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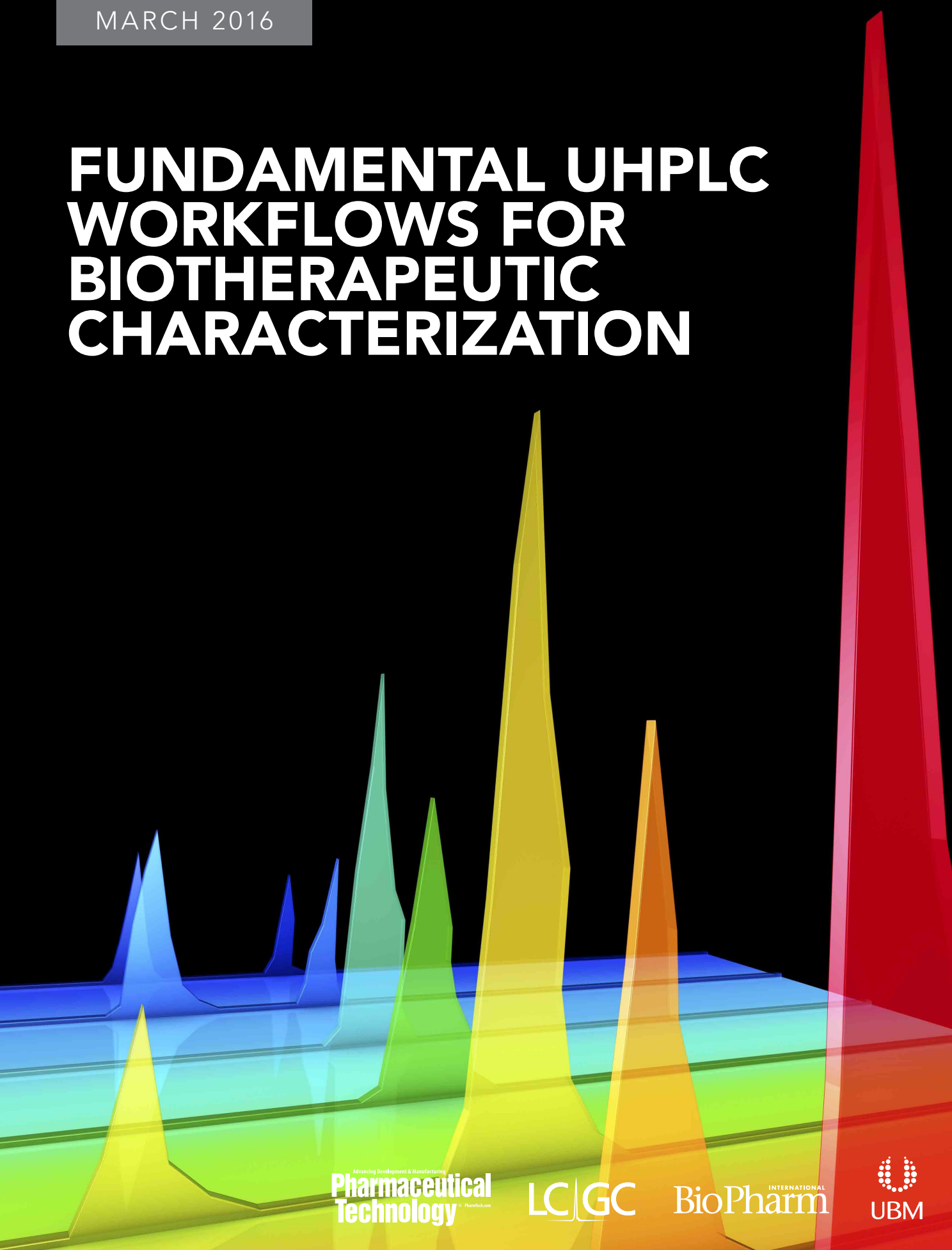
FUNDAMENTAL UHPLC WORKFLOWS FOR BIOTHERAPEUTIC CHARACTERIZATION



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INCREASING PRODUCTIVITY WITH UHPLC

Ultra high-pressure liquid chromatography (UHPLC) is enabling faster product development and production batch approvals with increased sensitivity.

By Cynthia A. Challener

Pharmaceutical laboratories are under pressure to increase productivity in order to speed up product development. The growing interest in process analytical technology has increased demand for more rapid analytical techniques that are appropriate for use in the manufacturing environment. More rapid analysis of production batches is needed to get material to market faster. Ultra high-pressure liquid chromatography (UHPLC), introduced in 2004, has become an important analytical tool for meeting these varied needs for higher throughput. Analytical instrument makers believe that further advances in the technology will enable UHPLC to have an even bigger impact on the pharmaceutical industry.

UHPLC meets many needs

"The field of separation science was

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revolutionized with the introduction of the first commercially available UHPLC system," asserts Eric Grumbach, senior product marketing manager for separations technologies with Waters Corporation. "Today, there are tens of thousands of these systems in use, clearly indicating that the inherent business and scientific benefits of UHPLC technology are being realized," he continues. Many factors are contributing to the demand for UHPLC. First, according to Grumbach, is the fact that the pharmaceutical and other industries, are under increasing pressure to improve overall profitability, from research and development through manufacturing and distribution, all the while facing the challenge of diversifying their product portfolio into new and unfamiliar territory. "One avenue towards improving profitability is to increase the throughput of their laboratory and manufacturing operations in an effort to decrease product development timelines and bring their products to market faster," he notes.

The need for quicker results, but with moderate sample sizes, is another driving force behind the implementation of high throughput UHPLC, according to Michael Frank, marketing manager for HPLC at Agilent Technologies. "For the release of a production batch, not only the one sample has to be analyzed, but easily up to 20 if counting blanks, standards, repeated analyses, etc. The approval process using conventional HPLC, with typical run times of 20 minutes, can take nearly 7 hours. UHPLC, which is often 5 to 10 times faster, allows a significant reduction in the analysis time, and thus, production batches can be released in much less time. As a result, produced goods can be packaged and shipped sooner, streamlining the complete process and saving money."

The online monitoring of products directly in the manufacturing facility is also much more feasible when using UHPLC compared to HPLC, according to Rainer Bauder, HPLC solutions manager for Thermo Fisher Scientific. "With UHPLC, the results are available within a few minutes rather than 30 or 45 minutes, which enables an immediate response to any undesired condition within the production cycle. Similar benefits can also be applied to process development and cleaning validation."

Multiresidue methods, including UHPLC, are also becoming the preferred way to monitor for drug residue contamination as the number of contaminants that are monitored increases, according to April DeAtley, LC product planner from PerkinElmer. "The combination tof more complex analyses and a growing number of samples means that longer runs cannot be tolerated in high throughput labs, where the emphasis is put on achieving the maximum chromatographic resolution in dramatically reduced times."

