Considerations in the HPLC Analysis of Biomolecules

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Introduction

Analysis of peptides and proteins by HPLC can be very complex due to the diversity of the compounds in biomolecule investigation. Carbohydrates, vitamins, nucleotides, nucleosides and multi-charged large protein species can all be involved.
Choice of stationary phase to match the analytes is only one variable that needs to be considered, the buffer selection, concentration and pH are all important. Gradient slope, flow rate and temperature all have to be optimum in order to achieve sufficient resolution.

In this poster we discuss the variability between stationary phases and the conditions that are used with those columns. We show applications of tryptic digests and larger biomolecules to highlight the effect that each of the conditions have on the resulting chromatography.

Biomolecule Interaction

Biomolecules come with a wide diversity of structure, size, confirmation and charges. This can make retention and resolution of complex mixtures difficult to achieve. In terms of chromatography the 2 main stationary phases used tend to revolve around hydrophobicity, C18 and C4 ligands.
Pore structure is very important in the separation of proteins and peptides since the ability to enter the pore and interact with the stationary phase is most necessary. In small molecule analysis a pore diameter around 100A is most common, with biomolecules above approximately 2000Da then a 300A pore diameter is much more common. Once the protein has entered the pore structure and retained on the hydrophobic ligand then the mechanism will generally follow a adsorption/desorption mechanism as opposed to partitioning with small molecules. The “hydrophobic foot” of the protein as dictated by the amino acid sequence and their confirmation plays a major role.

FIGURE 1. Pore structure and adsorption/desorption

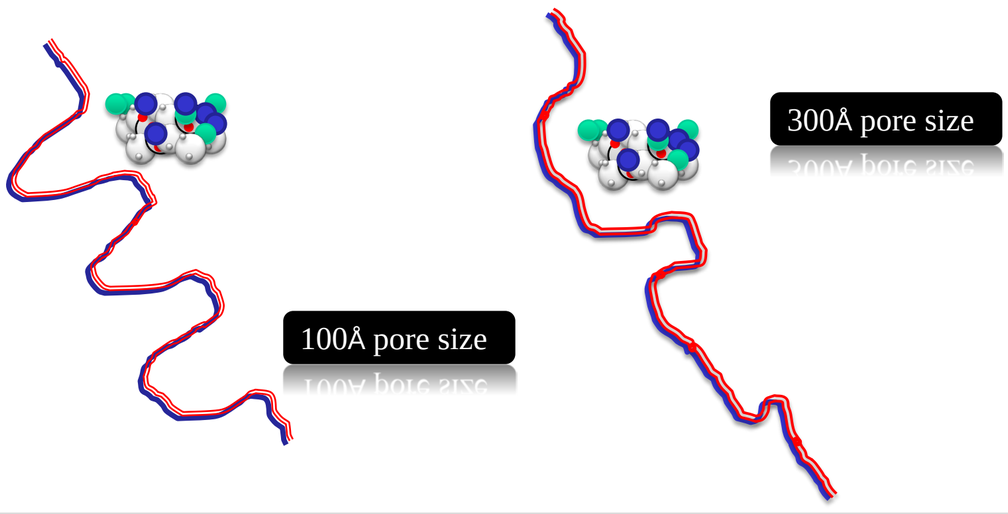
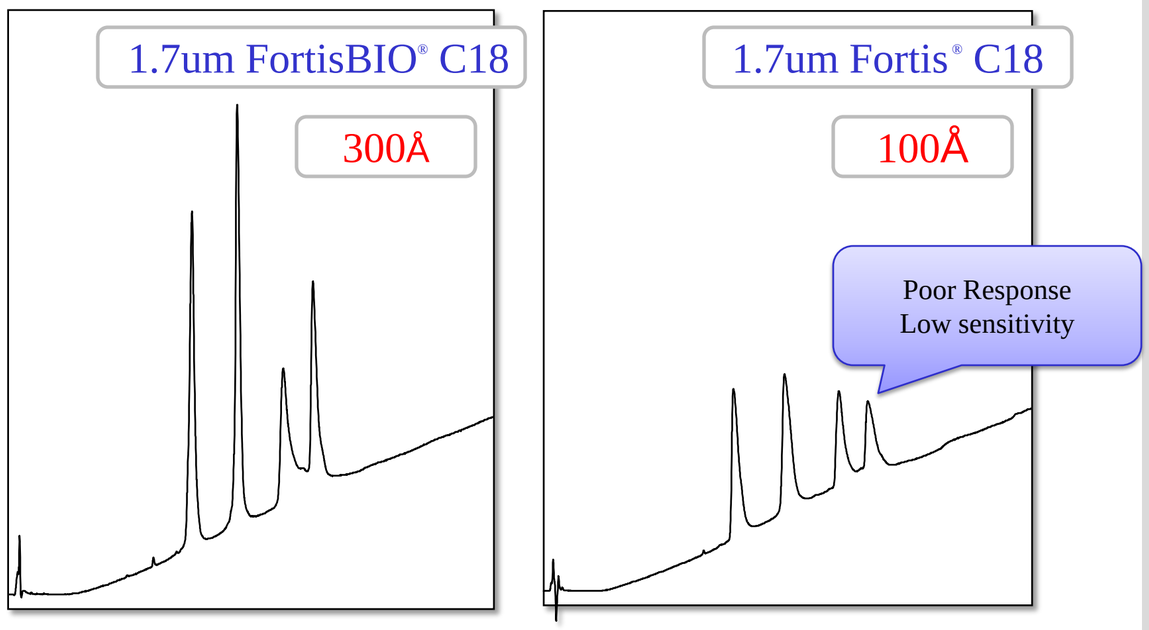


