Reversed-Phase Liquid Chromatography for the Separation and Purification of Peptides and Proteins

The analysis of peptides and proteins is an important and expanding area and involves the application of a range of chromatographic techniques. This short article focuses on the role of reversed-phase liquid chromatography and how it can be used to provide helpful information in biomolecule characterisation workflows.

Introduction

The characterisation of biomolecules is an important activity to ensure the quality and safety of bioproducts. It is estimated that there are currently over 400 biopharmaceuticals in the clinical trial phase of development [1]. Due to the complexity of biomolecules, analytical chromatography plays an important role in providing information such as confirming product identity, structure and for the detection of related impurities.

A combination of chromatographic techniques are typically used to characterise proteins, both in their native and fragmented states (e.g. enzymatic digestions of therapeutic proteins). Techniques including ion exchange (IEX), size exclusion (SEC), reversed-phase liquid chromatography (RPLC), hydrophilic interaction liquid chromatography (HILIC), along with affinity chromatography and hydrophobic interaction chromatography (HIC) may all be used. Whilst it is important to employ a range of chromatographic techniques in parallel to provide comprehensive characterisation of these complex biomolecules, this article focuses on the application of RPLC to intact proteins and their constituent peptides and discusses some of the recommended column requirements for successful chromatography.

Reversed-phase LC Separations of Peptides and Proteins

RPLC is a powerful and widely used tool for the analysis of both intact and fragmented proteins and can provide helpful characterisation data. Many analytical challenges exist in the analysis of peptides and proteins, including their highly diverse and complex structures, the need to separate closely related variants and their existence in complex heterogeneous samples. These challenges require robust separation techniques that use a variety of detection methods including mass spectrometry (MS) to provide useful information.

The use of high purity silica which is very inert offers advantages in terms of peak shape and efficiency for biomolecule analysis. The presence of metal ion impurities can result in undesirable interactions with analyte molecules and also increases the acidity of residual silanol groups at the stationary phase surface. This can result in excessive peak tailing, low separation performance and reproducibility challenges. It is therefore always advisable to use high-purity silica based columns for protein and peptide analyses. Additionally, due to the reduced secondary interactions with high-purity silicas providing better peak shapes, lower concentrations of mobile phase additives may be used for analyses. Reducing the concentration of mobile phase additives provides detection benefits in UV (reduced background absorption therefore better sensitivity) and also MS (reduced ion suppression leading to increased MS signal / sensitivity). Reducing the concentration of mobile phase additives with lower-purity silicas typically leads to an accelerated loss in peak shape and performance (see Figure 1).

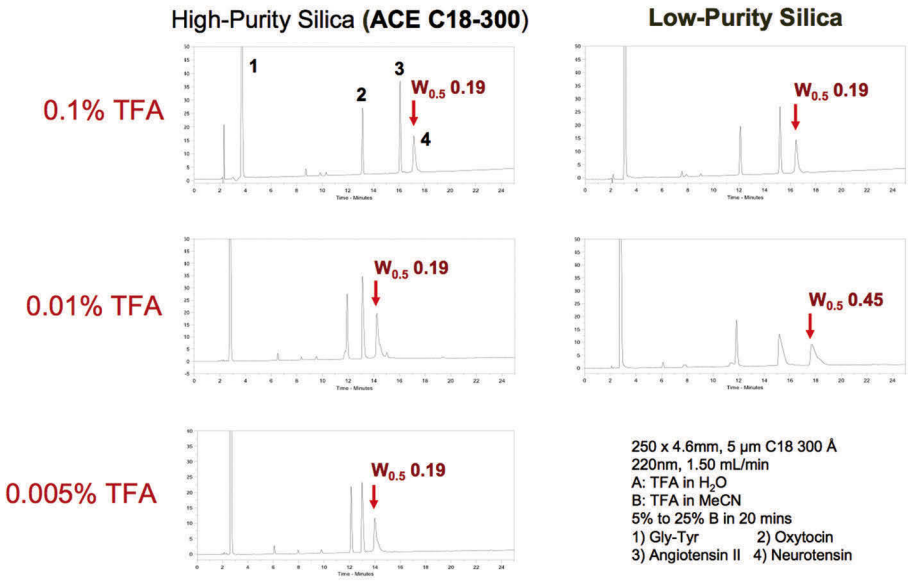


Figure 1: The effect of lowering the concentration of an ion-pairing additive (TFA) with low- and high-purity silica based columns.

