Optimising Protein and Peptide Speed and Resolution with Superficially Porous Columns

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Proteins and peptides are large biological molecules consisting of a chain of amino acids. They have a wide variety of functions within a physiological system, ranging from structure formation, cell signalling, enzymatic to transportation processes, with the only difference between the different molecules being the order and number of the backbone amino acid structure. Since in a biological entity there are only a finite number of amino acids this can result in structures that are very similar. Both of these types of compounds can be highly hydrophilic in nature and so to achieve a retention in a chromatographic environment, a suitable chemistry has to be chosen. The size of these types of molecules is something that also has to be considered, since their effective diffusion rates are very slow compared to the much smaller molecules that were traditionally analysed within the pharmaceutical industry. The low diffusion rate affects the physical dispersion processes that occur within a column and these have to be carefully considered when designing the stationary phase, to ensure that mass transfer effects are minimised. In particular the pore structure has to be carefully designed to ensure that surface area is maintained but that minimal dispersion due to mass transfer effects occurs.

Column Technology to Aid Speed Advances

The use of superficially porous materials can reduce the effect of mass transfer effects, by reducing the pore depth [1,2]; however it is important that the pore diameter is large enough to cope with the analysis of these larger molecules. The column technology to aid the separation scientist to achieve these goals has been developed and in particular the use of superficially porous columns with, 300 Å pore diameters for proteins and 120 Å pore diameters for peptides, addresses the challenges associated the mass transfer effects. The work presented here demonstrates how the use of the correct column chemistry coupled with the correct pore size can ensure that fast high-resolution separations of protein and peptides are obtained. Optimisation of the separation also provides increased resolving power for peptide mapping.

Another advantage that is provided by the use of superficially porous materials is the low impedance, which means that the highly efficient chromatography can be obtained without the need to go to ultra high pressure chromatographic systems [3]. To illustrate the impact of this technology, optimised chromatographic methods compatible with standard 400 bar HPLC system pressures have been developed to enable the rapid profiling of IgG1, degraded Insulin and EPO protein.

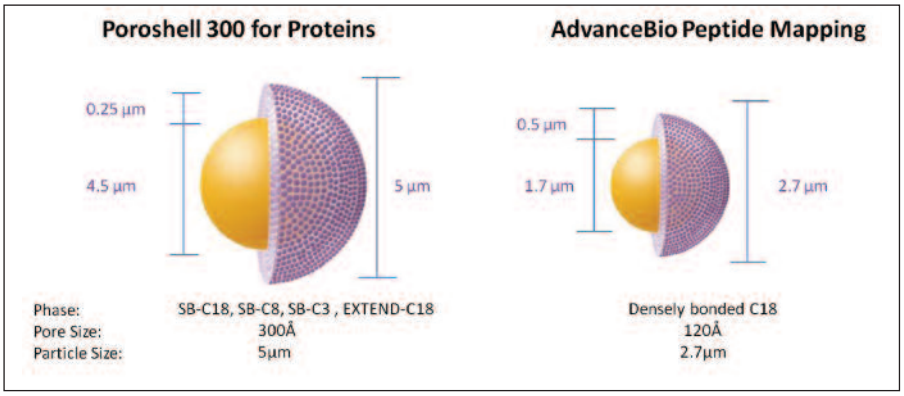


Figure 1: Examples of superficially porous column technology

Materials and Methods

The samples that were analysed were IgG1 which was used to highlight the separation of a protein and its impurities, and an enzyme digested recombinant human EPO as an example of the peptide separation, both purchased from Creative Biolab, Shirley, NY.

EPO Digestion Procedure

Trypsin protease was added to a solution containing approximately 4.2 mg (2.1 mg/ml, 2 ml) EPO. The ratio of substrate and enzyme was 50:1(w:w). The mixed solution was incubated at 37°C for 12h. The digestion was quenched by storing the sample at -70°C. After BCA analysis, 3.78 mg (2.1 mg/ml, 1.8 ml) of digested EPO was obtained.

IgG1 Papain Digestion

To prepare the FC and Fab fragments, a Pierce Fab Micro Preparation Kit was used. The final Fab and Fc clean-up used microcentrifugation with a spin column to separate from the immobilised papain. The final concentration of the fragments was 2 µL / µg.

Instrumentation

Intact and fragmented Protein Analysis: Agilent 1200 and Agilent Infinity 1290 Infinity LC system with auto injector (HiP-ALS), binary pump, thermostatted oven (TCC) and diode array detector (DAD).