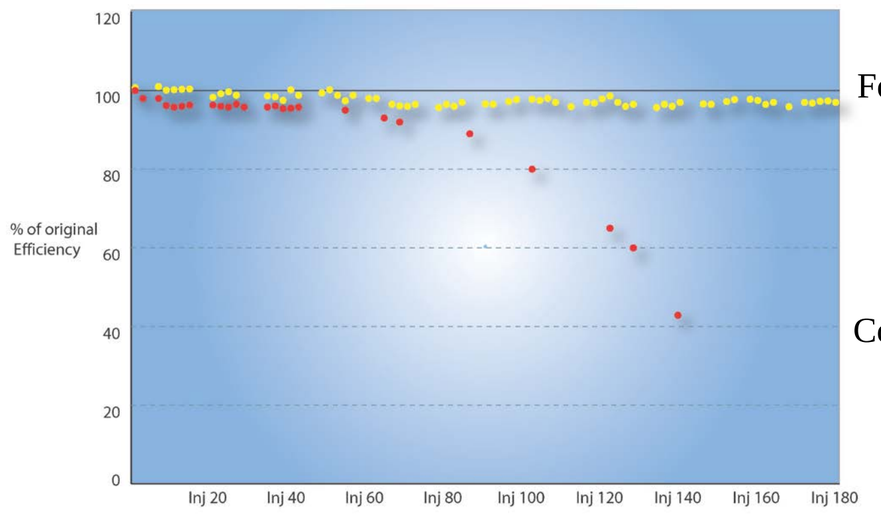
FIGURE 2. Pore Size – 300A vs 100A



1. Ribonuclease A 2. Cytochrome C 3. Holo-Transferrin 4. Apomyoglobin

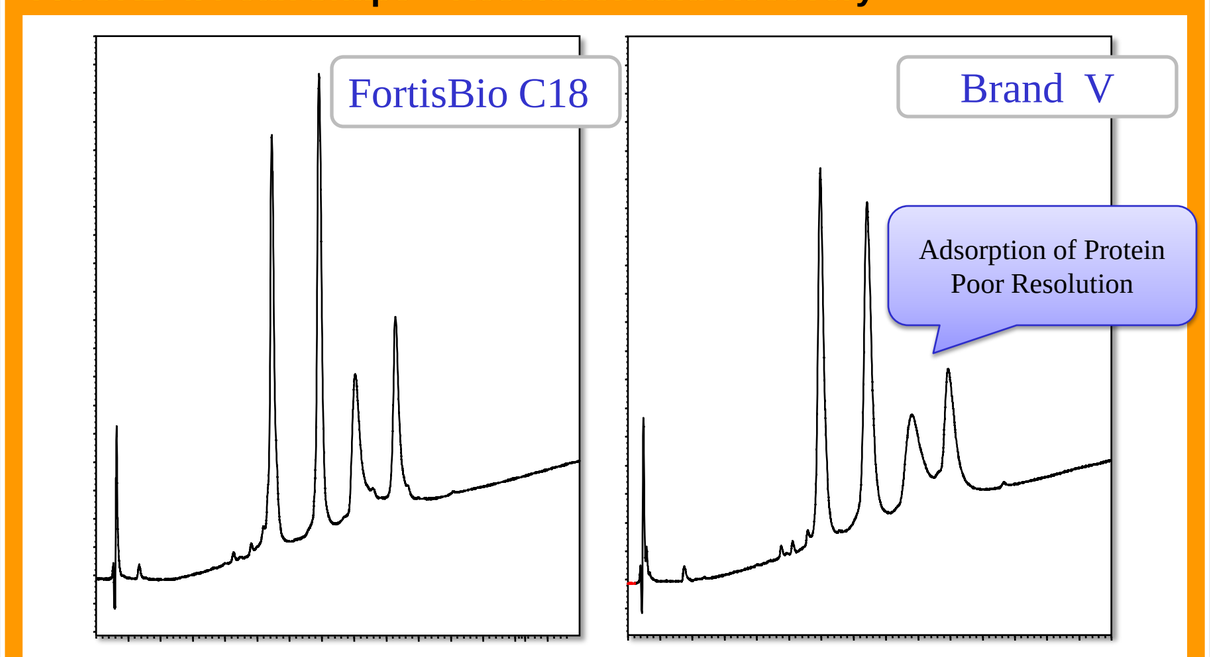
Once the biomolecule has entered the pore structure then the stationary phase will interact, if a C18 or C4 is used then the stability of the ligand has to be robust to mobile phase choice, since TFA is a very common additive in bioanalysis. Figure 3. highlights the stability of FortisBIO C4 ligand to acid hydrolysis.

FIGURE 3. Acid Stability – 0.1% TFA



Not all commercial alkyl chain stationary phases will perform in the same way. If we compare 2 different C18 BIO columns (Fig 4.) with a range of proteins, we see that if peak shape of the proteins is not optimal then resolution is lost, and recovery of the protein will be compromised. (Figure 4.)

FIGURE 4. Peak Shape – Resolution and Recovery



Buffer Considerations

Choice of buffer and concentration in bioanalysis can have big effects on the separation of peptides and proteins. TFA is commonly used due to its low UV adsorption, suitability in MS ionisation as well as its ion-pair characteristic in neutralising basic functionality of peptides/proteins. Typically used at concentrations of 0.1% to 0.5% for Tryptic digests. Concentrations below 0.1% may degrade peak shape and resolution (Fig 6).