Peptide mapping is an important characterisation technique involving enzymatic digestion of the parent peptide or protein to generate a series of peptide fragments. Separation by RPLC, and comparison to peptide maps from reference proteins, enables confirmation of a protein's primary structure and determination of any modifications to the amino acid sequence. Peptide mapping can be readily achieved using columns packed with high-purity, high performance silica-based particles with a pore size of ≥ 100 Å (e.g. ACE Excel 100 Å columns). For protein analysis, larger pore size silicas are typically required (e.g. ≥ 300 Å – discussed later). Whilst C18 modified silicas are most popular for smaller peptide fragment analysis, shorter alkyl chains (e.g. C8, C4), can also be used.

Sometimes, it can be beneficial to employ column chemistries with different selectivities for peptide analysis to help resolve closely eluting peaks in complex peptide mixtures. Figure 2 demonstrates the effect of 6 stationary phase chemistries with a simple mixture of peptides. The first four chemistries (C18, C18-AR, C18-PFP, SuperC18) give little change in retention or selectivity with this particular peptide mixture. However, the last 2 column chemistries (C18-Amide, CN-ES) offer differences in elution order (C18-Amide) and retention (CN-ES) that may be helpful for the analysis. Despite the first 4 column chemistries showing little difference for this particular peptide sample, it is always recommended to screen sample mixtures against chemistries with different retention mechanisms when performing characterisation work.

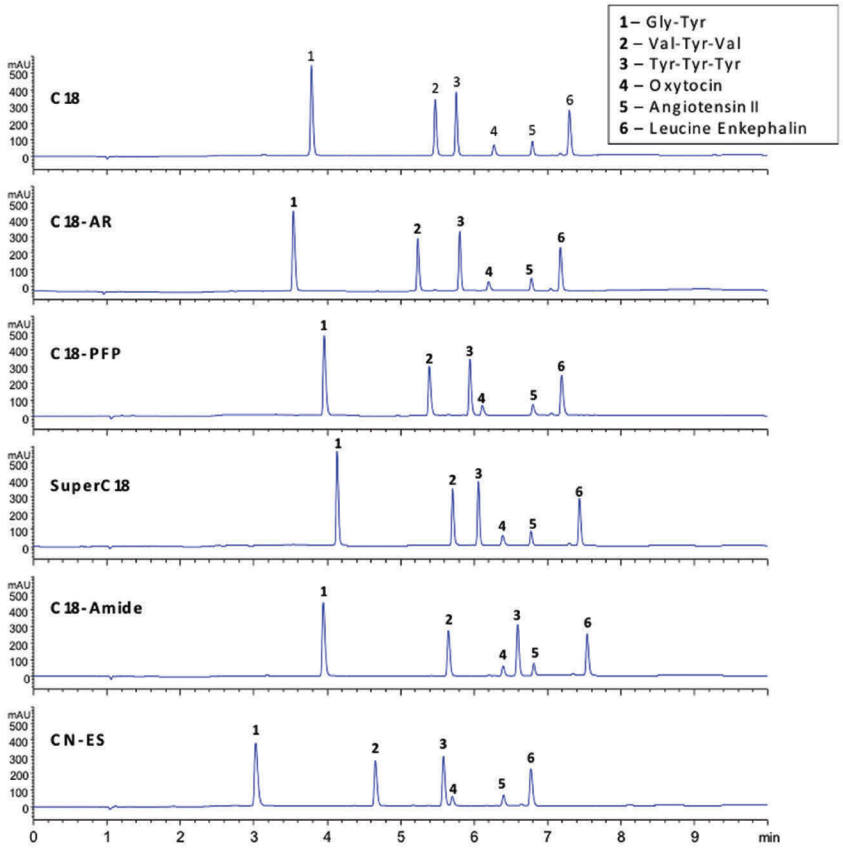


Figure 2: Separation of six peptides on a range of reversed-phase columns with different selectivity. Columns: ACE Excel 2µm, 100 x 3.0 mm, mobile phase: A = 0.05 % TFA (aq), B = 0.05 % TFA in MeCN, gradient: 5 – 40 % B in 7 mins with 1 minute isocratic hold at start, flow rate: 0.6 mL/min, temperature: 22°C, detection: UV, 220 nm.