Peptide Mapping: Agilent Infinity 1260 Bio-

inert LC system with auto injector (HiP-ALS), binary pump, thermostatted oven (TCC) and diode array detector (DAD)

Columns

Protein columns

The following columns were used for the analysis of the protein samples;

- Agilent Poroshell 300SB-C3, 2.1 x 75 mm, 5 µm (p/n 660750-909)
- Agilent Poroshell 300SB-C18, 2.1 x 75 mm, 5 µm (p/n 660750-902)

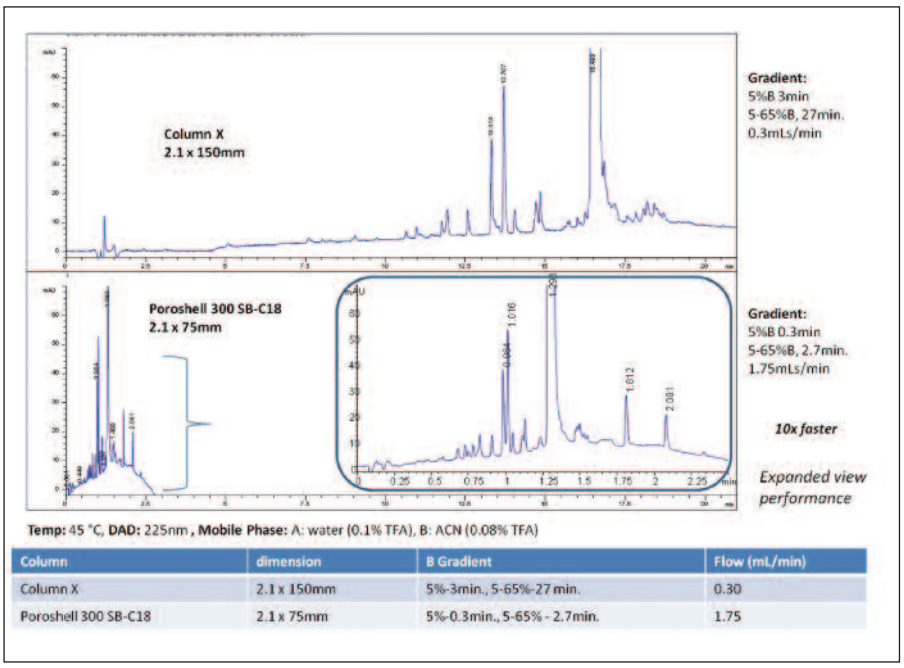
Peptide columns

The following columns were used for the analysis of the peptides samples;

- Agilent AdvanceBio Peptide Mapping Column, 2.1 x 150 mm, 2.7 mm (p/n 653750-902)
- Agilent AdvanceBio Peptide Mapping Column, 2.1 x 250 mm, 2.7 mm (p/n 651750-902)
- Agilent AdvanceBio Peptide Mapping Column, 3.0 x 150 mm, 2.7 mm (p/n 653950-302)

Optimising Methods for Improved Speed and Resolution for Intact Protein Separations

During the development of a protein biopharmaceutical, the need for rapid profiling methods are growing as throughput demands increase. A forced degradation study is one means to predict / evaluate changes in protein heterogeneity during monitoring and formulation. The advantage of the low back pressure experienced when using a superficially porous material is shown in Figure 2. The top chromatogram in Figure 2 shows a separation performed on a 3.6 mm superficially porous material. The low back pressure and high peak capacity associated with the superficially porous material allow for a substantial reduction in the analysis time [4,5], which is not always achievable with a fully porous material. The rapid separation of the degraded Insulin (Figure 2, lower chromatogram) was generated 10 times faster than a traditional separation (top chromatogram). This was achieved by using a shorter column, increasing the flow and reducing the gradient time. The fast separation maintained peak shapes of the degradant products which was achieved without the need to increase the backpressure above 300 bar, which would not be possible if a



Column	dimension	B Gradient	Flow (mL/min)
Column X	2.1 x 150mm	5%-3min., 5-65%-27 min.	0.30
Poroshell 300 SB-C18	2.1 x 75mm	5%-0.3min., 5-65% - 2.7min.	1.75

Figure 2: An example of rapid HPLC profiling of degraded insulin using Agilent Poroshell 300SB-C18, 2.1 x 75 mm, 5 µm, column in comparison to an alternative superficially porous column, C18 phase, 2.1 x 150mm, 3.6 µm using the same instrumentation, Agilent 1260 Infinity Bio-Inert LC System.