The chemistry of UHPLC

UHPLC is based on stationary phases using smaller particles. While conventional

HPLC assays use 2.5–5 µm separation media, UHPLC assays use smaller 1.7–1.8 µm column chemistries that offer up to a three-fold higher separation efficiency. Thus, the same exact separation can be achieved in a column that is threefold shorter, which directly translates into higher throughput due to shorter analysis times. Alternatively, better chromatographic resolution can be achieved with UHPLC compared to conventional HPLC if the same method conditions are used. Bauder adds that in addition to faster results, the power of UHPLC as a technique lies in its extreme versatility, the opportunity to reduce operation costs and solvent consumption, and the ability to develop higher resolving methods for new products, which mitigates the risk of missing problematic impurities during drug development or production. Nearly all instrument suppliers offer online calculators for establishing equivalent conditions for UHPLC and HPLC. Thus, the analytical chemist now has the flexibility to decide between the same resolution and higher speed or the same speed and higher resolution, according to Frank.

Advancing the technology

While it may seem contradictory, one recent advance in UHPLC technology has been the introduction of the ability to run conventional HPLC methods on UHPLC systems. "Pharmaceutical customers need to be able to run legacy HPLC methods because these methods have been validated for approved drugs, and any change in the QC method requires revalidation, a halt in production, and an interruption of the revenue stream," explains Grumbach.

There have also been a number of developments with respect to separation column technologies, according to Bauder. "New sub-2 micron particle and core-enhanced column technologies boost separation efficiencies and are triggering the development of new generations of column chemistries that offer improved selectivity for previously problematic target molecules, such as small, highly charged ions and mixtures of acidic, neutral and basic analytes. Grumbach points to the development of alternatives to U(H)PLC systems based on supercritical fluid chromatography (SFC), which he believes has enabled the development of a truly exceptional selectivity tool that is orthogonal to traditional reversed-phase methodologies. "This mode of chromatography has proven to be a clear replacement for normal-phase chromatography for most applications, and one that is ideally suited for chiral analysis. As an added benefit, the reduced solvent usage has significantly reduced assay cost and allows organizations to meet sustainability initiatives."

The ability of the instrumentation to withstand increasingly higher pressures is also critical to the advancement of UHPLC technology. "Entire systems are now able to tolerate greater pressures, including pump backpressures and

detector flowcells. As a result, UHPLC systems are increasing in their overall performance," notes DeAtley. Bauder adds that the development of novel detection techniques fully supporting UHPLC-type separation deliver additional leverage for faster and more in-depth sample assaying. Thermo Fisher Scientific has also focused on improving workflows around and within UHPLC instruments, including the introduction of automation tools, such as its x2 dual gradient UHPLC pumps and autosamplers that perform sample injection and fraction collection.

Challenges to overcome

tst, in fact, is one of the factors limiting the ability of pharmaceutical companies to fully leverage UHPLC technology. "A consistent theme we have heard from many of our customers is that the bottleneck of their workflow has moved from analyzing their samples, to preparing those samples prior to analysis. There is a need to make sample preparation workflows more efficient such that they can keep pace with the higher throughput analysis that UHPLC technology inherently provides," Grumbach observes. Frank believes, however, that automated liquid-handling systems and the ability to make automated changes with different ultra high-pressure valve solutions for rapid switching to different solvents and columns is increasing the flexibility of UHPLC systems.

Other limitations of UHPLC are being addressed with column technology, according to DeAtley. "Solid core column technology is allowing for very fast runs, incredible resolution, and lower pressures, which takes much of the stress off of the system," he comments. Bauder believes that, in addition to needed advances in system control and sample management, there remains significant improvement potential with respect to data processing and detection technologies, and that new and exciting column technologies are also showing great promise.

Anticipating the future

Clearly, much more can be expected from UHPLC in the future. DeAtley believes that column technology coupled with UHPLC capabilities will work together for lower pressure UHPLC applications with the benefit of fast run times and overall cost of ownership benefits. "We are definitely continuing to explore the boundaries of UHPLC technology. Further advances, however, may not come in the form of smaller particles (e.g. 1-µm particles) and higher pressures, but rather through the use of miniaturization, microfluidics, and different particle technologies," says Grumbach. Concludes Bauder, "The ultimate goal for UHPLC is to reduce the variety of separation methods required by end users while optimizing run time and sample throughput. Near-universal separation methods, as well as near-universal or (alternatively), highly selective detection technologies, have the potential to transform the chromatography lab and make UHPLC the methodology of choice."

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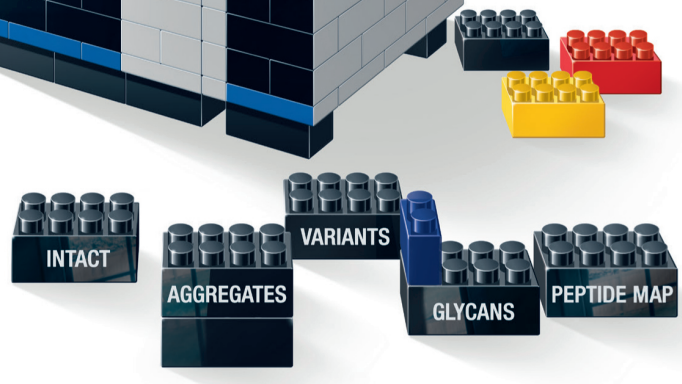

GLYCAN ANALYSIS: A PRIMER

NIBRT's Pauline Rudd on what to expect when performing glycan analysis.

By BioPharm International Editors

VIDEO

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The NIBRT Perspective

VIDEO

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Biopharmaceutical characterization demands advanced detection capabilities

In this fourth part of a series of primers with training experts from the National Institute for Bioprocessing Research and Training (NIBRT), Pauline Rudd, PhD, professor of glycobiology at University College Dublin (UCD), discusses glycan analysis. NIBRT provides training, educational, and research solutions for the international bioprocessing industry in state-of-the-art facilities. Located in South Dublin, it is based on an innovative collaboration between UCD, Trinity College Dublin, Dublin City University, and the Institute of Technology Sligo.

KEY DEVELOPMENT AND MANUFACTURING CONSIDERATIONS

BioPharm: Can you provide a brief overview of what exactly glycan analysis targets and its importance in bioprocessing?

Rudd: Most glycoproteins, and almost all of the new biological drugs, are proteins with sugars attached to them. These sugars are important for the safety and efficacy of drugs, so it is necessary to be able to control the processing

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of the sugar structures to make sure that the drug is as effective as possible. There are many aspects of developing and processing a drug that require having analytical technologies for glycosylation. For example, in the beginning of a drug-development process, one needs to understand the role of the sugars on the protein being used. On erythropoietin, for example, the sugars must be multiantennary and fully capped with sialic acid; otherwise, the erythropoietin will only be in the patient for a few minutes, whereas if it is completely sialylated, it will be present in the patient for 3 hours or more, during which time it will be able to be effective in stimulating the production of red blood cells. It is key to monitor the production process to make sure that the erythropoietin has the sugar structures that provide the full benefits of glycosylation. It is always important for biologics developers to understand exactly how the sugars in their product are going to modulate the functions of the drug in the patient. Once this knowledge is obained, drug developers can define an optimal glycan profile.

The next stage of bioprocessing, clonal selection, requires identifying a high-producing clone that has the ability to fully glycosylate the molecules with the optimal sugars. When selecting clones, glycan analysis enables the producer to determine the complement of glycoenzymes that are operating within a particular clone.