For the RPLC analysis of large intact proteins, a surface modified high-purity wide-pore silica is typically used to improve access of the analyte protein to the stationary phase pores. Column manufacturers typically include columns with pore sizes of ≥ 300 Å for such protein analysis. Intact protein separations are usually performed using water:acetonitrile gradients with the addition of TFA as an ion-pairing agent. Higher temperature analysis (≥ 60 °C) is not uncommon to improve peak shape, resolution and increase recovery of larger proteins. Figure 3 demonstrates the

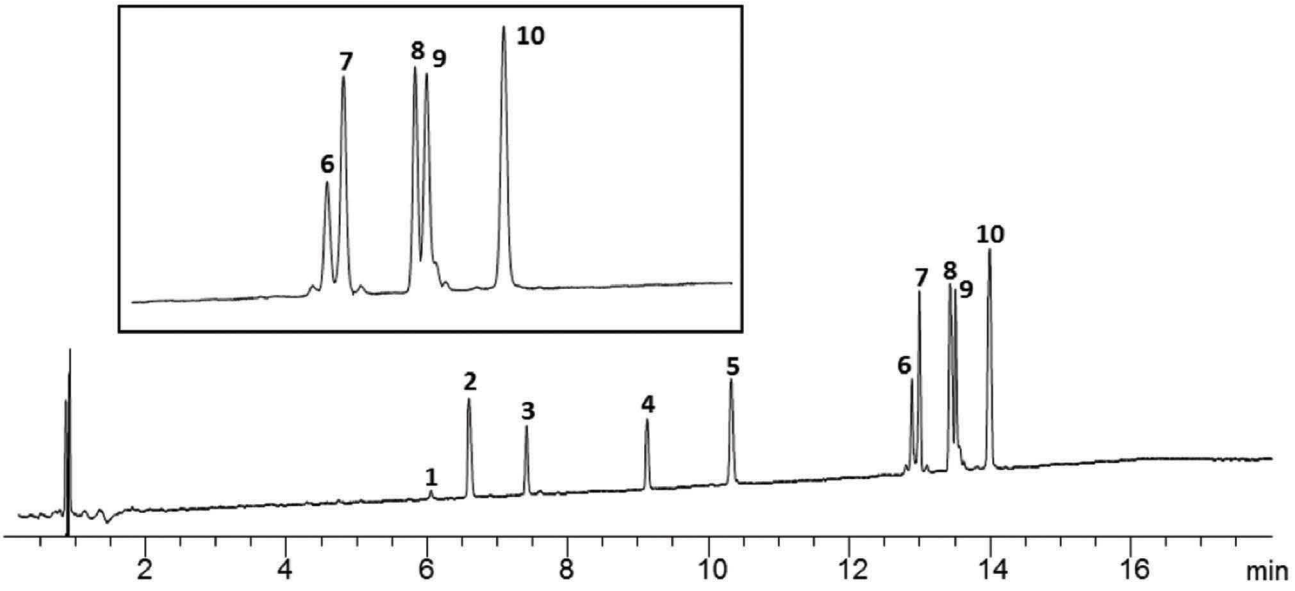
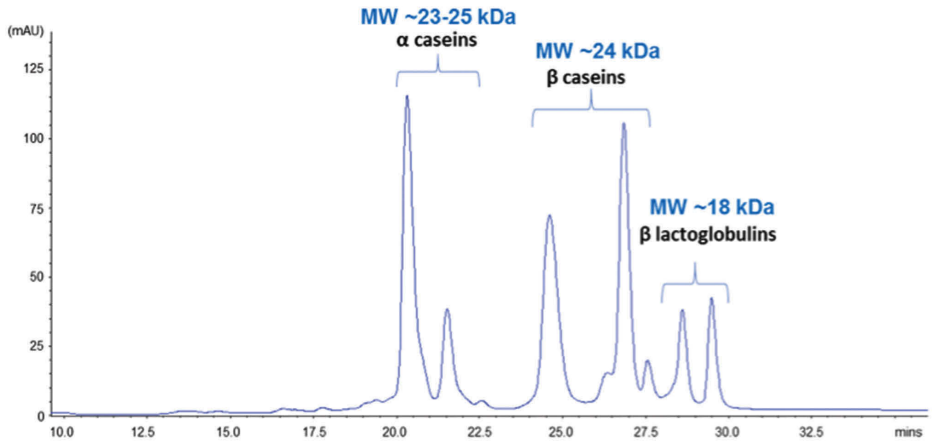