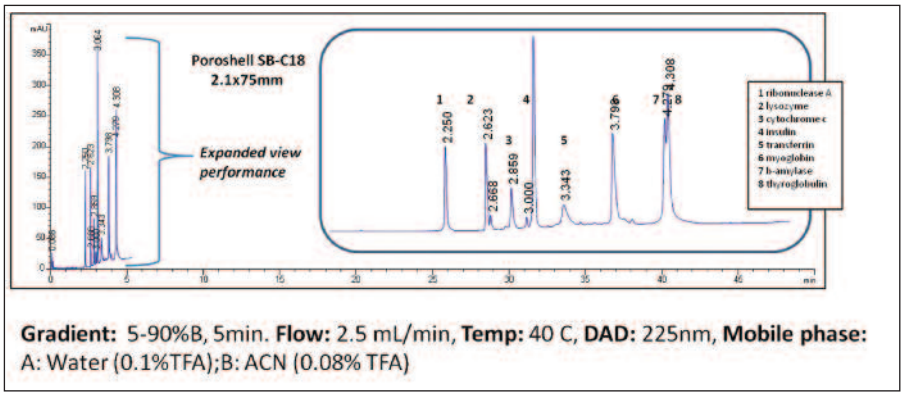


Figure 3: Eight intact proteins resolved in under five minutes using Agilent Poroshell 300SB-C18

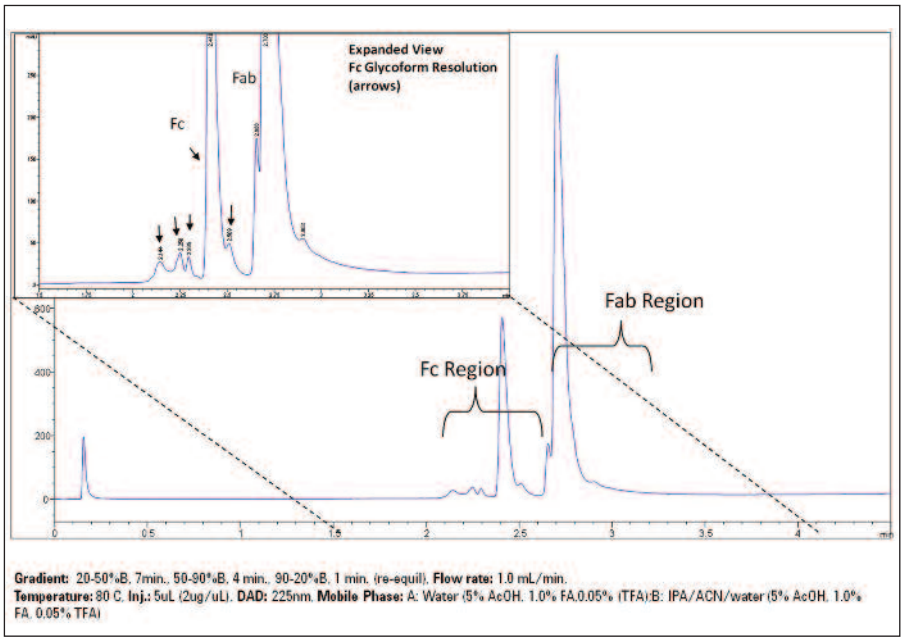


Figure 4: Reversed-phase separation of IgG1 after papain digestion showing two primary peaks of the Fc and Fab fragments. The inset details partially resolved peaks representing variants of the Fc and Fab fragments (arrows)