The next aspect of glycosylation that must be checked is the potential introduction of an antigenic epitope. If one is making a product in a nonhuman cell line, such as mouse cells, it is necessary to check the levels of alpha-galactose and N-glycolyl-neuraminic acid residues, which may be antigenic to humans. After the candidate clones are selected, the process of producing the protein from the cells must be monitored. Taking samples during the process allows one to assess whether the media composition is optimal for producing the desired glycosylation profile. In process development, optimizing the media is necessary to produce high levels of correctly folded proteins as well as the desired post-translational modifications, including glycosylation.

BioPharm: What role do glycans play in the manufacturing stage?

Rudd: When making a biologic product, one needs to track the glycans, which will help to determine the best time to harvest the product. When one reaches the downstream processing stage, high-performance liquid chromatography (HPLC) is often used to select subfractions of the product. Being able to analyze the glycans to ensure that their subfractions are correctly glycosylated is crucial. This information will be used during conversations with regulatory authorities so that the agency can agree on specifications for the drug product.

Glycan analysis will be part of that specification because it will be part of the regulators assessment of the drug's safety and efficacy. It will also be necessary to demonstrate to regulators that the process is robust and can be reproduced for batch and lot release.

Then comes the consideration of long-term storage. Although in general, glycans are quite stable, it is important to ensure that glycans do not change by testing after degradation and stability studies. Functional assays are also important to determine whether a product, IgG, for example, is able to bind to the desired receptor and not to those receptors that can cause side effects in the patient. Some of these questions require an understanding of how glycosylation modulates activity of the drug.

Overall, there are many reasons to perform glycan analysis and many ways to approach it. It is therefore important to understand the question being asked before deciding which method to use.

COMMON CHALLENGES IN CONTROL AND TECHNIQUE

BioPharm: What common challenges is the industry facing today when performing glycan analysis?

Rudd: Glycan processing is difficult to control because it involves a complex process that involves the expression of genes (the genes carry the code for the glycosylating enzymes) as well as the delivery of monosaccharides on nucleotide donors to grow the glycans. There are many things to control, and nearly 600 proteins are required to build the glycans as well as to transport the glycoproteins into the correct organelle for complete glycan processing.

If one is over-expressing a protein, it is possible to exhaust some of the glycosylation machinery. It is not uncommon to find incomplete structures attached to a protein, which indicate that the processes to build the sugars has not operated on all copies of the glycoprotein going through the secretory pathway. Understanding how to get the cells to express at a level where the rest of the machinery can cope is just one challenge. Another difficulty is determining what to do when the glycosylation is going wrong. One needs to understand in depth how media components and metal ions, for example, can alter glycan analysis. The natural cell is exquisitely tuned and responds very finely to its environment. It is difficult to replicate this robustness in a bioprocesssor.

BioPharm: You mentioned that industry uses various techniques for glycan analysis. What are the most commonly used instruments and techniques?

Rudd: Glycan analysis depends on a series of separations technologies that exploit different physical properties of the oligosaccharides. There are several ways to approach it. If you are focused on

analyzing released glycans, you need to have an optimized method for releasing them. There are many glycoconjugates, but from the point of view of the pharmaceutical companies the most commons ones are N-linked glycans. There is an enzyme called PNGase F that can be used to remove them from proteins of most species. After that stage, various separations technologies can be used to separate glycans according to mass/charge, charge, size, and lipophilicity by techniques such as hydrophilic interaction chromatography (HILIC), ion-exchange chromatography, reverse-phase chromatography, or capillary electrophoresis.

BioPharm: What gaps still exist in glycan-related technology and instrumentation?

Rudd: NIBRT uses a lot of special instrumentation, but we are working to get the analytical technologies miniaturized and as straightforward as we can. We have a robot that can accommodate 96 well plates, for example, so that one can put the samples on the instrument and come back later to put the released sugars on a HPLC machine or use mass spectrometry for analysis. We also have the capability to run linear samples, meaning that we can take samples one at a time from a bioprocesser every few hours. Going forward, industry will be looking to miniaturization, automation, and, particularly, automated data analysis.

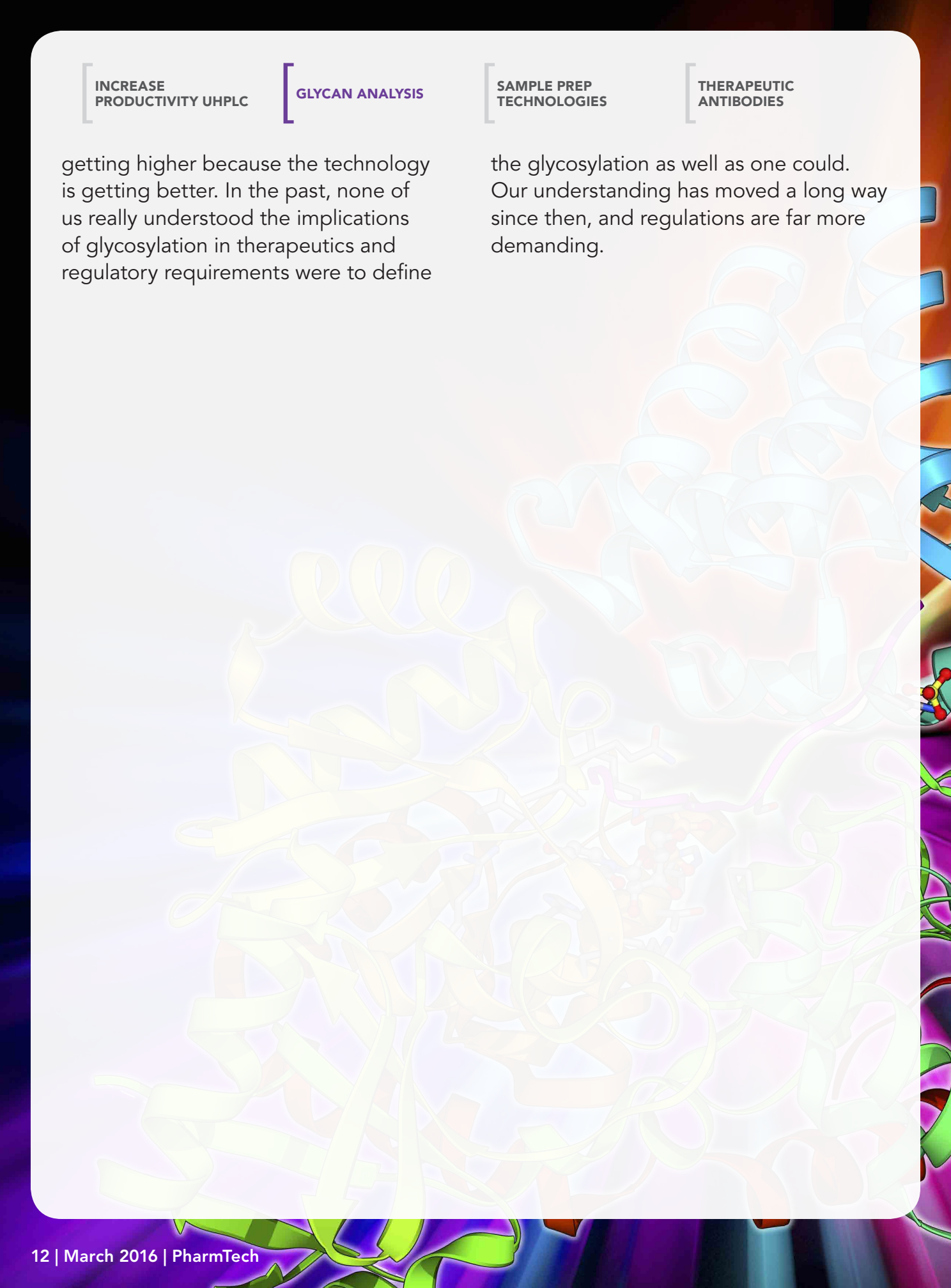
REGULATORY ISSUES

BioPharm: What regulatory expectations exist for glycan analysis when developing a biologic compared with a legacy product?

