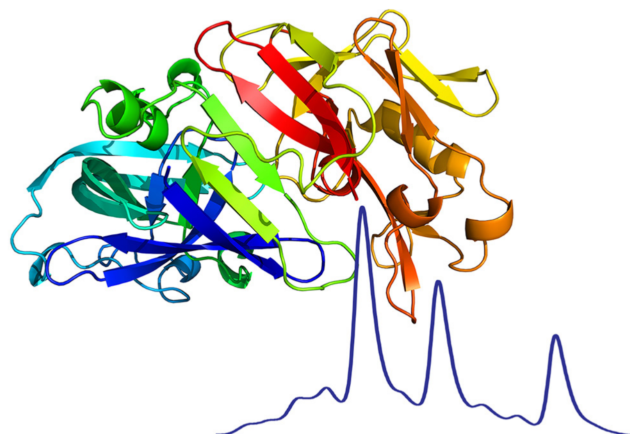
White Paper 70848

How does the analysis of biomolecules benefit from UHPLC?

Evert-Jan Sneekes, Remco Swart, and Mauro De Pra
Thermo Fisher Scientific, Germering, Germany

Executive Summary

Ultra high performance liquid chromatography (UHPLC) provides improved separation speed, throughput, and sensitivity by employing stationary phase particles of around 2 µm or smaller. UHPLC has found widespread use in the analysis of small molecules in pharmaceutical, food, and environmental areas. Are these analytical benefits also applicable to the separation of larger molecules, such as proteins and peptides? Biomolecules have, apart from their size, other differences from small molecules (e.g. charges, complex structures) that make the application of UHPLC not as straightforward as for small molecules. This article discusses the rationale of using UHPLC in life sciences and demonstrate where the potential is. It also discusses the requirements needed for a true bio UHPLC system and shows examples of relevant biomolecule separations.



Keywords

Proteins; Peptides; bio UHPLC, Biopharmaceuticals, Throughput

Introduction

An important physical parameter in the discussion of chromatographic performance of large molecules is the diffusion coefficient. As a consequence, chromatography, being a diffusion-controlled process, is highly influenced by the molecular size of the analyte. The increase of flow rates typically applied to increase throughput in UHPLC methods works for small molecules, but appears counter intuitive for the analysis of slower-diffusing molecules, such as proteins.

The most obvious difference between small and large molecules is their size, which is not only defined by the molecular weight (MW), but in the case of complex biomolecules also by their structure and hydrodynamic radius. The diffusion constants will decrease with molecular size.

Theoretically the effect of the difference in diffusion constants on the chromatographic efficiency can be visualized by the Van Deemter curves in Figure 1A. Here, a theoretical comparison on the optimal chromatographic conditions (i.e. optimal mobile phase velocity in the Van Deemter curve) for two analytes with a 100 times difference in molecular weight is given. For the same type of stationary phase the flow optimum is much lower for the protein and the range in which this performance is achieved is much narrower (see Figure 1A).

Thermo SCIENTIFIC

The Van Deemter curve has three components and the main underlying parameter for the differences between small and large molecules is the component that defines the interaction of the analyte with the stationary phase (also called C-term). This is why UHPLC and smaller particles are typically mentioned together; the shorter diffusion paths for smaller particles have a positive effect by lowering the C-term contribution. In practice this gives sharper peaks or allows higher flow rates with the same performance.[1]