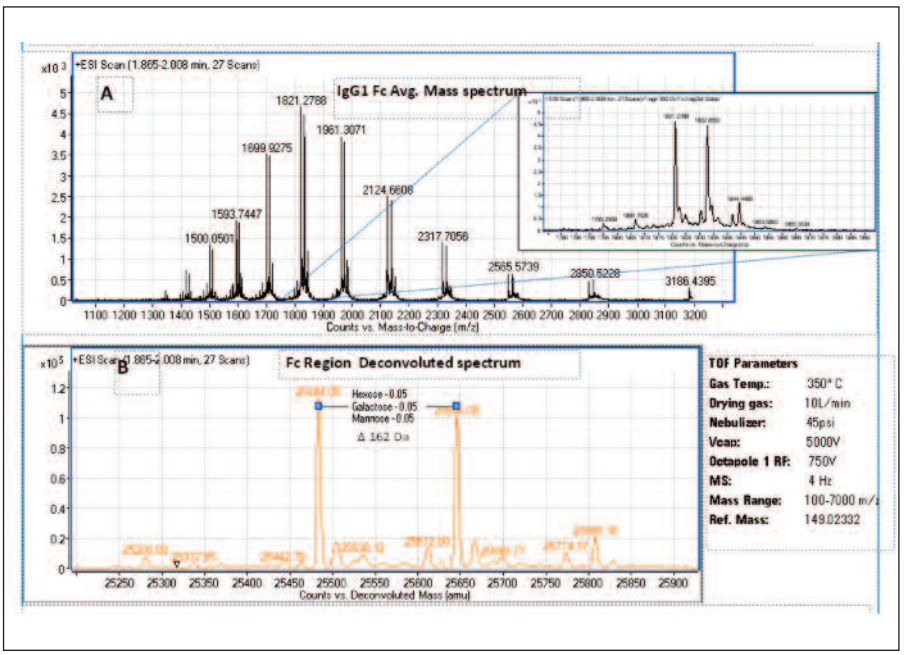


Figure 5: LC/MS analysis of Fc Region – Glycosylation Profiling. A and B show averaged mass spectrum and deconvoluted spectrum of the IgG1 Fc fragment from the separation.

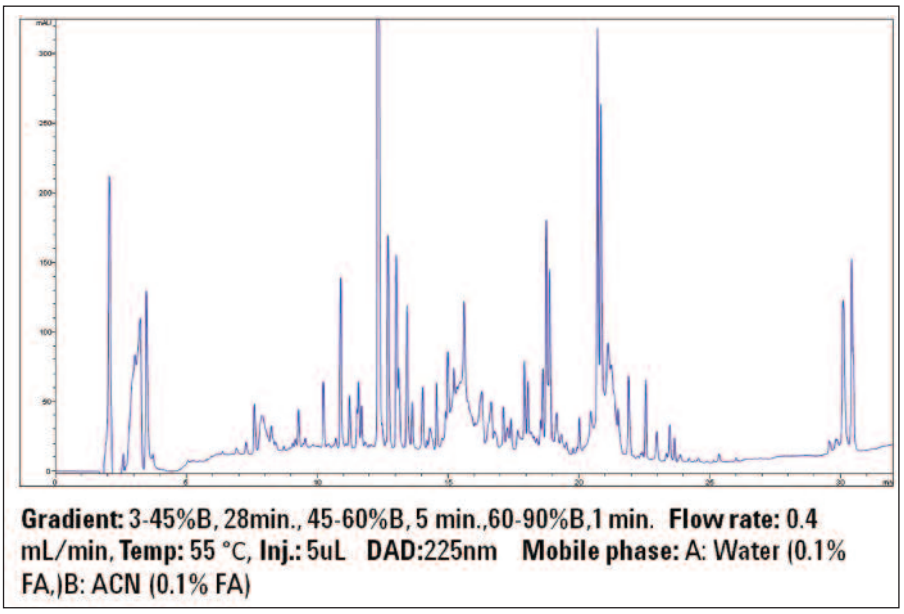


Figure 6: Tryptic Digest rhEPO Protein 2.1 x 250mm AdvanceBio Peptide Mapping Column

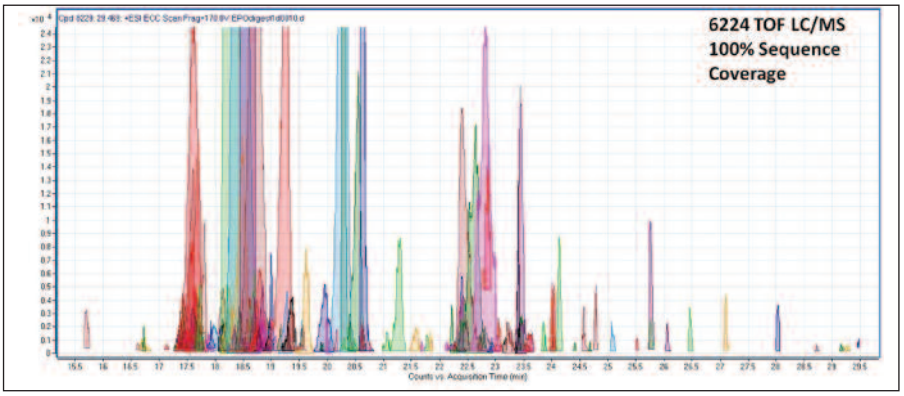


Figure 7: 100% sequence coverage using MassHunter Molecular Feature Extractor (MFE).

fully porous material was being used.

Large molecules diffuse much more slowly than small molecules, which results in the typical optimal flow rate for protein separations to be lower than for smaller molecules [6,7]. The lower diffusion rates result in a greater radial equilibration effect, so called 'c' term. Therefore, a column with a shorter diffusion path for protein migration becomes much more desirable. Agilent Poroshell 300 columns offer this shorter diffusion distance due to their superficially porous particles, making it possible to operate steeper gradients at higher flow rates without extra band effects [8,9]. Figure 3 shows a separation achieved in under five minutes, at 2.5 mL flow rates, demonstrating this advantage.