Rudd: Actually, there is a debate at the moment. Regulators need companies to report critical features of the glycosylation, but in some cases, it is not clear what "critical" features include. There is a need for more basic research to clarify these questions. For some molecules, such as IgG, it is known that the Fc glycosylation modulates effector function, so the regulators can ask for a full glycan analysis of IgG. One can report the sialylated structures, the levels of galactosylated, fucosylated, and bisected structures, because it is known that each of these features can modulate a function. To get the information, teams need to perform a complete analysis of IgG and present the data in a way that answers the questions about critical features that affect the safety and efficacy of their product.

If one is working with erythropoietin, it is necessary to report the percentages of different antennary structures as well as the extent of sialylation, because this critically affects the pharmacokinetcs of the drug. One needs to report levels of N-glycol-neuraminic acid, alpha(1,3) linked galactose as well as levels of xylose and alpha(1-3)-linked fucose, because these are potential antigenic epitopes. In general, regulatory expectations are

getting higher because the technology is getting better. In the past, none of us really understood the implications of glycosylation in therapeutics and regulatory requirements were to define the glycosylation as well as one could. Our understanding has moved a long way since then, and regulations are far more demanding.



SAMPLE PREPARATION TECHNOLOGIES FOR IMPROVED PEPTIDE QUANTITATION WORKFLOWS

By: Mike Oliver, Product Manager, Sample Preparation, Thermo Fisher Scientific

New sample preparation technologies can bring considerable benefit in terms of higher sample throughput and data quality to peptide quantitation. When these are combined with advances across the entire workflow, better quality, analysis speed, and greater return on investment are all achievable.

New sample preparation technologies can bring considerable benefit in terms of higher sample throughput and data quality to peptide quantitation. When these are combined with advances across the entire workflow, better quality, analysis speed, and greater return on investment are all achievable.

Within the life cycle of bringing a drug to market, there are several stages and associated workflows. Each workflow stage within discovery development or QA/QC follows a similar pattern: sample preparation, separation, detection, and data interpretation **Figure 1**.

Each of these stages can have a dramatic effect on the overall workflow output. Throughout all of these workflows, reproducibility and confidence in results are of paramount importance to ensure success of a candidate drug and compliance to regulation. In addition, speed and sample throughput are major considerations as they have a significant

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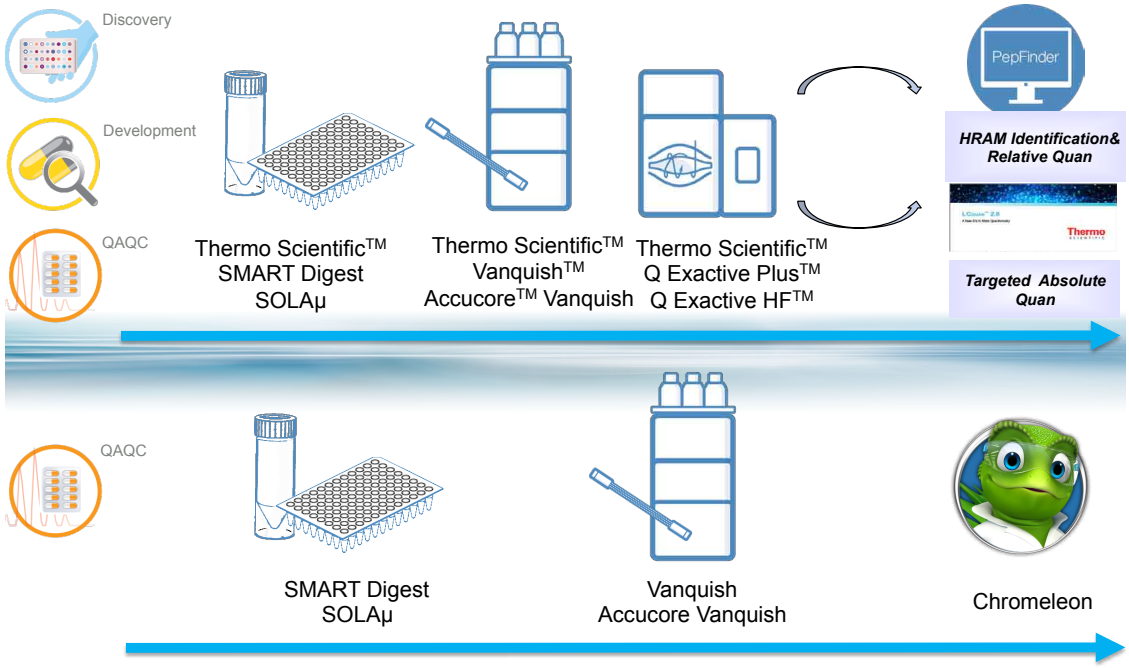
Figure 1: Bio-Pharma peptide analysis workflows



bearing on the overall cost and the return on investment of a project.

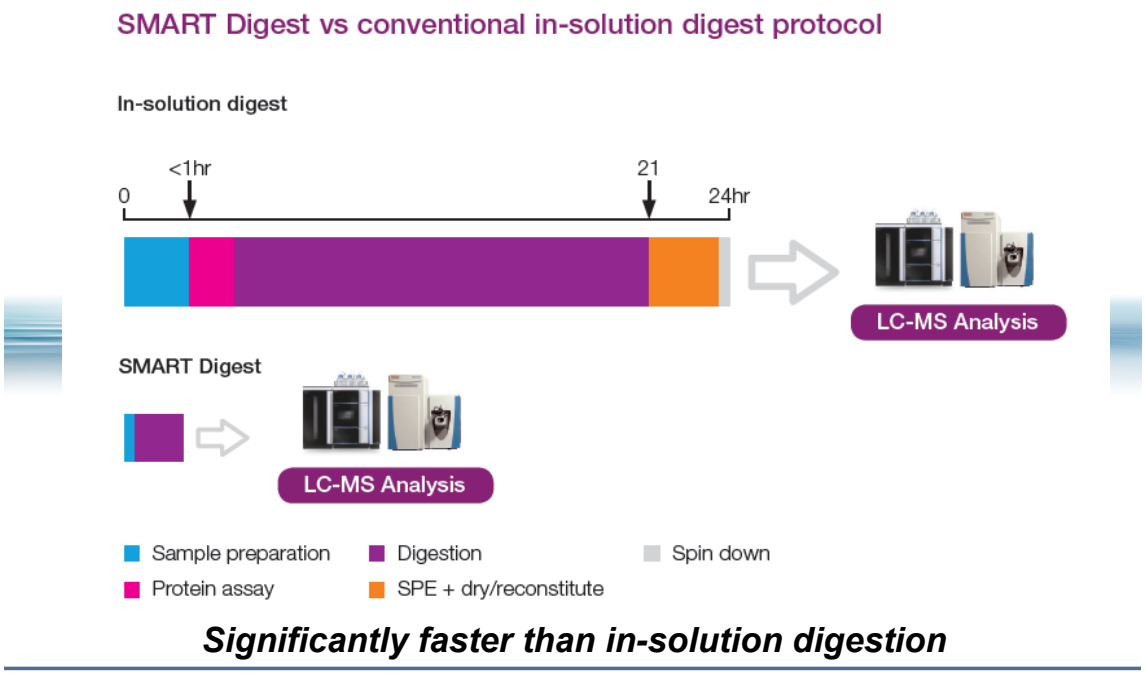
Challenges for Peptide Quantitation within Biopharma