Figure 3: Reversed-phase separation of a range of peptides and proteins with varying molecular weights on a 300 Å C4 column. Column: ACE 3 C4-300, 150 x 2.1 mm, mobile phase: A = 0.1% TFA (aq), B = 0.1% TFA in MeCN:water 80:20 v/v, gradient: 10 to 50% B in 15 minutes, flow rate: 0.5 mL/min, temperature: 60°C, detection: UV, 220 nm. Sample: 1. Oxytocin (1007 Da), 2. Bradykinin (1060 Da), 3. Angiotensin II (1046 Da), 4. Angiotensin I (1296 Da), 5. Ribonuclease A (13.7 kDa), 6. Cytochrome C (12 kDa), 7. Bovine Insulin (5733 Da), 8. Human Insulin (5808 Da), 9. Porcine Insulin (5777 Da), 10. Lysozyme (14.3 kDa).



Gradient Profile:

Time (mins)	%B
0.0	33
5.0	33
9.0	35
18.0	37
22.0	40
27.5	41
28.0	41
43.0	43

Figure 4: Separation of milk proteins using a 300 Å C18 column. Column: ACE 5 C18-300, 150 x 2.1 mm, mobile phase: A = 0.01% TFA (aq), B = 0.01% TFA in MeCN, gradient: as shown above, flow rate: 0.2 mL/min., temperature: 45°C, detection: UV, 214 nm.

high-efficiency separation of a peptides / protein mixture with varying molecular weights (1.0kDa – 13.7kDa) using a broad scouting gradient run on a 300 Å C4 column. Of note is the separation of the three insulin variants of human, bovine and porcine origin. These insulins are structurally very similar, with only slight variations in their amino acid sequences and each having a molecular weight of approximately 5,800 Da.

The separation of closely related protein variants is a common theme in protein characterisation, often requiring the use of shallow gradient profiles to provide enhanced resolution. Figure 4 shows how this approach can be used for the analysis of milk proteins. In this example, a much shallower acetonitrile:water gradient was employed on a 300 Å C18 column to elute a series of casein and lactoglobulin proteins between 20 and 30 minutes. The high efficiency and excellent peak shape of the 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, as a high-purity silica based column was being used, the concentration of TFA in the mobile phase was reduced by a factor of 10 to just 0.01% whilst chromatographic performance for these large molecules was still acceptable. This reduced background UV absorption (and would possibly improve sensitivity if MS detection was employed).

Conclusion

Reversed-phase liquid chromatography is a powerful and versatile, high resolution technique which can be applied to the analysis of both intact and fragmented proteins. This short discussion has highlighted some of the column stationary phase considerations that can be helpful to develop successful protein and peptide separations, along with some useful method conditions and parameters.

Acknowledgements: Figure 4 is reproduced with permission of The Chemical Analysis Facility, University of Reading, UK.

References:

1.LCGC Editors (2016) Reversed Phase HPLC for the Analysis of Biomolecules [online] Available at: http://www.chromatographyonline.com/reversed-phase-hplc-analysis-biomolecules-0 [Accessed October 2018]