In biotherapeutic development, the number of samples submitted for product quality analyses, such as glycosylation profiling during cell line and clone selection and cell culture optimisation studies, can be enormous. At the same time, data turn-around time needs to be fast, due to precise process development timelines [9]. The chromatogram and expanded view inset detail in Figure 4 show the rapid and efficient reversed-phase separation of papain digested IgG1 Fab/Fc fragments using a 2.1 x 75 mm Agilent Poroshell 300SB-C3 column which helps to facilitate the fast data turnaround times necessary. The Fab and Fc chromatogram details the excellent resolving power needed for profiling the glycosylated variant peaks [10]. Figures 5A and 5B show the averaged mass spectrum and deconvoluted spectrum of the IgG1 Fc fragment from the separation in Figure 4. Partial digestion of the intact IgG1 into Fc & Fab fragments allow for a more efficient analysis for enhance glycoform profiling.

Faster Peptide Mapping

A peptide map is a fingerprint of a protein and the end product of several processes that provide a comprehensive understanding of the protein being analysed. It involves four major steps: isolation and purification of the protein; selective cleavage of the peptide bonds; chromatographic separation of the peptides; and validated analysis of the peptides.

Often peptide maps using a 2.1 x 250 mm column can take two hours or longer to produce the necessary resolution. Additionally, re-equilibration and run-to-run cycle times can add extended analysis time, significantly affecting laboratory production. The conditions outlined here enable a fast analysis of a rhEPO

digest providing very narrow peak widths, excellent sensitivity and unique selectivity. Additionally, the optimised chromatographic conditions including use of formic acid ion-pair enabled 100% sequence coverage (Figure 6) and identification of 43 unique glycopeptides (Figure 7) [9]. This 34-minute LC/MS method resulted in 100% sequence coverage of the rhEPO protein.

Evaluating Column Ruggedness

The mobile phases routinely used for reversed phase analysis of proteins are acidic (low pH), containing either trifluroacetic acid or formic acid. A densely bonded phase will deliver exceptional peak shapes and reproducible performance under these conditions. Figure 8 shows chromatographic overlays of 200 injections, with consistent column performance using low pH conditions using such a column (Agilent AdvanceBio Peptide Mapping).

Conclusions

Superficially porous columns are a useful tool for delivering ultra-fast high-resolution protein and peptide separations. In combination with optimised chromatographic methods, difficult to manage proteins such as IgG1 and EPO can be efficiently resolved during fast run times, with separations achieved at HPLC system pressures.

It has been shown that the selection of the correct column will ensure phase stability during operation at low pH and elevated temperature. The conditions described here are highly compatible for LC/MS analysis and provide a robust solution for achieving repeatable, rapid and robust biopharmaceutical analyses.

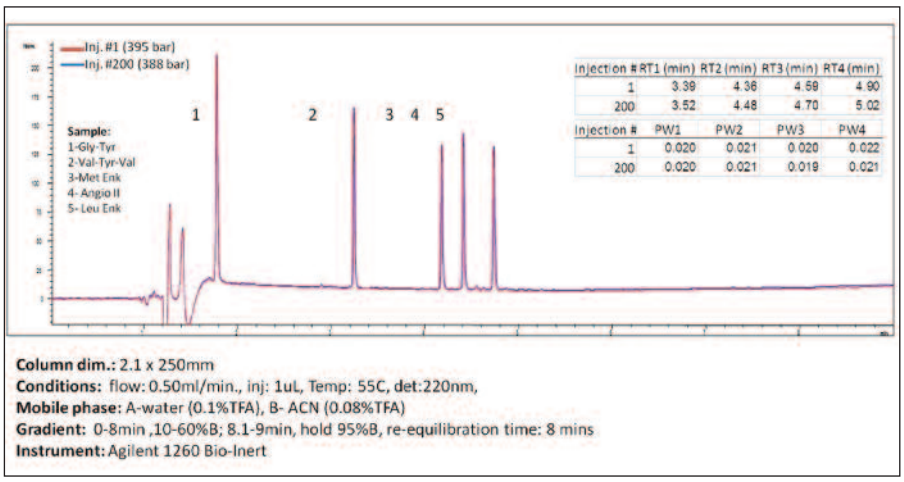


Figure 8: Agilent AdvanceBio Peptide Mapping column performance over 200 repeated injections at pH 2.2 at 55°C – continuous HPLC operation for 2.5 days with pressures under 400 bar.

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