A particular challenge is during the sample preparations stage, where proteins are digested into their constituent peptides for analysis. This stage typically involves in-solution digestion, which is multi-faceted and complex, and involves many steps to achieve a final result. The process is time-consuming and typically takes overnight or longer, depending on the size of the protein. The technique is also inherently irreproducible due to autolysis and the addition of unexpected post-translational modifications. This leads to variable data quality, which will manifest in poor reproducibility, sensitivity, and low sequence coverage.

There are also challenges with post-digestion sample cleanup protocols. These include sample integrity, which is of particular importance when dealing with peptides that are subject to non-specific binding and/or solvation, issues with reproducibility, and blocking when dealing with viscous biological samples.

Finally, there are challenges within LC-MS and data interpretation. Especially how systems are positioned for sensitivity and accuracy to enable reliable identification and quantitation of low-abundant peptides. Also consider if this is achievable with complex datasets that can be present when analyzing peptides.

Figure 2: Fast sample digestion



This all needs to be achieved while adhering to high throughput needs within the biopharmaceutical environment.

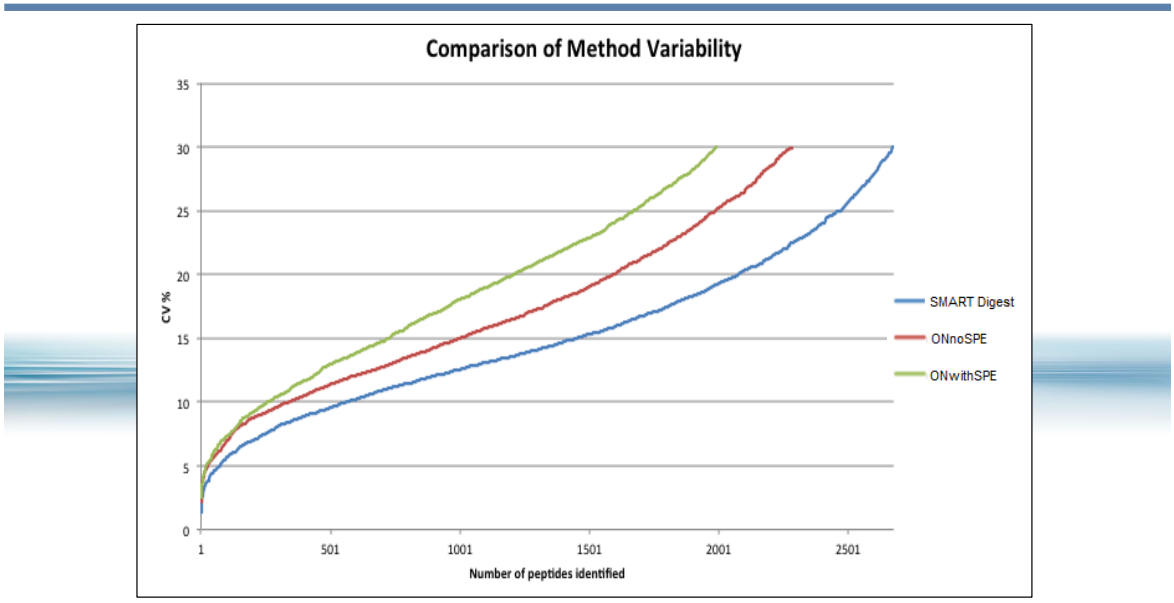
Addressing These Challenges

Tackling these challenges can be difficult when using a standard in-solution digest, which tend to be multifaceted, laborious, require multiple vendor components, and are prone to error **Figure 2**. Fortunately, there is an alternative that addresses these limitations: the SMART digest kit. This offers a simple, fast protocol with very high data quality. The simple process involves adding the sample and buffer to the PCR tube containing immobilized trypsin. The tube is then placed into a heater/shaker device for typically less than an hour, depending on the size and complexity of the sample. Once this is complete, the sample can be cleaned by centrifugation, filtration or SPE, depending on its matrix complexity, prior to analysis.

The digestion time for proteins such as IgG is typically less than an hour. Smaller proteins such as insulin can take just a few minutes. The process also removes the need for reduction or alkylation. As a result, the overall processing time using the SMART digest protocol can significantly reduce sample processing times resulting in the ability to increase throughput.

However, it's not just about speed and simplicity; this needs to be achieved in combination with reproducibility. Reproducibility of digestion can be

Figure 3: Smart digest provides greater sensitivity



More peptides identified with SMART Digest

difficult, whether between digested samples of the same protein or between users.

In addition to speed and reproducibility, the SMART digest kit can identify more peptides compared to in-solution digest protocols. **Figure 3** is a representation comparing the SMART digest protocol to an in-solution digest and to a post-SPE process to clean the samples. The SMART digest kit identified more than 2,500 peptides. The in-solution digest protocol with an SPE step identified around 2,200 peptides, and with the in-solution digest identified slightly less than 2,000 peptides. More peptides are identified with the SMART digest kit because it provides greater sensitivity.

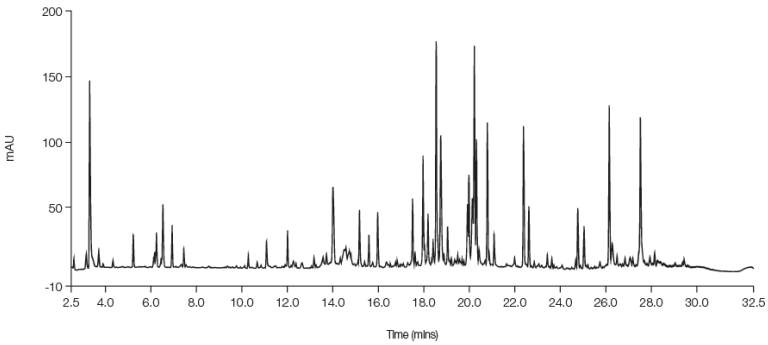
Sample Clean-up

While improvements can be made in the area of digestion, there is also the issue of sample clean-up. With complex matrices, a micro-elution SPE protocol is often employed post-digestion. Issues occur with conventional SPE designs, due to the presence of frits which are present to prevent the loss of sorbent material. In addition, the loose sorbent can compress when liquid is flowing through the bed. When dealing with viscous biological samples, this can lead to blocking and poor reproducibility. In conjunction with the SMART digest kit, the SOLAμ fritless SPE technology can be used to overcome these issues.