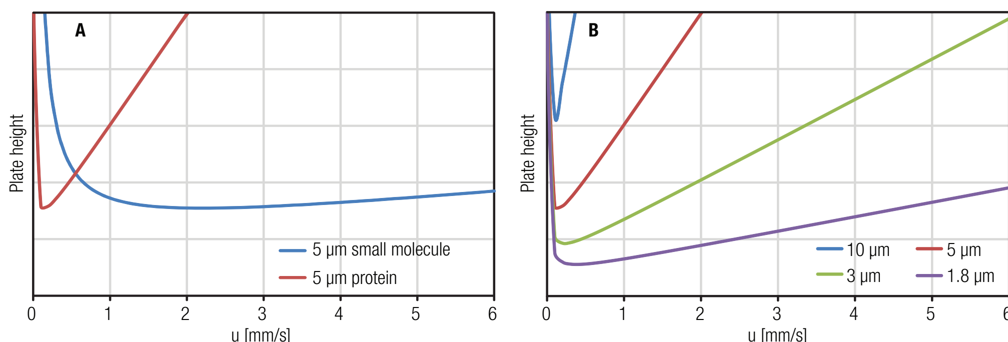


Figure 1. (A) Theoretical comparison between Van Deemter curve two analytes with 100× MW difference ("small molecule" vs "protein") for the same column. (B) Comparison of optimal flow change with particle sizes for a protein.

Fortunately, this trend with smaller particle sizes is the same for proteins; the range for optimal performance increases (Figure 1B) as a result of the shorter diffusion pathway. The degree of improvement is much smaller for larger MW species. Therefore, UHPLC brings analysis speed for proteins into the ballpark of HPLC analysis speed of small molecules. In practice this means a difference in analysis time between e.g., 60 min and 15 min—but not bringing analysis time below 1 minute. (See the SCX separation example in Figure 4.)

In addition, there are other means to shorten the diffusion paths. The use of non-porous particle technology has a long tradition in protein separations. The tradeoff is that the sample–loading capacity is lower as a result of the smaller surface area. A compromise combining the best of both worlds are the solid core particles, where a small porous layer on the particles provides minimized diffusion paths and still provides acceptable sample loading capacity.

In all cases the use of smaller particles will result in increased pressure requirements from the system and that is where bio inert UHPLC equipment is required.

Bio UHPLC Instrumentation

Biomolecules typically have multiple charges as well as a complex three dimensional structure. These properties allow and often necessitate the application of separation principles other than reversed phase. Therefore a bio UHPLC system does not only need to support UHPLC pressures but also the application of a wide range of chromatographic methods. A majority of analyses applied to proteins use solvents with extremes in pH and/or high salt concentrations. Any residual iron under these conditions will interfere with the analysis, foul the column, and in the end cause equipment malfunction.

The Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system is a dedicated bio UHPLC system, with an iron-free flow path to ensure stable operation under these harsh solvent conditions up to 1000 bar. This extends also to the columns, which are available in PEEK™-lined stainless steel, where the solvents and analytes are only in contact with inert PEEK and the stainless steel reinforcement allows the use of pressures that cannot be achieved by PEEK alone. All connections are made with a new addition to the Thermo Scientific™ Dionex™ Viper™ fingertight fittings family, offering the same performance and ease of use, but now with full biocompatibility.

Figure 2. UltiMate 3000 BioRS system, detail of the autosampler inert injection valve, and the Viper connection.

UHPLC Separations of Biomolecules

A technology requires a goal in order to be useful. Bio UHPLC is most relevant for biopharmaceutical analysis where in depth product characterization is required. Generally multiple complementary techniques are required to characterize an entity or just a part of the biomolecule. The last section of this document will show some application examples of the UltiMate 3000 BioRS system in analyses typical for biopharmaceutical characterization.

Peptide Mapping – Optimizing Analysis Time and Resolution

Peptide mapping is the analysis of enzymatically generated protein fragments. In combination with mass spectrometric detection peptide mapping is a powerful tool for elucidating the primary amino acid sequence. After enzymatic digestion, each protein is easily represented by 50 or more peptides, which have to be separated first to be identified. High resolution peptide mapping can be performed on the UltiMate 3000 BioRS system as shown in Figure 2. The use of a stationary phase with small particles will allow fine tuning for throughput or separation performance. The chromatograms in Figure 3 show the 5 minute and 30 minute separation with respective peak capacities