FIGURE 5. Choice of Buffer TFA vs Formic acid

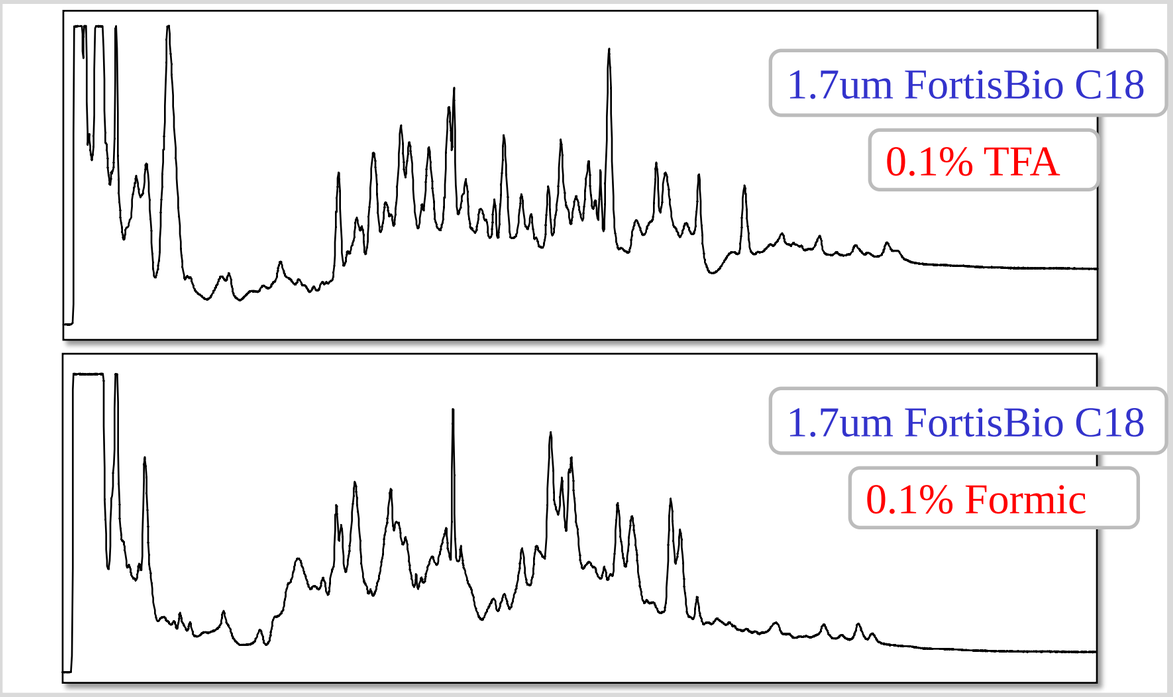
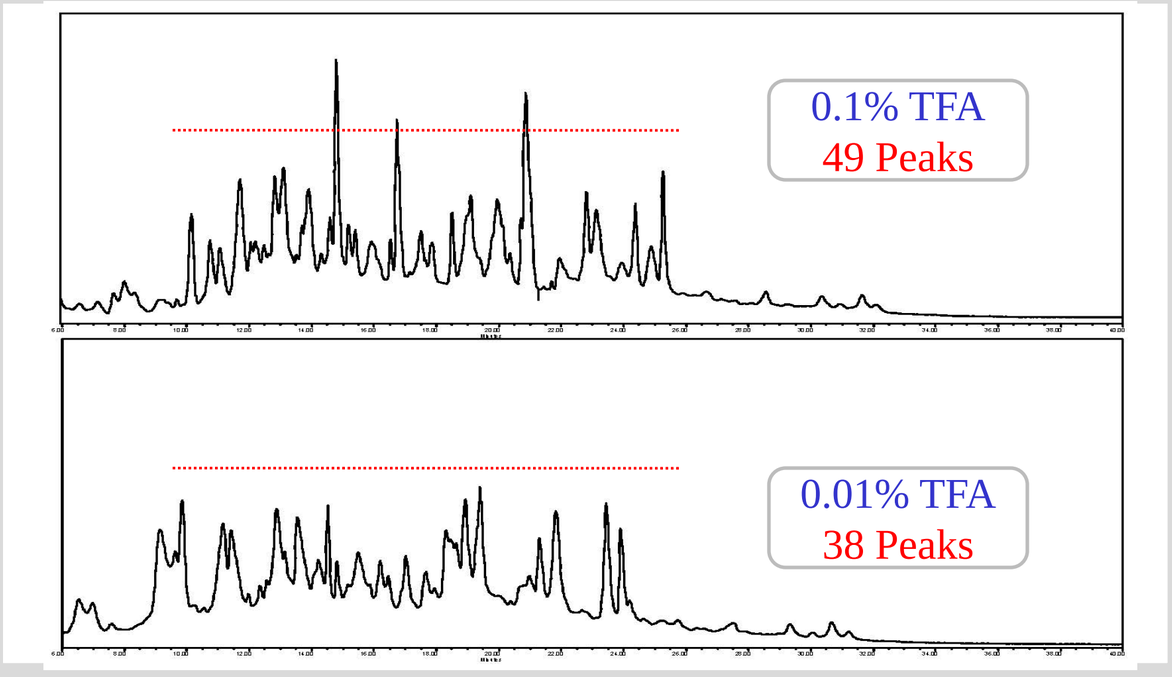


FIGURE 6. Different concentration of buffer



Conclusion

In this poster we have shown some of the variables that occur in the analysis of biomolecules. We have shown that it is necessary to have a well bonded, stable, stationary phase producing good peak shapes and therefore excellent recovery of proteins.
We highlight the need for a suitable pore structure in order for peptides/proteins to be able to ingress and therefore interact with the ligand.
We show how TFA is a popular buffer for bioanalysis and how the correct choice of concentration can help to maintain a neutralised state for molecules thereby allowing hydrophobic adsorption/desorption to occur.

In our other poster “UHPLC analysis of Biomolecules“ we discuss the use of a new 1.7um UHPLC particle for high sensitive, high resolution analysis of biomolecules.