The frit- less, macro-porous SPE structure ensures the sorbent is held

Figure 4: Vanquish UHPLC & Smart Digest – high reproductibility

Peak #	RT (min)	RT-RSD (%)
3	3.315	0.082
9	5.231	0.065
14	6.532	0.017
15	6.937	0.023
19	10.290	0.021
23	12.013	0.012
31	14.011	0.013
39	15.177	0.012
42	15.589	0.010
51	17.511	**0.007**
55	17.969	0.011
61	18.546	0.010
83	20.798	0.010
85	21.095	0.012
87	22.386	**0.009**
96	24.774	0.012
103	26.155	**0.009**
106	26.155	**0.009**
109	27.529	0.010



- 13 overlaid chromatograms of mAb digestions
- Reproducibility of digestion/reproducible results

rigidly in place and prevents blockages, provide consistent flow, and ensure a far more reproducible clean-up phase. Micro-elution also removes the blow down step associated with larger bed weight SPE products. This saves time, increases throughput, and maintains sample integrity as peptide-type molecules are susceptible to loss during the blow down phase.

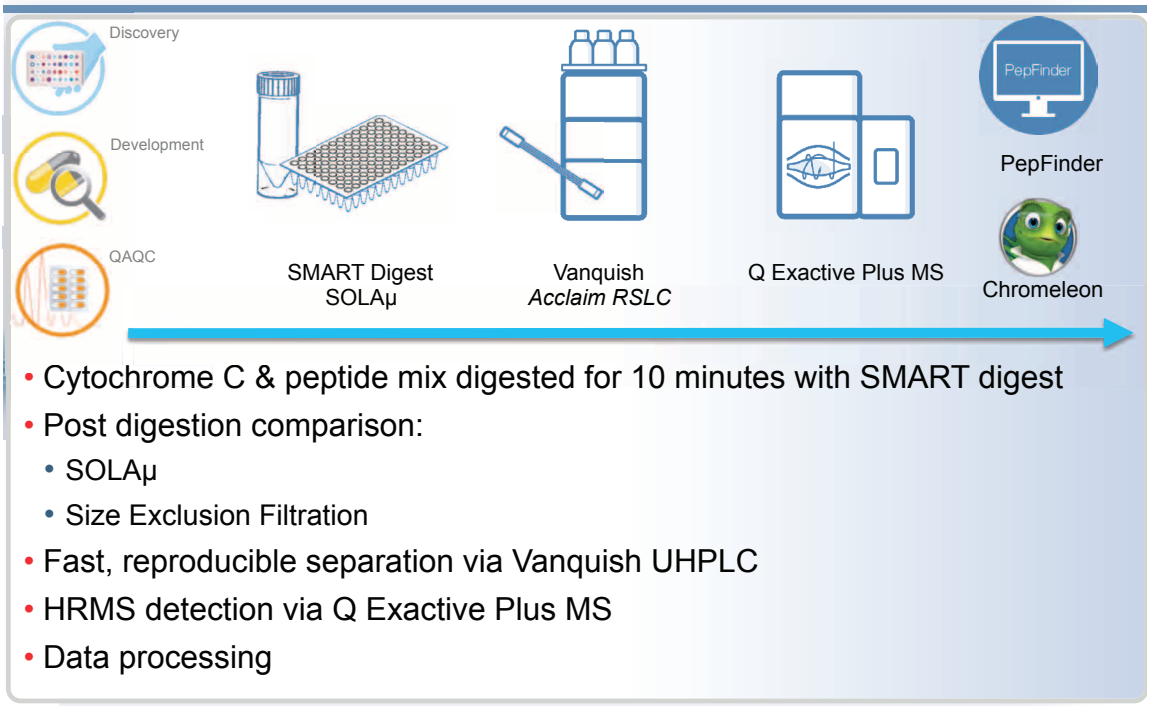
An additional benefit of the SOLAμ SPE product is the ability to pre-concentrate the sample up to 20-fold due to the high load and low elution volume capabilities, further helping to quantify low-abundant peptides.

Sample Analysis

Sample analysis requires that analytical systems complement sample preparation and workflow objectives. Peptide analysis requires highly reproducible separation of complex samples. The next-generation Vanquish UHPLC system with 1,500 bar capabilities provides high reproducibility and separation of complex samples, quickly and with high throughput capabilities.

The reproducibility delivered by the Vanquish is exemplified in **Figure 4**. Thirteen overlaid chromatograms of a monoclonal antibody are shown which are digested with a SMART digest kit. High levels of reproducibility are provided by the Vanquish, which is complemented by the SMART digest technology. This combination allows additional confidence when analyzing samples.

Figure 5: Bio-Pharma Peptide Workflows



The Benefits of High-Res Mass Spectrometry

We see how new sample preparation and UHPLC technology brings considerable benefit to the workflow, but how else can we achieve greater confidence in our results and our workflow capabilities?

High-resolution mass spectrometry provides additional tools to achieve confidence in results. High resolution allows greater separation of compounds by mass difference, allowing for greater confidence in quantification and qualification compared to nominal mass systems. Full scan methods can be employed and allow for interrogation of complete datasets, and the scan speeds are fast enough to achieve a required number of data points across the peak for accurate quantitation. High-resolution mass spectrometry separates compounds that are not possible via single or triple quad technologies, allowing for more accurate quantitation and qualification.

In the example it is not possible to separate two peptides with lower resolution MS systems. However, with the Q Exactive MS, the high-resolution allows compound separation, and thus accurate quantitation and qualification. PepFinder data processing software also accurately quantifies, and characterizes the dataset easily and effectively with a flexibility to identify post-translational modifications, identify disulphide bonds, and perform de novo sequencing.

Peptide Quantitation

How do all of these technologies combine in a real-life example? **Figure 5** shows cytochrome C and a peptide mix that was digested for 10 minutes with a SMART digest kit. The sample was cleaned separately via a SOLAμ SPE protocol and also with a size exclusion filtration step for the purpose of comparison. Separation via the Vanquish UHPLC system, and detection and data processing via the Q Exactive high-res mass spectrometer were then performed.

The SMART digest protocol involved taking 20 µg of cytochrome C and diluting to 50 µL with ultrapure water, further diluted to 200 µL with the SMART digest buffer. Samples were placed into a 70°C heater/shaker device at 1,400rpm for 10 minutes. Samples were then cleaned with the SOLAμ SPE device.

An alternative clean up would be to use a simple size exclusion filtration step, which provides a quicker and easier workflow, but sacrifices recoveries and demonstrates a much higher variability on the level of peptide recovered from the clean-up. SOLAμ, on the other hand, provides a more robust clean-up with high recoveries across the peptides. High precision gives increased confidence that each peptide will be recovered to the same level on multiple extractions.