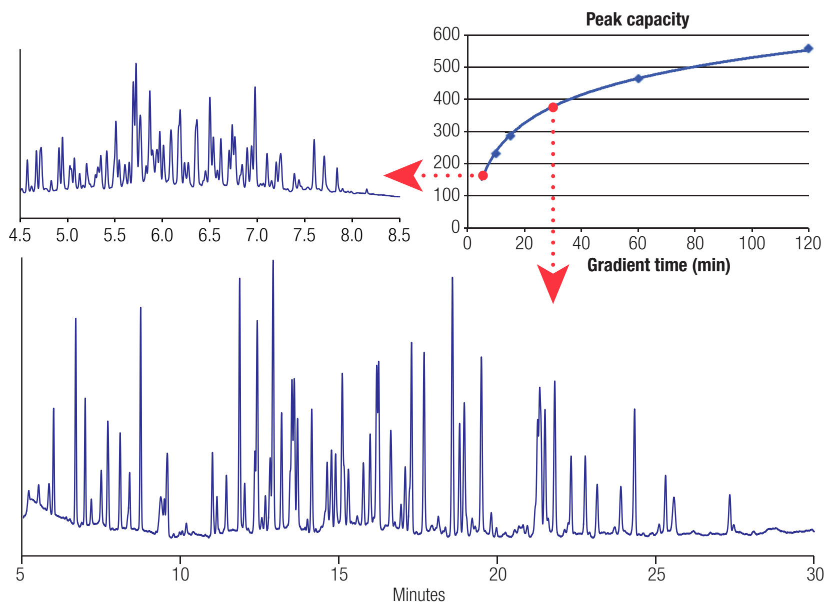


Figure 3. Peak capacity vs gradient time variation for a BSA digest separation on 2.1 × 250 mm Thermo Scientific™ Acclaim™ RSLC 2.2 µm C18 column.

Protein Charge Variant Analysis – Method Speed up

A common analysis in monoclonal antibody (MAb) characterization is charge variant analysis. Here ion exchange chromatography is used to separate different charge states that can result from sequence truncations or differences in glycan structures. Figure 4 shows the separation of the same antibody sample on the Thermo Scientific™ MabPac™ SCX column with either 10 µm or 3 µm particles. The application of 3 µm particles allowed the analysis to speed up without sacrificing resolution between the three major lysine truncation peaks or the detail between the major components. In this particular example the sample throughput has been increased by a factor of 4 using UHPLC.

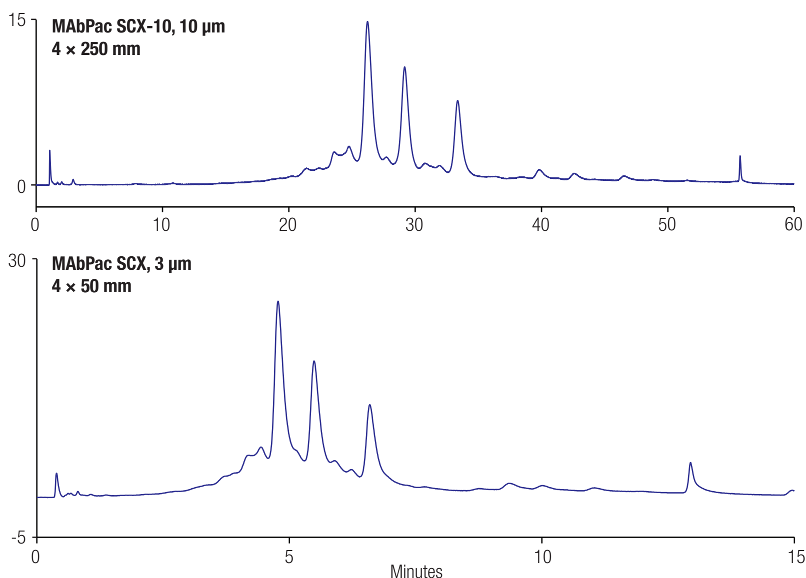


Figure 4. Comparison of SCX separation on 10 µm and 3 µm particle columns.

