR® ACE® ULTRACORE BIO COLUMNS FOR INTACT PROTEIN ANALYSIS

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 presents the specifications for the different phases available. 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. [6]

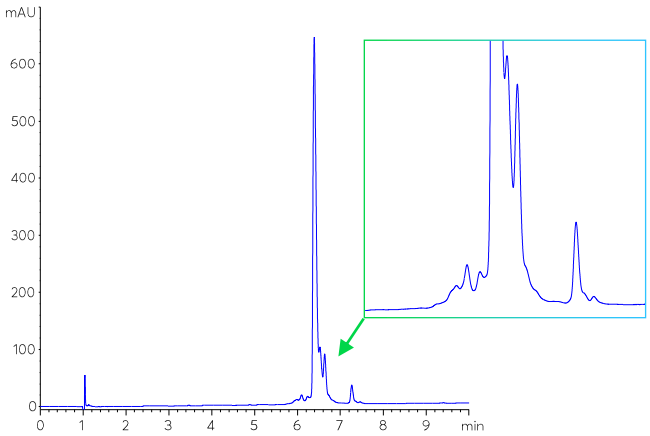


Figure 4: Analysis of α-chymotrypsinogen on an Avantor® ACE® UltraCore BIO C4-500 column.
Column dimensions: 100 x 3.0 mm; Mobile phases: A: 0.1% TFA in H_2O, B: 0.1% TFA in MeCN/H_2O 90:10 v/v; Gradient: 40 to 50% B in 10 minutes, then 50 to 100% B in 2 minutes, hold at 100% B for 2 minutes; Flow rate: 0.43 mL/min; Injection volume: 5 µL; Temperature: 80 °C; Detection: UV, 220 nm.

Table 1: Specifications of Avantor® ACE® UltraCore BIO columns.

Phase	USP listing	Functional group	End capped	Particle size (µm)	Pore size (Å)	Surface area (m^2/g)	Carbon load (%)	pH range	Low pH max temp.
UltraCore BIO C18	L1	Octadecyl	Yes	3.5	300	16	1.0	1-8	90 °C
UltraCore BIO C18	L1	Octadecyl	Yes	2.5	500	23	1.4	1-8	90 °C
UltraCore BIO C4	L26	Butyl	Yes	3.5	300	16	0.4	2-9	90 °C
UltraCore BIO C4	L26	Butyl	Yes	2.5	500	23	0.6	2-9	90 °C
UltraCore BIO Phenyl2	L11	Diphenyl	Yes	3.5	300	16	0.7	2-9	90 °C
UltraCore BIO Phenyl2	L11	Diphenyl	Yes	2.5	500	23	1.0	2-9	90 °C

Figure 4 shows an example of an ACE UltraCore BIO C4 column for the analysis of α-chymotrypsinogen (25.6 kDa). Using 0.1% TFA as the modifier, along with an elevated temperature of 80 °C, a rapid, high-resolution separation of the parent protein from closely eluting impurities is readily achieved. As protein molecular weight increases, achieving high-resolution, high-throughput analyses can become more challenging due to slow molecular diffusion and hindered pore access, as discussed above. The particle morphology of ACE UltraCore BIO means that highly efficient separations can be achieved for even mAbs. Figure 5 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 similar conditions, a very similar separation is obtained with this larger protein, with sample impurities clearly discernible from the main API peak.

An additional benefit to the ACE UltraCore BIO range is the inclusion of multiple stationary phases, which can be used to obtain optimised separations of intact proteins. Figure 6 shows how all three phases can provide high-resolution separations of trastuzumab. In this example, clear differences can be observed between the three phases. The C4 is the least retentive phase, followed by the C18 phase. The Phenyl2 phase provides additional retention of trastuzumab and enhanced separation of the mAb from closely eluting impurities, due to additional pi-pi interactions between aromatic amino acid side chains and the stationary phase ligand.