Summary

Advances in sample preparation technologies for protein digestion and cleanup allow for highly reproducible sample sets, which are quick, simple to implement, and robust. When these are combined with highly reproducible column, UHPLC and high-resolution mass spectrometer systems allows for workflows positioned to deliver higher reproducibility and confidence in our results, increased speed of analysis, and increased sample throughput, as well as reduced cost and increased return on investment.

REVERSED-PHASE SEPARATION OF INTACT THERAPEUTIC ANTIBODIES USING THE VANQUISH FLEX UHPLC SYSTEM

Mauro De Pra and Carsten Paul Thermo Fisher Scientific, Germering, Germany

APPLICATION BRIEF

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High salt gradient analysis of post-translational modifications – deamidation monitoring

VIDEO

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Biopharmaceuticals are complex; analyzing them just got a lot easier

In biopharmaceutical early analytical development, characterization of monoclonal antibodies is required to support process development. Separation techniques such as hydrophobic interaction chromatography, size-exclusion chromatography, ion-exchange chromatography, and reversed-phase chromatography are routinely applied to profile the therapeutic protein during this stage of development. Reversed-phase chromatography can be run with mass spectrometry (MS)-compatible mobile phase, hence the method can be easily transferred to MS characterization laboratories when required.

Besides providing separation of impurities based on hydrophobicity, reversed-phase chromatography is an excellent tool for protein quantitation of main compound and minor variants. Reversed-phase separation of intact proteins is typically run at high temperatures

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to improve peak shape and recovery of proteins. Thus, high-resolution columns, packed with temperature-stable material are required. In addition, the method should be sufficiently fast, in order to allow the processing of a large number of samples in a reasonable time. An initial stability evaluation of the new biological entities has to be provided by early development laboratories. The analytical methods for the early stability assessment need to be able to indicate, and approximately quantify, sample degradation.

The MAbPac RP column is dedicated to separations of intact proteins. It is based on supermacroporous 4 µm polymer particles with exceptional thermal stability. The Vanquish Flex UHPLC system offers column thermostatting up to 120 °C and features a low-dispersion active pre-column eluent heater. This device actively regulates the thermal balance between the mobile phase and the stationary phase. The accurate temperature control allows avoiding loss of efficiency due to temperature mismatch between the column and the incoming solvent.

In this work, the MAbPac-RP column was operated with the Vanquish Flex UHPLC system for the reversed-phase chromatography of several intact therapeutic antibodies. To assess the suitability for stability studies, the chromatograms of a reference and a stressed mAb were compared.

Experimental

Instrumentation

Vanquish Flex UHPLC system, equipped with:

- System Base (P/N VH-S01-A)
- Quarternary Pump F (P/N VF-P20-A)
- Split Sampler FT (P/N VF-A10-A)
- Column Compartment H (P/N VH-C10-A) with Active Pre-heater VH-C1 (P/N 6732.0110) and Post-column Cooler 1 µL VH-C1 (P/N 6732.0510)
- Diode Array Detector HL (P/N VH-D10-A) equipped with LightPipe™ Standard Flowcell (P/N 6083.0100)

Chromatographic Conditions

Column:	MAbPac RP (2.1 x 50 mm) (P/N 088648)	
Mobile phase A:	0.1:100 TFA/water (v/v)	
Mobile phase B:	0.1:90:10 TFA/acetonitrile/water (v/v/v)	
Flow rate:	300 µL/min	
Column compartment temperature settings:	80 °C Forced air mode Active pre-heater: Post column cooler:	Column compartment: 80 °C 50 °C
Detector settings:	Detection wavelength: Data acquisition rate: Response time:	280 nm 10 Hz 0.4 s

Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ Chromatography Data System software, version 7.2

Results and Discussion

Four commercial antibodies were eluted with a 10 minute linear gradient. The linear increase of acetonitrile in the mobile phase was (9%)/min, in the case of trastuzumab (Figure 1), and (3.6%)/min

for cetuximab (Figure 4). In all cases, the elution of the intact antibodies resulted in very sharp peaks. The peak width at half height spanned from 1.6 seconds to 3.1 seconds for the steepest and the shallowest gradient, respectively. Peak symmetry was excellent for all mAbs, as it can be observed in Figures 1–4.

Detailed views of the intact antibodies chromatograms revealed that the

Figure 1: Injection of 4.2 µg of trastuzumab. Gradient 0–100% B in 10 minutes. Full view (a) and enlarged view (b).

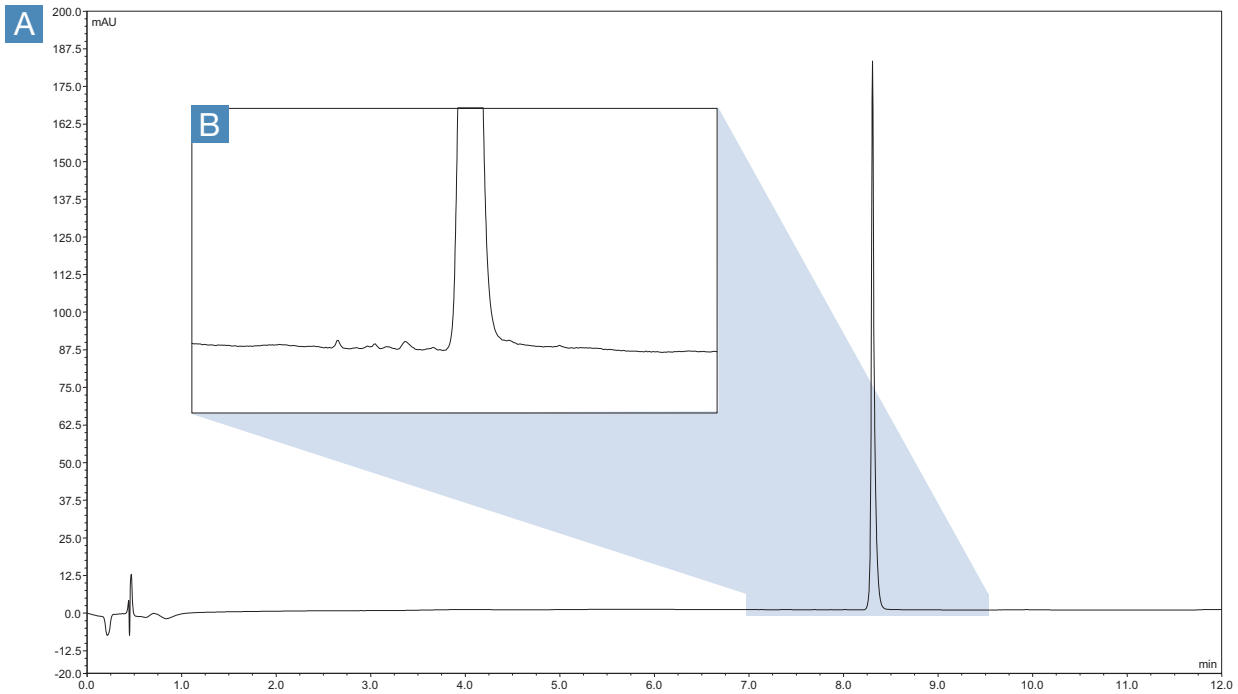


Figure 2: Injection of 25 µg of bevacizumab. Gradient 10–60% B in 10 minutes. Full view (a) and enlarged view (b).