Charge Variant Analysis with pH Gradients

The example in Figure 4 is a salt-based elution with buffered eluents. Given the pI range of MAbs it is not uncommon that mobile phase systems have to be optimized for pH and salts when analyzing different antibodies. This optimization takes time and the method change reduces throughput. In addition all new methods need to be thoroughly validated before use in the QC environment.

An alternative way to separate MAbs is to utilize pH gradient.[2] The MAb is loaded on the SCX column at a pH below the pI to ensure a positive charge on the protein. The proteins will bind to the negatively charged surface of the stationary phase. With a gradient the pH is increased over time, effectively reducing the overall positive charge until the pI of the MAb is reached, and at this point the MAb elutes from the column. The major benefit of this method is that it eliminates the need for pH optimization.

Figure 5 compares the salt with pH-based gradient separation of the same MAb on identical columns. Not only is the pH gradient method more generic and applicable to a wider range of samples but also, in this case, noticeably more detail is observed in the separated sample.

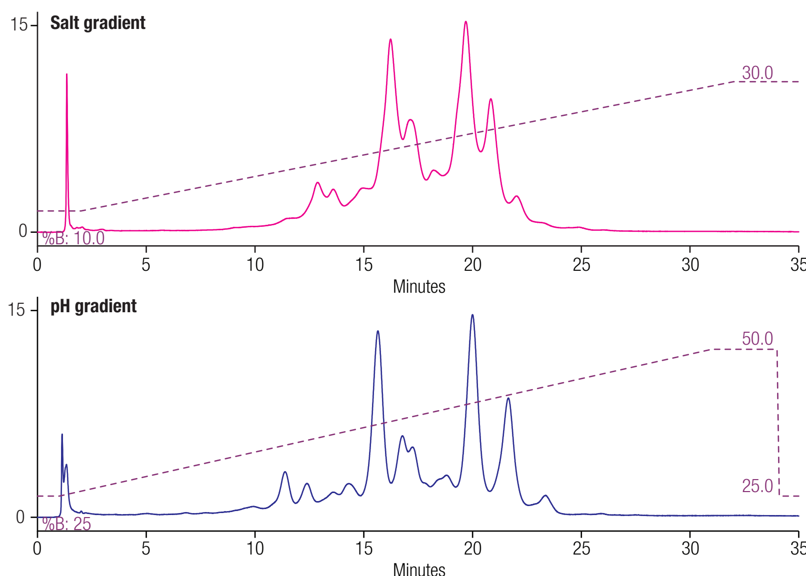


Figure 5. Comparison of salt-based vs pH gradient-based elution for the same MAb sample on identical MabPac SCX columns.

Since pH gradient methods use multiple buffer components that are not readily prepared, Thermo Scientific has developed a ready-to-use buffer kit. The buffer solutions have a specific make up that provide a linear pH variation when a linear AB gradient is programmed. The UltiMate 3000 BioRS system can also be equipped with a pH monitoring module (Thermo Scientific™ Dionex™ UltiMate™ 3000 PCM-3000 monitor) that can be used to validate the pH gradient formation.

Glycan Separation

The last example is in the field of glycan analysis[3] and features the analysis of glycans by LC-MS. The Thermo Scientific™ GlycanPac™ AXH-1 columns provide a mixed mode chemistry for the separation of glycans. The charge form sialytion, as well as the general retention, are employed and these columns are available with sub 2 micron particles. Figure 6 shows the separation of bovine fetuin glycan structures after enzymatic release from the protein. The distinct sialyted groups are visible as well as the separation within each group.