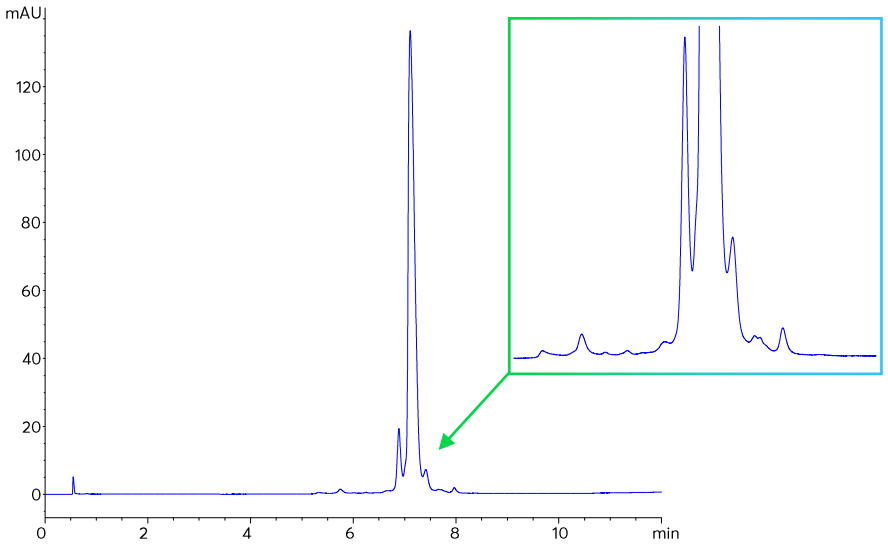
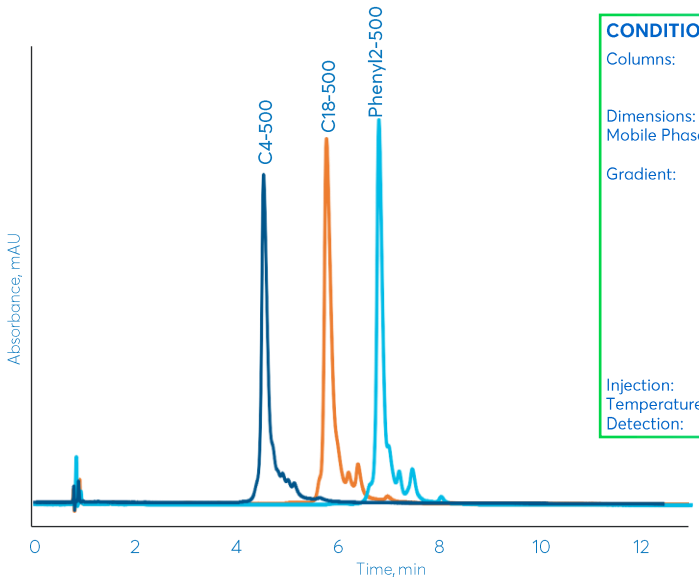


Figure 5: Analysis of NIST mAb on an Avantor® ACE® UltraCore BIO C4-500 column.

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.



CONDITIONS

Columns: Avantor® ACE® UltraCore BIO C4-500
Avantor® ACE® UltraCore BIO C18-500
Avantor® ACE® UltraCore BIO Phenyl2-500
Dimensions: 100 x 2.1 mm, 2.5 µm
Mobile Phases: A: 0.1% TFA in H_2O
B: 0.085% TFA in MeCN/H_2O (80:20 v/v)
Gradient:

Time (mins)	%B	Flow Rate (mL/min)
0	40	0.4
12	47.5	0.4
12.5	47.5	0.4
13	100	0.4
13.5	100	0.7
16	100	0.7

Injection: 2 µL
Temperature: 80 °C
Detection: UV, 280 nm

Figure 6: Analysis of Trastuzumab on 500 Å Avantor® ACE® columns bonded with different stationary phase ligands.

CONCLUSION

The analysis of intact proteins by RPLC is a critical workflow component in the discovery, development and quality control of protein-based therapeutics. Advantages of the technique include the ability to generate higher-resolution separations than other chromatographic techniques and the ability to readily hyphenate to MS. Compared to small molecule analysis, the analysis of proteins requires specialist wide-pore stationary phases to ensure unhindered analyte pore access. The Avantor® ACE® UltraCore BIO range of columns have been developed specifically for this type of analysis. The solid-core particle architecture reduces the impact of slow analyte diffusivity to provide the analyst with rapid, high-resolution separations of this challenging analyte class, as demonstrated by the various applications included.

REFERENCES

1. Avantor ACE® Technical® Note #022 "High Resolution Seperation of BSA Tryptic Digest using Coupled Avantor® ACE® Columns".
2. D. Guillarme, "Analytical Characterisation of Biopharmaceuticals," *LCGC Special Issues*, vol. 35, no. 8, pp. 496-498, 2017.
3. Avantor ACE® Technical® Note #003 "Avantor® ACE® 300 Å Wide Pore HPLC Columns for the Separation and Purification of Proteins".
4. S. Fekete, M. Rodriguez-Aller, A. Cusumano, R. Hayes, H. Zhang, T. Edge, J.-L. Veuthey and D. Guillarme, "Prototype sphere-on-sphere silica particles for the separation of large biomolecules," *Journal of Chromatography A*, vol. 1431, pp. 94-102, 2016.
5. Avantor ACE® Technical® Note #0010 "Chromatographic band broadening and the van Deemter equation".
6. D. Ran, G. D. Pipes, D. M. Hambly, P. V. Bondarenko, M. J. Treuheit, D. N. Brems and H. S. Gadgil, "Reversed-phase liquid chromatography of immunoglobulin G molecules and their fragments with the diphenyl column," *Journal of Chromatography A*, vol. 1175, pp. 63-68, 2007.