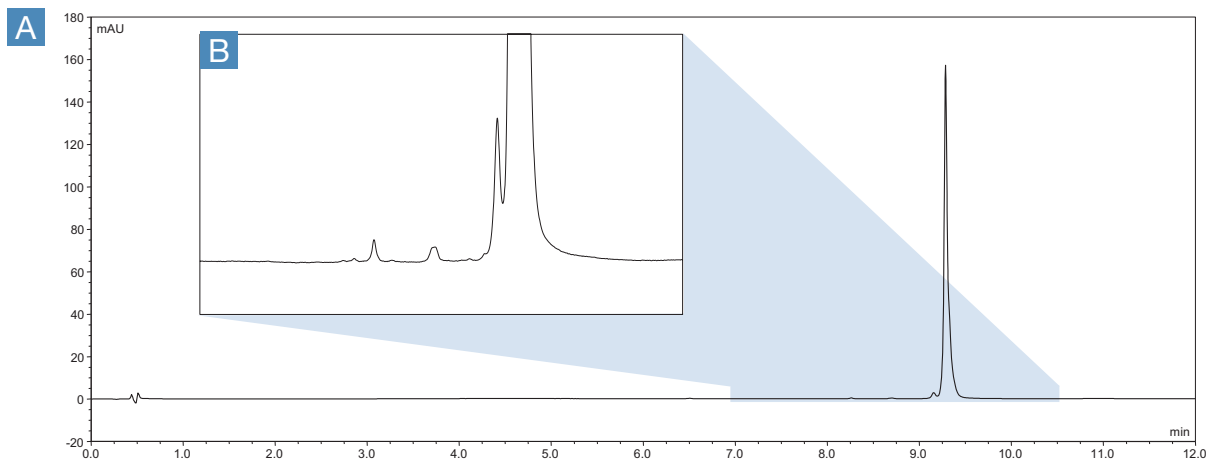


Figure 3: Injection of 1.25 µg of cetuximab. Gradient 20–45% B in 10 minutes. Full view (a) and enlarged view (b).

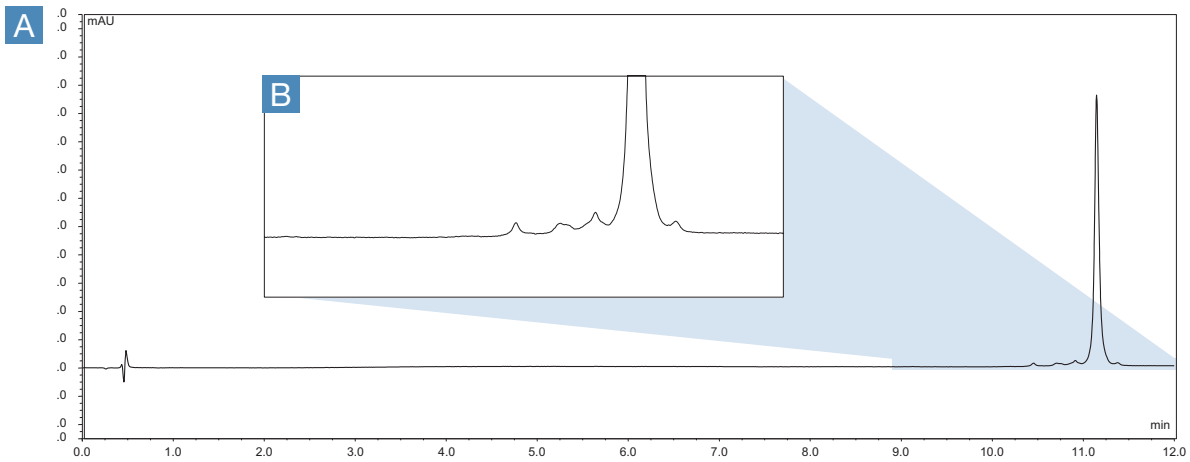
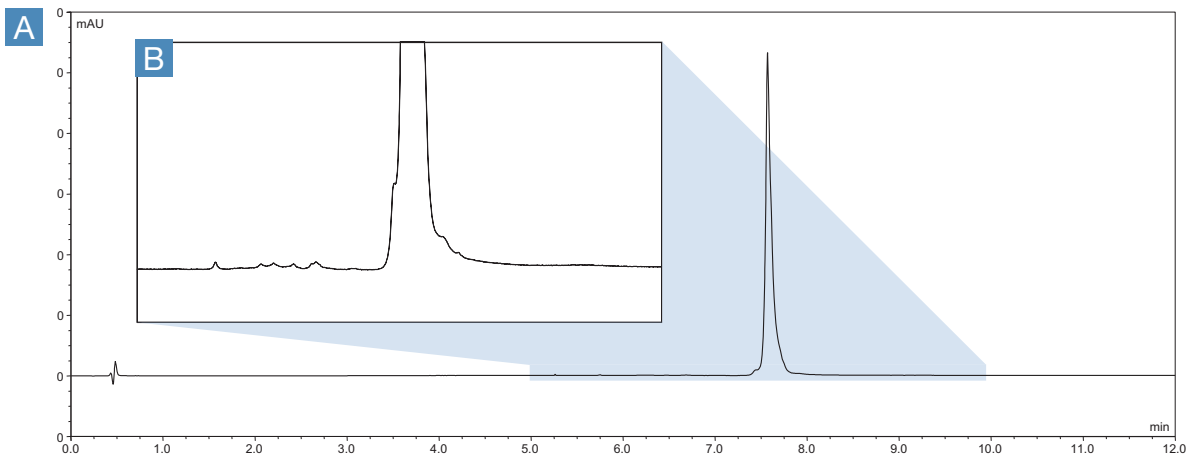


Figure 4: Injection of 1 µg of rituximab. Gradient 22–60% B in 10 minutes. Full view (a) and enlarged view (b).



MAbPac RP column provides selectivity to separate minor variants, even with relatively simple and fast gradient programs. This feature can be exploited in cases such as preliminary stability studies. In Figure 5, the chromatogram of a reference antibody is compared to a stressed one. The sample was donated by a customer and the stressing conditions were not disclosed. Here, the effects of stress-related degradation of an antibody are observed by running a simple 10 minute gradient from 0 to 100% B. The increased relative area of the impurities eluting before the main peak of the stressed mAb, confirmed the degradation of the sample. Additionally the degradation/denaturation of the sample can be estimated by the increased width

of the main peak. The width of the main peak at half height was 2.0 seconds for the reference, and 3.3 seconds for the stressed sample. This effect is likely caused by close-eluting species present in the stressed antibody but not in the reference one.

Conclusion

Reversed-phase chromatography is a powerful and convenient tool for the characterization of intact antibodies. With the extended thermostatting temperature range of the Vanquish Flex system and the new MAbPac RP column, very fast and efficient separations are achieved. Chromatograms with sharp symmetrical peaks are obtained that can be used to assess antibody purity in a very straightforward way.

Figure 5: Comparison between a reference (black trace) and a stressed antibody (blue trace). Gradient: 0–100% B in 10 minutes. Full view (a) and enlarged view (b).