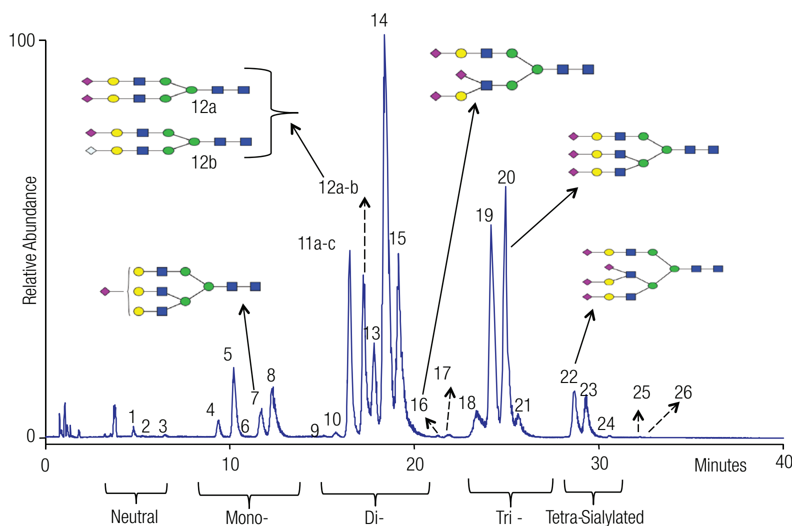


Figure 6. Separation of released glycans on GlycanPac AXH-1 columns and detected by LC-MS.

Conclusion

UHPLC is a technique with great acceptance for the analysis of small molecules. The title question "How does the analysis of biomolecules benefit from UHPLC?" was posed to explore and demonstrate that UHPLC can indeed offer advantages to the analysis of bio molecules. Apart from smaller particles, special solvents and separation principles beyond reversed phase also need to be supported. The UltiMate™ 3000 BioRS system represents a platform that is designed with multiple modules to allow configuration for in depth multidimensional analysis or high throughput quality control. The extensive column portfolio extends UHPLC capabilities beyond reversed phase by offering IEX or glycan separation materials in smaller particles and longer columns. Additionally, a ready-to-use buffer set is available to perform pH gradient charge variant analysis. The buffer system allows application of linear gradients to achieve a linear pH profile. With all these capabilities, the UltiMate 3000 BioRS is the system that will work in development, characterization, and QC.

References

1. Howard, J.W.; Kay, R.G.; Pleasance, S.; Creaser, C.S. *Bioanalysis* **2012,** *4* (24), 2971–2988.

2. Farnan , D.; Moreno , G. T. *Anal. Chem.* **2009,** *81* (21), 8846–8857.

3. Fernandes, D.L. *Eur. Biopharm. Review,* Spring **2006,** 100–104.

White Paper 70848

www.thermofisher.com/BioRS

©2016 Thermo Fisher Scientific Inc. All rights reserved. PEEK is a trademark of Victrex PLC. All other trademarks are the property of Thermo Fisher Scientific and its subsidiaries. This information is presented as an example of the capabilities of Thermo Fisher Scientific products. It is not intended to encourage use of these products in any manners that might infringe the intellectual property rights of others. Specifications, terms and pricing are subject to change. Not all products are available in all countries. Please consult your local sales representative for details.

Africa +43 1 333 50 34 0
Australia +61 3 9757 4300
Austria +43 810 282 206
Belgium +32 53 73 42 41
Brazil +55 11 3731 5140
Canada +1 800 530 8447
China 800 810 5118 (free call domestic) 400 650 5118
Denmark +45 70 23 62 60
Europe-Other +43 1 333 50 34 0
Finland +358 9 3291 0200
France +33 1 60 92 48 00
Germany +49 6103 408 1014
India +91 22 6742 9494
Italy +39 02 950 591
Japan +81 6 6885 1213
Korea +82 2 3420 8600
Latin America +1 561 688 8700
Middle East +43 1 333 50 34 0
Netherlands +31 76 579 55 55
New Zealand +64 9 980 6700
Norway +46 8 556 468 00
Russia/CIS +43 1 333 50 34 0
Singapore +65 6289 1190
Sweden +46 8 556 468 00
Switzerland +41 61 716 77 00
Taiwan +886 2 8751 6655
UK/Ireland +44 1442 233555
USA +1 800 532 4752

Thermo SCIENTIFIC
A Thermo Fisher Scientific Brand

WP70848-EN 0716S